MARIANA IANELLO GIASSETTI

Expressão de marcadores moleculares em espermatogônias

São Paulo
2015

MARIANA IANELLO GIASSETTI

Expressão de marcadores moleculares em espermatogônias

Tese apresentada ao Programa de Pós-Graduação em Reprodução Animal da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo - Brasil e Faculdade de Veterinária da Universidade de Zaragoza - Espanha para obtenção da Dupla Titulação de Doutor em Ciências.

Departamento:

Reprodução Animal

Área de concentração:

Reprodução Animal

Orientadores:

Prof. Dr. José Antônio Visintin

Prof. Dr. Pedro Muniesa Lorda

De acordo:_____

Orientador

São Paulo
2015

VERSÃO CORRIGIDA

Obs: A versão original se encontra disponível na Biblioteca da FMVZ/USP

Autorizo a reprodução parcial ou total desta obra, para fins acadêmicos, desde que citada a fonte.

DADOS INTERNACIONAIS DE CATALOGAÇÃO-NA-PUBLICAÇÃO

(Biblioteca Virginie Buff D'Ápice da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo)

T.3131
FMVZ

Giassetti, Mariana Ianello
Expressão de marcadores moleculares em espermatogônias / Mariana Ianello Giassetti. -- 2015.
163 f. : il.

Tese (Doutorado com Dupla Titulação em Ciência) - Universidade de São Paulo. Faculdade de Medicina Veterinária e Zootecnia. Departamento de Reprodução Animal, São Paulo, Brasil e Faculdade de Veterinária, Universidade de Zaragoza, Zaragoza, Espanha, 2015.

Programa de Pós-Graduação: Reprodução Animal.

Área de concentração: Reprodução Animal.

Orientadores: Prof. Dr. José Antônio Visintin.
Prof. Dr. Pedro Muniesa Lorda.

1. SSC. 2. Célula germinativa indiferenciada. 3. Marcadores moleculares. 4. Idade. 5. Yin Yang 2. I. Título.

UNIVERSIDADE DE SÃO PAULO

FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA

Comissão de Ética no Uso de Animais

CERTIFICADO

Certificamos que o Projeto intitulado "Expressão de marcadores moleculares em espermatôgonias", protocolado sob o nº 2509/2011, utilizando 30 (trinta) bovinos e 24 (vinte e quatro) camundongos, sob a responsabilidade do Prof. Dr. José Antonio Visintin, está de acordo com os princípios éticos de experimentação animal da "Comissão de Ética no Uso de Animais" da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo e foi aprovado em reunião de 15/2/2012.

We certify that the Research "Expression of molecular markers for espermatogonia", protocol number 2509/2011, utilizing 30 (thirty) cattle and 24 (twenty for) mouse, under the responsibility Prof. Dr. José Antonio Visintin, agree with Ethical Principles in Animal Research adopted by "Ethic Committee in the Use of Animals" of the School of Veterinary Medicine and Animal Science of University of São Paulo and was approved in the meeting of day 2/15/2012.

São Paulo, 27 de junho de 2014.

Denise Tabacchi Fantoni
Presidente

Av. Prof. Dr. Orlando Marques de Paiva, nº87
Cidade Universitária "Armando de Salles Oliveira"
São Paulo/SP – Brasil
05508-270

Fone: + 55 11 3091-7671/7676/0904
Fax: +55 11 3032-2224
E-mail: ceuavet@usp.br
http://www.fmvz.usp.br

FOLHA DE AVALIAÇÃO

Nome: Giassetti, Mariana Ianello

Título: Expressão de marcadores moleculares em espermatogônias

Tese apresentada ao Programa de Pós-Graduação em Reprodução Animal da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo - Brasil e Faculdade de Veterinária da Universidade de Zaragoza - Espanha para obtenção da Dupla Titulação de Doutor em Ciências

Data: 03/06/2015

Banca Examinadora

Prof. Dr._____

Instituição: _____ Julgamento: _____

Prof. Dr._____

Instituição: _____ Julgamento: _____

Prof. Dr._____

Instituição: _____ Julgamento: _____

Prof. Dr._____

Instituição: _____ Julgamento: _____

Prof. Dr._____

Instituição: _____ Julgamento: _____

Meu pai chama João Gato,

Minha mãe gata Maria,

Meus irmãos são todos gatos,

Ai meu Deus...que gataria.

Vô Orlando

Dedico com carinho aos meus pais, ao Augusto Cesar, a Tia Fina

e a minha família.

ACKNOWLEDGEMENTS

Agradeço ao Prof. Dr José Antônio Visintin, a Profa. Dra. Mayra Elena Ortiz D´Ávila Assumpção, ao Prof. Dr. Pedro Muniesa Lorda, ao Dr. Jon Schoorlemmer e a suas equipes de trabalho por todos esses anos de colaboração, apoio e ensinamentos. Meus mais sinceros agradecimentos.

Agradeço a colaboração e auxílio da Profa. Maria Climent, do Prof. Ricardo Pereira, do Prof. Pablo Ross e das suas equipes. Também agradeço aos pós-doutorandos que foram funamentais para minha formação e que são meus exemplos. Especialmente agradeço ao Dr. Marcelo Demarchi Goissis, sua dedicação exemplar foi fundamental para a execussão e melhoria dos projetos relacionados a essa tese. Obrigada por dispender seu tempo em minha orientação e formação como cientísta. Também agradeço as pós-doutorandas Dra. Flávia Regina Oliveira de Barros e Dra. Raquel Pérez Palacios, obrigada meninas por tudo.

Em nome do Prof. Dr. Enrico Ortolani e da Sra. Harumi, agradeço a Faculdade de Medicina Veterinária da Universidade de São Paulo e ao Departamento de Reprodução Animal pela infraestrutura, apoio financeito e resursos humanos. Obrigada! Em nome da do Prof. Dr. Paulo Maiorca e da Dra. Nicole Queiroz, agradeço ao Departamento de Patologia da FMVZ-USP.

Em nome no Profa. Dra. Rosário Osta e Sra. Carmen Ramos, agradeço a Faculdade de Medicina Veterinária da Universidade de Zaragoza e ao Deparamento de Anatomia e Embriologia pela infraestrutura, apoio financeiro e resursos humanos. Agradeço ao Serviço Aragonês de Saúde e Instituto Aragonês de Ciências da Saúde (IACS/CIBA) pela infraestrutura, apoio financeiro e resursos humanos.

A elaboração desta foi possível graças à Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) pelo apoio financeiro concedido por meio de Bolsa de Doutorado no País (proc. 2011/20115-1), Bolsa de Estágio de Pesquisa no Exterior (proc. 2012/05474-8) e Auxílio à pesquisa (proc. 13/03495-0 e proc. 10/51654-2).Obrigada.

Agradeço à todos que participaram de alguma forma dessa tese!

“Somewhere, something incredible is waiting to be known.”

Carl Sagan

RESUMO

GIASSETTI, M. I**. Expressão de marcadores moleculares em espermatogônias**. [Expression of molecular markers in spermatogonia]. 2015. 163 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2015. Tese (Doutorado em Ciências) - Faculdade de Veterinária, Universidade de Zaragoza, Zaragoza, 2015.

Em mamíferos, a espermatogênese é mantida pela autorrenovação e diferenciação das células-tronco espermatogoniais (SSC). Apesar da grande importância do SSC para a fertilidade masculina, em Bos taurus pouco se sabe sobre a sua identificação e biologia celular. Para roedores, mais de 30 marcadores para células germinativas indiferenciadas já foram descritos. No entanto, ainda não é conhecido um marcador específico apenas para SSC. Quase todos são também expressos por gonócitos, espermatogônias mais diferenciadas ou mesmo células somáticas. *Yin Yang 2* (YY2) é um factor de transcrição expresso nas células com a morfologia de gonócitos e SSC, sendo um candidato a marcador de SSC. Assim, a identificação de novos marcadores para SSC e factores que afectam a sua expressão, tais como a idade, são fundamentais para o desenvolvimento da biotecnologia como transgenia e tratamento de infertilidade, nos quais as SSC poderiam ser ferramentas biológicos importantes. Assim, nesta tese temos duas hipóteses principais: 1) a idade do dador afeta a expressão de marcadores moleculares específicos de SSC bovinas assim como potencial de células-tronco dessas células e que as sequências de DNA em que se associa YY2 regulam a expressão génica de SSC em camundongos. Os objetivos específicos, organizados em 4 artigos científicos, foram: identificar a melhor plaqueamento diferencial para enriquecer SSC bovina (artigo 1), verificar se a expressão de marcadores moleculares de SSC bovina difere entre adultos pré-púberes (artigo 2 e 3), identificar novos marcadores específicos para SSC em *Bos taurus* (artigo 3), verificar que a idade afeta o potencial de célula-tronco de SSC bovinas (artigo 3), descrever YY2 como um marcador específico para SSC em camundongos e verificar se as sequências DNA associadas YY2 são *loci* de importância para SSC. Assim, definimos o melhor plaqueamento diferencial para o enriquecimento de SSC bovinas, que idade afeta a expressão marcadores já estabelecidos assim como genes específicos do transcriptoma de SSC bovinas e que idade também afeta o seu potencial de células-tronco (ensaio de repopulação).

Concluímos também que YY2 é um marcador para SSC de camundongo em cultivo, em animais adultos e que as sequências do genoma que se associam YY2 possivelmente tem capacidade de regulação génica em SSC murina.

Palavras-chave: SSC. Célula germinativa indiferenciada. Marcadores moleculares. Idade. Yin Yang 2.

RESUMEN

GIASSETTI, M. I**. Expresión de marcadores moleculares en espermatogonias**. [Expression of molecular markers in spermatogonia]. 2015. 163 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2015. Tese (Doutorado em Ciências) - Faculdade de Veterinária, Universidade de Zaragoza, Zaragoza, 2015.

En mamíferos la espermatogénesis es mantenida por la auto-renovación y diferenciación de las células madre espermatogoniales (SSC). A pesar de la gran importancia de las SSC para la fertilidad masculina, en *Bos taurus* poco se sabe acerca de su identificación y biología celular. En ratones se han descrito más de 30 marcadores para células germinativas indeferenciadas pero todavía no se ha descrito un marcador específico para SSC. Casi todos marcadores de SSC son también expresados por gonocitos, espermatogonias más diferenciadas o hasta células somáticas. *Yin Yang 2* (YY2) es un factor de transcripción que se expresa en células con la morfología y la localización propia de gonocitos y SSC, siendo un candidato a marcador para SSC. La identificación de nuevos marcadores para SSC y de factores que afectan su expresión, como la edad, son fundamentales para el desarrollo de biotécnicas en que se puedan utilizar las SSCs como herramientas biológicas en transgenesis animal y tratamiento de infertilidad. Este trabajo tiene dos hipótesis principales: 1) la edad del bovino donante afecta la expresión de marcadores moleculares específicos de las SSC así como su potencial de célula madre y 2) que YY2 tiene funciones importantes en SSC, y que las secuencias genómicas a las que se asocia YY2 representan *loci* de importancia para SSC. Los objetivos específicos fueron organizados en cuatro artículos científicos: identificar el mejor plaqueamento diferencial para enriquecer SSC bovinas (articulo 1), *c*omprobar que la expresión de marcadores moleculares de las SSC bovinas difiere entre animales prepúberes y adultos (articulo 2 y 3), identificar nuevos marcadores específicos para SSC de *Bos taurus (articulo 2 y 3),* comprobar que la edad afecta el potencial de célula madre de las SSC bovinas (articulo 3), describir YY2 como un marcador específico para SSC en ratón y testar si las secuencias de DNA a las que se asocia YY2 (identificados previamente) tienen capacidad de regulación génica en SSC. Como conclusiones, definimos el mejor plaqueamento diferencial para el enriquecimiento de SSC bovinas, aportamos pruebas que la edad afecta la

expresión de marcadores ya consagrados, afecta un número pequeño la expresión de un número restringido de genes dentro del transcriptoma de SSC bovino, y comprobamos que la edad también afecta su potencial de célula madre (ensayo de repoblación). Aportamos a la identificación de YY2 como marcador de SSC en cultivo, de ratones adultos, y demostramos la capacidad de regulación genética en SSC de ratón de las secuencias a las que se asocia YY2.

Palabras clave: SSC. Células germinales indiferenciadas. Marcadores moleculares. Edad. Yin Yang 2.

ABSTRACT

GIASSETTI, M I**. Expression of molecular markers in spermatogonia**. 2015. 163 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2015. Tese (Doutorado em Ciências) - Faculdade de Veterinária, Universidade de Zaragoza, Zaragoza, 2015.

Mammalian spermatogenesis is sustained by self-renewal and differentiation of spermatogonial stem cells (SSC). Despite the importance of the SSC for male fertility, in *Bos taurus* little is known about their identification identity and cell biology. For rodents, more than thirty markers for undifferentiated germ cells have already been described. However, none of these represents a marker specific for SSC, as most are also expressed in gonocytes, differentiated spermatogonia or even somatic cells. *Yin Yang 2* (YY2) is a transcription factor specifically expressed in cells with the morphology of gonocytes and SSC in the mouse, being a candidate marker for SSC. For the use of SSC as an important biological tool in the development of biotechnology such as transgenesis and the treatment of infertility, it is important to identify new markers for SSC and factors that affect their expression, such as the age of donors. Therefore, the experimental work described in this thesis was based on two main hypothesis: (1) the donor age affects the expression of specific molecular markers in SSC as well their potential as stem cells and 2) YY2 exerts important functions in SSC and genomic targets correspond to *loci* relevant for gene regulation or genome management in SSC. The specific goals have been organized in 4 manuscripts as follows: optimization of differential plating to enrich for bovine SSC (Article 1), check if the expression of molecular markers of bovine SSCs differs between prepubertal and adult donors (Article 2 and 3), identify new markers specific for SSC in Bos taurus (Article 3), continuation of the initial description of YY2 as a specific marker for SSC in mice and check if sequences bound by YY2 *in vivo* harbor the capacity to influence gene expression in SSC. As conclusions, we present an optimized differential plating protocol for the enrichment of bovine SSC, we conclude that age effects the expression of SSC markers, the expression of specific genes of bovine SSC and that age also affects their potential as stem cells (measured in repopulation assays). We also contribute to the description of the restricted expression of YY2 in prepuberal and adult SSC in mice and we show that YY2 binding sites represent genomic sequences relevant for control of gene expression.

Keywords: SSC. Undifferentiated germ cells. Molecular markers. Age. Yin Yang

INDEX

1 INTRODUCTION

Spermatogenesis is a classical and complex biological stem cell-dependent process. In a correct environment, called the testicular niche, few spermatogonial stem cells (SSC) maintain the undifferentiated cell pool by self-renewal and some committed cells differentiate to the spermatic cell line. In domestic animals, SSC characterization and properties are not as elucidated as in rodents. In adult mice testis, SSC are a rare cell type (TEGELENBOSCH; DE ROOIJ, 1993); however, for the percentage of SSCs in adult testis is not clear in domestic animals. The development of biotechnological tools to identify unique properties of SSC and to purify those cells with high efficiency is fundamental to better understand SSC molecular biology and to apply SSC in animal transgenesis or infertility treatment.

Until recently, the highest rate of purified SSC was described in mice (BELLVÉ et al., 1977), which also is the species with highest number of specific markers described for undifferentiated spermatogonia (MEINHARDT et al., 1998; PHILLIPS; GASSEI; ORWING, 2010; ZHENG et al., 2014). More than thirty specific markers for mice spermatogonia were described (PHILLIPS; GASSEI; ORWING, 2010) such as ITGA6 (SHINOHARA et al., 1999), OCT4 (GUAN et al., 2009), GFRA1 (MENG et al., 2000), THY1 (KUBOTA; AVARBOCK; BRINSTER, 2003), CD9 (KANATSU-SHINOHARA; TOYOKUNI; SHINOHARA, 2004), PLZF (BUAAS et al., 2004) and CXCR4 (YANG et al., 2013). Yin Yang 2 (YY2) is a DNA-binding transcription factor that expression was reported in almost all cell types, tissues and embryo developmental stages but YY2 expression is not ubiquitous (LUO, J. et al., 2006; CHENG et al., 2008). Recently, high expression of YY2 was observed in undifferentiated mice spermatogonia in vivo and in vitro, indicating that YY2 might be a new candidate marker for undifferentiated spermatogonia in mice (PEREZ-PALACIOS et al., 2014[1]).

New candidates for SSC molecular markers have been proposed for mice, but transposition of knowledge from mice to domestic animals have some limitations in replicability of methods to enrich SSC, to understand self-renewal pathways and to

[1] PEREZ-PALACIOS, R.; GIASSETTI, M. I.; ACÍN, C.; CLIMENT, M.; GUALLAR, D.; MUNIESA, P.; SCHOORLEMMER, J. On a possible role for YY2 in male germline, [2014]. Data not published.

obtain long-term cell culture (HE et al., 2015). Such as in other domestic animals, only few specific markers for undifferentiated spermatogonia were described in cattle: THY1 (REDING et al., 2010), PLZF (REDING et al., 2010), UCHL1 (GOEL et al., 2010), NANOG, POU5F1, VASA (FUJIHARA et al., 2011), DBA affinity (IZADYAR et al., 2002) and CXCR4 (GOISSIS et al., 2015[2]).

Adult stem cells support tissues and organs maintenance and homeostasis, but tissue regeneration dramatically decrease with age (BOYLE et al., 2007). The age effect on SSCs functionality and properties are not complete clear for mouse and other mammalian species (JOHNSON et al., 1991; KOKKINAKI et al., 2010; FUJIHARA et al., 2011). In flies, the total number of germline stem cells, germline stem cell division and self-renewal factors decrease with aging (BOYLE et al., 2007; CHENG et al., 2008). In mice, SSC transcriptome is affected by age and consequently, by sexual maturation (KOKKINAKI et al., 2010). In livestock, offspring genetic gain is highly correlated with the male ancestor which most of times is a breeding adult male with previously approved genetic merit. However, the effects of sexual maturation in stem germ cells are unclear in mammals with long prepubertal phase, such as the bovine.

The present doctoral thesis goal is to study the expression of new and previously described specific spermatogonial markers for bovine and mice. We are also interested to understand the effect of age/sexual maturation in the expression of markers and stem cell functionality of bovine SSC. For better understanding, the objectives and results of this study were divided into four chapters organized as scientific articles. In chapter 1 (Comparison of spermatogonial stem cell markers after diverse differential plating methods of bovine testicular cells), we proposed to evaluate three differential plating methods to enrich spermatogonia based on the expression of interspecific molecular markers ITGA6, GFRA1, CXCR4 and THY1. In chapter 2 (Effect of age on expression of spermatogonial markers in bovine testis and isolated cells), we aimed to evaluate effect of age on the expression of undifferentiated spermatogonial markers in testis and in enriched testicular cells from prepubertal calves and adult bulls. Chapter 3 (Comparison of molecular and functional elements of CXCR4+ germ cells in pre-pubertal and adult bulls) we aimed

[2] GOISSIS, M. D.; GIASSETTI, M. I.; WORST, R. A.; MENDES, C. M.;. MOREIRA, P. V.; ASSUMPÇÃO, M. E. O. A.; VISINTIN, J. A. Spermatogonial stem cell potential of CXCR4-positive cells from prepubertal bovine testis, [2015]. Data not published.

to verify if sexual maturation affect CXCR4+ germ cell function and gene expression. In chapter 4 (Selected YY2 binding sites positively mediate transcriptional activity in Spermatogonial Stem cells) we aimed to clarify questions regarding the expression of YY2 in postpuberal mouse testis, regarding the intracellular localization of YY2 in SSC and verify YY2 and REX1 binding sequences ability to influence gene expression and transcriptional control at a distance.

2 LITERATURE REVIEW

2.1 TESTICULAR DEVELOPMENT: PRIMORDIAL GERM CELLS AND GONOCYTES

In the initial gonads, primordial germ cells (PGCs) originated from the epiblast migrate by a chemoattractive gradient to the mesenchymal tissue adjacent to mesonephros and that undifferentiated genitalia is called genital ridge (PARKER; SCHIMMER, 2006). Cell-cell contact, stem cell/c-kit signaling pathway, chemokine (C-X-C motif) ligand 12 (CXCL12) (ARA et al., 2003) and its receptor CXCR4 (MOLYNEAUX et al., 2003) are critical factors for PGC migration and/or proliferation (PARKER; SCHIMMER, 2006). After the migration to the genital ridge, PGCs are referred as gonocytes (either spermatogonia or oogonia) (PARKER; SCHIMMER, 2006). The dimorphic differentiation is controlled by presence or absence of Y chromosome, which contains the SRY gene. In males, SRY is expressed by somatic cells, which coordinate the differentiation of bipotential gonad to testes. Testes is histologically distinguished when testis cord appear, containing fetal Sertoli cells and PGC, being the precursors of seminiferous tubules (PARKER; SCHIMMER, 2006).

After birth, immature germ cells (gonocytes) proliferate and migrate in direction to testicular basal membrane (KERR et al., 2006). Two subpopulations have distant developmental potential at birth; round gonocytes are predicted to undergo apoptosis while pseudopod gonocytes migrate along the seminiferous tubule parenchyma and differentiate to spermatogonia stem cell (SSC) (ORWIG et al., 2002). SSC attach to the basal membrane and are surrounded by Sertoli cells (SPRADLING; DRUMMOND-BARBOSA; KAI, 2001). A simple terminology classified gonocytes as undifferentiated germ cells that are centrally allocated in seminiferous tubule and SSC are germ stem cells attached in the basal membrane (KERR et al., 2006). The gonocyte conversion to SSC happens at 0-6 dpp in mice but is not completed until 6 months of age in bovine (CURTIS; AMANN, 1981). SSCs self-renewal and differentiation support spermatogenesis in adult male however, undifferentiated

spermatogonia and pseudopod gonocytes have similar stem cell potential (ORWIG et al., 2002) and share several molecular markers (PHILLIPS; GASSEI; ORWING, 2010; ZHENG et al., 2014). The easy way to differentiate pseudopod gonocytes from round gonocytes and from SSC is by evaluation of morphologic parameters and localization in seminiferous tubule (ORWIG et al., 2002).

2.2 TESTICULAR ANATOMY AND CYTOLOGY

The anatomy and histology of the testis was previously reviewed (KERR et al., 2006; JOHNSON; THOMPSON; VARNER, 2008). The testis is covered by a dense connective tissue capsule called tunica albuginea, which is covered on its anterior and lateral aspects by tunica vaginalis. In the dorsal portion of testis, tunica albuginea is thicker to form the mediastinum testis. Those septa divide testicular parenchyma in several incomplete spaces called lobules. Each lobule is filled with blood vessels, lymphatic vessels, loose connective tissue, interstitial cells (Leydig cells) and seminiferous tubules. Three populations of cells within the seminiferous tubule: myoepithelial cells, germ cells and the Sertoli cells.

Successful testicular formation and spermatogenesis depend of a somatic element, namely the Sertoli cell. Sertoli cells are adhered in the basal membrane and display columnar shape, long and thin mitochondria, lipid deposits at the base, nucleus exhibiting a variety of shapes but with euchromatin and evident nucleolus (JOHNSON; THOMPSON; VARNER, 2008). Sertoli cells are fundamental to testis development, as during the embryo development Sertoli cells capture PGC inside of seminiferous cord inhibiting their proliferation and meiosis (JOHNSON; THOMPSON; VARNER, 2008). In adult, Sertoli cells plays a central role in regulation of the spermatogenic niche controlling SSC self-renewal or differentiation (GRISWOLD, 1998; JOHNSON; THOMPSON; VARNER, 2008). Other functions correlated with Sertoli cells are providing support and nutrition for all germ cells types, formation of blood testis barrier by tight junctions with surrounding Sertoli cells, phagocytosis of residual bodies and degenerated cells, production of regulatory proteins in response of pituitary release to influence the mitotic activity, chemotactic response of

spermatogonia and control of spermatogenesis (BELLVÉ et al., 1977; DYM; RAJ, 1977; FEIG et al., 1980; JUTTE et al., 1982; TRES et al., 1986; BELLVÉ; ZHENG, 1989; JOHNSON et al., 1991; JOHNSON; THOMPSON; VARNER, 2008)

2.3 SPERMATOGENESIS AND SPERMATOGONIAL CELL

Spermatogenesis is a complex and specific process in which a high number of spermatozoa are produced daily along the male life by SSC self-renewal and differentiation. The major constitutional elements of spermatogenesis are: SSC self-renewal and amplification by mitosis, SSC differentiation, chromosomal number reduction by meiosis and, transformation of an undifferentiated cell in a highly specialized and morphologically specific structure of spermatozoon by a process named spermiogenesis (KERR et al., 2006). Thus, SSCs are the most undifferentiated germ cell present is the adult testis. Studying SSCs characteristics and molecular physiology is complex because SSCs are present in few number in testis and a unique molecular characteristic has not been reported until now (PHILLIPS; GASSEI; ORWING, 2010). In the adult testis, SSC are usually recognized as a single cell arranged and attached along the basal membrane of seminiferous tubule that preferentially are adjacent to intertubular tissue; However, is well known that SSC are mobile and they can move 60 μm per day along the tubule length (reviewed by KERR et al., 2006) SSCs undergo many mitosis cycles without suffering differentiation prior entering mitosis. Once the committed cells enter mitosis they proceed into spermatogenic cycle until the spermatozoon is formed.

The history of spermatogonial classification was previously reviewed (KERR et al., 2006). First, spermatogonial cell were classified in rat as dusty and crusty cells. Dusty spermatogonial showed a nucleus with fine palely stained chromatin granulation. However, crusty spermatogonia had nuclei with coarse granules of heavily stained chromatin close to the nuclear membrane. Nowadays, a new nomenclature is frequently used; dusty and crusty were renamed to type A and type B spermatogonia, respectively. In human, type A spermatogonia is divided in pale and dark.

Spermatogonia type A could be divided in A_s (single), A_{pr} (paired) and A_{al} (aligned). In mice, almost 0.02-0.03% of total germ cells are stem cells and during the spermatogenic cycle the number of A_s cell fluctuates between 9 to 14 cells per 1000 Sertoli cells (TEGELENBOSCH; DE ROOIJ, 1993). A_s divides to produce two new A_s (self-renewal) or intracellular connections are maintained producing four A_{al} cells in the next division. In the next divisions, large chains with 8 until 32 cells become differentiated A_{al} spermatogonia. Historically, A_s spermatogonia were considered the only SSCs (DE ROOIJ; RUSSELL, 2000; SINGH et al, 2011). Nevertheless, new studies have demonstrated that both A_{pr} and A_{al} can revert to become As by fragmentation, therefore A_s, A_{pr} and A_{al} could act as SSCs (NAKAGAWA et al, 2010; YOSHIDA et al, 2007). Committed spermatogonia (A_1, A_2, A_3, A_4, intermediate and B) divide in a synchronized manner in the seminiferous tubule epithelium. Type B spermatogonia could in turn give a rise to primary spermatocytes and then secondary spermatocytes, which progress into meiosis in spermatogenic cycle to spermatids. Those haploid cells are transformed during spermiogenesis to finally become spermatozoa (HUCKINS, 1971; OAKBERG, 1971; PHILLIPS; GASSEI; ORWING, 2010).

2.4 TESTICULAR NICHE AND MOLECULAR MARKERS FOR UNDIFFERENTIATED SPERMATOGONIA

SSC are fundamental for spermatogenesis and a rare type of cell (TEGELENBOSCH; DE ROOIJ, 1993). Since a study performed in the late 90s (SHINOHARA et al., 1999), the current strategy to study SSC is to pre-selected those cells by expression of a membrane protein (marker+ and marker-) and perform transplantation with both cell types in a recipient testes (without SSC) to predict their stem cell potential. In the last 20 years, several markers for SSC have been reported and more than thirty were described just for mouse (PHILLIPS; GASSEI; ORWING, 2010). In mice, undifferentiated germ cells positively express integrin alpha six (ITGA6) (SHINOHARA et al., 1999), integrina beta 1 (SHINOHARA et al., 1999), Small Cell Lung Carcinoma Cluster 4 Antigen (CD24) (KUBOTA; AVARBOCK;

BRINSTER, 2003), , Neurogenin 3 (NGN3) (YOSHIDA et al., 2004), promyelocytic leukaemia zinc-finger (PLZF) (BUAAS et al., 2004), CD9 molecule (CD9) (KANATSU-SHINOHARA; TOYOKUNI; SHINOHARA, 2004), Cadherin Type 1 (CDH1) (TOKUDA et al., 2007), Nanos Homolog 3 (NANOS3) (LOLICATO et al., 2008) POU Class 5 Homeobox 1 (POU5F1/OCT4) (DANN et al., 2008), GDNF family receptor alpha 1 (GFRA1) (MENG et al., 2000) and Chemokine C-X-C motif receptor (CXCR4) (YANG et al., 2013). Notably, previously described markers for undifferentiated germ cells not only identify SSC but also most differentiate stages of spermatogonia (PHILLIPS; GASSEI; ORWING, 2010; ZHENG et al., 2014).

In almost all tissues, stem cells are present and generally have adhesive contact to the basement membrane surrounded by stromal cells producing a microenvironment called niche (SPRADLING; DRUMMOND-BARBOSA; KAI, 2001). The niche regulates SSC self-renewal or differentiation (SCADDEN, 2006) and, it is basically formed by SSC, Sertoli cells, Leydig cells and peritubular myoid cells (KOKKINAKI et al., 2009). In mammalian, the strongest evidence of niche-based regulation comes from spermatogenesis (SPRADLING; DRUMMOND-BARBOSA; KAI, 2001). Beta-1 integrin and ITGA6, that are localized in SSCs membrane, help SSCs to attach on basement membrane binding to laminin (SHINOHARA et al., 1999). However, in rodent testis the ITGA6 expression is not exclusive from SSC being also described in Sertoli cells (SALANOVA et al., 1995; BEARDSLEY; ROBERTSON; O´DONNELL, 2006) and in spermatic release during the spermiation (BEARDSLEY; ROBERTSON; O´DONNELL, 2006). Leydig cell and myoid cells affect SSC niche producing colony-stimulating factor 1 (CSF1), which acts stimulating SSC self-renewal (OATLEY et al., 2009). Leydig cells also are fundamental for SSC niche because they participate of testosterone production. In the SSC niche, glial cell line-derived neurotrophic factor (GDNF) is express by Sertoli cells and acts in receptors present in SSC membrane (RET and GFRA1) forming a complex in surface of A_s, A_{pr} and A_{al} spermatogonia (MENG et al., 2000; PHILLIPS; GASSEI; ORWING, 2010). Transgenic loss-of-function and overexpression models of GDNF and its receptor GFRA1 was used to observed that GDNF is essential for SSC *in vivo* self-renewal (MENG et al., 2000). In vitro, the combination of GDNF and GFRA1 is very favorable for SSC self-renewal (KUBOTA et al., 2004a; b). Sertoli cells also produce CXCL12 that interacts with germ cell receptor CXCR4 being associated with maintenance and self-renewal of SSC in postnatal life (PAYNE et al., 2010;

KANATSU-SHINOHARA et al., 2012; YANG, KIM, et al., 2013). So, CXCL12 and GDNF are produced by Sertoli cells and interacted specifically to SSC receptors (CXCR4 and GFRA1/RET, respectively) playing a pivotal role in SSC chemotaxis (in vivo and in vitro) (KANATSU-SHINOHARA et al., 2012) and self-renewal (MENG et al., 2000; KUBOTA et al., 2004a; b; YANG et al., 2013).

Recently, it was observed that YY2 is expressed in vivo and in vitro by gonocytes and undifferentiated mice germ cells (PÉREZ-PALACIOS et al., 2014[3]). YY2 is highly expressed in SSCs in culture and binds to retroviral elements present in genomic DNA of SSC (PÉREZ-PALACIOS et al., 2015[4]). However, the role of YY2 in undifferentiated germ cells has not been defined so far. YY2 is a transcription factor present in almost cell types in embryo and after birth, however YY2 expression is not ubiquitous (LUO, J. et al., 2006; CHENG et al., 2008). YY2 is highly expressed in gonocytes, SSC and spermatocytes (LUO, C. et al., 2006). YY2 and REX1 are generated by retroposition from Yin Yang 1 (YY1). YY1, YY2 and REX1 are from same family of transcription factors and have high homology to zinc-finger DNA-binding region (MONGAN; MARTIN; GUDAS, 2006; KIM; FAULK; KIM, 2007), indicating that these three transcription factors might share functions. The high expression of YY2 in undifferentiated germ cells suggest that YY2 plays a role in spermatogenesis and thus, YY2 might be a candidate for SSC marker in mice.

In domestic animals, a few number of specific marker for undifferentiated spermatogonia have been described in comparison to rodents (Chart 1)**.** Ubiquitin carboxyl-terminal hydrolase L1 (UCHL1 or PGP9.5) is expressed by mouse spermatogonia and activated Sertoli cells (KON; ENDOH; IWANAGA, 1999). Nucleotide sequence, protein's structure and immune-reactive epitopes of UCHL1 are conserved across mammalian species (KON; ENDOH; IWANAGA, 1999), being express in prepubertal testis from cattle, pigs, buffalo and goat, (KON; ENDOH; IWANAGA, 1999; LUO et al., 2006; HERRID; DAVEY; HILL, 2007; ANGLIN et al., 2010; GOEL et al., 2010; HEIDARI et al., 2012). In adult bovine testis, UCHL1 is a specific marker for type A spermatogonia and it is not express by Sertoli cell (FUJIHARA et al., 2011). In vitro, UCHL1 also is express by colonies of spermatogonia type A from adult donor (FUJIHARA et al., 2011). In domestic

[3] PÉREZ-PALACIOS, R.; GIASSETTI, M. I.; ACÍN, C.; CLIMENT, M.; GUALLAR, D.; MUNIESA, P.; SCHOORLEMMER, J. On a possible role for YY2 in male germline, [2014]. Data not published.
[4] PÉREZ-PALACIOS, R.; GIASSETTI, M. I.; ACÍN, C.; CLIMENT, M.; GUALLAR, D.; MUNIESA, P.; SCHOORLEMMER, J. On a possible role for YY2 in male germline, [2014]. Data not published.

animals, UCHL1 is consider an optimal marker for spermatogonia whereas it does not show affinity for somatic cells and is express by pre-meiotic male germ cell (KON; ENDOH; IWANAGA, 1999). Positive UCHL1 bovine germ cell co-express other undifferentiated spermatogonial indirect marker such as affinity to Dolichos Biflorus agglutinin (DBA) (HERRID et al., 2007). However, the affinity of SSC to DBA it is not constant over the domestic animals species and mice (ZHENG et al., 2014). DBA is frequently used to identify gonocytes and spermatogonia in large animals but cannot be used in sheep (GOEL et al., 2007; ZHENG et al., 2014). Furthermore, evidences indicate that spermatogonial DBA binding is lost along the male life (BORJIGIN et al., 2010).

THY1 is a high conserved SSC marker for different species and THY1 is described as conserved marker for undifferentiated spermatogonia for rat, mouse, pig and goat (KUBOTA; AVARBOCK; BRINSTER, 2003; RYU et al., 2004; REDING et al., 2010; ABBASI et al., 2013; WU et al., 2013; ZHENG, HE, et al., 2014). In pre-pubertal bulls, SSCs are THY+ testicular cells (REDING et al., 2010). GFRA1 is one of the most important molecular marker in mice but in domestic animals its expression was reposted only for pig gonocytes (LEE et al., 2013) and equid spermatogonia (COSTA et al., 2012). As GFRA1, CXCR4 expression in undifferentiated spermatogonia is unclear for most domestic animals. However, recently we observed that bovine undifferentiated cells also express CXCR4 and CXCR4+ germ cells have the stem cell ability to recolonize mice testis after xenotransplant (GOISSIS et al., 2015[5]). NANOG and POU5F1 are consecrated markers for embryonic stem cells (ES) and dynamic expression of both markers was observed through male germ cell development in domestic animals (Chart 1)**.** PLZF is an essential transcriptional factor to maintenance and self-renewal of SSC in mice (BUAAS et al., 2004) and it is expressed by undifferentiated germ cells in several species (Chart 1). Is important to remember that the expression of some previously described markers for SSC also are expressed by somatic cells and thus, it is recommended a functional assay as SSC xenotransplantation to verify the stem cell ability to proliferate in the recipient testes (ZHENG et al., 2014).

[5] GOISSIS, M. D.; GIASSETTI, M. I.; WORST, R. A.; MENDES, C. M.;. MOREIRA, P. V.; ASSUMPÇÃO, M. E. O. A.; VISINTIN, J. A. Spermatogonial stem cell potential of CXCR4-positive cells from prepubertal bovine testis, [2015]. Data not published.

Chart 1 - Molecular markers of spermatogonia in domestic animals, adapted from ZHENG et al., 2014

Continue

Marker	Species	Approaches	Cell type	Reference
UCHL1 (PGP9.5)	Pig	IHC, ICC	Gonocytes and spermatogonia	(LUO, J. et al., 2006)
	Cattle	IHC, ICC and WB	Gonocytes and spermatogonia	(HERRID et al., 2007; Fujihara et al., 2011)
	Buffalo	IHC and ICC	Prepubertal spermatogonia	(GOEL et al., 2010)
	Goat	IHC and ICC	Gonocytes	(HEIDARI et al., 2012)
DBA	Pig	IHC and ICC	Gonocytes	(GOEL et al., 2007)
	Cattle	IHC, ICC and XenoTP	Gonocytes and spermatogonia	(ERTL; WROBEL, 1992;HERRID; DAVEY; HILL, 2007; FUJIHARA et al., 2011)
	Buffalo	IHC and ICC	Prepubertal spermatogonia	(GOEL et al., 2010)
	Sheep	IHC	No expression	(BORJIGIN et al., 2010)

Continue

Marker	Species	Approaches	Cell type	Reference
THY1 (CD90)	Pig	IHC, ICC, WB, MACS and qPCR	Gonocytes and spermatogonia	(ZHENG;HE, et al., 2014)
	Cattle	IHC, ICC, WB, MACS, qPCR, FACS and XenoTP	Gonocytes and spermatogonia	(REDING et al., 2010)
	Goat	IHC, ICC, WB, MACS and qPCR	Spermatogonia	(ABBASI et al., 2013; WU et al., 2013)
GFRA1	Pig	IHC, ICC and MACS	Gonocytes	(LEE et al., 2013)
	Equid	IHC and WB	Spermatogonia	(COSTA et al., 2012)
CXCR4	Cattle	IHC, MACS, qPCR and XenoTP	Gonocytes and Spermatgonia	(GOISSIS et al., 2015[6]))

[6] GOISSIS, M. D.; GIASSETTI, M. I.; WORST, R. A.; MENDES, C. M.;. MOREIRA, P. V.; ASSUMPÇÃO, M. E. O. A.; VISINTIN, J. A. Spermatogonial stem cell potential of CXCR4-positive cells from prepubertal bovine testis, [2015]. Data not published.

Continue

Marker	Species	Approaches	Cell type	Reference
NANOG2	Pig	IHC	Gonocytes and undifferentiated spermatogonia (prepubertal)	(GOEL et al., 2008)
	Cattle	IHC, ICC and WB	Gonocytes, spermatogonia and differentiated spermatic cells	(FUJIHARA et al., 2011)
POU5F1	Pig	IHC	Undifferentiated spermatogonia (prepubertal) and differentiated germ cells	(GOEL et al., 2008)
	Cattle	ICH, ICC and WB	Gonocytes, spermatogonia (adult) differentiated germ cell	(FUJIHARA et al., 2011)
	Buffalo	IHC, ICC and WB	Gonocytes, spermatogonia (prepubertal) and spermatids (adult)	(MAHLA et al., 2012)
SSEA1	Pig	IHC, ICC, FACS and XenoTP	Spermatogonia	(KIM et al., 2013)
Claudin-8	Cattle	IHC, IHH, FACS and XenoTP	Spermatogonia	(MCMILLAN et al., 2014)
CSF1R	Equid	IHC and WB	Spermatogonia	(COSTA et al., 2012)

Marker	Species	Approaches	Cell type	Reference
ZBTB16 (PLZF)	Pig	IHC and ICC	Gonocytes and spermatogonia	(LUO, C. et al., 2006; LUO, J. et al., 2006; GOEL et al., 2007)
	Cattle	IHC, ICC, WB and qPCR	Gonocytes and spermatogonia	(REDING et al., 2010)
	Equid	IHC and WB	Spermatogonia	(COSTA et al., 2012)
	Sheep	IHC and ICC	Gonocytes and spermatogonia	(BORJIGIN et al., 2010)
	Goat	IHC, ICC and qPCR	Gonocytes and spermatogonia	(SONG et al., 2013)

IHC, immunohistochemistry; ICC, immunocytochemistry; WB, western blot; qPCR, quantitative real-time PCR; XenoTP, xenotransplantation; FACS, fluorescence-activated cell sorting; MACS, magnetic-activated cell sorting

2.5 ISOLATION AND ENRICHMENT PROCESS OF UNDIFFERENTIATED SPERMATOGONIA

One of the most used methods to isolate SSC from testes is the two-step enzymatic incubation process (BELLVÉ et al., 1977; IZADYAR et al., 2002). Briefly, testis is first dissected, the tunica albuginea is removed and the seminiferous tubules are dispersed by collagenase and then, trypsin digestion leads to the single cell suspension (BRINSTER; AVARBOCK, 1994). The addition of DNase in the two-step digestion process improves the protocol efficiency because DNase digest DNA from damaged cells reducing viscosity (GUAN et al., 2009). The two-step enzymatic digestion process has a high reproducibility, is economical, simple (HE et al., 2015) and can be associated with others methodologies to improve the purification of SSC.

The methodologies to enrich SSC are divided in: physical methods such as differential plating and discontinuous Percoll density gradient centrifugation selection; or immunological methods such as fluorescence-activated cell sorting (FACS) and magnetic-activated cell sorting (MACS) selection, which depend on molecular marker expression by SSC (HE et al., 2015). The differential platting concept is based on positive or negative selection of cells adhesive properties to different subtracts. Laminin is an extracellular matrix that binds to ITGA6 receptor present on undifferentiated spermatogonia. Thus, laminin provides a positive selection of SSC in adherent fraction (GUAN et al., 2009). The other approach to differential platting is to select the somatic cells on the adherent fraction and thus, enriching SSCs on the supernatant fraction. In this situation, differential plating could be performed with non-coated or BSA-coated cell dishes (HERRID et al., 2009). The somatic cells adhere faster than SSC and part of somatic cells can be eliminated based on this time gap. However, a large amount of SSC may be lost if the time for adhesion is not judged correctly or the operator does not have experience (HE et al., 2015). The discontinuous Percoll density gradient centrifugation selection is currently used for SSC enrichment in domestic animals (IZADYAR et al., 2002; HERRID et al., 2009; GOEL et al., 2010; DE BARROS et al., 2012; AHMAD et al., 2013). In this method, cells isolated from testis are placed onto the top layer of discontinuous Percoll gradient and after centrifugation the purified SSC are retained in one fraction of

Percoll gradient (HE et al., 2015). However, distinguishing the layers is not simple and a trained operator is necessary (HE et al., 2015). The physical methods for SSC enrichment are widely used because they are simple and not expensive (HE et al., 2015); however, they have low efficiency and association with an immunological method is recommended (HERRID et al., 2009; AHMAD et al., 2013).

In MACS technology, a specific antibody for a SSC marker is associated with a magnetic bead that are retained by positive magnetic force in a column placed in a magnetic stand. Thus, cells that express the SSC marker may be retained in the column and SSC are then enriched. However, after a positive sorting the complex antibody-antigen is not dissociated and interferes with a adhesion of another antibody for the antigen (HE et al., 2015). FACS allows a highly efficient selection of SSC by one or several markers at the same time (GUAN et al., 2009; HE et al., 2015). However, FACS is an expensive technology and a trained operator is necessary and, generally a high number of initial cells are needed and target cells may be damaged after the sorting (HE et al., 2015). Thus, based on the literature, the two-step enzyme digestion and MACS are the fast way to isolate and purify SSC because MACS is fast, simple, does not requires large equipment and is not limited by initial cell number (HE et al., 2015).

