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Characterization of the testicular transcriptome of transgenic rat overexpressing regucalcin: insights into (in)fertility

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Resumo

A regucalcina (RGN) é uma proteína de ligação ao cálcio (Ca^{2+}) altamente conservada, a qual foi inicialmente identificada no fígado de rato, desempenhando um papel na homeostase do Ca^{2+} intracelular. Nos últimos anos, a RGN foi identificada em vários tecidos não reprodutivos e reprodutivos e, devido à sua capacidade de modular os níveis de Ca^{2+}, e de regular a atividade de enzimas independentes e dependentes de Ca^{2+}, a panóplia de funções conhecidas da RGN foi sendo alargada. A RGN foi assim sendo envolvida também na regulação da sinalização intracelular, stress oxidativo e metabolismo, assim como em processos biológicos importantes, como proliferação e apoptose.

A RGN é codificada pelo gene *Rgn,* que foi identificado como um gene alvo dos androgénios, o qual é amplamente expresso em todos os tipos de células testiculares, o que pela primeira vez relacionou a RGN com a reprodução masculina. Mais recentemente, foi demonstrado que ratos transgénicos que sobreexpressam a RGN (Tg-RGN) apresentam maior viabilidade espermática, com menor incidência de defeitos na cauda, resistência ao stress oxidativo e apoptose induzida por produtos químicos ou radiação. Apesar das diferentes evidências que suportam o papel benéfico do RGN na espermatogénese, os agentes moleculares subjacentes às suas ações ainda não são conhecidos.

O presente estudo teve como objetivo identificar as vias e os agentes moleculares subjacentes às ações citoprotetoras da RGN na espermatogénese.

Com este propósito, foi analisado o transcriptoma testicular de ratos Tg-RGN comparativamente com ratos wild-type (Wt) usando uma abordagem de sequenciamento de RNA (RNA-seq). Os genes diferencialmente expressos foram agrupados de acordo com o seu padrão de expressão, ontologia genética (GO) e análises de enriquecimento. Um total de 1064 genes foram diferencialmente expressos em ratos Tg-RGN, tendo-se encontrado os maiores *scores* de enriquecimento para termos GO de processos biológicos como transporte de iões ou ciclo celular meiótico.

A informação obtida foi então filtrada considerando o fold-change, o nível de expressão e a potencial relevância para a espermatogénese, e foram selecionados 10 genes para validação dos resultados do RNA-seq por reação em cadeia da polimerase quantitativa em tempo real (qPCR). Os genes *Atp10b*, *Orai1*, *Sfrp2* e *Tnni1* foram validados como sendo regulados positivamente nos testículos de ratos Tg-RGN, enquanto os genes *Fign* e *Sycp1* foram regulados negativamente, o que sugere o seu provável papel como parceiros da RGN na modulação da espermatogénese.

Posteriormente, procedeu-se à análise da expressão dos 10 genes selecionados em diferentes tipos de células testiculares. Com este intuito, utilizou-se uma linha celular com características de células estaminais espermatogoniais (células GC-6spg), e células de Sertoli (SCs) primárias isoladas de ratos Wt. A expressão génica foi avaliada por transcrição reversa por PCR (RT-PCR). Todos os genes diferencialmente expressos, com exceção do *Atp10b*, foram expressos em SCs; os genes *Eng*, *Fign*, *Orai1*, *Star*, *Sycp1* e *Tnni1* foram também detetados nas células GC-6spg.

No geral, os resultados obtidos na presente tese deram uma nova visão sobre os mecanismos moleculares por detrás das ações da RGN nos testículos, e forneceram a base para investigação futura no sentido de explorar e aprofundar o conhecimento sobre o papel da RGN na regulação da espermatogénese e na (in)fertilidade masculina.

Palavras-chave

Regucalcina; Transcriptoma testicular; Espermatogénese; Infertilidade masculina.

Resumo Alargado

A espermatogénese é a base da fertilidade masculina, sendo que defeitos neste processo e danos provocados por agentes externos são causas de infertilidade. Assim sendo, a compreensão de seu controlo molecular e a descoberta de potenciais agentes citoprotetores contra fatores danosos é fundamental para o desenvolvimento de novas estratégias mais eficientes para a preservação da fertilidade.

A regucalcina (RGN) é uma proteína de ligação ao cálcio (Ca^{2+}) altamente conservada em toda a linha evolutiva, e entre eucariotas e procariotas. A RGN foi inicialmente identificada no fígado de ratos, e a sua função melhor documentada é a que se associa com a manutenção da homeostase do Ca^{2+}. No entanto, e muito devido à sua capacidade de regular várias proteínas relacionadas com o Ca^{2+}, a RGN tem vindo a ser descrita como uma proteína multifuncional com vários efeitos biológicos. Constatou-se que a RGN possui efeitos citoprotetores, sendo anti-apoptótica e antiproliferativa, bem como capaz de regular as vias de sinalização intracelular, o metabolismo e o stress oxidativo.

Ao longo dos anos, para além da sua presença no fígado, a RGN foi descoberta em vários tecidos e fluídos, incluindo na mama, próstata, testículos, fluído dos túbulos seminíferos e fluído epididimal. Para além disso, o gene *Rgn*, que codifica a proteína RGN, foi indicado como um gene que é alvo de androgénios na próstata e testículo, o que no conjunto constituíram os primeiros factos a relacionar esta proteína com a função reprodutiva. Curiosamente, a expressão de RGN nos testículos é verificada tanto em células somáticas, como germinativas, e esta parece ter um efeito citoprotetor na espermatogénese. Um modelo de rato transgénico que sobrexpressa a RGN (Tg-RGN) apresentou maior viabilidade espermática e menor incidência de defeitos espermáticos, assim como menor stress oxidativo, e resistência das células testiculares à apoptose induzida por agentes químicos ou radiação ionizante. No entanto, os agentes e vias moleculares subjacentes às ações citoprotetores da RGN na espermatogénese não são totalmente conhecidos.

Assim sendo, a presente dissertação teve como objetivo identificar as vias de sinalização e agentes moleculares que estão por detrás das ações da RGN nos testículos, e qual o seu papel na espermatogénese e fertilidade masculina.

Para isto, procedeu-se à análise do transcriptoma testicular de ratos Tg-RGN comparativamente ao de ratos wild-type (Wt) através de sequenciamento de RNA (RNA-seq), de modo a identificar genes diferencialmente expressos neste modelo transgénico, que possam estar a agir em conjunto com a RGN na modulação da espermatogénese. Os genes identificados foram posteriormente sujeitos a análises de enriquecimento de Gene Ontology (GO) e de vias da Kyoto

Encyclopedia of Gene and Genome (KEGG) para identificação dos processos biológicos e vias de sinalização enriquecidos no modelo transgénico, e que, consequentemente, poderão estar envolvidos nas ações da RGN nos testículos.

A análise por RNA-seq revelou que 1064 genes foram diferencialmente expressos nos testículos de ratos Tg-RGN comparativamente aos controlos Wt, sendo que a maioria dos genes (714 genes) foram negativamente regulados. As análises de enriquecimento demonstraram que vários processos biológicos e vias da fisiologia testicular estavam enriquecidas nos ratos Tg-RGN, existindo vários que são associados a processos relevantes para a espermatogénese, nomeadamente transporte, homeostase e sinalização de Ca^{2+}, biossíntese de hormonas esteroides, ciclo meiótico, recombinação homóloga, entre outros.

Os resultados obtidos foram ainda filtrados considerando o fold-change, o nível de expressão, a análise de enriquecimento e a potencial relevância de cada gene no processo espermatogénico. Do total de 1064 genes foram assim selecionados 10 genes para validação dos resultados do RNA-seq por reação em cadeia da polimerase quantitativa em tempo real (qPCR). Estes genes foram: *Atp10b*, *Eng*, *Fign*, *Lum*, *Orai1*, *Plcb1*, *Sfrp2*, *Star, Sycp1* e *Tnni1*, que são genes envolvidos em processos biológicos como mitose, meiose, proliferação e apoptose, sinalização intracelular, regulação de canais de Ca^{2+} e da síntese de hormonas esteroides. A expressão de 6 destes genes (*Atp10b*, *Fign, Orai1, Sfrp2, Sycp1* e *Tnni1*) foi confirmada como significantemente diferencial nos testículos de ratos Tg-RGN comparativamente com o grupo de controlo na análise por qPCR, o que, tendo em conta a sua função e grupos de enriquecimento, sugere o seu papel como parceiros moleculares das ações da RGN na espermatogénese. Adicionalmente, foi encontrada uma correlação linear entre os resultados de RNA-seq e qPCR (com $r = 0,71$ e valor de $p < 0,001$), o que também valida os resultados de expressão diferencial encontrados nos testículos de ratos Tg-RGN por RNA-seq.

Tendo em conta a heterogeneidade celular do testículo, a presença dos 10 genes selecionados foi ainda investigada por transcrição reversa por PCR (RT-PCR) em diferentes tipos de células testiculares. Para este propósito, utilizou-se uma linha celular de rato com características de células estaminais espermatogoniais (células GC-6spg), e células de Sertoli (SCs) isoladas de ratos Wt. Verificou-se que a expressão dos genes *Eng, Fign, Orai1, Star, Sycp1* e *Tnni1* foi detetada tanto nas células GC-6spg como em SCs, enquanto os genes *Lum, Plcb1* e *Sfrp2* foram detetados apenas em SCs; o gene *Atp10b* não foi detetado em nenhum destes tipos celulares.

O presente estudo caraterizou o transcriptoma testicular de ratos Tg-RGN, identificando genes diferencialmente expressos, assim como processos biológicos e vias de sinalização enriquecidos neste modelo transgénico, o que forneceu uma nova base para a investigação e compreensão dos processos, funções e vias nas quais a RGN estará envolvida nos testículos, com enfâse na espermatogénese. Assim, a presente dissertação permitiu obter uma nova visão sobre os mecanismos moleculares por detrás das ações da RGN nos testículos e forneceu a base para

investigação futura sobre o papel da RGN na regulação da espermatogénese e da (in)fertilidade masculina. Tendo em conta os efeitos citoprotetores da RGN, é razoável considerar que a manipulação dos níveis de RGN nos testículos poderá potenciar a preservação da fertilidade masculina ou vir a ter utilidade como eventual estratégia terapêutica.

Abstract

Regucalcin (RGN) is a highly-conserved calcium (Ca^{2+})-binding protein, which was initially identified in the rat liver, playing a role in intracellular Ca^{2+} homeostasis. Over the last years, RGN was identified in several non-reproductive and reproductive tissues and, due to its modulation of Ca^{2+} levels, and regulation of Ca^{2+}-dependent and -independent enzymes, the panoply of RGN known functions has been widening. RGN has thus also been involved in the regulation of intracellular signaling pathways, oxidative stress and metabolism, as well as important biological processes such as cell proliferation and apoptosis.

RGN is encoded by the *Rgn* gene, identified as an androgen-target gene broadly expressed in all testicular cell types, which for the first linked RGN with male reproduction. More recently, transgenic rats overexpressing RGN (Tg-RGN) have been shown to display increased sperm viability with lower incidence of tail defects, as well as resistance to oxidative stress and to chemical- or radiation-induced apoptosis. Despite the different evidences supporting the beneficial role of RGN in spermatogenesis, the molecular players underlying its actions are not yet known.

The present study aimed to identify the pathways and molecular players underlying the cytoprotective actions of RGN in spermatogenesis.

For this purpose, the testicular transcriptome of Tg-RGN rats compared to wild-type (Wt) rats was analyzed using an RNA sequencing approach (RNA-seq). Differentially expressed genes were clustered according to their expression pattern, gene ontology (GO) and pathway enrichment analysis. A total of 1064 genes were differentially expressed in the Tg-RGN rats with higher enrichment scores found for GO terms for biological processes such as ion transport or meiotic cell cycle.

The obtained information was then filtered considering fold-change, expression level and the potential relevance for spermatogenesis, and 10 genes were selected for real-time quantitative reverse transcription polymerase chain reaction (qPCR) validation of the RNA-seq results. *Atp10b*, *Orai1*, Sfrp2 and *Tnni1* genes were validated as being up-regulated genes in the testis of Tg-RGN rats, whereas *Fign* and *Sycp1* genes were down-regulated, which suggests their likely role as RGN partners in modulating spermatogenesis.

Afterwards, the expression of the 10 selected genes in different types of testicular cells was investigated. For this purpose, a cell line with spermatogonia stem cell characteristics (GC-6spg cells) and primary Sertoli Cells (SCs) isolated from Wt rats were used. Gene expression was assessed by reverse transcription PCR (RT-PCR). All differentially expressed genes, with

the exception of *Atp10b*, were expressed in SCs; *Eng, Fign, Orai1, Star, Sycp1* and *Tnni1* genes were also detected in GC-6spg cells.

Overall, the findings obtained in the present thesis gave a new insight into the molecular mechanisms behind RGN' actions in the testes and provided the basis for future research exploring and deepening the knowledge about the role of RGN in the regulation of spermatogenesis and male (in)fertility.

Keywords

Regucalcin; Testicular transcriptome; Spermatogenesis; Male infertility.

