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A metabolização da glucose e da glutamina em células testiculares de ratos que sobre-expressam regucalcina

Inês Filipa Dias Simões Mateus

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Orientadora: Doutora Sara Correia
Co-orientadora: Profª. Doutora Sílvia Socorro

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Resumo

As células de Sertoli (SCs) possuem a capacidade fenomenal de fornecerem fatores de crescimento e nutrientes às células da linha germinativa. Apesar de consumirem vários tipos de substratos, incluindo aminoácidos como a glutamina, as SCs preferencialmente metabolizam glucose. Ao fazê-lo, estas células produzem grandes quantidades de lactato, aquele que é considerado pela maioria dos autores, o substrato preferencial das células germinativas. A Regucalcina (RGN) é uma proteína de ligação ao cálcio expressa nas SCs e que estudos anteriores associaram à regulação do metabolismo celular. Neste trabalho, avaliou-se a metabolização de glucose e glutamina nas SCs de ratos transgénicos que sobre expressam regucalcina (Tg-RGN) e dos seus homólogos *wild-type* (Wt). Dos testículos destes animais procedeu-se ao isolamento de SCs primárias, que foram mantidas em cultura durante 24 horas. No final deste período experimental foram analisados vários parâmetros metabólicos, tais como a expressão proteica e atividade de vários intervenientes na glicólise e glutaminólise. Observou-se que, apesar de consumirem menos glucose, as SCs dos animais Tg-RGN produzem e exportam mais lactato. Estas observações foram concomitantes com o aumento da expressão de alanina aminotransferase e com o aumento da taxa de consumo/oxidação de glutamina, o que sugere a existência de vias alternativas à glucose a contribuirde forma significativa para o aumento da produção de lactato nas SCs dos ratos Tg-RGN. Os resultados obtidos demonstram um metabolismo distinto das SCs entre ratos Wt e Tg-RGN, o que alarga o espectro das possíveis funções da RGN ao nível da espermatogénese. Para além disso, as observações registadas demonstram a enorme plasticidade do metabolismo das SCs, uma característica que pode ser de extrema relevância no contexto da fertilidade masculina.

Palavras-chave

Células de Sertoli, Espermatogénese, Glucose, Glutamina, Metabolismo, Regucalcina

Resumo alargado

A espermatogénese é um processo altamente coordenado e complexo através do qual os espermatozóides são produzidos, e que se inicia com a diferenciação das espermatogónias estaminais. Este processo compreende três fases principais: mitose, meiose e espermiógenese. Na fase proliferativa as espermatogónias sofrem uma série de divisões mitóticas diferenciando-se posteriormente em espermatócitos primários. Estes iniciam a divisão meiótica, para originar espermatócitos secundários, que por sua vez sofrem uma segunda divisão meiótica transformando-se em espermátides. Na terceira fase, espermiogénese, ocorre um rearranjo da estrutura das células e reorganização do citoplasma com diferenciação das espermátides em espermatozóides. A fertilidade masculina assenta fundamentalmente numa espermatogénese bem sucedida, a qual é dependente do suporte das células de Sertoli (SCs), as células somáticas presentes nos túbulos seminíferos (SeT). Para além de serem responsáveis pela formação da barreira hemato-testicular (BTB), as SCs desempenham um conjunto de funções de suporte físico e bioquímico relativas à manutenção da linha germinativa o que lhes concedeu, ao longo dos anos, a designação de “nurse cells”. De entre estas capacidades destaca-se o fornecimento de fatores de crescimento e nutrientes. A maioria dos investigadores sugere que, apesar de consumirem vários tipos de substratos, as células germinativas têm como substrato preferencial o lactato. No entanto, apesar de possuírem todas as enzimas do metabolismo glicolítico, as células germinativas em desenvolvimento não são capazes de metabolizar glucose, dependendo do suporte nutricional das SCs. Para tal, estas “nurse cells” captam a glucose externa através de transportadores de glucose (GLUTs) e metabolizam-na numa série de reações, denominada glicólise, cuja velocidade de reação depende da enzima fosfofrutoquinase 1 (PFK1). No final da glicólise, toda a glucose foi convertida em piruvato que, por sua vez, pode ser convertido em lactato por ação da enzima lactato desidrogenase (LDH), ou em alanina por ação da enzima alanina aminotransferase (ALT). O lactato produzido é depois exportado para o espaço extracelular pela ação de transportadores de monocarboxilato (MCTs), para poder ser utilizado pela linha germinativa. Para além disso, as SCs possuem a capacidade de metabolizar aminoácidos, tais como a glutamina, o que de alguma forma pode também levar à produção de lactato. Por forma a degradar glutamina, as SCs necessitam primeiro de captá-la do meio exterior através do transportador de aminoácidos ASC tipo 2 (ASCT2). Contudo, a importância da glutamina para as SCs e o impacto resultante para as células germinativas ainda requer clarificação. A compreensão dos mecanismos que controlam o metabolismo destas “nurse cells” é assim fundamental para o desenvolvimento futuro de tratamentos de infertilidade.

A regucalcina (RGN) é uma proteína de ligação ao cálcio (Ca^{2+}) que tem um papel importante na homeostase da concentração intracelular deste ião atuando através da modulação da atividade de canais e transportadores de Ca^{2+} na membrana celular, retículo endoplasmático e mitocôndria. Recentemente, foi demonstrado que a RGN é expressa em vários tecidos do trato reprodutor masculino, incluindo o testículo. Ao nível testicular, a RGN pode ser

encontrada quer nas SCs, quer nas células germinativas. Vários estudos acerca desta proteína demonstraram o seu envolvimento na regulação de várias funções celulares, nomeadamente, proliferação, apoptose e stress oxidativo. É de salientar, ainda, a função que tem vindo a ser atibuída à RGN ao nível do metabolismo celular. Um estudo do nosso grupo de investigação demonstrou que na próstata, a RGN parece controlar o metabolismo celular através da regulação da expressão e atividade de várias enzimas e transportadores glicolíticos. No entanto, esta associação entre a RGN e o controlo do metabolismo celular no testículo permanece por esclarecer. Assim sendo, o objectivo da presente dissertação passa por avaliar o papel da RGN no metabolismo glicolítico e na glutaminólise das SCs.

Para tal, foram recolhidos os testículos de ratos com três meses de idade, quer transgénicos que sobre-expressam a RGN (Tg-RGN) quer dos seus homólogos selvagens (Wt), tendo-se procedido ao isolamento de culturas primárias de SCs. Após 24 horas em cultura analisou-se o consumo de glucose e glutamina, a produção de lactato, os conteúdos de glucose e lactato nas SCs e nos fluídos testiculares (fluído intersticial e fluído dos SeT), bem como a expressão de vários reguladores do metabolismo glicolítico e da glutaminólise. Para além disso foi avaliada, espetrofotometricamente, a atividade enzimática da LDH.

Observou-se que as SCs dos animais Tg-RGN apresentam um metabolismo glicolítico distinto dos seus homólogos Wt. Apesar de terem um menor consumo de glucose, as SCs dos ratos Tg-RGN produzem mais lactato. Embora não concordante com os níveis diminuídos de LDH observados, a aumentada produção de lactato foi consistente com o aumento da expressão de MCT4 e de ALT. Para além disso, a expressão dos GLUTs permite explicar o menor consumo de glucose encontrado nos ratos Tg-RGN. Isto porque, apesar da expressão de GLUT1 e GLUT3, os transportadores mais associados ao consumo de glucose nas SCs, se encontrar aumentada nestes animais, a expressão de GLUT2 apresentava uma diminuição de ~70%. Ainda que este transportador já tenha sido identificado nas SCs, este foi o primeiro estudo a sugerir o seu envolvimento no metabolismo glicolítico destas células. O baixo consumo de glucose foi seguido pelos baixos níveis de glucose intracelular que podem também ser explicados pelos elevados níveis de expressão da PFK1 encontrados nas SCs transgénicas. Para além disso, foi observado um aumento do consumo de glutamina, assim como dos níveis de expressão quer do ASCT2, quer da glutaminase (GLS) nas SCs dos animais Tg-RGN quando comparadas com o grupo Wt. O aumento da taxa de consumo e oxidação deste aminoácido, juntamente com a expressão aumentada de ALT, sugerem a existência de vias alternativas à glicólise para garantir a produção de lactato quando o consumo de glucose estava diminuído. De uma forma geral, os resultados obtidos indicam que a sobre-expressão da RGN pode ser a responsável pela estimulação da via glicolítica e da glutaminólise, estabelecendo a RGN como um importante modulador do metabolismo da glucose e da glutamina nas SCs. Para além disso, estes dados demonstram a grande plasticidade do metabolismo das SCs mantendo a produção de lactato em diferentes situações, o que pode ser extremamente relevante para a manutenção da fertilidade masculina. Apesar de serem necessários mais estudos, estes

resultados despertam a curiosidade para o papel que a RGN desempenha ao nível do testículo, concretamente na regulação da espermatogénese.

Abstract

Sertoli cells (SCs) possess the outstanding capability to provide the germ line with growth factors and nutrients. Despite consuming several types of substrate, including amino acids such as glutamine, SCs preferentially metabolize glucose. By doing so, SCs produce high amounts of lactate, the substrate considered by most authors as the preferred of germ cells. Regucalcin (RGN) is a calcium binding protein expressed in the SCs that has been previously associated to the regulation of cell metabolism. On this dissertation we have evaluated glucose and glutamine handling in the SCs of transgenic rats overexpressing regucalcin (Tg-RGN) and their *wild-type* (Wt) homologous. Primary SCs were isolated from adult Wt and Tg-RGN animals and maintained in culture for 24 hours. Afterwards, several metabolic parameters, such as the protein expression of several metabolic intervenients, were analysed. We have observed that, despite consuming less glucose, the SCs of Tg-RGN animals produce and export more lactate. These observations were underpinned by increased expression of alanine transaminase and augmented rates of glutamine consumption/oxidation, which suggests the existence of alternative routes to glucose that significantly contribute to the available lactate pool in the SCs of Tg-RGN rats. The results obtained clearly present a completely distinct metabolic profile between the SCs of Tg-RGN and Wt animals, which widens the roles that RGN is likely to play in the control of spermatogenesis. Moreover, the registered observations display the enormous plasticity of SCs' metabolism, a capacity that might be of extreme relevance in the context of male fertility.

Keywords

Glucose, Glutamine, Metabolism, Regucalcin, Sertoli cells, Spermatogenesis

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List of abbreviations

ABP	Androgen-binding protein
AIS	Androgen insensitivity syndrome
ALT	Alanine aminotransferase
AMH	Anti-Mullerian hormone
AR	Androgen receptor
ASCT2	Asc-type amino acid transporter 2
ATP	Adenosine triphosphate
BSA	Bovine Serum Albumin
BTB	Blood testis barrier
Ca^{2+}	Calcium
CO_2	Carbon dioxide
EDTA	Ethylenediamine tetraacetic acid
ER	Oestrogen receptor
FBS	Fetal bovine serum
FSH	Follicle-stimulating hormone
FSH-R	Follicle-stimulating hormone receptor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GnRH	Gonadotropin releasing hormone
GLDH	Glutamate dehydrogenase
GLUT	Glucose transporter
GLS	Glutaminase
HBSS	Hank´s buffered salt solution
HDL	High-density lipoprotein
HepG2	Cloned human hepatoma cells
ITS	Insulin, transferrin and sodium selenite suplement
KO	Knockout mice
LC	Leydig cell
LDH	Lactate dehydrogenase
LH	Luteinizing hormone
LH-R	Luteinizing hormone receptor
Mac-	Macrophage-1 antigen
MCT	Monocarboxylate transporter
MPO	Myeloperoxidase
NIH	National Institutes of Health
PFK	Phosphofructokinase
PMSF	Phenylmethylsulfonyl fluoride pNA p-nitro-aniline
PVDF	Polyvinylidene difluoride
RIPA	Radioimmunoprecipitation assay

RGN	Regucalcin
SC	Sertoli cell
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SeT	Seminiferous tubules
Sry	Sex-determining region y
SCs	Sertoli cells
SeT	Seminiferous tubules
SSCs	Spermatogonial stem cell
T	Testosterone
TBHP	Tert-butyl hydroperoxide
TCA cycle	Tricarboxylic or citric acid cycle
Tg-RGN	Transgenic rats overexpressing RGN
TNFα	Tumour-necrosis-factor-α
T3	Triiodothyronine
WB	Western Blot
Wt	Wild-type
Zn	Zinc

I. Introduction

1. Male physiology: an inside out perspective of the testis and epididymis

The testicles are two oval structures suspended by the spermatic cord into the scrotum (Figure I.1), a special dual-chambered sac characteristic of humans and other land-dwelling mammals [1–3].

On their anterior and lateral surfaces the testicles are covered by a thin serous membrane, the *tunica vaginalis* (Figure I.1). Several nerves and vessels enter and exit the testis through a fibrous connective tissue capsule deep to the *tunica vaginalis*, the *tunica albuginea*. This capsule thickens and projects into the parenchyma of the testis to form the mediastinum. Numerous, thin connective tissue septa extend from the *tunica albuginea* toward the mediastinum, dividing the testis in several hundred incomplete pyramidal lobules that contain the seminiferous tubules (SeT) (Figure I.1) [4, 5].