2.6 SSC TRANSPLANTATION

The stem cell concept describes the ability of cells to reestablish the functionality of a tissue/system from which they were derived and, transplantation is a golden standard method to analyze stem cell function (OATLEY; BRINSTER, 2008). The functional activity of SSC was analyzed for the first time by transplantation in Ralph Brinster's studies (BRINSTER; AVARBOCK, 1994; BRINSTER; ZIMMERMANN, 1994). In the SSC transplantation assay, SSC suspension is injected in a seminiferous tubule of a recipient male, which endogenous germ cells were depleted by chemotoxic treatment or are naturally not present (OATLEY; BRINSTER, 2008; HE et al., 2015). SSC have the ability to repopulate the recipient testis, proliferate and reestablish the spermatogenesis. In a xenotransplant assay, SSC

from domestic animals could colonize and proliferate in recipient mouse testis, but could not complete spermatogenesis (OATLEY et al., 2002; HERRID; DAVEY; HILL, 2007; REDING et al., 2010; ZHENG et al., 2014). Currently, the SSC transplantation is the only unequivocal method to evaluate SSC functionality (OATLEY; BRINSTER, 2008). However, SSC transplantation requires more manpower, more time, use of host animals and it is expensive (HE et al., 2015).

2.7 APPLICATIONS OF UNDIFFERENTIATED SPERMATOGONIA IN BIOTECHNOLOGIES

In recent years, great progress has been made in isolation, purification and characterization of SSC, especially in rodents. Thus, that knowledge provides theoretical basis to explore SSC characterization in domestic animals and application of those SSC in biotechnologies such as in vitro culture or animal transgenesis. SSC might be a powerful and revolutionary tool in reproductive biotechnology of large animals (ZHENG et al., 2014). However, the transposition of knowledge about SSC from rodent to large animals have some limitations such as low replicability of SSC enrichment methods efficiency, molecular markers and biological control of self-renewal, which are fundamental for SSC in vitro culture (HE et al., 2015). SSC could be very useful in animal reproduction and gene transfection but homologous transplantation is a fundamental to produce transgenic animals with high productivity and commercial value (ZHENG et al., 2014).

In vitro culture of SSC is a crucial biological tool to expand and manipulate a very rare cell population in vivo and thus, a long term in vitro culture is necessary to better understand mechanisms of SSC self-renewal and differentiation (ZHENG et al., 2014). In contrast to rodents (KANATSU-SHINOHARA et al., 2003; KUBOTA et al., 2004b) and humans (KOKKINAKI; DJOURABTHI; GOLESTANE, 2011), a long term culture of SSC was not well established for domestic animals (OATLEY; REEVES; MCLEAN, 2004; APONTE et al., 2006; LUO et al., 2006; GOEL et al., 2007., 2010; FUJIHARA et al., 2011; HEIDARI et al., 2012; NASIRI et al., 2012). SSC in domestic animals can be easily isolated and primary culture is obtained fast;

however, SSC proliferation drastically decrease and over time SSC differentiation and apoptosis dominate the cell culture (ZHENG et al., 2014). In bovine, SSC long-term culture has been considered a problematic issue mainly because of the absence of specific markers to identify and purify SSC with high efficiency (reviewed by NASIRI et al., 2012). Also, a limited knowledge about in vitro culture requirements such as media, factors required and presence of feeder layers for promoting bovine SSC self-renewal and proliferation (reviewed by NASIRI et al., 2012). Thus domestic animals long-term SSC culture not has been reported, but probably in a near future SSC culture will help to bridge the gap between basic research and real biotechnological application of SSC from domestic animals in animal reproduction (ZHENG et al., 2014).

2.8 EFFECT OF AGE AND SEXUAL MATURATION ON UNDIFFERENTIATED GERM CELLS

After birth, the seminiferous cord parenchyma development depends of the time span until sexual maturation (reviewed by KERR et al., 2006). In mammals with a long prepubertal period (bovine, nonhuman primate and humans) little changes in the parenchyma are observed since birth until sexual maturation (reviewed by KERR et al., 2006). During testis development and sexual maturation process, gonocytes differentiate into SSC but both cell types share stem cell activity (ORWIG et al., 2002) and molecular markers (ZHENG et al., 2014). After puberty, the spermatogenic cycle is active and rate of undifferentiated germ cells decreases in testicular parenchyma; thus, SSC becomes a rare cell type in the adult testes (TEGELENBOSCH; DE ROOIJ, 1993).

Adult stem cells support tissue maintenance and homeostasis but tissue regeneration dramatically decrease with age; however, the role of aging in organs and tissues is not yet fully understood (BOYLE et al., 2007). In mice, the effects of age in stem cells that have been described are: loss of function, low proliferation and reduced ability to differentiate (ZHANG et al., 2006; CHAMBERS et al., 2007; CHENG et al., 2008; GEIGER; RUDOLPH, 2009; KOKKINAKI et al., 2010). In the

human male, advanced age affects sperm DNA damage, chromatin integrity, gene mutation and aneuploidies (WYROBEK et al., 2006). However, few studies were performed to understand the effect of aging on mammalian SSCs. In flies, the total number of germline stem cells, germline stem cell division and self-renewal factors decrease with aging (BOYLE et al., 2007; CHENG et al., 2008). In mice, some genes are expressed specifically in SSCs from oldest mice such as Selp and Icam1 (KOKKINAKI et al., 2010). However, the molecular mechanisms involved in age influences of SSCs functional and properties are unclear for mouse and other mammalian species.

SSC characterization and properties are not so elucidated in domestic animals as in rodents. However, an elegant study showed that UCHL1 is expressed by gonocytes in prepubertal Bos taurus taurus calves and by spermatogonia type A in adult bulls testis (FUJIHARA et al., 2011). In situ, testicular parenchyma from bulls (2 year old) had high number of UCHL1 +, DBA+, NANOG+, VASA+ and OCT4+ cells per seminiferous tubule than younger (1.5 week, 2.5, 3 and 5.5 months) (FUJIHARA et al., 2011). Thus, it was observed that age affects expression of SSC markers in bovine but the effect of sexual maturation in undifferentiated germ cell with stem cell properties (gonocytes and SSC) is still unclear in domestic animals with long prepubertal phase, such as bovine.

3 HYPOTHESIS AND OBJECTIVES

Based on the data presented, this study proposes the following principal hypotheses:

Age affects the expression of molecular markers and stem cell potential of bovine SSC

YY2 is a specific molecular marker for pospubertal mice SSC and, YY2 and REX1 binding sequences have genetic regulation capacity.

The general objective is:

Find and describe molecular markers with potential applicability in bovine SSC.

The specific objectives of this study are:

- ✓ Verify which differential platting is most efficient to bovine SSC enrichment,
- ✓ Identify new molecular markers for undifferentiated spermatogonia in mice and bovine,
- ✓ Verify if age/sexual maturity affects on expression of specific bovine undifferentiated spermatogonia molecular markers,
- ✓ Verify if age/sexual maturity affects on stem cell capacity and transcriptome of bovine undifferentiated spermatogonia,
- ✓ Identify YY2 genomic targets genes associated with YY2 in mice SSC,
- ✓ Test the specificity of "reporter genes" for YY2 and REX1 genomic targets to control gene expression in embryonic stem cells and SSC.

4 CHAPTER 1: COMPARISON OF SPERMATOGONIAL STEM CELL MARKERS AFTER DIVERSE DIFFERENTIAL PLATING METHODS OF BOVINE TESTICULAR CELLS

4.1 SUMMARY

Spermatogonial stem cells (SSC) have important applications in domestic animal reproduction and advanced biotechnologies, such as animal transgenesis. Differential plating is one of the most common methods used for SSC enrichment. The goal of this study was to identify the most efficient differential plating method for the isolation of bovine SSC. In this matter, testicular parenchyma from prepubertal calves was minced and isolated cells were obtained after two enzymatic digestions. We tested three coating methods for differential plating: laminin (20ng/ml), BSA (0.05mg/ml) and PBS. Cells were incubated at 37°C in 5% CO2 in air for 15 min onto laminin coated dishes or for 2 hours onto BSA or PBS coated dishes. Cell viability was assessed by Trypan Blue solution exclusion method. Recovered cells were analyzed for expression of SSC molecular markers by quantitative RT-PCR (*GFRA1, CXCR4 ITGA6, THY1*) and flow cytometry (GFRA1, CXCR4 and ITGA6). Cells at time 0, attached on laminin and non-attached cells from BSA and PBS groups had the same cell viability (p=0.0655). *GFRA1, CXCR4* and *THY1* relative gene expression were higher (p=0.0402, p=0.0007, p=0.0117, respectively) for non-adherent cells selected in PBS group. Flow cytometry analysis revealed that presence of GFRA positive (GFRA+) cells was higher in non-adherent cells from BSA and PBS groups (p<0.001). However, laminin adherent cells had higher number of ITGA6+ cells (p<0.001) and lowest presence of CXCR4+ cells (p=0.0012). In conclusion, differential platting is an effective method for enrichment of bovine undifferentiated spermatogonia and higher expression of SSC markers is obtained without laminin or BSA coating.

Keywords: Bovine. Spermatogonia. Molecular marker. Differential plating. Laminin.

4.2 INTRODUCTION

Spermatogenesis is a complex and specific process in which a high number of spermatozoa are produced daily. Spermatogonial stem cells (SSC) self-renewal and differentiation are responsible for maintaining sperm production throughout the adult male lifespan. SSC are the most undifferentiated germ cell present is the adult testis. Studying SSC molecular characteristics and physiology is complex because SSC are present in few number in testis and its unique molecular characteristics were not reported until recently (PHILLIPS; GASSEI; ORWING, 2010). In domestic animals, the percentage of SSC in adult animals is not well defined as in rodents. This is due in part to lack of studies using reliable molecular markers of SSC. To better understand specific markers for undifferentiated spermatogonial cells, processes such as differential plating are required to enrich undifferentiated spermatogonial stem cells from isolated testicular cells. Thus, improving the enrichment is essential for further studies with bovine spermatogonial cells.

In mice, GFRA1 is a known SSC/progenitor cell marker expressed by most undifferentiated type A spermatogonia and some A paired and A aligned (KOKKINAKI et al., 2010). GFRA1 is also required for SSC self-renewal (MENG et al., 2000; HOFMANN; BRAYDICH-STOLLEL; DYM, 2005; KOKKINAKI et al., 2010). THY1 was described as a conserved marker for undifferentiated spermatogonia for rat, mouse, pre-pubertal bulls and goat (KUBOTA; AVARBOCK; BRINSTER, 2003; RYU et al., 2004; REDING et al., 2010; ABBASI et al., 2013). Recently, CXCR4 was described as a membrane receptor in murine SSC necessary for stem cell maintenance by regulation of self-renewal (YANG et al., 2013).

In bovine buffalos and pig, the affinity to Dolichos Biflorus agglutinin (DBA) is an indirect marker for type A spermatogonia cells (ERTL; WROBEL, 1992; IZADYAR et al., 2002;HERRID; DAVEY; HILL, 2007; KLISCH et al., 2011; AHMAD et al., 2013). In domestic animals, the epitope-N-acetyl-D-galactosamine present in

gonocytes and spermatogonial cells is recognized by the DBA (IZADYAR et al., 2002) is frequently used to identify these cells in livestock animals but it does not show constantly expression over domestic animals species (GOEL et al., 2007; ZHENG et al., 2014) and it was not correlated with more specific molecular markers such as GFRA1 and THY1. Unlike GFRA1 and THY1, the affinity to DBA is not observed in murine SSC. Furthermore, evidences indicate that spermatogonial DBA binding is lost along the male lifespan (BORJIGIN et al., 2010).

Differential plating can be performed with non-coated, BSA-coated or laminin-coated cell culture dishes. Spermatogonia can be enriched through negative selection based on adhesion of other cells on non-coated or BSA-coated dishes, while they adhere to laminin due to expression of ITGA6 (SHINOHARA et al., 1999; SHINOHARA et al., 2000). It was previously shown that BSA coating was more efficient than laminin coating to enrich bovine spermatogonia (HERRID et al., 2009). Results were similar to enrichment after differential plating without coating (IZADYAR et al., 2002). However, in bovine studies only DBA staining was used to evaluate the efficiency of enrichment methods (IZADYAR et al., 2002, HERRID et al., 2009).

We propose to evaluate three differential plating methods to enrich spermatogonia based on the expression of interspecific molecular markers ITGA6, GFRA1, CXCR4 and THY1. Due to the specificity of laminin and ITGA6 interaction, we hypothesized that differential plating with laminin is more efficient to enrich undifferentiated bovine spermatogonia. In this study we performed non-coated, BSA-coated or laminin-coated differential plating of testicular cells from pre-pubertal bulls and evaluated the selection by quantitative PCR and flow cytometry analysis of spermatogonial molecular markers.

4.3 MATERIAL AND METHODS

All chemicals were supplied by Sigma Chemical Company (St. Louis, Missouri, USA) unless otherwise stated. All procedures were approved by Bioethics Committee for use of animals of College of Veterinary Medicine and Animal Science of the University of Sao Paulo.

4.3.1 Animals, enzymatic isolation and cell cryopreservation

Prepubertal bull calves with 5 months of age (n=5) were anesthetized (0.2 mg of xylazine hydrochloride/ Kg) (Virbaxyl 2%, Virbac do Brasil Ind. E com, Sao Paulo, Brazil) and testes were surgically removed. Testicular cells were isolated by two-step enzymatic digestion as described previously with minor modifications (Reding et al., 2010). All testicular parenchyma was minced and incubated in collagenase solution (1 mg/ml collagenase type V and 7 mg/ml DNase I) for 30 min at 37°C with continuous agitation. Digested seminiferous tubules were washed three times with Phosphate-buffered saline (PBS; 100 x g/ 1min) and incubated in trypsin solution [0.25% (w/v) trypsin, 2.21 mM EDTA and 7 mg/ml DNase I] for 5 min at 37°C. Enzymatic activity was blocked by addition of 10% fetal bovine serum (FBS; Gibco®, Life Technologies, Carlsbad, CA, USA). Cells were passed through a 100 mm cell strainer (BD Biosciences, Durham, NC, USA), centrifuged (600 x g/ 7 min) and the cell pellet was suspended in PBS. Cell concentration and viability were assessed by Trypan Blue solution exclusion method (5 mg/ml) and cells were cryopreserved (10x106 viable cells/ml) in DMEM medium (Life Technologies,) with 10% (v/v) FBS, 10% (v/v) DMSO and, 0.25% (w/v) sucrose at -80ºC using the Mr. Frosty container (Nalgene®, Waltham, Massachusetts, USA) and then placed into liquid nitrogen at -196 ºC.

4.3.2 Differential plating

Laminin-coated dishes (60 mm, Falcon, BD Biosciences, North, NSW, Australia) were prepared as described previously (GUAN et al., 2006) after incubation with 3 ml of laminin (20 μg/ml) at 37°C overnight. Nonspecific binding was prevented by pre-incubation with BSA solution (0.5 mg/ml) for 1 hour at 37°C. The dishes were washed 3 times with PBS before cell plating. BSA-coated dishes and non-coated control dishes were prepared adding 3 ml of BSA solution (0.5 mg/ml in PBS) or PBS only in 60 mm cell culture dish and incubated overnight at 37°C. Coating solution was removed immediately before cell plating.

Testicular cells were thawed, washed with DMEM medium (Life Technologies) with 10% (v/v) FBS and centrifuged (400 x g /min). Cells were suspended in DMEM-BSA medium [DMEM medium with 0.2 % (w/v) BSA and 0.1% (v/v) non-essential amino acids and cell viability was assessed by Trypan Blue solution exclusion method. A total of $3x10^6$ viable cells in duplicate were plated in each dish previously coated with laminin, BSA or PBS (control) at 37°C in 5% CO_2 incubator. Cells were incubated onto laminin for 15 minutes (GUAN et al., 2006) or 2 hour for BSA and control groups (REDING et al., 2010). Non-adherent cells in the supernatant fraction were collected in a conical tube and cell concentration and viability was performed. Adhered cells fraction were washed three times with PBS and isolated by 0.25 % (w/v) trypsin solution for 1 min at 37°C. Enzyme activity was blocked by addition of DMEM medium with 10% (v/v) FBS. Adhered cells were removed by pipetting, collected in conical tube and concentration of viable cells was performed.

4.3.3 Quantitative real time RT-PCR

Gene expression was performed with 0.2 x 10^6 viable cells for each sample. RNA was obtained from total cells previously to any differential plating (time 0),

adherent cells fraction on laminin, non-adherent cells fraction on BSA and non-adherent cells fraction from control group. Total RNA was extracted by Illustra RNAspin mini according to manufacturer's instructions (GE Healthcare®, Buckinghamshire, United Kingdom) and quantified at Nanodrop ND-1000 (Nanodrop Technologies, Inc., Wilmington, Delaware, EUA). cDNA synthesis was performed using SuperScript® First Strand Synthesis System (Life Technologies) according to manufacturer's instructions and cDNA concentration was determined using Qubit dsDNA BR Assays kit (Life Technologies) by fluorometry (Qubit 2.0 Fluorometer, Life Technologies). cDNA concentration was standardized at 200 ng/ml and the quantitative RT-PCR (Mastercycler Ep Realplex Thermal Cycler, Eppendorf AG, Hamburg, Germany) was performed using SYBR GreenER qPCR Supermix Universal (Life Technologies) in a two-step reaction: 2 min at 50ºC, 10 min at 95ºC, 40 cycles at 95ºC for 15 s and 60ºC for 1 min; dissociation curve was performed in the end of cycle. All reactions were performed in triplicate with a negative control. Primers were previously standardized and efficiency curve was performed with cDNA obtained from pre-pubertal bull testis. For this study, the housekeeping genes analyzed were: Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (forward primer: 3'TGACCCCTTCATTGACCTTC 5'; reverse primer: 5' TACTCAGCACCAGCATCACC 3'; NCBI Reference Sequence NM_001034034.2) and beta-actin (*ACTB*) (forward primer: 5'AGGGATGTGGAGACGACAAC 3'; reverse primer: 5' TCTTTGGTGGGATTCTTTGG 3'; NCBI Reference Sequence NM_001109981). SSC target genes analyzed were: *ITGA6* (forward primer: 5' AGGGATGTGGAGACGACAAC 3'; reverse primer: 5' TCTTTGGTGGGATTCTTTGG 3'; NCBI Reference Sequence NM_001109981), *GFRA1* (forward primer: 5' CAAGTGGAGCACATCTCGAA 3'; reverse primer: 5' GGCAGGAACAGAAGAGCATC 3'; NCBI Reference Sequence NM_001105411), *CXCR4* (forward primer: 5' GGACTTGAGTAGCCGGTAGC 3'; reverse primer: 5' CATAGTCACCCGAGCCCAAG 3'; NCBI Reference Sequence NM_174301) and *THY1* (forward primer: 5' TGCTAACAGTCTTACAGGTGGC 3'; reverse primer: 5' TCTTTGTGTCACGGGTCAGG 3'; NCBI Reference Sequence NM_001034765). Sertoli target gene analyzed was *GDNF* (forward primer: 5' AACTCGTGCCCCTAACCTG 3';reverse primer: 5' GACAGCCACGACATCCATA 3'; NCBI Reference Sequence XM_005221583.1)

4.3.4 Flow cytometry

After determining the optimal concentration for immunolabeling, cells before (time 0) and after differential plating were suspended in PBS with 10% (v/v) FBS and incubated with primary antibody: 1:500 anti-GFRA1 (ab84106, Abcam, Cambridge, UK), 1:40 Anti-ITGA6/ALEXA488 (313608, Biolegends, San Diego, CA) and 1:500 anti-CXCR4 (ab7199, Abcam) for 30 min at room temperature, washed in PBS (950 x g/ 3min) and suspended in 3% (w/v) BSA in PBS. Samples previously incubated with anti-GFRA1 and anti-CXCR4 were incubated with a secondary antibody conjugated with FITC (1:200; Ab9750, Abcam) for 30 min at room temperature. All samples had two control groups: without any antibody incubation and incubation just with secondary antibody. Flow Cytometry analysis was perfomed (FACSCalibur, Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) and at least 10 000 events were obtained for each sample. Data were analyzed by FlowJo software (TreeStar Data Analysis Software, Ashland, OR, EUA). Percentage of positive cells for each sample was obtained by comparison with its control groups (without antibody and incubation just with secondary antibody) to control unspecific staining.

4.3.5 Statistical Analyses

All statistical procedures were performed by SAS 9.3 software (SAS Institute, Cary, NC, EUA). In this study, cells before differential platting, adherent cells in laminin, cells from supernatant of BSA or control group were considered as independent variables. Flow Cytometry data was transformed by Log_{10} when the assumptions of normality and homogeneity of variances were not respected. Data were analyzed by PROC GLM and Tukey post hoc test was performed. qPCR data was analyzed by PROC MIXED as described (STEIBEL et al., 2009). Data in graphs are shown in log2 distribution but in the text the results are described in fold change to facilitate interpretation. The level of statistical significance was set at α=0.05.

4.4 RESULTS

4.4.1 Cell viability after isolation and differential plating

Testes obtained after surgical orchiectomy were dissected and the average weight of the parenchyma was 18,348±1,715 g. After enzymatic isolation it was obtained $1{,}16x10^{7}\pm3{,}8x10^{6}$ viable cell per gram of testicular parenchyma, with more than 85% of viability. Testicular cells were frozen, stored in liquid nitrogen and thawed in the day of experiment. The cell viability rate observed was 39.54±7.99 % just after thawing. Differential plating was performed using dishes coated with laminin, BSA and control (PBS). Selection of cells with high viability is fundamental for biotechnological applications of SSC. We observed a higher cell viability ($p<0.001$) and total number of viable cells ($p=0.0016$) in non-adherent fraction after the differential plating; However, interaction effect between coating and fraction was observed just for total number of viable cells ($p=0.0039$) (Table 1). We observed the same cell viability and total number of viable cells for all type of coating (Table 1). We observed the same cell viability ($p=0.0655$) for cell obtained at time 0, attached on laminin and non-attached cells from BSA and PBS groups.

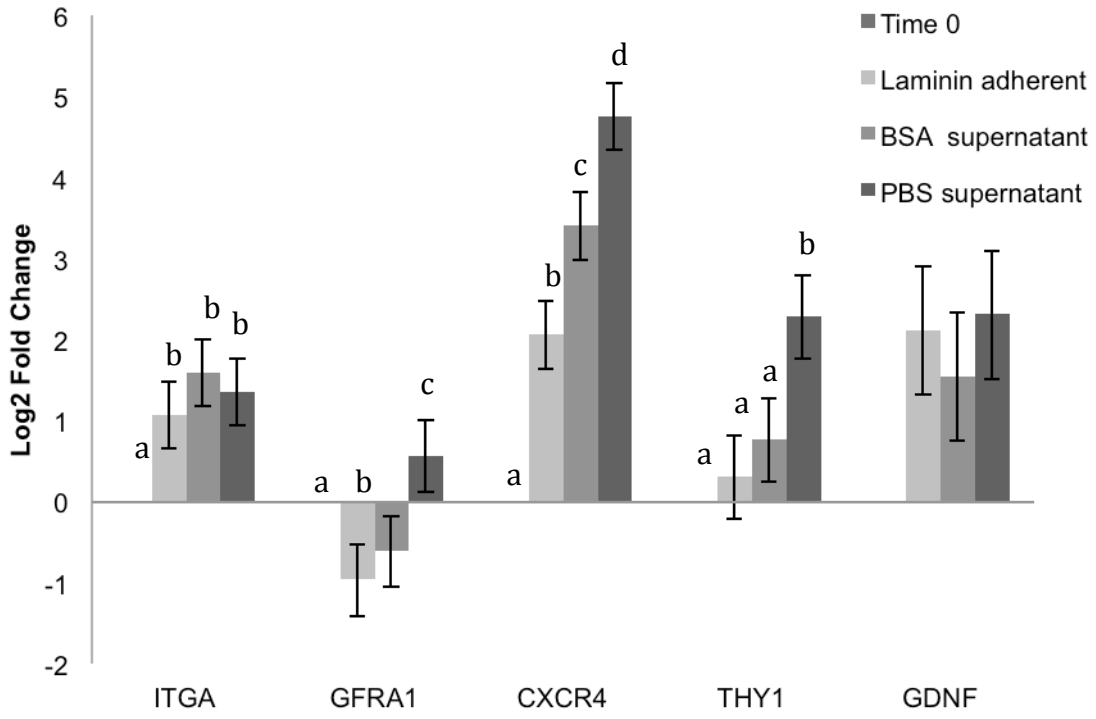
Table 1 - Percentage (mean ± S.E.M) of positive cells (CXCR4, ITGA6 and GFRA1) in adherent and supernatant fractions and enrichment fold on supernatant compared to adherent fraction

	Laminin		BSA		PBS		P value		
	Adhered	Supernatant	Adhered	Supernatant	Adhered	Supernatant	Coating	Fraction	Coating*fraction
Total of viable cell ($x10^6$)	2,666 ± 1.580	2,649 ± 2.344	0,236 ± 0,152	6,24 ± 2.817	1,875 ± 1.460	3,164 ± 1,791	0.0517	0.0016	0.0039
Cell viability (%)	74.440 ± 29.255	30.461 ± 24.793	95 ± 11.180	32.130 ± 8.025	95.745 ± 4.636	28.771 ± 7.354	0.3518	<0.0001	0.2911

4.4.2 Expression of molecular markers by quantitative PCR

In order to determine the efficiency of the three differential plating methods, we assessed gene expression levels of bovine spermatogonial markers commonly used in murine species, such as *ITGA6, GFRA1, CXCR4* and *THY1*. We also verified the expression levels of *GDNF* to identify the presence of Sertoli cells. *ITGA6* was more expressed (P=0.039) in all enriched groups when compared to time 0 (Figure 1) *GFRA1, CXCR4* and *THY1* were more expressed (p=0.0402, p=0.0007, p=0.0117, respectively) by non-adherent cells selected in PBS group (Figure 1). Curiously, *GFRA1* expression was reduced in cells adhered to laminin when compared to non-adherent cells in control group and when compared to time 0 (p=0.0402). *CXCR4* was also upregulated in the comparison of non-adherent cells from PBS group to non-adherent cells on BSA and cells adhered to laminin (p=0.0007). Expression of *GDNF* was not affected (p=0,0517) by differential plating (Figure 1).

Figure 1: Relative gene quantification (*ITGA6, GFRA1, CXCR4, THY1* and *GDNF*) before differential plating (time 0) or after (Laminin adherent, BSA supernatant or PBS supernatant). Values are presented as $\log_2$ of Fold Change ± Ct standard deviation. Different letters in each gene indicate significant statistical difference (*$P \leq 0.05$).

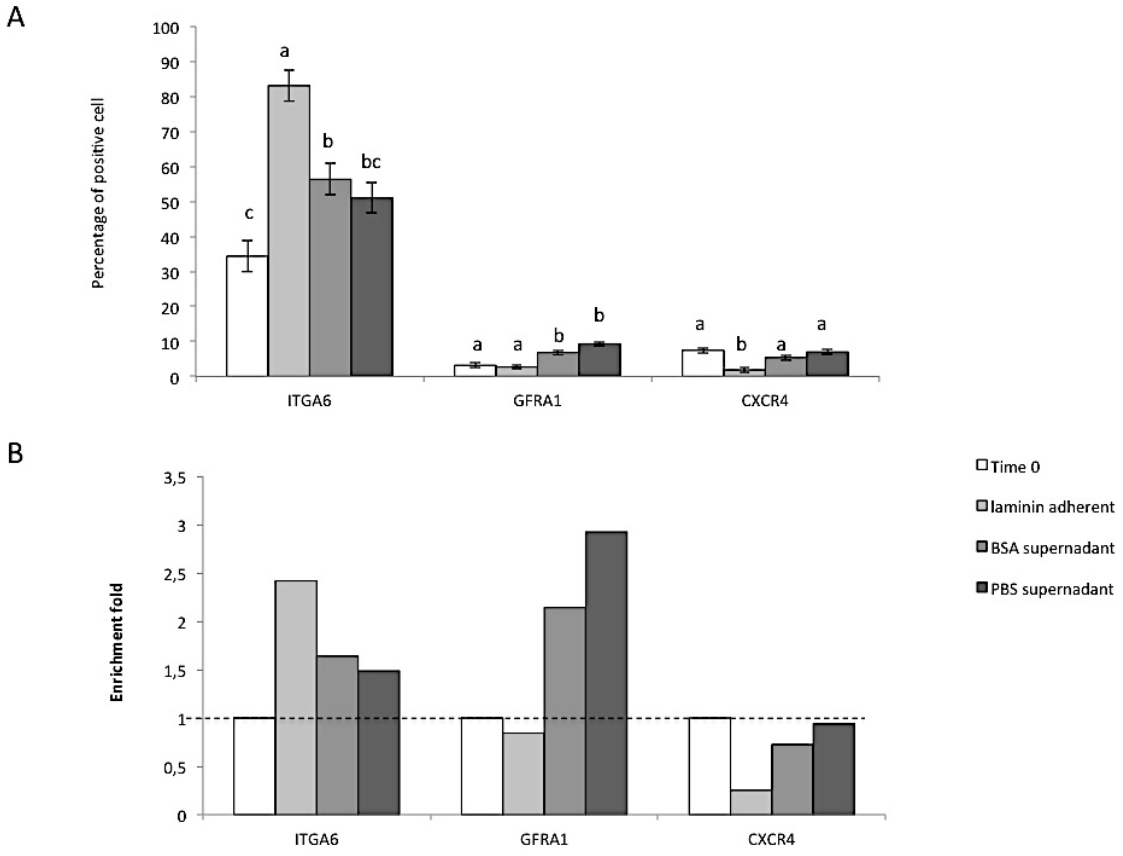


4.4.3 Flow Cytometry

Flow cytometry analysis was performed to assess the enrichment of bovine spermatogonial cells, based on the number of cells that expressed markers ITGA6, GFRA1 and CXCR4. At first, enrichment efficiency was evaluated by comparing the number of positive cells for ITGA6, GFRA1 and CXCR4 (ITGA6+, GFRA1+ and CXCR4+; respectively) at time 0 and positively selected cells after differential plating (laminin adherent cells, non-adherent cells in dish coated by BSA or PBS) Laminin adherent cells showed the highest presence of ITGA+ cells (83.074%, $p<0.001$) and the lowest presence of CXCR4+ cells (1.874%, $p=0.0012$), which was even lower than cells at time 0 (7.438% - Figure 2A). The highest number of GFRA1+ cells were

observed in non-adherent cells after BSA (6.824%, p<0.001) or control differential plating (9.316% - Figure 2A). The percentage of CXCR4+ cells was not different in non-adherent cells when compared to time 0 (Figure 2A). We normalized ITGA+, GFRA1+ and CXCR4+ cell populations by each positive staining before differential plating (time 0) to best visualize the enrichment fold for each differential platting (Figure 2B).

Figure 2: Percentage of positive cells (ITGA6, GFRA1and CXCR4) before differential plating (time 0) or after (Laminin adherent, BSA supernatant or PBS supernatant). Variables (mean ± S.E.M) and P-value. Different letters indicate significant statistical difference, significance level of 0.05.



However, to evaluate the enrichment efficiency inside each type of differential platting we calculated the enrichment fold change between adherent cells and non-adherent cells for each marker (

Table 2). The highest non-adhered enrichment fold was observed for non-coated differential plating, which was over 17-fold considering GFRA1 and over 5-fold considering CXCR4. Considering ITGA6, enrichment fold change was reduced in non-adherent cells after laminin, as expected (
Table 2).

Table 2 - Percentage (mean ± S.E.M) of positive cells (CXCR4, ITGA6 and GFRA1) on adherent and supernatant fraction and enrichment fold on supernatant compared to adherent fraction

		% of positive cell (average)				
Differential plating	Marker	Adherent	Non-adherent	Supernatant enrichment fold	Std Error	Pvalue
Laminin	ITGA6	83.0748	44.5902	0.537	9.295	0.0263
BSA	ITGA6	25.9644	56.3382	2.170	7.051	0.0194
PBS	ITGA6	57.2288	51.078	0.893	6.415	0.6592
Laminin	GFRA1	2.288	10.51	4.594	1.706	0.0024
BSA	GFRA1	0.8426	6.824	8.099	1.075	<.0001
PBS	GFRA1	0.522	9.316	17.847	1.620	<.0001
Laminin	CXCR4	1.874	6.438	3.435	0.869	0.0009
BSA	CXCR4	1.6225	5.398	3.327	0.891	0.0086
PBS	CXCR4	1.255	7	5.578	1.211	0.0012

4.5 DISCUSSION

Isolation and enrichment of spermatogonial stem cells is required for basic studies on cell biology or applied research on generation of transgenic livestock. In the present study, we exploited diverse differential plating methods to enrich bovine undifferentiated spermatogonia based on the ability of testicular cells to adhere on laminin, BSA or PBS coated dishes and tested the hypothesis that differential plating with laminin is more efficient to enrich bovine spermatogonial stem cells from isolated testicular cells.

The successful use of SSC in downstream applications depends of cell viability and purity after the enrichment process (ZHENG et al., 2014). Our findings show a positive effect of differential plating on laminin-enriched cells viability, probably because only live cells can attach to a substrate. Non-attached cells viability was around 32.13 and 28.77 %, respectively for BSA and PBS group, and did not differ from cell viability before differential platting. The cell viability for unattached cells reported in current work is lower than previously reported previously (IZADYAR et al., 2002; HERRID et al., 2009). We strong believe that the low viability observed originated from the cryopreservation and thawing process. However, the same cryopreservation protocol was previously performed to bovine testicular cells and, spermatogonial colonies were observed after transplant of these cells to immunocompromised mice testis previously treated with busulfan (IZADYAR et al., 2002).

Previously, an elegant study compared bovine non-germline cells attachment on coated flasks with a specific matrix lectin for Sertoli cell binding Datura stramonium agglutinin (DSA), BSA and laminin (HERRID et al., 2009). As we observed in this study, Herrid et al. (2009) showed highest presence of undifferentiated spermatogonia (DBA+ cells, 5.3 fold) in the non-adherent cellular fraction from BSA group (HERRID et al., 2009). However, the affinity for DBA is not used as marker for mice and human type A spermatogonia.

In the current study, we choose to work with markers of undifferentiated stages of spermatogonial cells, such as ITGA6, GFRA1, CXCR4 and THY1,

previously described in other species and with a known function in SSC physiology (SHINOHARA et al., 1999; MENG et al., 2000; SHINOHARA; BRINSTER, 2000; ABBASI et al., 2013; YANG et al., 2013; HERMANN et al., 2015). As we observed at present study, the relative expression of THY1 was previously observed in isolated and short-term culture cells provide from prepubertal calves testicular cells enriched by BSA/2h differential plating (NASIRI et al., 2012). In the present study, we observed highest relative expression of GFRA1, THY1 and CXCR4 transcripts in non-attached cells from PBS group. The expression of these three genes was detected in enriched samples, confirming the presence of SSC. This suggests a better outcome for control differential plating than the other two methods and also then use of discontinuous percoll gradient, as in the latter method no differences in gene expression of ITGA6 and GFRA1 were observed before or after purification (DE BARROS et al., 2012). In addition, the increased expression of ITGA6 in cells adhered on laminin combined with lower expression of GFRA1 and THY1 suggests that ITGA6 may not be a reliable marker of undifferentiated bovine spermatogonia.

After gene expression analysis, we evaluated presence of cellular populations expressing protein markers of undifferentiated spermatogonial cells. We obtained enriched sub-population of ITGA6+ cells in all groups. However, we only observed enriched GFRA1+ cells in BSA coated or control differential plating. In previous studies, almost 40% of positive cells in supernatant after differential plating without coating were DBA+ (IZADYAR et al. 2002, HERRID et al. 2009). In mice, just 0.02-0.03% of total germ cells are stem cells (TEGELENBOSCH; DE ROOIJ, 1993) and isolated cells from juvenile mice (10dpp) testis contained 21-24% of GFRA+ cells (STUKENBORG et al., 2008). We observed less than 11% of GFRA1+ cells before and after all differential platting, which suggest that GFRA1 is a more specific marker of undifferentiated bovine spermatogonia than DBA.

Contrary to our hypothesis, laminin-based differential plating was not efficient in enriching more undifferentiated spermatogonial cells GFRA1+ or CXCR4+ cells. Laminin differential plating was effective for enrichment of ITGA6+ cells, as it was expected. ITGA6 was described as marker for undifferentiated spermatogonial cells in mice (SHINOHARA et al., 1999). However it is also is expressed in other cells in seminiferous tubules (TOKUDA et al., 2007), such as myofibroblasts (KOKKINAKI; DJOURABTHI; GOLESTANEHN, 2011), reducing specificity of selection.

BSA-coated dishes did not improve the differential platting efficiency to enrich GFRA1+ cells when compared to non-coated dishes, suggesting BSA is not essential for non-germline cells attachment. Moreover, we also observed the highest enrichment fold for GFRA1+ (17.85 fold) and CXCR4+ (5.58 fold) for non-coated group. Thus, in agreement with previous studies (IZADYAR et al. 2002, HERRID et al. 2009), differential plating without laminin is a better option for enrichment of undifferentiated spermatogonial cells.

Differential plating is a simple and not expensive technique that could be associated to other methods such as MACS (READING et al .2011) to improve bovine SSC isolation efficiency. Here we compared diverse differential plating methods using more specific approaches to assess enrichment of undifferentiated spermatogonial cells. In summary, we observed specific markers (GFRA1, CXCR4, THY1 and ITGA6) in attached and non-attached cells by qPCR and flow cytometry. We observed highest relative gene expression of *GFRA1, THY1* and *CXCR4* transcripts in non-attached cells from non-coated group. Presence of GFRA1+ cells was higher in non-attached cells from BSA and PBS groups while attached cells on laminin had highest presence of ITGA6+ cells and less presence of CXCR4+ cells. Based on these data we rejected our hypothesis that differential plating using laminin would be more efficient to enrich bovine spermatogonial stem cells from isolated testicular cells.

4.6 CONCLUSION

Differential plating is an effective technique to enrichment of undifferentiated spermatogonial stem cell being more efficient when performed without laminin coating.

Acknowledgements

This research was funding supported by São Paulo Research Foundation (FAPESP) and National Counsel of Technological and Scientific Development (CNPq). Authors would like to thank Prefeitura do Campus de Pirassununga for providing the bull calves used in this experiment; Profa. Cristina de Oliveira Massoco Salles Gomes and Dra. Nicolle Queiroz in the Department of Pathology for assistance with flow cytometry.

REFERENCES

ABBASI, H.; TAHMOORESPUR, M.; HOSSEINI, S. M.; NASIRI, Z.; BAHADORANI, M.; HAJIAN, M. ; NASIRI, M. R.; NASR-ESFAHANI, M. H. THY1 as a reliable marker for enrichment of undifferentiated spermatogonia in the goat. **Theriogenology,** v. 80, n. 8, p. 923-32, Nov 2013. ISSN 1879-3231. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/23987985 >. Acessed at: 24. march. 2015.

AHMAD, S.; XIAO, Y.; HAN, L.; HUA, H.; RIAZ, H.;LIANG, A.; YANG, L.G.. Isolation, identification and enrichment of type a spermatogonia from the testis of chinese cross-bred buffaloes (swamp × river). **Reprod Domest Anim,** v. 48, n. 3, p. 373-81, Jun 2013. ISSN 1439-0531. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/22928737 >. Acessed at: 24. march. 2015.

BORJIGIN, U.; DAVEY, R.; HUTTON, K.; HERRID, M. Expression of promyelocytic leukaemia zinc-finger in ovine testis and its application in evaluating the enrichment efficiency of differential plating. **Reprod Fertil Dev,** v. 22, n. 5, p. 733-42, 2010. ISSN 1031-3613. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/20450825 >. Acessed at: 24. march. 2015.

ERTL, C.; WROBEL, K. H. Distribution of sugar residues in the bovine testis during postnatal ontogenesis demonstrated with lectin-horseradish peroxidase conjugates. **Histochemistry,** v. 97, n. 2, p. 161-71, 1992. ISSN 0301-5564. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/1559848 >. Acessed at: 24. march. 2015.

GOEL, S.; SUGIMOTO, M.; MINAMI, N.; YAMADA, M.;KUME, S.; IMAI, H. I. Identification, isolation, and in vitro culture of porcine gono cytes. **Biol Reprod,** v. 77, n. 1, p. 127-37, Jul 2007. ISSN 0006-3363. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/17377141 >. Acessed at: 24. march. 2015.

GUAN, K. ;NAYERNIA, K.; MAIER, L. S.; WAGNER, S.; DRESSEL, R.; LEE, J. H.; NOLTE, J.; WOLF, F.; LI, M.; ENGEL, W.; HASENFUSS, G.. Pluripotency of spermatogonial stem cells from adult mouse testis. **Nature,** v. 440, n. 7088, p. 1199-203, Apr 2006. ISSN 1476-4687. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/16565704 >. Acessed at: 24. march. 2015.

HERMANN, B. P.; MUTOJI, K. N.; VELTE, E. K.; KO, D.; OATLEY, J. M.; GEYER, C. B.; MCCARREY, J. R.. Transcriptional and Translational Heterogeneity among Neonatal Mouse Spermatogonia. **Biol Reprod**, Jan 2015. ISSN 1529-7268. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/25568304 >. Acessed at: 24. march. 2015.

HERRID, M.; DAVEY, R. J.; HILL, J. R. Characterization of germ cells from pre-pubertal bull calves in preparation for germ cell transplantation. **Cell Tissue Res,** v.

330, n. 2, p. 321-9, Nov 2007. ISSN 0302-766X. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/17593396 >. Acessed at: 24. march. 2015.

HERRID, M.; DAVEY, R. J.; HUTTON, K.; COLDITZ, I. G.; HILL, J. R.. A comparison of methods for preparing enriched populations of bovine spermatogonia. **Reprod Fertil Dev,** v. 21, n. 3, p. 393-9, 2009. ISSN 1031-3613. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/19261216 >. Acessed at: 24. march. 2015.

HOFMANN, M. C.; BRAYDICH-STOLLE, L.; DYM, M. Isolation of male germ-line stem cells; influence of GDNF. **Dev Biol,** v. 279, n. 1, p. 114-24, Mar 2005. ISSN 0012-1606. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/15708562 >. Acessed at: 24. march. 2015.

IZADYAR, F.; MATTHIJS-RIJSENBILT, J. J.; DEN OUDEN, K.; CREEMERS, L. B.; WOELDERS, H.; DE ROOIJ, D. G. Development of a cryopreservation protocol for type A spermatogonia. **J Androl,** v. 23, n. 4, p. 537-45, 2002 Jul-Aug 2002. ISSN 0196-3635. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/12065461 >. Acessed at: 24. march. 2015.

IZADYAR, F.; SPIERENBERG, G.T.; CREEMERS, L.B.; DEN OUDEN, K.; DE ROOIJ, D.G. Isolation and purification of type A spermatogonia from the bovine testis. **Reproduction,** v. 124, n. 1, p. 85-94, Jul 2002. ISSN 1470-1626. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/12090922 >. Acessed at: 24. march. 2015.

KLISCH, K.; CONTRERAS, D. A. ;SUN, X.; BREHM, R.; BERGMANN, M.; ALBERIO, R.. The Sda/GM2-glycan is a carbohydrate marker of porcine primordial germ cells and of a subpopulation of spermatogonia in cattle, pigs, horses and llama. **Reproduction,** v. 142, n. 5, p. 667-74, Nov 2011. ISSN 1741-7899. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/21896636 >. Acessed at: 24. march. 2015.