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List of Abbreviations

AAA	ATPases associated with diverse cellular activities
Actb	Beta-actin
Akt-1	Serine/Threonine Kinase 1
ANOVA	Analysis of variance
ATP	Adenosine 5´-triphosphate
ATP10B	ATPase phospholipid transporting 10B
ATPase	Adenosine 5´-triphosphatease
ß2M	Beta-2-microglobulin
BMP	Bone morphogenetic proteins
BTB	Blood-testis barrier
Ca^{2+}	Calcium
$CaCl_2$	Calcium chloride
CaM	Calmodulin
cAMP	Cyclic adenosine monophosphate
CaSR	Calcium sensing receptor
cDNA	Complementary deoxyribonucleic acid
CRAC	Calcium release–activated calcium channel
Cts	Treshold cycles
DAG	Diacylglycerol
DE	Differential expression
ENG	Endoglin
FC	Fold-change
FDR	False discovery rate
FIGN	Fidgetin, microtubule severing factor
FSH	Follicle-stimulating hormone
GnRH	Gonadotropins releasing hormone
GO	Gene ontology
GO-BP	Gene ontology terms for biological processes
IP2	Phosphatidylinositol 4,5-bisphosphate
IP3	Inositol 1,4,5-trisphosphate
KEGG	Kyoto Encyclopedia of Gene and Genome
LC	Leydig cell
LH	Luteinizing hormone
LUM	Lumican
Mg^{2+}	Magnesium
Mn^{2+}	Manganese
mRNA	Messenger ribonucleic acid
NEM	N-ethylmaleimide
NIH	United States National Institutes of Health
NOS	Nitric oxide synthase
ORAI1	Ca^{2+} release-activated Ca^{2+} modulator 1
PCR	Polymerase chain reaction
PKC	Protein kinase C
PLCB1	Phospholipase C beta 1

qPCR	Quantitative real-time reverse transcription polymerase chain reaction
RAAS	Renin-angiotensin-aldosterone system
RGN	Regucalcin
RNA-seq	Ribonucleic acid sequencing
ROS	Reactive oxygen species
RPKM	Reads per kilobase of exon model per million mapped reads
RT-PCR	Reverse transcription polymerase chain reaction
SC	Sertoli cell
SEM	Standard error of the mean
SeT	Seminiferous tubules
SFRP	Secreted frizzled-related protein
SFRP2	Secreted frizzled-related protein 2
SLRP	Small leucine-rich proteoglycans
SMP30	Senescence marker protein-30
SOCE	Store-operated calcium entry
SOD	Superoxide dismutase
SSC	Spermatogonial stem cell
STAR	Steroidogenic acute regulatory protein
STIM1	Stromal interaction molecule 1
SYCP1	Synaptonemal complex protein 1
TGF- ß	Transforming growth factor ß
Tg-RGN	Transgenic overexpressing regucalcin
TNF-α	Tumor necrosis factor α
TNFR	TNF-α receptor;
TNNI1	Troponin I1, slow skeletal type
TPM	Transcripts per million
WNT	Putative wingless-related MMTV integration site protein
Wt	Wild-type
Zn^{2+}	Zinc

1. Introduction

1.1 The mammalian spermatogenic process: brief overview

The testes are the essential organs in male reproductive physiology, having the central functions of synthesizing and secreting steroid hormones, and producing the male gametes (1). Each testis has an ovoid shape and is located outside of the abdomen, in the scrotum that provides a cooler environment compared to body temperature, which is mandatory for sperm production to occur (1).

The testicular dual function is supported by the structural organization of the testis, with steroidogenesis taking place in the interstitium and germ cell development occurring in the seminiferous tubules (SeT), the highly convoluted structures that are the functional units of the testis (1) (Figure 1). The interstitium, residing between SeT, contains a great diversity of cells and structures such as leukocytes, macrophages, mesenchymal cells, nerves, blood vessels and Leydig cells (LCs) (Figure 1), which are the testosterone producing cells (2). The SeT represent about 90% of the testicular mass (2) and are composed of diverse germ cells that form numerous concentric layers penetrated by a single somatic cell type, the Sertoli cell (SC) (3) (Figure 1).

The spermatogenic process, also known as spermatogenesis, is a complex and tightly regulated biological process, involving mitosis, meiosis and cell differentiation events that promote the transformation of diploid spermatogonial stem cells (SSCs) into male haploid germ cells (Figure 1). This is a continuous process and is the basis of male fertility, ensuring the production of male gametes, the spermatozoa (3). During spermatogenesis, the expression of a large number of genes is developmentally regulated at both transcriptional and translational level, which maintains a temporal and stage-specific expression pattern of different regulatory proteins (4). Transcriptomic analyses of the successive germ cell subtypes reveal dynamic transcription of over 4000 genes during human spermatogenesis, with the testis being one of the organs with the most complex transcriptome (4).

The organization of the SeT epithelium is fundamental for spermatogenesis, with this process beginning at the basal compartment and moving towards the lumen of the SeT (3) (Figure 1). SSCs are localized at the basal compartment of SeT and have self-renewal and differentiation capability, which plays a key role in the maintenance of spermatogenesis cycle since they are able to divide mitotically giving origin to daughter cells, the spermatogonia (5). Furthermore, the presence of SCs in the SeT ensures germ cells support, nourishing and maintaining cell associations throughout the several steps of spermatogenesis, as the SCs cytoplasm extends as thin arms around the developing germ cells (3,6).The establishment of tight-junctions between adjacent SCs forms the blood-testis barrier (BTB), which divides the SeT in basal and adluminal compartments and provides a protected environment for the developing germ cells. The BTB prevents autoimmune responses and the passage of cytotoxic agents to the adluminal compartment (6). Other SCs functions include the secretion of intratesticular fluid, proteins and several growth factors, as well as the phagocytosis of the degenerating germ cells and of

the excess cytoplasm (residual body) that remains after spermatid differentiation and sperm release (3,6).

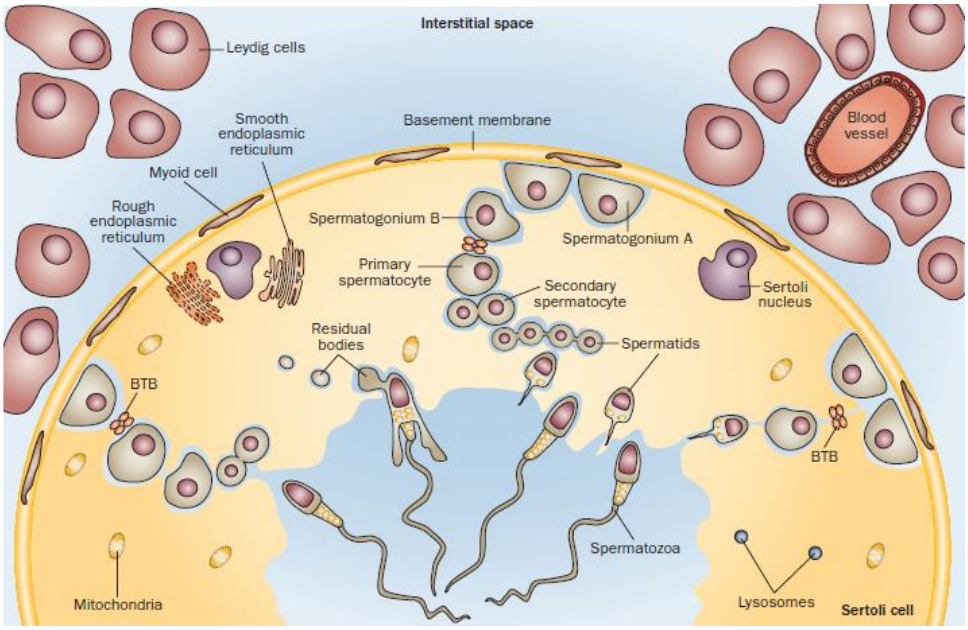

Figure 1 - Schematic representation of the organization of the seminiferous tubules (SeT) and the mammalian spermatogenic process. Spermatogenesis occurs in the SeT, in close contact with the somatic Sertoli cells (SCs). This process begins with the mitotic division of spermatogonia that originates spermatogonia type A or type B. Spermatogonia type A maintain the spermatogonial population whereas spermatogonia type B are committed with spermatogenesis and differentiate into primary spermatocytes, which pass the tight-junctions formed by adjacent SCs, evolving into secondary spermatocytes through meiosis I. Meiosis II yields four equalized spermatids that suffer a process called spermiogenesis and migrate towards the lumen where the fully formed spermatozoa are finally released. Leydig cells (LCs), the endocrine cells in the testis, reside in the interstitial space between tubules. BTB: blood-testis barrier formed by the tight-junctions between adjacent SCs (7).

Each spermatogenic cycle essentially involves four sequential phases: proliferation of spermatogonia by mitotic divisions, meiosis, differentiation of haploid spermatids and release of mature spermatids from SCs into the lumen of SeT (8) (Figure 1).

The spermatogonia are diploid cells (2n) that are able to divide by mitosis, originating 2 cell types, spermatogonia type A or type B. Of the two types, only spermatogonia B will differentiate into spermatocytes, whereas spermatogonia A remain close to the tubule wall as stem cells and maintain the spermatogonia population ensuring a constant supply for spermatogenesis progression (9). Spermatogonia B, after a species-specific fixed number of mitotic divisions, differentiate into primary spermatocytes (2n) (3,9). Primary spermatocytes then move to the adluminal compartment and proceed to the first meiotic division originating 2 haploid secondary spermatocytes (n), which undergo the second meiotic division, becoming in turn spermatids (n) (3,8). In theory, one spermatogonia originates 4 spermatids, but, due to germ cell loss during meiosis (10), only 2 spermatids are in practice originated by each spermatogonia (8).

Afterwards, spermatids enter a process called spermiogenesis, which consists on the differentiation of round to elongated spermatids, and then finally to mature spermatozoa (3,8).

The SeT cycle ends with the release of mature non-motile spermatozoa into the tubular lumen, in a process called spermiation (8,11). It is thought that the process of spermiation is promoted by peristaltic waves produced by the peritubular myoid cells that surround the SeT (Figure 1). Spermiation is determinant of the number of spermatozoa entering the epididymis and, thus, critical for normal sperm content in the ejaculate (11). Throughout all the spermatogenic process, germ cells remain connected by bridges of cytoplasm to allow synchronous development (8).

Spermatozoa leaving the testis are non-functional gametes and it is only during passage through the long-convoluted tubule of the epididymis that they acquire the ability to move progressively and to fertilize, in a process called maturation (12).

As previously mentioned, spermatogenesis is dependent on the existence of a tight regulation and a controlled environment that ensures testicular germ cells survival (6,13,14). Due to their high mitotic rate, the developing germ cells are highly susceptible to endogenous and exogenous damage (15,16), and factors such as abnormal hormone or oxidative stress levels, exposure to local testicular heating, toxicants and radiation, can damage germ cells and consequently trigger apoptosis of defective cells (15–18).

Therefore, the correct development of germ cells, the germline homeostasis and the quality control of gametes are ensured by the tight balance between germ cell proliferation, differentiation and apoptosis, which is fundamental for normal spermatogenesis (13). In normal conditions, approximately 75% of testicular germ cells undergo apoptosis as a mean to control the overproliferating population or discard unfit cells (17–19).

Hormonal regulation is needed for the proper development and function of somatic testicular cells and, consequently, of germ cells (20). Hormones exert their effects by endocrine, paracrine, juxtracrine and autocrine signaling mechanisms (14). The major player in the hormonal regulation of spermatogenesis is the hypothalamic-pituitary-gonadal axis (Figure 2), referring to the intricate interaction between hormones from the hypothalamus and anterior pituitary with the hormones produced in the gonads (14). Overall, the hypothalamus releases gonadotropin releasing hormone (GnRH), which stimulates the anterior pituitary to release gonadotropins, namely, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). In turn, LH acts on LCs stimulating their steroidogenic activity and synthesis of gonadal testosterone, while FSH acts on SCs inducing the production of several growth factors and other factors required for spermatogenesis, such as androgen binding protein and inhibin. Lastly, testosterone diffuses into the SeT where, together with FSH, exerts a stimulatory effect on the activity of SCs (21). In the testis, LH, FSH and testosterone are germ cell survival factors (18). It is noteworthy to mention that LCs, SCs and the peritubular cells are the cell types that express receptors for testosterone in the testis, with germs cells being devoid of androgen receptors (22). Thus, LCs and SCs coordinated actions play a crucial role in the regulation of the spermatogenic process, being paramount for male fertility.

The ideal concentration of hormones and of other essential factors for spermatogenesis is maintained through negative feedbacks (14). Testosterone exerts a negative feedback mechanism on the hypothalamus and pituitary, with high levels of testosterone resulting in the inhibition of the release of GnRH and LH. Moreover, inhibin, a member of the transforming growth factor ß (TGF-ß) superfamily produced by SCs in response to FSH, can block the production and release of FSH by the pituitary, controlling the output of spermatogenesis (14,23) (Figure 2).

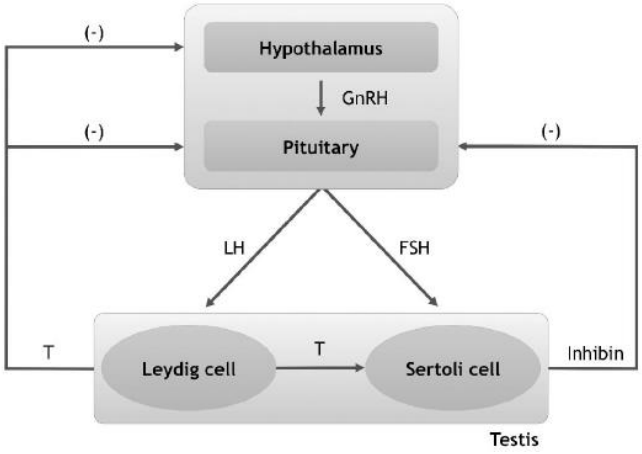

Figure 2 - Hormonal regulation of spermatogenesis. The hypothalamus releases the gonadotropin-releasing hormone (GnRH), which stimulates the anterior pituitary to secrete the follicle stimulating hormone (FSH) and the luteinizing hormone (LH). FSH stimulates the activity of Sertoli cells (SCs) while LH acts on Leydig cells (LCs), inducing the production of 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 subject to a negative feedback (-) by inhibin, which is secreted by SCs (24).