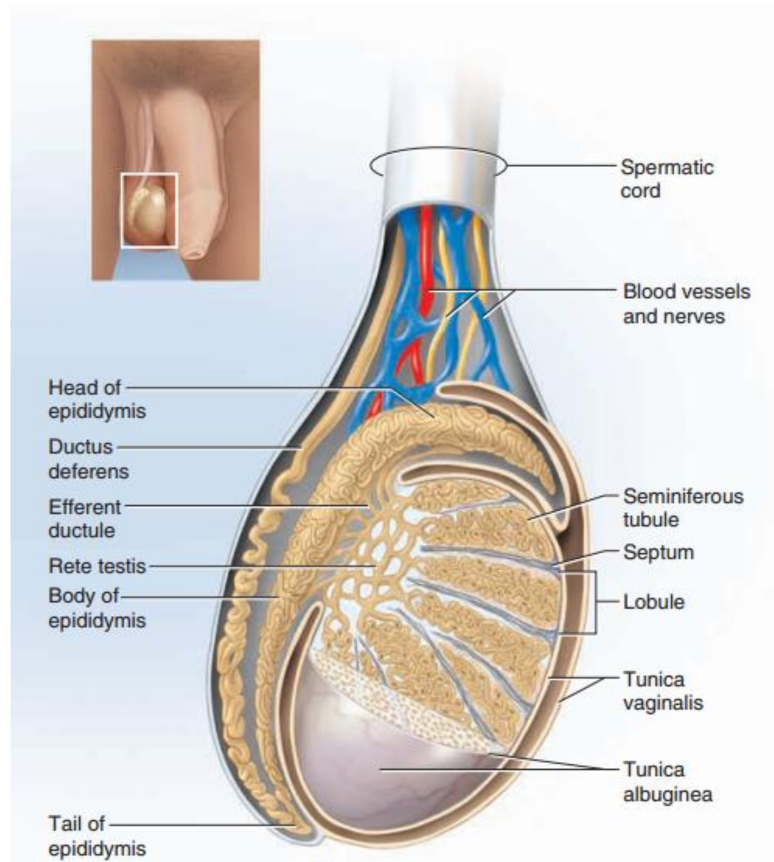

Figure I.1. Schematic representation of the mammalian testis and associated structures. The scrotum consists of a suspended sac that contains three major structures: the testis, the epididymis and the spermatic cord. The testis is covered by two connective tissue capsules, the *tunica albuginea* on the inside and the *tunica vaginalis* on the outside. Several fibrous septa extend from the tunica dividing the testis in several lobules containing the seminiferous tubules (SeT). The SeT converge to form the *rete testis* that promotes their connection to the efferent ductules. The head of the epididymis receives the testicular secretions from the efferent ductules(adapted from [4]).

In humans, the SeT have hundreds of metres long and a couple hundred micrometres in diameter, representing more than 80% of all testicular mass [6, 7]. On the outside, the SeT are surrounded by mesenchymal cells (such as peritubular myoid cells) and on the inside are composed by germ line cells and the somatic Sertoli cells (SCs), which penetrate several layers of germ cells providing them with a protective and nurturing environment [8, 9]. These SCs form tight junctions with each other creating the blood-testis-barrier (BTB) that divides the seminiferous epithelium into two separated compartments (Figure I.2): the basal and the adluminal [5, 10].

The testicular interstitium residing between SeT is quite diverse and contains Leydig cells (LCs), macrophages, leukocytes, mesenchymal cells and complex networks of nerves and blood capillaries [10].

The anatomical organization of the testicles reflects its functional role allowing the separation between the endocrine and the gamete compartments without compromising the coordination between them [11]. The main functions accomplished by the adult testis are: spermatogenesis and steroidogenesis [12, 13]. Despite their phonetical similarities, both processes conceal different goals and take place at different sites. Spermatogenesis is the process of production of the male germ cells (spermatozoa) and develops within the functional units of the testis, the SeT (Figure I.1) [7]. Steroidogenesis, on the other hand, is a process that mainly occurs in the testicular interstitium and corresponds to the synthesis and secretion of steroid hormones [14]. LCs are the main endocrine cells in the testis and the primary source of testosterone (T), an hormone that is essential for male sexual differentiation, expression of the male secondary characteristics and initiation/maintenance of spermatogenesis [15, 16].

On their posterior surface, the testis is associated with the epididymis and spermatic cord (Figure I.1), the latter incorporating the ductus deferens and the testicular neurovascular pedicle. The terminal ends of the SeT are connected to the *rete testis* trough the *tubuli recti*, which in turn are linked to the efferent ductules (Figure I.1). These efferent ductules are a series of conductive tubules that are unique in the male reproductive tract because they are lined by a ciliated epithelium. Several functions have been described for the efferent ductules, such as the reabsorption of large volumes of fluid and the transport of the sperm from the *rete testis* to the epididymis [17, 18].

The epididymis consists of a single highly compartmentalized duct to where all efferent ductules converge [19]. In most species, the epididymis can be divided into three major regions known as, from proximal to distal, the caput (head), corpus (body), and cauda (tail) [20]. Located in these regions are epithelial cells with specific functions and morphological characteristics that establish a very particular luminal microenvironment for the maturation, concentration, and storage of spermatozoa cells. The caput and corpus regions perform early and late sperm maturation events, respectively, while the cauda stores the functionally mature spermatozoa [21].

2. Sperm production: behind scenes

a) The spermatogenic process

Spermatogenesis is a complex and extremely coordinated process that is astonishingly similar in even very different animals, with the genes responsible for its control being highly conserved throughout the evolutive line [22]. The total duration of this process is about 64 days for the man and 50 days for the rat [23].

In the testicles of mice and other mammals, spermatogenesis occurs inside the SeT (Figures I.1 and I.2) [24, 25]. The spermatogenic process begins at the basal compartment and moves towards the lumen of the SeT (Figure I.2) [26, 27]. In mammals, each spermatogenic cycle encompasses three main phases: mitotic proliferation of spermatogonia, meiosis of spermatocytes, and differentiation of haploid spermatids (Figure I.2) [28, 29]. This cycle is maintained by the self-renewal of the population of spermatogonial stem cells (SSCs) and its differentiation into spermatogonia, both residents of the basal compartment [30]. As aforementioned, spermatogonia divide by mitosis. One daughter cell from each division remains close to the tubule wall, as a stem cell called the type A spermatogonium. Type A spermatogonia serve as lifetime suppliers of stem cells, thus preserving male fertility throughout old age [4]. The other daughter cell, called the type B spermatogonium, migrates slightly away from the wall on its way to becoming spermatozoa. Type B spermatogonia are committed to develop, enlarge and become primary spermatocytes. Given the fact that these cells are about to undergo meiosis and become genetically different from other cells of the body, they must be protected from the immune system. In order for this to happen, the SCs tight junctions ahead of the primary spermatocytes are dismantled, whilst new tight junctions are formed behind it. Once protected by BTB, the spermatocyte is free to move forward towards the lumen of the tubule [4]. The primary spermatocyte undergoes the first phase of meiosis, or meiosis I, a cell division that reduces the chromosome number by half. The newly formed daughter cells, called secondary spermatocytes, are therefore haploid, since they have 23 unpaired chromosomes, each consisting of two genetically identical chromatids. These secondary spermatocytes undergo the second phase of meiosis, or meiosis II, in which each chromosome splits into separate chromatids [4]. The result of this process is four daughter cells with 23 single-stranded chromosomes each, called round spermatids. These new cells divide no further, but rather undergo a process named spermiogenesis, in which they differentiate into a single spermatozoon [4]. The fundamental changes in spermiogenesis are a loss of excess cytoplasm and the growth of a tail (flagellum), restructuring the round-spermatids, first, into elongated-spermatids and, second, into light-weighted, “mobile” cells, the spermatozoa that are released in the lumen of SeT [25].

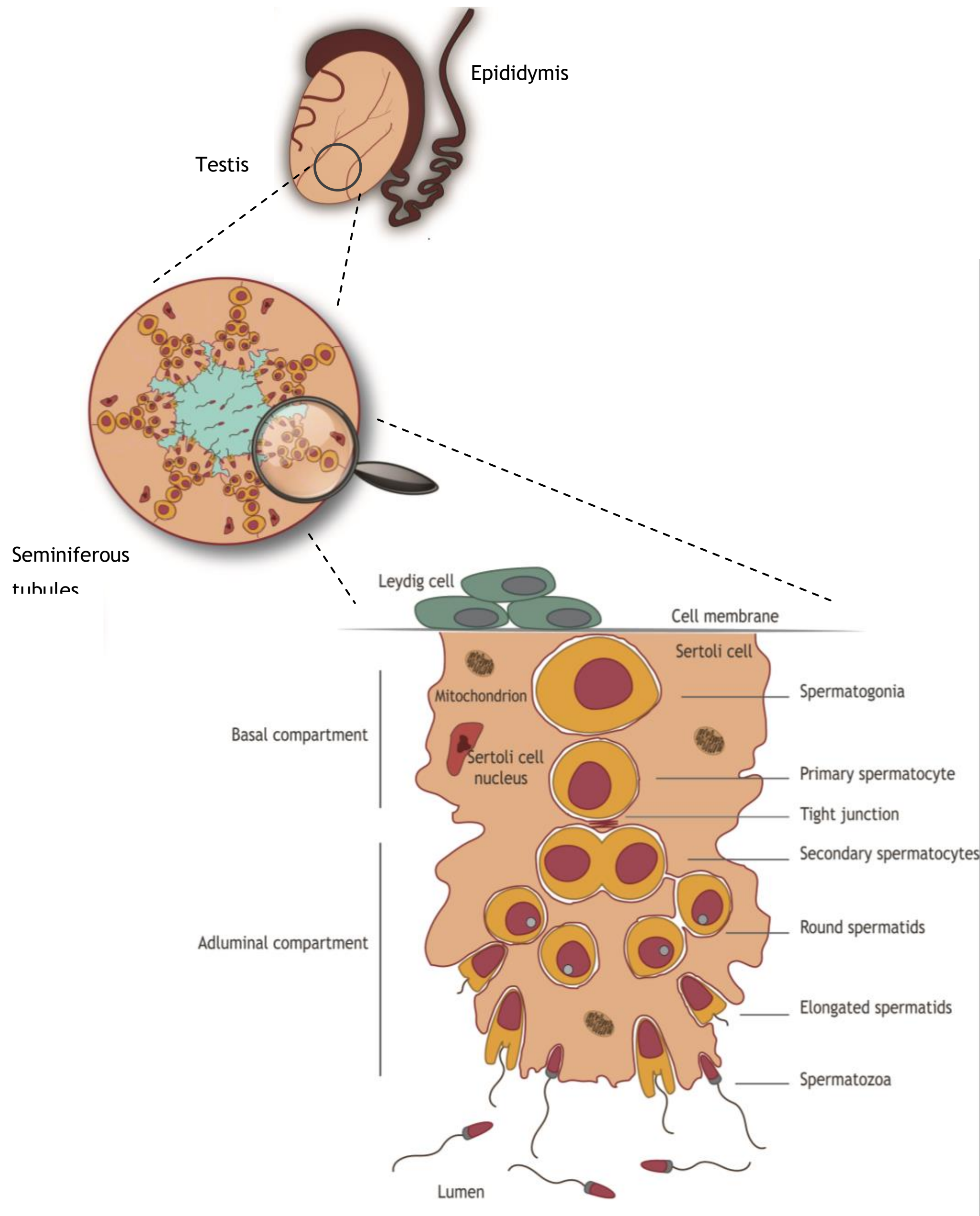

Figure I.2. Schematic representation of the testicular histology and mammalian spermatogenesis. Spermatogenesis occurs within the seminiferous tubules (SeT) in close contact to the only somatic cell type present, the Sertoli cells (SCs). This process begins with the differentiation of spermatogonia into primary spermatocytes, which then pass the tight junctions formed by adjacent SCs, evolving into secondary spermatocytes. These secondary spermatocytes differentiate even further, to the stage of spermatids, which then suffer a process called spermiogenesis originating the spermatozoa.

When spermatozoa are released from the seminiferous epithelium, they are immotile and unable to fertilize an oocyte [31]. Spermatozoa's full fertilization capacity is acquired during their sequential passage through the epididymal regions [31]. Spermatozoa reach the epididymis via efferent ductules (Figure I.1), enter the caput of the epididymis, progress to the corpus, and finally reach the caudal region, the spermatozoa's storage unit [32]. Alongside the way, mainly through the activity of caput and corpus, spermatozoa suffer a

number of biochemical and metabolic changes involving several signalling cascades, such as, cAMP and Ca^{2+} pathways [33]. These modifications result in the acquisition of hyperactivity and acrosomal responsiveness that, when in the presence of fluids of female reproductive tract, allow spermatozoa to penetrate the secondary oocyte [34].

b) Sertoli cells, the "full time" nurses

After a period of undifferentiated development, the gonadal ridges start differentiating into testicles upon the activation of specific genes located on the Y chromosome [35]. The expression of those genes cause the undifferentiated foetal cell precursors to develop into SCs [36]. Since SCs are the very first testicular cell-type to differentiate, every other cell lineage depends upon them [37].

This earliest differentiation triggers several events in the intra-uterine space. One of these events is the regression of the Mullerian ducts as a consequence of the secretion of the anti-Mullerian hormone (AMH) by immature SCs [38]. Furthermore, SCs' differentiation causes the formation of the seminiferous cord [39] and the seclusion of primordial germ cells (gonocytes) inside of it [40]. All these occurrences dictate the formation of the male reproductive tract and sexual differentiation.

Male fertility requires a very complex and dynamic process of interactions between germ cells and SCs in the epithelium of the SeT. The evolution of this epithelium is a very elongated procedure that begins in the early stages of foetal development. When completely formed, the SeT are lined by a stratified epithelium composed by SCs and germ cells, where 17-20% of all the volume is occupied by mature SCs [41].

In addition to the role in the testis formation, SCs play a pivotal role when it comes to the successful progression of germ cells into spermatozoa. SCs' ability to synthesize critical factors to facilitate and mediate spermatogenesis is unparalleled. Such critical factors may be in the form of physical support, junctional complexes or barriers, or biochemical stimulators, such as nutrients and growth factors [42–45].

The glycoproteins secreted by the SCs can be grouped in several categories based on their known biochemical properties: the first category includes transport or bioprotective proteins that are secreted in relative high abundance, such as, metal ion transporters (transferrin and ceruloplasmin) [46]; the second category includes proteases and protease inhibitors, allegedly important in tissue remodelling processes during spermiogenesis [46]; the third category includes the glycoproteins that form the basement membrane between the SCs and the peritubular cells, such as, collagen type IV and laminin [46]; and finally, the fourth category includes glycoproteins that can be made in very low abundance and still carry out their biochemical roles [46].