KOKKINAKI, M.; DJOURABTCHI, A.; GOLESTANEH, N. Long-term Culture of Human SSEA-4 Positive Spermatogonial Stem Cells (SSCs). **J Stem Cell Res Ther,** v. 2, n. 2, Nov 2011. ISSN 2157-7633. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/24466499 >. Acessed at: 24. march. 2015.

KOKKINAKI, M.; LEE, T. L.; HE, Z.; JIANG, J.; GOLESTANEH, N.; HOFMANN, M. C.; CHAN, W. Y.; DYM, M. .Age affects gene expression in mouse spermatogonial stem/progenitor cells. **Reproduction,** v. 139, n. 6, p. 1011-20, Jun 2010. ISSN 1741-7899. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/20371641 >. Acessed at: 24. march. 2015.

KUBOTA, H.; AVARBOCK, M. R.; BRINSTER, R. L. Spermatogonial stem cells share some, but not all, phenotypic and functional characteristics with other stem cells. **Proc Natl Acad Sci U S A,** v. 100, n. 11, p. 6487-92, May 2003. ISSN 0027-8424. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/12738887 >. Acessed at: 24. march. 2015.

MENG, X.; LINDAHL, M.; HYVÖNEN, M. E.; PARVINEN, M.; DE ROOIJ, D. G.; HESS, M. W.; RAATIKAINEN-AHOKAS, A.; SAINIO, K.; RAUVALA, H.; LAKSO, M.;

PICHEL, J. G.; WESTPHAL, H.; SAARMA, M.; SARIOLA, H. Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. **Science,** v. 287, n. 5457, p. 1489-93, Feb 2000. ISSN 0036-8075. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/10688798 >. Acessed at: 24. march. 2015.

NASIRI, Z.; HOSSEINI, S. M.; HAJIAN, M.; ABEDI, P.; BAHADORANI, M.; BAHARVAND, H.; NASR-ESFAHANI, M. Effects of different feeder layers on short-term culture of prepubertal bovine testicular germ cells in-vitro. **Theriogenology,** v. 77, n. 8, p. 1519-28, May 2012. ISSN 1879-3231. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/22289219 >. Acessed at: 24. march. 2015.

PHILLIPS, B. T.; GASSEI, K.; ORWIG, K. E. Spermatogonial stem cell regulation and spermatogenesis. **Philos Trans R Soc Lond B Biol Sci,** v. 365, n. 1546, p. 1663-78, May 2010. ISSN 1471-2970. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/20403877 >. Acessed at: 24. march. 2015.

READING, S. C.; STEPNOSKI, A. L.; CLONINGER, E. W.; OATLEY, J. M.. THY1 is a conserved marker of undifferentiated spermatogonia in the pre-pubertal bull testis. **Reproduction,** v. 139, n. 5, p. 893-903, May 2010. ISSN 1741-7899. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/20154176 >. Acessed at: 24. march. 2015.

RYU, B. Y.; ORWIG, K. E.; KUBOTA, H.; AVARBOCK, M. R.; BRINSTER, R. L. Phenotypic and functional characteristics of spermatogonial stem cells in rats. **Dev Biol,** v. 274, n. 1, p. 158-70, Oct 2004. ISSN 0012-1606. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/15355795 >. Acessed at: 24. march. 2015.

SHINOHARA, T.; AVARBOCK, M. R.; BRINSTER, R. L. beta1- and alpha6-integrin are surface markers on mouse spermatogonial stem cells. **Proc Natl Acad Sci U S A,** v. 96, n. 10, p. 5504-9, May 1999. ISSN 0027-8424. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/10318913 >. Acessed at: 24. march. 2015.

SHINOHARA, T.; BRINSTER, R. L. Enrichment and transplantation of spermatogonial stem cells. **Int J Androl,** v. 23 Suppl 2, p. 89-91, 2000. ISSN 0105-6263. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/10849507 >. Acessed at: 24. march. 2015.

SHINOHARA, T.; ORWIG, K. E.; AVARBOCK, M. R.; BRINSTER, R. L. Spermatogonial stem cell enrichment by multiparameter selection of mouse testis cells. **Proc Natl Acad Sci U S A,** v. 97, n. 15, p. 8346-51, Jul 2000. ISSN 0027-8424. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/10900001 >. Acessed at: 24. march. 2015.

STEIBEL, J. P.; POLETTO, R.; COUSSENS, P. M.; ROSA, G. J. A powerful and flexible linear mixed model framework for the analysis of relative quantification RT-PCR data. **Genomics,** v. 94, n. 2, p. 146-52, Aug 2009. ISSN 1089-8646. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/19422910 >. Acessed at: 24. march. 2015.

STUKENBORG, J. B.; WISTUBA, J.; LUETJENS, C. M.; ELHIJA, M. A.; HULEIHEL, M.; LUNENFELD, E.; GROMOLL, J.; NIESCHLAG, E.; SCHLATT, S. Coculture of

spermatogonia with somatic cells in a novel three-dimensional soft-agar-culture-system. **J Androl,** v. 29, n. 3, p. 312-29, 2008 May-Jun 2008. ISSN 1939-4640. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/18046051 >. Acessed at: 24. march. 2015.

TEGELENBOSCH, R. A.; DE ROOIJ, D. G. A quantitative study of spermatogonial multiplication and stem cell renewal in the C3H/101 F1 hybrid mouse. **Mutat Res,** v. 290, n. 2, p. 193-200, Dec 1993. ISSN 0027-5107. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/7694110 >. Acessed at: 24. march. 2015.

TOKUDA, M.; KADOKAWA, Y.; KURAHASHI, H.; MARUNOUCHI, T. CDH1 is a specific marker for undifferentiated spermatogonia in mouse testes. **Biol Reprod,** v. 76, n. 1, p. 130-41, Jan 2007. ISSN 0006-3363. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/17035642 >. Acessed at: 24. march. 2015.

YANG, Q. E.; KIM, D.; KAUCHER, A.; OATLEY, M.J.; OATLEY, J.M.. CXCL12-CXCR4 signaling is required for the maintenance of mouse spermatogonial stem cells. **J Cell Sci,** v. 126, n. Pt 4, p. 1009-20, Feb 2013. ISSN 1477-9137. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/23239029 >. Acessed at: 24. march. 2015.

ZHENG, Y.; ZHANG, Y.; QU, R.; HE, Y.; TIAN, X.; ZENG, W. Spermatogonial stem cells from domestic animals: progress and prospects. **Reproduction,** v. 147, n. 3, p. R65-74, Mar 2014. ISSN 1741-7899. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/24357661 >.

5 CHAPTER 2: EFFECT OF AGE ON EXPRESSION OF SPERMATOGONIAL MARKERS IN BOVINE TESTIS AND ISOLATED CELLS

5.1 SUMMARY

Spermatogenesis is a classical model of stem cell differentiation along the male life. Spermatogonial stem cells are the most undifferentiated germ cell present in adult male testes and, it is responsible to maintain the spermatogenesis. Age has a negative effect over stem cell, but the aging effect on SSC is not elucidated for mammals with long prepubertal period, as bovine. The present study aim to evaluate the effect of age on the expression of undifferentiated spermatogonial markers in testis and in enriched testicular cells from prepubertal calves and adult bulls. In this matter, testicular parenchyma from prepubertal calves (3-5 months) (n=5) and adult bulls with 3 years of age (n=5) were minced and, isolated cells were obtained after two enzymatic digestions. Differential platting with BSA was performed and cells were incubated at 37°C in 5% CO2 for hours onto BSA coated dish. Cell viability was assessed by Trypan Blue solution exclusion method and testicular cells enriched for SSC was evaluated by expression of specific molecular markers by qRT-PCR (*POU5F1, GDNF, CXCR4, UCHL1, ST3GAL, SELP, ICAM1 and ITGA6*) and flow cytometry (GFRA1, CXCR4 and ITGA6). CXCR4 and UCHL1 expression was evaluated in fixated testes by immunohistochemistry. We observed that age just affected the expression of selective genes [SELP (Fold Change=5.61; p=0.0023) and UCHL1 (Fold Change=4.98; p=0.0127)]. By flow cytometry, age affected only the proportion of ITGA6+ cells (P<0.001), which was higher in prepubertal calves when compared to adult bulls. In situ, we observed an effect of age on the number of UCHL1+ (p=0.0006) and CXCR4+ (p=0.0139) cells per seminiferous tubule. At conclusion, age affects gene expression and the population of cells expressing specific spermatogonial markers in the bovine testis

Keywords: Bovine. Spermatogonia. Markers. Age. Undifferentiated germ cell.

5.2 INTRODUCTION

Normally, a bull produces about 5 to 6 million sperm cells daily during the first ten years of its life (AMANN et al., 1974). Spermatogenesis depends on spermatogonial stem cells (SSC) self-renewal and differentiation during the male reproductive life. In non-primates mammalian, just type A single spermatogonia (A_s) are considered true SSC (DE ROOIJ, 2001). SSC have the ability to colonize testes and promote spermatogenesis after transplantation (BRINSTER; AVARBOCK, 2004; BRINSTER; ZIMMERMANN, 2004). SSC have been considered a powerful biotechnological tool for reproductive biology in large animals but the gap between basic research and potential applications need to be overcome (ZHENG et al., 2014). Studies on bovine SSC often use prepubertal calves as cell sources, including SSC cryopreservation (IZADYAR et al., 2002; OATLEY; REEVES; MCLEAN, 2004), in vitro culture (APONTE et al., 2006; FUJIHARA et al., 2011), molecular marker characterization (REDING et al., 2010), differentiation (QASEMI-PANAHI et al., 2011), homologous transplant (IZADYAR et al., 2003) and heterologous transplant (IZADYAR et al., 2002; OATLEY et al., 2002) However, the reproductive performance and generation of offspring with superior genetic merit can only be proved in proven bulls.

Adult stem cells support tissues and organs homeostasis, but tissue regeneration dramatically decrease with age (BOYLE et al., 2007). In most strains of mice, aging is associated with hematopoietic stem cell loss of function and number (CHAMBERS et al., 2007; GEIGER; RUDOLPH, 2009). Also, reduced differentiation of hematopoietic stem cells to lymphoid lineage cell might be strong correlated with senescence (CHAMBERS et al., 2007; GEIGER; RUDOLPH, 2009). In the human male, aging affects sperm DNA damage, chromatin integrity, gene mutations and aneuploidies (WYROBEK et al., 2006). However, few studies were performed to understand the effect of aging on mammalian SSC. In flies, the total number of germline stem cells, germline stem cell division and self-renewal factors decrease with aging (BOYLE et al., 2007; CHENG et al., 2008). In mice, some genes are expressed specifically in SSC from oldest mice such as SELP and ICAM1 (KOKKINAKi et al., 2010). However, the molecular mechanisms involved in age

influences of SSC functional and properties are still unclear for mouse and other mammalian species.

In domestic animals, SSC characterization and properties are not as elucidated as in rodents and few SSC markers were studied. In bovine, UCHL1 (PGP9.5) was previously reported to be only expressed by type A spermatogonia in adult testis (FUJIHARA et al., 2011). In domestic animals, UCHL1 is considered an optimal marker for spermatogonia as it does not show affinity for somatic cells and is expressed by pre-meiotic male germ cell (KON; ENDOH; IWANAGA, 1999). The number of positive cells per seminiferous tubule for UCHL1, OCT4 or for affinity to Dolichos Biflorus agglutinin (DBA; indirect marker for undifferentiated germ cells in bovine) is higher in adult bulls than in prepubertal calves, reveling that bovine undifferentiated germ cells have different characteristics during the development of testis from neonatal to adult life (FUJIHARA et al., 2011).

In almost all tissues, stem cells generally have adhesive contact to the basement membrane and are surrounded by stromal cells producing a microenvironment called niche (SPRADLING; DRUMMOND-BARBOSA; KAI, 2001). Beta-1 integrin (ITGB1) and alpha-6 integrin (ITGA6), help SSC attachment on the basement membrane by binding to laminin (SHINOHARA et al., 1999). In the spermatogonial niche, Sertoli cells express GDNF and CXCL12. These factors bind respectively to receptor GFRA1 and CXCR4 in stem cell membrane promoting SSC self-renewal and maintenance (MENG et al., 2000; KOKKINAKI et al., 2009; YANG. In bovine, the number of As and Apr is higher in GDNF treated in vitro culture (APONTE et al., 2005).

In livestock breeding programs, just adult bulls have proven genetic merit due to offspring assessment. Homologous transplantation of SSC from proven breeding bull donors to low genetic merit bulls could be an interesting tool to improve breeding systems. However, effect of age on the testicular niche and on SSC is not clear for domestic animals. Thus, we aimed to evaluate the effect of age on the expression of undifferentiated spermatogonial markers in testis and in enriched testicular cells from prepubertal calves and adult bulls. We observed that age affected the expression of some markers but not all, indicating that age influenced some particular characteristics of bovine undifferentiated spermatogonia.

5.3 MATERIAL AND METHODS

All animal procedures were approved by the Bioethics Committee of the School of Veterinary Medicine and Animal Science of the University of Sao Paulo. All chemicals were supplied by Sigma Chemical Company (St. Louis, Missouri, USA) unless otherwise stated.

5.3.1 Animals and Immunohistochemistry

Testes from prepubertal bull calves (PUSP-P, University of Sao Paulo, Pirassununga, Sao Paulo, Brazil) with 5 months of age (n=5) were surgically removed Testes from adult bulls with 3 years of age (n=5) were obtained in a commercial slaughterhouse. Testes were weighted and decapsulated prior processing in the laboratory. Testicular fragments (0.3 mm^3) from each sample were fixed in Methacarn for 24 hours for immunohistochemistry analysis. After fixation, external edges were removed and all fragments were transferred to absolute ethanol until paraffin embedding. Sections (5 μm thick) were dried over silanized glass slides and stored at room temperature until staining. Expression of UCHL1 and CXCR4 was evaluated by immunohistochemistry in testis from prepubertal calves (n=5) and adult bulls (n=5).

Sections were rehydrated (xylol for 20 min, xylol for 20 min, absolute ethanol for 5 min, 95% ethanol for 5 min, 70% ethanol for 5 min and distillated water for 5 min). Peroxidase blocking was performed with incubation for 30 min at room temperature with 30% (v/v) hydrogen peroxidase in ethanol. Slides were washed with PBS (three times of 3 min) and non-specific reaction was blocking with 5% (w/v) non-fat powdered milk in PBS for 1 hour at room temperature. Slides were washed with PBS (three times of 3 min) and incubated overnight with primary antibody 1:500 anti-CXCR4 (Ab7199, Abcam, Cambridge, MA, EUA) and 1:200 anti-PGP9.5- (Ab72911, Abcam) at 4ºC. After incubation, slides were washed and incubated with ADVANCE

HRP Link (ADVANCE^{TM} HRP, DAKO, Carpinteria, CA, EUA) for 30 min at room temperature without light exposition. After wash, slides were incubated with ADVANCE HRP Enzyme (ADVANCE^{TM} HRP, DAKO,) for 30 min. Visualization of specific immunolocalization by DAB were obtained by Liquid DAB+ Substrate Chromogen (DAKO,). Morphology and specific staining was analyzed in ten representative images that were obtained by optical microscopy (Olympus IX81) at 400 x magnification by Image Plus Software (Olympus) for each animal. We obtained the number of positive cells per seminiferous tubule to normalize the presence of positive cells as previously described (FUJIHARA et al., 2011)

5.3.2 Enzymatic isolation and cell cryopreservation

Testicular cell isolation was performed as previously described (REDING et al., 2010) with minor modifications. All testicular parenchyma was minced and digested with collagenase (1 mg/ml collagenase type V and 7 mg/ml DNase I) for 30 min at 37°C with continuous agitation. Digested testicular parenchyma were washed three times with Phosphate-buffered saline (PBS; 100 x g/ 1min) and digested with trypsin [0.25% (w/v) trypsin, 2.21 mM EDTA and 7 mg/ml DNase I] for 5 min at 37°C. 10% fetal bovine serum (FBS; Gibco®, Life Technologies, Carlsbad, CA, USA) was used to block enzymes activity. To remove cell clusters, 100 mm cell strainer (BD Biosciences, Durham, NC, USA) was used. Cells were centrifuged (600 x g/ 7 min) and cell pellet was suspended in PBS. Cell concentration and viability were assessed by Trypan Blue solution (5 mg/ml) exclusion method. Cells were cryopreserved ($10x10^6$ viable cells/ml) in DMEM medium (Life Technologies, Carlsbad, California, USA) with 10% (v/v) FBS, 10% (v/v) DMSO and, 0.25% (w/v) sucrose at -80ºC using the Mr. Frosty container (Nalgene®, Waltham, Massachusetts, USA) and then placed into liquid nitrogen at -196 ºC.

5.3.3 Differential plating

Differential plating with BSA-coated dishes (0.5 mg/ml) was performed as described (HERRID et al., 2009). 100 mm cell culture dish were coated overnight by 0.5 mg/ml BSA at 37°C. Cells were thawed and total number of viable cell evaluated by Trypan Blue staining exclusion. Approximately $10x10^6$ viable cells were plated per dish for each animal, placed at 37°C in 5% CO_2 incubator for 2 hours. Non-attached cells from supernatant were recovered and cell viability performed. Cells from non-adherent fraction were used for flow cytometry evaluation and cell pellets with $2x10^5$ cells were stored at -80°C until gene expression analysis.

5.3.4 Quantitative RT-PCR

Total RNA from $2x10^5$ cells per sample was extracted by Illustra RNAspin mini (GE Healthcare®, Buckinghamshire, United Kingdom), quantified by Nanodrop ND-1000 (Nanodrop Technologies, Inc., Wilmington, Delaware, EUA) and cDNA synthesis performed by SuperScript® Vilo (Invitrogen®, Carlsbad, California, USA). cDNA quantification was performed using Qubit dsDNA BR Assays kit (Life Technologies, Carlsbad, California, USA) by fluorometry (Qubit 2.0 Fluorometer, Life Technologies). Manufacturer's instructions were followed for all procedures.

cDNA concentration was normalized to 200 ng/ml and qPCR performed with SYBR GreenER™ qPCR Supermix Universal (Invitrogen®, Carlsbad, California, USA) in a two-step cycle reaction: 2 min at 50ºC, 10 min at 95ºC, 40 cycles at 95ºC for 15 s and 60ºC for 1 min; plus dissociation curve (Mastercycler Ep Realplex Thermal Cycler, Eppendorf AG, Hamburg, Germany). Primers (Chart 2) were previously standardized and efficiency curves were performed with cDNA from bull testis

Chart 2 - Primers sequences for quantitative RT-PCR analyses

Gene	**Primer sequence**	**GenBank Reference**
ACTB	F - GTCCACCTTCCAGCAGATGT	NM_173979
	R - GTCACCTTCACCGTTCCAGT	
GAPDH	F - TGACCCCTTCATTGACCTTC	NM_001034034.2
	R - TACTCAGCACCAGCATCACC	
POU5F1	F - TACTGTGCGCCGCAGGTTGG	NM_174580
	R - GCTTTGATGTCCTGGGACTCCTCA	
GDNF	F - AACTCGTGCCCCTAACCTG	XM_005221583.1
	R - GACAGCCACGACATCCATA	
CXCR4	F - GGACTTGAGTAGCCGGTAGC	NM_174301
	R - CATAGTCACCCGAGCCCAAG	
GFRA1	F - CAAGTGGAGCACATCTCGAA	NM_001105411
	R - GGCAGGAACAGAAGAGCATC	
UCHL1	F - TGCTGAACAAAGTGCTGACC	NM_001046172.2
	R - GATGATGGAACCGAGATGCT	
ST3GA	F - AGGATGAATCAGGCACCGAC	NM_001002892.2
	R - ACTGGGGCATAGGTGATTCG	
SELP	F - ATATGTCAGAGAGTGCGGCG	NM_174183.2
	R - GCACTGGACAGCTACACACT	
ICAM1	F - CCTGCCGTCCTCCTTACAAT	NM_174348.2
	R - TGTTGTAAGACGTGAGGGGC	
ITGA6	F – AGGGATGTGGAGACGACAAC	NM_001109981
	R - TCTTTGGTGGGATTCTTTGG	

5.3.5 Flow cytometry

Enriched cells from supernatant were incubated for 30 min at room temperature with primary antibodies 1:500 anti-GFRA1 (Ab84106, Abcam, Cambridge, MA, EUA), 1:40 Anti-ITGA6/ALEXA488 (313608, Biolegends, San Diego, CA) and 1:500 anti-CXCR4 (Ab7199, Abcam) in PBS with 10% (v/v) FBS. After incubation, cells were washed with PBS (950 x g/ 3min) and suspended in 3% (w/v) BSA in PBS. Cells previously incubated with antibody anti-GFRA1 and anti-CXCR4 were incubated with secondary antibody labeled with FITC (1:200; Ab9750, Abcam, Cambridge, MA, EUA) for 30 min. Each sample had two controls: cells incubated only with secondary antibody (unspecific labeling) and without any antibody (auto fluorescence control). Flow cytometry analysis was performed by FACScalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) and data analyzed by software FlowJo (TreeStar Data Analysis Software, Ashland, OR, EUA). All antibodies had the optimal concentration previously standardized.

5.3.6 Statistical Analyses

All statistical procedures were performed by SAS 9.3 software (SAS Institute, Cary, NC, EUA). In this study, age (prepubertal and adult) was considered as an independent variable. When the assumptions of normality and homogeneity of variances were not respected, dependent variables were transformed by log10. Data presenting a normal distribution were evaluated by PROC TTEST. PROC NPAR1WAY was used to evaluate data with non-normal distribution. qPCR data was analyzed by PROC MIXED as previously described (STEIBEL et al., 2009). Data in graphs are shown in log2 distribution but in the text the results are described in fold change. The level of statistical significance was set at α=0.05.

5.4 RESULTS

5.4.1 Immunohistochemistry

The presence and the morphological localization on testicular parenchyma of positive cell for UCHL1 (UCHL1+) and CXCR4 (CXCR4+) were established by immunohistochemistry. We also observed morphological differences between testicular parenchyma from prepubertal calves and adult bulls. Prepubertal bulls had more seminiferous tubule by image area at 400 x magnification ($p<0.001$) than adults (Figure 3 and Figure 4). Presence of UCHL1+ cell by image area was not affected by age ($p=0.1426$). However, CXCR4+ cell were not detected by immunohistochemistry in adult testis ($p=0.0356$) (Figure 3 and Figure 4). We observed an effect of age on the number of UCHL1+ ($p=0.0006$) and CXCR4+ ($p=0.0139$) cells per seminiferous tubule and UCHL1+/tubule was higher in adults ($p=0.0006$) and CXCR4+/tubule higher in prepubertal calves ($p=0.0139$) (Figure 4).

Figure 3 - Immunolocalization of undifferentiated germ cell markers (UCHL1 and CXCR4) in bovine testis at 5 months (prepubertal) and 2 years old (adult), 400X image magnification. Staining was performed with DAB (brown) and hematoxylin. Enhanced (1000X) magnification is included in the lower right corner. Arrow indicates positive cells

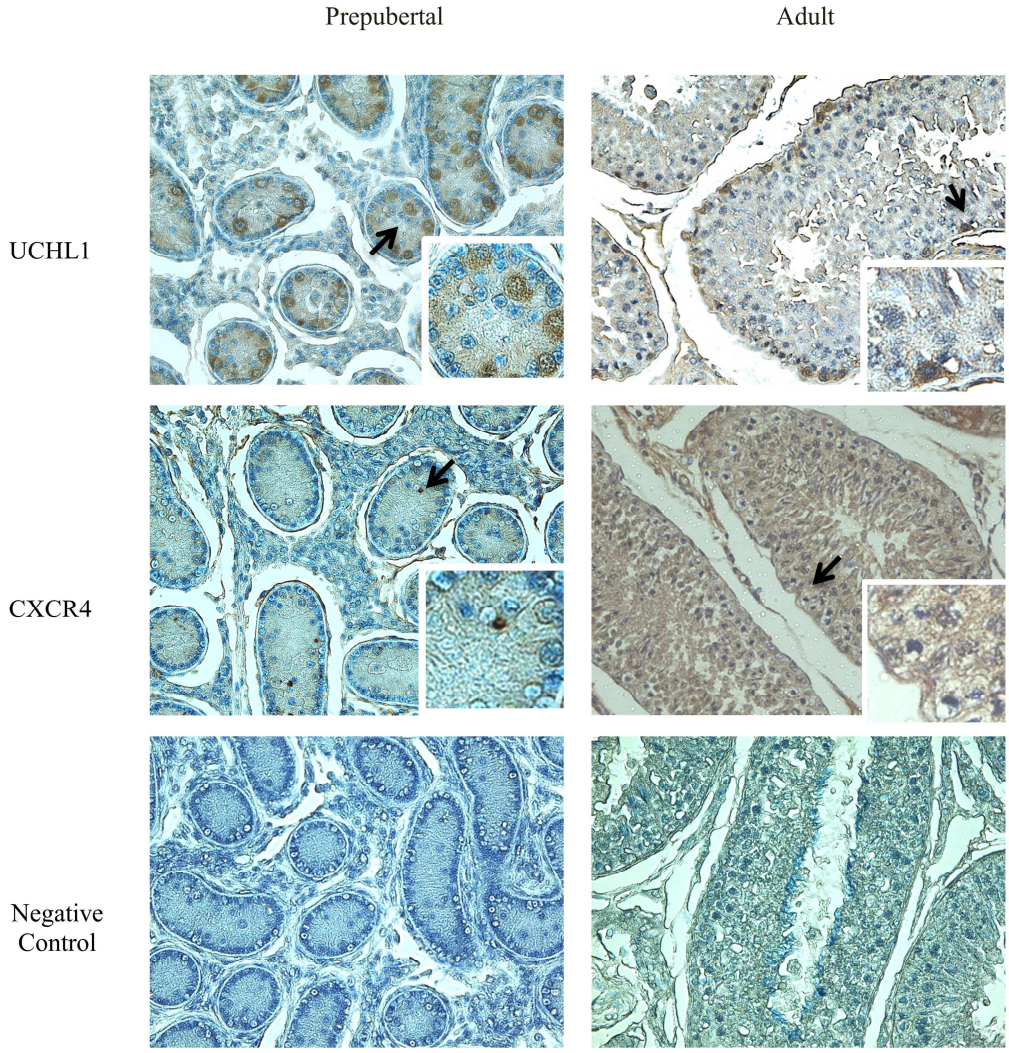
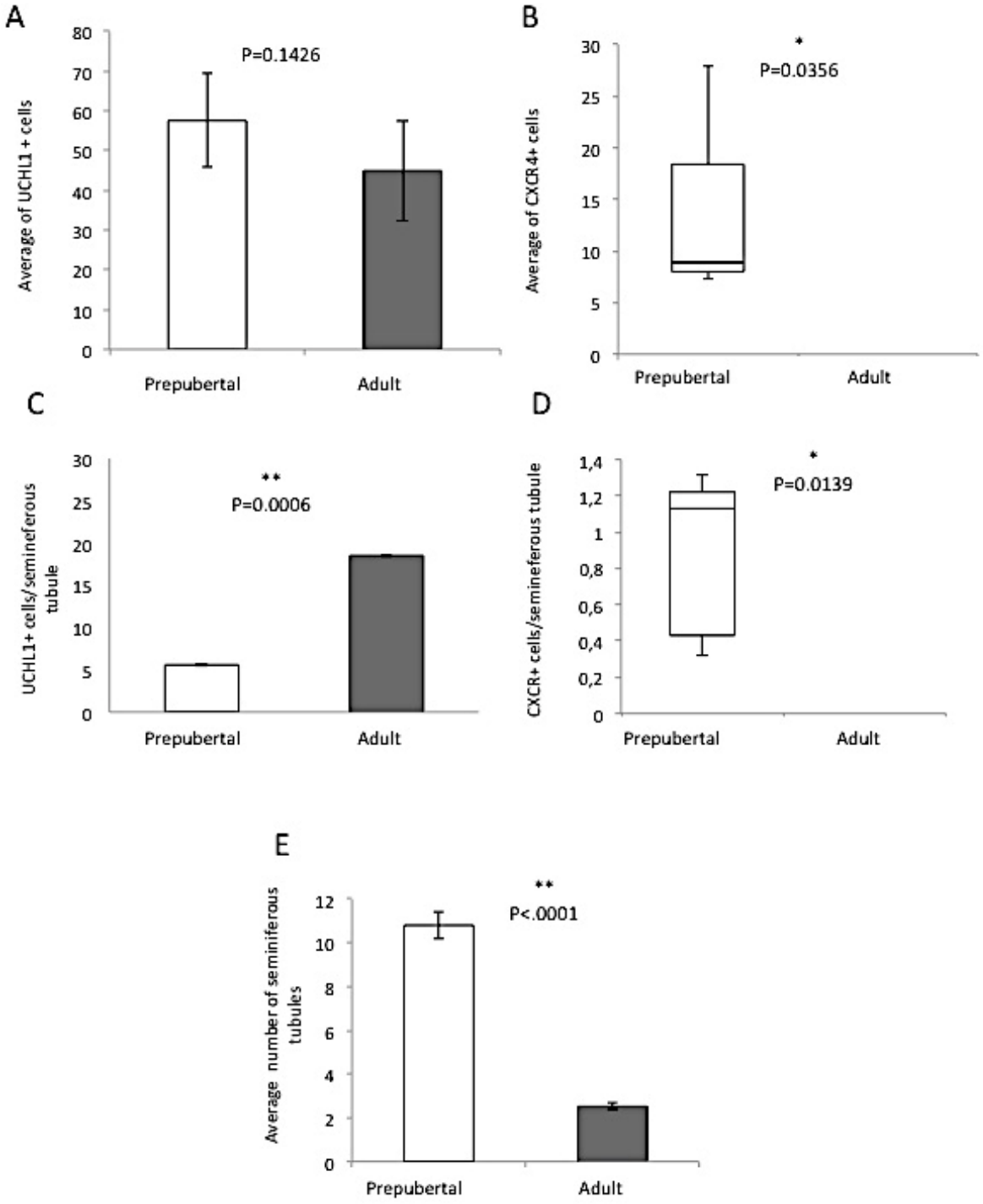


Figure 4 - Change in total of positive cells for UCHL1 (A) and CXCR4 (B), immunolocalization and positive cell by seminiferous tubule for UCHL1 (C) and CXCR4 (D) and number of seminiferous tubule (E) in bovine testis at 5 months (prepubertal) and 2 old (adult) Values (mean + s.e.m) with different superscript letter are different (*p<0.05 and **p<0.001), n=10



5.4.2 Enzymatic isolation

Age has been suggested to play a role in SSC properties such as gene and protein expression. In order to verify age-related differential expression in bovine, we isolated cells from testicular fragments of prepubertal calves and adult bulls. Cell viability and total number of viable cell was established after enzymatic digestion, after thawing and after differential platting. In this study, we observed that after enzymatic isolation age affected cell viability (p=0.0005) and number of viable cells recovered per gram of testicular tissue (p=0.0453).

Table 3 - Changes on, cell viability after enzymatic isolation, number of viable cells after thawing an after differential platting by age (prepubertal and adult). Within each column, values (mean+s.e) with different superscript letter are different (p<0.05), n=10

	Prepubertal	Adult	Pvalue
Testicular weight (g)	10.039 ± 0.3 [a]	271.872 ± 1.713 [b]	<0.0001
Cell viability after enzymatic isolation	0.221 ± 0.00 [a]	0.664 ± 0.077 [b]	0.0005
Viable cell (x106)/testicular weight (g)	6.127 ± 0.925 [a]	3.101 ± 0.881[b]	0.0453
Total of viable cell after thawing	9.14 ± 2.253	10.15± 1.507	0.7192
Total of viable cell after differential platting	4.245 ± 1.131	9.015 ±1.755	0.0517

5.4.3 Differential Platting

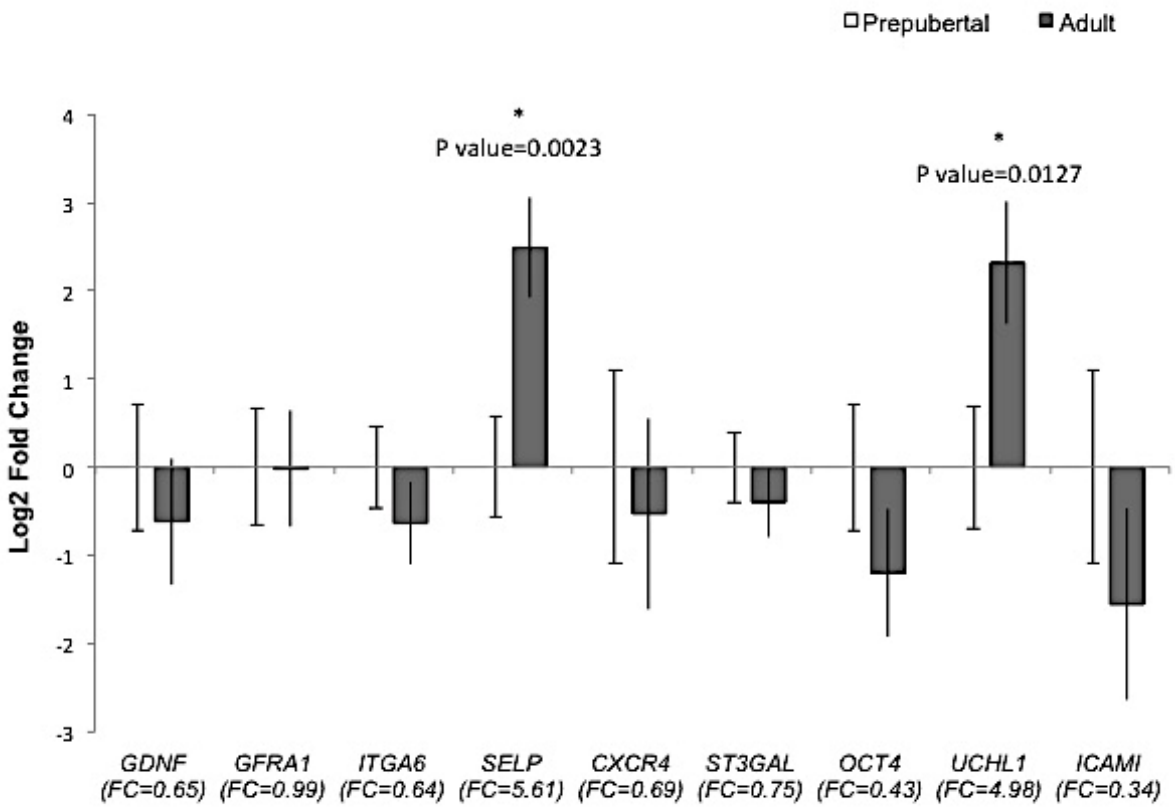
Spermatogonial cells are present in different proportion in testicular parenchyma from prepubertal calves and adult bulls. To study spermatogonial markers expression, previously we obtained an enriched SSC sub-population by BSA differential platting. We observed that age not affected number of viable cells after

thawing (p=0.7192), however there was a trend to affect viable cells after differential platting (p=0.0517; Table 3).

5.4.4 Qualitative RT-PCR

To evaluate the effect of age on undifferentiated spermatogonia gene expression we performed q-RT-PCR and determined the relative expression of *GFRA1, ITGA6, SELP, CXCR4, ST3GAL, OCT4, UCHL1* and *ICAM1* after differential plating (Figure 5). Age affected expression of *SELP* (Fold Change=5.61; p=0.0023) and *UCHL1* (Fold Change=4.98; p=0.0127), which were more expressed in adult cells when compared to prepubertal testicular cells. Age did not affect *GFRA1* (p=0.9801), *ITGA6* (p=0.2054), *CXCR4* (p=0.6403), *ST3GAL* (p=0.3455), *OCT4* (p=0.1341) and Icam1 (p=0.1915) relative expression. Expression of *GDNF* by Sertoli cells was not affected by age (Fold Change=0.65; p=0.4156) (Figure 5).

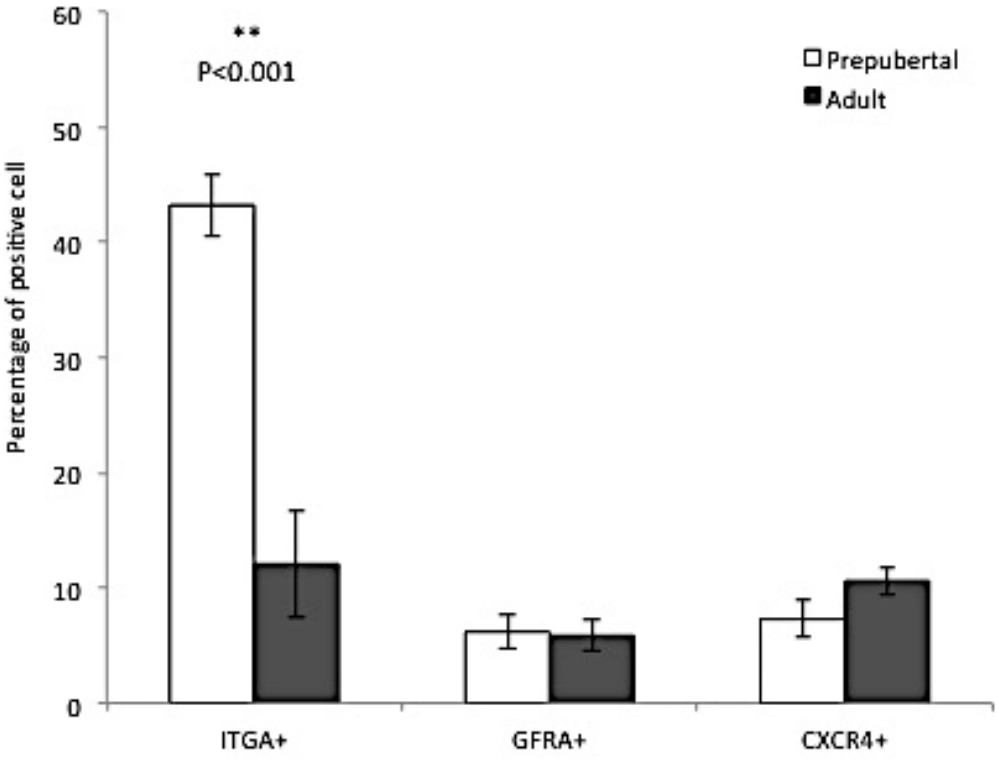
Figure 5 - Relative gene quantification by qPCR (ITGA6, GFRA1, CXCR4, SELP, ST3GAL, OCT4, UCHL1, ICAM and GDNF) Values are presented as log_2 of Fold Change ± Ct standard deviation. Different letters in each gene indicate significant statistical difference (*P ≤ 0.05)



5.4.5 Flow Cytometry

Membrane proteins are important markers to identify and characterize undifferentiated stem cells. In this study, we evaluated the expression of three membrane proteins specific for SSC and undifferentiated spermatogonia by flow cytometry analysis. Percentage of positive cells for ITGA6 (ITGA6+), GFRA1 (GFRA+) and CXCR4 + were established by flow cytometry for isolated and enriched testicular cells (Figure 6). Age affected only the proportion of ITGA6+ cells (P<0.001), which was higher in prepubertal calves when compared to adult bulls. No effect of age was observed on GFRA+ (P=0.8777) and CXCR4+ (P=0.1315) cell populations.

Figure 6 - Percentage of positive cells (ITGA6, GFRA1and CXCR4) in isolated testicular cell from bovine testis at 5 months (prepubertal) and 2 years old (adult). Values (mean + s.e.m) with different superscript letter are different (*p<0.05 and **p<0.001), n=10



5.5 DISCUSSION

Mammalian spermatogenesis is a classic adult stem cell-dependent process that is supported by SSC self-renewal and differentiation throughout the male fertile life. Aging effect was previously associated with changes in properties of stem cells in other species and tissues (BOYLE et al., 2007). However aging effect on SSC properties is unclear in the bovine species. The present study aimed to evaluate the effect of age on gene expression and subpopulations of bovine undifferentiated spermatogonia expression. We observed a specific aging influence on the

expression of spermatogonial stem cell markers in bovine isolated and enriched testicular cells.

Our finds show that more cells expressed UCHL1 in adult bulls than prepubertal calves testicular parenchyma (number of positive cells/ seminiferous tubule). Consistent with our results, a previous study (FUJIHARA et al., 2011) showed that testicular parenchyma from bulls (2 year old) had higher number of UCHL1+ cells per seminiferous tubule and high seminiferous tubule area than younger animals (5 months). We also observed an age effect on UCHL1 transcript expression in isolated cell enriched by differential platting. We observed that UCHL1 transcripts were more expressed in cells from adult bulls testes than from prepubertal calves. UCHL1 is express by mouse testicular germ cell, mainly spermatogonia and Sertoli cells, and it's physiology function is not well elucidate. Moreover, the overexpressing of UCHL1 arrest the spermatogonia to spermatogenesis (Wang et al., 2006). However, the same expression profile was not observed for other markers for undifferentiated spermatogonia such as ITGA6 and GFRA1. This results support our hypothesis that age influence specific properties of bovine undifferentiated spermatogonia.

In mice, ITGA6 is not a specific marker for SSC as it is also expressed in Sertoli cells (SALANOVA et al., 1995; BEARDSLEY; ROBERTSON; O´DONNELL, 2006) and in spermatic release during the spermiation (BEARDSLEY; ROBERTSON; O´DONNELL, 2006). Sertoli cells represent a higher percentage of testicular cell population in prepubertal than in adult individuals (SALANOVA et al., 1995; BEARDSLEY; ROBERTSON; O´DONNELL, 2006), which could explain the higher presence of ITGA6+ cells in the enriched fraction from prepubertal calves when compared to adult bulls. Prepubertal seminiferous tubule is almost composed only by Sertoli cell, gonocytes and SSC (KERR et al., 2006). As differential platting could have not completely removed somatic cell from supernatant, it was plausible that there are Sertoli cells in enriched cell pool, especially from calves. We detected the presence of Sertoli cells in the enriched pool by observing GDNF expression by qPCR. GDNF expression was shown to be affected by age as it was more expressed by Sertoli cells from fertile aging mice than young fertile ones (RYU et al., 2006). Since we did not observe differences in GDNF expression as expected, it is indeed possible that more Sertoli cells were present after enrichment in the prepubertal group, thus increasing the number of ITGA6+ cells.

SSC proliferation and differentiation is a complex and niche-dependent process, Sertoli produces GDNF that promotes stem cell self-renewal by GFRA1 receptors systems (DE ROOIJ, 2001). Age effect on this complex system is unclear and results are controversial. Taken together, results concluded that the decline in function of supporting cells is one of major reasons for age-related infertility (RYU et al., 2006). However, this does not agree with another study which shows that SSC and somatic environment are involved in aging process (ZHANG et al., 2006). GFRA1 expression was detected in young and aging fertile mice (RYU et al., 2006; KOKKINAKi et al., 2010), results that corroborate with our data. CXCR4 also play a role in spermatogonial stem cell niche and are associated with SSC self-renewal (MENG et al., 2000; YANG et al., 2013). Unpublished data from our laboratory corroborates the stem cell potential of bovine CXCR4+ cells. In enriched cells, we observed that CXCR4 expression was not affected by age. We also observed that CXCR4 expression is not ubiquitous in prepubertal testis, however it was not detected in adult testis suggesting that this expression might be niche-controlled. Nonetheless, flow cytometry experiments did not detect an age effect on the population of CXCR4+ cells. The immunophenotypical characterization of germ cells by techniques dependent on microscopy is a widely used tool, but flow cytometry has advantages in cell analysis such as speed, objective measurements, analysis of large number of cells providing a high statistical significance and unbiased cell sampling (JANCA; JOST; EVERSON, 1986). We also evaluated the expression of two genes previously correlated with age effect in murine SSC, Selp and Icam1 (KOKKINAKI et al., 2010). We observed no age effect in ICAM1 expression but SELP was more expressed in cells isolated from adult bulls. SELP is a cell surface adhesion molecule, which is a marker for physiological stress such as aging, inflammatory process and cardiovascular disease (ZOU et al., 2004; CHAMBERS et al., 2007; KOKKINAKI et al., 2010). In addition SELP expression is up-regulated by aging in hematopoietic stem cells (CHAMBERS et al., 2007). However, SELP is expressed in the equatorial region of acrosome-react human sperm (FUSI et al., 1996) and in a acrosome membrane of porcine sperm (GENG et al., 1997). Thus the high expression of SELP could be also explained by presence of some differentiated germ cells in enriched cell pool.