Interestingly, the testes also have the ability to irreversibly convert androgens into estrogens (25). Although androgens and FSH are broadly recognized as the main regulators of spermatogenesis (26,27), being fundamental for germ cells survival, maturation and sperm production (21), the role of estrogens in spermatogenesis is still not fully understood. It is known that SCs and LCs are able to produce 17ß-estradiol and that rodent and human testis express nuclear and membrane estrogen receptors (28). Furthermore, germ cells express estrogen receptors, which suggests that estrogens produced by SCs may act directly in these cells (29). Estrogens may also be able to decrease testosterone production through direct action on LCs (30) or by exerting a negative feedback on the hypothalamus or pituitary (31). It was proven that disruption of estrogen-dependent mechanisms can cause defects in spermatogenesis (28,32). However, while some studies support the estrogens' role as male germ cell survival factors others associate their actions with testicular apoptosis and diminution of germ cell number (28,29,33). Thus, the action of estrogens in the testis is a matter that still needs further research.

Non-hormonal factors are also involved in the regulation of spermatogenesis. For instance, the germ cell cycle and movement along the SeT involves distinct mechanisms that include the

activation of several families of kinases and phosphatases in response to growth factors and cytokines (34). One non-hormonal factor that has been implicated in spermatogenesis, and is of a special relevance to this study, is the ion calcium (Ca^{2+}). Ca^{2+} is a second messenger involved in diverse cellular functions of both somatic and germ cells, which can mediate the transcription of important genes for spermatogenesis, such as the ones specific for meiosis and postmeiotic differentiation (35), and the responses to endocrine hormones and local regulators (36,37). Furthermore, studies show that deregulation of Ca^{2+} homeostasis can cause reversible infertility, which was observed when administration of Ca^{2+} channel blockers resulted in defective steroidogenesis and spermatogenesis (38–40), altered gene expression in the testis (39) and diminished sperm fertilizing potential (41,42). Therefore, Ca^{2+} homeostasis appears to be crucial for mammalian spermatogenesis and sperm function (43–46).

1.2 The regulcalcin protein: general structure and function

1.2.1 Regucalcin gene and protein structure

Regucalcin (RGN) is a Ca^{2+}-binding protein that was initially discovered in the rat liver by M. Yamaguchi (47). This protein is encoded by the *Rgn* gene, an X-linked gene containing seven exons and six introns (48), which is localized in the p11.3–q11.2 and q11.1–12 segments of the human and rat X chromosome, respectively (49,50). Human and rodent RGN proteins encoded by the full-length mRNA transcripts contain 299 amino acid residues and display a molecular weight of approximately 33 kDa (49,51–54). For this reason, and because RGN expression was shown to markedly decrease with aging, it was also named senescence marker protein-30 (SMP30) (49,53–55). Noteworthy, alternative splicing for the R*gn* gene has been described, existing transcript variants that can result in proteins with different molecular weights (56–58).

Unlike other proteins involved in Ca^{2+}-binding, RGN does not possess the typical EF-hand Ca^{2+}-binding motif (52), but it contains 24 ß-strands forming six ß-sheets. These make a closed circular arrangement around a central solvent filled tunnel with one metal binding site, capable of binding divalent metal ions, namely Ca^{2+}, zinc (Zn^{2+}), magnesium (Mg^{2+}) and manganese (Mn^{2+}), which have a catalytic role (59). Interestingly, kinetic studies of RGN using gluconolactone as a substrate showed a preference of binding divalent cations in the order $Zn^{2+} > Mn^{2+} > Ca^{2+} > Mg^{2+}$. However, among all metal ions, Ca^{2+} had a significantly higher dissociation constant, which suggests that the RGN Ca^{2+}-bound form may be relevant in stress conditions where elevated free Ca^{2+} level is present (59).

Besides the well-characterized down-regulation of RGN expression with aging (49,53–55), several other agents regulate its expression, including regulatory transcription factors, Ca^{2+}-dependent and independent mechanisms, and hormonal factors (60). Consensus regulatory binding sites for transcription factors that exist upstream of the 5' flanking region of the *Rgn* gene (namely activator protein 1, nuclear factor I-A1, b-catenin and RGN gene promoter region-related protein) stimulate the *Rgn* promoter activity, enhancing this gene transcription in

response to cell-signaling factors (reviewed in (61)). However, RGN regulation appears to be tissue-specific, considering that different responses to the same cell-signaling factor can be observed in different tissues (60,61).

Cell-signaling factors that have been described to regulate RGN expression are Ca^{2+} (62–65), oxidative stress (66), insulin (67,68), and non-steroid and steroid hormones, such as calcitonin (69), parathyroid hormone (70), aldosterone (71), estrogens and androgens (57). This multitude of cell-signaling regulatory agents indicates the intricacy and multifactorial nature of the regulation of RGN expression.

One of the most important and studied cell-signaling factors is Ca^{2+}, since this ion is involved in signaling pathways that regulate a wide range of cellular and physiological processes. The administration of Ca^{2+} chloride ($CaCl_2$) was shown to increase RGN messenger ribonucleic acid (mRNA) expression in rat and mice liver (62–65,68,69), as well as in rat kidney cortex (72). Furthermore, Ca^{2+} regulation of RGN expression was also demonstrated in human cells lines, namely in LNCaP and PNT1A prostate cells (73). The proposed mechanism behind Ca^{2+} regulation of RGN is hypothesized to be mediated by a Ca^{2+}/calmodulin (CaM) signaling pathway (63,68), and was suggested to involve protein kinase C (PKC) activation in rat H4-II-E hepatoma cells (74). In addition, a dependency on calcitonin action was also proposed, which is possible, considering that parathyroid and thyroid hormones play an important role in the maintenance of Ca^{2+} homeostasis (69).

Interestingly, insulin regulation of the expression of RGN in human hepatoma cells and in rat liver suggests a role for this protein in liver metabolism (67,68). Moreover, the regulation of RGN by oxidative stress and steroid hormones may explain the characteristic RGN down-regulation with aging, since this process is associated with increased oxidative stress and diminished sex steroid hormones levels. The fact that *Rgn* is an estrogen and androgen target gene in the testis supports its involvement in male reproductive processes (24,75).

1.2.2 Regucalcin tissue expression pattern

Although RGN was first shown to be highly expressed in the liver and kidney (47,76), over the years several reports have described its presence in other non-reproductive and reproductive tissues and body fluids, as well as, in several pathological and non-pathological conditions. Namely, RGN has been detected in the brain (77), heart (78), lung (76), bone (79), diaphragm muscle (80), breast and prostate (57), testis (75), pea aphid saliva (81), plasma (82), SeT fluid (75), among others, with altered expression being associated with different diseases (62,83,84). In terms of cellular localization, RGN protein has been shown to reside in the nucleus, cytoplasm, perinuclear space (85–87), mitochondrial fractions and microsomal membranes (88).

As previously indicated, RGN expression diminishes with aging (49,53–55). Studies in rat liver and kidney revealed that, in these tissues, the maximum of expression for RGN is reached within

the first month after birth and is maintained up to 3 or 6.5 months, respectively, after which there is a marked decrease of its expression with aging progression (55). The age-related down-regulation of RGN expression was later corroborated in other tissues, including the prostate and the testis (75,89). Curiously, RGN tissue expression was also shown to be gender-dependent with the liver expression being lower in female rats compared to males (76,90), which first suggested that its tissue levels are modulated by the hormonal environment.

From an evolutionary perspective, RGN gene and protein are highly conserved from prokaryotes to eukaryotes, having been detected in a broad range of species, including vertebrates, invertebrates, fungi and bacteria, which supports RGN involvement in basic and important biological functions (60,91,92). Therefore, it is not surprising that altered expression of RGN has been associated with distinct pathological conditions.

RGN expression was reported to be up-regulated in human brain of Parkinson's disease patients (84,93). On the other hand, RGN was down-regulated in a muscular dystrophy mouse model (80) and in liver tissues of patients with non-alcoholic fatty liver disease (94) and acute liver failure, although RGN levels were high in the serum of the latter (82). Interestingly, RGN was also shown to be underexpressed in several cancer cell lines and tissues (60,95), which suggests the RGN role as a tumor suppressor protein. Collectively, this evidence suggests that the deregulation of RGN may play a role in the pathophysiological processes of these diseases and may be a useful biomarker for their diagnosis.

Besides the 33 kDa RGN protein, other transcripts originated by alternative splicing have been described in several tissues, including in the breast, prostate (57,96), testis (91,97) and spermatozoa (98). Maia *et al*. 2009 described two transcripts in human (non-neoplastic and neoplastic) breast and prostate tissues and cell lines, that resulted from the deletion of exon 4 (Δ4 RGN) and exon 4 and 5 (Δ4,5 RGN) of RGN mRNA, and, therefore, were originated by exon skipping mechanisms (57). The heterogeneity in the 5'-untranslated regions of human *rgn* gene had already been described in the liver (56), but these new transcripts were shown to differ in their coding region, giving origin to different proteins if translated. Namely, Δ4 RGN is thought to encode a protein with 227 amino acids and 25 kDa and Δ4,5 RGN can encode a protein with 183 amino acids and 20 kDa (57,58). The presence of these same two alternative transcripts was later described in the human testis (91), liver, kidney, brain, lung (58) and pancreas (99), although, curiously, these two transcripts have not been detected in rat and mouse tissues (58). It is not known if the proteins encoded by these transcripts have normal functions, being even speculated that they can lack the ability for Ca^{2+} binding, because the region where putative functional domain related to Ca^{2+} binding is located in RGN (52) is predicted to be deleted in these (57). However, in human normal and tumor tissues, in accordance with the results of Maia *et al.*, 2009 (57), it was verified that the expression of full length RGN and of these alternatively spliced RGN transcripts was decreased in the human tumor tissues compared with healthy ones (58). This suggested that RGN is associated with cancer development and that the

alternative splicing event of RGN has biological meaning in normal and pathologic physiology (57,58). In this regard, a study by Yamaguchi *et al.*, 2016, demonstrated *in vitro*, with human pancreatic cancer MIA PaCa-2 cells, that the full length RGN was able to suppress proliferation, cell death and migration in MIA PaCa-2 cells, but, conversely, these effects were not seen with the alternatively spliced variants Δ4 and Δ4,5 RGN (99). Nevertheless, the precise function of RGN spliced variants in normal and pathologic condition needs to be further explored.

1.2.3 Regucalcin expression in the male reproductive system

Although RGN expression has been studied in non-reproductive tissues for a long time, in the years it was determined that this protein was also shown to be present in the female and male reproductive tracts. Initially, studies showed that RGN was present in the ovary (100), breast and prostate (57,96). However, more recently the presence of RGN in the male reproductive tract was extensively studied. RGN is broadly expressed in the male reproductive system, being detected in the accessory glands, epididymis, testis, and spermatozoa, as well as also in the reproductive fluids of several mammalian species, namely *Homo sapiens* (human), *Rattus norvegicus* (rat), *Bos Taurus* (cow) and *Bubalus bubalis* (buffalo) (Table 1).

Table 1 – RGN expression in the male reproductive tissues and fluids of distinct mammalian species.

Tissue	Cell localization	Species	References
Bulbourethral Glands	Epithelium	Cow Buffalo	(97,101)
Prostate	Epithelium	Human Rat Cow Buffalo	(57,75,96,97,101)
Seminal Vesicles	Epithelium	Rat Buffalo	(75,97)
Vas Deferens	Epithelium of the ampulla	Buffalo	(97)
Epididymal Fluid	N/A	Rat	(102)
Seminal Vesicular Fluid	N/A	Buffalo	(97,103)
Seminiferous Tubule Fluid	N/A	Rat	(75)
Epididymis	Epithelium Smooth muscle Connective tissue	Rat Buffalo	(75,97,102)
Testis	Leydig Cells Sertoli Cells Spermatogonia Spermatocytes Spermatids Spermatozoa	Human Rat Cow Buffalo	(75,76,97,101)
Cauda and Ejaculated Spermatozoa	Acrosome	Buffalo	(98)

Legend: N/A indicates that no information was available for RGN cellular localization in the tissue.

The male accessory glands of mammals include the prostate, the seminal vesicles and the bulbourethral glands (also called Cowper's glands). These structures produce fluids that are essential for the motility, nourishment and protection of sperm. RGN mRNA and protein expression have been identified by means of PCR analysis and immunostaining in the male accessory glands of different species (Table 1). RGN was detected in human, rat, cow and buffalo prostate (57,75,96,97,101), in rat and buffalo seminal vesicles (75,97) and in cow and buffalo bulbourethral glands (97,101). RGN was also found in the ampulla of buffalo *vas deferens* (97), the duct that transports sperm from the epididymis to the ejaculatory ducts previous to ejaculation. RGN immunoreactivity in the majority of these tissues is mainly confined to the cytoplasm and to some nucleus of the glandular epithelial cells (57,75,96,97,101). Curiously, in bovine bulbourethral glands and in the *vas deferens*, contrary to other tissues, RGN was primarily found in the nuclei (97,101).

RGN had already been identified as a secreted protein in different fluids, namely in pea aphid saliva (81), murine (82,104), rat (105,106) and human plasma (82), but currently RGN protein expression also has been reported in the rat SeT and epididymal fluids (75,102), as well as in buffalo seminal vesicular fluid (97,103).

The presence of RGN in epididymal and seminal vesicular fluids probably results from the secretory activity of epididymis and seminal vesicular epithelial cells (97,102,103). The RGN present in SeT fluid may be a secretion product of SCs (75), considering that this fluid is essentially produced due to the secretory activity of these somatic cells (107).

The SeT fluid is fundamental for germ cell development and maturation (107), meanwhile epididymal fluid is necessary for sperm maturation and storage (12,108), and seminal vesicular fluid, constituting the major part of seminal plasma, is essential for spermatozoa function and survival (109). Therefore, RGN localization in reproductive fluids points out a possible action of this protein in sperm physiology, which is likely to be associated with RGN Ca^{2+}-binding function, as this ion appears to be crucial for mammalian spermatogenesis and sperm function (43–46). In fact, Ca^{2+} modulation is involved in sperm capacitation and acrosome reaction, as Ca^{2+}-adenosine 5'-triphosphatease (ATPase) function is inhibited during capacitation, leading to an increase of acrosomal Ca^{2+} that results in the triggering of acrosomal exocytosis (43). However, so far only a probable role of RGN in seminal vesicles fluid as a suppressor of premature sperm capacitation was suggested (97). Other roles of RGN in reproductive fluids may be associated with suppression of apoptosis and oxidative stress (102,110,111), but these, although interesting, remain to be determined.