The glial cell line-derived neurotrophic factor (GDNF) is a distant member of the transforming growth factor-p family that promotes the survival and differentiation of several types of neurons in the nervous system [47] and regulates ureteric branching in the embryonic kidney [48]. *In vitro*, GDNF promotes SCs' differentiation, whereas *in vivo*, GDNF dosage modulates cell fate decisions of undifferentiated spermatogonial cells [49]. Mice with decreased GDNF expression (with one null-allele) survive to adulthood and are fertile, but their spermatids are found in abnormal positions, which compromises their reproductive potential. On the other hand, mice overexpressing GDNF display several clusters of undifferentiated spermatogonia, that start to degenerate after puberty [49, 50].

The role of the stem cell factor (SCF) in germ cell development has long been established [51]. *In situ* hybridization studies have shown that the expression of the SCF gene is developmentally related and that it appears to follow the process of SCs' proliferation and differentiation during postnatal life [52]. Moreover, a significant increase in DNA synthesis in spermatogonia was detected when tubule segments were cultured in the presence of high concentrations of SCF, suggesting that SCF is a SC-produced paracrine regulator acting as a survival factor for spermatogonia in the adult rat seminiferous epithelium in a stage-specific manner [52]. Further studies have corroborated this premise. Mutant mice for the locus encoding the c-kit receptor (W), the specific SCF receptor, or the locus encoding the SCF (Sl) possess fewer germ cells, if any, due to an impairment of the proliferation and migration of the primordial germ cells [53, 54]. In the SeT of mutant mice lacking SCF, transplanted donor germ cells were able to proliferate and form colonies of undifferentiated type A spermatogonia, that were unable to differentiate any further. Extra analyses have shown that these type A spermatogonia were c-kit negative, which could implicate that SCF is a prerequisite for maintenance of c-kit-positive differentiated germ cells [55]. However, when placed in the SeT of mice expressing both the c-kit and SCF, type A spermatogonia resumed their differentiation, which could indicate that the stimulation of c-kit receptor by its ligand is necessary for the maintenance of differentiated type A spermatogonia [55].

One other growth factor related to spermatogenesis is the transforming growth factor-β (TGF-β). TGF-β is a major initiator of fibrotic reaction, inducing fibroblast cell growth and stimulating the expression, synthesis and release of collagen and fibronectin [56]. The basement membrane of the SeT contains a common set of proteins that includes laminin and type IV collagen. TGF-β has been associated to the function and migration of peritubular cells [57], whose capacity to synthesize products to the extracellular matrix is involved in the maintenance of SeT structural integrity and spermatogenesis promotion [58]. One study has demonstrated that the intracellular isoform of TGF-β was mainly expressed in the SCs and germ cells, thus suggesting that TGF-β is related to fibrosis of SeT and may lead to spermatogenic disruption [59]. Moreover, some studies have shown that TGF-β modulates LC steroidogenesis [60] and proliferation [61], which itself can indirectly modulate spermatogenesis.

Iron (Fe) and copper (Cu) are trace elements that constitute an essential ecophysiological component of cells and tissues present in the male reproductive system. Transferrin, an Fe transporter, is a major secretory product of the SCs and it is present in high concentrations in seminal plasma of fertile men [62]. Transferrin is part of a SCs' proposed shuttle system that effectively transports Fe around the tight junction complexes for the developing germ cells [63]. The proposed model includes basal transferrin receptors on SCs, movement of Fe through the cell, secretion of ferric ions associated with a newly synthesized testicular transferrin and incorporation of Fe in the newly synthesized transferrin into ferritin in the developing germ cells [42, 64]. Ceruloplasmin is the primary copper-binding protein and, within the testicles, approximately 80% of seminal ceruloplasmin is located in the SCs [65]. Each molecule of ceruloplasmin binds to six Cu ions and the remaining Cu is bound to metallothioneins (MTs), storage proteins for both Cu and zinc (Zn) [66]. These MTs are known to detoxify a variety of heavy metals in the male reproductive system of mice, rats and humans [67]. Two major MT isoforms and their corresponding mRNAs have been shown to be expressed primarily in SCs and spermatogenic cells to protect the germinal epithelium [68]. Therefore, it is currently used as an early marker of male germ line differentiation.

Mature and immature SCs display two completely distinct morphological and biochemical profiles. Upon their way to fully differentiate, SCs stop their proliferation [69], causing their nucleus to enlarge and their nucleolus to become more prominent. Several factors have been described to be modulators/stimulators of SCs' maturation, namely, thyroid hormone (T_3), follicle stimulating hormone (FSH) and androgens. These various components seem to act together in order to stimulate SCs' maturation. FSH was found to increase SCs' rate of proliferation [70] whereas thyroid hormones were found to diminish it [71]. Moreover, patients with complete androgen insensitivity syndrome (AIS) usually exhibit SCs that show various features of immaturity, such as the persistence of AMH expression [72]. Furthermore, *in vitro* studies in rats have shown that FSH and T_3 both induce androgen receptor (AR) expression in immature SCs [73].

Mature SCs produce smaller amounts of oestrogens when compared to immature cells, since the enzyme involved in oestrogens' synthesis, aromatase [74], is mainly expressed in the immature SCs and hormonally regulated by FSH [75]. Other biomarkers of SCs' maturity are the decrease in the expression of cytokeratin-18 [76], the decrease in neural-cell-adhesion-molecule expression rates [77], the augmentation in the laminin-alpha5 expression [78], the increase in the expression of GATA-1 [79] and increased levels of p27 [80].

c) Hormonal control of spermatogenesis

The series of complex cellular events occurring in spermatogenesis require the precise and timely involvement of a complex assortment of regulatory peptides and hormones. These hormonal messengers regulate the germ cell differentiation and the proliferation/functioning of the somatic cell types required for the proper development and function of the testis [81]. Their actions are exerted by endocrine, paracrine, juxtacrine and autocrine signalling mechanisms, under the major control of the hypothalamic-pituitary-gonadal axis [81].

The endocrine axis begins with the pulsatile secretion of gonadotropin releasing hormone (GnRH) from the hypothalamus (Figure I.3). This hormone acts on the anterior pituitary, triggering the synthesis and secretion of the luteinizing hormone (LH) and FSH [81]. In the testis, LH stimulates the secretion of T by LCs whereas FSH induces the production of several growth factors and other stimulatory players of spermatogenesis by the intermediary action of SCs, including the androgen-binding protein (ABP) [82–84]. Both FSH and LH exert their actions in the testis via specific G protein coupled receptors: the FSH receptor (FSH-R), mainly expressed in the SCs, and the LH receptor (LH-R), mainly expressed in LCs [85–87].

The T synthesized by LCs diffuses into the SeT where, conjointly with FSH, exerts stimulatory effects on the activity of SCs, which is decisive for germ cells' maturation and spermatozoa production [83, 88]. T is also related to a tight control of the hypothalamic-pituitary axis by a negative feedback loop, that inhibits the release of GnRH and LH [89–91].

One other mechanism that regulates the endocrine axis is the synthesis of the hormone inhibin by the SCs in response to FSH (Figure I.3) [92]. This protein is a member of the transforming growth factor β superfamily and acts in a negative feedback manner to diminish/suppress the synthesis and release of FSH from the anterior pituitary gland [93, 94]. The combined effect of the negative feedback exercised by androgens and inhibin regulates the gonadotropin production during the progression of puberty to the adult stage [95]. Furthermore, in the past few years, the role that oestrogens play in the control of the development and maintenance of the male reproductive tract, ergo spermatogenesis, has become more and more evident [96–98]. In the mammalian testis, oestrogens such as 17β-estradiol, are synthesized through the aromatization of androgens (mainly T and androstenedione) by cytochrome-c P450 aromatase enzyme.

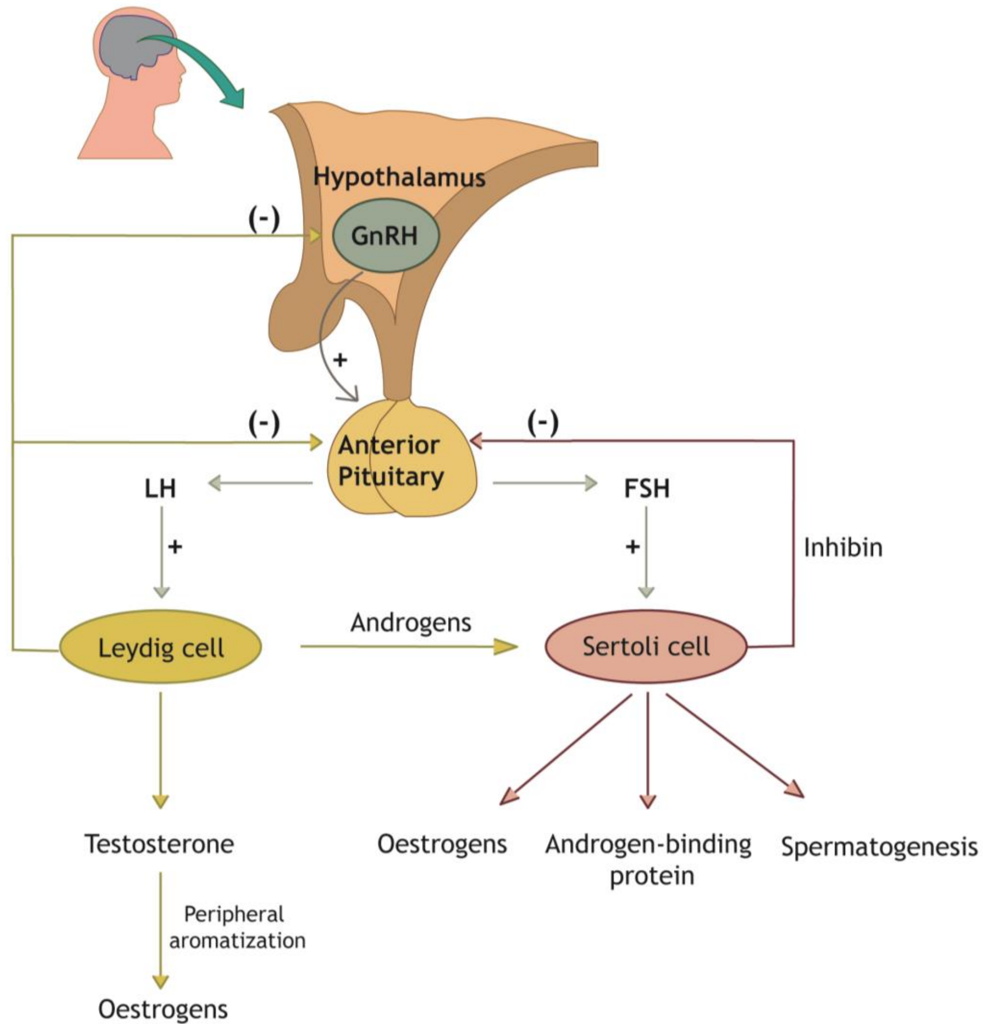

Figure I.3. Hormonal regulation of the testicular function and spermatogenesis. The release of gonadotropin-releasing hormone (GnRH) from the hypothalamus stimulates the pituitary to secrete two gonadotropins, the follicle-stimulating hormone (FSH) and the luteinizing hormone (LH). FSH stimulates the activity of Sertoli cells (SCs) and LH acts on Leydig cells (LCs), inducing the production of androgens, namely testosterone (T). A negative feedback (-) by T on the hypothalamus and pituitary regulates the levels of GnRH, LH and FSH, although its main action is to decrease secretion of LH. FSH secretion is also a subject of a negative feedback (-) by inhibin secreted by the SCs.

Despite the controversial topic that these hormones' actions in spermatogenesis represent to most authors, one incontrovertible fact was established: oestrogens exert their effects through their interaction with specific receptors. Both subtypes of the nuclear oestrogen receptor (ER), ERα and ERβ, as well as all the subtypes of the membrane bound receptor (GPR30) have been identified in the SCs [99–102]. Moreover, the oestrogens produced by the SCs are capable of directly stimulate the germ line, since germ cells express ERs [103]. Oestrogens have been described to affect LCs directly, through the inhibition of T production [104]. In addition, these hormones have been associated to a modulation of the apoptotic

signalling pathways, being also related to the stimulation of spermatogenesis through the decline of apoptosis in post-meiotic germ cells and the adjustment of germ cells' proliferation [74, 103, 105, 106]. Another evidence of oestrogens importance to a successful spermatogenesis is the fact that knock-out (KO) male mice for the ER (ERKO) display lower epididymal spermatozoa counts and defective spermatozoa function [107].

These data, accompanied by the high levels of oestrogens described in the testicular interstitial fluid [108], suggest that these hormones play an active role in the control of spermatogenesis and the male reproductive function.

3. Sertoli and germ cells metabolism: implications for a successful spermatogenesis

Among the several duties that SCs have toward the development of the germ line, the provision of energy and nutritional support becomes imperative. Most authors, consider glucose the universal energy substrate nearly used by all cells, including the ones from the germ line. However, despite the fact that all germ cells express every enzyme of the glycolytic pathway, some of them depend upon the nutrients provided by the secretory activity of SCs [109, 110]. The majority of the glucose consumed by SCs is metabolized into lactate, with only 25% being oxidized via the citric acid cycle (TCA) [111]. According to literature, this lactate production increases as SCs differentiate during the pubertal development [112].

The glycolytic process has been conserved among species since the beginning of their evolution, but some enzymes have testis-specific isoforms that are expressed largely on some spermatogenic cells rather than others [113–115]. In the earliest stage of development, spermatogonia possess all the enzymes needed to perform glycolysis, and thus these cells preferentially use, as energy source, glucose. This very same principle is observed in the final stage' cells, spermatozoa [109]. In fact, from all germ cells, spermatozoa display the lowest TCA activity and the highest glycolytic activity [116]. However, germ cells in the stage of spermatocyte or spermatid, despite possessing all glycolytic enzymes, have their glycolytic apparatus inactivated and so they disregard glucose as a substrate, giving emphasis to lactate as their primary energy source [117, 118]. This lactate is not only considered an extremely important energy source, as it is associated to the stimulation of RNA and protein synthesis in spermatids [109] and to an antiapoptotic effect on germ cells [118, 119]. Why germ cells differ in their metabolic needs is not completely understood, but it might be related to the position they occupy inside the testis itself. The BTB separates spermatogonia on the outer portion of the SeT and spermatocytes and spermatids in the inner portion of these tubules. In such manner, spermatogonia can access to the glucose flowing in the blood, whereas spermatocytes and spermatids depend upon SCs' metabolism to be provided with substrate. Circulating free in the lumen of the SeT and on the outside of the BTB, spermatozoa can also access to the glucose present in their midst [120].