In summary, we observed a higher number of UCHL1 positive cells in the adult testis and increased gene expression of *UCHL1* and *SELP* in isolated and

enriched adult testicular cells. The effect of age on genes and proteins described is this study may be useful to understand aging outcome on the spermatogenesis of domestic animals and enhance adult bull SSC applications in reproductive biotechnologies.

5.6 CONCLUSION

Age affects gene expression and the population of cells expressing specific spermatogonial markers in the bovine testis.

Acknowledgements

This research was funding supported by São Paulo Research Foundation (FAPESP) and National Counsel of Technological and Scientific Development (CNPq). Authors would like to thank Prefeitura do Campus de Pirassununga for providing the bull calves used in this experiment; Prof. Cristina de Oliveira Massoco Salles Gomes and Dr. Nicolle Queiroz in the Department of Pathology for assistance with flow cytometry, Prof. Paulo Maiorka in the Department of Pathology and Prof. Ricardo J.G. Pereira in Department of Animal Reproduction for assistance with sample preparation for immunohistochemistry

REFERENCE

AMANN, R. P.; KAVANAUGH, J. F.; GRIEL, L. C.; VOGLMAYR, J. K. Sperm production of Holstein bulls determined from testicular spermatid reserves, after cannulation of rete testis or vas deferens, and by daily ejaculation. **J Dairy Sci,** v. 57, n. 1, p. 93-9, Jan 1974. ISSN 0022-0302. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/4810488 >. Acessed at: 24. march. 2015.

APONTE, P. M.; SODA, T.; VAN DE KANT, H. J.; DE ROOIJ, D. G. Basic features of bovine spermatogonial culture and effects of glial cell line-derived neurotrophic factor. **Theriogenology,** v. 65, n. 9, p. 1828-47, Jun 2006. ISSN 0093-691X. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/16321433 >. Acessed at: 24. march. 2015.

APONTE, P.M.; VAN BRAGT, M.P.; DE ROOIJ, D.G.; VAN PELT, A.M. Spermatogonial stem cells: characteristics and experimental possibilities. **APMIS,** v. 113, n. 11-12, p. 727-42, 2005 Nov-Dec 2005. ISSN 0903-4641. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/16480445 >. Acessed at: 24. march. 2015.

BEARDSLEY, A.; ROBERTSON, D. M.; O'DONNELL, L. A complex containing alpha6beta1-integrin and phosphorylated focal adhesion kinase between Sertoli cells and elongated spermatids during spermatid release from the seminiferous epithelium. **J Endocrinol,** v. 190, n. 3, p. 759-70, Sep 2006. ISSN 0022-0795. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/17003277 >. Acessed at: 24. march. 2015.

BOYLE, M.; WONG, C.; ROCHA, M.; JONES, D. L.. Decline in self-renewal factors contributes to aging of the stem cell niche in the Drosophila testis. **Cell Stem Cell,** v. 1, n. 4, p. 470-8, Oct 2007. ISSN 1875-9777. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/18371382 >. Acessed at: 24. march. 2015.

CHAMBERS, S. M.; SHAW, C. A.; GATZA, C.; FISK, C. J.; DONEHOWER, L. A.;GOODELL, M. A. Aging hematopoietic stem cells decline in function and exhibit epigenetic dysregulation. **PLoS Biol,** v. 5, n. 8, p. e201, Aug 2007. ISSN 1545-7885. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/17676974 >. Acessed at: 24. march. 2015.

CHENG, J.; TÜRKEL, N.; HEMATI, N.; FULLER, M. T.; HUNT, A. J.; YAMASHITA, Y. M. Centrosome misorientation reduces stem cell division during ageing. **Nature,** v. 456, n. 7222, p. 599-604, Dec 2008. ISSN 1476-4687. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/18923395 >. Acessed at: 24. march. 2015.

DE ROOIJ, D. G. Proliferation and differentiation of spermatogonial stem cells. **Reproduction,** v. 121, n. 3, p. 347-54, Mar 2001. ISSN 1470-1626. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/11226060 >. Acessed at: 24. march. 2015.

FUJIHARA, M.; KIM, S. M.; MINAMI, N.; YAMADA, M.; IMAI, H. Characterization and in vitro culture of male germ cells from developing bovine testis. **J Reprod Dev,** v. 57, n. 3, p. 355-64, Jun 2011. ISSN 1348-4400. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/21289464 >. Acessed at: 24. march. 2015.

FUSI, F. M.; MONTESANO, M.; BERNOCCHI, N.; PANZERI, C.; FERRARA, F.; VILLA, A.; BRONSON, R. A.. P-selectin is expressed on the oolemma of human and hamster oocytes following sperm adhesion and is also detected on the equatorial region of acrosome-reacted human spermatozoa. **Mol Hum Reprod,** v. 2, n. 5, p. 341-7, May 1996. ISSN 1360-9947. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/9238701 >. Acessed at: 24. march. 2015.

GEIGER, H.; RUDOLPH, K. L. Aging in the lympho-hematopoietic stem cell compartment. **Trends Immunol,** v. 30, n. 7, p. 360-5, Jul 2009. ISSN 1471-4981. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/19540806 >. Acessed at: 24. march. 2015.

GENG, J. G.; RAUB, T. J.; BAKER, C. A.; SAWADA, G. A.; MA, L.; ELHAMMER, A. P. Expression of a P-selectin ligand in zona pellucida of porcine oocytes and P-selectin on acrosomal membrane of porcine sperm cells. Potential implications for their involvement in sperm-egg interactions. **J Cell Biol,** v. 137, n. 3, p. 743-54, May 1997. ISSN 0021-9525. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/9151678 >. Acessed at: 24. march. 2015.

HE, Y.; CHEN, X.; ZHU, H.; WANG, D. Developments in techniques for the isolation, enrichment, main culture conditions and identification of spermatogonial stem cells. **Cytotechnology**, Mar 2015. ISSN 0920-9069. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/25749914 >. Acessed at: 24. march. 2015.

HERRID, M.; DAVEY, R. J.; HUTTON, K.; COLDITZ, I. G.; HILL, J. R. A comparison of methods for preparing enriched populations of bovine spermatogonia. **Reprod Fertil Dev,** v. 21, n. 3, p. 393-9, 2009. ISSN 1031-3613. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/19261216 >. Acessed at: 24. march. 2015.

IZADYAR, F.; DEN OUDEN, K.; STOUT, T. A.; STOUT, J.; CORET, J.; LANKVELD, D. P.; SPOORMAKERS, T. J.; COLENBRANDER, B.; OLDENBROEK, J. K.; VAN DER PLOEG, K. D.; WOELDERS, H.; KAL, H. B.; DE ROOIJ, D. G. Autologous and homologous transplantation of bovine spermatogonial stem cells. **Reproduction,** v. 126, n. 6, p. 765-74, Dec 2003. ISSN 1470-1626. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/14748695 >. Acessed at: 24. march. 2015.

IZADYAR, F.; MATTHIJS-RIJSENBILT, J. J.; DEN OUDEN, K.; CREEMERS, L. B.; WOELDERS, H.; DE ROOIJ, D. G. Development of a cryopreservation protocol for type A spermatogonia. **J Androl,** v. 23, n. 4, p. 537-45, 2002 Jul-Aug 2002. ISSN 0196-3635. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/12065461 >. Acessed at: 24. march. 2015.

IZADYAR, F.; SPIERENBERG, G.T.; CREEMERS, L.B.; DEN OUDEN, K.; DE ROOIJ, D.G. Isolation and purification of type A spermatogonia from the bovine

testis. **Reproduction,** v. 124, n. 1, p. 85-94, Jul 2002. ISSN 1470-1626. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/12090922 >. Acessed at: 24. march. 2015.

JANCA, F. C.; JOST, L. K.; EVENSON, D. P. Mouse testicular and sperm cell development characterized from birth to adulthood by dual parameter flow cytometry. **Biol Reprod,** v. 34, n. 4, p. 613-23, May 1986. ISSN 0006-3363. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/3708046 >. Acessed at: 24. march. 2015.

KERR, J. B.; LOVELAND, K. L.; O'BRYAN, M. K.; DE KRETSER, D. M. Cytology of the Testis and Intrinsic Control Mechanisms. In: NEILL, J. D. (Ed.). **Knobil and Neill's Physiology of Reproduction**. 3ed. Oxford, UK: Elsevier Inc, v.1, 2006. chap. Cytology of the Testis and Intrinsic Control Mechanisms, p.827-948. (Male Reproductive System).

KOKKINAKI, M.; LEE, T. L.; HE, Z.; JIANG, J.; GOLESTANEH, N.; HOFMANN, M. C.; CHAN, W. Y.; DYM, M**.** The molecular signature of spermatogonial stem/progenitor cells in the 6-day-old mouse testis. **Biol Reprod,** v. 80, n. 4, p. 707-17, Apr 2009. ISSN 0006-3363. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/19109221 >. Acessed at: 24. march. 2015.

KOKKINAKI, M.; LEE, T. L.; HE, Z.; JIANG, J.; GOLESTANEH, N.; HOFMANN, M. C.; CHAN, W. Y.; DYM, M. Age affects gene expression in mouse spermatogonial stem/progenitor cells. **Reproduction,** v. 139, n. 6, p. 1011-20, Jun 2010. ISSN 1741-7899. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/20371641 >. Acessed at: 24. march. 2015.

KON, Y.; ENDOH, D.; IWANAGA, T. Expression of protein gene product 9.5, a neuronal ubiquitin C-terminal hydrolase, and its developing change in sertoli cells of mouse testis. **Mol Reprod Dev,** v. 54, n. 4, p. 333-41, Dec 1999. ISSN 1040-452X. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/10542373 >. Acessed at: 24. march. 2015.

MENG, X.; LINDAHL, M.; HYVÖNEN, M. E.; PARVINEN, M.; DE ROOIJ, D. G.; HESS, M. W.; RAATIKAINEN-AHOKAS, A.; SAINIO, K.; RAUVALA, H.; LAKSO, M.; PICHEL, J. G.; WESTPHAL, H.; SAARMA, M.; SARIOLA, H. Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. **Science,** v. 287, n. 5457, p. 1489-93, Feb 2000. ISSN 0036-8075. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/10688798 >. Acessed at: 24. march. 2015.

OATLEY, J. M.; DE AVILA, D. M.; MCLEAN, D. J.; GRISWOLD, M. D.; REEVES, J. J.Transplantation of bovine germinal cells into mouse testes. **J Anim Sci,** v. 80, n. 7, p. 1925-31, Jul 2002. ISSN 0021-8812. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/12162661 >. Acessed at: 24. march. 2015.

OATLEY, J. M.; REEVES, J. J.; MCLEAN, D. J. Biological activity of cryopreserved bovine spermatogonial stem cells during in vitro culture. **Biol Reprod,** v. 71, n. 3, p. 942-7, Sep 2004. ISSN 0006-3363. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/15151932 >. Acessed at: 24. march. 2015.

QASEMI-PANAHI, B.; TAJIK, P.; MOVAHEDIN, M.; MOGHADDAM, G.; BARZGAR, Y.; HEIDARI-VALA, H. Differentiation of bovine spermatogonial stem cells into osteoblasts. **Avicenna J Med Biotechnol,** v. 3, n. 3, p. 149-53, Jul 2011. ISSN 2008-2835. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/23408761 >. Acessed at: 24. march. 2015.

REDING, S. C.; STEPNOSKI, A. L.; CLONINGER, E. W.; OATLEY, J. M. THY1 is a conserved marker of undifferentiated spermatogonia in the pre-pubertal bull testis. **Reproduction,** v. 139, n. 5, p. 893-903, May 2010. ISSN 1741-7899. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/20154176 >. Acessed at: 24. march. 2015.

RYU, B. Y.; ORWIG, K. E.; OATLEY, J. M.; AVARBOCK, M. R.; BRINSTER, R. L. Effects of aging and niche microenvironment on spermatogonial stem cell self-renewal. **Stem Cells,** v. 24, n. 6, p. 1505-11, Jun 2006. ISSN 1066-5099. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/16456131 >. Acessed at: 24. march. 2015.

SALANOVA, M.; STEFANINI, M.; DE CURTIS, I.; PALOMBI, F. Integrin receptor alpha 6 beta 1 is localized at specific sites of cell-to-cell contact in rat seminiferous epithelium. **Biol Reprod,** v. 52, n. 1, p. 79-87, Jan 1995. ISSN 0006-3363. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/7711187 >. Acessed at: 24. march. 2015.

SHINOHARA, T.; AVARBOCK, M. R.; BRINSTER, R. L. beta1- and alpha6-integrin are surface markers on mouse spermatogonial stem cells. **Proc Natl Acad Sci U S A,** v. 96, n. 10, p. 5504-9, May 1999. ISSN 0027-8424. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/10318913 >. Acessed at: 24. march. 2015.

SPRADLING, A.; DRUMMOND-BARBOSA, D.; KAI, T. Stem cells find their niche. **Nature,** v. 414, n. 6859, p. 98-104, Nov 2001. ISSN 0028-0836. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/11689954 >. Acessed at: 24. march. 2015.

STEIBEL, J. P.; POLETTO, R.; COUSSENS, P. M.; ROSA, G. J. A powerful and flexible linear mixed model framework for the analysis of relative quantification RT-PCR data. **Genomics,** v. 94, n. 2, p. 146-52, Aug 2009. ISSN 1089-8646. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/19422910 >. Acessed at: 24. march. 2015.

WANG, Y. L.; LIU, W.; SUN, Y. J.; KWON, J.; SETSUIE, R.; OSAKA, H.; NODA, M.; AOKI, S.; YOSHIKAWA, Y.; WADA, K. Overexpression of ubiquitin carboxyl-terminal hydrolase L1 arrests spermatogenesis in transgenic mice. **Mol Reprod Dev,** v. 73, n. 1, p. 40-9, Jan 2006. ISSN 1040-452X. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/16177983 >. Acessed at: 24. march. 2015.

WYROBEK, A. J.; ESKENAZI, B.; YOUNG, S.; ARNHEIM, N.; TIEMANN-BOEGE, I.; JABS, E. W.; GLASER, R. L.; PEARSON, F. S.; EVENSON, D. Advancing age has differential effects on DNA damage, chromatin integrity, gene mutations, and aneuploidies in sperm. **Proc Natl Acad Sci U S A,** v. 103, n. 25, p. 9601-6, Jun 2006. ISSN 0027-8424. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/16766665 >. Acessed at: 24. march. 2015.

YANG, Q. E.; KIM, D.; KAUCHER, A.; OATLEY, M.J.; OATLEY, J.M. CXCL12-CXCR4 signaling is required for the maintenance of mouse spermatogonial stem

cells. **J Cell Sci,** v. 126, n. Pt 4, p. 1009-20, Feb 2013. ISSN 1477-9137. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/23239029 >. Acessed at: 24. march. 2015.

ZHANG, X.; EBATA, K. T.; ROBAIRE, B.; NAGANO, M. C.. Aging of male germ line stem cells in mice. **Biol Reprod,** v. 74, n. 1, p. 119-24, Jan 2006. ISSN 0006-3363. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/16177220 >. Acessed at: 24. march. 2015.

ZHENG, Y.; ZHANG, Y.; QU, R.; HE, Y.; TIAN, X.; ZENG, W. Spermatogonial stem cells from domestic animals: progress and prospects. **Reproduction,** v. 147, n. 3, p. R65-74, Mar 2014. ISSN 1741-7899. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/24357661 >. Acessed at: 24. march. 2015.

ZOU, Y.; JUNG, K. J.; KIM, J. W.; YU, B. P.; CHUNG, H. Y**.** Alteration of soluble adhesion molecules during aging and their modulation by calorie restriction. **FASEB J,** v. 18, n. 2, p. 320-2, Feb 2004. ISSN 1530-6860. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/14688195 >. Acessed at: 24. march. 2015.

6 CHAPTER 3: COMPARISON OF MOLECULAR AND FUNCTIONAL ELEMENTS OF CXCR4+ GERM CELLS IN PRE-PUBERTAL AND ADULT BULLS

6.1 SUMMARY

CXCR4 and CXCL12 are fundamental factors for gonocyte migration, SSCs proliferation and, thus development of normal testes. The sexual maturation effect on CXCR4+ cells is not clear in mammals with a long pre-pubertal phase, such as bovine. In this study, we aimed to verify if sexual maturation affect in gene expression and stem cell potential of CXCR4+ testicular cells from pre-pubertal calves and adult bulls. Isolated testicular cells from pre-pubertal calves and adult bulls were sorted by CXCR4 expression using magnetic-activated cell sorting (MACS). Relative expression of six genes were performed in a factorial design (n=12) based on MACS fraction (CXCR4+ and CXCR4-) and age (pre-pubertal and adult). Age effect on stem cell potential of CXCR4+ cells was evaluated by heterologous transplantation into mouse recipient testes pre-treated with Busulfan. We observed a specific age effect on gene expression of CXCR4+ cells and *LIMS2,* a gene previously associated to negative regulation of cell migration was overexpressed in adult cells. We also observed high proliferating colonies after the heterologous transplantation of CXCR4+ cells from pre-pubertal and adult donors. The total number of colonies observed after transplant was higher for adults. In conclusion, gene expression of CXCR4+ testicular cells is affected by sexual maturation. Moreover, CXCR4 is a conserved marker for undifferentiated germ cell from calves and adult bulls.

Keywords: CXCR4. Bovine. Age. Spermatogonia. SSC markers.

6.2 INTRODUCTION

Spermatogenesis is a classical model of stem cell differentiation occurring throughout the male reproductive life. After birth, immature germ cells called gonocytes proliferate and migrate towards the basal membrane (KERR et al., 2006). Round gonocytes are predicted to enter apoptosis, but pseudopod gonocytes migrate along the seminiferous cord parenchyma and differentiate into spermatogonial stem cell (SSC) in a correct niche (ORWIG et al., 2002) surrounded by Sertoli cells (SPRADLING; DRUMMOND-BARBOSA; KAI, 2001). This gonocyte differentiation happens at 0-6 dpp in mice but is not completed until 6 months of age in bovine (CURTIS; AMANN, 1981). Despite that SSCs self-renewal and differentiation support spermatogenesis in adult male, undifferentiated spermatogonia and pseudopods gonocytes have similar stem cell potential (ORWIG et al., 2002).

Gonocytes and undifferentiated spermatogonial cells not only share functional properties as they also share specific biological markers (ZHENG et al., 2014) such as GDNF family receptor alpha-1 (GFRA1) (MENG et al., 2000; NAUGHTON et al., 2006; COSTA et al., 2012; LEE et al., 2013), Thy-1 Cell Surface Antigen (THY1) (KUBOTA; AVARBOCK; BRINSTER, 2003; RYU et al., 2004; HERMANN et al., 2009; REDING et al., 2010; ZHENG et al., 2014) and Chemokine (C-X-C Motif) Receptor 4 (CXCR4) (YANG et al., 2013; WESTERNSTRÖER et al., 2014). Normal primordial germ cell migration to the gonads is regulated by CXCL12-CXCR4 interaction (MOLYNEAUX et al., 2003; YANG et al., 2013). In mice, CXCR4 expression also is detected in gonocytes, pre-pubertal and adult undifferentiated germ cells (MCIVER et al., 2013; YANG et al., 2013; WESTERNSTRÖER et al., 2014) and is associated with SSC self-renewal and maintenance (YANG et al., 2013). Recently, we observed that bovine CXCR4+ cells from pre-pubertal calves hold SSCs potential (GOISSIS et al., 2015[7]).

Undifferentiated germ cells have potential biotechnological applications in domestic animals (ZHENG et al., 2014). In livestock populations the offspring genetic gain is highly correlated with the male ancestor, thus breeding programs generally

[7] GOISSIS, M. D.; GIASSETTI, M. I.; WORST, R. A.; MENDES, C. M.;. MOREIRA, P. V.; ASSUMPÇÃO, M. E. O. A.; VISINTIN, J. A. Spermatogonial stem cell potential of CXCR4-positive cells from prepubertal bovine testis, [2015]. Data not published.

use adult males with previously approved genetic merit. In mammals with a long pre-pubertal period, such as bovine, nonhuman primates and humans, little changes in parenchyma are observed until sexual maturation (KERR et al., 2006). Recently the age effect on stem cells was reported for hematopoietic cells (CHAMBERS et al., 2007) and THY1+ SSCs from mice (KOKKINAKI et al., 2010), and in both studies Intercellular Adhesion Molecule 2 (ICAM2) and Selectin P (SELP) are overexpressed in oldest mice compared with youngest ones (6, 21 and 60 days after birth, for SSC) (KOKKINAKI et al., 2010). However, the effect of sexual maturation in undifferentiated germ cell with stem cell properties is unclear in mammals with long pre-pubertal phase, such as the bovine. Thus, we aimed to verify if sexual maturation affect CXCR4+ germ cell function and gene expression

6.3 MATERIAL AND METHODS

All chemicals were supplied by Sigma Chemical Company (St. Louis, Missouri, USA) unless otherwise stated. All procedures were approved by Bioethics Committee for use of animals of the School of Veterinary Medicine and Animal Science of the University of Sao Paulo.

6.3.1 Animals, enzymatic isolation and cell cryopreservation

Pre-pubertal bull calves with 5 months of age (n=7; PUSP-P, University of Sao Paulo, Pirassununga, Sao Paulo, Brazil) were anesthetized (0.2 mg of xylazine hydrochloride/ Kg) (Virbaxyl 2%, Virbac do Brazil Ind. E com, Sao Pauo, Brazil) and testes were surgically removed. Adult bull testes with 3 years old (n=5) were obtained from slaughterhouse. Testicular cells were isolated by enzymatic digestion as described previously with minor modifications (REDING et al., 2010). All testicular parenchyma was minced and incubated in collagenase solution (1 mg/ml

collagenase type V and 7 mg/ml DNase I) for 30 min at 37°C with continuous agitation. Digested seminiferous tubules were washed three times with Phosphate-buffered saline (PBS; 100 x g/ 1min) and incubated in trypsin solution [0.25% (w/v) trypsin, 2.21 mM EDTA and 7 mg/ml DNase I] for 5 min at 37°C. Enzymatic activity was blocked by addition of 10% fetal bovine serum (FBS; Gibco®, Life Technologies, Carlsbad, CA, USA). Cells were passed through 100 mm cell strainer (BD Biosciences, Durham, NC, USA), centrifuged (600 x g/ 7 min) and cell pellet was suspended in PBS. Cell concentration and viability were assessed by Trypan Blue solution exclusion method (5 mg/ml). Isolated testicular cells were cryopreserved as previously described (IZADYAR et al., 2002) with minor modifications. Isolated cells were cryopreserved ($10x10^6$ viable cell/ml) in DMEM medium (Life Technologies, Carlsbad, California, USA) with 10% (v/v) FBS, 10% (v/v) DMSO and, 0.07 M sucrose using non-controlled rate freezing protocol (Mr. Frosty container, Nalgene®, Waltham, Massachusetts, USA), manufacturer instructions were following.

6.3.2 Obtaining CXCR4+ cell from isolated cells

To elucidate age effect on bovine undifferentiated germ cell, magnetic activated cell sorting (MACS, Miltenyi-biotec, Teterow, Germany) was performed. At present study, we used cells from pre-pubertal and adult bovine in order to observe gene differential expression and ability to repopulate testis after heterologous transplantation. We employed a 2x2 factorial design (n=12) considering age effect (pre-pubertal or adult) and cell fraction after MACS (positive selection or negative selection) as independent variables. Briefly, cells were thawed and washed (200 x g/5 min) in DMEM-BSA medium [DMEM medium with 0.2 % (w/v) BSA and 0.1% (v/v) non-essential amino acids] and suspended in PBS with 10% BSA . Cells were passed through 100 mm cell strainer (BD Biosciences, Durham, NC, USA) and incubated with 1:500 anti-CXCR4 (ab7199, Abcam, Cambridge, UK) for 30 min at room temperature. Cells were washed (300 x g/ 5min), resuspended in cold PBS with 0.5% (w/v) BSA and 2mM EDTA and incubated with Anti-rabbit IgG microbeads (1:20; Miltenyi Biotec). The cell suspension was subjected to separation using MS

Columns (Miltenyi Biotec). Flow though cells were considered the unsorted fraction (CXCR4-) and cell recovered from the column considered as CXCR4 positive (CXCR4+) sorted cells.

6.3.3 RNASeq library

RNA library was obtained from ours previously study (GOISSIS et al., unpublished data) which compared bovine testis cell transcriptome [CXCR4+ (n=5) and CXCR4- (n=5) from pre-pubertal calves (5 months age). Briefly, RNA was extracted by PicoPure RNA Isolation Kit (Applied Biosystems, Carlsbad, CA) including DNAse (Qiagen, Valencia, CA) treatment and manufacturer instructions were followed. Total RNA from CXCR4+ (n=5) and CXCR4- (n=5) testes cell were analyzed using Agilent RNA Pico 6000 (Agilent Technologies, Santa Clara, CA) and 146ng was used as input for TruSeq Stranded mRNA Sample Prep Kit. cDNA was analyzed an Agilent DNA 1000 assay (Agilent Technologies) and quantified using Qubit DNA BR Assay kit (Life Technologies) in Qubit Fluorometer (Life Technologies). Libraries were sequenced at the BGI@UC Davis core on a HiSeq 2000 apparatus (Illumina). RNA-Seq tool from of CLC Genomics Workbench 7.5 (CLCbio, Aarhaus, Denmark) was used for read mapping and gene expression analysis.

6.3.4 Gene Ontology Analysis

Gene classification and biological function were online analyzed by DAVID data base v6.7 (HUANG; SHERMANBT; LEMPICKI, 2009). Genes upregulated in CXCR4 samples (p adjusted<0,01) with a fold-change higher than two were analyzed by functional annotation tool. Gene ontology was analyzed by Biological Function (BP_FAT) using human database as reference. The first one hundred genes with lower adjusted p value were evaluated by cell type expression using online search on

Gene Cards (http://www.genecards.org) and also by RNA expression in nine bovine different tissues using Atlas Expression from European Bioinformatics Institute part of European Molecular Biology Laboratory (KAPUSHESKY et al., 2012; PETRYSZAK et al., 2014).

6.3.5 Quantitative real time RT-PCR

Gene expression was performed with 0.1 x 10^6 viable cells for each sample. RNA was obtained from CXCR4+ and CXCR4- testicular cells from pre-pubertal calves and adult bulls, in a 2x2 factorial design (n=12, 3 samples for each age and MACS fraction association). Total RNA was purified by PicoPure RNA Isolation Kit (Applied Biosystems, Carlsbad, CA) including DNAse (Qiagen, Valencia, CA) treatment and quantified at Nanodrop ND-1000 (Nanodrop Technologies, Inc., Wilmington, Delaware). cDNA synthesis was performed using SuperScript® III Reverse Transcriptase (Life Technologies) according to manufacturer's instructions and cDNA concentration was determined by Qubit dsDNA BR Assays kit (Life Technologies) by fluorometry (Qubit 2.0 Fluorometer, Life Technologies). cDNA concentration was standardized at 200 ng/ml and the quantitative RT-PCR (Mastercycler Ep Realplex Thermal Cycler, Eppendorf AG, Hamburg, Germany) was performed using SYBR GreenER qPCR Supermix Universal (Life Technologies) in a two-step reaction: 2 min at 50ºC, 10 min at 95ºC, 40 cycles at 95ºC for 15 s and 60ºC for 1 min; a dissociation curve was performed in the end of cycle. All reactions were performed in triplicate with a negative control. Primers were previously standardized and efficiency curve was performed with cDNA obtained from pre-pubertal bull testis. For this study, the housekeeping genes analyzed were: Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (forward primer: 3'TGACCCCTTCATTGACCTTC5'; reverse primer: 5'TACTCAGCACCAGCATCACC3'; NCBI Reference Sequence NM_001034034.2) and beta-actin (*ACTB)* (forward primer: 5'AGGGATGTGGAGACGACAAC 3'; reverse primer: 5'TCTTTGGTGGGATTCTTTGG3'; NCBI Reference Sequence NM_001109981). We

selected genes from RNA sequencing library (GOISSIS et al., 2015[8]) that are up-regulated in sorted cells, with fold change over than 2 with adjusted p value< 0.001, that are express in undifferentiated germ cell or are high express in bovine testes: *FST* (forward primer: 5'ACCTATGCTAGCGAGTGTGC3'; reverse primer: 5'GTGTCTTCCGAAATGGAGTTGC3'; NCBI Reference Sequence XM_005221516.1), *LIMS2* (forward primer: 5'ACCATGACGGGAAGCAACAT3'; reverse primer: 5'CTCGCAGTACTTCCGACCTT3'; NCBI Reference SequenceNM_001130751.1), *EFNA1* (forward primer: 5'CCAGGAAGACCGTTGCTTGA3'; reverse primer: 5'GGGTCATCTGCTGGAAGTCTC3'; NCBI Reference Sequence NM_001034292.1), *PCDH1* (forward primer: 5'GAGTCGGCTCTCCTGATTCTG3'; reverse primer: 5'GGCTCCCGATGAGGGTATTG3'; NCBI Reference Sequence NM_001083655.1), *SELP* (forward primer: 5'ATATGTCAGAGAGTGCGGCG 3'; reverse primer: 5'GCACTGGACAGCTACACACT3'; NCBI Reference SequenceNM_174183.2) and *THY1* (forward primer: 5'TGCTAACAGTCTTACAGGTGGC3'; reverse primer: 5'TCTTTGTGTCACGGGTCAGG 3'; NCBI Reference Sequence NM_001034765). To validate RNA sequencing results, gene expression was previously tested in sorted (CXCR4+) and unsorted (CXCR4-) testicular cells from prepubertal bulls (n=7).

6.3.6 Bovine testicular cell transplantation

Sorted (CXCR4+) and unsorted (CXCR4-) cells from adult bulls and pre-pubertal calves were transplanted into testis from nude mice previously (40-44 days) treated with bulsulfan (44mg/Kg) (TANG; RODRIGUEZ-SOSA; DOBRINSKI, 2012) as described before (OATLEY et al., 2002) with minor modifications. After MACS, sorted and unsorted cells were stained with PKH26 Fluorescent Cell Linker Kits following manufacturer's instructions for membrane labeling. Recipient mice were treated with 4mg/kg morphine SC (Hipolabor, Belo Horizonte, MG, Brazil) and

[8] GOISSIS, M. D.; GIASSETTI, M. I.; WORST, R. A.; MENDES, C. M.;. MOREIRA, P. V.; ASSUMPÇÃO, M. E. O. A.; VISINTIN, J. A. Spermatogonial stem cell potential of CXCR4-positive cells from prepubertal bovine testis, [2015]. Data not published.

anesthesized using inhaled isoflurane (Biochimico, Itatiaia, RJ, Brazil) and testes were exteriorized. Cells were suspended at $10x10^6$ cells/ml and20 μl placed in glass micropipette for injection. The efferent duct was cannulated with a glass micropipette and injection was performed until 60-95% of visible seminiferous tubules were filled. After 28 days of transplant, number of colonies derived from donor spermatogonia was examined in recipient mice testis by fluorescence microscopy with 551-567 nm excitation wavelength (IX80, Olympus, Tokyo, Japan). The number of colonies with high proliferation were counted in each transplanted testicle and normalized by total of injected cells. The total injected cells were obtained from the injected volume of cell suspension.

6.3.7 Statistical Analyses

Statistical analysis was performed by SAS 9.3 software (SAS Institute, Cary, NC, EUA). In this study, age and MACS fraction were considered as independent variables. MACS and transplantation data was transformed by Log_{10} when the assumptions of normality and homogeneity of variances were not respected. Data were organized as a 2x2 factorial (MACS fraction and age) and analyzed by PROC GLM and Tukey post hoc test was performed. qPCR data was analyzed by PROC MIXED as described (STEIBEL et al., 2009). Data in graphs are shown in log2 distribution but in the text the results are described in fold change to facilitate interpretation. The level of statistical significance was set at α=0.05.

6.4 RESULTS

6.4.1 Testicular cells isolation

Cell viability and total number of viable cell was established after enzymatic digestion of pre-pubertal or adult testis. In this study, we observed that age affected cell viability after enzymatic isolation (p=0.001) but not number of viable cells recovered per gram of testicular tissue (p=0.079) (Table 4) and, highest values were observed in samples provide from adult bulls.

Table 4 - Isolation of testicular cells from pre-pubertal calves and adult bulls. Testicular weight, cell viability after enzymatic isolation (%) and number of viable cells ($x10^6$)/testicular weight. Variables (mean ± S.E.M) and P-value. *P ≤ 0.05and **P ≤ 0.01.

	Prepubertal	Adult	Pvalue
Testicular weight (g)	13.341±4.661	236.84±24.604	<0.00001**
	53.051±11.514	86.21±1.745	0.001**
Cell viability after enzymatic isolation (%)			
	4.84±2.47	11.75±7	0.079
Viable cell ($x10^6$)/testicular weight (g)			

6.4.2 Enrichment of CXCR4+ by MACS

CXCR4 is a specific marker for undifferentiated germ cells in pre-pubertal bulls. However, effect of sexual maturation on CXCR4+ germ cells is not known. First, MACS was performed to obtain samples for qPCR and xenotransplantation. In pre-pubertal calves we observed an enrichment of 8.06-fold comparing total cell number in sorted versus unsorted cells. Percentage and average of total number of cells for unsorted (CXCR4-) cells are 92.909 % ($7.482 \pm 1.856 \times 10^6$ cells), respectively, and CXCR4+ cells are just 7.090% ($0.574 \pm 0.294 \times 10^6$ cells) ($p=0.001$ and $p<0.001$,). We evaluated effect of age, MACS fraction and their interaction in percentage and total number of cells in sorted and unsorted fractions for pre-pubertal calves and adult bulls (Table 5). We observed that total number of cells CXCR4- or CXCR4+ ($p<0.0001$) and percentage of these cells after MACS ($p=0.038$) was affected by age. That way, we observed the highest values for prepubertal animal (Table 5). However, interaction effect was observed for age and MACS fraction to percentage of cell after MACS ($p=0.028$) indicating that percentage of cell after MACS should be analyzed for influence of both factors at same time (Table 5). Total number of cells ($p<0.0001$) and percentage of cell after MACS ($p<0.0001$) were different when comparing CXCR4+ and CXCR4- fraction in both age groups (Table 5).

Table 5 - Percentage of sorted (CXCR4+) and unsorted (CXCR4-) cells for from pre-pubertal calves and adult bulls testicular cells. Variables (mean ± S.E.M) and P-value. *P ≤ 0.05and **P ≤ 0.01

	Prepubertal		Adult		Pvalue		
	Unsorted (CXCR4-)	Sorted (CXCR4+)	Unsorted (CXCR4-)	Sorted (CXCR4+)	Age	MACS	Age*MACS
Cell Percentage after MACS (%)	92.044 ±4.917	10.661 ±2.365	95.130 ±2.734	4.870 ±2.634	0.038 *	<0.0001 **	0.028 *
Total number of cell ($x10^6$)	8.275 ± 2.123	0.98 ± 0.34	2.330 ± 1.438	0.1 ± 0.0646	<0.0001 **	<0.0001 **	0.076

6.4.3 Overview of RNA-seq results and functional annotation of CXCR4+ germ cells

The RNA-Seq library used in this study was provide from our previously study (GOISSIS et al., 2015[9]). 24,617 gene reads were obtained in the original library and just 822 genes were considered differentially expressed (adjusted p value <0,001) between sorted (CXCR4+) and unsorted (CXCR4-) population. We selected from these genes those that were upregulated by 2-fold or more in CXCR4+ cell (697 reads). Gene ontology was evaluated using DAVID database v6.7 (HUANG; SHERMANBT; LEMPICKI, 2009) and divided in three categories base on gene ontology percentage: 0,1%, 0,2% and >0.2% (respectively Figure 7, 8 and 9).

[9] GOISSIS, M. D.; GIASSETTI, M. I.; WORST, R. A.; MENDES, C. M.;. MOREIRA, P. V.; ASSUMPÇÃO, M. E. O. A.; VISINTIN, J. A. Spermatogonial stem cell potential of CXCR4-positive cells from prepubertal bovine testis, [2015]. Data not published.

Figure 7 - Functional classification (≥0.4%) of genes up-regulated by at least 2-fold in and adjusted Pvalue<0.01 from CXCR4+ testicular cells pre-pubertal calves

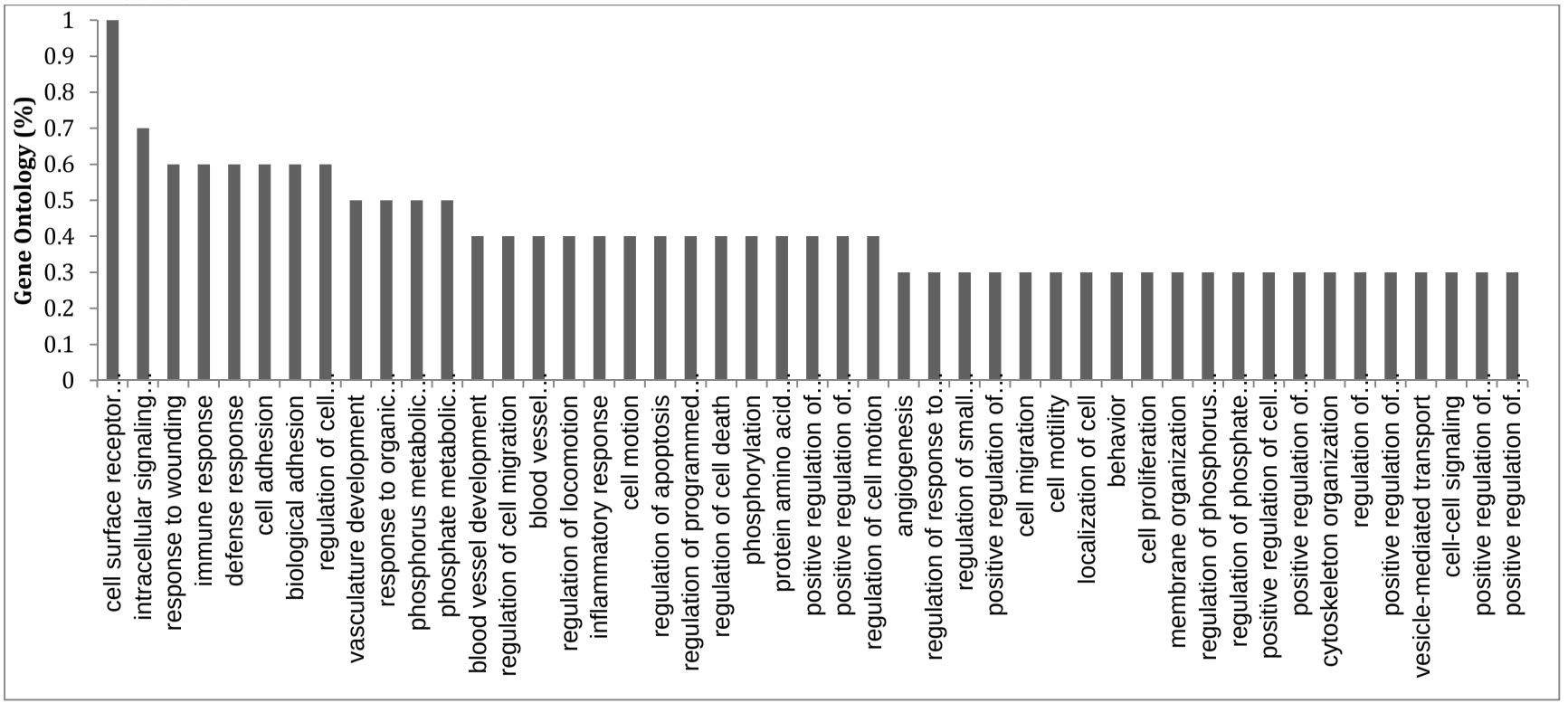


Figure 8 - Functional classification (0.2%≥x<0.4%) of genes up-regulated by at least 2-fold in and adjusted Pvalue<0.01 from CXCR4+ testicular cells pre-pubertal calves

Figure 9 - Functional classification (<0.2%) of genes up-regulated by at least 2-fold in and adjusted Pvalue<0.01 from CXCR4+ testicular cells pre-pubertal calves

We observed that within selected genes biological functions are correlated with regulation of cell motion, adhesion, migration, inflammatory response, positive regulation of lymphocytes, cell-cell adhesion, cell adhesion, cell migration, chemotaxis and integrin-mediated signaling pathway (Chart 3). The first one hundred genes with lowest adjusted p value were evaluated by cell type and tissue expression.