As a strategy to investigate the potential role of RGN in spermatogenesis and sperm function, RGN expression has been analyzed also in the testis, epididymis and spermatozoa of several mammalian species (Table 1). RGN was detected in human, rat, cow and buffalo testis (75,76,97,101), and in rat and buffalo epididymis (75,97) and spermatozoa (75,98).

In the testis, within the SeT, RGN mRNA and protein was identified in human, rat and buffalo SCs, as well as in all development stages of germ cells, namely spermatogonia, spermatocytes and spermatids, and spermatozoa with all cells displaying strong positivity for RGN in the cytoplasm but weak in the nucleus (75,97). In the case of cow testis, RGN protein was identified only in spermatogonia (101). Outside the tubules, human, rat, cow and buffalo LCs were positive for RGN expression, although expression appears to be weaker in the buffalo (75,97,101).

Hence, the RGN protein is widely expressed in the testis, being detected in both somatic cells and germ cells from different species (Table 1). The common interspecies localization of RGN found in the testis suggests that it may be a key player in testicular physiology. Moreover, localization in SC corroborates that RGN protein presence in SeT fluid may be a secretion product from these cells (75).

It should also be mentioned that, in concordance with the characteristic downregulation of RGN expression during aging in the liver and kidney (55), RGN mRNA expression in the rat prostate and testis also decreases with aging. In the prostate, RGN expression reached a peak in young rats with 3 months, maintaining a plateau until the age of 6 months, after which RGN expression decreased (89). Similarly, in the rat testis, RGN mRNA expression reached a maximum expression level at 120 days, which corresponds to rat adulthood, and decreased afterwards with rat aging (75).

In the epididymis of rat and buffalo, RGN was mainly detected in epithelial cells, which suggests this protein is secreted (75,97), and thus, may explain its presence in the epidydimal fluid (102). However, RGN also was localized in smooth muscle cells and connective tissue of the epididymis (75,97,102). In the buffalo epididymis, the caput region contained RGN in greater abundance than the corpus or cauda regions (97), but in rat epididymis, the corpus was the region with the highest levels of RGN protein (102). Immature spermatozoa are transformed into mature spermatozoa through the sequential passage and interaction with the environment created by the epididymis epithelium. Thus, the regional differences found in the different segments of epididymis are essential for the establishment of the required environment for sperm maturation. Gene expression analysis and the identification of targets that differ by at least 2-fold variation between adjacent segments of the epididymis was proposed as a way to emphasize their relative importance in the function of the distinct epididymis regions (112). The identification of RGN as a protein expressed in a region-specific manner along the epididymis emphasizes its likely importance in sperm maturation (102).

Overall, RGN cell localization is essentially cytoplasmic, but nuclear staining is also apparent, which supports that RGN is able to translocate to the nucleus to regulate DNA, RNA synthesis and gene expression (113–116) as it has been suggested.

Regarding the localization of RGN in sperm cells, the available studies are in buffalo, with RGN being found in the cytoplasm and acrosome of testicular spermatozoa, whereas in caudal and ejaculated spermatozoa RGN was specifically localized in the acrosomal region (98). This suggests that as sperm advances towards maturation, RGN relocates from the cytosol to the acrosomal region (98), which is a potent Ca^{2+}-storing organelle of spermatozoa (45,117). However, it is still necessary to verify if RGN's presence in spermatozoa is of endogenous origin only, or if an exogenous origin can also be involved. RGN presence in the secretions of male reproductive tract (75,97,102,103) may indicate its translocation from these fluids to spermatozoa, which would be in agreement with previous reports that show the translocation of exogenous RGN to different sites, namely the nucleus of osteoblasts (118) and liver cells (87,119).

1.3 Regucalcin functions and roles

1.3.1 Calcium homeostasis

Ca^{2+} is an ubiquitous second messenger involved in the activation of diverse signaling cascades, regulating a wide range of biological functions including transcriptional activation, cell cycle control, cell proliferation, migration and death (120–122). The maintenance of low cytosolic concentrations of Ca^{2+} is fundamental for proper cell signaling, since prolonged cytoplasmic elevation of free Ca^{2+} is toxic and triggers cell death (122). Thus, Ca^{2+} homeostasis is essential in normal physiology and its deregulation is associated with pathophysiologic conditions (123). Under normal circumstances, Ca^{2+} cytoplasmic levels oscillate between a resting (~100 nM) and an activated state (~500nM – 1µM). These are achieved by the conjoined effort of three classes of membrane transport proteins, namely Ca^{2+}-channels, -exchangers and -pumps. These proteins handle the transport of Ca^{2+} across membranes, in and out of the cell or to the intracellular storages organelles, namely the sarco/endoplasmic reticulum and mitochondria (microsomes), being essential for intracellular Ca^{2+} homeostasis (37,123).

The RGN most well-established function is as a regulator of Ca^{2+} homeostasis through the enhancement of Ca^{2+} pumping activity at the plasma membrane, sarco/endoplasmic reticulum and mitochondria of several cell types (Figure 3). Ca^{2+} pumps remove Ca^{2+} to the extracellular space or accumulate it inside of intracellular organelles, being able to transport Ca^{2+}, at the expense of energy, against the transmembrane electrochemical gradient driving the entry of this ion into cells (124).

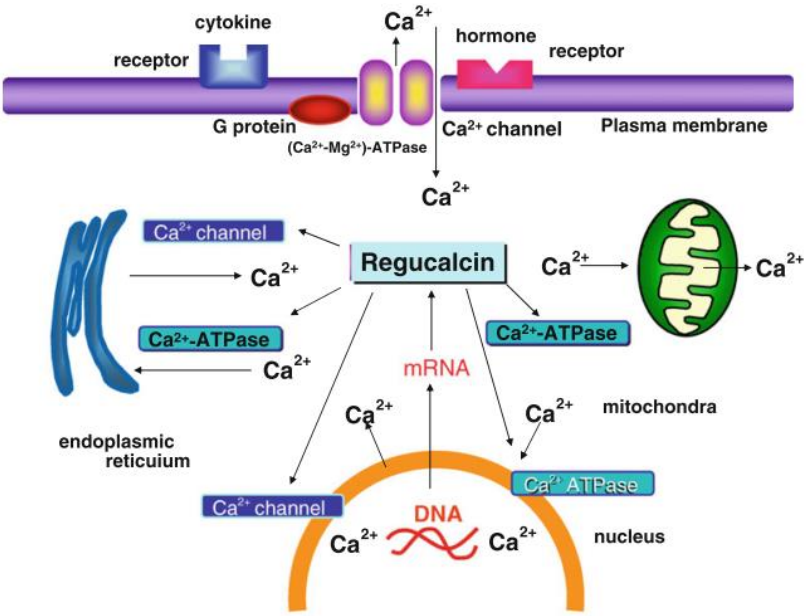

Figure 3 - RGN role as a regulator of several Ca^{2+}-handling proteins involved in the maintenance of Ca^{2+} homeostasis. RGN increases plasma membrane Ca^{2+}-Mg^{2}-ATPase and microsomal Ca^{2+}-ATPase activities in cells. Conversely, RGN decreases nuclear Ca^{2+}-ATPase activity. Moreover, RGN was shown to promote Ca^{2+} release from the nucleus and microsomes, which implies that it is able to modulate Ca^{2+} channel activity (125).

In rat liver, RGN was shown to regulate the expression and activity of Ca^{2+}-ATPase on the membranes of microsomes (126,127) and of Ca^{2+}-Mg^{2+}-ATPase (128,129) at the plasma membrane (Figure 3) of several tissues, namely the liver, kidney, brain and heart (125). The addition of RGN to rat liver microsomes significantly increased Ca^{2+}-ATPase activity (126,127), an effect that was inhibited by N-ethylmaleimide (NEM), a modifying reagent of sulfhydryl groups, or by digitonin, a solubilizing reagent of membrane lipids (127). These results suggest that the regulation of Ca^{2+}-ATPase activity by RGN may involve the regulation of ATPase sulfhydryl groups and direct binding to the microsomal membrane (127). Similar results were obtained for the Ca^{2+}-Mg^{2+}-ATPase in the plasma membrane (128,129), confirming that RGN is also capable of activating plasma membrane Ca^{2+}-Mg^{2+}-ATPase through the same mechanism. Some reports also suggest that RGN action regulating ATPases is Ca^{2+}-dependent and involves CaM (60).

Besides Ca^{2+} uptake into the mitochondria promoted by activation of Ca^{2+}-ATPase activity, RGN was also proposed to stimulate Ca^{2+} uptake through the regulation of the mitochondrial Ca^{2+} uniporter, since the addition of ruthenium red or lanthanum chloride (both inhibitors of the mitochondria Ca^{2+} uniporter) markedly inhibited Ca^{2+} uptake into the mitochondria of rat liver (130) and kidney cortex (131).

Interestingly, RGN had a contrasting effect in the nuclear outer membrane ATPases of microsomes (Figure 3). Nuclear Ca^{2+}-ATPase activity was significantly reduced by the addition of RGN in rat liver, but the presence of an anti-RGN monoclonal antibody caused a significant

elevation of this pump activity (132). Also, accumulated Ca^{2+} in the nuclei was significantly released by the addition of RGN in a dose-dependent manner, which seems to imply that RGN promotes nuclear Ca^{2+} release (133). Furthermore, although RGN activates microsomal Ca^{2+}-ATPase, other studies revealed that RGN also stimulates Ca^{2+} release from rat liver microsomes (134). These results imply that RGN influences Ca^{2+} channels, since these are responsible for Ca^{2+} release, while ATPase promotes Ca^{2+} uptake. Presumably, RGN may stimulate microsomal Ca^{2+} uptake when cytosolic Ca^{2+} concentration is raised but promote Ca^{2+} release to regulate Ca^{2+}-related microsomal functions (125).

Besides the effects of RGN in Ca^{2+} pumps, there is also evidence that this protein is able to modulate the extracellular Ca^{2+}-sensing receptor (CaSR) (73,86) and L-Type Ca^{2+} channels (86), which may represent another mechanism for RGN intervention in Ca^{2+} homeostasis. RGN overexpression in normal rat kidney proximal tubular epithelial NRK52E cells lead to a remarkable decrease in CaSR and L-type Ca^{2+} channel mRNA expression (86). Recently, it was also shown that the prostate of transgenic rats overexpressing RGN (Tg-RGN) exhibits low levels of CaSR, and that RGN-knockdown in prostate epithelial PNT1A cells increases the CaSR expression, which suggests that RGN has a role in suppressing the signaling pathways triggered by CaSR (73).

In sum, RGN is able to lower intracellular cytoplasmic Ca^{2+} levels by stimulation or inhibition of Ca^{2+} handling proteins (Figure 3), which may be related with its inhibitory role in Ca^{2+} signaling (124,135).

1.3.2 Cytoprotective effects: as a gluconolactonase and antioxidant protein

Several lines of evidence have been suggesting the protective actions of RGN against cell damage. One of the mechanisms identified to contribute to the cytoprotective effect of RGN is its ability to reduce the cell oxidative stress levels.

RGN was shown to have an enzymatic function as a gluconolactonase in rats and mice, having an essential role in the penultimate step of L-ascorbic acid biosynthesis in the liver, namely by catalyzing the lactonization of L-gulonic acid (136). L-ascorbic acid, also known as vitamin C, is a well-known antioxidant that scavenges free radicals and other reactive oxygen species (ROS) resultant from cell metabolism, being a potent agent against oxidative stress. Almost all animals have the ability to synthesize L-ascorbic acid, but due to evolutionary genetic mutations affecting the enzyme required for the last step of biosynthesis, humans, non-human primates and guinea pigs have lost this capability, needing to obtain vitamin C through diet (137). The establishment of an RGN-knockout model allowed the generation of mice that were shown to lose the ability to synthesize L-ascorbic acid. These RGN knockout mice developed scurvy when fed with a vitamin C-deficient diet (136) and were shown to be more susceptible

to oxidative stress (138,139), which confirms RGN gluconolactonase activity and antioxidant effect.

In addition, other antioxidant properties of RGN that are not associated with L-ascorbic acid biosynthesis have been reported, namely its capability to influence the activity of enzymes involved in the generation of oxidative stress and antioxidant defense, therefore being able to maintain physiological levels of oxidative stress and prevent oxidative damage (140–142). RGN overexpression in mouse embryonic carcinoma P19 cells (140) and in SeT of rats (110) was shown to exert a protective effect against *tert*-butylhydroperoxide induced oxidative stress, increasing cell viability. Furthermore, in human hepatic carcinoma HepG2 cells transfected with RGN (142), diminished ROS levels were detected both in mitochondria and post-mitochondrial fractions, as well as reduced lipid peroxidation, suggesting that RGN suppresses the generation of ROS. On the other hand, in the liver (141) and heart cytosol of normal rats (143), the addition of exogenous RGN caused a significant increase in the activity of the antioxidant enzyme superoxide dismutase (SOD), being proposed that RGN directly activates this enzyme. The activity of glutathione, another antioxidant enzyme involved in oxidative stress regulation, as well as general antioxidant capacity were also found to be significantly increased in the prostate of Tg-RGN rats (89). Lastly, RGN was shown to exert a suppressive effect over the activity of nitric oxide synthase (NOS), the enzyme responsible for the free radical nitric oxide production, in several rat tissues and cell lines (144,145).

Overall, available studies have shown that due to suppression of ROS production and increase of the activity of antioxidant enzymes, RGN is able to counteract oxidative stress and, consequently, reduce cell death, in several tissues, including the liver (141), heart (143), lung (138), prostate (89), testis (110) and epididymis (102).