Glucose enters the SCs via specific glucose transporters (GLUTs) (Figure I.4) [121]. To this date there have been identified four GLUTs isoforms in the SCs: GLUT1, GLUT2, GLUT3 and GLUT8 [122–124]. Though GLUT1 and GLUT3 have been reported to play a crucial role in the SCs' metabolism, GLUT2 has not yet been observed in purified SCs' preparations [123] and GLUT8 has been identified in the endoplasmic reticulum membrane of SCs but not in the plasma membrane, thus not being involved in glucose uptake [125]. Once inside the cell, glucose undergoes a series of conversion steps (Figure I.4) named glycolysis, at the end of which it has been fully converted into pyruvate with the net gain of two molecules of ATP

[126]. In the first steps of glycolysis there is actually energy consumption, where ATP is used to phosphorylate glucose, first, into glucose-6-phosphate and, second, into fructose-6-phosphate [127]. The enzymes responsible for these two reactions are the hexokinase and the phosphofructokinase 1 (PFK1), respectively. The rate limiting step of the glycolytic process is catalysed by PFK1, which is inhibited by high levels of ATP [127]. In cases where PFK1 is inhibited, glucose-6-phosphate is accumulated in the cell, resulting in the inhibition of hexokinase activity. Thus, when the cell has an adequate supply of metabolic energy available in the form of ATP, the breakdown of glucose is inhibited [127].

Part of the pyruvate produced during glycolysis is catalysed into lactate by the lactate dehydrogenase enzyme (LDH) [118]. Once synthesized, this lactate exits the SCs via specific monocarboxylate transporters (MCTs) [128]. In SCs, MCT4 expression has been previously confirmed and, from the MCT family, MCT4 is the member required for lactate export [129].

Due to their high glycolytic activity, SCs are able to adapt their metabolism under the condition of glucose deprivation, so that they can keep supporting germ cells' development [130]. In order to do so, SCs direct their resources into the metabolism of lipids [131], amino acids [132] and even glycogen [133], all of them able to maintain the production of lactate and ATP. According to Xiong and collaborators [131], SCs preferentially use lipid β-oxidation to produce ATP. In spite of being able to maintain ATP production when glycolysis is interrupted, SCs cannot produce ATP when β-oxidation is blocked. One curious fact that corroborates this theory is that SCs use the residual bodies from germ cells' phagocytosis to produce lipids that then are directed into β-oxidation for ATP production [131]. Evidence point out that rat spermatozoa sustain extensive lipid remodelling during their course on to the epididymis [134]. Furthermore, spermatozoa require a large amount of free phospholipids to constantly renew their cell membrane, although saturated fats and/or trans-fatty acids, as well as sugars, negatively affect testicular lipid metabolism and, by extension, spermatogenesis [135–137].

Amino acids can also represent an important source of energy to SCs. From this perspective, the oxidation of glutamine by glutaminase (GLS) has been proven to represent the major energy supplier that these cells require [132]. The process by which this amino acid is oxidized is named glutaminolysis, and firstly depends on glutamine entering the cell. This task is achieved by the specific solute glutamine transporter, the ASCT2, that uptakes glutamine from the extracellular space [138]. Furthermore, glutamine prevents the incorporation of alanine into proteins in SCs, which is critical given the fact that alanine is another very effective amino acid that can be converted to pyruvate, being an intermediary for lactate production (Figure I.4) [132].

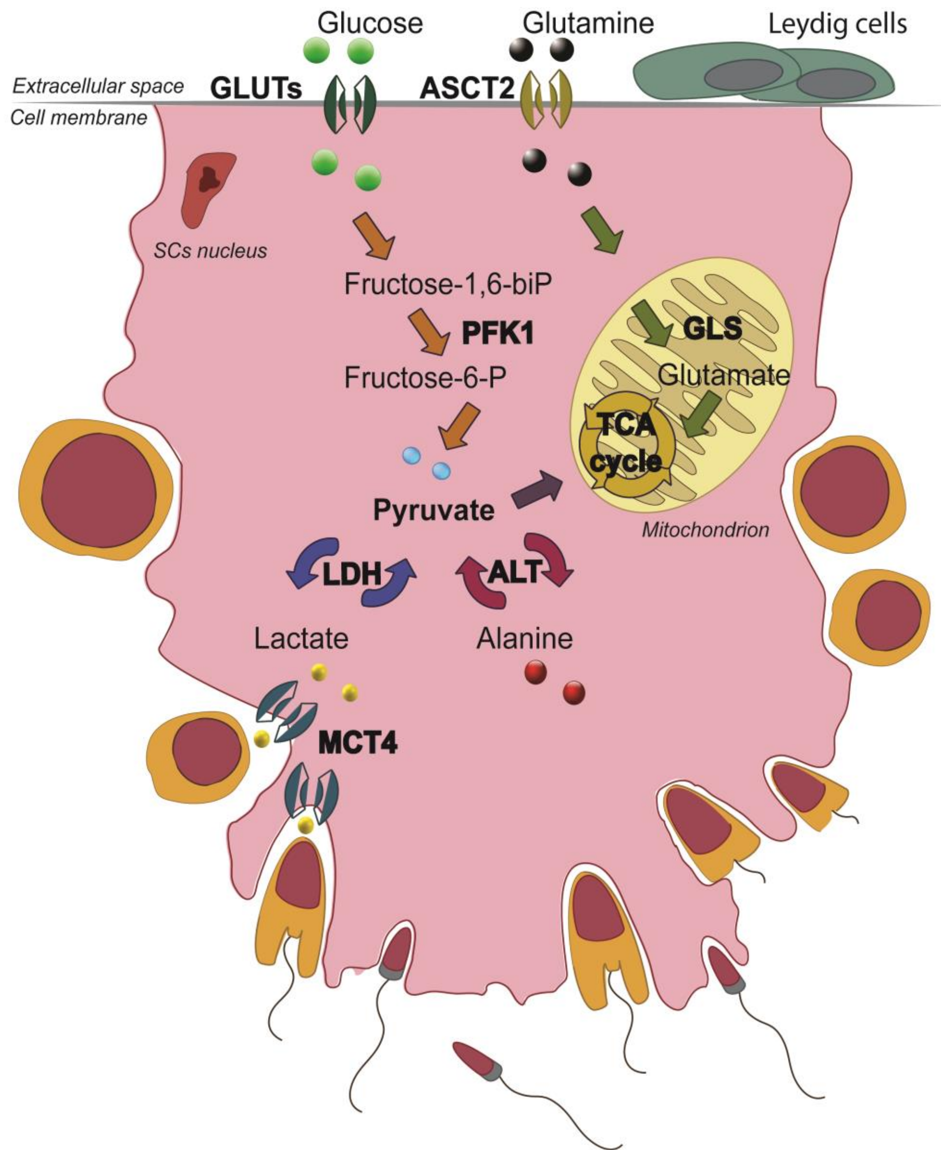

Figure I.4. Schematic representation of the glucose and glutamine metabolizing pathways in Sertoli cells (SCs). The exogenous glucose is uptaken by SCs via specific glucose transporters (GLUTs), namely, the GLUT1 and GLUT3, being then converted to pyruvate by glycolysis (orange arrows) through the sequential action of several enzymatic players. This includes the phosphofructokinase-1 (PFK1) that catalyses a rate limiting step of glycolysis converting fructose 6-phosphate to fructose 1,6-bisphosphate. Pyruvate can either be directed into the mitochondrion to regenerate acetyl-CoA, or can be converted into lactate by lactate dehydrogenase (LDH). Pyruvate can also be obtained via alanine by the reversible reaction catalysed by alanine transaminase (ALT), respectively. SCs seem preferentially use pyruvate to produce lactate that is exported onto the extracellular space via monocarboxylate transporters (MCTs), specifically by the MCT4. Among other substrates, the SCs also present the ability to metabolize glutamine (green arrows). This amino acid enters the cell via specific glutamine transporters (ASCT2) and is directed to the citric acid cycle (TCA) where it is oxidized by glutaminase (GLS) for ATP generation.

SCs' metabolism and lactate production are under the control of several regulators, such as, growth factors [139, 140], FSH [140, 141], cytokines [139, 142], insulin [141], sex steroid hormones [105], tumour-necrosis-factor-α (TNFα) [143], mammalian target of rapamycin (mTOR) [144] and GATA4 [145]. Over the past few years, SCs' metabolism has emerged as an

important modulator of male fertility, since the stimulation of the glycolytic pathway boosts the development of both SCs and germ cells.

4. Regucalcin, the "handyman" protein

It has been established by many authors that the proliferation and differentiation of germ cells require balanced intracellular Ca^{2+} levels, in more than just one way [146, 147]. Germ cells generate Ca^{2+} fluxes that achieve their bulk when spermatogonia develop into early spermatids [148] showing that the tight control of Ca^{2+} homeostasis is a critical factor for spermatogenesis. Also, SCs demand a tight control of Ca^{2+} homeostasis in order to maintain the integrity of the tight junctions, ergo the BTB [149]. The process of steroidogenesis that occurs in LCs is too under the control of Ca^{2+} levels [150] and even the post-spermatogenesis events of spermatozoa capacitation, motility and acrosome reaction require Ca^{2+} in order to properly occur [151, 152].

RGN is a Ca^{2+}-binding protein first described in 1978 [153] that differs from calmodulin and other Ca^{2+}-related proteins in the absence of an EF-hand motif as a Ca^{2+}-binding motif [154]. In humans and rodents, the RGN gene encodes a 299 amino acidic protein with an estimated molecular weight of 33 kDa [155] that seems to play a very important biological role, since, from an evolutionary point of view, it's sequence is highly conserved [156].

This protein's expression has been identified in numerous tissues such as the liver [157], the kidney cortex [158], the heart [159], the bone [160], the prostate [161], the breast [162], the ovary [163] and the testis [164]. The localization of RGN mRNA in adult rat testis was verified in the SCs, LCs and various types of germ cells, namely, spermatogonia, spermatocytes and round spermatids. RGN protein was identified in all the aforementioned types of cells, plus the elongating spermatids and spermatozoa [165]. The expression pattern of RGN was very similar in human testicular tissue [165].

A number of factors such as, Ca^{2+}, oxidative stress, thyroid, parathyroid and steroid hormones have been shown to regulate RGN gene expression in a variety of tissues. Several reports have shown that rats treated with Ca^{2+} chloride ($CaCl_2$) present augmented levels of RGN mRNA at 30, 60 and 120 min after the compound administration [166, 167]. The thyroid hormone, T3, has shown to increase RGN mRNA and protein levels when injected in female rats for up to 12 h of stimulation [168]. However, no effects were observed in response to T4 treatment [169]. Insulin has also been reported to stimulate the expression of RGN in cloned human hepatoma cells (HepG2) *in vitro* [170]. The effect of sex steroid hormones on RGN expression has been assessed in various tissues and cell lines and in the past few years, this protein has been granted with the recognition of an androgen-target gene in the male reproductive tract [162, 165, 171]. In rat liver, RGN's expression was not altered by orchiectomy or treatment with testosterone, suggesting that RGN expression in the liver is androgen-independent [172]. Moreover, ovariectomized female rats did not present a significant alteration of their RGN mRNA levels in the liver [173]. However, one report demonstrated that E_2 decreases RGN mRNA levels in rat kidney [174]. Previous findings from our research group have shown that in rat prostate and mammary gland, RGN's expression levels are downregulated by the E_2 [161].

Moreover, RGN is underexpressed in breast and prostate cancer cases and, while E_2 upregulates RGN mRNA expression in MCF-7 cells, DHT downregulates RGN mRNA expression in LNCaP cells [162]. In order to assess how DHT levels influence RGN's expression in the SeT, a predefined dosage of DHT was administered to rat SeT cultured *ex vivo* [165]. This treatment caused the mRNA expression levels of RGN to increase remarkably, an effect blocked in the presence of flutamide (an AR antagonist), which suggests the involvement of a classical genomic mechanism of regulation of gene expression through the AR [165]. Furthermore, a study conducted in the prostate of Tg-RGN rats showed that the *in vivo* stimulation with DHT causes RGN expression levels to decrease in this tissue [171].

RGN's cellular functions have been associated to the regulation of several biological processes, mainly, the control of cell death and proliferation, the regulation of intracellular Ca^{2+} levels, the modulation of several Ca^{2+}-dependent enzymes (such as tyrosine kinase) and the control of the oxidative stress [165, 175, 176].

In the prostate of Tg-RGN animals both cell proliferation and apoptotic pathways seem to be inhibited under overexpression of RGN, which demonstrates this protein's role maintaining prostate growth balance [171]. These findings followed previous evidence in several cell line models. For instance, in the liver, the proliferation of rat hepatoma H4-II-E2 cells is stunted by the overexpression of endogenous RGN in an apoptosis-independent process [177]. NRK52E cells overexpressing RGN also present a lower index of proliferation than mock-transfected cells [178]. Also, the enhancement of DNA fragmentation in these NRK52E cells after an incubation with Bay K 8644, thapsigargin, or lipopolysaccharide (LPS) seems to be successfully suppressed by RGN overexpression [178]. In addition, an intracellular increase in RGN's expression downregulates mRNA expression of *c-myc* and *H-ras*, while it upregulates p53 and p21 [177–179]. This suggests that RGN suppresses cell proliferation through the modulation of the expression of proto-oncogenes and tumour suppressor genes [177–179]. Moreover, it is of the uttermost importance to refer that the diminished expression of RGN found in both rodent and human cancer tissues is associated to the degree of cellular differentiation of breast, prostate and liver carcinomas [180, 181]. Nevertheless, the anti-proliferative action of RGN and its contribution to tissue homeostasis was definitely demonstrated by a report describing that Tg-RGN animals are resistant to the development of carcinogen-induced mammary gland tumours, and that the large majority of developed tumours were of non-invasive phenotype with low cell proliferation rates [182].