Chart 3 - Functional classification of genes up-regulated by at least 2-fold and with adjusted Pvalue<0.01 from CXCR4+ testicular cells pre-pubertal calves

continue

Biological process	Up regulated genes
Aging	*NOTCH4, ADA, CP, C1QB, ENG, INPP5D, LOXL2, NOS3, PTGS1, TGFB1*
Regulation of cell motion	*ARAP3, EGFL7, TEK, THY1, ACTN1, ACTN4, ADA, ACE, BDKRB1, CDH13, CXCL16, DLC1, DLL4, EDN1, EDN3, FLT1, HDAC7, ITGA2, JAG2, KDR, LAMA3 LAMA4, LAMA5, MMP9, PDGFB, RRAS, ROBO4, SELP, SEMA3F, S1PR1, THBS1, TGFB1, TIE1, ETS1, LYN*
Regulation of cell migration	*ABI3, ARAP3, EGFL7, TEK, THY1, ADA, ACE, BDKRB1, CDH13, CXCL16, DLC1, DLL4, EDN1, EDN3, FLT1, HDAC7, ITGA2, JAG2, KDR, LAMA3, LAMA4, LAMA5, MMP9, PDGFB, RRAS, ROBO4, SELP, S1PR1, THBS1, TGFB1, TIE1*
Regulation of cell adhesion	*FXYD5, ARHGDIB, RND1, ADA, CDH13, CX3CL1, COL8A1, DLL1, DMP1, ENG, EMCN, ITGA2, JAG2, LAMA3, LAMA4, LAMA5, PODXL, SPP1, S1PR1, THBS1, TGFB1, TGM2*
Cell adhesion	*ADAM15, CD34, CD72, CD93, CD97, F11R, FAT2, TEK, THY1, ACTN1, CDH13, CDH5, CADM3, CCL4, CX3CL1, CX3CR1, CLDN5, COL8A1, DLC1, EMR1, ENG, EMCN, ESAM, EPDR1, FN1, HSPG2, HAPLN3, ITGA1, ITGA2, ITGA2B, ITGA5, ITGA8, ITGB4, IBSP, ICAM2, LAMA3, LAMA4, LAMA5, LOXL2, MCAM, MSN, PARVB, PECAM1, PCDH1, PCDH12, PCDH17, PCDH19, SCARF1, SPP1, SELP, S1PR1, SNED1, THBS1, VWF*
Cell-cell adhesion	*CD93, FAT2, TEK, THY1, CDH13, CDH5, CADM3, CLDN5, EMCN, ESAM, ITGA5, ITGA8, ICAM2, MSN, PCDH1, PCDH12, PCDH17, PCDH19*

Biological process	Up regulated genes
Chemotaxis	*CCL14, CCL16, CCL3, CCL4, CCL8, CXCL11, CXCL16, CXCL3, CXCL9, CX3CL1, CX3CR1, C5AR1 ,ENG, ECSCR, EDN3, ITGA1, PLAU, PTAFR, PDGFB, SEMA3F*
Cell migration	*LHX6, S100A2, CDH13, CAV2, CXCL16, CXCL3, CX3CL1, ENG, EDN3, FN1, FLT1, ITGA1, ITGA5, KDR, LAMA5, MSN, NOS3, PLAU, PDGFB, PODXL, SELP, SEMA3F, THBS1, TGFB1*
Integrin-mediated signaling pathway	*ITGA1, ITGA2, ITGA2B, ITGA5, ITGA8, ITGB4, LAMA5*
Regulation of inflammatory response	*ADA, ADRB2, CX3CL1, IDO1, ITGA2, PPARG, C3, SBNO2, TLR4, TGM2*
Positive regulation of lymphocyte differentiation	*CD74, HLX, ADA, ITPKB, INPP5D, PNP, TGFBR2*

6.4.4 qPCR validation of RNA-seq results

To evaluate sexual maturation effect on CXCR4+ germ cells we selected six genes (*THY1, SELP, PCDH1, LIMS2, EFNA1* and *FST)* which RNA expression is correlated with undifferentiated germ cell or testicular development/spermatogenesis (Figure 10 A). RNA expression of *THY1, SELP, LIMS2, EFNA1* was previously detected in bovine testes (Figure 10 B). We then validated RNA-seq results by qPCR. The relative expression of *THY1* (fold change=3.946; p=0.0022), *SELP* (fold change=3.247; p=0,2448), *PCDH1* (fold change=2.912; p=0.0413), LIMS2 (fold change=5,317; p=0.004), *EFNA1* (fold change=2.532; p=0.029), and *FST* (fold change=2.584; p=0.033) were evaluated in sorted (CXCR4+) and unsorted (CXCR4) cells from pre-pubertal calves (Figure 11) Therefore, *THY1, PCDH1, LIMS2, EFNA1* and *FST* were higher expressed in CXCR4+ cells (Figure 11).

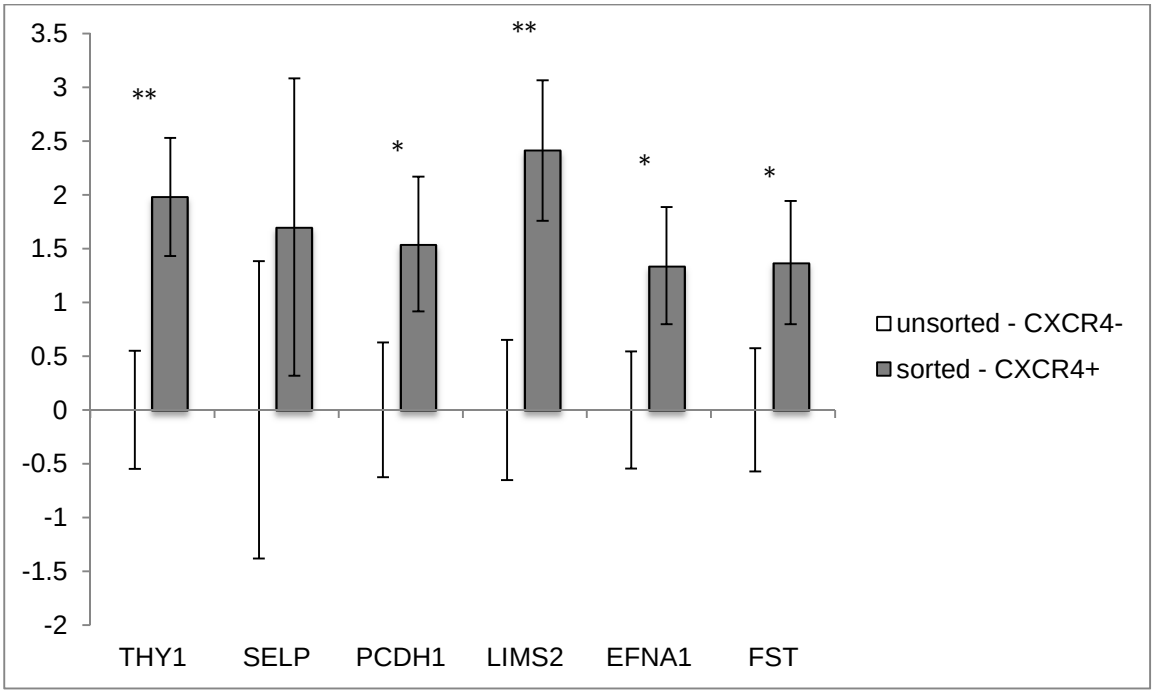
Figure 10 - Description of cellular and bovine tissue expression of selected genes *THY1, SELP, PCDH1, LIMS2, EFNA1* and *FST*. A) Fold Change and adjusted p value of each selected up-regulated gene with at least 2-fold in and adjusted Pvalue<0.01 from CXCR4+ testicular cells pre-pubertal calves and description note about type cell expression or biological process involved in spermatogenesis. B) Bovine tissue expression of *THY1, SELP, PCDH1, LIMS2, EFNA1* and *FST* with expression levels by iRAP method (data and image adopted by Atlas Expression from European Bioinformatics Institute part of European Molecular Biology Laboratory (KAPUSHESKY et al., 2012; PETRYSZAK et al., 2014)

A

Gene	Fold Change	Pvalue	Descriptional notes		
			Cell	Species	Author
THY	3,759	$2,11x10^{-08}$	Gonocytes and spermaotognia	Bovine	Reading et al. (2010)
			Gonocytes and spermaotognia	Pig	Zheng et al. (2013)
			Spermatogonia	Goat	Abbasi et al. (2013)
SELP	13,25	$6,24x10^{-09}$	Spermatogonia	Mouse	Kokkinaki et al. (2010)
			Spermatozoa	Human	Fuis et al. (1998)
PCDH1	2,276	$4,92x10^{-05}$	Primordial Germ Cell	Mouse	Hayashi et al. (2011)
LIMS2	5,154	$8,30x10^{-10}$	Testicular development/Spermatogenesis	Human	Liu et al. (2007)
EFNA1	4,2	$1,81x10^{-09}$	Primordial Germ Cell	Mouse	Hayashi et al. (2011)
FST	5,027	$1,85x10^{-10}$	type B spermatogonia/primary spermatocytes	Rat	Meinhard et al. (1998)

B

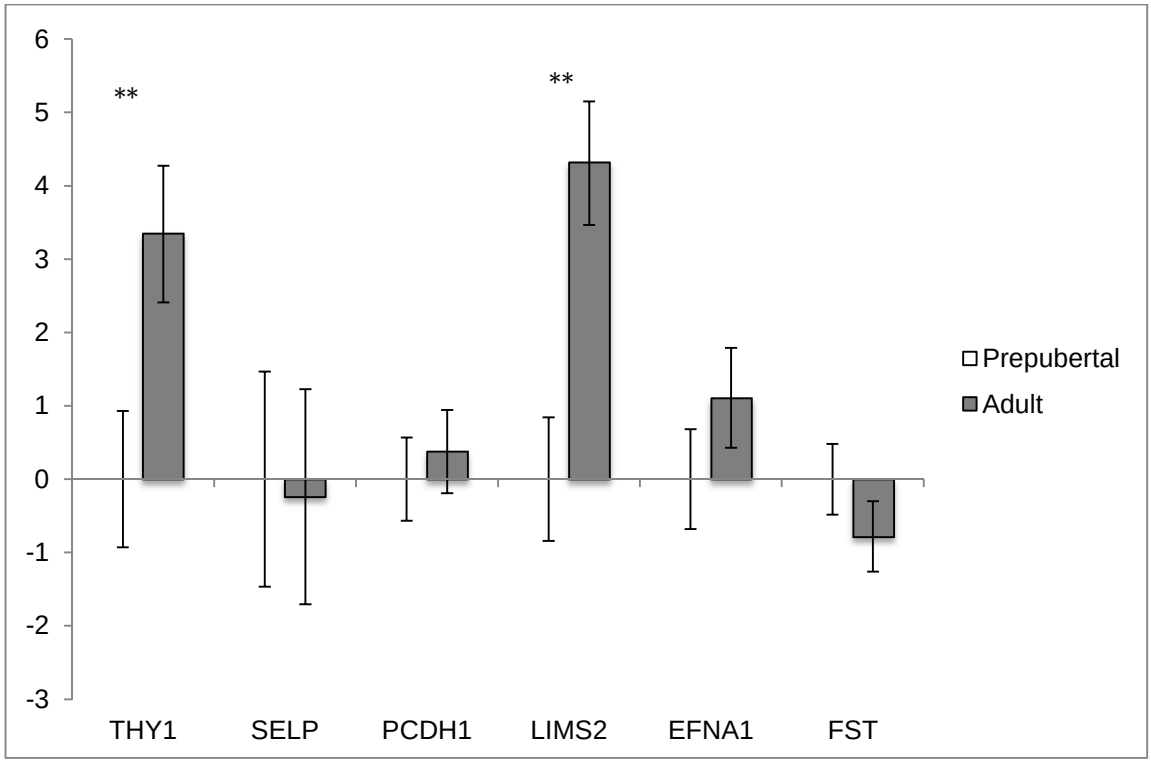
Figure 11 - Relative gene quantification (*THY1, SELP, PCDH1, LIMS2, EFNA1* and *FST* A) of unsorted (CXCR4-) and sorted (CXCR4-) cells from pre-pubertal calves testis. Values are presented as log_2 of Fold Change ± Ct standard deviation. Different letters in each gene indicate significant statistical difference (*P ≤ 0.05 or **P ≤ 0.01)



6.4.5 Differential gene expression between pre-pubertal calves and adult bulls

RNA-Seq library provided us a list of gene names list that are associated with important biological functions of CXCR4+ germ cell such as adhesion, migration, aging and chemotaxis. After the RNAseq validation, we evaluated the relative expression of *THY1* (fold change=10.143), *SELP* (fold change=0.847), *PCDH11* (fold change=1,296), *LIMS2* (fold change=19.824), EFNA11 (fold change=2,155), and *FST* (fold change=0.582) in sorted (CXCR4+) cells from pre-pubertal calves and adult bulls (Figure 7). We observed a punctual effect of age on *THY1* (p=0.001) and *LIMS2* (p=0.0071) that were higher expressed in adults (Figure 12). In this experiment, we performed a 2x2 factorial design (n=12) and we observed interaction between age and MACS fraction for relative expression of LIMS2 (p=0.0009) and Thy1 (p=0.0370). *LIMS2* and *THY1* were higher expressed in adult testicular cells but their expression were affected by MACS fraction thus, *LIMS2* and *THY1* were more expressed in +CXCR4 cells (Figure 11 and 12).

Figure 12 - Relative gene quantification (*THY1, SELP, PCDH1, LIMS2, EFNA1* and *FST* A) of sorted (CXCR4-) cells from pre-pubertal calves and adult bulls testes. Values are presented as log_2 of Fold Change ± Ct standard deviation. Different letters in each gene indicate significant statistical difference (**P ≤ 0.01)



6.4.6 CXCR4+ cells transplantation

Transplantation is the golden standard functional assay to characterize stem cell activity of SSC and gonocytes. In this study, we observed that CXCR4+ germ cells from pre-pubertal and adult bulls also were able to colonize nude testes (Figure 13 and Figure 14). Total number and of colonies was affected by age, by MACS fraction but was not affected by age and MACS fraction interaction Normalized number of colonies (colonies/10^4 injected-cells) was also affected by age, by MACS fraction but was not affected by interaction age and MACS fraction (Figure 14). However, we did not observe effect of age on fold enrichment of the normalized colony number comparing sorted and unsorted population (p=0.1530; Figure 15)

Figure 13 - Stem cell capacity of CXCR+ testicular cells from pre-pubertal calves and adult bulls. Representative image of high proliferating cell colonies transplanted germ cells (red; PKH26 Fluorescent Cell Linker) in testis of infertile recipient mice

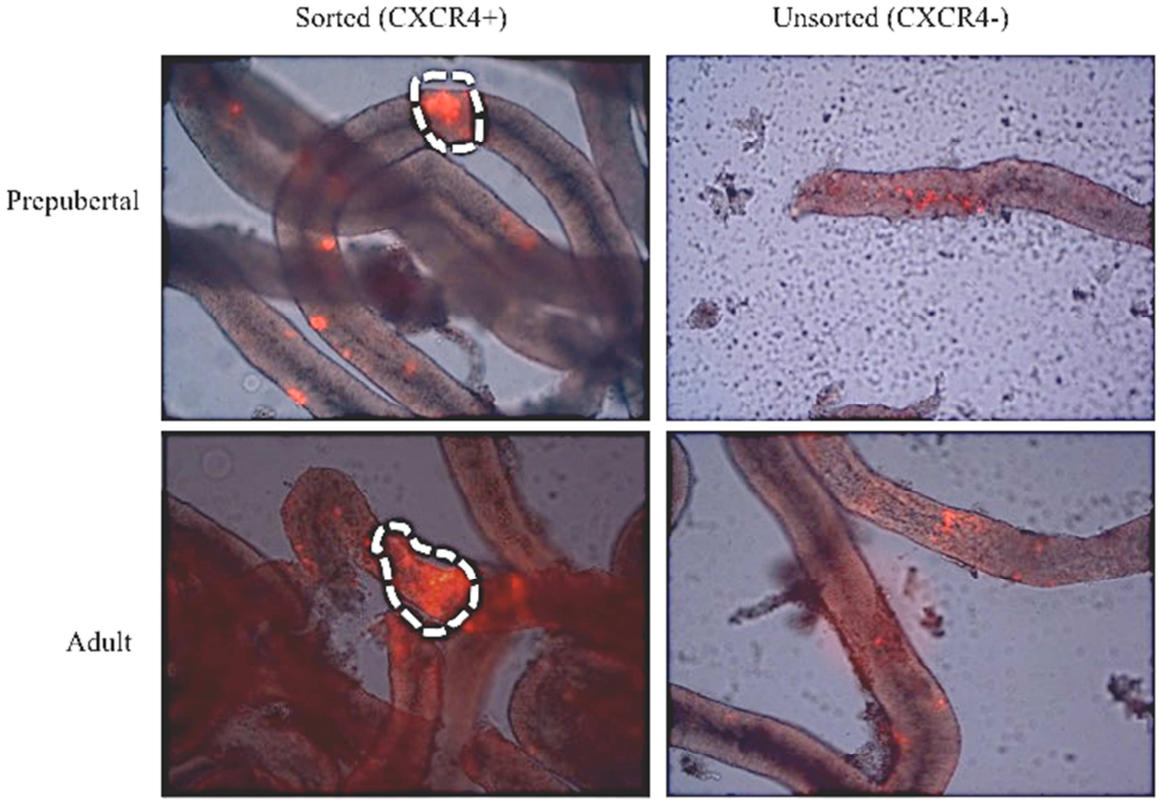


Figure 14 - Stem cell capacity of CXCR+ testicular cells from pre-pubertal calves and adult bulls. Effect of cell enrichment by MACS (CXCR+ or CXCR-) and sexual maturation (pre-pubertal and adult) in total number of high proliferating colonies per testes and adjusted number of high proliferating colonies by number of injected cells (10^4) per testis. Total number of colonies was affected by age (p=0.0016), by MACS fraction (p=0.0392) but was not affected by interaction age and MACS fraction (p=0.6722). Total number of colonies/10^4 injected-cells was affected by age (p=0.0036), by MACS fraction (p=0.0306) but was not affected by iteration age and MACS fraction (p=0.6078). Variables (mean ± S.E.M) *P ≤ 0.05 and **P ≤ 0.01

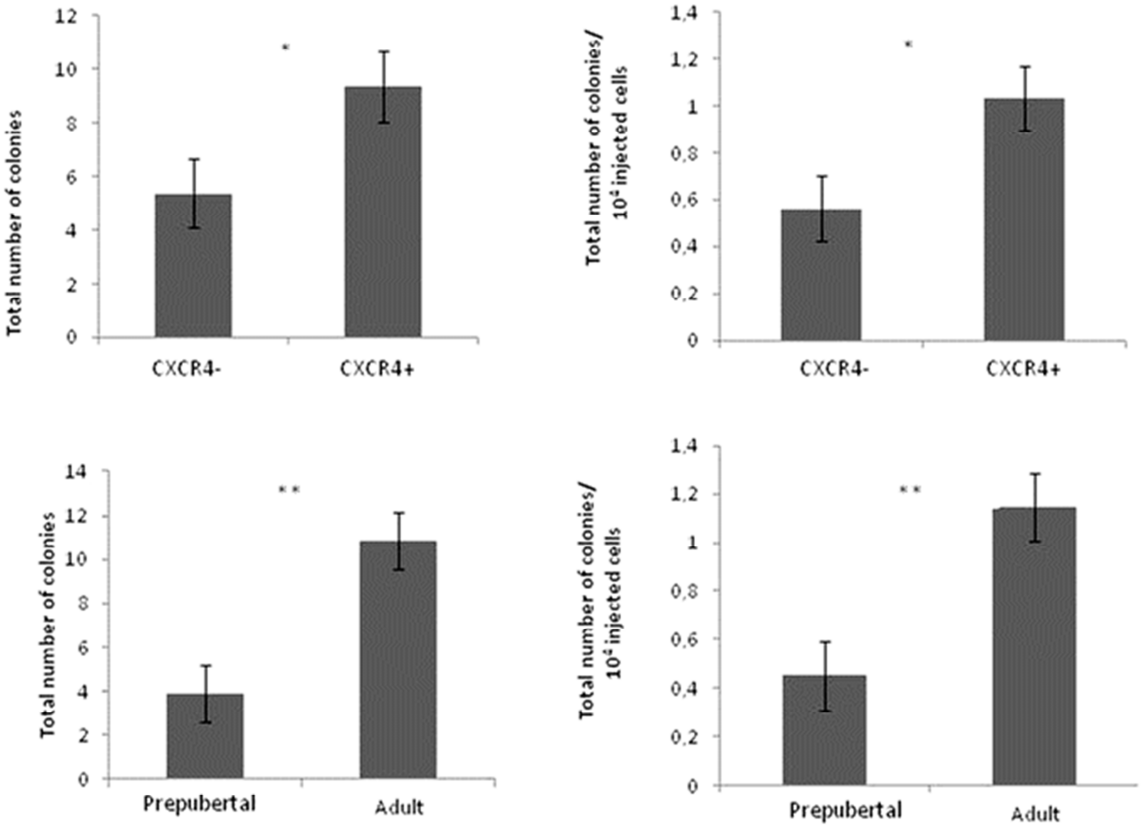
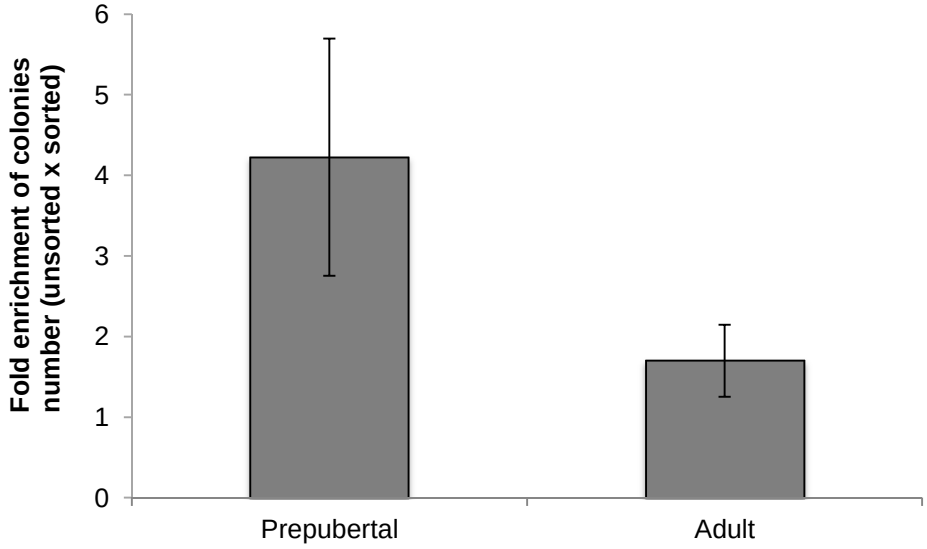


Figure 14 - Fold enrichment of colonies number (total number of colonies from CXCR4+ by total number of colonies CXCR4-) from pre-pubertal and adult isolated cells. Variables (mean ± S.E.M) *$P \leq 0.05$ and **$P \leq 0.01$



6.5 DISCUSSION

Gonocytes migrate to peripheral basement membrane of the seminiferous cord during the pre-pubertal phase. In the correct niche, gonocytes differentiate into SSC helping to provide the sexual maturation. In a normal testis, CXCR4 and CXCL12 are pivotal factors for gonocytes migration, SSCs proliferation and development of normal testes (WU et al., 2011; YANG et al., 2013). However the sexual maturation effect on CXCR4+ cells is not clear in mammals with a long pre-pubertal phase. In this study, we aimed to verify if sexual maturation affect gene expression and stem cell potential of CXCR4+ cells from pre-pubertal calves and adult bulls testes.

We performed global transcriptome analysis of CXCR4+ and CXCR4- pre-pubertal cells. This analysis revealed that biological functions of differentially expressed genes are correlated with regulation of cell motion, adhesion, migration, inflammatory response, positive regulation of lymphocytes, cell-cell adhesion, cell adhesion, cell migration, chemotaxis and integrin-mediated signaling pathway were up-regulated in CXCR4+ germ cells. CXCL12-CXCR4 signaling is involved in cell locomotion and adhesion (KUCIA et al., 2004), chemotaxis of several cells type such

as lymphocytes (BLEUL et al., 1996) and it is also a pivotal factor between normal and tumor stem cell migration (KUCIA et al., 2005; MCIVER et al., 2013) being an essential factor in tumor metastases (MÜLLER et al., 2001). CXCL12-CXCR4 interaction regulates primordial germ cells migration to gonads, SSC maintenance and self-renewal, playing an important role in spermatogenesis (MOLYNEAUX et al., 2003; YANG et al., 2013)

CXCR4 is expressed by different types of undifferentiated germ cells in testes of pre-pubertal calves and adult bulls. It is well known that transcriptional expression of germ cell are affected by spermatogenesis (LIANG et al., 2014). We observed that sexual maturation affect expression of *LIMS2* and *THY1* that were highly expressed in CXCR4+ positive cells from adult bulls, which also are able to colonize recipient testes In human, *LIMS2* expression was up-regulated in adult when compared to fetal testes and other tissues thus, *LIMS2* should be correlated to spermatogenesis development (LIU et al., 2007). The overexpression of *LIMS2* in adult CXCR4+ should be associated to the functional changes of gonocytes to SSCs such as lost of migration ability. LIMS2 is a negative regulator of LIMS1-ILK-parvin complex, which is correlated with cell spreading and migration via integrin at focal adhesions (KIM et al., 2006). We observed that *LIMS2* is overexpressed in bull CXCR4+ germ cells with stem cell properties. Gonocytes, migratory germ cells with stem cell properties, had low expression of *LIMS2.* Thus we speculates that the aging effect at expression of LimS2 should be correlated with migratory state and might be considered as marker for undifferentiated germ cell movement.

We also observed that CXCR4+ germ cells from adult express more *THY1* transcripts than pre-pubertal cells; however, we did not observe a MACS fraction effect on THY1 expression by prepubertal testicular cells. We observed that *THY1* are overexpress by spermatogonia in oldest animals. Our results agree with that previously reported for UCHL1, DBA and OCT4, which are more expressed in seminiferous cord from adult bulls (2 years old) than pre-pubertal calves (1.5 week to 5.5 months) (FUJIHARA et al., 2011), indicating that expression of markers for undifferentiated germ cells are indeed affected by age .

CXCL12 and CXCR4 transcripts are expressed by undifferentiated germ cells thought testicular development being up-regulated adult mice. We demonstrated that CXCR4+ germ cells (gonocytes and spermatogonia) from pre-pubertal calves (5

months age) were able to recolonize nude mice testes (GOISSIS et al., 2015[10]). In the present study, we observed that age of bovine donor (pre-pubertal and adult) affects the colonization efficiency after transplantation. A higher number of colonies were observed in adult when compared to pre-pubertal donors probably because CXCR4+ gonocytes are pooled with round and pseudopod morphologies, which present different stem cell potential. The characterization of round and pseudopod gonocytes depends of their cellular morphology and not protein expression and, their sorting is generally performed by micromanipulation that is the most effective method to isolate round to the pseudopod gonocytes (ORWIG et al., 2002). Thus, CXCR4 could be considered also as a conserved SSC marker for adult bulls.

In summary, the effect of sexual maturation on CXCR4+ germ cells described is this study may be useful to understand selected gene function outcome on mammalian spermatogenesis with long pre-pubertal phase duration such bovines, nonhumans primates and humans.

6.6 CONCLUSION

CXCR4 is a conserved marker for undifferentiated germ cell from calves and adult bull. CXCR4+ cells transcriptome from pre-pubertal calves and adult bulls testes is affected by sexual maturation.

.

Acknowledgements

This research was funding supported by São Paulo Research Foundation (FAPESP) and National Counsel of Technological and Scientific Development (CNPq). Authors would like to thank Prefeitura do Campus de Pirassununga for providing the bull calves used in this experiment; Carolina D. N. Pereira for

[10] GOISSIS, M. D.; GIASSETTI, M. I.; WORST, R. A.; MENDES, C. M.;. MOREIRA, P. V.; ASSUMPÇÃO, M. E. O. A.; VISINTIN, J. A. Spermatogonial stem cell potential of CXCR4-positive cells from prepubertal bovine testis, [2015]. Data not published.

assistance with mouse anesthesia Profa. Cristina de Oliveira Massoco Salles Gomes and Dra. Nicolle Queiroz in the Department of Pathology for assistance with flow cytometry, Prof. Paulo Maiorka in the Department of Pathology and Prof. Ricardo J. G. Pereira in Department of Animal Reproduction for assistance with sample preparation for immunohistochemistry

.

REFERENCE

BLEUL, C. C.; FARZAN, M.; CHOE, H.; PAROLIN, C.; CLARK-LEWIS, I.; SODROSKI, J.; SPRINGER, T. A. The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry. **Nature,** v. 382, n. 6594, p. 829-33, Aug 1996. ISSN 0028-0836. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/8752280 >. Acessed at: 24. march. 2015.

CHAMBERS, S. M.; SHAW, C. A.; GATZA, C.; FISK, C. J.; DONEHOWER, L. A.;GOODELL, M. A. Aging hematopoietic stem cells decline in function and exhibit epigenetic dysregulation. **PLoS Biol,** v. 5, n. 8, p. e201, Aug 2007. ISSN 1545-7885. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/17676974 >. Acessed at: 24. march. 2015.

COSTA, G. M.; AVELAR, G. F.; REZENDE-NETO, J. V.; CAMPOS-JUNIOR, P. H.; LACERDA, S. M.; ANDRADE, B. S.; THOMÉ, R. G.; HOFMANN, M. C.; FRANCA, L. R. Spermatogonial stem cell markers and niche in equids. **PLoS One,** v. 7, n. 8, p. e44091, 2012. ISSN 1932-6203. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/22937157 >. Acessed at: 24. march. 2015.

CURTIS, S. K.; AMANN, R. P. Testicular development and establishment of spermatogenesis in Holstein bulls. **J Anim Sci,** v. 53, n. 6, p. 1645-57, Dec 1981. ISSN 0021-8812. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/7341622 >. Acessed at: 24. march. 2015.

FUJIHARA, M.; KIM, S. M.; MINAMI, N.; YAMADA, M.; IMAI, H. Characterization and in vitro culture of male germ cells from developing bovine testis. **J Reprod Dev,** v. 57, n. 3, p. 355-64, Jun 2011. ISSN 1348-4400. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/21289464 >. Acessed at: 24. march. 2015.

HERMANN, B. P.; SUKHWANI, M.; SIMORANGKIR, D. R.; CHU, T.; PLANT, T. M.; ORWIG, K. E**.** Molecular dissection of the male germ cell lineage identifies putative spermatogonial stem cells in rhesus macaques. **Hum Reprod,** v. 24, n. 7, p. 1704-16, Jul 2009. ISSN 1460-2350. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/19336441 >. Acessed at: 24. march. 2015.

HUANG, D. W.; SHERMAN, B. T.; LEMPICKI, R. A. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. **Nat Protoc,** v. 4, n. 1, p. 44-57, 2009. ISSN 1750-2799. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/19131956 >. Acessed at: 24. march. 2015.

IZADYAR, F.; MATTHIJS-RIJSENBILT, J. J.; DEN OUDEN, K.; CREEMERS, L. B.; WOELDERS, H.; DE ROOIJ, D. G. Development of a cryopreservation protocol for type A spermatogonia. **J Androl,** v. 23, n. 4, p. 537-45, 2002 Jul-Aug 2002. ISSN 0196-3635. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/12065461 >. Acessed at: 24. march. 2015.

KAPUSHESKY, M.; ADAMUSIAK, T.; BURDETT, T.; CULHANE, A.; FARNE, A.; FILIPPOV, A.; HOLLOWAY, E.; KLEBANOV, A.; KRYVYCH, N.; KURBATOVA, N.; KURNOSOV, P.; MALONE, J.; MELNICHUK, O.; PETRYSZAK, R.; PULTSIN, N.; RUSTICI, G.; TIKHONOV, A.; TRAVILLIAN, R. S.; WILLIAMS, E.; ZORIN, A.; PARKINSON, H.; BRAZMA, A. Gene Expression Atlas update--a value-added database of microarray and sequencing-based functional genomics experiments. **Nucleic Acids Res,** v. 40, n. Database issue, p. D1077-81, Jan 2012. ISSN 1362-4962. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/22064864 >. Acessed at: 24. march. 2015.

KERR, J. B.; LOVELAND, K. L.; O'BRYAN, M. K.; DE KRETSER, D. M**.** Cytology of the Testis and Intrinsic Control Mechanisms. In: NEILL, J. D. (Ed.). **Knobil and Neill's Physiology of Reproduction**. 3ed. Oxford, UK: Elsevier Inc, v.1, 2006. chap. Cytology of the Testis and Intrinsic Control Mechanisms, p.827-948. (Male Reproductive System).

KIM, S. K.; JANG, H. R.; KIM, J. H.; NOH, S. M.; SONG, K. S.; KIM, .M. R.; KIM, S. Y.; YEOM, Y. I.; KIM, N. S.; YOO, H. S.; KIM, Y. S The epigenetic silencing of LIMS2 in gastric cancer and its inhibitory effect on cell migration. **Biochem Biophys Res Commun,** v. 349, n. 3, p. 1032-40, Oct 2006. ISSN 0006-291X. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/16959213 >. Acessed at: 24. march. 2015.

KOKKINAKI, M.; LEE, T. L.; HE, Z.; JIANG, J.; GOLESTANEH, N.; HOFMANN, M. C.; CHAN, W. Y.; DYM, M. Age affects gene expression in mouse spermatogonial stem/progenitor cells. **Reproduction,** v. 139, n. 6, p. 1011-20, Jun 2010. ISSN 1741-7899. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/20371641 >. Acessed at: 24. march. 2015.

KUBOTA, H.; AVARBOCK, M. R.; BRINSTER, R. L. Spermatogonial stem cells share some, but not all, phenotypic and functional characteristics with other stem cells. **Proc Natl Acad Sci U S A,** v. 100, n. 11, p. 6487-92, May 2003. ISSN 0027-8424. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/12738887 >. Acessed at: 24. march. 2015.

KUCIA, M.; JANKOWSKI, K.; RECA, R.; WYSOCZYNSKI, M.; BANDURA, L.; ALLENDORF, D. J.; ZHANG, J.; RATAJCZAK, J.; RATAJCZAK, M. Z. CXCR4-SDF-1 signalling, locomotion, chemotaxis and adhesion. **J Mol Histol,** v. 35, n. 3, p. 233-45, Mar 2004. ISSN 1567-2379. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/15339043 >. Acessed at: 24. march. 2015.

KUCIA, M.; RECA, R.; MIEKUS, K.; WANZECK, J.; WOJAKOWSKI, W.; JANOWSKA-WIECZOREK, A.; RATAJCZAK, J.; RATAJCZAK, M. Z. Trafficking of normal stem cells and metastasis of cancer stem cells involve similar mechanisms: pivotal role of the SDF-1-CXCR4 axis. **Stem Cells,** v. 23, n. 7, p. 879-94, Aug 2005. ISSN 1066-5099. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/15888687 >. Acessed at: 24. march. 2015.

LEE, K. H.; LEE, W. Y.; KIM, J. H.; YOON, M. J.; KIM, N. H.; UHM, S. J.; KIM, D. H.; CHUNG, H. J.; SONG, H. Characterization of GFRα-1-positive and GFRα-1-negative spermatogonia in neonatal pig testis. **Reprod Domest Anim,** v. 48, n. 6, p. 954-60,

Dec 2013. ISSN 1439-0531. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/23808407 >. Acessed at: 24. march. 2015.

LIANG, M.; LI, W.; TIAN, H.; HU, T.; WANG, L.; LIN, Y.; LI, Y.; HUANG, H.; SUN, F. Sequential expression of long noncoding RNA as mRNA gene expression in specific stages of mouse spermatogenesis. **Sci Rep,** v. 4, p. 5966, 2014. ISSN 2045-2322. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/25097017 >. Acessed at: 24. march. 2015.

LIU, Y.; LIU, J.; CHEN, J.; CHENG, L.; CAO, Q.; ZHU, L.; SUN, Y.; LIU, Q.; LI, J. Molecular cloning and characterization of a novel splice variant of the LIM domain family gene, PINCH 2, in human testis. **Mol Biotechnol,** v. 35, n. 2, p. 109-18, Feb 2007. ISSN 1073-6085. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/17435276 >. Acessed at: 24. march. 2015.

MCIVER, S. C.; LOVELAND, K. L.; ROMAN, S. D.; NIXON, B.; KITAZAWA, R.; MCLAUGHLIN, E. A**.** The chemokine CXCL12 and its receptor CXCR4 are implicated in human seminoma metastasis. **Andrology,** v. 1, n. 3, p. 517-29, May 2013. ISSN 2047-2927. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/23495012 >. Acessed at: 24. march. 2015.

MENG, X.; LINDAHL, M.; HYVÖNEN, M. E.; PARVINEN, M.; DE ROOIJ, D. G.; HESS, M. W.; RAATIKAINEN-AHOKAS, A.; SAINIO, K.; RAUVALA, H.; LAKSO, M.; PICHEL, J. G.; WESTPHAL, H.; SAARMA, M.; SARIOLA, H. Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. **Science,** v. 287, n. 5457, p. 1489-93, Feb 2000. ISSN 0036-8075. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/10688798 >. Acessed at: 24. march. 2015.

MOLYNEAUX, K. A.; ZINSZNER, H.; KUNWAR, P. S.; SCHAIBLE, K.; STEBLER, J.; SUNSHINE, M. J.; O'BRIEN, W.; RAZ, E.; LITTMAN, D.; WYLIE, C.; LEHMANN, R. The chemokine SDF1/CXCL12 and its receptor CXCR4 regulate mouse germ cell migration and survival. **Development,** v. 130, n. 18, p. 4279-86, Sep 2003. ISSN 0950-1991. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/12900445 >. Acessed at: 24. march. 2015.

MÜLLER, A.; HOMEY, B.; SOTO, H.; GE, N.; CATRON, D.; BUCHANAN, M. E.; MCCLANAHAN, T.; MURPHY, E.; YUAN, W.; WAGNER, S. N.; BARRERA, J. L.; MOHAR, A.; VERÁSTEGUI, E.; ZLOTNIK, A.l. Involvement of chemokine receptors in breast cancer metastasis. **Nature,** v. 410, n. 6824, p. 50-6, Mar 2001. ISSN 0028-0836. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/11242036 >. Acessed at: 24. march. 2015.

NAUGHTON, C. K.; JAIN, S.; STRICKLAND, A. M.; GUPTA, A.; MILBRANDT, J. Glial cell-line derived neurotrophic factor-mediated RET signaling regulates spermatogonial stem cell fate. **Biol Reprod,** v. 74, n. 2, p. 314-21, Feb 2006. ISSN 0006-3363. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/16237148 >. Acessed at: 24. march. 2015.

OATLEY, J. M.; DE AVILA, D. M.; MCLEAN, D. J.; GRISWOLD, M. D.; REEVES, J. J.l. Transplantation of bovine germinal cells into mouse testes. **J Anim Sci,** v. 80, n.

7, p. 1925-31, Jul 2002. ISSN 0021-8812. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/12162661 >. Acessed at: 24. march. 2015.

ORWIG, K. E.; RYU, B. Y.; AVARBOCK, M. R.; BRINSTER, R. L. Male germ-line stem cell potential is predicted by morphology of cells in neonatal rat testes. **Proc Natl Acad Sci U S A,** v. 99, n. 18, p. 11706-11, Sep 2002. ISSN 0027-8424. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/12185252 >. Acessed at: 24. march. 2015.

PETRYSZAK, R.; BURDETT, T.; FIORELLI, B.; FONSECA, N. A.; GONZALEZ-PORTA, M.; HASTINGS, E.; HUBER, W.; JUPP, S.; KEAYS, M.; KRYVYCH, N.; MCMURRY, J.; MARIONI, J. C.; MALONE, J.; MEGY, K.; RUSTICI, G.; TANG, A. Y.; TAUBERT, J.; WILLIAMS, E.; MANNION, O.; PARKINSON, H. E.; BRAZMA, A. Expression Atlas update--a database of gene and transcript expression from microarray- and sequencing-based functional genomics experiments. **Nucleic Acids Res,** v. 42, n. Database issue, p. D926-32, Jan 2014. ISSN 1362-4962. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/24304889 >. Acessed at: 24. march. 2015.

REDING, S. C.; STEPNOSKI, A. L.; CLONINGER, E. W.; OATLEY, J. M. THY1 is a conserved marker of undifferentiated spermatogonia in the pre-pubertal bull testis. **Reproduction,** v. 139, n. 5, p. 893-903, May 2010. ISSN 1741-7899. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/20154176 >. Acessed at: 24. march. 2015.

RYU, B. Y.; ORWIG, K. E.; KUBOTA, H.; AVARBOCK, M. R.; BRINSTER, R. L. Phenotypic and functional characteristics of spermatogonial stem cells in rats. **Dev Biol,** v. 274, n. 1, p. 158-70, Oct 2004. ISSN 0012-1606. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/15355795 >. Acessed at: 24. march. 2015.

SPRADLING, A.; DRUMMOND-BARBOSA, D.; KAI, T. Stem cells find their niche. **Nature,** v. 414, n. 6859, p. 98-104, Nov 2001. ISSN 0028-0836. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/11689954 >. Acessed at: 24. march. 2015.

STEIBEL, J. P.; POLETTO, R.; COUSSENS, P. M.; ROSA, G. J. A powerful and flexible linear mixed model framework for the analysis of relative quantification RT-PCR data. **Genomics,** v. 94, n. 2, p. 146-52, Aug 2009. ISSN 1089-8646. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/19422910 >. Acessed at: 24. march. 2015.

TANG, L.; RODRIGUEZ-SOSA, J. R.; DOBRINSKI, I. Germ cell transplantation and testis tissue xenografting in mice. **J Vis Exp**, n. 60, 2012. ISSN 1940-087X. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/22330955 >. Acessed at: 24. march. 2015.

WESTERNSTRÖER, B.; TERWORT, N.; EHMCKE, J.; WISTUBA, J.; SCHLATT, S.; NEUHAUS, N. Profiling of Cxcl12 receptors, Cxcr4 and Cxcr7 in murine testis development and a spermatogenic depletion model indicates a role for Cxcr7 in controlling Cxcl12 activity. **PLoS One,** v. 9, n. 12, p. e112598, 2014. ISSN 1932-6203. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/25460567 >. Acessed at: 24. march. 2015.

WU, X.; GOODYEAR, S. M.; TOBIAS, J. W.; AVARBOCK, M. R.; BRINSTER, R. L. Spermatogonial stem cell self-renewal requires ETV5-mediated downstream activation of Brachyury in mice. **Biol Reprod,** v. 85, n. 6, p. 1114-23, Dec 2011. ISSN 1529-7268. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/21816850 >. Acessed at: 24. march. 2015.

YANG, Q. E.; KIM, D.; KAUCHER, A.; OATLEY, M.J.; OATLEY, J.M. CXCL12-CXCR4 signaling is required for the maintenance of mouse spermatogonial stem cells. **J Cell Sci,** v. 126, n. Pt 4, p. 1009-20, Feb 2013. ISSN 1477-9137. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/23239029 >. Acessed at: 24. march. 2015.

ZHENG, Y.; HE, Y.; AN, J.; QIN, J.; WANG, Y.; ZHANG, Y.; TIAN, X.; ZENG, W. THY1 is a surface marker of porcine gonocytes. **Reprod Fertil Dev,** v. 26, n. 4, p. 533-9, 2014. ISSN 1031-3613. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/23683542 >. Acessed at: 24. march. 2015.
ZHENG, Y.; ZHANG, Y.; QU, R.; HE, Y.; TIAN, X.; ZENG, W. Spermatogonial stem cells from domestic animals: progress and prospects. **Reproduction,** v. 147, n. 3, p. R65-74, Mar 2014. ISSN 1741-7899. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/24357661 >. Acessed at: 24. march. 2015.

7 CHAPTER 4: SELECTED YY2 BINDING SITES POSITIVELY MEDIATE TRANSCRIPTIONAL ACTIVITY IN SPERMATOGONIAL STEM CELLS

7.1 SUMMARY

Genes encoding YY2 and REX1 are uniquely present in placental mammals, and have both been generated by retrotransposition from the ancestral gene YY1. REX1 is a well known marker of embryonic stem (ES) cells, which however is also expressed in placenta and testis. Moreover, our initial results indicated that YY2 may be expressed in undifferentiated germ cells and spermatogonia stem cells (SSC). This study aims to clarify outstanding questions regarding the presence of YY2 in SSCs in postpuberal mouse and in culture, using immunohistochemistry and immunofluorescence. We observed that YY2 was specifically expressed in undifferentiated germ cell in situ and with a nuclear localization in vitro. However, the role of YY2 in undifferentiated germ cells or during spermatogenesis has not been defined sofar. SSC might have a future use as a tool in transgenesis, facilitating fertility treatments. However, molecular markers that allow unequivocal identification of these cells are still lacking, limiting its present use. A second aim of this study is to validate the possibility that YY2 (and to lesser extent REX1) and their target genes, may represent more specific markers. Reporter vectors driven by REX1 and YY2 binding sites recently identified in vivo have been constructed. These vectors have been used to verify that genomic sequences containing REX1 and/or YY2 binding sites may control gene expression in SSC. We observed that selected REX1 and YY2 binding sites, positively control the activity of a reporter gene in ES and SSC, respectively. In conclusion, YY2 is highly expressed in vitro in mouse SSCs and in vivo in undifferentiated spermatogonia after birth. YY2 may play a role in genome management at these stages concerning the control of retroviral elements. In conclusion, our data are compatible with a function for YY2 SSC and with the hypothesis that genomic loci bound by YY2 might indicate sequences relevant for gene expression in SSC.