It is also established that RGN has a protective role against the development of age-related pathologies by preventing changes in oxidative stress (89,138). This was shown by the increase in the activity of glutathione and the antioxidant capacity in Tg-RGN rats prostate, which occurred in response to the age-associated increase in oxidative stress (89). However, although the decreased levels of RGN observed in older individuals are correlated with the increased levels of ROS formed with aging progression, it is not yet clear if RGN down-regulation with aging is the cause or the consequence of higher ROS levels, considering that oxidative stress is also a regulator of RGN expression.

1.3.3 Regulation of calcium-dependent intracellular signaling pathways

Beyond the ability to modulate Ca^{2+} cytosolic levels, RGN also plays a role in the regulation of intracellular signaling pathways and cell metabolism by modulating the activity of Ca^{2+}-dependent enzymes, such as cyclic adenosine monophosphate (cAMP) phosphodiesterase (83,146), kinases and phosphatases (125), glycogen phosphorylase a (147), fructose 1,6-

diphosphatase (148), glucose-6-phosphatase (149) and mitochondrial succinate dehydrogenase (150).

cAMP, along with Ca^{2+}, is an ubiquitous second messenger essential to the control of cellular homeostasis (151). The levels of cAMP are regulated by the balance between the activity of adenylyl cyclase's, responsible for cAMP synthesis, and cAMP phosphodiesterase's, which degrade cAMP (151,152). Both molecules are activated by Ca^{2+}/CaM signaling, but the addition of RGN was shown to inhibit Ca^{2+}/CaM-dependent activation of cAMP phosphodiesterase's in rat liver (83) and kidney (146), which was suggested to involve Ca^{2+} binding. Thus, RGN has a role in regulating cAMP levels.

The majority of enzymes regulated by RGN are protein kinases and phosphatases, which in turn regulate the activity of other proteins, allowing RGN to exert an indirect effect in diverse signaling pathways (125). One of the most studied protein kinases which is affected by RGN is PKC. RGN was found to significantly decrease Ca^{2+} or phospholipid-stimulated cytosolic PKC activity in rat liver cytosol and in the nucleus (153,154). Similar results were found in rat kidney cortex, where RGN also inhibited this enzyme by preventing its activation through Ca^{2+}/CaM (155). Regarding phosphatases, RGN was shown to significantly reduce cytosolic and nuclear phosphatase activities of CaM-dependent serine/threonine phosphatase, such as calcineurin (125). It was found that phosphotyrosine, phosphoserine and phosphothreonine activities were suppressed by RGN addition to the reaction mixture in rat liver and kidney cortex (156,157).

Other enzymes modulated by RGN include NOS, which as previously mentioned is inhibited by RGN resulting in decreased oxidative stress (144,145). It also includes calpains, a family of Ca^{2+}-dependent activated neutral cysteine proteases involved in several cell processes, such as cell cycle progression, death, proliferation, differentiation, migration, meiosis and mitosis, and related to numerous diseases, that are modulated by RGN (158). On the contrary to other enzymes, calpains proteolytic activity is enhanced by RGN and not inhibited, in a mechanism that seems to be associated with sulfhydryl groups of cysteinyl-proteases and Ca^{2+} independent (159,160).

Also, RGN has an inhibitory effect on enzymes involved in metabolic pathways, such as glycolysis, glycogenolysis, gluconeogenesis and oxidative phosphorylation, reverting the increase of enzymatic activity promoted by Ca^{2+}/CaM. It was found that the activities of rat liver pyruvate kinase (161), and of rat prostate lactate dehydrogenase and phosphofructokinase 1 (162) (all enzymes involved in glycolysis), were diminished by RGN overexpression, which demonstrates the overall capacity of RGN in the suppression of the glycolytic metabolism. Ca^{2+}-induced activity of glycogen phosphorylase a, an enzyme that catalyzes a rate-limiting step in glycogenolysis (147), was also reverted by RGN action. Furthermore, fructose 1,6-diphosphatase (148) and microsomal glucose-6-phosphatase enzyme activity (149) (both enzymes catalyze gluconeogenesis and are activated by Ca^{2+}), was diminished by the addition

of RGN. At the metabolic level, RGN also demonstrated the regulation of mitochondrial succinate dehydrogenase, an enzyme involved in oxidative phosphorylation participating both in the citric acid cycle and the electron transport chain (163). This enzyme activity is increased by Ca^{2+}, but RGN presence induces its decrease, having an indirect effect on cell energy production (150).

Altogether, the available data indicate that RGN has diverse regulatory roles on enzyme activity, exerting its inhibitory effects mainly through direct action on the regulation of Ca^{2+} or CaM, which permits the regulation of signaling and metabolic pathways by this protein.

1.3.4 Cell proliferation and apoptosis

Another relevant function of RGN is its capability of influencing cell survival and death, displaying both anti-apoptotic and anti-proliferative effects (60,95,164,165). Considering the aforementioned actions of RGN, it is not surprising that this protein has the ability to influence cell proliferation and apoptosis. However, the molecular mechanisms underlying the RGN control of cell fate are still not fully understood, though they have been linked to the control of DNA synthesis and fragmentation, as well as with the modulation of the expression of oncogenes, tumor suppressor genes and cell cycle regulators (95,113,166–168).

RGN control of proliferation and apoptosis seems to occur through the modulation of gene expression and the activity of proteins involved in these processes (60,95,164,165,169). As a regulator of intracellular Ca^{2+} levels RGN modulates Ca^{2+}, which is involved as a second messenger in several transduction pathways (37,170), and several Ca^{2+}-dependent enzymes, which in turn can regulate the activity of phospho-proteins and, consequently, protein phosphorylation/ dephosphorylation processes (171). Studies show that RGN has a suppressive effect on the activity of proliferation and apoptosis modulators, such as protein kinases (153,172) and phosphatases (119,173,174), cAMP phosphodiesterase (83,146), NOS (144,145,175) and Ca^{2+}-dependent endonuclease (176).

Studies showed that the exogenous administration or overexpression of endogenous RGN had anti-proliferative action in several tissues and cell lines, namely by decreasing the proliferation index in human pancreatic cancer MIA PaCa-2 cells (99,177) and Pt45P1 cells (177), in breast cancer MDA-MB-231 bone metastatic cells (178), in mouse embryonic carcinoma P19 cells (140), in rat hepatoma H4-II-E cells (167), kidney NRK52E cells (179) and prostate cells (180). Contrastingly, overexpression of RGN in rat cardiomyocytes (181), NRK52E (182), HepG2 (168) and H4-II-E cells (183–185), and in mouse P19 cells (140) lead to decreased cell death.

The RGN anti-proliferative effects may be caused by its suppressive effect on various signaling pathways active in proliferating cells, from the cytoplasm to the nucleus (60,125,186). In addition, the intracellular increase of RGN down-regulated the mRNA expression of proto-oncogenes such as c-myc and H-ras, while up-regulating tumor suppressor genes p53 and p21,

which suggests that RGN suppresses cell proliferation by modulation of these regulators (60,180). RGN's ability to translocate to the nucleus (57,75,87,97) leads to its capability of inhibiting RNA (115) and DNA synthesis (113,114), which is associated with the control of cell proliferation (60,186).

On the other hand, RGN anti-apoptotic actions may be due to inhibition of DNA fragmentation (176), to RGN down-regulation of caspase-3 expression and activity, and up-regulation of Bcl-2 and Akt-1 expression, which are anti-apoptotic. Thus, RGN may decrease apoptosis in a mechanism likely to include the activation of the Akt survival pathway (111,180,182). Moreover, the RGN anti-apoptotic effect is also proposed to include the inhibition of the death-inducing Fas pathway, since the livers of RGN-knockout mice are highly susceptible to tumor necrosis factor (TNF-α) and Fas-mediated apoptosis, with caspase-8 activity being two-fold increased comparatively to wild-type (Wt) animals (169). RGN knockout mice also presented enhanced liver injury in response to administration of anti-Fas antibody, while $RGN^{+/-}$ mice had an intermediate susceptibility (169). Another possible mechanism involved in RGN anti-apoptotic action is the TGF-ß pathway. The knockout mice for *Smad3* (a key downstream mediator of TGF-ß), with interrupted TGF-ß signaling, showed significantly increased RGN levels in the liver concomitant with increased resistance to radiation-induced apoptosis (187).

Altogether, these findings suggest that RGN can modulate cell survival and death pathways, having opposite functions as a suppressor of both cell proliferation and apoptosis. The general mechanisms of action were systematically review by Marques *et al.*, 2013 (60) (Figure 4).

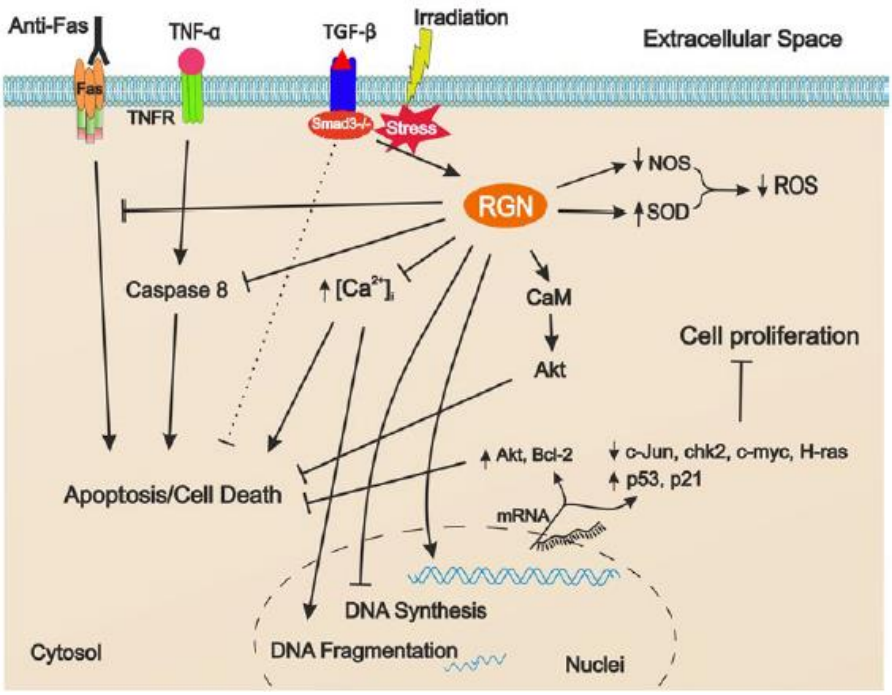

Figure 4 - Schematic representation of the mechanisms involved in RGN modulation of cell proliferation and apoptosis. *Arrows* represent activation and *bar-headed lines* indicate inhibition. *Dashed bar-headed line* represents the inhibition of apoptosis in *Smad3* knockout animals, concomitantly with increased RGN levels. RGN increases the expression of p53 and p21 proteins while suppressing the expression of c-Jun, chk2, c-myc and H-ras genes, thus blocking cell proliferation. In turn, RGN decreases the production of ROS, blocks increase of intracellular Ca^{2+}, inhibits caspase-8 activity, enhances activity of apoptosis inhibitors Akt-1 and Bcl-2 and blocks the Fas system, leading to inhibition of apoptosis. TNF-α, tumor necrosis factor; TNFR, TNF-α receptor; TGF-ß, transforming growth factor ß; NOS, nitric oxide synthase; SOD, superoxide dismutase; ROS, reactive oxygen species; CaM, calmodulin (60).

1.3.5 Tumor suppressor protein

In line with its multifunctional role regulating Ca^{2+} homeostasis, oxidative stress, intracellular signaling, metabolism, cell proliferation and apoptosis, RGN has recently emerged as a tumor suppressor protein. The continuous increase of oxidative stress, the aberrant capacity to proliferate, evasion from apoptosis and the reprogramming of energy metabolism are all processes that promote tumorigenesis and are identified as the hallmarks of cancer (95). Therefore, RGN protective role against these processes may be indicative of RGN having biological significance in tumor suppression. The ability of RGN as a modulator of the expression of oncogenes, tumor suppressor genes and cell cycle regulators is also telling, supporting the relevance of RGN in cancer (95,166,167,188).

Age is a major risk factor for the development of the majority of neoplastic diseases. As RGN expression is demonstrated to decrease with age (49,53–55), the loss of its cytoprotective effects are likely to contribute to the deterioration of cell function, favoring the onset and progression of tumorigenesis (95). RGN is expected to prevent the characteristic uncontrolled cell proliferation of tumor cells, as well as avoid cell escape to malignant transformation by reducing the effects of noxious stimuli (95). Accordingly, RGN overexpression protected from carcinogen-induced mammary gland tumor development and was linked with reduced

proliferation and increased apoptosis in this tissue (188). Furthermore, RGN was shown to prevent the hyper-glycolytic metabolism of prostate cells, having a protective role against the development of cancer metabolic phenotype (162).

Altered expression of RGN was found in several animal and human cancer cell lines and tissues, namely the liver, kidney, brain, lung, breast and prostate (reviewed in (95)). In these neoplastic tissues, RGN expression was diminished, which corroborates this protein role as a suppressor of tumorigenesis, as RGN loss appears to be necessary for cancer progression. Noteworthy, diminished expression of RGN was associated with histological grade of infiltrating ductal carcinoma of breast (57,188) and with cellular differentiation of prostate adenocarcinoma (57), pointing to the potential use of RGN as a diagnosis/prognosis biomarker. The use of RGN as a biomarker had previously also been shown in cases of liver failure, where RGN elevated plasma levels have prognostic value (82). However, there is still need of confirmation on whether RGN down-regulation is one of the causes contributing for cancer onset, or a consequence of the cancer status itself.

1.4 Regucalcin in male reproduction

Among the pleiotropic effects of RGN its role in spermatogenesis and sperm maturation has also been suggested (91,102). RGN is broadly expressed in the male reproductive system (Table 1), and its tissue expression levels are regulated by sex steroid hormones with RGN being indicated as an androgen-target gene in the testis and prostate (75,180). These facts, along with the RGN established functions, lead our research group and others to study the mechanisms that link RGN with male reproduction. RGN actions in testicular cells have been linked with Ca^{2+} homeostasis (91), suppression of apoptosis in response to injury (111,189) and maintenance of antioxidant balance (91,102,110).