The most well-established role of RGN is related to the maintenance of intracellular Ca^{2+} homeostasis in many types of cells through the regulation of Ca^{2+}-pumps localized on the plasma membrane, endoplasmic reticulum and mitochondria [183–187]. In the particular case of male reproductive tract, previous findings from our research group using $^{45}Ca^{2+}$ in epididymal tissue cultures, have found diminished rates of Ca^{2+} influx in the epididymis of Tg-RGN rats, which was suggested to be an indicative of unbalanced Ca^{2+} concentrations in the epididymal lumen of these animals [188]. Interestingly, analysis of spermatozoa parameters

of Tg-RGN rats has shown that these animals display fewer but more viable and normal spermatozoa, which also suggested a role for RGN in spermatozoa maturation [188].

Considering oxidative stress, RGN has been identified as an antioxidant protein diminishing the levels of reactive oxygen species and enhancing the activity of antioxidant defence enzymes [189–191]. The antioxidant activity of RGN has been demonstrated by several experimental approaches including the highly demonstrative studies in RGN KO mice. In the brain of these RGN KO mice was observed that the synthesis of reactive species (RS) and NADPH oxidase activities were significantly elevated [192]. Moreover, these animals demonstrated augmented levels of modified proteins and higher activity of Mac-1 protein and myeloperoxidase (MPO) [192]. Furthermore, the SeT of Tg-RGN animals displayed a significantly higher antioxidant capacity and diminished levels of oxidative stress upon stimulation with tert-butyl hydroperoxide (TBHP), a pro-oxidant stimulus, when compared with the Wt controls [193]. Regarding the antioxidant defence system, a significant increase in the activity of glutathione-S-transferase was found in the SeT of Tg-RGN whereas no differences were observed in superoxide dismutase activity throughout experimental conditions [193]. Also, it was shown that RGN suppressed thapsigargin- and actinomycin D-induced apoptosis in SeT by modulating the expression and activity of key apoptotic and antiapoptotic factors, which supports the idea that RGN overexpression protects germ cell from apoptosis induced by noxious stimuli [194].

Aside these functions, it has been demonstrated that RGN acts as a regulator of cell metabolism, particularly by studies using animal models and cell line cultures, with under- or overexpression of RGN [195–197]. *In vivo* studies have identified bone loss in Tg-RGN rats associated to the occurrence of hyperlipidaemia. It was found an augmentation in serum triglycerides, free fatty acids and high-density lipoprotein (HDL)-cholesterol concentrations [195, 198], accompanied by higher levels of serum Ca^{2+} [199]. Another important characteristic that has been identified for RGN is its ability to regulate insulin function. In H4-II-E cells cultured with TNF-α and insulin, both modulators of insulin resistance, RGN exhibited a differentially expression pattern [200] suggesting that RGN may be linked to insulin resistance. In fact, glucose and insulin tests demonstrated that an insufficiency in RGN expression levels may induce glucose intolerance [201]. RGN was also identified as an gluconolactonase, an enzyme involved in the synthesis of L-ascorbic acid in mammals [202]. RGN KO mice treated with L-ascorbic acid had blood glucose levels increased and insulin levels decreased upon glucose administration when compared to Wt counterparts [201, 203]. In H4-II-E cells overexpressing RGN the production of triglycerides and free fatty acids was stimulated in the absence of insulin and with or without the supplementation of glucose in the medium [170]. This suggests that RGN may stimulate lipid production, which is linked to glucose metabolism.

Indeed, RGN has also been associated to the control of the glycolytic metabolism by studies showing its influence on the regulation of several transporters and glycolytic enzymes. A

lower glucose content was found in the prostate of Tg-RGN animals when compared to the Wt group [129]. This lower glucose levels were accompanied by the diminished expression of GLUT3 and PFK1 demonstrating a suppressed glycolytic metabolism under overexpression of RGN. Moreover, the prostate of Tg-RGN animals also displayed lower lactate levels in consequence of the diminished expression and activity of LDH [129]. On the opposite, in bone marrow cell cultures RGN increased the consumption of glucose and lactate production [204]. Despite all the evidence associating RGN to the modulation of cell metabolism in several tissues, the role that this protein plays in the regulation of glycolytic metabolism in testicular cells remains completely unknown.

II. Aim of this dissertation

For several years now, SCs are acknowledged with the function of providing germ cells with nutritional and physical support. These roles, amongst others, have granted SCs the epithet “nurse cells”. SCs are responsible for uptaking and metabolizing the external glucose into lactate, which is then provided to germ cells, in order for them to develop. Lactate is not only considered by most authors as the preferred energy source of the germ line, as it appears to display antiapoptotic effects over germ cells. However, other substrates have been indicated as energy sources for the SCs. Such is the case of alanine and glutamine. In fact, some authors have reported that glutamine alone can yield most of the energy required by the SCs.

Regucalcin is a Ca^{2+}-binding protein identified in several cell types in the testis, namely, the SCs. This protein has been associated to the regulation of Ca^{2+} homeostasis, cell proliferation and apoptosis, but several other studies have linked RGN to the modulation of cell metabolism. Indeed, it was demonstrated that transgenic rats overexpressing RGN (Tg-RGN) suffer from osteoporosis and hyperlipidaemia. Moreover, *in vitro* approaches demonstrated that RGN enhances the utilization of glucose through the modulation of the expression of several transporters and glycolytic enzymes. Despite the evidence associating RGN to the regulation of testis physiology and protection of the germ line, its influence over the metabolism of testicular cells has not yet been studied. The present work aimed to characterize glucose and glutamine metabolism in the SCs of Tg-RGN rats comparatively with their wild-type (Wt) littermates. For this purpose, primary SCs cultures were established from 3 months-old Wt and Tg-RGN Sprague-Dawley rats. After 24 hours of culture, the following parameters were evaluated in both experimental groups:

- ✓ Glucose and glutamine consumption, and lactate production;
- ✓ Glucose and lactate levels in the testicular interstitial fluid and seminiferous tubules (SeT) fluid;
- ✓ Expression and activity of several modulators of glycolytic metabolism and glutaminolysis.

III. Materials and Methods

1. Animals and Tissue Collection

Three-month-old Wt and Tg-RGN Sprague-Dawley rats (*Rattus norvegicus*) were used in this study. Tg-RGN animals were originally generated by Yamaguchi M [198] by oocyte-transgene-pronuclear-injection technique and were purchased from Japan SLC (Hamamatsu, Japan), while Wt animals were purchased from Charles River (Barcelona, Spain).

The animals were maintained with food and water *ad libitum* in a constant room temperature (20 ± 2°C) on a 12-hour cycle of artificial lighting. All experiments complied with the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH Publication No. 85-23, revised on 1996) and the European Union rules for the care and handling of laboratory animals (Directive 2010/63/EU).

Wt and Tg-RGN rats (n=6 in each group) were euthanized by cervical dislocation under anesthesia (Clorketam 1000, Vetoquinol, Lure, France) and the testes were removed, trimmed free of fat and washed in cold Hank's balanced salt solution (HBSS). One testis from each animal was used for fluids' collection while the contralateral testis was used for SCs' isolation.

2. Testicular Fluids Collection

After testicular excision, a cruciate incision was made in the tunica albuginea of the distal pole of the testis, followed by a centrifugation of 100 *g*, at 4°C for 25 minutes. Testicular interstitial fluid was transferred to a new tube, frozen on liquid nitrogen and stored at -80°C. Thereafter, SeT were exposed and rinsed four times in HBSS in order to remove residual interstitial fluid, and the tubules extruded through the hub of a 3 mL syringe into a tube. After a 6000 *g* centrifugation at 4°C for 15 minutes, the SeT fluid (supernatant) was collected, frozen on liquid nitrogen and stored at -80°C.

3. Primary *SCs* Culture

SCs were isolated using an adaptation of the enzymatic procedure described by [205]. Briefly, testes were decapsulated and washed in cold HBSS with antibiotic and antimicotic solution (HBSS,10 000 units/ml of penicillin, 10 mg/ml of streptomycin and 25 µg/ml of amphotericin B, pH 7.4 and Ca^{2+}, Mg^{2+} free). Thereafter, extruded SeT were incubated in a collagenase solution (0,5 mg/mL in 1X HBSS pH 7.4) at 34 ºC for 10-15 min under shaking (80 oscillation/min) and allowed to settle. The tubule fragments were then washed three times in HBSS and incubated in a trypsin solution (0,5 mg/mL in 1X HBSS pH 7.4) at 37 ºC for 5-10 min with gentle shaking. The SCs suspension was collected by centrifugation (250-300 *g* for 3-4 min), washed in HBSS and resuspended in SCs' culture medium (DMEM:F-12) (Sigma-Aldrich, St. Louis, Missouri, USA) supplemented with 50 IU/ml penicillin, 50 mg/ml streptomycin sulfate, 0.5 mg/ml Fungizone, 50 µg/ml gentamicin, and 10% (v/v) heat-inactivated FBS (Biochrom, Berlin, Germany)). The cell suspension was forced through a 10 ml syringe and plated in culture flasks (Cell+; Sarstedt, Nümbrecht, Germany) containing pre-warmed SCs' culture medium. The cultures were incubated at 37 °C in an atmosphere of 5% CO_2 until a 90-95% confluence was achieved. Culture medium was then replaced by serum-free medium supplemented with ITS (insulin, transferrin and sodium selenite) (Sigma-Aldrich) and the SCs were left undisturbed for 24 hours.

4. Quantification of Glucose and Lactate

The concentration of glucose and lactate in the culture medium of SCs and testicular fluids (interstitial and SeT fluid) of Tg-RGN and Wt rats was determined through spectrophotometric assays using commercial kits (Spinreact, Girona, Spain) as previously described [129]. Calculations were performed to determine glucose consumption and lactate production by SCs of both groups over 24 hours of culture.

Polar and non-polar metabolites were extracted from SCs cultured for 24 hours by a methanol/chloroform/water extraction. Briefly, cells were instantly quenched in liquid nitrogen followed by the addition of 1 ml of cold methanol and 500 µl of chloroform. After defrosting on ice, samples were vortexed for 60 s and sonicated. Chloroform and ice-cold water (500 µl for both) were then added to each sample, which were vortexed and centrifuged at 5000 *g* for 15 min at 4 ºC. The upper layer composed by the water-soluble metabolites was collected for quantification of glucose and lactate concentrations. All

measurements complied with manufacturers' instructions and were normalized for the total number of cells in each experimental condition.

5. Quantification of Glutamine

The quantification of L-glutamine in the culture medium of SCs was determined using a commercial kit (NZYTech, Lisbon, Portugal) according to the manufacturers' instructions. Succinctly, the concentration of this amino acid was calculated by measuring the amount of NADP+ formed through the combined action of GLS and glutamate dehydrogenase (GLDH). The amount of NADP+ formed, measured at 340 nm, is stoichiometric to the amount of L-glutamine and ammonia in the sample's volume. The amount of L-glutamine per sample was normalized for the total number of cells in each experimental condition.

6. Total protein Extraction and Quantification

Total protein was isolated from rat SCs using RIPA buffer (150 mM NaCl, 1% Nonidet-P40 substitute, 0.5% Na-deoxycholate, 0.1% SDS, 50 mM Tris pH 8 and 1 mM EDTA) supplemented with a protease inhibitors cocktail (Sigma-Aldrich) and 10% of phenylmethylsulfonyl fluoride (PMSF). The cell lysates were homogenized, centrifuged at 14000 *g*, 20 min, 4 ° C, in a Hettich Mikro 200R centrifuge, and the supernatant containing the protein was collected and kept on ice. Afterward, protein concentration was determined by the Bradford assay (Bio-Rad, Hercules, CA, USA) using Bovine Serum Albumin (BSA) as a standard.

7. Western Blot

Proteins (50 µg of each protein extract) were resolved in a 12% gel by SDS-PAGE and transferred to a PVDF membrane (Bio-Rad). Membranes were incubated overnight at 4 ºC with rabbit anti-GLUT1 (1:500, CBL242, Millipore, MA, USA), rabbit anti-GLUT2 (1:500, sc-9117, Santa Cruz Biotechnology, Dallas, TX, USA), rabbit anti-GLUT3 (1:1000,sc-30107, Santa Cruz Biotechnology), rabbit anti-phosphofructokinase 1 (PFK1) (1:500, sc-67028, Santa Cruz Biotechnology), rabbit anti-MCT4 (1:1000, sc-50329, Santa Cruz Biotechnology), rabbit anti-LDH (1:10000, Ab52488, Abcam, Cambridge, MA, USA), mouse anti-alanine transaminase (ALT) (1:200, sc-374501, Santa Cruz Biotechnology), rabbit anti-GLS (1:1000, ab93434, Abcam) and rabbit anti-ASCT2 (1:1000, V501, Cell signalling technology, Danvers, MA, USA) primary antibodies. A mouse anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:10000, AB2302, Millipore, Darmstadt, Germany) antibody was used for protein loading control in all WB analyses. Goat anti-rabbit IgG-HRP (1:5000, NIF1317; Santa Cruz Biotechnology) or goat anti-mouse IgG + IgM-HRP (1:5000, Santa Cruz Biotechnology) were used as secondary antibodies. The membranes were incubated with ECL substrate (Bio-Rad) for 5 min and immune-reactive proteins were scanned with the ChemiDoc™ MP Imaging System (Bio-Rad). The density of the bands was obtained according to standard methods using the Image Lab 5.1 software (Bio-Rad) and normalized by division with the respective GAPDH band density. Results are presented as fold-variation relatively to the control Wt group.

8. LDH enzymatic activity

The enzymatic activity of LDH in SCs was determined using a commercial kit (Spinreact) according to the manufacturers' instructions. This enzyme catalyses the reduction of pyruvate by NADH and the rate of decrease in concentration of NADPH, measured photometrically, is proportional to the catalytic concentration of LDH present in the sample. The enzymatic activity was calculated by measuring the variation on the absorbance (340 nm) of samples. The method was calibrated using an LDH Positive Control included in the kit. The activities were calculated as µg/µL.

9. Statistical analysis

The statistical significance of differences between Wt and Tg-RGN experimental groups was evaluated through an unpaired Student´s t-test using GraphPad Prism v6.00 (GraphPad Software, San Diego, CA, USA). The differences were considered significant when $P < 0.05$. Experimental data are shown as mean ± SEM ($n \geq 4$ for each experimental condition).