Keywords: YY2. REX1. Spermatogonia. SSC. Gene expression

7.2 INTRODUCTION

Rex1/Zfp42 encodes a zinc finger protein predominantly expressed in Embryonic Stem (ES) cells (ROGERS; HOSLER; GUGAS, 1991; MASUI *et al.*, 2008). Expression at lower levels has also been described throughout pre-implantation mouse development (CLIMENT et al., 2013), in early trophectoderm derivatives and placenta in the mouse (ROGERS M *et al*., 1991; KIM *et al*., 2011) and during spermatogenesis (REZENDE et al. 2011). Although deficiency of *Rex1/Zfp42* does not display a severe phenotype in ES cells (GUALLAR et al., 2011, MASUI *et al*., 1998) expression is frequently used to identify pluripotent stem cells. REX1 controls the initiation of XCI in female ES cells (GONTAN et al., 2012), through regulation of the non-coding RNA *Tsix* (NAVARRO et al., 2010) regulates imprinted gene expression (KIM *et al*., 2011) and expression of Endogenous Retroviral Elements especially *MERVL* (GUALLAR et al., 2011). Weak expression of *Rex1* has also been reported in spermatocytes undergoing meiosis (ROGERS; HOSLER; GUGAS, 1991) and Rex1-deficient mice show abnormal testis morphology, age-associated decrease in sperm counts and abnormal sperm morphology (REZENDE et al., 2011). Although deregulation of some testis-specific genes was demonstrated (REZENDE et al., 2011), Rex1-regulated gene expression underlying this phenotype was only partially characterized.

Yin Yang 2 (Yy2) is a DNA-binding transcription factor reportedly expressed in almost all cell types, tissues and stages throughout the entire E11.5 embryo suggesting ubiquitous expression (LUO *et al.*, 2006; CHENG *et al.*, 2008). Varying levels of expression among tissues have been reported, with high levels in testis (DREWS et al., 2009). RNA expression studies indicated that the *Yy2* gene was highly expressed in all layers of spermatocytes, but was not detected in sperm cells (LUO et al., 2006). However, immunohistochemistry on testis sections indicated a more restricted presence of YY2 protein in 3 day-old mouse gonocytes (PEREZ-

PALACIOS et al., 2014[11]). These preliminary results suggested that YY2 expression is not ubiquitous, as in the testis YY2 is highly expressed in spermatogonia but we did not observe YY2 in differentiating spermatocytes (PEREZ-PALACIOS et al., 2014[12]).

YY2 and REX1 are members of the same protein family and have been generated by retroposition from the–well-conserved zinc finger gene *Yin Yang 1 (YY1)*. The retroposition events giving rise to *Rex1* and *Yy2*, respectively, occurred after divergence of the lineage of placental mammals and thus REX1 and YY2 expression are exclusive for placental mammals (LUO *et al.*, 2006). YY1, YY2 and REX1 share 95% homology in the zinc-finger DNA-binding region (MONGAN; MARTIN; GUDAS, 2006; KIM; FAULK; KIM, 2007) indicating these three transcription factors might share functions. YY1 controls transcription of several genes but its function as an activator or as a repressor depends on the particular YY1 binding site and on its interaction with other transcription factors (THOMAS; SETO, 1999) and a variety of chromatin-modifying complexes (reviewed in ATCHISON *et al.*, 2011; SCHOORLEMMER *et al.*, 2014). Among those, YY1 is a member of the Polycomb protein Group of genes (PcG), which controls plasticity of the pluripotent state and stem cell development (SCHUETTENGRUBER *et al.*, 2007; KÖHLER; VILLAR, 2008).

Spermatogenesis is a classical example of stem cell differentiation throughout male reproductive life. After birth, immature germ cells called gonocytes proliferate, migrate towards the basal membrane (KERR *et al.*, 2006) along the seminiferous cord parenchyma and differentiate into spermatogonial stem cells (SSC). In their definitive niche, SSC remain attached to basal membrane (ORWIG *et al.*, 2002) surrounded by Sertoli cells (SPRADLING; DRUMMOND-BARBOSA; KAI, 2001). In mice, the conversion of gonocytes to SSC happens between 0-6 dpp (CURTIS; AMANN, 1981). However, a simple terminology classified gonocytes as undifferentiated germ cells that are centrally allocated in seminiferous tubules and SSC are those attached to the basal membrane (KERR *et al.*, 2006). In spermatogenesis, spermatogonia type A cells can be divided in A_s (single), A_{pr} (paired) and A_{al} (aligned) type cells. In mice, only 0.02-0.03% of total germ cells are

[11] PEREZ-PALACIOS, R.; GIASSETTI, M. I.; ACÍN, C.; CLIMENT, M.; GUALLAR, D.; MUNIESA, P.; SCHOORLEMMER, J. On a possible role for YY2 in male germline, [2014]. Data not published.
[12] PEREZ-PALACIOS, R.; GIASSETTI, M. I.; ACÍN, C.; CLIMENT, M.; GUALLAR, D.; MUNIESA, P.; SCHOORLEMMER, J. On a possible role for YY2 in male germline, [2014]. Data not published.

stem cells (TEGELENBOSCH; DE ROOIJ, 1993). A_s cells either divide to produce two new, separate A_s cells (self-renewal), or generate daughter cells whose intracellular connections are maintained, producing four A_{al} cells in the next division. Thus, large chains with 8 up to 32 connected and differentiated cells are generated and referred to as A_{al} spermatogonia. Committed spermatogonia (A1, A2, A3, A4, intermediated and B) divide in a synchronized manner in seminiferous tubule epithelium. Type B spermatogonia in turn give rise to primary spermatocytes which progress in the spermatogenic cycle, initiate meiosis and finally turn into spermatozoa (HUCKINS, 1971; OAKBERG, 1971; PHILLIPS; GASSEI; ORWING, 2010).

Pseudopods gonocytes and undifferentiated spermatogonia have similar stem cell ability (ORWIG *et al.*, 2002) and also share specific biological markers (ZHENG *et al.*, 2014) such as GDNF family receptor alpha-1 (GFRA1) (MENG *et al.*, 2000; NAUGHTON *et al.*, 2006; COSTA *et al.*, 2012; LEE *et al.*, 2013), Thy-1 Cell Surface Antigen (THY1) (KUBOTA; AVARBOCK; BRINSTER, 2003; RYU *et al.*, 2004; HERMANN *et al.*, 2009; REDING *et al.*, 2010; ZHENG, HE*, et al.*, 2014) and Chemokine (C-X-C Motif) Receptor 4 (CXCR4) (YANG *et al.*, 2013; WESTERNSTRÖER *et al.*, 2014). Recently, we observed that YY2 is expressed in gonocytes/undifferentiated germ cells, as well as in SSC in culture and that YY2 specifically binds to selected Retroviral Elements in cultured SSC cells (PÉREZ-(PEREZ-PALACIOS et al., 2014[13]). However, the role of YY2 in undifferentiated germ cells or during spermatogenesis has not been defined sofar. The aim of this study is to clarify questions regarding the expression of YY2 in postpuberal mouse testis and regarding the intracellular localization of YY2 in SSC. Genomic in vivo targets of YY2 in mouse SSC cells have recently been reported (PEREZ-PALACIOS et al., 2014[14]). A second aim of this study is to verify their ability to influence gene expression and transcriptional control at a distance. As we report REX1 expression both in premeiotic spermatocytes (PEREZ-PALACIOS et al., 2014[15]). and in SSCs in culture, we also tested the possibility that DNA elements bound by REX1 in ES cell display SSC-specific activity.

[13] PEREZ-PALACIOS, R.; GIASSETTI, M. I.; ACÍN, C.; CLIMENT, M.; GUALLAR, D.; MUNIESA, P.; SCHOORLEMMER, J. On a possible role for YY2 in male germline, [2014]. Data not published.
[14] PEREZ-PALACIOS, R.; GIASSETTI, M. I.; ACÍN, C.; CLIMENT, M.; GUALLAR, D.; MUNIESA, P.; SCHOORLEMMER, J. On a possible role for YY2 in male germline, [2014]. Data not published.
[15] PEREZ-PALACIOS, R.; GIASSETTI, M. I.; ACÍN, C.; CLIMENT, M.; GUALLAR, D.; MUNIESA, P.; SCHOORLEMMER, J. On a possible role for YY2 in male germline, [2014]. Data not published.

7.3 MATERIALS AND METHODS

All chemicals were supplied by Sigma Chemical Company (St. Louis, Missouri, USA) unless otherwise stated. All procedures were approved by Ethics Committee for Animal Experiments from the University of Zaragoza under the Project Licenses Pl11/12 and PI25/09

7.3.1 Immunohistochemistry

Testes were dissected from 3 weeks old CD1 mice, fixed in 4 % paraformaldehyde and maintained in PBS at 4°C until embedding in paraffin. Sections were rehydrated, peroxidase activity blocked and antigen retrieval was performed using PTLink (DAKO, Carpinteria, CA, EUA). Slides were washed with PBS and incubated with αYY2IS antibody (1:3000) at RT. Specific immunolocalization was visualized by Envision System-HRP labeled Polymer anti-Rabbit (DAKO, Carpinteria, CA, EUA). No primary antibody was used in negative control. Morphology and specific staining was analyzed by inverted optical microscopy (Nikon, Tokyo, Japan) with Leica DFC420 image system (Leica Image Software, Wetzlar, Germany).

7.3.2 SSC culture

Testicular cells were isolated from 129 strain mice and laminin-selected cells were maintained as SSCs in culture as described with minor modifications (GUAN *et al.*, 2009). Briefly, SSCs were co-cultivated with a γ-irradiated mouse embryonic fibroblasts (MEFs) monolayer in αMEM media supplemented with 1x B27 without

vitamin A (Invitrogen, Carlsbad, CA), 1x penicillin/streptomycin (Gibco®, Life Technologies, Carlsbad, CA, USA), 1 x NEAA (Gibco®, Life Technologies, Carlsbad, CA, USA), 0.07% fresh prepared β-ME, 1 x N2-2, GDNF (20ng/ul; Gibco®, Life Technologies, Carlsbad, CA, USA), LIF (10ng/ul), bFGF (1ng/ul), GFRα1 (150 ng/ul; R&D Systems, Minneapolis, MN, USA) at 37 C and 5% CO2. 50 x N2-1 solution [insulin (1.25 mg/ml, Invitrogen, Carlsbad, CA), transferrin (5 mg/ml), progesterone (9.5 mM), Puterescine (3 mM), sodium selenite (1.5 mM) and BSA (2.5 mg/ml; Gibco®, Life Technologies, Carlsbad, CA, USA) was prepared as described (Guan *et al.*, 2009). Media was changed every two days and after attaining semi-confluence, cells were passaged by enzymatic digestion with 0.125% (w/v) trypsin for 1 min at 37°C. MEFs were removed by incubation over 0.1% gelatin for 20-40 min at 37°C and 5% CO2. Cells were reseeded at a 1:2 ratio in 12 wells dishes previously covered with MEF every week.

7.3.3 FACS analysis

SSC at confluence (two days after a 1:1 passage), were individualized by trypsin digestion and suspended in PBS. Biotynilated primary antibody anti-THY1 (NBP1-28033, Novus Biological, Littleton, CO; Ontario, Canada) was added in three different concentrations (0.25, 0.083 and 0.027 μg/μl) in PBS supplemented with 0.5% BSA and 2mM EDTA, incubated for 30 min at room temperature. Cells were washed (600 x g/ 5min) with PBS and incubated for 30 min with a streptavidin-associated Alexa Fluor 488 (1:200) secondary antibody in PBS supplemented with 0.5% BSA and 2mM EDTA. A negative control sample was treated identically without the addition of primary antibody. Labeling of the cells was analyzed by flow cytometry in DB FACS Aria equipment with FACS DIVA Software.

7.3.4 Single SSC immunocytocheminal staining

The immunofluorescence of single cultured SSC was performed as described (YANG *et al.*, 2013) with modifications. After enzymatic isolation with trypsin, cells from the SSC enriched fraction (non-adherent on gelatin) were selected or not by laminin (GUAN *et al.*, 2009) and seeded (50.000 cell/well) onto poly-L-lysine-coated coverslips. Previously, coverslips were placed in 24 wells and incubated overnight with 1:100 poly-L-lysine solution. After seeding the cells, the plate was centrifuged (1,000 rpm for 10 min), media was removed, cells were washed with PBS and fixed with 4% (w/v) paraformaldehyde solution for 10 min at room temperature. Fixed cells were stored at 4°C in 0.1% Triton X-100 until the staining. Cells were permeabilized with 0.5% Triton-X100 for 10 minutes at RT, nonspecific antibody binding was blocked by incubation for 1 hour with PGBA (0.1% gelatin, 1% BSA, 0.05% NaAzide in PBS, pH 7.4) and cells incubated with anti YY2 IS antibody (1:4800) in PGBA/FBS (PGBA plus 10% of fetal calf serum). No primary antibody was added in negative control. In the next day, coverslips were washed with PBS and incubated with anti-rabbit biotin antibody (1:500) in PGBA for 30 minutes and washed. Cells were incubated with streptavidin-Alexa 488 (1:200) for 45 minutes in PGBA and nucleus stained by DAPI (10 µg/ml). Coverslips was mounted with glycerol and images were obtained in fluorescence microscope (Nikon, Tokyo, Japan).

7.3.5 Plasmids construction

To generate reporter vectors, primers were designed to amplify the relevant sequences (Table 1) (yielding 200-500 bp fragments). The specific amplification was performed by conventional PCR (Taq polymerase recombinant, Invitrogen, Carlsbad, CA). Forward primers were designed to incorporate a linker (5' ATGTCC3’ or 5’TACCGT3') at 5’ position followed by a BamH1 restriction site (5’GGATCC 3’) (Chart 4). Reverse primers were designed to incorporate at the 5’ position a linker (5’ATGTCC 3’) followed by a SalI restriction site (5’ GTCGAC 3’) (Chart 4). All fragments were gel-purified (GeneClean Turbo Cartridges, MP Biomedicals, Santa Ana, California, USA) and ligated into the BamHI-SalI digested backbone derived from Oct4PPLuc (Chart 4). This reporter contains the proximal promoter region of OCT4, a Luciferase gene whose expressed can be modulated by insertion of fragments at a distance (CHEN *et al.*, 2008). All constructs were verified by DNA sequencing

Chart 4 - Construction of reporter vectors driven by sequences identified either as REX1 binding sites in embryonic stem cell (ES) (Guallar et al., unpublished data) or as YY2 binding sites in spermatogonial stem cells (SSC) (Pérez-Palacios, Giassetti et al., unpublished data). Primers were designed to amplify the sequences surrounding peaks (200-500 bp). Primers were designed to create overhangs compatible with BamHI (5' GATC 3') and SalI (5' TCGA 3') sites, fragments were inserted into the BamHI-SalI digested backbone derived from Oct4PPLucEMyc. Chromosmal localization of the putative binding sites is indicated, as well as names used for the resulting plasmids

			Primer		
Binding protein	Cell type	Chromossome	Forward	Reverse	Reporter vector
REX1	ES	18	TACCGTGGATCCTGTCAGCTCTGTATGGCTGC	ATGTCCGTCGACAAATCGTAGGCAGAGGGCTG	O4Luc1
REX1	ES	19	TACCGTGGATCCCACCTCCGGAGTGTTGGTC	ATGTCCGTCGACCGCAAAGTATAAGGTGCCCG	O4Luc2
REX1	ES	15	TACCGTGGATCCCTGGACTTCACGGGCATTCT	ATGTCCGTCGACCGGTGCTTCTCTTCACGACT	O4Luc3
REX1	ES	10	TACCGTGGATCCGGGCATCCATACCGTGTACC	ATGTCCGTCGACTGGTGGGTCAGAGATTCCCA	O4Luc4
REX1	ES	12	TACCGTGGATCCCCATAAGTGTGGACCAAGCCT	ATGTCCGTCGACGTCCTTGGCCTTCTACCCAC	O4Luc5
REX1	ES	2	TACCGTGGATCC AAATGACGCCTCTGGGTCC	ATGTCCGTCGACTTGCTGGCAGTCATCCCAAG	O4Luc6
REX1	ES	7	TACCGTGGATCCCGAGCCCATAGAGGAAGCAG	ATGTCCGTCGACGCCTTGTAAGGCAGGGATGA	O4Luc7
YY2	SSC	5	ATGTCCGGATCCTCTGGAGATGAGGCCAGACA	ATGTCCGGATCCTGGCTGAGTTTCATGCCTGT	O4Luc8
YY2	SSC	2	ATGTCCGGATCCCTCCTGGGTAGAAAAGGGGC	ATGTCCGGATCCCCCACCTCCAGGTCTGTCTA	O4Luc9
YY2	SSC	Y	ATGTCCGGATCCCTGGCCCAGTTGTCAAATAAGG	ATGTCCGGATCCGTCTGGCATGATCCAGGAACC	O4Luc10

7.3.6 Embryonic stem cells culture, transfection, luciferase and beta-galactosidase measurement

The embryonic stem cell line E14T (AUBERT *et al.*, 2002) was maintained in gelatin-coated dishes in GMEM medium supplemented with 10% fetal calf serum, LIF and 2i (YING *et al.*, 2008). The day before transfection, 0.25 x10^6 ES cells were seeded per well of a 24 well culture plate pre-coated with gelatin. After 16 hour, cells were transfected with a total of 1µg of exogenous DNA (0.5 µg of reporter vector or luciferase control plus 0.5 µg of pRSVZ, which provides beta-galactosidase expression that is used as a control for transfection efficiency) using Lipofectamin 2000 Reagent (Invitrogen, Carlsbad, CA, USA) in OPTI-MEM medium (Gibco®, Life Technologies, Carlsbad, CA, USA) at 37ºC and 5% CO2. After 3 hours, the transfection mixture was removed and replaced with regular culture medium. After 16 hours, cells were lysed and luciferase activities were measured using Luciferase Reporter Gene Detection Kit and detected by spectrophotometry (Synergy HT, Biotek, Winooski, USA). Transfection with Oct4PPLucONS, which provide high expression of luciferase (CHEN *et al.*, 2008), was considered as positive control for luciferase expression. Other controls [pSV2Luc (commercial vector to express luciferase), Oct4PPLucEMyc (intermediate luciferase expression, (CHEN *et al.*, 2008) and pGL1 (promoterless control reporter was devoid of activity)] were used. To quantitate beta-galactosidase activity, 25 µl of cell extract and 2 x Beta-galactosidase buffer [200mM PBS (pH 7.3), 2mM $MgCl_2$, 1 mM $MgSO_4$ e 100mM beta-mercaptoethanol] were mixed, 10µl of ONPG (4mg/ml) was added, followed by incubation for 2 h at 37°C. Activity was quantitated by colorimetric analysis on a spectrophotometry (Synergy HT, Biotek, Winooski, USA). Luciferase expression was normalized against beta-galactosidase expression.

7.3.7 Transfection of SSC and reporter assays

SSC transfection was performed as described (YANG *et al.*, 2013) with modifications. Briefly, after attaining semi-confluence cells were split 1:1 in a well of a 12 well plate (day 1) and cultured for 2 days until the transfection on day 3. On the day of transfection, each well (0.7-0,8-1x10^6 SSC cells) was transfected with 2µg of exogenous DNA and 3µl Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) in serum free media supplemented with GDNF (regular serum free SSC medium). The next day, cells were digested with trypsin and left onto gelatinized culture dishes for 20-40 min at 37°C to allow feeders to attach and obtain a non-adherent SSC enriched fraction. Cells were lysed and luciferase activity was measured according to manufacturer´s instructions (Luciferase Reporter Gene Detection Kit; Luc1, Sigma). Luciferase activity was quantified by spectrophotometry (Synergy HT, Biotek, Winooski, USA). We tested beta-galactosidase (pRSVZ) or GFP (pCAGIPeGFP) reporter plasmids for use as a control for transfection efficiency. Their use was abandoned as no consistent expression of either plasmid could be detected either 24 or 48 hours after transfection. Therefore, the luciferase activity detected after transfection of each individual construct was normalized against the activity measured in MEF transfected under the same conditions and in parallel of co-cultured SSC.

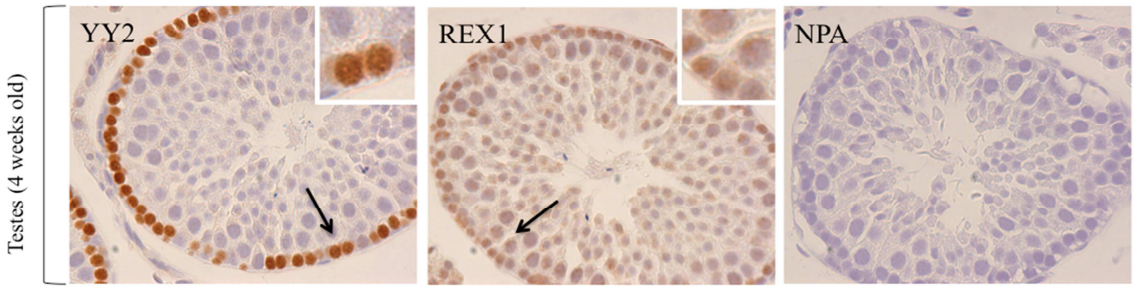
7.4 RESULTS

7.4.1 Restricted expression of YY2 in undifferentiated germ cells.

Rex1 (ROGERS; HOSLER; GUGAS, 1991) and Yy2 mRNAs are expressed in germ cells during spermatogenesis (LUO, J. et al., 2006; DREWS et al., 2009). We

evaluated the expression of both proteins in the testes (Figure 15). In the testes from 4-week-old mice, we observed that YY2 is specifically expressed in undifferentiated germ cells attached to the basal membrane of seminiferous tubules, which include the SSCs. However, we observed a more diffuse expression of REX1 in testicular parenchyma, including both undifferentiated and differentiated germ cells (Figure 15). These results confirm that YY2 expression is not ubiquitous, as in the testis YY2 is highly restricted to spermatogonia (Figure 15; R. PEREZ-PALACIOS et al., 2014[16]).

Figure 15 - Immunolocalization of YY2 and REX1 in mice testis at 4 weeks (600X image magnification) Staining was performed with DAB (brown) and hematoxylin. Enhanced magnification is included at the right corner and positive cells are indicated by arrows. Negative control staining carried out in the absence of primary antibody (NPA)



7.4.2 SSC lineage: nuclear localization of REX1 and YY2

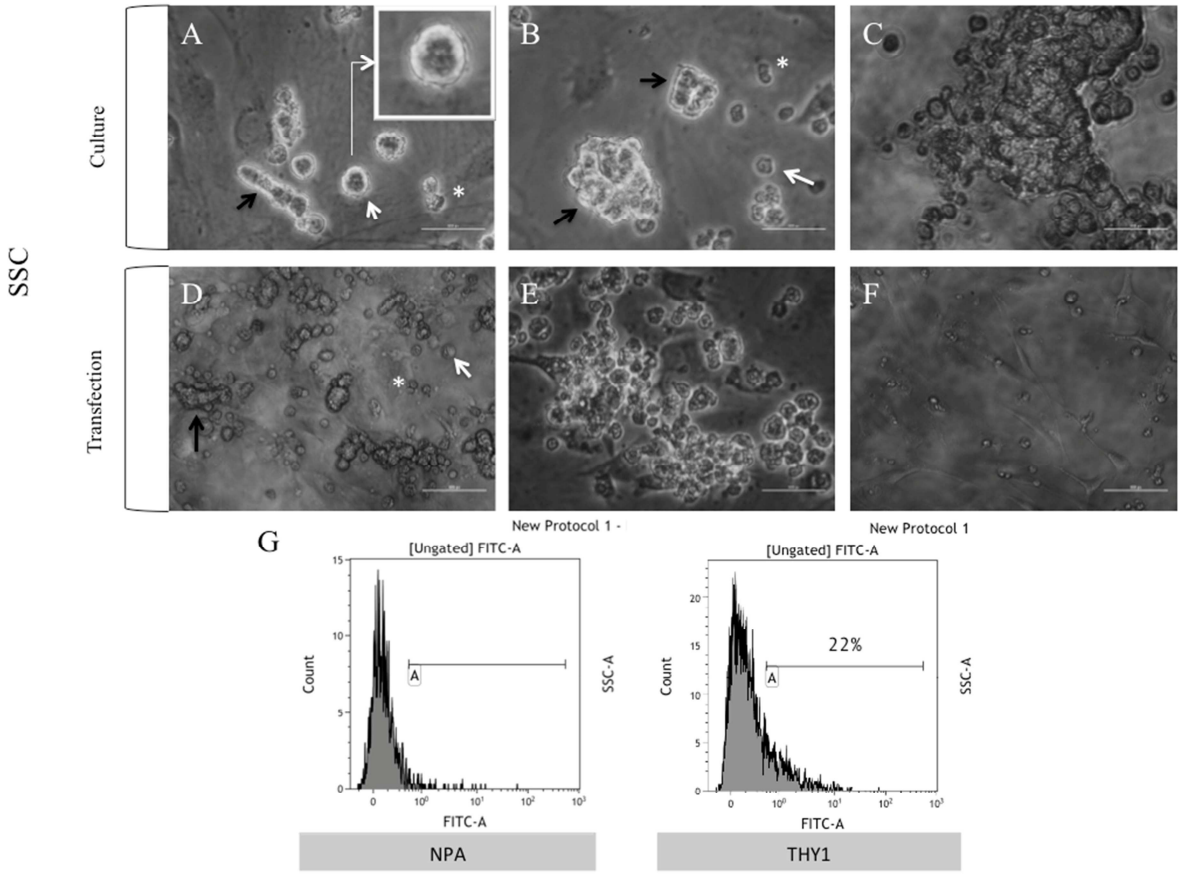
In vivo, SSC are a rare cell population, and long-term culture of SSC is a crucial biological tool to expand and manipulate these cells (ZHENG et al., 2014). In the present study, we worked with SSC isolated as described (GUAN et al., 2009). These cells express specific SSC markers, and display growth curves and morphology (ACÍN, 2013[17]; PEREZ-PALACIOS et al., 2014[18]) typical to SSC (GUAN

[16] PEREZ-PALACIOS, R.; GIASSETTI, M. I.; ACÍN, C.; CLIMENT, M.; GUALLAR, D.; MUNIESA, P.; SCHOORLEMMER, J. On a possible role for YY2 in male germline, [2014]. Data not published.
[17] ACÍN, S. Establishment of several cell line of mouse SSC, [2013]. Data not published.

et al., 2009). Also, we observed by flow cytometry that 22% of cultured SSC express THY1 (Figure 16 G). Two days after passage, we observed that the largest cell population consisted of single SSCs (A_s) with bright edges, while paired SSC (A_{pr}) with bright edge and short chain of SSC (A_{al}) were also observed (Figure 16). By 5 days after passage, we continued to observe the presence of both A_s and A_{pr} SSCs, but median chains of SSC (A_{al}) in the format of medium cell clusters had appeared. In the confluent culture at 7 days after passage, we observed the predominant presence of median and long chain of SSCs (A_{al}) in the format of cell clusters (Figure 16).

[18] PEREZ-PALACIOS, R.; GIASSETTI, M. I.; ACÍN, C.; CLIMENT, M.; GUALLAR, D.; MUNIESA, P.; SCHOORLEMMER, J. On a possible role for YY2 in male germline, [2014]. Data not published.

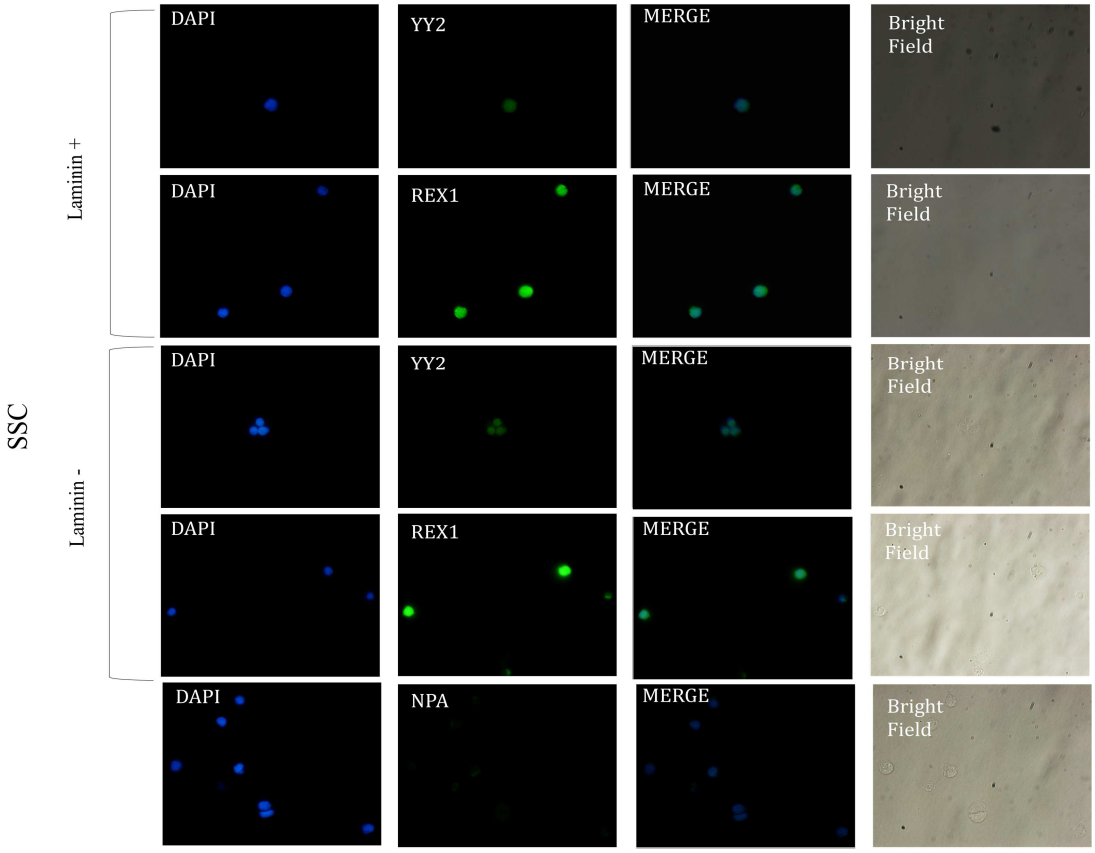
Figure 16 - SSC co-culture on a feeder layer of gamma irradiated MEF in long-term culture (A-C) and after transfection (D-E). Representative images at 400X magnification. A) SSC at 2 days after passage, white arrow indicates single SSC (A_s) with bright edge, asterisks indicates paired SSC (A_{pr}) with bright edge and black arrow indicates short chain of SSC (A_{al}); B) SSC at 5 days after passage, white arrow indicates single SSC (A_s), asterisks indicates paired SSC (A_{pr}) and black arrow indicates median chain of SSC (A_{al}); C) SSC at 7 days after passage, presence of a long chain of SSC (A_{al}); D) SSC at 24 hours after transfection with lipofectamine and reporter gene, white arrow indicates single SSC (A_s), asterisks indicates paired SSC (A_{pr}) and black arrow indicates short chain of SSC (A_{al}); E) SSC at 48 hours after transfection with lipofectamine and reporter gene, presence of short, median and long chains of SSC (A_{al}). F) Gamma irradiated MEF cells after at 48 hours after transfection with lipofectamine and reporter gene, presence of lived and attached with long pseudopods (scale of 500px and 400x image magnification)



Having observed the presence of REX1 and YY2 protein in germ cells of the mouse testis (Figure 15), we tested their presence in SSC. We separated SSC (two days after passage) into laminin positive (attached to laminin) and negative (non-attached to laminin) cells (Figure 17). Both populations SSC were stained for REX1 and YY2. Results (Figure 17) showed a specific immunolocalization of REX1 and YY2 in the nucleus of either population of SSC (Figure 17). Surprisingly, the signals obtained for REX1 appeared stronger compared to YY2. No apparent difference was

observed between Laminin positive and negative cells.

Figure 17 - Immunofluorescent staining of YY2 and REX1 in SSC long-term culture after positive (attached) and negative (non-attached) selection on laminin. Representative images (400X image magnification) of staining using sera directed against either YY2 or REX1 (visualized using an Alexa Fluor 488 dye as secondary antibody, green) and nucleus (DAPI, blue). No primary antiboby (NPA)



7.4.3 Gene regulatory capacities of REX1 chromatin binding sites

REX1 has been identified as an ES cell-specific transcription factor, contributing to the regulation of XCI and retroviral elements (reviewed in SCHOORLEMMER et al., 2014). In addition to well-described chromatin binding sites in the Tsix locus (NAVARRO et al., 2010) and MERVL elements (GUALLAR et al.,

2011), additional genome-wide binding sites of REX1 have been identified by ChIPseq (GUALLAR et al., 2012[19]). To evaluate a potential function for REX1 as a transcriptional activator, we wanted to test gene regulatory activity of its chromatin binding sites. Reporter genes were constructed that contain several of these fragments in a distal location to an Oct4-promoter-driven Luciferase reporter gene (Chart 4). Reporters driven by seven different REX1 binding sites were named O4Luc1-7 (Chart 4).

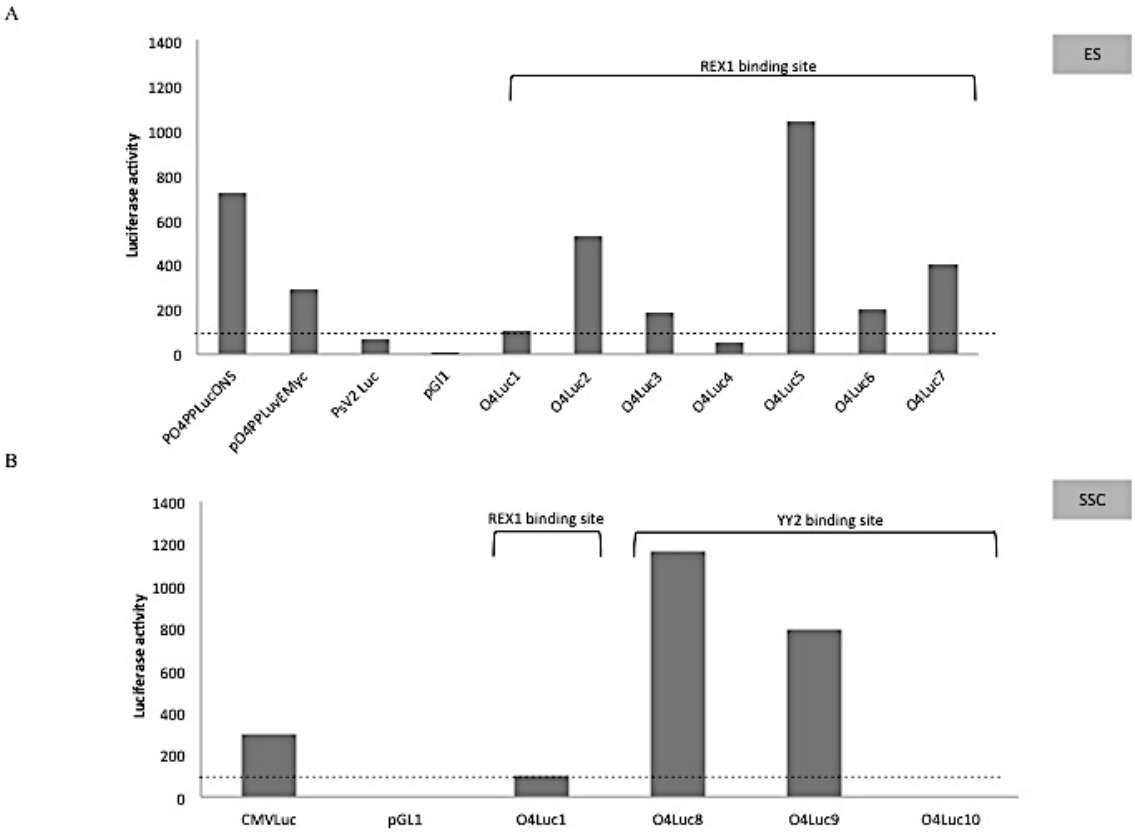
We transfected ES cells, and the next day Luciferase expression was measured in cell lysates and normalized against the activity of a co-transfected beta-galactosidase gene. Results are shown in Figure 18 A. An empty, promoterless control reporter (pGL1) was devoid of activity. Reporters (described by CHEN et al., 2008) with an identical structure to O4Luc1-7 with high (pO4PPLucONS) or intermediate (pO4PPLucEMYC) activities were tested alongside our novel reporters. High luciferase activity was observed for these positive controls [pO4PPLucONS; Fold=7.21, pO4PPLucEMYC; Fold=2.87). Weak but detectable activity in ES was also observed for the SV2Luc positive control. The activity of the O4Luc1-7 reporters driven by REX1 binding sites (Chart 4) varied widely from very high to very low (Figure 18). Compared to the ONS and Myc reporters, the activity of several of our reporters qualify as high: O4Luc2 (Fold=5.22), O4Luc5 (Fold=10.35) and O4Luc7 (Fold=3.97) (Figure 19 A). By contrast, various others display low or even almost non-existent activities: O4Luc3 (Fold=1.86), O4Luc4 (Fold=0.52) and O4Luc6 (Fold=1.99). These results indicate that REX1 binding sites do not necessarily represent sequences that confer high activity in ES cells.

Among the reporters tested in ES cells, several are driven by sequences bordering testis-specific genes. As REX1 is expressed in SSC (Figure 15), we tested the possibility that such reporters are active in SSC, driving the expression of testis-specific genes. The transfection procedure did not visibly affect the morphology of SSC after 24 and 48 hour of treatment with Lipofectamin and reporter gene plasmids (Figure 16). SSC were transfected in co-culture with MEF cells. Therefore, the luciferase activities in transfected cultured SSC were compared to those measured in a parallel well of MEF cells in identical culture conditions. The activities measured in SSC were normalized by subtracting the values obtained in MEF. Results are shown

[19] GUALLAR, D.; CLIMENT, M; PEREZ-PALACIOS, R.;. I.;.MUNIESA, P.; SCHOORLEMMER, J. Análisis funcional de Rex1 en las células troncales embrionarias de ratón, [2012]. Data not published

in Figure 18 B. In duplicate experiments, comprising multiple parallel transfections, we consistently measured a low but detectable activity of the O4Luc1 construct, above the background values determined after transfection of SSC with pGL1 or in similarly transfected MEF.

Figure 18 - Luciferase activities of a set of reporter genes driven by either REX1 (pO4PPLuc1-7) or YY2 (pO4PPLuc8-10) binding sequences (Table1) and several positive control reporters (CMVLuc, SV2Luc, O4LucONS, O4LucMyc). pGL1 is a promoterless Luciferase control vector. Reporters were transfected in embryonic stem cells (A) or spermatogonial stem cell (B). Luciferase activity was measured in cell lysates and normalized against the activity of a co-transfected beta-galactosidase gene (A). Luciferase activity in SSC cells was normalized against the activity in MEFs (B). Luciferase activities are represented as a percentage relative to the activity of O4Luc1 (reporter gene for REX1 binding sequence with lowest expression)



7.4.4 Reporter gene activation by YY2 chromatin binding sites

We had shown restricted expression of YY2 in undifferentiated germ cells attached to the basal membrane (Figure 15) and high level expression in SSC (Figure 16). As these results suggested a potential function of YY2 in the maintenance of undifferentiated germ cells, genomic targets for YY2 in SSC were determined using massively parallel sequencing of ChIPped DNA (ChIpseq) (PEREZ-PALACIOS et al., 2014[20]This procedure identified almost 500 binding sites with high statistical significance and $\log_2$ enrichment values ≥2. Several of the sequences identified also scored positive in posterior ChIP assays (PEREZ-PALACIOS et al., 2014[21]. Surprisingly, the majority of these binding sites do not coincide with gene promoters (data not shown). We therefore initiated studies to determine the capacity of selected YY2 binding sites to regulate gene expression in SSC. To do so, reporter genes were constructed that contain YY2 binding sites for SSC chromatin in a distal location to the Luciferase reporter gene described above (Chart 4). Three different reporters termed O4Luc8-10 were transfected into SSC, and luciferase activity was measured as described above. While no activity was observed after transfections performed with a Luciferase control vector lacking regulatory sequences (pGL1) (Figure 18 B), activity of a positive control construct CMVLuc was easily demonstrated (Fold=3.99). Even higher luciferase activities were observed in SSC cells transfected with two of the three reporters tested i.e. O4Luc8 (Fold=11.64) and O4Luc9 (Fold=7.95) (Figure 18 B). No detectable luciferase activity was observed for cells transfected with the O4Luc10 reporter (Figure 18 B). Thus, the sequences comprising selected YY2 binding sites were able to confer high expression onto reporter genes in SSC.

[20] PEREZ-PALACIOS, R.; GIASSETTI, M. I.; ACÍN, C.; CLIMENT, M.; GUALLAR, D.; MUNIESA, P.; SCHOORLEMMER, J. On a possible role for YY2 in male germline, [2014]. Data not published.
[21] PEREZ-PALACIOS, R.; GIASSETTI, M. I.; ACÍN, C.; CLIMENT, M.; GUALLAR, D.; MUNIESA, P.; SCHOORLEMMER, J. On a possible role for YY2 in male germline, [2014]. Data not published.

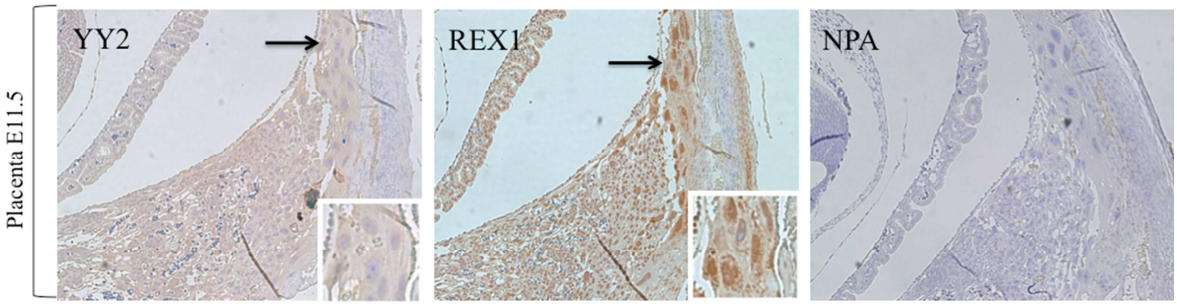
7.5 DISCUSSION

REX1 and YY2 don't share same patterns of expression in tissues during embryonic development and after birth (ROGERS; HOSLER; GUGAS, 1991; NGUYEN et al., 2004; LUO, C. et al., 2006; LUO, J. et al., 2006; MASUI et al., 2008; KIM et al., 2011; REZENDE et al., 2011; GUALLAR et al., 2012; CLIMENT et al., 2013). However, REX1 and YY2 are members of same transcription factor family and share high homology in the zinc finger region (MONGAN; MARTIN; GUDAS, 2006; KIM; FAULK; KIM, 2007) and when co-expressed those factors could share function, such as control of gene transcription. The aim of this study is to describe tissue-specific YY2 expression, compare the location of REX1 or YY2 expression, and to verify the ability of their genomic targets to influence gene expression and transcriptional control at a distance. For that purpose, we performed immunolocalization of REX1 and YY2 in situ (testes) and in SSC cultures. We also tested the activity of reporter genes driven by binding sites for either REX1 or YY2, to determine their ability to regulate luciferase expression ex vivo.

REX1 is most notoriously expressed in Embryonic Stem cells, and also during spermatogenesis, throughout pre-implantation mouse development, and in early trophectoderm derivatives and placenta during embryonic development (ROGERS; HOSLER; GUGAS, 1991; KIM et al., 2011; REZENDE et al., 2011; CLIMENT et al., 2013). Here we observed a diffuse expression of REX1 across testicular parenchyma (Figure 16). In contrast, in the placenta we observed that REX1 was highly expressed in a limited subset of Trophoblast Giant Cells (CLIMENT et al., 2012[22], data not shown; Figure 19).