Ca^{2+} homeostasis is crucial for mammalian spermatogenesis and sperm function (43–46), being necessary for maintenance of LC steroidogenesis (190,191), SC function (192,193), tight-junctions structure (194) and remodeling (195), as well as in the regulation of sperm motility, hyperactivation (196,197), chemotaxis (198), capacitation (199,200) and acrosome reaction (201–205), leading to sperm maturation and fertilization. Thus, RGN action as a regulator of Ca^{2+} homeostasis through the enhancement of Ca^{2+} pumping activity in the plasma and microsome membrane (124) and its presence in spermatozoa (98), suggest its involvement, not only in spermatogenesis, but also in sperm maturation in the epididymis (102) and in other pre-fertilization Ca^{2+}-dependent events (97,98). The identification of RGN in the acrosomal region of mammalian spermatozoa sustains the hypothesis that RGN is involved in pre-fertilization events being fundamental to the fertilizing potential of spermatozoa (98).

Considering that the success of spermatogenesis depends on the tight balance between germ cell survival and death (13), the RGN dual function as anti-proliferative and anti-apoptotic

factor in combination with its presence in all testicular cell types (75), leads to the high probability of this protein' involvement in proliferation/apoptosis control in the testis. Moreover, RGN is androgen-responsive and androgens are the well-known regulators of germ cell survival (206–208). Therefore, it is likely that RGN takes part in the mechanisms by which androgens regulate testicular cell death, favoring cell survival (91).

The importance of RGN for the spermatogenic output was investigated in testicular biopsies of infertile men with abnormal spermatogenesis phenotypes (91). RGN expression was significantly increased in the testis of individuals with hypospermatogenesis, a condition resulting from deregulation of germ cell proliferation and apoptosis (91). This finding allowed to theorize that RGN augmented expression can be blocking cell proliferation, or, conversely, be a physiological response trying to suppress apoptosis induced by other factors.

Studies in the Tg-RGN model confirmed that RGN overexpression is able to exert anti-proliferative and anti-apoptotic effects in the rat male reproductive tract. Overexpression of RGN lead to a decreased proliferation index in the prostate tissues of 3 month old rats, while increasing the Bcl-2 (antiapoptotic)/Bax (proapoptotic) protein ratio (209), and decreasing caspase-3 activity (180). In Tg-RGN rats testis, RGN overexpression lead to the suppression of thapsigargin- and actinomycin D-induced apoptosis in SeT, also accompanied by the augmented Bcl-2/Bax protein ratio, and diminished caspase-3 activity (111). Moreover, these results were in agreement with those described in the prostate of Tg-RGN rats (180), which strongly implies RGN as a suppressor of apoptosis in response to injury.

Besides its protective effect against chemical-induced apoptosis, RGN also exerted a protective effect against radiation-induced testicular cell damage. The testicular status and the epididymal sperm parameters of Tg-RGN rats were evaluated after exposure to radiation comparatively with the Wt counterparts (189). Tg-RGN rats displayed higher gonodosomatic index, sperm viability, sperm motility and diminished incidence of head-defects following irradiation and contrastingly with the more severe effects found in Wt animals (189). The responses in the testis of Tg-RGN rats were accompanied by a lower rate of apoptosis, as evidenced by the decreased activity of caspase-3, lower levels of caspase-8 and increased Bcl-2/Bax ratio (189). Interestingly, RGN expression was augmented after irradiation in both Tg-RGN and Wt rats, which suggests that its expression increases in response to cell damage (189). This result is in agreement with a previous study demonstrating that the generation of radioresistant cell lines using fractionated irradiation is accompanied by a concomitant overexpression of RGN (210).

It has also been proven that RGN has antioxidant effects, being a suppressor of oxidative stress (110,139–142). Oxidative stress is the main environmental factor that causes DNA damage (211,212) and apoptosis of germ cells (213), leading to defective spermatogenesis and interfering with sperm function (214). Furthermore, spermatozoa are particularly susceptible

to oxidative stress due to the requirement of energy by the sperm-motility apparatus, that demands a high level of respiratory activity, leading to ROS production (215), and because their plasma membranes consist of polyunsaturated fatty acids, prone to oxidative injury (216).

In the SeT of Tg-RGN rats, overall antioxidant capacity was naturally higher than in Wt control, while thiobarbituric acid reactive substances were diminished, which indicates decreased lipid peroxidation (110). This effect might be derived from RGN regulation of Ca^{2+} concentration, seeing as Ca^{2+} deregulation is associated with generation of oxidative stress (217). However, RGN action as gluconolactonase is also noteworthy, because it means that RGN can influence L-ascorbic acid biosynthesis in rats (136), which is a powerful antioxidant present in the testis (137,218), with a role in cell survival and death balance (219–221). In humans, ascorbic acid is present in sperm, acting against oxidative stress damage, but it comes from dietary sources upon ingestion (222). In the presence of *tert*-butylhydroperoxide and cadmium chloride, both pro-oxidant stimuli, the overexpression of RGN resulted in increased glutathione s-transferase activity and decreased caspase-3 activity, what illustrates a higher antioxidant defense and resistance to chemical-induced apoptosis in Tg-RGN animals (110). Moreover, Ca^{2+} levels were higher in the SeT fluid of Tg-RGN rats, supporting the idea that lowers levels of oxidative stress also result from lower intracellular Ca^{2+} concentrations (110).

Considering the supportive evidence of RGN cytoprotective action in the testis, due to its anti-proliferative, anti-apoptotic or anti-oxidative properties, it would be expected that the reproductive phenotype of the RGN-knockout and knockin models would be altered. However, although RGN knockout animals are more susceptible to apoptosis (169,223), display higher levels of oxidative stress (139) and have shorter lifespans (224) than their Wt counterparts, they appear to have normal fertilization capability (169). Nevertheless, a thorough study of the spermatogenic status of these animals has not been done yet, and the existence of mild or later age alterations in spermatogenesis might have not been accounted for. In the case of knockin animals (Tg-RGN) their reproductive phenotype was investigated. Tg-RGN rats seem to be fertile and breed normally (173), but they display an altered morphology of epididymis, as well as lower sperm counts and motility, which may be consequence of the increased Ca^{2+} concentrations found in the epididymal lumen. However, this apparently subfertility phenotype is countered by the higher sperm viability and frequency of normal sperm, with a diminished incidence of tail defects, that may be explained by lower oxidative stress levels in these animals (102). The altered morphology of the epididymis and higher concentrations of Ca^{2+} in the epididymal lumen observed in Tg-RGN rats suggests a role for RGN on Ca^{2+} homeostasis during epididymal sperm maturation (102).

Recently, RGN was also proposed to act on sperm capacitation (97). Capacitation is the process that occurs in the female reproductive tract prior to acrosome reaction, that enables spermatozoa to acquire fertilizing ability (225). It depends on the progressive activation of a cAMP-protein kinase A-dependent signaling pathway mediating protein tyrosine

phosphorylation, that has Ca^{2+} as a modulator (199). Furthermore, it was also demonstrated that Ca^{2+}-ATPases play an important role in this process (226). RGN was recognized as an anti-capacitatory component of buffalo seminal vesicular fluid, that is later degraded in the seminal plasma of ejaculated semen (97). Thus, RGN appears to prevent premature sperm capacitation until it is depleted by ejaculation, which may involve Ca^{2+} homeostasis and intracellular signaling mechanisms. This is also of relevant and practical interest in assisted reproductive techniques to achieve the on-time sperm capacitation improving success outcomes.

Also, and following the rationale of the RGN cytoprotective functions, the effect of this protein on sperm cryopreservation was investigated. Although sperm cryopreservation has come a long way over the years, this technique still causes undesirable damage to spermatozoa by exposing them to physical and chemical factors that induce apoptosis and reduce spermatozoa fertilization rate (227). In cryopreservation, two of the main factors that contribute to poor fertility are an elevated intracellular Ca^{2+} concentration (228,229) and a diminished antioxidant activity that makes spermatozoa more susceptible to ROS damage (230–232). Furthermore, it is possible that cryopreservation damages sperm DNA integrity by causing DNA fragmentation (227). Thus, in an effort to overcome these problems, the use of sperm extenders containing cryoprotectants has become a standard in cryopreservation. In this regard, RGN has emerged as a possible cryoprotectant of spermatozoa, that can exert Ca^{2+}-regulating and antioxidant effects (233). Recently, Harikrishna *et al*. 2017 (233), concluded that the use of recombinant RGN (1 μM) in a sperm extender containing Tris, citric acid, egg yolk and glycerol, improved the general performance and overall characteristics of the post-thaw fertility of buffalo spermatozoa (233). In this study, three different concentrations of recombinant RGN (20, 40 and 60 μg/ml) were used and post-thaw progressive motility, acrosome integrity and zona-pellucida binding of spermatozoa metrics were analyzed. Post-thaw progressive motility was significantly increased with the supplementing recombinant RGN in semen extender at 40 μg/ml concentration, but was decreased at 60 μg/ml concentration (233). This is in accordance with the reduced motility of epididymal spermatozoa in Tg-RGN rats (102), and could be due to an inhibitory, concentration-dependent effect of the protein on sperm motility or on physical/biochemical disruption. The acrosome integrity and number of zona-pellucida bind spermatozoa were significantly increased in the presence of recombinant RGN in semen extenders at 40 and 60 μg/ml concentrations (233). Thus, overall RGN appears to have a cryoprotective effect in buffalo spermatozoa at 40 μg/ml, that may be due to the Ca^{2+} binding and/or regulating activity of RGN, and to its positive impact in antioxidant activity (233).

Gathering together the available information, it can be accepted that RGN has a role in spermatogenesis and sperm function, emerging as a protector of germ cells against noxious stimuli, and possibly improving sperm maturation, capacitation and cryopreservation. Overall, the cytoprotective role of RGN may be explored as a useful tool in the treatment of male infertility.

2. Aim of the study

RGN is a highly-conserved Ca^{2+}-binding protein whose primary function is the maintenance of intracellular Ca^{2+} homeostasis and regulation of Ca^{2+}-dependent enzymes. However, further research over the years has shown that RGN also has important antioxidant, anti-apoptotic and anti-proliferative effects, being associated with a likely cytoprotective role.

The identification of RGN as an androgen-target gene broadly expressed in all testicular cell types was the first link of this protein with male reproduction, which was further detailed by additional research performed by our research group in the last years. Making use of the Tg-RGN rat model it was shown that RGN can be associated with increased sperm viability with lower incidence of tail defects, as well as with resistance to oxidative stress, and chemical- or radiation-induced apoptosis, which supports its involvement in the spermatogenic process, protecting cells against noxious stimuli.

However, the signaling pathways and the molecular players underlying the RGN actions in spermatogenesis and male fertility remain to be clarified. The present study aims to characterize the testicular transcriptome of Tg-RGN rats comparatively with their Wt counterparts to identify the potential molecular partners of RGN in the spermatogenic process and clarify the mechanisms underlying the cytoprotective actions of RGN with impact in (in)fertility.

For this purpose, an RNA sequencing (RNA-seq) approach analyzed the testicular transcriptome of Tg-RGN rats comparatively with their Wt counterparts and differentially expressed genes were clustered according to their expression pattern, gene ontology and pathway enrichment analysis. Results were further validated through real-time quantitative polymerase chain reaction (qPCR) analysis.

3. Materials and Methods

3.1 Animals

Three-months old Wt and Tg-RGN Sprague Dawley (*R. norvegicus*) rats were obtained from Charles River (Barcelona, Spain) and Japan SLC (Hamamatsu, Japan), respectively. The Tg-RGN rats were originally generated by M. Yamaguchi by means of oocyte transgene pronuclear injection with a complementary deoxyribonucleic acid (cDNA) construct encoding rat RGN (173).

Animals were handled in compliance with the US National Institutes of Health (NIH) guidelines, established in the "Guide for the Care and Use of Laboratory Animals", and the Portuguese legislation (DL 113/2013) and European Union rules for the care and handling of laboratory animals (Directive number 86\609\EU). They were housed under a 12 h light:12 h darkness artificial lighting cycle, at a constant temperature of 20 ± 2ºC, and with food and water available *ad libitum*.

Wt and Tg-RGN rats (n=5, for each group) were euthanized under anesthesia (Clorketam 1000, Vetoquinol, Lure, France) and testicles were removed, dissected to remove fat, and immediately frozen in liquid nitrogen and stored at -80ºC until RNA extraction.

3.2 RNA sequencing (RNA-seq), data analysis and annotation

Total RNA was isolated using TRIZOL (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. Testis RNAs of each individual were pooled for Wt and Tg-RGN groups (n=5, 1ug per animal). RNA concentration and integrity were assessed using an Agilent 2100 Bioanalyser (Agilent Technologies, USA), which confirmed that both pooled samples had an RNA integrity number above 8.5.

Library preparation was conducted at Stabvida NGS laboratory (Caparica, Portugal), using a TruSeq Stranded mRNA Library Preparation Kit (Illumina, USA). The two libraries were subjected to 100 bp paired-end sequencing using an HiSeq 2500 system (Illumina, USA).

The bioinformatics analysis of the generated raw data was carried out using CLC Genomics Workbench 9.0.1 (CLC Bio/Qiagen, Denmark) and IlluminaClip (Illumina), which included the trimming of low quality reads (maximum 0.01 error and minimum phred score of Q20), of ambiguity reads (with stretches of more than 2 Ns) and of reads shorter than 30 nucleotides, as well as trimming of adapter (Illumina RNA TruSeq) sequences.

High quality reads were mapped against the reference genome (*R. norvegicus*, Rnor 6.0) using length fraction of 0.95 (minimum percentage of the alignment that matched the reference sequence) and similarity fraction of 0.95 (minimum percentage of identity between the read and the reference in aligned regions).