IV. Results

1. Glucose and lactate content were altered in the SCs and testicular fluids of Tg-RGN rats

a) Glucose consumption and lactate production

Given that SCs use glucose as a substrate for the production of lactate, the preferential energy source of germ cells [206], and that RGN is expressed in the SCs, we have decided to investigate if the presence of RGN modulates the glycolytic metabolism of SCs. For that purpose, we started by measuring the content of glucose and lactate in primary SCs of Wt and Tg-RGN animals, and in the respective culture medium, after a culture period of 24 hours.

Glucose consumption (Figure IV.1A) was found to be significantly lower in the SCs of Tg-RGN rats when compared to their Wt littermates (8,05 ± 0,69 vs. 10,85 ± 0,65 pmol/cell, $p<0.05$). On the other hand, lactate production (Figure IV.1B) was significantly augmented in the SCs of Tg-RGN rats comparatively to the control group (9,77 ± 0,41 vs. 6,62 ± 0,35 pmol/cell, $p<0.001$).

Regarding the intracellular concentration of glucose (Figure IV.1C), a significant decrease was found in the SCs of Tg-RGN animals, when compared to their Wt homologous (31,27 ± 3,55 vs. 83,82 ± 5,62 pmol/cell, $p<0.001$). In spite on that, no variations were found in the intracellular lactate content (Figure IV.1D) between Wt and Tg-RGN rats SCs after 24 hours of culture.

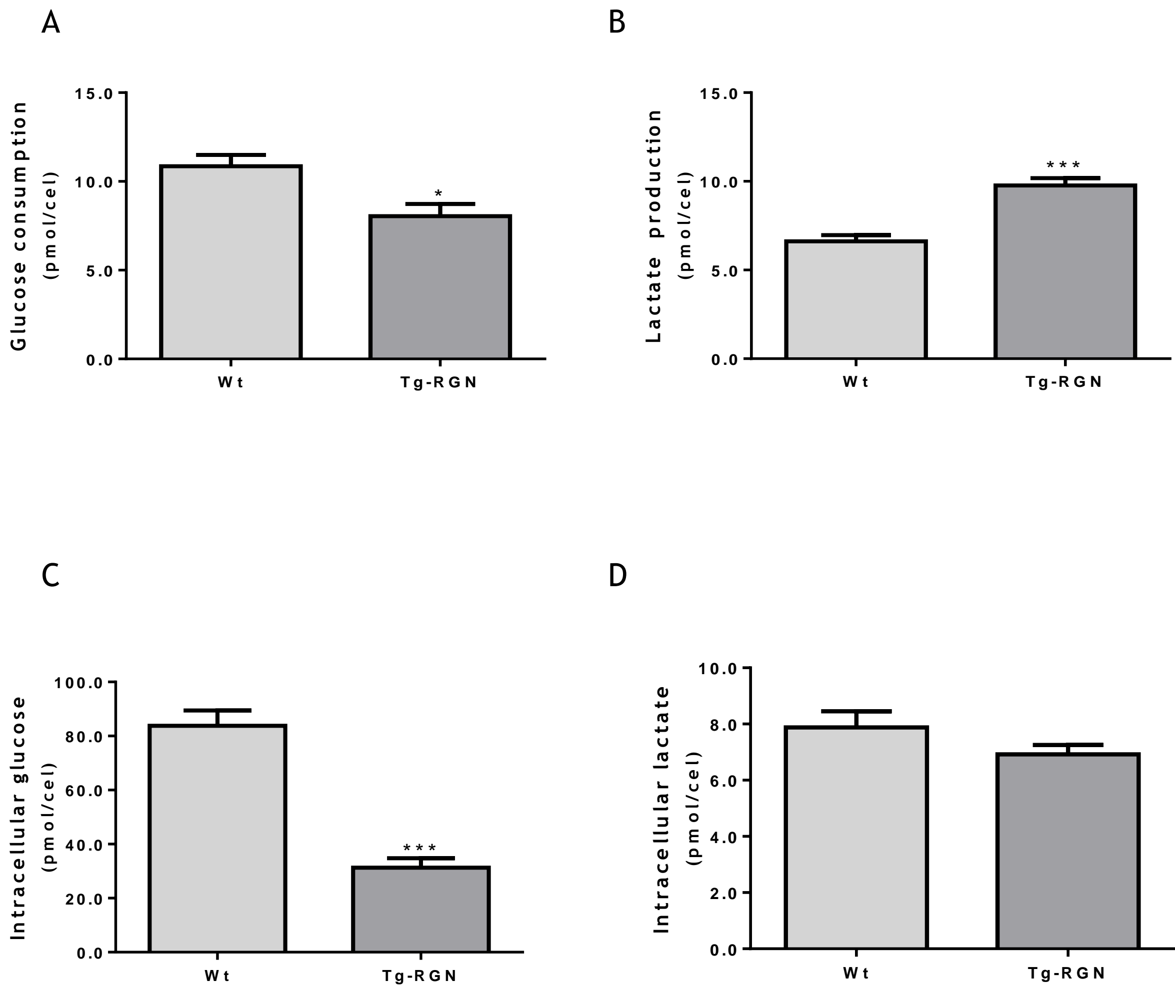

Figure IV.1. Glucose consumption (A), lactate production (B), and intracellular concentration of glucose (C) and lactate (D) in primary SCs of Wt and Tg-RGN cultured for 24 hours. Data are presented as mean ± S.E.M. (n≥4 in each group). * $p < 0.05$, *** $p < 0.001$

b) Glucose and lactate contents in the interstitial and SeT fluids

In the rat testis, the interstitial fluid bathes and separates the LCs and the SeT. Therefore, this fluid is likely to provide a reliable indication of the intratesticular (extratubular) medium composition [207].

Moreover, SCs play a key role in the establishment of an adequate luminal environment in the SeT, which is vital for the normal occurrence of spermatogenesis. This SeT fluid not only provides the perfect milieu for germ cells to develop properly, as it also represents a means of transportation for spermatozoa to exit the testis [208].

Some differences between the glucose and lactate content in the testicular fluids of Wt and Tg-RGN animals were observed. Regarding the interstitial fluid, Tg-RGN animals displayed higher levels of glucose comparatively to their Wt counterparts (4,95 ± 0,23 vs 3,65 ± 0,17, $p<0.01$, Figure IV.2A). However, the lactate concentration found on the interstitial fluid of Tg-RGN rats was lower than that of the control group (1,97 ± 0,09 vs. 2,30 ± 0,10, $p<0.05$, Figure IV.2B).

The same pattern was found for the content of these metabolites in the SeT fluid. When compared to the control animals, the SeT fluid of Tg-RGN rats displayed increased glucose (3,19 ± 0,10 vs 2,87 ± 0,06, $p<0.05$, Figure IV.2C) and diminished lactate concentration (1,42 ± 0,05 vs 1,70 ± 0,10, $p<0.05$, Figure IV.2D).

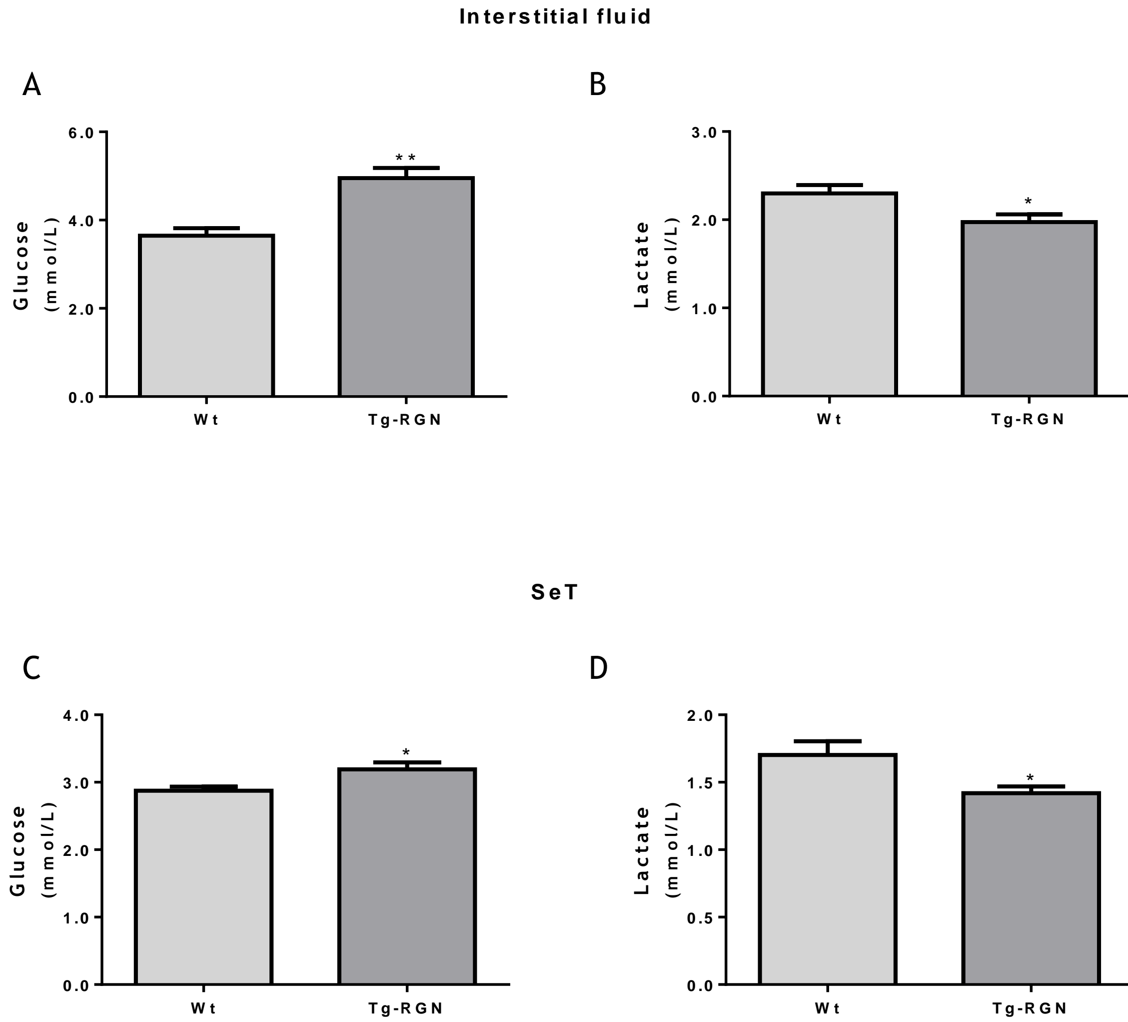

Figure IV.2. Glucose and lactate content in interstitial (A, B) and SeT (C, D) fluid of Wt and Tg-RGN rats. Data are presented as mean ± S.E.M. (n ≥4 in each group). * $p < 0.05$, ** $p < 0.01$

2. Transgenic overexpression of RGN alters the glycolytic metabolism of SCs

a) Decreased expression of GLUT2 and increased expression of GLUT1 and GLUT3 in the SCs of Tg-RGN animals

In order to provide lactate for the germ line, the SCs require several glycolytic enzymes and transporters to uptake the external glucose, convert it into lactate and then export that lactate into the extracellular milieu.

GLUTs' family encompasses several isoforms with specific biochemical features and a tissue-specific expression pattern [209]. Four GLUT isoforms have been identified in the SCs: GLUT1, GLUT2, GLUT3 and GLUT8 [123, 210]. These isoforms are characterized by displaying substrate specificity, proper kinetic characteristics and tissue-specific expression pattern [209].

After 24 hours of culture the protein levels of GLUT1 and GLUT3 were significantly increased in the SCs of Tg-RGN rats when compared to the control group (2,21 ± 0,10 fold variation, $p<0.001$, Figure IV.3A and 1,51 ± 0,05 fold variation, $p<0.01$, Figure IV.3C, respectively). However, the expression of GLUT2 was deeply decreased in the SCs of Tg-RGN animals when compared to the control group (0,30 ± 0,04 fold variation, $p<0.05$, Figure IV.3B).