[22] CLIMENT, M.; PEREZ-PALACIOS, R..; GUALLAR, D.; MUNIESA, P.; SCHOORLEMMER, J Función biológica de Rex1/Zpf42 en el desarrollo del ratón. Regulación de la expresión génica en embriones de ratón en período preimplantacional, [2012]. Data not published.

Figure 19 - Immunolocalization of YY2 and REX1 in placenta from embryos at 11.5 dpc (100X image magnification) as described in the legend to Figure 16.



YY2 expression has been described throughout the entire E11.5 embryo suggesting ubiquitous expression (brain, lung, heard and in testis) (LUO, J. et al., 2006; DREWS et al., 2009). YY2 mRNA expression was observed by in situ hybridization though the testes parenchyma and the highest expression was described in spermatocytes but, it was not observed in sperm (LUO, J. et al., 2006). However, recently our research group observed that YY2 is expressed in embryos at early stages of development, in gonocytes and other types of undifferentiated germ cells after birth (in vivo) and in cultured SSC (in vivo) (PEREZ-PALACIOS et al., 2014[23].).

In line with these data, in the present study we describe a specific localization of YY2 in undifferentiated germ cells in testis from 4 weeks old mice (Figure 16). YY2 was expressed only in undifferentiated germ cells attached to the seminiferous tubule basal membrane. As previously described (PEREZ-PALACIOS et al., 2014[24]). no positive staining for YY2 was observed in testicular somatic cells (Sertoli cells, myoid or Leydig cells) or more differentiated germ cells (spermatocytes, spermatids or sperm). However, YY2 displayed a low and rather diffuse expression pattern in placenta (Figure 20), further demonstrating non-ubiquitous expression. Also, in the testis YY2 expression is non-ubiquitous, and YY2 protein and mRNA expression

[23] PEREZ-PALACIOS, R.; GIASSETTI, M. I.; ACÍN, S.; CLIMENT, M.; GUALLAR, D.; MUNIESA, P.; SCHOORLEMMER, J. On a possible role for YY2 in male germline, [2014]. Data not published.
[24] PEREZ-PALACIOS, R.; GIASSETTI, M. I.; ACÍN, S.; CLIMENT, M.; GUALLAR, D.; MUNIESA, P.; SCHOORLEMMER, J. On a possible role for YY2 in male germline, [2014]. Data not published.

differ. Moreover, the distinct expression patterns of REX1 and YY2 might indicate distinct functions of REX1 and YY2 in placenta and testis, respectively.

In adult mice testis, SSC are a rare cell type (TEGELENBOSCH; DE ROOIJ, 1993) and, in vitro culture of SSC is a crucial biological tool to expand and manipulate those in vivo rare cell (ZHENG et al., 2014). Thus, a long-term culture is necessary to better understand the biological mechanisms involved in SSC self-renewal and differentiation (HE et al., 2015). The SSC used in our studies, have been characterized as SSCs using marker gene expression. RNA expression studies showed they express several markets typically associated with SSCs such as ITGA6, GFRA1, OCT4, NGN3, cRET and DDX4 (ACÍN, 2013[25]). Using Flow Cytometry (Figure 17), we showed that 7-22% of cells in our cultures were positive for THY1.1, a specific marker for SSC (HERMANN et al., 2015). Moreover, initial IF studies showed the cells to stain positive for several SSC markers i.e. OCT4 and CD9 (PEREZ-PALACIOS et al., 2014[26]). Among the YY1 family members, mRNA encoding Yy2 had been previously shown to be highly expressed in SSC (PEREZ-PALACIOS et al., 2014[27]). Expression of Yy1 was easily detectable and expression of Rex1 was low but above the detection limit (PEREZ-PALACIOS et al., 2014[28]). YY2 protein had also been detected in SSC cells by indirect immuno-fluorescence, but mostly in the cytoplasm with some nuclear presence as well (PEREZ-PALACIOS et al., 2014[29]). The localization of YY2 in SSC cells was now re-assessed using an altered fixation protocol, based on spinning down cells onto lysine-coated coverslips (see M&M). Using this protocol, YY2 now was easily detected in predominantly nuclear localization (Figure 18). So, we described that REX1 and YY2 were localized with SSC chromatin, as is expected for an active transcription factor.

Previously, our group observed that REX1 binds specific sequences within ES cell chromatin (GUALLAR et al., 2012[30]). As REX1 is best known as an ES cell-specific transcription factor, we tested the hypothesis that REX1 binding sites

[25] ACÍN, S. Establishment of several cell line of mouse SSC, [2013]. Data not published.
[26] PEREZ-PALACIOS, R.; GIASSETTI, M. I.; ACÍN, S.; CLIMENT, M.; GUALLAR, D.; MUNIESA, P.; SCHOORLEMMER, J. On a possible role for YY2 in male germline, [2014]. Data not published.
[27] PEREZ-PALACIOS, R.; GIASSETTI, M. I.; ACÍN, S.; CLIMENT, M.; GUALLAR, D.; MUNIESA, P.; SCHOORLEMMER, J. On a possible role for YY2 in male germline, [2014]. Data not published.
[28] PEREZ-PALACIOS, R.; GIASSETTI, M. I.; ACÍN, S.; CLIMENT, M.; GUALLAR, D.; MUNIESA, P.; SCHOORLEMMER, J. On a possible role for YY2 in male germline, [2014]. Data not published.
[29] PEREZ-PALACIOS, R.; GIASSETTI, M. I.; ACÍN, S.; CLIMENT, M.; GUALLAR, D.; MUNIESA, P.; SCHOORLEMMER, J. On a possible role for YY2 in male germline, [2014]. Data not published.
[30] GUALLAR, D.; CLIMENT, M; PEREZ-PALACIOS, R.;. I.;.MUNIESA, P.; SCHOORLEMMER, J. Análisis funcional de Rex1 en las células troncales embrionarias de ratón, [2012]. Data not published

enhance gene expression in these cells. Indeed, several of the binding sequences for REX1 confer high levels of luciferase acivity on reporter constructs (Figure 19). Although the REX1 binding sites identified are generally located far away from gene promoters, these data are compatible with a role for REX1 binding sites in positive control of transcription in ES. The reporter genes created will allow future experiments to define the precise binding site within the 500 bp reporter fragments, and show direct regulation of these sequences by REX1. Among the binding sites incorporated in reporters with low activity (Figure 19), we also observed that several are bordering testis-specific genes. We focused on a reporter with a REX1 binding site upstream of the testis-specific Spata24 gene (O4Luc1 construct). Indeed, this reporter was weakly active in SSC cells (Figure 19 B). This result suggests that REX1 binding sites in ES cells may be important for regulation in more differentiated cells.

Specific YY2 binding sites in SSC chromatin have been determined by our research group, using genome-wide approaches (PEREZ-PALACIOS et al., 2014[31]). Similar to previous experiments using REX1 binding sites in ES cells, we evaluated the activity of reporter genes driven by YY2 binding sequences in SSC cells. We identified two reporters with high expression of luciferase. Although the YY2 binding sites identified are generally located far away from gene promoters, these preliminary data are compatible with a regulatory role for these sequences in long-range transcriptional control in SSC. Future studies will address the tissue-specificity of these reporters and the function of YY2 in their activity. At present the biochemical or biological output resulting from YY2 binding to the binding sites remains undefined. However, the majority of binding sites do not coincide with gene promoters, raising the question as to why YY2 is binding to those sequences.

Recently, an important role of all YY1-related members was described in the transcription of retroviral elements (REs) (GUALLAR et al., 2012[32]).. Transposable elements (TEs) are DNA sequences that have the ability to move, changing its chromosomal position in the genome (FEDOROFF, 2001) and include several families of sequences i.e. LINE, SINE, ERV (JERN;COFFIN, 2008). Around 10% of mammalian genomes is occupied by a family of RE called Endogenous Retrovirus

[31] PEREZ-PALACIOS, R.; GIASSETTI, M. I.; ACÍN, S.; CLIMENT, M.; GUALLAR, D.; MUNIESA, P.; SCHOORLEMMER, J. On a possible role for YY2 in male germline, [2014]. Data not published.
[32] GUALLAR, D.; CLIMENT, M; PEREZ-PALACIOS, R.;. I.;.MUNIESA, P.; SCHOORLEMMER, J. Análisis funcional de Rex1 en las células troncales embrionarias de ratón, [2012]. Data not published

Elements (ERVs), which are the remnants of retroviral genomes inserted into the mammalian germline that have mostly mutated into non-functional RE elements (LÖWER et al., 1996; MAKSAKOVA et al., 2006). For a long time, TE/ERVs have been considered “junk” DNA, however they are now known to be important for pathogenic processes, to provide genetic variability within species, to regulate gene transcription, cause alternative splicing and promote genomic plasticity (JERN; COFFIN, 2008; SCHOORLEMMER et al., 2014).

Previously, we not only described that YY1 and REX1 bind to REs but also observed that REX1 regulates the expression of particular ERV sequences in ES cells and mouse embryos (GUALLAR et al, 2012). YY2 also specifically binds to some REs in mouse trophectoderm stem cells (TS) (GUALLAR et al, 2012) and in Spermatogonial Stem Cells (SSCs) (PEREZ-PALACIOS et al., 2014[33]). Inspection of the top YY2 binding sites we discovered in SSC revealed that 13/14 of those overlap with Transposable Elements. To be precise, they overlap with DNA transposons (MER5A), sequences from different ERV families (ERV1, ERVK and ERVL-MaLR) as well as SINE and LINE elements. We do not know the biological or functional significance of the fenomenon, but consider this information relevant. The binding of YY2 to such sequences may reflect they are the mayor targets of regulation by YY2. Alternatively, YY2 may regulate tissue-specific gene expression in testis and SSC through the contribution of TE to alternative promoters or intron/exon selection. At present, no YY2 gene is annotated in the Bos Taurus genome. However, we easily found a gene with Zinc Fingers and genomic structure similar to mouse Yy2 in the genome of Bos taurus (breed Hereford, X chromosome). Importantly, the binding site in reporter 8 is well conserved among mammals, including Bos taurus. In theory, such a reporter may aid the identification or characterization of the so far elusive bovine SSC.

[33] PEREZ-PALACIOS, R.; GIASSETTI, M. I.; ACÍN, C.; CLIMENT, M.; GUALLAR, D.; MUNIESA, P.; SCHOORLEMMER, J. On a possible role for YY2 in male germline, [2014]. Data not published.

7.6 CONCLUSION

As YY2 is highly expressed *in vitro* in mouse SSCs and *in vivo* in gonocytes and in undifferentiated spermatogonia after birth (PEREZ-PALACIOS et al., 2014[34]; Figure 16), YY2 may play a role in genome management at these stages concerning the control of retroviral elements.

Acknowledgements

This research was funding supported by São Paulo Research Foundation (FAPESP) and National Counsel of Technological and Scientific Development (CNPq).

[34] PEREZ-PALACIOS, R.; GIASSETTI, M. I.; ACÍN, C.; CLIMENT, M.; GUALLAR, D.; MUNIESA, P.; SCHOORLEMMER, J. On a possible role for YY2 in male germline, [2014]. Data not published.

REFERENCE

ATCHISON, M.; BASU, A.; ZAPRAZNA, K.; PAPASANI, M. Mechanisms of Yin Yang 1 in oncogenesis: the importance of indirect effects. **Crit Rev Oncog,** v. 16, n. 3-4, p. 143-61, 2011. ISSN 0893-9675. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/22248052 >. Acessed at: 24. march. 2015.

AUBERT, J.; DUNSTAN, H.; CHAMBERS, I.; SMITH, A**.** Functional gene screening in embryonic stem cells implicates Wnt antagonism in neural differentiation. **Nat Biotechnol,** v. 20, n. 12, p. 1240-5, Dec 2002. ISSN 1087-0156. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/12447396 >. Acessed at: 24. march. 2015.

CHEN, X.; XU, H.; YUAN, P.; FANG, F.; HUSS, M.; VEGA, V. B.; WONG, E.; ORLOV, Y. L.; ZHANG, W.; JIANG, J.; LOH, Y. H.; YEO, H. C.; YEO, Z. X.; NARANG, V.; GOVINDARAJAN, K. R.; LEONG, B.; SHAHAB, A.; RUAN, Y.; BOURQUE, G.; SUNG, W. K.; CLARKE, N. D.; WEI, C. L.; NG, H. H. Integration of external signaling pathways with the core transcriptional network in embryonic stem cells. **Cell,** v. 133, n. 6, p. 1106-17, Jun 2008. ISSN 1097-4172. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/18555785 >. Acessed at: 24. march. 2015.

CHENG, J.; TÜRKEL, N.; HEMATI, N.; FULLER, M. T.; HUNT, A. J.; YAMASHITA, Y. M. Centrosome misorientation reduces stem cell division during ageing. **Nature,** v. 456, n. 7222, p. 599-604, Dec 2008. ISSN 1476-4687. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/18923395 >. Acessed at: 24. march. 2015.

CLIMENT, M.; ALONSO-MARTIN, S.; PÉREZ-PALACIOS, R.; GUALLAR, D.; BENITO, A. A.; LARRAGA, A.; FERNÁNDEZ-JUAN, M.; SANZ, M.; DE DIEGO, A.; SEISDEDOS, M. T.; MUNIESA, P.; SCHOORLEMMER, J**.** Functional analysis of Rex1 during preimplantation development. **Stem Cells Dev,** v. 22, n. 3, p. 459-72, Feb 2013. ISSN 1557-8534. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/22897771 >. Acessed at: 24. march. 2015.

COSTA, G. M.; AVELAR, G. F.; REZENDE-NETO, J. V.; CAMPOS-JUNIOR, P. H.; LACERDA, S. M.; ANDRADE, B. S.; THOMÉ, R. G.; HOFMANN, M. C.; FRANCA, L. R. Spermatogonial stem cell markers and niche in equids. **PLoS One,** v. 7, n. 8, p. e44091, 2012. ISSN 1932-6203. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/22937157 >. Acessed at: 24. march. 2015.

CURTIS, S. K.; AMANN, R. P. Testicular development and establishment of spermatogenesis in Holstein bulls. **J Anim Sci,** v. 53, n. 6, p. 1645-57, Dec 1981. ISSN 0021-8812. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/7341622 >. Acessed at: 24. march. 2015.

DREWS, D.; KLAR, M.; DAME, C.; BRÄUER, A. U. Developmental expression profile of the YY2 gene in mice. **BMC Dev Biol,** v. 9, p. 45, 2009. ISSN 1471-213X. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/19638226 >. Acessed at: 24. march. 2015.

FEDOROFF, N. How jumping genes were discovered. **Nat Struct Biol,** v. 8, n. 4, p. 300-1, Apr 2001. ISSN 1072-8368. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/11276245 >. Acessed at: 24. march. 2015.

GUALLAR, D.; PÉREZ-PALACIOS, R.; CLIMENT, M.; MARTÍNEZ-ABADÍA, I.; LARRAGA, A.; FERNÁNDEZ-JUAN, M.; VALLEJO, C.; MUNIESA, P.; SCHOORLEMMER, J. Expression of endogenous retroviruses is negatively regulated by the pluripotency marker Rex1/Zfp42. **Nucleic Acids Res,** v. 40, n. 18, p. 8993-9007, Oct 2012. ISSN 1362-4962. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/22844087 >. Acessed at: 24. march. 2015.

GUAN, K.; WOLF, F.; BECKER, A.; ENGEL, W.; NAYERNIA, K.; HASENFUSS, G. Isolation and cultivation of stem cells from adult mouse testes. **Nat Protoc,** v. 4, n. 2, p. 143-54, 2009. ISSN 1750-2799. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/19180086 >. Acessed at: 24. march. 2015.

HE, Y.; CHEN, X.; ZHU, H.; WANG, D. Developments in techniques for the isolation, enrichment, main culture conditions and identification of spermatogonial stem cells. **Cytotechnology**, Mar 2015. ISSN 0920-9069. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/25749914 >. Acessed at: 24. march. 2015.

HERMANN, B. P.; MUTOJI, K. N.; VELTE, E. K.; KO, D.; OATLEY, J. M.; GEYER, C. B.; MCCARREY, J. R. Transcriptional and Translational Heterogeneity among Neonatal Mouse Spermatogonia. **Biol Reprod**, Jan 2015. ISSN 1529-7268. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/25568304 >. Acessed at: 24. march. 2015.

HERMANN, B. P.; SUKHWANI, M.; SIMORANGKIR, D. R.; CHU, T.; PLANT, T. M.; ORWIG, K. E. Molecular dissection of the male germ cell lineage identifies putative spermatogonial stem cells in rhesus macaques. **Hum Reprod,** v. 24, n. 7, p. 1704-16, Jul 2009. ISSN 1460-2350. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/19336441 >. Acessed at: 24. march. 2015.

HUCKINS, C. The spermatogonial stem cell population in adult rats. 3. Evidence for a long-cycling population. **Cell Tissue Kinet,** v. 4, n. 4, p. 335-49, Jul 1971. ISSN 0008-8730. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/5127357 >. Acessed at: 24. march. 2015.

JERN, P.; COFFIN, J. M. Effects of retroviruses on host genome function. **Annu Rev Genet,** v. 42, p. 709-32, 2008. ISSN 0066-4197. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/18694346 >. Acessed at: 24. march. 2015.

KERR, J. B.; LOVELAND, K. L.; O'BRYAN, M. K.; DE KRETSER, D. M. Cytology of the Testis and Intrinsic Control Mechanisms. In: NEILL, J. D. (Ed.). **Knobil and Neill's Physiology of Reproduction**. 3ed. Oxford, UK: Elsevier Inc, v.1, 2006. chap. Cytology of the Testis and Intrinsic Control Mechanisms, p.827-948. (Male Reproductive System).

KIM, J. D.; FAULK, C.; KIM, J. Retroposition and evolution of the DNA-binding motifs of YY1, YY2 and REX1. **Nucleic Acids Res,** v. 35, n. 10, p. 3442-52, 2007. ISSN 1362-4962. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/17478514 >. Acessed at: 24. march. 2015.

KIM, J. D. ; KIM, H.; EKRAM, M. B.; YU, S.; FAULK, C.; KIM, J. Rex1/Zfp42 as an epigenetic regulator for genomic imprinting. **Hum Mol Genet,** v. 20, n. 7, p. 1353-62, Apr 2011. ISSN 1460-2083. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/21233130 >. Acessed at: 24. march. 2015.

KUBOTA, H.; AVARBOCK, M. R.; BRINSTER, R. L. Spermatogonial stem cells share some, but not all, phenotypic and functional characteristics with other stem cells. **Proc Natl Acad Sci U S A,** v. 100, n. 11, p. 6487-92, May 2003. ISSN 0027-8424. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/12738887 >. Acessed at: 24. march. 2015.

KÖHLER, C.; VILLAR, C. B. Programming of gene expression by Polycomb group proteins. **Trends Cell Biol,** v. 18, n. 5, p. 236-43, May 2008. ISSN 1879-3088. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/18375123 >. Acessed at: 24. march. 2015.

LEE, K. H.; LEE, W. Y.; KIM, J. H.; YOON, M. J.; KIM, N. H.; UHM, S. J.; KIM, D. H.; CHUNG, H. J.; SONG, H LEE, K. H. et al. Characterization of GFRα-1-positive and GFRα-1-negative spermatogonia in neonatal pig testis. **Reprod Domest Anim,** v. 48, n. 6, p. 954-60, Dec 2013. ISSN 1439-0531. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/23808407 >. Acessed at: 24. march. 2015.

LUO, C.; LU, X.; STUBBS, L.; KIM, J. Rapid evolution of a recently retroposed transcription factor YY2 in mammalian genomes. **Genomics,** v. 87, n. 3, p. 348-55, Mar 2006. ISSN 0888-7543. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/16377127 >. Acessed at: 24. march. 2015.

LUO, J.; MEGEE, S.; RATHI, R.; DOBRINSKI, I**.** Protein gene product 9.5 is a spermatogonia-specific marker in the pig testis: application to enrichment and culture of porcine spermatogonia. **Mol Reprod Dev,** v. 73, n. 12, p. 1531-40, Dec 2006. ISSN 1040-452X. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/16894537 >. Acessed at: 24. march. 2015.

LÖWER, R.; LÖWER, J.; KURTH, R. The viruses in all of us: characteristics and biological significance of human endogenous retrovirus sequences. **Proc Natl Acad Sci U S A,** v. 93, n. 11, p. 5177-84, May 1996. ISSN 0027-8424. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/8643549 >. Acessed at: 24. march. 2015.

MAKSAKOVA, I. A.; ROMANISH, M. T.; GAGNIER, L.; DUNN, C. A.; VAN DE LAGEMAAT, L. N.; MAGER, D. L. Retroviral elements and their hosts: insertional mutagenesis in the mouse germ line. **PLoS Genet,** v. 2, n. 1, p. e2, Jan 2006. ISSN 1553-7404. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/16440055 >. Acessed at: 24. march. 2015.

MASUI, S.; OHTSUKA, S.; YAGI, R.; TAKAHASHI, K.; KO, M. S.; NIWA, H. Rex1/Zfp42 is dispensable for pluripotency in mouse ES cells. **BMC Dev Biol,** v. 8, p. 45, 2008. ISSN 1471-213X. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/18433507 >. Acessed at: 24. march. 2015.

MENG, X.; LINDAHL, M.; HYVÖNEN, M. E.; PARVINEN, M.; DE ROOIJ, D. G.; HESS, M. W.; RAATIKAINEN-AHOKAS, A.; SAINIO, K.; RAUVALA, H.; LAKSO, M.; PICHEL, J. G.; WESTPHAL, H.; SAARMA, M.; SARIOLA, H. Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. **Science,** v. 287, n. 5457, p. 1489-93, Feb 2000. ISSN 0036-8075. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/10688798 >. Acessed at: 24. march. 2015.

MONGAN, N. P.; MARTIN, K. M.; GUDAS, L. J. The putative human stem cell marker, Rex-1 (Zfp42): structural classification and expression in normal human epithelial and carcinoma cell cultures. **Mol Carcinog,** v. 45, n. 12, p. 887-900, Dec 2006. ISSN 0899-1987. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/16865673 >. Acessed at: 24. march. 2015.

NAUGHTON, C. K.; JAIN, S.; STRICKLAND, A. M.; GUPTA, A.; MILBRANDT, J. Glial cell-line derived neurotrophic factor-mediated RET signaling regulates spermatogonial stem cell fate. **Biol Reprod,** v. 74, n. 2, p. 314-21, Feb 2006. ISSN 0006-3363. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/16237148 >. Acessed at: 24. march. 2015.

NGUYEN, N.; ZHANG, X.; OLASHAW, N.; SETO, E. Molecular cloning and functional characterization of the transcription factor YY2. **J Biol Chem,** v. 279, n. 24, p. 25927-34, Jun 2004. ISSN 0021-9258. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/15087442 >. Acessed at: 24. march. 2015.

OAKBERG, E. F. Spermatogonial stem-cell renewal in the mouse. **Anat Rec,** v. 169, n. 3, p. 515-31, Mar 1971. ISSN 0003-276X. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/5550531 >. Acessed at: 24. march. 2015.

ORWIG, K. E.; RYU, B. Y.; AVARBOCK, M. R.; BRINSTER, R. L. Male germ-line stem cell potential is predicted by morphology of cells in neonatal rat testes. **Proc Natl Acad Sci U S A,** v. 99, n. 18, p. 11706-11, Sep 2002. ISSN 0027-8424. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/12185252 >. Acessed at: 24. march. 2015.

PHILLIPS, B. T.; GASSEI, K.; ORWIG, K. E. Spermatogonial stem cell regulation and spermatogenesis. **Philos Trans R Soc Lond B Biol Sci,** v. 365, n. 1546, p. 1663-78, May 2010. ISSN 1471-2970. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/20403877 >. Acessed at: 24. march. 2015.

REDING, S. C.; STEPNOSKI, A. L.; CLONINGER, E. W.; OATLEY, J. M. THY1 is a conserved marker of undifferentiated spermatogonia in the pre-pubertal bull testis. **Reproduction,** v. 139, n. 5, p. 893-903, May 2010. ISSN 1741-7899. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/20154176 >. Acessed at: 24. march. 2015.

REZENDE, N. C.; LEE, M. Y.; MONETTE, S.; MARK, W.; LU, A.; GUDAS, L. J.. Rex1 (Zfp42) null mice show impaired testicular function, abnormal testis morphology, and aberrant gene expression. **Dev Biol,** v. 356, n. 2, p. 370-82, Aug 2011. ISSN 1095-564X. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/21641340 >. Acessed at: 24. march. 2015.

ROGERS, M. B.; HOSLER, B. A.; GUDAS, L. J. Specific expression of a retinoic acid-regulated, zinc-finger gene, Rex-1, in preimplantation embryos, trophoblast and spermatocytes. **Development,** v. 113, n. 3, p. 815-24, Nov 1991. ISSN 0950-1991. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/1821852 >. Acessed at: 24. march. 2015.

RYU, B. Y.; ORWIG, K. E.; KUBOTA, H.; AVARBOCK, M. R.; BRINSTER, R. L. Phenotypic and functional characteristics of spermatogonial stem cells in rats. **Dev Biol,** v. 274, n. 1, p. 158-70, Oct 2004. ISSN 0012-1606. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/15355795 >. Acessed at: 24. march. 2015.

SCHOORLEMMER, J.; PÉREZ-PALACIOS, R.; CLIMENT, M.; GUALLAR, D.; MUNIESA, P. Regulation of Mouse Retroelement MuERV-L/MERVL Expression by REX1 and Epigenetic Control of Stem Cell Potency. **Front Oncol,** v. 4, p. 14, 2014. ISSN 2234-943X. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/24567914 >. Acessed at: 24. march. 2015.

SCHUETTENGRUBER, B.; CHOURROUT, D.; VERVOORT, M.; LEBLANC, B.;CAVALLI, G Genome regulation by polycomb and trithorax proteins. **Cell,** v. 128, n. 4, p. 735-45, Feb 2007. ISSN 0092-8674. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/17320510 >. Acessed at: 24. march. 2015.

SPRADLING, A.; DRUMMOND-BARBOSA, D.; KAI, T. Stem cells find their niche. **Nature,** v. 414, n. 6859, p. 98-104, Nov 2001. ISSN 0028-0836. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/11689954 >. Acessed at: 24. march. 2015.

TEGELENBOSCH, R. A.; DE ROOIJ, D. G. A quantitative study of spermatogonial multiplication and stem cell renewal in the C3H/101 F1 hybrid mouse. **Mutat Res,** v. 290, n. 2, p. 193-200, Dec 1993. ISSN 0027-5107. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/7694110 >. Acessed at: 24. march. 2015.

THOMAS, M. J.; SETO, E. Unlocking the mechanisms of transcription factor YY1: are chromatin modifying enzymes the key? **Gene,** v. 236, n. 2, p. 197-208, Aug 1999. ISSN 0378-1119. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/10452940 >. Acessed at: 24. march. 2015.

WESTERNSTRÖER, B.; TERWORT, N.; EHMCKE, J.; WISTUBA, J.; SCHLATT, S.; NEUHAUS, N. Profiling of Cxcl12 receptors, Cxcr4 and Cxcr7 in murine testis development and a spermatogenic depletion model indicates a role for Cxcr7 in controlling Cxcl12 activity. **PLoS One,** v. 9, n. 12, p. e112598, 2014. ISSN 1932-6203. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/25460567 >. Acessed at: 24. march. 2015.

YANG, Q. E.; KIM, D.; KAUCHER, A.; OATLEY, M.J.; OATLEY, J.M. CXCL12-CXCR4 signaling is required for the maintenance of mouse spermatogonial stem cells. **J Cell Sci,** v. 126, n. Pt 4, p. 1009-20, Feb 2013. ISSN 1477-9137. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/23239029 >. Acessed at: 24. march. 2015.

YANG, Q. E.; RACICOT, K. E.; KAUCHER, A. V.; OATLEY, M. J.; OATLEY, J. M. MicroRNAs 221 and 222 regulate the undifferentiated state in mammalian male germ cells. **Development,** v. 140, n. 2, p. 280-90, Jan 2013. ISSN 1477-9129. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/23221369 >. Acessed at: 24. march. 2015.

YING, Q. L.; WRAY, J.; NICHOLS, J.; BATLLE-MORERA, L.; DOBLE, B.; WOODGETT, J.; COHEN, P.; SMITH, A. The ground state of embryonic stem cell self-renewal. **Nature,** v. 453, n. 7194, p. 519-23, May 2008. ISSN 1476-4687. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/18497825 >. Acessed at: 24. march. 2015.

ZHENG, Y.; HE, Y.; AN, J.; QIN, J.; WANG, Y.; ZHANG, Y.; TIAN, X.; ZENG, W. THY1 is a surface marker of porcine gonocytes. **Reprod Fertil Dev,** v. 26, n. 4, p. 533-9, 2014. ISSN 1031-3613. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/23683542 >. Acessed at: 24. march. 2015.

ZHENG, Y.; ZHANG, Y.; QU, R.; HE, Y.; TIAN, X.; ZENG, W. Spermatogonial stem cells from domestic animals: progress and prospects. **Reproduction,** v. 147, n. 3, p. R65-74, Mar 2014. ISSN 1741-7899. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/24357661 >. Acessed at: 24. march. 2015.

8 CONCLUSIONS

Based on the presented data, this study has two main conclusions matching with our principal hypothesis. The first principal conclusion is: age/sexual maturity affects stem cell potential of bovine undifferentiated germ cells, as observed in the specific expression of SSC molecular markers and genes associated with SSC cellular biology. Specifically, we observed that differential plating is an effective technique to enrichment of undifferentiated spermatogonial stem cell being more efficient when performed without laminin coating (article 1), age/sexual maturity affects expression of consecrated undifferentiated spermatogonia molecular markers for bovine such as UCHL1 (article 2) and age/sexual maturity affects expression of specific genes (*SELP* and *UCHL1)* (article 2). We also observed, that CXCR4 is a specific molecular marker for SSC and its expression is conserved in SSC provided from prepubertal calves and adult bulls (article 3). However, age/sexual maturity affects expression of specific genes (*THY1* and *LIMS2*) and the stem cell potential of bovine CXCR4+ SSC (article 3).

The second main conclusion of present study is: YY2 is a specific molecular marker for pospubertal mice SSC (article 4) and possibly YY2 could be considered as interspecific candidate gene for SSC. Complementing that conclusion, we observed that YY2 binding sequences possibly influence gene expression and transcriptional control at a distance in SSC (article 4). Moreover, REX1 binding sequences also possibly control gene expression in embryonic stem cells (article 4).

REFERENCE

ABBASI, H.; TAHMOORESPUR, M.; HOSSEINI, S. M.; NASIRI, Z.; BAHADORANI, M.; HAJIAN, M. ; NASIRI, M. R.; NASR-ESFAHANI, M. H. THY1 as a reliable marker for enrichment of undifferentiated spermatogonia in the goat. **Theriogenology,** v. 80, n. 8, p. 923-32, Nov 2013. ISSN 1879-3231. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/23987985 >. Acessed at: 24. march. 2015.

AHMAD, S.; XIAO, Y.; HAN, L.; HUA, H.; RIAZ, H.;LIANG, A.; YANG, L.G. Isolation, identification and enrichment of type a spermatogonia from the testis of chinese cross-bred buffaloes (swamp × river). **Reprod Domest Anim,** v. 48, n. 3, p. 373-81, Jun 2013. ISSN 1439-0531. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/22928737 >. Acessed at: 24. march. 2015.

AMANN, R. P.; KAVANAUGH, J. F.; GRIEL, L. C.; VOGLMAYR, J. K Sperm production of Holstein bulls determined from testicular spermatid reserves, after cannulation of rete testis or vas deferens, and by daily ejaculation. **J Dairy Sci,** v. 57, n. 1, p. 93-9, Jan 1974. ISSN 0022-0302. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/4810488 >. Acessed at: 24. march. 2015.

ANGLIN, E.; DAVEY, R.; HERRID, M.; HOPE, S.; KURKURI, M.; PASIC, P.; HOR, M.; FENECH, M.; THISSEN, H.;VOELCKER, N. H. Cell microarrays for the screening of factors that allow the enrichment of bovine testicular cells. **Cytometry A,** v. 77, n. 9, p. 881-9, Sep 2010. ISSN 1552-4930. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/20803736 >. Acessed at: 24. march. 2015.

APONTE, P. M.; SODA, T.; VAN DE KANT, H. J.; DE ROOIJ, D. G.l. Basic features of bovine spermatogonial culture and effects of glial cell line-derived neurotrophic factor. **Theriogenology,** v. 65, n. 9, p. 1828-47, Jun 2006. ISSN 0093-691X. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/16321433 >. Acessed at: 24. march. 2015.

APONTE, P.M.; VAN BRAGT, M.P.; DE ROOIJ, D.G.; VAN PELT, A.M. Spermatogonial stem cells: characteristics and experimental possibilities. **APMIS,** v. 113, n. 11-12, p. 727-42, 2005 Nov-Dec 2005. ISSN 0903-4641. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/16480445 >. Acessed at: 24. march. 2015.

ARA, T.; NAKAMURA, Y.; EGAWA, T.; SUGIYAMA, T.; ABE, K.; KISHIMOTO, T.; MATSUI, Y. ; NAGASAWA, T. Impaired colonization of the gonads by primordial germ cells in mice lacking a chemokine, stromal cell-derived factor-1 (SDF-1). **Proc Natl Acad Sci U S A,** v. 100, n. 9, p. 5319-23, Apr 2003. ISSN 0027-8424. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/12684531 >. Acessed at: 24. march. 2015.

ATCHISON, M.; BASU, A.; ZAPRAZNA, K.; PAPASANI, M. Mechanisms of Yin Yang 1 in oncogenesis: the importance of indirect effects. **Crit Rev Oncog,** v. 16, n. 3-4, p. 143-61, 2011. ISSN 0893-9675. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/22248052 >. Acessed at: 24. march. 2015.

AUBERT, J.; DUNSTAN, H.; CHAMBERS, I.; SMITH, A**.** Functional gene screening in embryonic stem cells implicates Wnt antagonism in neural differentiation. **Nat Biotechnol,** v. 20, n. 12, p. 1240-5, Dec 2002. ISSN 1087-0156. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/12447396 >. Acessed at: 24. march. 2015.

BEARDSLEY, A.; ROBERTSON, D. M.; O'DONNELL, L. A complex containing alpha6beta1-integrin and phosphorylated focal adhesion kinase between Sertoli cells and elongated spermatids during spermatid release from the seminiferous epithelium. **J Endocrinol,** v. 190, n. 3, p. 759-70, Sep 2006. ISSN 0022-0795. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/17003277 >. Acessed at: 24. march. 2015.

BELLVÉ, A. R. et al. Spermatogenic cells of the prepuberal mouse. Isolation and morphological characterization. **J Cell Biol,** v. 74, n. 1, p. 68-85, Jul 1977. ISSN 0021-9525. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/874003 >. Acessed at: 24. march. 2015.

BELLVÉ, A. R.; MILLETTE, C. F.; BHATNAGAR, Y. M.; O'BRIEN, D. A. Dissociation of the mouse testis and characterization of isolated spermatogenic cells. **J Histochem Cytochem,** v. 25, n. 7, p. 480-94, Jul 1977. ISSN 0022-1554. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/893996 >. Acessed at: 24. march. 2015.

BELLVÉ, A. R.; ZHENG, W. Growth factors as autocrine and paracrine modulators of male gonadal functions. **J Reprod Fertil,** v. 85, n. 2, p. 771-93, Mar 1989. ISSN 0022-4251. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/2649665 >. Acessed at: 24. march. 2015.

BLEUL, C. C.; FARZAN, M.; CHOE, H.; PAROLIN, C.; CLARK-LEWIS, I.; SODROSKI, J.; SPRINGER, T. A The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry. **Nature,** v. 382, n. 6594, p. 829-33, Aug 1996. ISSN 0028-0836. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/8752280 >. Acessed at: 24. march. 2015.

BORJIGIN, U.; DAVEY, R.; HUTTON, K.; HERRID, M.. Expression of promyelocytic leukaemia zinc-finger in ovine testis and its application in evaluating the enrichment efficiency of differential plating. **Reprod Fertil Dev,** v. 22, n. 5, p. 733-42, 2010. ISSN 1031-3613. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/20450825 >. Acessed at: 24. march. 2015.

BOYLE, M.; WONG, C.; ROCHA, M.; JONES, D. L.l. Decline in self-renewal factors contributes to aging of the stem cell niche in the Drosophila testis. **Cell Stem Cell,** v. 1, n. 4, p. 470-8, Oct 2007. ISSN 1875-9777. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/18371382 >. Acessed at: 24. march. 2015.

BRINSTER, R. L.; AVARBOCK, M. R. Germline transmission of donor haplotype following spermatogonial transplantation. **Proc Natl Acad Sci U S A,** v. 91, n. 24, p. 11303-7, Nov 1994. ISSN 0027-8424. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/7972054 >. Acessed at: 24. march. 2015.

BRINSTER, R. L.; ZIMMERMANN, J. W. Spermatogenesis following male germ-cell transplantation. **Proc Natl Acad Sci U S A,** v. 91, n. 24, p. 11298-302, Nov 1994. ISSN 0027-8424. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/7972053 >. Acessed at: 24. march. 2015.

BUAAS, F. W.; KIRSH, A. L.; SHARMA, M.; MCLEAN, D. J.; MORRIS, J. L.; GRISWOLD, M. D.;DE ROOIJ, D. G.; BRAUN, R. E. Plzf is required in adult male germ cells for stem cell self-renewal. **Nat Genet,** v. 36, n. 6, p. 647-52, Jun 2004. ISSN 1061-4036. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/15156142 >. Acessed at: 24. march. 2015.

CHAMBERS, S. M.; SHAW, C. A.; GATZA, C.; FISK, C. J.; DONEHOWER, L. A.;GOODELL, M. A. Aging hematopoietic stem cells decline in function and exhibit epigenetic dysregulation. **PLoS Biol,** v. 5, n. 8, p. e201, Aug 2007. ISSN 1545-7885. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/17676974 >. Acessed at: 24. march. 2015.

CHEN, X.; XU, H.; YUAN, P.; FANG, F.; HUSS, M.; VEGA, V. B.; WONG, E.; ORLOV, Y. L.; ZHANG, W.; JIANG, J.; LOH, Y. H.; YEO, H. C.; YEO, Z. X.; NARANG, V.; GOVINDARAJAN, K. R.; LEONG, B.; SHAHAB, A.; RUAN, Y.; BOURQUE, G.; SUNG, W. K.; CLARKE, N. D.; WEI, C. L.; NG, H. H.. Integration of external signaling pathways with the core transcriptional network in embryonic stem cells. **Cell,** v. 133, n. 6, p. 1106-17, Jun 2008. ISSN 1097-4172. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/18555785 >. Acessed at: 24. march. 2015.

CHENG, J.; TÜRKEL, N.; HEMATI, N.; FULLER, M. T.; HUNT, A. J.; YAMASHITA, Y. M. Centrosome misorientation reduces stem cell division during ageing. **Nature,** v. 456, n. 7222, p. 599-604, Dec 2008. ISSN 1476-4687. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/18923395 >. Acessed at: 24. march. 2015.

CLIMENT, M.; ALONSO-MARTIN, S.; PÉREZ-PALACIOS, R.; GUALLAR, D.; BENITO, A. A.; LARRAGA, A.; FERNÁNDEZ-JUAN, M.; SANZ, M.; DE DIEGO, A.; SEISDEDOS, M. T.; MUNIESA, P.; SCHOORLEMMER, J. Functional analysis of Rex1 during preimplantation development. **Stem Cells Dev,** v. 22, n. 3, p. 459-72, Feb 2013. ISSN 1557-8534. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/22897771 >. Acessed at: 24. march. 2015.

COSTA, G. M.; AVELAR, G. F.; REZENDE-NETO, J. V.; CAMPOS-JUNIOR, P. H.; LACERDA, S. M.; ANDRADE, B. S.; THOMÉ, R. G.; HOFMANN, M. C.; FRANCA, L. R. Spermatogonial stem cell markers and niche in equids. **PLoS One,** v. 7, n. 8, p. e44091, 2012. ISSN 1932-6203. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/22937157 >. Acessed at: 24. march. 2015.

CURTIS, S. K.; AMANN, R. P. Testicular development and establishment of spermatogenesis in Holstein bulls. **J Anim Sci,** v. 53, n. 6, p. 1645-57, Dec 1981. ISSN 0021-8812. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/7341622 >. Acessed at: 24. march. 2015.

DANN, C. T.; ALVARADO, A. L.; MOLYNEUX, L. A.; DENARD, B. S.; GARBERS, D. L.; PORTEUS, M. H. Spermatogonial stem cell self-renewal requires OCT4, a factor

downregulated during retinoic acid-induced differentiation. **Stem Cells,** v. 26, n. 11, p. 2928-37, Nov 2008. ISSN 1549-4918. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/18719224 >. Acessed at: 24. march. 2015.

DE BARROS, F. R.; WORST, R. A.; SAURIN, G. C.; MENDES, C. M.; ASSUMPÇÃO, M. E.; VISINTIN, J. A. α-6 integrin expression in bovine spermatogonial cells purified by discontinuous Percoll density gradient. **Reprod Domest Anim,** v. 47, n. 6, p. 887-90, Dec 2012. ISSN 1439-0531. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/22320406 >. Acessed at: 24. march. 2015.

DE ROOIJ, D. G. Proliferation and differentiation of spermatogonial stem cells. **Reproduction,** v. 121, n. 3, p. 347-54, Mar 2001. ISSN 1470-1626. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/11226060 >. Acessed at: 24. march. 2015.

DREWS, D.; KLAR, M.; DAME, C.; BRÄUER, A. U. Developmental expression profile of the YY2 gene in mice. **BMC Dev Biol,** v. 9, p. 45, 2009. ISSN 1471-213X. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/19638226 >. Acessed at: 24. march. 2015.

DYM, M.; RAJ, H. G. Response of adult rat Sertoli cells and Leydig cells to depletion of luteinizing hormone and testosterone. **Biol Reprod,** v. 17, n. 5, p. 676-96, Dec 1977. ISSN 0006-3363. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/597535 >. Acessed at: 24. march. 2015.

ERTL, C.; WROBEL, K. H. Distribution of sugar residues in the bovine testis during postnatal ontogenesis demonstrated with lectin-horseradish peroxidase conjugates. **Histochemistry,** v. 97, n. 2, p. 161-71, 1992. ISSN 0301-5564. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/1559848 >. Acessed at: 24. march. 2015.

FEDOROFF, N. How jumping genes were discovered. **Nat Struct Biol,** v. 8, n. 4, p. 300-1, Apr 2001. ISSN 1072-8368. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/11276245 >. Acessed at: 24. march. 2015.

FEIG, L. A.; BELLVÉ, A. R.; ERICKSON, N. H.; KLAGSBRUN, M. Sertoli cells contain a mitogenic polypeptide. **Proc Natl Acad Sci U S A,** v. 77, n. 8, p. 4774-8, Aug 1980. ISSN 0027-8424. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/6933525 >. Acessed at: 24. march. 2015.

FUJIHARA, M.; KIM, S. M.; MINAMI, N.; YAMADA, M.; IMAI, H l. Characterization and in vitro culture of male germ cells from developing bovine testis. **J Reprod Dev,** v. 57, n. 3, p. 355-64, Jun 2011. ISSN 1348-4400. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/21289464 >. Acessed at: 24. march. 2015.

FUSI, F. M.; MONTESANO, M.; BERNOCCHI, N.; PANZERI, C.; FERRARA, F.; VILLA, A.; BRONSON, R. A. P-selectin is expressed on the oolemma of human and hamster oocytes following sperm adhesion and is also detected on the equatorial region of acrosome-reacted human spermatozoa. **Mol Hum Reprod,** v. 2, n. 5, p. 341-7, May 1996. ISSN 1360-9947. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/9238701 > Acessed at: 24. march. 2015..