After mapping to the rat genome, gene expression levels were calculated for each library as Reads Per Kilobase of exon model per Million mapped reads, calculated as RPKM = [total number of reads mapping a particular gene] / [number of total mapped reads to the transcriptome (Millions) x the length of that gene (Kb)]. In addition, expression levels were also calculated in Transcripts per Million (TPM).

Differential expression was established based on the proportion of reads that mapped to a gene in each sample (relative expression levels in TPM) using the "Differential Expression for RNA-Seq" tool (CLC Bio/Qiagen, Denmark), a multi-factorial statistical analysis tool based on a negative binomial model. A generalized linear model approach influenced by the multi-factorial edgeR method (234) was used. Genes were considered differential expressed for Benjamini–Hochberg corrected for false discovery rate (FDR) p-Values ≤ 0.05 and fold change > 2 (up-regulation) or < -2 (down-regulation).

3.3 Enrichment analysis

Enrichment analyses based on the Gene Ontology (GO) classifications and the Kyoto Encyclopedia of Gene and Genome (KEGG) pathways were carried out using Cytoscape v 3.5.1 and the ClueGO plug-in v 2.3.2 with Cluepedia v1.3.2 (235–237). All differentially expressed genes were mapped using the gene annotations from the rat genome in the Ensembl database.

GO terms for Biological Processes (GO-BP) were updated on 15/11/2016 and used between levels 3-8. Benjamini–Hochberg FDR correction was applied and only terms with FDR ≤ 0.05 and a minimum representation of three genes/4% were considered significant.

For GO term grouping, initial group size was set as 1 and group merging at 50%, with Kappa-statistics score threshold set at 0.4. Leading terms for each group were selected based on the highest significance.

3.4 cDNA synthesis and quantitative real time RT-PCR (qPCR)

cDNA was synthesized from 1 µg of Wt and Tg-RGN testis total RNA using the NZY First-Strand cDNA Synthesis Kit (MB12501, NZYTech, Lisboa, Portugal) in a final volume of 20 µL, according to the manufacturer's instructions. Quantitative real time reverse transcription polymerase chain reaction (qPCR) was carried out on a StepOnePlus qPCR thermocycler (Applied Biosystems, UK) to measure relative transcript abundance of 10 selected genes (Table 2) in the testis, as a validation of the RNA-seq analysis. The relative standard curve method and the EvaGreen chemistry were used. Reactions were performed in duplicate and contained 1x Sso Fast EvaGreen Supermix (Bio-Rad), 300 nM of each specific primer (Table 2) and 2 µL of each cDNA (diluted 1:10) in a final volume of 10 µL. Cycling conditions consisted of initial 30 s at 95 ºC for denaturation, followed by 40 cycles of 5 s at 95 ºC and 10 s at the optimized annealing temperature for each respective primer (Table 2). A final melting curve between 60 and 95 ºC

confirmed that all qPCR reactions had a single product melt curve and primer specificity was confirmed by sequencing all amplicons.

Table 2 - List of the amplified transcripts and the oligonucleotides sequences, annealing temperatures and amplicon sizes used for analysis of gene expression by qPCR and RT-PCR.

Gene symbol		Sense / Anti-sense	Primer Sequence (5' – 3')	AT (°C)	Amplicon size (bp)
Target genes	*Atp10b*	Sense	AGGATGGTAGTGTATTAC	52	133
		Anti-sense	TGAAGAAGAGATTGAAGA		
	Eng	Sense	CACCACCCTTCTGCTTCCC	60	182
		Anti-sense	CACCGTTACCGTCACTGTCC		
	Fign	Sense	TCAGCAATTATTCAGACA	52	110
		Anti-sense	AACACAGTTCATAGGATAA		
	Lum	Sense	GGTCCGCTCCCAAAGTCC	60	112
		Anti-sense	GGTTGTGTTGAAGGTAAATGAAGG		
	Orai1	Sense	GCCTGGTGTTTATCGTCTTT	58	77
		Anti-sense	CTCCTGGAACTGTCGGTC		
	Plcb1	Sense	GTCACACAAGTCGTCAGAGGGAAG	64	150
		Anti-sense	TCATCATCGTCGTCATCGTCATCAC		
	Sfrp2	Sense	GGAGACAAAGAGCAAGACCATTTAC	62	131
		Anti-sense	GACCAGATAGGGAGCGTTGATG		
	Star	Sense	TCTCAACTGGAAGCAACACTCTAC	60	164
		Anti-sense	CCTGGCACCACCTTACTTAGC		
	Sycp1	Sense	AGTTGCCACTATCAGGAAGGAG	58	134
		Anti-sense	TTCAGTTCAGACTCTATGCTCAC		
	Tnni1	Sense	GGAGCAGGAGCACGAGGAG	64	181
		Anti-sense	GTTGTGGAGGCATTTGGCTTCG		
Reference genes	*Actb*	Sense	ATGGTGGGTATGATGCAG	60	79
		Anti-sense	CAATGCCGTGTTCAATGG		
	B2M	Sense	ATGAGTATGCCTGCCGTGTG	62	149
		Anti-sense	CAAACCTCCATGATGCTGCTTAC		

Legend: AT, annealing temperature; bp, base pairs. For gene names see Table 5.

Initially, to estimate the relative abundance of each transcript in the testicles, pools of cDNAs from individual samples (n = 5 per group) were analyzed. Transcript expression of selected genes that demonstrated detectable levels of expression was then quantified by qPCR in individual cDNA samples.

Standard curves were prepared from serial dilutions of quantified amplicons for each gene and were included in all qPCR plates to permit product quantification and determination of the reaction efficiency, which ranged between 79% and 116%, with $R^2 > 0.99$. The relation between amplification cycle to initial template copy number is established by the standard curve. Copy number of target or reference genes was estimated using the following equation: number of copies = (X / NA)/(Y x 1 x 109 x 650), where X is the initial template amount (ng of the amplicon fragment), NA is Avogadro's number, Y is the template length (bp of each amplicon), and 650 (Da) is the average weight of a base pair (238,239).

The stability of two reference genes, Beta-Actin (*Actb*) and Beta-2-Microglobulin (*B2M*), was evaluated in the testicles of Wt and Tg-RGN animals by analyzing the obtained threshold cycles (Cts) using RefFinder (available at 150.216.56.64/referencegene.php), that integrates the GeNorm (240), Normfinder (241), BestKeeper (242) and Comparative delta-Ct (243) methods.

As they did not vary significantly, all data was normalized by the geometric mean of these two genes (target genes normalized expression was calculated by dividing the obtained gene copy number by the geometric mean of the copy number of the two reference genes). Finally, relative changes in gene expression were expressed as fold-change comparatively to the control, defined as the Wt group.

Statistical significance of differences between groups on the relative transcript expression determined by qPCR was assessed by one-way analysis of variance (one-way ANOVA), followed by a post-hoc Tukey test, performed with the SigmaPlot software (SigmaStat v.3.50, Systat Software, USA). Statistical significance was set at $p < 0.05$ (*) or $p < 0.01$ (**). All results were expressed as the mean ± standard error of the mean (SEM) for expression levels relative to the Wt group, defined as control.

Pearson correlations between the qPCR data (average normalized expression of each gene in each experimental group) and relative expression levels obtained by RNA-seq (in TPM) were evaluated after log2 transformation of both variables, with significance levels set as $p < 0.05$.

3.5 Reverse transcription polymerase chain reaction (RT-PCR)

After validation through qPCR analysis, the expression of the selected genes was investigated in the rat gonadal cell-6 spermatogonia (GC-6spg) and in Wt SCs to determine if the selected genes were present in these specific testicular cell populations. GC-6spg is a rat cell line with SSCs characteristics generated by Doctor Ans Van Pelt, Academic Medical Center (AMC), The Netherlands (244). The SCs were isolated from Wt rats testis for previous studies done by our research group (245).

GC-6spg and SCs RNA extraction and cDNA synthesis were performed as described in sections 3.2 and 3.4. Reverse transcription-polymerase chain reaction (RT-PCR) was conducted using a

T100™ thermal cycler (Bio-Rad) and each reaction mixture contained 1x DreamTaq Green PCR Master Mix (K1081, Thermo Scientific), 0.5 mM $MgCl_2$ (A351H, Promega, Madison WI, USA), 400 nM of each specific primer (Table 2) and 1µl of GC-6spg or SCs cDNA in a final volume of 25 µL. After an initial incubation at 95°C for 5 min, 35 cycles of the following temperature conditions were used: 95°C for 30 s, the optimized annealing temperature (Table 2) for 30 s and 72°C for 30 s. A final extension at 72°C for 5 min was performed.

PCR products were analyzed by electrophoresis in agarose gels stained with GreenSafe Premium (MB13201, NZYTech, Lisboa, Portugal).

4. Results and Discussion

4.1 Transcriptome analysis revealed 1064 differentially expressed genes in the testis of Tg-RGN rats comparatively with Wt controls

The characterization of a transcriptome (analyzing the identity and quantity of all transcripts expressed in a tissue or cell) is an essential tool for the interpretation of the functional elements of the genome and represents the key connection between the genomic information and phenotype (246). The use of the Tg-RGN rat model has been allowing to investigate RGN's role in male reproduction, considering that the altered processes found in this model result from a direct or indirect action of this protein. Our research group previously showed that the reproductive phenotype of Tg-RGN rats differs from Wt controls, with RGN overexpression being related to an altered morphology of epididymis, as well as lower sperm counts and motility, which in opposition was accompanied by an higher sperm viability and frequency of normal sperm, with a diminished incidence of tail defects (102). The lower sperm counts and motility were proposed to be a consequence of the increased Ca^{2+} concentrations found in the epididymal lumen of Tg-RGN rats. On the other hand, the higher sperm viability and the diminished incidence of tail defects are likely a result of the cytoprotective action of RGN related with protection from oxidative stress, since an higher antioxidant potential was found in the epididymal fluid of Tg-RGNs. The cytoprotective actions of RGN in the testis were also verified in other studies performed in this model. The SeT of Tg-RGN rats display a naturally higher overall antioxidant capacity comparatively with Wt controls, and lower levels of lipid peroxidation as indicated by the diminished quantities of thiobarbituric acid reactive substances (110). In addition, a protective effect against chemical- (110,111) and radiation (189) - induced apoptosis was also observed, with RGN overexpression preventing testicular cell damage. Other studies also found that RGN may prevent premature sperm capacitation (97) and protect sperm from damage during cryopreservation (233). This is likely due to RGN activity regulating Ca^{2+} levels and promoting antioxidant activity, and may be of usefulness improving the outcomes of assisted reproductive techniques

Although all gathered information concurs for an important role of RGN in male reproduction, the molecular mechanisms underlying RGN cytoprotective actions in spermatogenesis are still poorly understood. In the present study, a high-throughput DNA sequencing method (RNA-seq) was used to determine whether the transcriptional activity network in the testis of Tg-RGN rats is modified comparatively with Wt controls. This strategy will be of paramount importance to reveal potential RGN' molecular partners and the molecular mechanisms underlying the physiological actions of RGN, with particular focus in spermatogenesis.

The RNA-seq approach is a powerful tool for mapping and quantifying the transcriptome, allowing a very high-throughput surveying of the whole transcriptome without prior genomic knowledge and showing high levels of reproducibility (246). To the best of our knowledge, this

is the first study presenting a comprehensive analysis of the testicular transcriptome of Tg-RGN rats, providing a deeper insight into the RGN molecular actions in the testis. Herein, a link between RGN and several molecular partners and pathways that are related with spermatogenesis was revealed. Therefore, the data presented is a fundamental basis for future research on the understanding of spermatogenesis and male infertility, but also for disclosing the general and likely conserved biologic functions of RGN.

The *R. norvegicus* (Rnor 6.0) genome was used as reference and a total of 106 million (M) reads (98% of the 108 M sequenced reads) could be mapped to the genome, after data cleaning using the required quality parameters. 99.9 M reads were mapped to unique locations in the genome and followed up for the differential expression analysis. The unique mapping rates were similar between the two samples (92.2-92.3%).

Statistical tests allowed to compare gene expression levels and to identify a total of 1064 genes with significant differential expression between the Tg-RGN and Wt testis samples, with a minimum of 2 fold-change differences and FDR ≤ 0.05. Of these, 67% (714 genes) were down-regulated and 33% (350 genes) were up-regulated (Figure 5). Considering more stringent levels of significance, it was also established that the majority of the genes were down-regulated, with 329 (70%) genes down-regulated out of 468 changing expression at least 3-fold, and 201 (69%) genes down-regulated more than 4-fold, out of a total of 289 differentially expressed genes at this level (Figure 5).

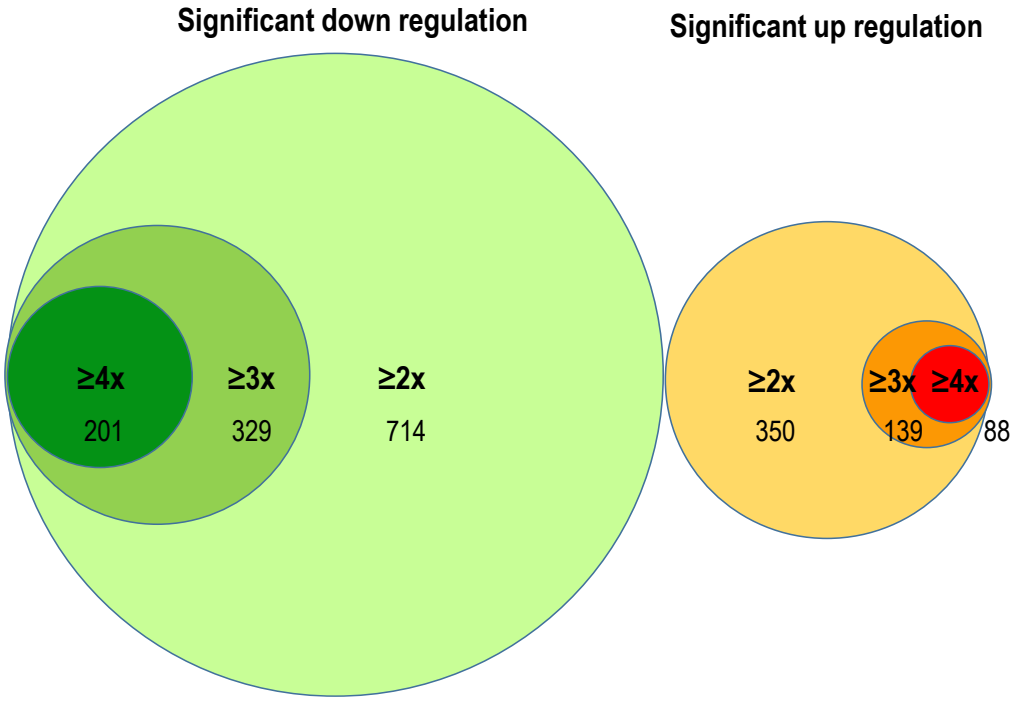

Figure 5 - Number of genes differentially expressed (up- or down-regulated) in the testis of transgenic rats overexpressing regucalcin (Tg-RGN) comparatively with Wt controls using different levels of significance. Significant differences in expression were considered at FDR ≤ 0.05 and fold-change ≥ than 2-fold (2x), 3x or 4x of up- or down-regulation.