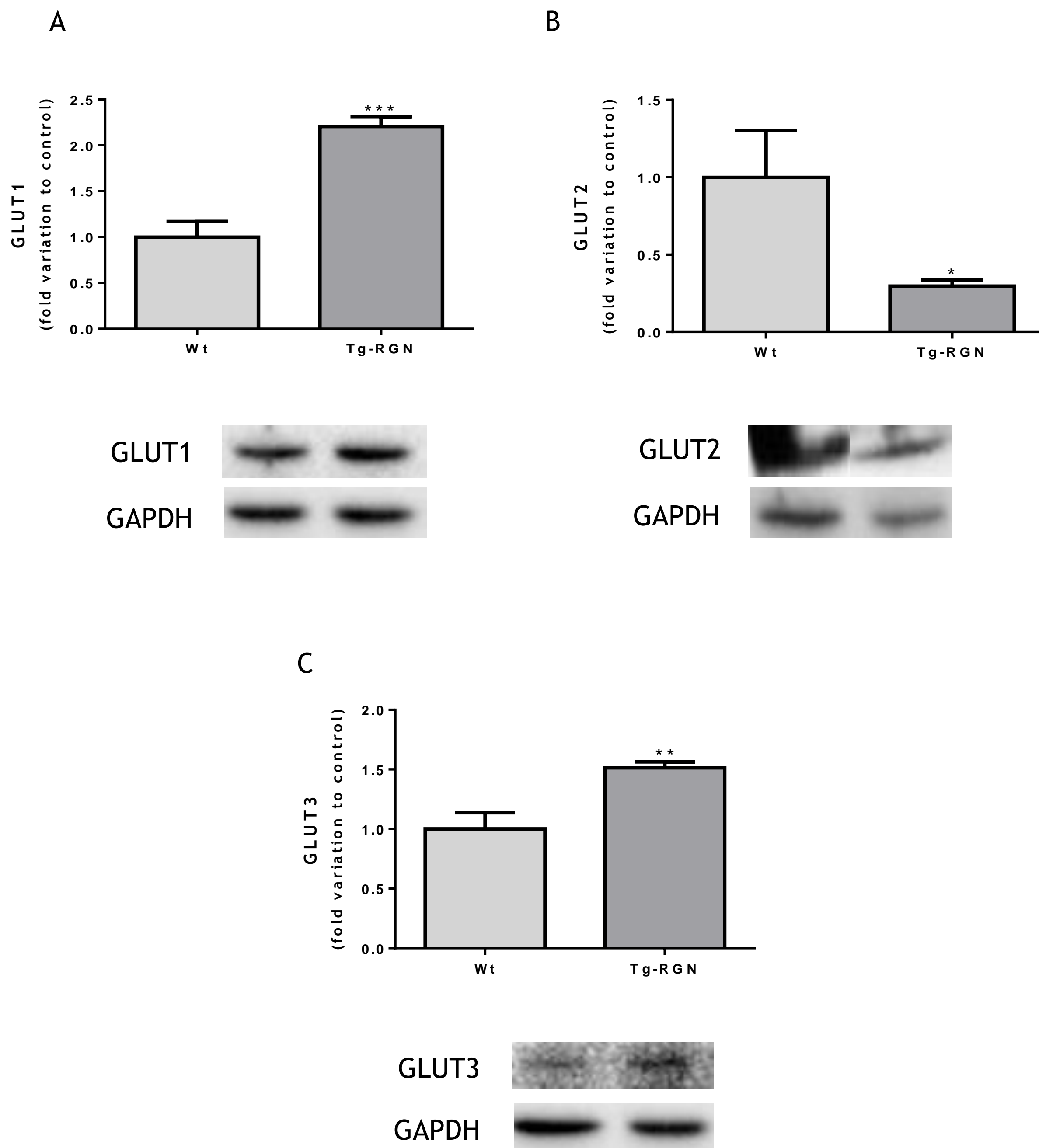

Figure IV.3. Expression of GLUT1 (A), GLUT2 (B) and GLUT3 (C) in primary SCs of Wt and Tg-RGN rats cultured for 24 hours. Data are presented as mean ± S.E.M. after normalization with GAPDH (n≥4 in each group). Results are expressed as fold-variation relatively to Wt animals. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Representative immunoblots are shown in bottom panels.

b) SCs of Tg-RGN rats presented augmented expression levels of PFK1, MCT4 and ALT

PFK1 is an extremely important regulatory enzyme long established to determine the flux through the glycolytic pathway [126]. The expression levels of this enzyme were significantly higher in the SCs of Tg-RGN animals when compared to the control group (3,78 ± 0,70 fold variation, $p<0.01$, Figure IV.4A).

MCT4 is the MCT family member that is required for lactate export in highly glycolytic cells and its presence in SCs has been previously confirmed [105, 211, 212]. The SCs of Tg-RGN rats showed a pronounced increase in MCT4 expression levels when compared to the control group (5,38 ± 0,85 fold variation, $p<0.01$, Figure IV.4B).

ALT is the enzyme responsible for the reversible catalysis of alanine into pyruvate, playing a key role in the intermediary metabolism of glucose and amino acids [213, 214]. The SCs of Tg-RGN animals presented higher expression levels of ALT when compared to the SCs of their Wt littermates after 24 hours of culture (1,73 ± 0,20 fold variation, $p<0.05$, Figure IV.4C).

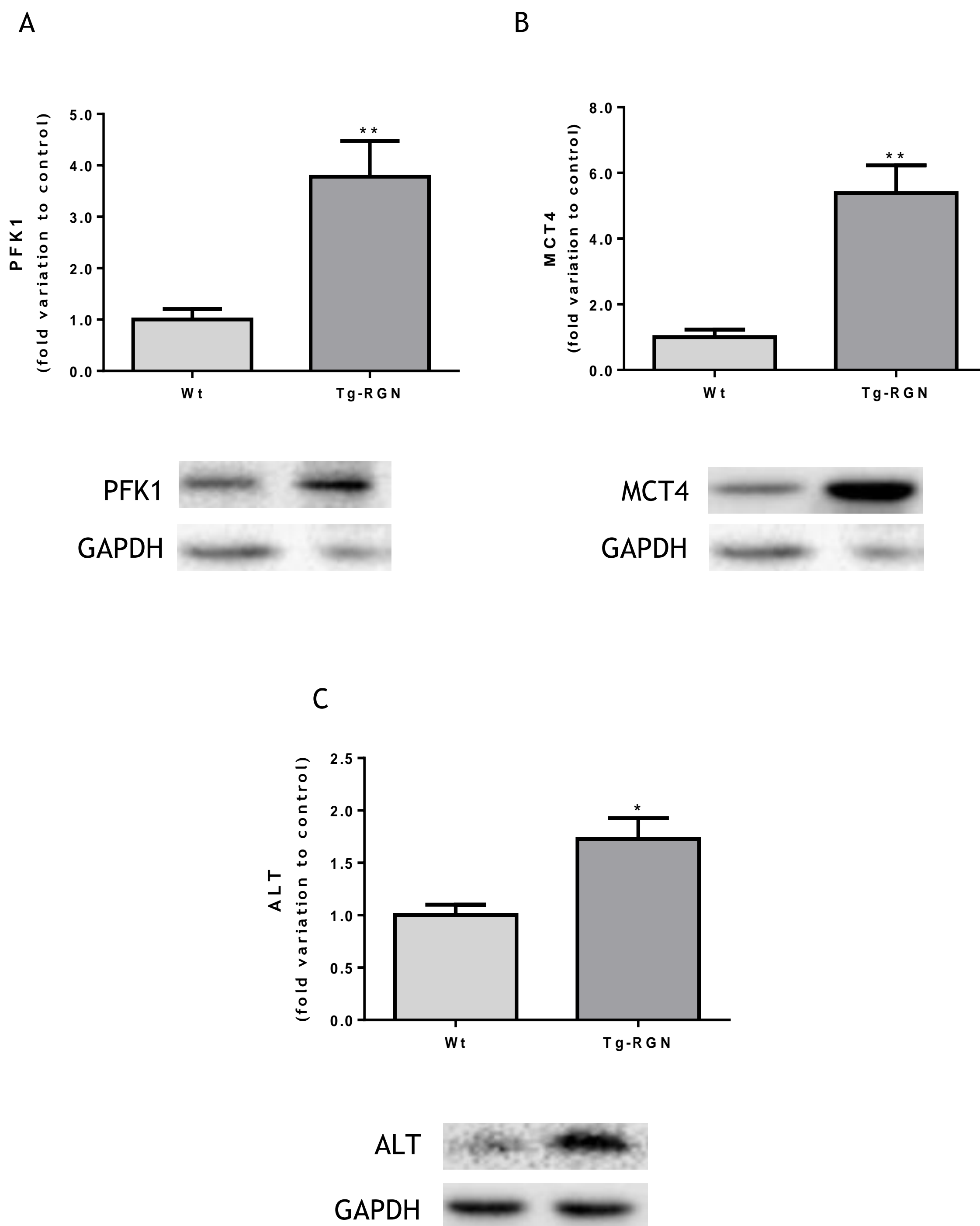

Figure IV.4. Expression of metabolism-related enzymes and transporters, PFK1 (A), MCT4 (B) and ALT (C) in primary SCs of Wt and Tg-RGN rats cultured for 24 hours. Data are represented as mean ± S.E.M. after normalization with GAPDH (n≥4 in each group Results are expressed as fold-variation relatively to Wt animals. * $p<0.05$, ** $p < 0.01$. Representative immunoblots are shown in bottom panels.

c) Protein expression and enzymatic activity of LDH were decreased in the SCs under RGN overexpression

LDH is the enzyme responsible for the reversible conversion of glycolysis end-product pyruvate into lactate [126, 215], the germ cells most preferred substrate [206]. For that matter, and to help characterizing RGN's role in SCs' metabolism, it became of the uttermost importance to evaluate the protein expression and enzymatic activity of LDH in the SCs of Tg-RGN and Wt animals (Figure IV.5).

The expression levels of LDH in the SCs of Tg-RGN animals after 24 hours of culture were found to be significantly lower (0,61 ± 0,08 fold variation to control, $p<0.05$, Figure IV.5A). Accordingly, the same pattern was found in the enzymatic activity of LDH. A significant decrease in the enzymatic activity of LDH was found in the SCs of Tg-RGN (19,51 ± 3,91 vs 31,68 ± 3,04 μg/μL, $p<0.05$, Figure IV.5B).

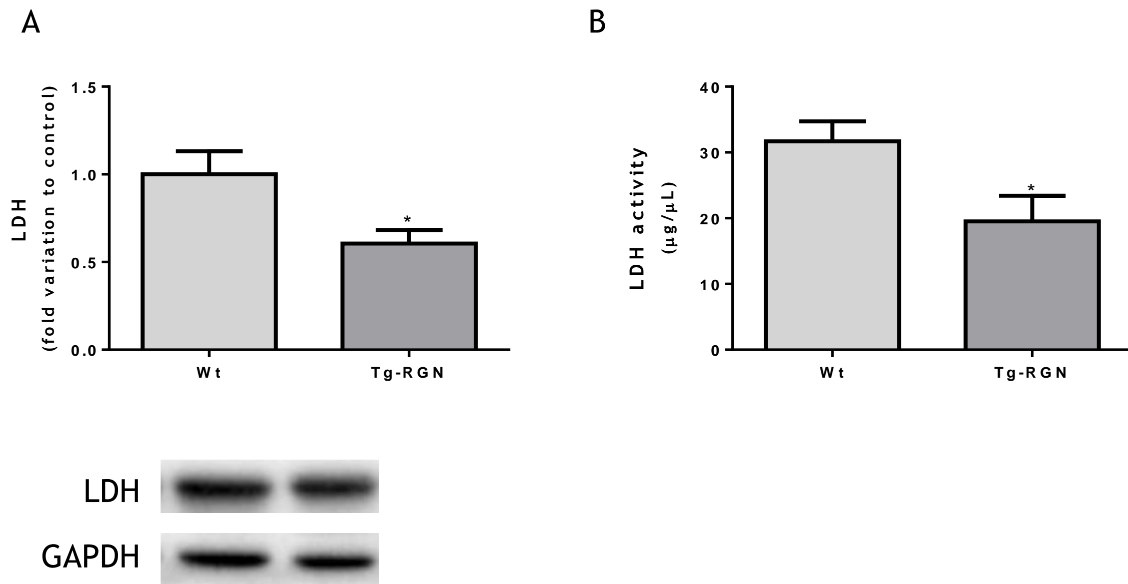

Figure IV.5. Protein expression (A) and enzymatic activity (B) of LDH in the SCs of Wt and Tg-RGN rats cultured for 24 hours. Data are represented as mean ± S.E.M. after normalization with GAPDH (n≥4 in each group). Results are expressed as fold-variation relatively to Wt animals. * $p < 0.05$. Representative immunoblots are shown as bottom panel.

3. Glutaminolysis is enhanced in the SCs of Tg-RGN animals

Glutamine is one of the most important substrates for SCs, being reported that the single oxidation of glutamine can yield much of the energy required by SCs [111]. However, the regulation of glutaminolysis in SCs remains fairly unknown.

Herein, the results obtained showed that glutamine consumption was augmented in the SCs of Tg-RGN animals when compared to the control group after 24 hours of culture (0,10 ± 0,02 vs 0,02 ± 0,004 pmol/cell, $p<0.05$, Figure IV.6A).

ASCT2 is a solute glutamine transporter required to uptake the extracellular glutamine into the cells [138]. We observed that the expression levels of ASCT2 were significantly higher in the SCs of Tg-RGN after 24 hours of culture when compared to the Wt group (1,66 ± 0,17 fold variation, $p<0.05$, Figure IV.6B).

Glutaminolysis relies on the crucial step of glutamine conversion into glutamate, a reaction catalysed by GLS [216]. In this work, the expression levels of GLS were significantly higher in the SCs of Tg-RGN cultured for 24 hours when compared to the control group (1,37 ± 0,09 fold variation, $p<0.05$, Figure IV.6C).

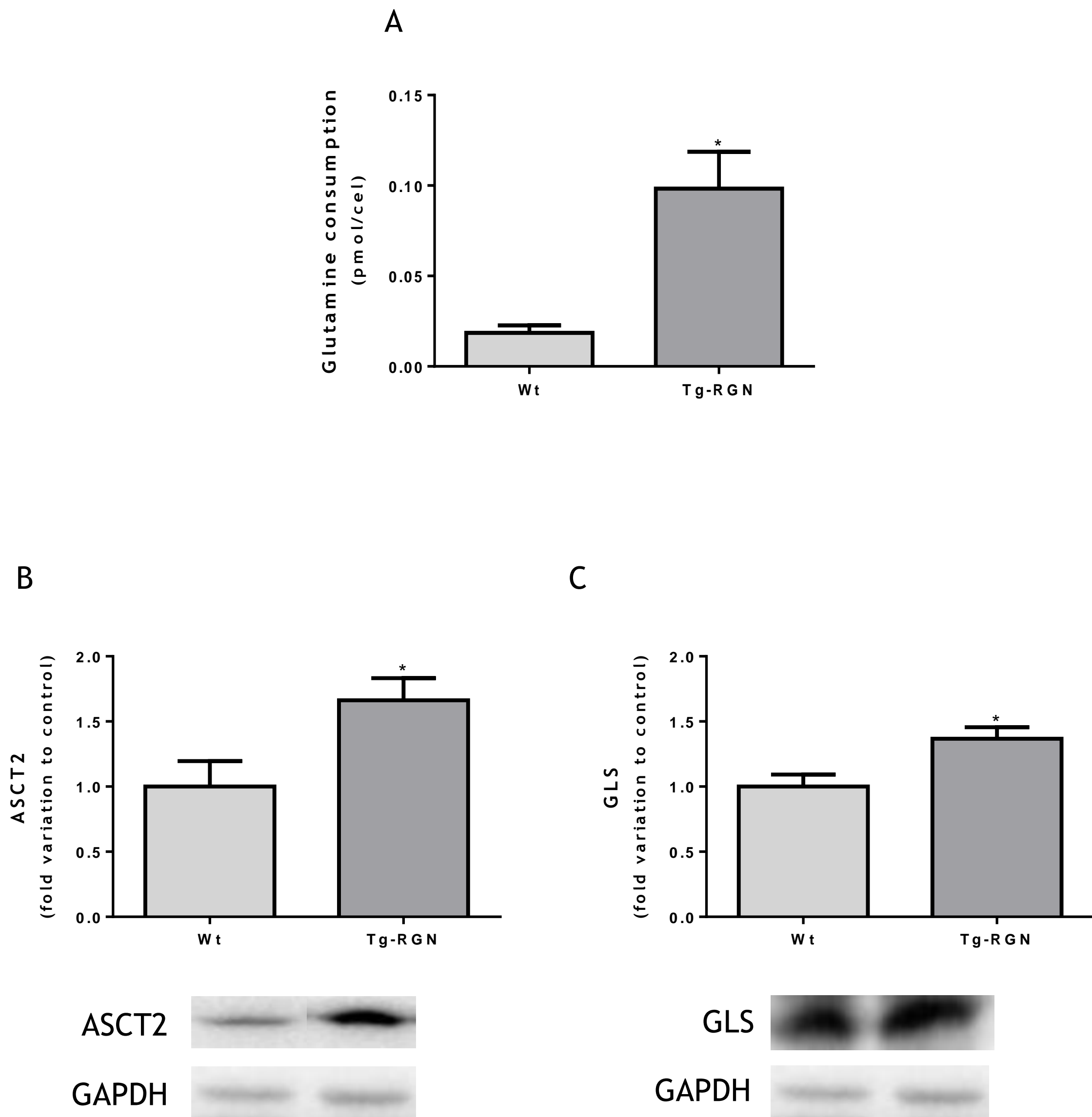

Figure IV.6. Glutamine consumption (A) and protein expression of glutaminolysis-related proteins, ASCT2 (B) and GLS (C) in the SCs of Tg-RGN rats vs Wt cultured for 24 hours. Data are represented as mean ± S.E.M. after normalization with GAPDH (n≥4 in each group). Results are expressed as fold-variation relatively to Wt animals. * $p < 0.05$. Representative immunoblots are shown in bottom panels.