GEIGER, H.; RUDOLPH, K. L. Aging in the lympho-hematopoietic stem cell compartment. **Trends Immunol,** v. 30, n. 7, p. 360-5, Jul 2009. ISSN 1471-4981. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/19540806 >. Acessed at: 24. march. 2015.

GENG, J. G.; RAUB, T. J.; BAKER, C. A.; SAWADA, G. A.; MA, L.; ELHAMMER, A. P. Expression of a P-selectin ligand in zona pellucida of porcine oocytes and P-selectin on acrosomal membrane of porcine sperm cells. Potential implications for their involvement in sperm-egg interactions. **J Cell Biol,** v. 137, n. 3, p. 743-54, May 1997. ISSN 0021-9525. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/9151678 >. Acessed at: 24. march. 2015.

GOEL, S.; FUJIHARA, M.; MINAMI, N.; YAMADA, M.; IMAI, H.. Expression of NANOG, but not POU5F1, points to the stem cell potential of primitive germ cells in neonatal pig testis. **Reproduction,** v. 135, n. 6, p. 785-95, Jun 2008. ISSN 1741-7899. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/18367503 > Acessed at: 24. march. 2015..

GOEL, S.; REDDY, N.; MANDAL, S.; FUJIHARA, M.; KIM, S. M.; IMAI, H. Spermatogonia-specific proteins expressed in prepubertal buffalo (Bubalus bubalis) testis and their utilization for isolation and in vitro cultivation of spermatogonia. **Theriogenology,** v. 74, n. 7, p. 1221-32, Oct 2010. ISSN 1879-3231. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/20708247 >. Acessed at: 24. march. 2015.

GOEL, S.; SUGIMOTO, M.; MINAMI, N.; YAMADA, M.;KUME, S.; IMAI, H. Identification, isolation, and in vitro culture of porcine gonocytes. **Biol Reprod,** v. 77, n. 1, p. 127-37, Jul 2007. ISSN 0006-3363. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/17377141 >. Acessed at: 24. march. 2015.

GRISWOLD, M. D. The central role of Sertoli cells in spermatogenesis. **Semin Cell Dev Biol,** v. 9, n. 4, p. 411-6, Aug 1998. ISSN 1084-9521. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/9813187 >. Acessed at: 24. march. 2015.

GUALLAR, D.; PÉREZ-PALACIOS, R.; CLIMENT, M.; MARTÍNEZ-ABADÍA, I.; LARRAGA, A.; FERNÁNDEZ-JUAN, M.; VALLEJO, C.; MUNIESA, P.; SCHOORLEMMER, J.l. Expression of endogenous retroviruses is negatively regulated by the pluripotency marker Rex1/Zfp42. **Nucleic Acids Res,** v. 40, n. 18, p. 8993-9007, Oct 2012. ISSN 1362-4962. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/22844087 >. Acessed at: 24. march. 2015.

GUAN, K.; NAYERNIA, K.; MAIER, L. S.; WAGNER, S.; DRESSEL, R.; LEE, J. H.; NOLTE, J.; WOLF, F.; LI, M.; ENGEL, W.; HASENFUSS, G. Pluripotency of spermatogonial stem cells from adult mouse testis. **Nature,** v. 440, n. 7088, p. 1199-203, Apr 2006. ISSN 1476-4687. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/16565704 >. Acessed at: 24. march. 2015.

GUAN, K.; WOLF, F.; BECKER, A.; ENGEL, W.; NAYERNIA, K.; HASENFUSS, G. Isolation and cultivation of stem cells from adult mouse testes. **Nat Protoc,** v. 4, n. 2, p. 143-54, 2009. ISSN 1750-2799. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/19180086 >. Acessed at: 24. march. 2015.

HE, Y.; CHEN, X.; ZHU, H.; WANG, D. Developments in techniques for the isolation, enrichment, main culture conditions and identification of spermatogonial stem cells. **Cytotechnology**, Mar 2015. ISSN 0920-9069. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/25749914 > Acessed at: 24. march. 2015..

HEIDARI, B.; RAHMATI-AHMADABADI, M.; AKHONDI, M. M.; ZARNANI, A. H.; JEDDI-TEHRANI, M.; SHIRAZI, A.; NADERI, M. M.; BEHZADI, B. Isolation, identification, and culture of goat spermatogonial stem cells using c-kit and PGP9.5 markers. **J Assist Reprod Genet,** v. 29, n. 10, p. 1029-38, Oct 2012. ISSN 1573-7330. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/22782689 > Acessed at: 24. march. 2015..

HERMANN, B. P.; MUTOJI, K. N.; VELTE, E. K.; KO, D.; OATLEY, J. M.; GEYER, C. B.; MCCARREY, J. R. Transcriptional and Translational Heterogeneity among Neonatal Mouse Spermatogonia. **Biol Reprod**, Jan 2015. ISSN 1529-7268. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/25568304 > Acessed at: 24. march. 2015..

HERMANN, B. P.; SUKHWANI, M.; SIMORANGKIR, D. R.; CHU, T.; PLANT, T. M.; ORWIG, K. E. Molecular dissection of the male germ cell lineage identifies putative spermatogonial stem cells in rhesus macaques. **Hum Reprod,** v. 24, n. 7, p. 1704-16, Jul 2009. ISSN 1460-2350. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/19336441 >. Acessed at: 24. march. 2015.

HERRID, M.; DAVEY, R. J.; HILL, J. R. Characterization of germ cells from pre-pubertal bull calves in preparation for germ cell transplantation. **Cell Tissue Res,** v. 330, n. 2, p. 321-9, Nov 2007. ISSN 0302-766X. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/17593396 >. Acessed at: 24. march. 2015.

HERRID, M.; DAVEY, R. J.; HUTTON, K.; COLDITZ, I. G.; HILL, J. R. A comparison of methods for preparing enriched populations of bovine spermatogonia. **Reprod Fertil Dev,** v. 21, n. 3, p. 393-9, 2009. ISSN 1031-3613. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/19261216 >. Acessed at: 24. march. 2015.

HOFMANN, M. C.; BRAYDICH-STOLLE, L.; DYM, M. Isolation of male germ-line stem cells; influence of GDNF. **Dev Biol,** v. 279, n. 1, p. 114-24, Mar 2005. ISSN 0012-1606. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/15708562 >. Acessed at: 24. march. 2015.

HUANG, D. W.; SHERMAN, B. T.; LEMPICKI, R. A. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. **Nat Protoc,** v. 4, n. 1, p. 44-57, 2009. ISSN 1750-2799. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/19131956 >. Acessed at: 24. march. 2015.

HUCKINS, C. The spermatogonial stem cell population in adult rats. 3. Evidence for a long-cycling population. **Cell Tissue Kinet,** v. 4, n. 4, p. 335-49, Jul 1971. ISSN 0008-8730. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/5127357 >. Acessed at: 24. march. 2015.

IZADYAR, F.; DEN OUDEN, K.; STOUT, T. A.; STOUT, J.; CORET, J.; LANKVELD, D. P.; SPOORMAKERS, T. J.; COLENBRANDER, B.; OLDENBROEK, J. K.; VAN DER PLOEG, K. D.; WOELDERS, H.; KAL, H. B.; DE ROOIJ, D. G. Autologous and homologous transplantation of bovine spermatogonial stem cells. **Reproduction,** v. 126, n. 6, p. 765-74, Dec 2003. ISSN 1470-1626. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/14748695 >. Acessed at: 24. march. 2015.

IZADYAR, F.; MATTHIJS-RIJSENBILT, J. J.; DEN OUDEN, K.; CREEMERS, L. B.; WOELDERS, H.; DE ROOIJ, D. G. Development of a cryopreservation protocol for type A spermatogonia. **J Androl,** v. 23, n. 4, p. 537-45, 2002 Jul-Aug 2002. ISSN 0196-3635. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/12065461 >. Acessed at: 24. march. 2015.

IZADYAR, F.; SPIERENBERG, G.T.; CREEMERS, L.B.; DEN OUDEN, K.; DE ROOIJ, D.G. Isolation and purification of type A spermatogonia from the bovine testis. **Reproduction,** v. 124, n. 1, p. 85-94, Jul 2002. ISSN 1470-1626. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/12090922 >. Acessed at: 24. march. 2015.

JANCA, F. C.; JOST, L. K.; EVENSON, D. P. Mouse testicular and sperm cell development characterized from birth to adulthood by dual parameter flow cytometry. **Biol Reprod,** v. 34, n. 4, p. 613-23, May 1986. ISSN 0006-3363. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/3708046 >. Acessed at: 24. march. 2015.

JERN, P.; COFFIN, J. M. Effects of retroviruses on host genome function. **Annu Rev Genet,** v. 42, p. 709-32, 2008. ISSN 0066-4197. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/18694346 >. Acessed at: 24. march. 2015.

JOHNSON, L.; THOMPSON, D. L.; VARNER, D. D. Role of Sertoli cell number and function on regulation of spermatogenesis. **Anim Reprod Sci,** v. 105, n. 1-2, p. 23-51, Apr 2008. ISSN 0378-4320. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/18242891 >. Acessed at: 24. march. 2015.

JOHNSON, L.; VARNER, D. D.; THOMPSON, D. L. Effect of age and season on the establishment of spermatogenesis in the horse. **J Reprod Fertil Suppl,** v. 44, p. 87-97, 1991. ISSN 0449-3087. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/1795306 >. Acessed at: 24. march. 2015.

JUTTE, N. H.; JANSEN, R.; GROOTEGOED, J. A.; ROMMERTS, F. F.; CLAUSEN, O. P.; VAN DER MOLEN, H. J. Regulation of survival of rat pachytene spermatocytes by lactate supply from Sertoli cells. **J Reprod Fertil,** v. 65, n. 2, p. 431-8, Jul 1982. ISSN 0022-4251. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/6808129 >. Acessed at: 24. march. 2015.

KANATSU-SHINOHARA, M.; OGONUKI, N.; INOUE, K.; OGURA, A.; TOYOKUNI, S.;SHINOHARA, T. Reconstitution of mouse spermatogonial stem cell niches in culture. **Cell Stem Cell,** v. 11, n. 4, p. 567-78, Oct 2012. ISSN 1875-9777. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/23040482 >. Acessed at: 24. march. 2015.

KANATSU-SHINOHARA, M.; OGONUKI, N.; INOUE, K.; MIKI, H.; OGURA, A.; TOYOKUNI, S.; SHINOHARA,T. Long-term proliferation in culture and germline

transmission of mouse male germline stem cells. **Biol Reprod,** v. 69, n. 2, p. 612-6, Aug 2003. ISSN 0006-3363. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/12700182 >. Acessed at: 24. march. 2015.

KANATSU-SHINOHARA, M.; TOYOKUNI, S.; SHINOHARA, T. CD9 is a surface marker on mouse and rat male germline stem cells. **Biol Reprod,** v. 70, n. 1, p. 70-5, Jan 2004. ISSN 0006-3363. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/12954725 > Acessed at: 24. march. 2015..

KAPUSHESKY, M.; ADAMUSIAK, T.; BURDETT, T.; CULHANE, A.; FARNE, A.; FILIPPOV, A.; HOLLOWAY, E.; KLEBANOV, A.; KRYVYCH, N.; KURBATOVA, N.; KURNOSOV, P.; MALONE, J.; MELNICHUK, O.; PETRYSZAK, R.; PULTSIN, N.; RUSTICI, G.; TIKHONOV, A.; TRAVILLIAN, R. S.; WILLIAMS, E.; ZORIN, A.; PARKINSON, H.; BRAZMA, A.. Gene Expression Atlas update--a value-added database of microarray and sequencing-based functional genomics experiments. **Nucleic Acids Res,** v. 40, n. Database issue, p. D1077-81, Jan 2012. ISSN 1362-4962. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/22064864 >. Acessed at: 24. march. 2015.

KERR, J. B.; LOVELAND, K. L.; O'BRYAN, M. K.; DE KRETSER, D. M. Cytology of the Testis and Intrinsic Control Mechanisms. In: NEILL, J. D. (Ed.). **Knobil and Neill's Physiology of Reproduction**. 3ed. Oxford, UK: Elsevier Inc, v.1, 2006. chap. Cytology of the Testis and Intrinsic Control Mechanisms, p.827-948. (Male Reproductive System). Acessed at: 24. march. 2015.

KIM, J. D.; FAULK, C.; KIM, J. Retroposition and evolution of the DNA-binding motifs of YY1, YY2 and REX1. **Nucleic Acids Res,** v. 35, n. 10, p. 3442-52, 2007. ISSN 1362-4962. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/17478514 >. Acessed at: 24. march. 2015.

KIM, J. D. ; KIM, H.; EKRAM, M. B.; YU, S.; FAULK, C.; KIM, J. Rex1/Zfp42 as an epigenetic regulator for genomic imprinting. **Hum Mol Genet,** v. 20, n. 7, p. 1353-62, Apr 2011. ISSN 1460-2083. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/21233130 >. Acessed at: 24. march. 2015.

KIM, S. K.; JANG, H. R.; KIM, J. H.; NOH, S. M.; SONG, K. S.; KIM, .M. R.; KIM, S. Y.; YEOM, Y. I.; KIM, N. S.; YOO, H. S.; KIM, Y. S. The epigenetic silencing of LIMS2 in gastric cancer and its inhibitory effect on cell migration. **Biochem Biophys Res Commun,** v. 349, n. 3, p. 1032-40, Oct 2006. ISSN 0006-291X. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/16959213 >. Acessed at: 24. march. 2015.

KIM, Y. H.; KIM, B. J.; KIM, B. G.; LEE, Y. A.; KIM, K. J.; CHUNG, H. J.; HWANG, S.; WOO, J. S.; PARK, J. K.; SCHMIDT, J. A.; PANG, M. G.; RYU, B. Y. Stage-specific embryonic antigen-1 expression by undifferentiated spermatogonia in the prepubertal boar testis. **J Anim Sci,** v. 91, n. 7, p. 3143-54, Jul 2013. ISSN 1525-3163. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/23572256 >. Acessed at: 24. march. 2015.

KLISCH, K.; CONTRERAS, D. A. ;SUN, X.; BREHM, R.; BERGMANN, M.; ALBERIO, R. The Sda/GM2-glycan is a carbohydrate marker of porcine primordial germ cells and of a subpopulation of spermatogonia in cattle, pigs, horses and llama.

Reproduction, v. 142, n. 5, p. 667-74, Nov 2011. ISSN 1741-7899. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/21896636 >. Acessed at: 24. march. 2015.

KOKKINAKI, M.; DJOURABTCHI, A.; GOLESTANEH, N. Long-term Culture of Human SSEA-4 Positive Spermatogonial Stem Cells (SSCs). **J Stem Cell Res Ther,** v. 2, n. 2, Nov 2011. ISSN 2157-7633. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/24466499 >. Acessed at: 24. march. 2015.

KOKKINAKI, M.; LEE, T. L.; HE, Z.; JIANG, J.; GOLESTANEH, N.; HOFMANN, M. C.; CHAN, W. Y.; DYM, M. The molecular signature of spermatogonial stem/progenitor cells in the 6-day-old mouse testis. **Biol Reprod,** v. 80, n. 4, p. 707-17, Apr 2009. ISSN 0006-3363. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/19109221 >. Acessed at: 24. march. 2015.

KOKKINAKI, M.; LEE, T. L.; HE, Z.; JIANG, J.; GOLESTANEH, N.; HOFMANN, M. C.; CHAN, W. Y.; DYM, M. Age affects gene expression in mouse spermatogonial stem/progenitor cells. **Reproduction,** v. 139, n. 6, p. 1011-20, Jun 2010. ISSN 1741-7899. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/20371641 >. Acessed at: 24. march. 2015.

KON, Y.; ENDOH, D.; IWANAGA, T. Expression of protein gene product 9.5, a neuronal ubiquitin C-terminal hydrolase, and its developing change in sertoli cells of mouse testis. **Mol Reprod Dev,** v. 54, n. 4, p. 333-41, Dec 1999. ISSN 1040-452X. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/10542373 >. Acessed at: 24. march. 2015.

KUBOTA, H.; AVARBOCK, M. R.; BRINSTER, R. L. Spermatogonial stem cells share some, but not all, phenotypic and functional characteristics with other stem cells. **Proc Natl Acad Sci U S A,** v. 100, n. 11, p. 6487-92, May 2003. ISSN 0027-8424. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/12738887 >. Acessed at: 24. march. 2015.

KUBOTA, H.; AVARBOCK, M. R.; BRINSTER, R. L. Culture conditions and single growth factors affect fate determination of mouse spermatogonial stem cells. **Biol Reprod,** v. 71, n. 3, p. 722-31, Sep 2004a. ISSN 0006-3363. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/15115718 >. Acessed at: 24. march. 2015.

KUBOTA, H.; AVARBOCK, M. R.; BRINSTER, R. L.. Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. **Proc Natl Acad Sci U S A,** v. 101, n. 47, p. 16489-94, Nov 2004b. ISSN 0027-8424. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/15520394 >. Acessed at: 24. march. 2015.

KUCIA, M.; JANKOWSKI, K.; RECA, R.; WYSOCZYNSKI, M.; BANDURA, L.; ALLENDORF, D. J.; ZHANG, J.; RATAJCZAK, J.; RATAJCZAK, M. Z. CXCR4-SDF-1 signalling, locomotion, chemotaxis and adhesion. **J Mol Histol,** v. 35, n. 3, p. 233-45, Mar 2004. ISSN 1567-2379. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/15339043 >. Acessed at: 24. march. 2015.

KUCIA, M.; RECA, R.; MIEKUS, K.; WANZECK, J.; WOJAKOWSKI, W.; JANOWSKA-WIECZOREK, A.; RATAJCZAK, J.; RATAJCZAK, M. Z. Trafficking of

normal stem cells and metastasis of cancer stem cells involve similar mechanisms: pivotal role of the SDF-1-CXCR4 axis. **Stem Cells,** v. 23, n. 7, p. 879-94, Aug 2005. ISSN 1066-5099. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/15888687 >. Acessed at: 24. march. 2015.

KÖHLER, C.; VILLAR, C. B. Programming of gene expression by Polycomb group proteins. **Trends Cell Biol,** v. 18, n. 5, p. 236-43, May 2008. ISSN 1879-3088. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/18375123 >. Acessed at: 24. march. 2015.

LEE, K. H.; LEE, W. Y.; KIM, J. H.; YOON, M. J.; KIM, N. H.; UHM, S. J.; KIM, D. H.; CHUNG, H. J.; SONG, H. Characterization of GFRα-1-positive and GFRα-1-negative spermatogonia in neonatal pig testis. **Reprod Domest Anim,** v. 48, n. 6, p. 954-60, Dec 2013. ISSN 1439-0531. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/23808407 >. Acessed at: 24. march. 2015.

LIANG, M.; LI, W.; TIAN, H.; HU, T.; WANG, L.; LIN, Y.; LI, Y.; HUANG, H.; SUN, F. Sequential expression of long noncoding RNA as mRNA gene expression in specific stages of mouse spermatogenesis. **Sci Rep,** v. 4, p. 5966, 2014. ISSN 2045-2322. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/25097017 >. Acessed at: 24. march. 2015.

LIU, Y.; LIU, J.; CHEN, J.; CHENG, L.; CAO, Q.; ZHU, L.; SUN, Y.; LIU, Q.; LI, J.. Molecular cloning and characterization of a novel splice variant of the LIM domain family gene, PINCH 2, in human testis. **Mol Biotechnol,** v. 35, n. 2, p. 109-18, Feb 2007. ISSN 1073-6085. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/17435276 >. Acessed at: 24. march. 2015.

LOLICATO, F.; MARINO, R.; PARONETTO, M. P.; PELLEGRINI, M.; DOLCI, S.; GEREMIA, R.; GRIMALDI, P. Potential role of Nanos3 in maintaining the undifferentiated spermatogonia population. **Dev Biol,** v. 313, n. 2, p. 725-38, Jan 2008. ISSN 1095-564X. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/18089289 >. Acessed at: 24. march. 2015.

LUO, C.; LU, X.; STUBBS, L.; KIM, J. Rapid evolution of a recently retroposed transcription factor YY2 in mammalian genomes. **Genomics,** v. 87, n. 3, p. 348-55, Mar 2006. ISSN 0888-7543. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/16377127 >. Acessed at: 24. march. 2015.

LUO, J.; MEGEE, S.; RATHI, R.; DOBRINSKI, I. Protein gene product 9.5 is a spermatogonia-specific marker in the pig testis: application to enrichment and culture of porcine spermatogonia. **Mol Reprod Dev,** v. 73, n. 12, p. 1531-40, Dec 2006. ISSN 1040-452X. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/16894537 >. Acessed at: 24. march. 2015.

LÖWER, R.; LÖWER, J.; KURTH, R. The viruses in all of us: characteristics and biological significance of human endogenous retrovirus sequences. **Proc Natl Acad Sci U S A,** v. 93, n. 11, p. 5177-84, May 1996. ISSN 0027-8424. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/8643549 >. Acessed at: 24. march. 2015.

MAHLA, R. S.; REDDY, N.; GOEL, S. Spermatogonial stem cells (SSCs) in buffalo (Bubalus bubalis) testis. **PLoS One,** v. 7, n. 4, p. e36020, 2012. ISSN 1932-6203. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/22536454 >. Acessed at: 24. march. 2015.

MAKSAKOVA, I. A.; ROMANISH, M. T.; GAGNIER, L.; DUNN, C. A.; VAN DE LAGEMAAT, L. N.; MAGER, D. L. Retroviral elements and their hosts: insertional mutagenesis in the mouse germ line. **PLoS Genet,** v. 2, n. 1, p. e2, Jan 2006. ISSN 1553-7404. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/16440055 >. Acessed at: 24. march. 2015.

MASUI, S.; OHTSUKA, S.; YAGI, R.; TAKAHASHI, K.; KO, M. S.; NIWA, H.l. Rex1/Zfp42 is dispensable for pluripotency in mouse ES cells. **BMC Dev Biol,** v. 8, p. 45, 2008. ISSN 1471-213X. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/18433507 >. Acessed at: 24. march. 2015.

MCIVER, S. C.; LOVELAND, K. L.; ROMAN, S. D.; NIXON, B.; KITAZAWA, R.; MCLAUGHLIN, E. A**.** The chemokine CXCL12 and its receptor CXCR4 are implicated in human seminoma metastasis. **Andrology,** v. 1, n. 3, p. 517-29, May 2013. ISSN 2047-2927. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/23495012 >. Acessed at: 24. march. 2015.

MCMILLAN, M.; ANDRONICOS, N.; DAVEY, R.; STOCKWELL, S.; HINCH, G.; SCHMOELZL, S. Claudin-8 expression in Sertoli cells and putative spermatogonial stem cells in the bovine testis. **Reprod Fertil Dev,** v. 26, n. 5, p. 633-44, Jun 2014. ISSN 1031-3613. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/23673210 >. Acessed at: 24. march. 2015.

MEINHARDT, A.; O'BRYAN, M. K.; MCFARLANE, J. R.; LOVELAND, K. L.; MALLIDIS, C.; FOULDS, L. M.; PHILLIPS, D. J.; DE KRETSER, D. M. . Localization of follistatin in the rat testis. **J Reprod Fertil,** v. 112, n. 2, p. 233-41, Mar 1998. ISSN 0022-4251. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/9640262 >. Acessed at: 24. march. 2015.

MENG, X.; LINDAHL, M.; HYVÖNEN, M. E.; PARVINEN, M.; DE ROOIJ, D. G.; HESS, M. W.; RAATIKAINEN-AHOKAS, A.; SAINIO, K.; RAUVALA, H.; LAKSO, M.; PICHEL, J. G.; WESTPHAL, H.; SAARMA, M.; SARIOLA, H. Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. **Science,** v. 287, n. 5457, p. 1489-93, Feb 2000. ISSN 0036-8075. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/10688798 >. Acessed at: 24. march. 2015.

MOLYNEAUX, K. A.; ZINSZNER, H.; KUNWAR, P. S.; SCHAIBLE, K.; STEBLER, J.; SUNSHINE, M. J.; O'BRIEN, W.; RAZ, E.; LITTMAN, D.; WYLIE, C.; LEHMANN, R. The chemokine SDF1/CXCL12 and its receptor CXCR4 regulate mouse germ cell migration and survival. **Development,** v. 130, n. 18, p. 4279-86, Sep 2003. ISSN 0950-1991. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/12900445 >. Acessed at: 24. march. 2015.

MONGAN, N. P.; MARTIN, K. M.; GUDAS, L. J. The putative human stem cell marker, Rex-1 (Zfp42): structural classification and expression in normal human

epithelial and carcinoma cell cultures. **Mol Carcinog,** v. 45, n. 12, p. 887-900, Dec 2006. ISSN 0899-1987. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/16865673 >. Acessed at: 24. march. 2015.

MÜLLER, A.; HOMEY, B.; SOTO, H.; GE, N.; CATRON, D.; BUCHANAN, M. E.; MCCLANAHAN, T.; MURPHY, E.; YUAN, W.; WAGNER, S. N.; BARRERA, J. L.; MOHAR, A.; VERÁSTEGUI, E.; ZLOTNIK, A. Involvement of chemokine receptors in breast cancer metastasis. **Nature,** v. 410, n. 6824, p. 50-6, Mar 2001. ISSN 0028-0836. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/11242036 >. Acessed at: 24. march. 2015.

NASIRI, Z.; HOSSEINI, S. M.; HAJIAN, M.; ABEDI, P.; BAHADORANI, M.; BAHARVAND, H.; NASR-ESFAHANI, M. Effects of different feeder layers on short-term culture of prepubertal bovine testicular germ cells in-vitro. **Theriogenology,** v. 77, n. 8, p. 1519-28, May 2012. ISSN 1879-3231. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/22289219 >. Acessed at: 24. march. 2015.

NAUGHTON, C. K.; JAIN, S.; STRICKLAND, A. M.; GUPTA, A.; MILBRANDT, J. Glial cell-line derived neurotrophic factor-mediated RET signaling regulates spermatogonial stem cell fate. **Biol Reprod,** v. 74, n. 2, p. 314-21, Feb 2006. ISSN 0006-3363. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/16237148 >. Acessed at: 24. march. 2015.

NGUYEN, N.; ZHANG, X.; OLASHAW, N.; SETO, E. Molecular cloning and functional characterization of the transcription factor YY2. **J Biol Chem,** v. 279, n. 24, p. 25927-34, Jun 2004. ISSN 0021-9258. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/15087442 >. Acessed at: 24. march. 2015.

OAKBERG, E. F. Spermatogonial stem-cell renewal in the mouse. **Anat Rec,** v. 169, n. 3, p. 515-31, Mar 1971. ISSN 0003-276X. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/5550531 >. Acessed at: 24. march. 2015.

OATLEY, J. M.; BRINSTER, R. L. Regulation of spermatogonial stem cell self-renewal in mammals. **Annu Rev Cell Dev Biol,** v. 24, p. 263-86, 2008. ISSN 1081-0706. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/18588486 >. Acessed at: 24. march. 2015.

OATLEY, J. M.; DE AVILA, D. M.; MCLEAN, D. J.; GRISWOLD, M. D.; REEVES, J. J. Transplantation of bovine germinal cells into mouse testes. **J Anim Sci,** v. 80, n. 7, p. 1925-31, Jul 2002. ISSN 0021-8812. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/12162661 >. Acessed at: 24. march. 2015.

OATLEY, J. M.; OATLEY, M. J.; AVARBOCK, M. R.; TOBIAS, J. W.; BRINSTER, R. L. Colony stimulating factor 1 is an extrinsic stimulator of mouse spermatogonial stem cell self-renewal. **Development,** v. 136, n. 7, p. 1191-9, Apr 2009. ISSN 0950-1991. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/19270176 >. Acessed at: 24. march. 2015.

OATLEY, J. M.; REEVES, J. J.; MCLEAN, D. J. Biological activity of cryopreserved bovine spermatogonial stem cells during in vitro culture. **Biol Reprod,** v. 71, n. 3, p.

942-7, Sep 2004. ISSN 0006-3363. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/15151932 >. Acessed at: 24. march. 2015.

ORWIG, K. E.; RYU, B. Y.; AVARBOCK, M. R.; BRINSTER, R. L. Male germ-line stem cell potential is predicted by morphology of cells in neonatal rat testes. **Proc Natl Acad Sci U S A,** v. 99, n. 18, p. 11706-11, Sep 2002. ISSN 0027-8424. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/12185252 >. Acessed at: 24. march. 2015.

PARKER, K. L.; SCHIMMER, B. P. S. Embryology and Genetics of the Mammalian Gonads and Ducts. In: JIMMY D. NEILL (Ed.). **Knobil and Neill's Physiology of Reproduction**. 3rd. London, UK: Elsevier, v.1, 2006. chap. 8, p.314-336. Acessed at: 24. march. 2015.

PAYNE, C. J.; GALLAGHER, S. J.; FOREMAN, O.; DANNENBERG, J. H.; DEPINHO, R. A.; BRAUN, R. E. Sin3a is required by sertoli cells to establish a niche for undifferentiated spermatogonia, germ cell tumors, and spermatid elongation. **Stem Cells,** v. 28, n. 8, p. 1424-34, Aug 2010. ISSN 1549-4918. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/20572009 >. Acessed at: 24. march. 2015.

PETRYSZAK, R.; BURDETT, T.; FIORELLI, B.; FONSECA, N. A.; GONZALEZ-PORTA, M.; HASTINGS, E.; HUBER, W.; JUPP, S.; KEAYS, M.; KRYVYCH, N.; MCMURRY, J.; MARIONI, J. C.; MALONE, J.; MEGY, K.; RUSTICI, G.; TANG, A. Y.; TAUBERT, J.; WILLIAMS, E.; MANNION, O.; PARKINSON, H. E.; BRAZMA, A Expression Atlas update--a database of gene and transcript expression from microarray- and sequencing-based functional genomics experiments. **Nucleic Acids Res,** v. 42, n. Database issue, p. D926-32, Jan 2014. ISSN 1362-4962. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/24304889 >. Acessed at: 24. march. 2015.

PHILLIPS, B. T.; GASSEI, K.; ORWIG, K. E. Spermatogonial stem cell regulation and spermatogenesis. **Philos Trans R Soc Lond B Biol Sci,** v. 365, n. 1546, p. 1663-78, May 2010. ISSN 1471-2970. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/20403877 >. Acessed at: 24. march. 2015.

QASEMI-PANAHI, B.; TAJIK, P.; MOVAHEDIN, M.; MOGHADDAM, G.; BARZGAR, Y.; HEIDARI-VALA, H. Differentiation of bovine spermatogonial stem cells into osteoblasts. **Avicenna J Med Biotechnol,** v. 3, n. 3, p. 149-53, Jul 2011. ISSN 2008-2835. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/23408761 >. Acessed at: 24. march. 2015.

REDING, S. C.; STEPNOSKI, A. L.; CLONINGER, E. W.; OATLEY, J. M. THY1 is a conserved marker of undifferentiated spermatogonia in the pre-pubertal bull testis. **Reproduction,** v. 139, n. 5, p. 893-903, May 2010. ISSN 1741-7899. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/20154176 >. Acessed at: 24. march. 2015.

REZENDE, N. C.; LEE, M. Y.; MONETTE, S.; MARK, W.; LU, A.; GUDAS, L. J. Rex1 (Zfp42) null mice show impaired testicular function, abnormal testis morphology, and aberrant gene expression. **Dev Biol,** v. 356, n. 2, p. 370-82, Aug 2011. ISSN 1095-564X. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/21641340 >. Acessed at: 24. march. 2015.

ROGERS, M. B.; HOSLER, B. A.; GUDAS, L. J. Specific expression of a retinoic acid-regulated, zinc-finger gene, Rex-1, in preimplantation embryos, trophoblast and spermatocytes. **Development,** v. 113, n. 3, p. 815-24, Nov 1991. ISSN 0950-1991. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/1821852 >. Acessed at: 24. march. 2015.

RYU, B. Y.; ORWIG, K. E.; KUBOTA, H.; AVARBOCK, M. R.; BRINSTER, R. L. Phenotypic and functional characteristics of spermatogonial stem cells in rats. **Dev Biol,** v. 274, n. 1, p. 158-70, Oct 2004. ISSN 0012-1606. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/15355795 >. Acessed at: 24. march. 2015.

RYU, B. Y.; ORWIG, K. E.; OATLEY, J. M.; AVARBOCK, M. R.; BRINSTER, R. L. Effects of aging and niche microenvironment on spermatogonial stem cell self-renewal. **Stem Cells,** v. 24, n. 6, p. 1505-11, Jun 2006. ISSN 1066-5099. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/16456131 >. Acessed at: 24. march. 2015.

SALANOVA, M.; STEFANINI, M.; DE CURTIS, I.; PALOMBI, F. Integrin receptor alpha 6 beta 1 is localized at specific sites of cell-to-cell contact in rat seminiferous epithelium. **Biol Reprod,** v. 52, n. 1, p. 79-87, Jan 1995. ISSN 0006-3363. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/7711187 >. Acessed at: 24. march. 2015.

SCADDEN, D. T. The stem-cell niche as an entity of action. **Nature,** v. 441, n. 7097, p. 1075-9, Jun 2006. ISSN 1476-4687. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/16810242 >. Acessed at: 24. march. 2015.

SCHOORLEMMER, J.; PÉREZ-PALACIOS, R.; CLIMENT, M.; GUALLAR, D.; MUNIESA, P. Regulation of Mouse Retroelement MuERV-L/MERVL Expression by REX1 and Epigenetic Control of Stem Cell Potency. **Front Oncol,** v. 4, p. 14, 2014. ISSN 2234-943X. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/24567914 >. Acessed at: 24. march. 2015.

SCHUETTENGRUBER, B.; CHOURROUT, D.; VERVOORT, M.; LEBLANC, B.;CAVALLI, G. Genome regulation by polycomb and trithorax proteins. **Cell,** v. 128, n. 4, p. 735-45, Feb 2007. ISSN 0092-8674. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/17320510 >. Acessed at: 24. march. 2015.

SHINOHARA, T.; AVARBOCK, M. R.; BRINSTER, R. L. beta1- and alpha6-integrin are surface markers on mouse spermatogonial stem cells. **Proc Natl Acad Sci U S A,** v. 96, n. 10, p. 5504-9, May 1999. ISSN 0027-8424. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/10318913 >. Acessed at: 24. march. 2015.

SHINOHARA, T.; BRINSTER, R. L. Enrichment and transplantation of spermatogonial stem cells. **Int J Androl,** v. 23 Suppl 2, p. 89-91, 2000. ISSN 0105-6263. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/10849507 >. Acessed at: 24. march. 2015.

SHINOHARA, T.; ORWIG, K. E.; AVARBOCK, M. R.; BRINSTER, R. L. Spermatogonial stem cell enrichment by multiparameter selection of mouse testis cells. **Proc Natl Acad Sci U S A,** v. 97, n. 15, p. 8346-51, Jul 2000. ISSN 0027-

8424. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/10900001 >. Acessed at: 24. march. 2015.

SONG, W.; ZHU, H.; LI, M.; LI, N.; WU, J.; MU, H.; YAO, X.; HAN, W.; LIU, W.; HUA, J. Promyelocytic leukaemia zinc finger maintains self-renewal of male germline stem cells (mGSCs) and its expression pattern in dairy goat testis. **Cell Prolif,** v. 46, n. 4, p. 457-68, Aug 2013. ISSN 1365-2184. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/23869766 >. Acessed at: 24. march. 2015.

SPRADLING, A.; DRUMMOND-BARBOSA, D.; KAI, T. Stem cells find their niche. **Nature,** v. 414, n. 6859, p. 98-104, Nov 2001. ISSN 0028-0836. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/11689954 >. Acessed at: 24. march. 2015.

STEIBEL, J. P.; POLETTO, R.; COUSSENS, P. M.; ROSA, G. J. A powerful and flexible linear mixed model framework for the analysis of relative quantification RT-PCR data. **Genomics,** v. 94, n. 2, p. 146-52, Aug 2009. ISSN 1089-8646. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/19422910 >. Acessed at: 24. march. 2015.

STUKENBORG, J. B.; WISTUBA, J.; LUETJENS, C. M.; ELHIJA, M. A.; HULEIHEL, M.; LUNENFELD, E.; GROMOLL, J.; NIESCHLAG, E.; SCHLATT, S. Coculture of spermatogonia with somatic cells in a novel three-dimensional soft-agar-culture-system. **J Androl,** v. 29, n. 3, p. 312-29, 2008 May-Jun 2008. ISSN 1939-4640. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/18046051 >. Acessed at: 24. march. 2015.

TANG, L.; RODRIGUEZ-SOSA, J. R.; DOBRINSKI, I. Germ cell transplantation and testis tissue xenografting in mice. **J Vis Exp**, n. 60, 2012. ISSN 1940-087X. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/22330955 >. Acessed at: 24. march. 2015.

TEGELENBOSCH, R. A.; DE ROOIJ, D. G. A quantitative study of spermatogonial multiplication and stem cell renewal in the C3H/101 F1 hybrid mouse. **Mutat Res,** v. 290, n. 2, p. 193-200, Dec 1993. ISSN 0027-5107. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/7694110 >. Acessed at: 24. march. 2015.

THOMAS, M. J.; SETO, E. Unlocking the mechanisms of transcription factor YY1: are chromatin modifying enzymes the key? **Gene,** v. 236, n. 2, p. 197-208, Aug 1999. ISSN 0378-1119. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/10452940 >. Acessed at: 24. march. 2015.

TOKUDA, M.; KADOKAWA, Y.; KURAHASHI, H.; MARUNOUCHI, T. CDH1 is a specific marker for undifferentiated spermatogonia in mouse testes. **Biol Reprod,** v. 76, n. 1, p. 130-41, Jan 2007. ISSN 0006-3363. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/17035642 >. Acessed at: 24. march. 2015.

TRES, L. L.; SMITH, E. P.; VAN WYK, J. J.; KIERSZENBAUM, A. L.. Immunoreactive sites and accumulation of somatomedin-C in rat Sertoli-spermatogenic cell co-cultures. **Exp Cell Res,** v. 162, n. 1, p. 33-50, Jan 1986. ISSN 0014-4827. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/2998847 >. Acessed at: 24. march. 2015.

WANG, Y. L.; LIU, W.; SUN, Y. J.; KWON, J.; SETSUIE, R.; OSAKA, H.; NODA, M.; AOKI, S.; YOSHIKAWA, Y.; WADA, K.. Overexpression of ubiquitin carboxyl-terminal hydrolase L1 arrests spermatogenesis in transgenic mice. **Mol Reprod Dev,** v. 73, n. 1, p. 40-9, Jan 2006. ISSN 1040-452X. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/16177983 >. Acessed at: 24. march. 2015.

WESTERNSTRÖER, B.; TERWORT, N.; EHMCKE, J.; WISTUBA, J.; SCHLATT, S.; NEUHAUS, N. Profiling of Cxcl12 receptors, Cxcr4 and Cxcr7 in murine testis development and a spermatogenic depletion model indicates a role for Cxcr7 in controlling Cxcl12 activity. **PLoS One,** v. 9, n. 12, p. e112598, 2014. ISSN 1932-6203. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/25460567 >. Acessed at: 24. march. 2015.

WU, J.;SONG, W.; ZHU, H.; NIU, Z.; MU, H.; LEI, A.; YANG, C.; PENG, S.; LI, X.; LI, G.; HUA, J.. Enrichment and characterization of Thy1-positive male germline stem cells (mGSCs) from dairy goat (Capra hircus) testis using magnetic microbeads. **Theriogenology,** v. 80, n. 9, p. 1052-60, Dec 2013. ISSN 1879-3231. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/24055398 >. Acessed at: 24. march. 2015.

WU, X.; GOODYEAR, S. M.; TOBIAS, J. W.; AVARBOCK, M. R.; BRINSTER, R. L.l. Spermatogonial stem cell self-renewal requires ETV5-mediated downstream activation of Brachyury in mice. **Biol Reprod,** v. 85, n. 6, p. 1114-23, Dec 2011. ISSN 1529-7268. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/21816850 >. Acessed at: 24. march. 2015.

WYROBE WYROBEK, A. J.; ESKENAZI, B.; YOUNG, S.; ARNHEIM, N.; TIEMANN-BOEGE, I.; JABS, E. W.; GLASER, R. L.; PEARSON, F. S.; EVENSON, D. Advancing age has differential effects on DNA damage, chromatin integrity, gene mutations, and aneuploidies in sperm. **Proc Natl Acad Sci U S A,** v. 103, n. 25, p. 9601-6, Jun 2006. ISSN 0027-8424. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/16766665 >. Acessed at: 24. march. 2015.

YANG, Q. E.; KIM, D.; KAUCHER, A.; OATLEY, M.J.; OATLEY, J.M. CXCL12-CXCR4 signaling is required for the maintenance of mouse spermatogonial stem cells. **J Cell Sci,** v. 126, n. Pt 4, p. 1009-20, Feb 2013. ISSN 1477-9137. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/23239029 >. Acessed at: 24. march. 2015.

YANG, Q. E.; RACICOT, K. E.; KAUCHER, A. V.; OATLEY, M. J.; OATLEY, J. M. MicroRNAs 221 and 222 regulate the undifferentiated state in mammalian male germ cells. **Development,** v. 140, n. 2, p. 280-90, Jan 2013. ISSN 1477-9129. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/23221369 >. Acessed at: 24. march. 2015.

YING, Q. L.; WRAY, J.; NICHOLS, J.; BATLLE-MORERA, L.; DOBLE, B.; WOODGETT, J.; COHEN, P.; SMITH, A. The ground state of embryonic stem cell self-renewal. **Nature,** v. 453, n. 7194, p. 519-23, May 2008. ISSN 1476-4687. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/18497825 >. Acessed at: 24. march. 2015.

YOSHIDA, S.; TAKAKURA, A.; OHBO, K.; ABE, K.; WAKABAYASHI, J.; YAMAMOTO, M.; SUDA, T.; NABESHIMA, Y. Neurogenin3 delineates the earliest stages of spermatogenesis in the mouse testis. **Dev Biol,** v. 269, n. 2, p. 447-58, May 2004. ISSN 0012-1606. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/15110712 >. Acessed at: 24. march. 2015.

ZHANG, X.; EBATA, K. T.; ROBAIRE, B.; NAGANO, M. C. Aging of male germ line stem cells in mice. **Biol Reprod,** v. 74, n. 1, p. 119-24, Jan 2006. ISSN 0006-3363. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/16177220 >. Acessed at: 24. march. 2015.

ZHENG, Y.; HE, Y.; AN, J.; QIN, J.; WANG, Y.; ZHANG, Y.; TIAN, X.; ZENG, W.. THY1 is a surface marker of porcine gonocytes. **Reprod Fertil Dev,** v. 26, n. 4, p. 533-9, 2014. ISSN 1031-3613. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/23683542 >. Acessed at: 24. march. 2015.

ZHENG, Y.; ZHANG, Y.; QU, R.; HE, Y.; TIAN, X.; ZENG, W. Spermatogonial stem cells from domestic animals: progress and prospects. **Reproduction,** v. 147, n. 3, p. R65-74, Mar 2014. ISSN 1741-7899. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/24357661 >. Acessed at: 24. march. 2015.

ZOU, Y.; JUNG, K. J.; KIM, J. W.; YU, B. P.; CHUNG, H. Y. Alteration of soluble adhesion molecules during aging and their modulation by calorie restriction. **FASEB J,** v. 18, n. 2, p. 320-2, Feb 2004. ISSN 1530-6860. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/14688195 >. Acessed at: 24. march. 2015.