V. Discussion

Although RGN's actions have been associated to the modulation of cellular metabolic processes [189, 194, 217, 218], its relationship with the glycolytic metabolism of SCs remains completely unexplored. In the present study, we began to investigate glucose metabolism in SCs under overexpression of RGN comparatively with that of Wt controls. It was found that the SCs of Tg-RGN animals consumed less glucose (Fig. IV.1A), whereas producing higher amounts of lactate (Fig. IV.1B).

In order to consume glucose, first, SCs require to uptake this sugar from the extracellular space, a process mediated by the GLUTs present at the cell membrane (Fig. I.4). At least four GLUTs isoforms have been identified in the SCs to this date (GLUT1, GLUT2, GLUT3 and GLUT8) [123, 210] though only GLUT1 and GLUT3 have been reported as playing a crucial role in glucose incorporation [219]. We have observed that both GLUT1 and GLUT3 expression levels were elevated in the SCs of Tg-RGN rats (Fig. IV.3A and Fig. IV.3C, respectively) which, at first sight, would appear not consistent with the diminished glucose consumption observed. However, we have also found that GLUT2 expression pattern was strongly diminished in the SCs of Tg-RGN animals (Fig. IV.3B), which was in agreement with the diminished glucose consumption observed. Although GLUT2 was previously identified in the SCs [220], its functional role has been mostly described in tissues handling the dietary sugars [221–223], such as the intestine, pancreas, kidney, and liver, with this study firstly indicating the involvement of GLUT2 in the glycolytic metabolism of SCs.

Concerning the energy metabolites in testicular fluids, higher glucose levels were detected in the interstitial fluid (extracellular *milieu*) of Tg-RGN animals (Fig. IV.2A); thus SCs of these animals have greater availability of glucose. It was reported that SCs possess the ability of increasing glucose consumption under glucose deprivation conditions, a mechanism that ensures the appropriate glycolytic flux [130, 212]. Hence, it is liable to assume that the diminished glucose consumption found in the SCs of Tg-RGN animals, concomitantly with the diminished expression of GLUT2, is driven by the augmented glucose levels in the interstitial fluid.

Interestingly, glucose levels were also higher in the SeT fluid of Tg-RGN rats (Fig. IV.2C). Since SeT fluid production and composition are determined by the activity of SCs [224, 225], the higher concentration of glucose in the SeT fluid of Tg-RGN animals is consistent with the diminished glucose consumption observed by the SCs of these animals.

The diminished glucose consumption detected in the SCs of Tg-RGN rats (Fig. IV.1A) was accompanied by a decrease in the intracellular glucose concentrations (Fig. IV.1C), and followed previous findings in other glycolytic tissues. Vaz et al. [129] showed that the *in vivo* overexpression of RGN in the liver, one of the body's reservoirs of glucose, brain, an organ with a great demand for glucose, and prostate, a tissue with very high glycolytic activity, caused tissue glucose concentrations to decrease. Moreover, the lower glucose concentration

found in prostatic tissues was accompanied by the augmented expression levels of PFK1 [129], an enzyme that catalyzes a rate-limiting step in glycolysis (Fig. I.4), which allowed authors to suggest that glucose was being actively metabolized. Indeed, higher expression rates of PFK1 have been linked to a high glycolytic activity which, in turn, is related to a greater glucose flux and metabolization in the cell [226]. Our results follow these observations. The expression levels of PFK1 in the SCs of Tg-RGN animals were deeply augmented (Fig. IV.4A) contributing to the diminished intracellular glucose levels, and sustaining a high glycolytic flux.

As previously mentioned, lactate is the central energy metabolite used by germ cells, being responsible for the stimulation of RNA and protein synthesis in spermatids [227]. SCs' ability to produce high amounts of lactate [118, 145, 224], and thus, fulfilling germ cells' metabolic needs throughout their development, is widely recognized and has granted these cells the epithet "nurse cells". We have noted that notwithstanding with the diminished glucose consumption, and despite no significant differences were found in the intracellular lactate levels (Fig. IV.1D), lactate production was augmented in the SCs of Tg-RGN animals (Fig. IV.1B). The enhanced export of this glycolytic metabolite was sustained by the increased expression levels of MCT4 (Fig. IV.4B), the membrane transporter required to export the lactate onto the extracellular space. However, the protein expression (Fig. IV.5A) and the enzymatic activity (Fig. IV.5B) of LDH were significantly decreased in the SCs of Tg-RGN rats. This lead us to hypothesize that other alternative substrates might be getting used for the production of lactate. It has been shown that in order to maintain high rates of metabolic activity SCs can use amino acids as energy sources, which includes alanine and glutamine but not glycine [132]. The oxidation of glycine by SCs has been considered non-significant for energy purposes [132].

Alanine is a substrate placed at the crossroad of glucose and amino acids metabolism by the activity of ALT, the enzyme that catalyses the reversible reaction of alanine conversion into pyruvate (Fig. I.4). We have found a pronounced increase in ALT's expression in the SCs of Tg-RGN animals (Fig. IV.4C), indicating that a significant part of the pyruvate produced by these cells may have its origin in the conversion of alanine by ALT and not in glycolysis.

Although the contribution of ALT for the production of pyruvate by SCs has been a matter that lacks directed studies, it has been shown that these cells can maintain lactate production even in the total absence of glucose [130]. This premise further supports the idea that the enhanced lactate production in the SCs of Tg-RGN rats may be driven by the augmented pyruvate production in consequence of increased ALT expression.

Besides being an energy substrate, lactate exerts antiapoptotic effects on germ cells [109], being also shown that the testicular infusion of lactate into adult cryptorchidic rat testis improves the spermatogenic process [228]. A previous study conducted in the whole testis

demonstrated that Tg-RGN rats present lower apoptotic rates in consequence of the diminished activity of the apoptosis-effector caspase-3 [229]. Also, it has been shown that RGN suppresses thapsigargin- and actinomycin D-induced apoptosis in SeT by modulating the expression and activity of key apoptotic and antiapoptotic factors, which could indicate that RGN overexpression protects germ cell from apoptosis induced by noxious stimuli [194]. Thereafter, it is liable to speculate that the antiapoptotic role played by RGN in the testis of Tg-RGN rats could be associated to the augmented levels of lactate made available for the germ cells in consequence of the enhanced production of SCs, as was demonstrated herein. Moreover, the results obtained are in line with previous findings from our research group that also described a higher spermatozoa viability in the Tg-RGN animals [188].

Considering glutamine, Grootegoed et al. [111] have demonstrated that the single oxidation of this amino acid yields most of the energy that SCs require. In order for glutaminolysis to occur, glutamine firstly requires to enter the cell, a task that is achieved by the glutamine transporter ASCT2 (Fig. I.4) [138]. The SCs of Tg-RGN animals have shown increased expression of the ASCT2 (Fig. IV.6B), which was very consistent with the higher glutamine consumption (Fig. IV.6A) observed. Aside supporting the production of antioxidant molecules (NADPH and glutathione), glutaminolysis is a mitochondrial pathway (Fig. I.4) that involves the initial deamination of glutamine by GLS [230]. Our results have shown that also the expression levels of GLS were increased in the SCs of Tg-RGN animals (Fig. IV.6C), suggesting a high rate of glutamine-oxidation. In sum, glutamine consumption and metabolization are increased in the SCs of Tg-RGN animals, which would be of the uttermost importance considering the diminished uptake of glucose. The specific role of this substrate to SCs' metabolism remains unknown, but it cannot be excluded from the discussion that it can contribute to the final pool of pyruvate/lactate. In both astrocytes [231] and enterocytes [232], glutamine utilization to produce lactate via the oxidative pathways of glutamate degradation has been described. In the case of SCs this ability requires further investigation, but it has been shown that glutamine prevents the incorporation of alanine into proteins [139], a quite relevant issue given the fact that alanine can be converted to pyruvate. Therefore, in an indirect manner, glutamine can be increasing pyruvate by augmenting the intracellular levels of alanine, which in the scenario of Tg-RGN SCs could be potentiated by the increased expression of ALT.

The mitochondrial degradation of glutamine's metabolite, glutamate, is initiated by its entry into the tricarboxylic acid (TCA) cycle and its conversion to α-ketoglutarate. This degradation is a partial process because glutamate cannot be fully degraded by the TCA cycle since this is a catalytic process in which two carbons enter as acetyl-CoA and two carbons are released as CO_2. A four-carbon molecule must leave the TCA cycle and be converted to pyruvate, which can have several possible fates: re-enter the TCA cycle for complete oxidative degradation of glutamate or be converted to lactate [233].

The existence of this relationship between glutamine metabolism and glycolysis, together with the augmented expression of ALT, explains the augmented lactate production in the SCs of Tg-RGN rats when the protein expression and enzymatic activity of LDH were diminished.

VI. Conclusions and Future Perspectives

Nowadays, male fertility has become one of the major health concerns of western civilizations. The maintenance of a fully functional spermatogenesis is of the uttermost importance to preserve the fertility potential of male. Since SCs are the key-players in the regulation of spermatogenesis, a tight control over these cells' functions and metabolic needs is constantly in order, and possible regulators of these cells' metabolism are paramount study goals.

In this dissertation we have established RGN as an important regulator of the SCs' glucose and glutamine metabolism. Despite consuming less glucose, the SCs of Tg-RGN animals displayed an adaptation of metabolism that maintained high rates of lactate production and exportation. The lower glucose uptake was compensated by the alanine and glutamine metabolism that can be driven to the production of pyruvate, fuelling the production of lactate. The understanding of the molecular mechanisms behind the control of glutaminolysis and glycolysis in the SCs, and how both amino acids contribute to the final pool of available lactate in these cells, will be of paramount importance. These scientific questions will be pursued in future *in vitro* studies using radio-labelled substrates, transporters and enzyme inhibitors and/or gene knockdown methodological approaches. Moreover, the precise mechanisms that alter the enzymatic activity of ALT and GLS in the SCs of Tg-RGN animals are very relevant aspects to be adressed in the future.

Overall, the present discoveries widened the array of RGN roles supporting a successful spermatogenesis and highlighted for the plasticity of SCs' metabolism, which could be explored in the context of male infertility. In the future, the full comprehension of the molecular mechanisms responsible for the supporting action of RGN in SCs' metabolism must be of the uttermost importance. Further clarification on this issue is expected to come from *in vivo* and *in vitro* studies using, respectively, the RGN knock-in rats already available in the lab or a gene-knocking down strategy (e.g. siRNA) for both RGN and metabolism-associated genes of interest. Also, the trasncriptomics, proteomics and metabolomics characterization of the SCs of Tg-RGN animals would produce crucial information to unreavel the mechanism of action of RGN. The thorough disclosure of how to enhance this protein's protective effects in the male reproductive tract, will be of exceeding importance to the development of new, more affordable and further efficient fertility-preserving strategies and treatments.

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VIII. Publications and Communications

1. Publication in International Peer-Reviewed Journal

Mateus I., Correia S.*, Espínola, L. M. and Socorro S.*[1] Glucose and glutamine handling in the Sertoli cells of transgenic rats overexpressing regucalcin: plasticity towards lactate production. *Scientific Reports*. (submitted, under revision). IF: 5.228

*contributed equally as senior authors; [1]corresponding author

2. Poster in International Congress

Mateus I., Correia S.*, Socorro S.* Glucose handling in testicular cells of transgenic rats overexpressing regucalcin. *I Congress in Health Sciences Research: Towards Innovation and Entrepreneurship - Trends in Endocrinology and Neurosciences*. 26th-28th November 2015, Covilhã, Portugal

*contributed equally as senior authors

3. Poster in International Meeting

Mateus I.*, Vaz C.*, Correia S.#, Socorro S.# Metabolic alterations on the crossroad of infertility and cancer. *Encontro com a ciência e tecnologia em portugal*. 4th-6th July 2016, Lisbon, Portugal

*contributed equally; #contributed equally as senior authors

4. Oral communication

Mateus I., Correia S.*, Socorro S.* Testicular cells of transgenic rats overexpressing regucalcin display a distinct glycolytic metabolism: from glucose uptake to lactate export. *XI Annual CICS-UBI Symposium*. 30th June and 1st July 2016, Covilhã, Portugal.

*contributed equally as senior authors