Among the global list of 1064 differentially expressed genes, the top 20 most up-regulated genes (Table 3) and the top 20 most down-regulated genes (Table 4) presented fold-change values higher that 10 and lower than -20, respectively. Interestingly, the top differentially expressed genes represent a combination of genes associated to RGN and testis expected functions, such as ion transport, DNA recombination and transcription. However, other highly regulated genes have roles not yet associated with testicular physiology and whose function is yet unknown. This reflects the capacity of RNA-seq analysis in identifying new targets with potential biological relevance, in this particular case considering the testis function and/or RGN's action.

Table 3 - Top 20 up-regulated genes in the testicular transcriptome of Tg-RGN rats comparatively to Wt controls.

Gene ID / symbol	Gene name / description	FC
AC125688.1	N/A	136,0
Myrfl	myelin regulatory factor-like	90,4
Spdef	SAM pointed domain containing ets transcription factor	54,4
AABR07024716.1	N/A	37,0
RGD1561853	similar to SET domain-containing protein	31,1
Sfrp2	secreted frizzled-related protein 2	27,2
AC098189.1	N/A	25,8
AABR07065600.1	N/A	24,7
Cml1	probable N-acetyltransferase CML1	22,2
AABR07002001.1	N/A	18,3
AABR07051670.1	N/A	17,6
Atp10b	ATPase phospholipid transporting 10B (putative)	17,6
AABR07026503.1	N/A	16,9
Lat	linker for activation of T cells	16,0
Tnni1	troponin I1, slow skeletal type	15,3
Mixl1	mix paired-like homeobox 1	14,7
Dctd	dCMP deaminase	14,4
AC095678.2	N/A	13,6
Snrpn	small nuclear ribonucleoprotein polypeptide N	13,1
Kcnmb3	potassium Ca^{2+} -activated channel subfamily M regulatory beta subunit 3	10,2

Legend: FC represents the fold change of up-regulation between Tg-RGN (in TPM) relative to the Wt group; N/A indicates that no annotation information was available for this gene in the rat genome.

Table 4 - Top 20 down-regulated genes in the testicular transcriptome of Tg-RGN rats comparatively to Wt controls.

Gene ID / symbol	Gene name / description	FC
AABR07000533.1	(novel lincRNA)	-21,2
Lamc2	laminin subunit gamma 2	-23,5
Rn60_20_0047.3	RT1 class I histocompatibility antigen, AA alpha chain	-23,8
AC115159.2	N/A	-25,4
Fign	fidgetin, microtubule severing factor	-26,8
Vom2r44	vomero+G3nasal 2 receptor 44	-28,3
AABR07065566.1	N/A	-30,4
Galnt13	polypeptide N-acetylgalactosaminyltransferase 13	-31,7
AABR07014512.1	N/A	-33,7
Rgs7bp	regulator of G-protein signaling 7 binding protein	-45,8
AABR07013095.1	N/A	-45,9
LOC100910237	N/A	-46,5
AC098190.1	N/A	-47,5
Rn50_20_0046.8	N/A	-82,1
P4ha3	prolyl 4-hydroxylase subunit alpha 3	-120,2
RGD1564698	similar to ribosomal protein S10	-323,8
LOC103690086	uncharacterized LOC103690086	-1007,5
Olr1226	olfactory receptor 1226	-1471,1
RGD1309362_2	N/A	-2047,8
Hmgb2	high mobility group box 2	-2568,0

Legend: FC represents the fold change of expression between Tg-RGN (in TPM) relative to the Wt group, with a negative signal "-" being used to indicate these changes were decreased in expression (down-regulation); N/A indicates that no annotation information was available for this gene in the rat genome.

4.2 Ca^{2+} transport and meiotic cell cycle are highly enriched biological processes in the testis of Tg-RGN animals

GO and KEGG pathway-based enrichment analysis can help us further understand the functional profile of a gene set, underlying the biological functions of genes that are over-represented (or under-represented) under certain experimental or endogenous biological conditions. GO terms for biological processes (GO-BP) permit the identification of one or more biological processes to which a gene or gene product contributes, describing the larger cellular or physiological role carried out by the gene coordinated with other genes (247). On the other hand, the KEGG pathway database contains information on networks of intracellular molecular interactions and their organism-specific variations (248). To identify the biological processes and pathways that are active in the testis of Tg-RGN rats, and consequently identify potential molecular mechanisms underlying RGN cytoprotective actions in the testis, all differentially expressed genes were mapped to reference GO-BP terms and KEGG canonical pathways using the gene annotations from the rat genome in the Ensembl database.

Initially, enrichment GO and KEGG analysis was performed for all 1064 genes differentially expressed in Tg-RGN testis. Analysis revealed 159 GO-BP terms (Figure 6 and Supplementary table S1) and 4 KEGG pathways (Figure 7 and Supplementary table S2) significantly enriched (FDR ≤ 0.05) in this list of genes, that were grouped into 24 and 4 groups of functionally related terms, respectively. Some genes contributed to the enrichment in multiple terms, as expected.

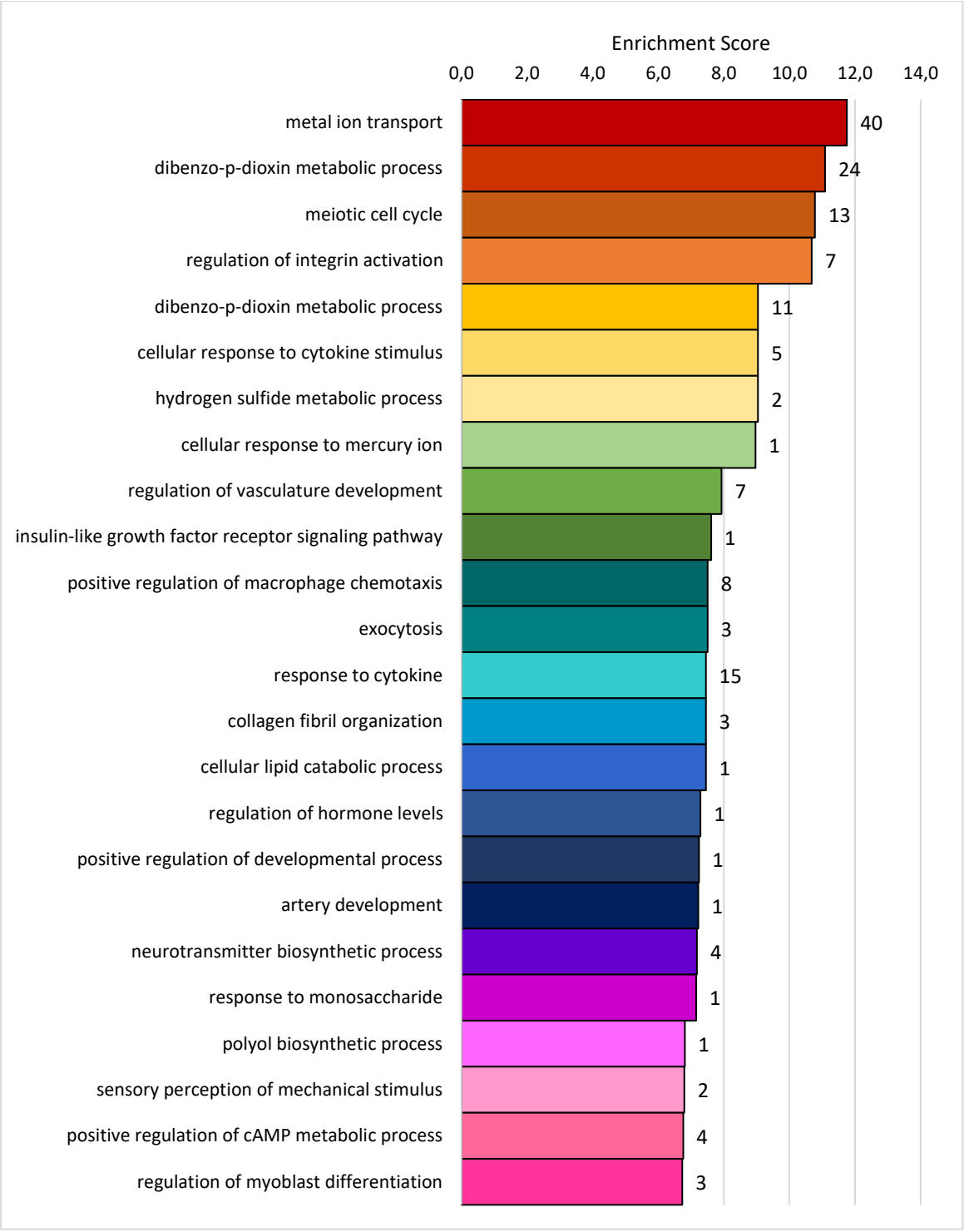

Figure 6 - Main functionally related networks (groups) of Gene Ontology - Biological Processes (GO-BP) enriched among the list of all 1064 differentially expressed genes in Tg-RGN testis, using ClueGO/Cytoscape. GO enriched groups are sorted by enrichment score (calculated as - Log2 (group FDR)) with the most significant enrichments (lower p-value) being shown on top. Each GO-BP group identified in the left panel summarizes a higher number of significant GO-BP terms (number of terms indicated at the end of each respective bar) and was named after its most significant term.

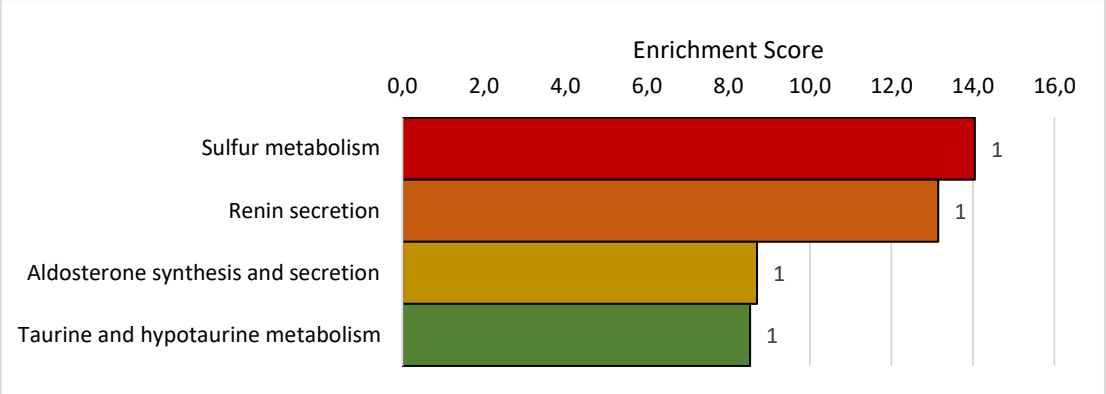

Figure 7 – Main functionally related networks (groups) of Kyoto Encyclopedia of Gene and Genome (KEGG) pathways enriched among the list of all 1064 differentially expressed genes in Tg-RGN testis, using ClueGO/Cytoscape. KEGG enriched groups are sorted by enrichment score (calculated as - Log2 (group FDR)) with the most significant enrichments (lower p-value) being shown on top. Each KEGG group identified in the left panel summarizes a higher number of significant KEGG terms (number of terms indicated at the end of each respective bar) and was named after its most significant term.

The most significantly over-represented GO-BP groups in the analysis of all differentially expressed genes mainly included “metal ion transport”, “dibenzo-p-dioxin metabolic process”, “meiotic cell cycle”, “regulation of integrin activation” and “cellular response to cytokine stimulus”, followed by other groups of enriched GO-BP terms of lower enrichment scores (Figure 6). Relative to the KEGG analysis, four pathways were significantly enriched (FDR ≤ 0.05), namely the “sulfur metabolism”, “renin secretion”, “aldosterone synthesis and secretion”, and “taurine and hypotaurine metabolism” pathways (Figure 7).

Thereafter, separate enrichment analysis for up-regulated and down-regulated genes was performed to detail the processes and pathways that have a positive or negative regulation in response to RGN action. This separate analysis also allowed the discovery of specific enriched biological processes and pathways that were not considered significantly enriched in the general GO-BP and KEGG enrichment analysis.

The list of up-regulated genes had a significant (FDR ≤ 0.05) enrichment in 374 GO-BP terms (Figure 8 and Supplementary table S3) and 9 KEGG pathways (Figure 9 and Supplementary table S4), grouped into 38 and 7 groups of functionally related terms, respectively. The majority of the “up-regulated-enriched” biological processes were related to Ca^{2+} transport, homeostasis and signaling, to the dibenzo-p-dioxin metabolism, the regulation of vasculature development and to cellular responses to several factors, including cytokines (Figure 8). Hence, RGN action appears to result in the overexpression of many genes involved in the mentioned processes.

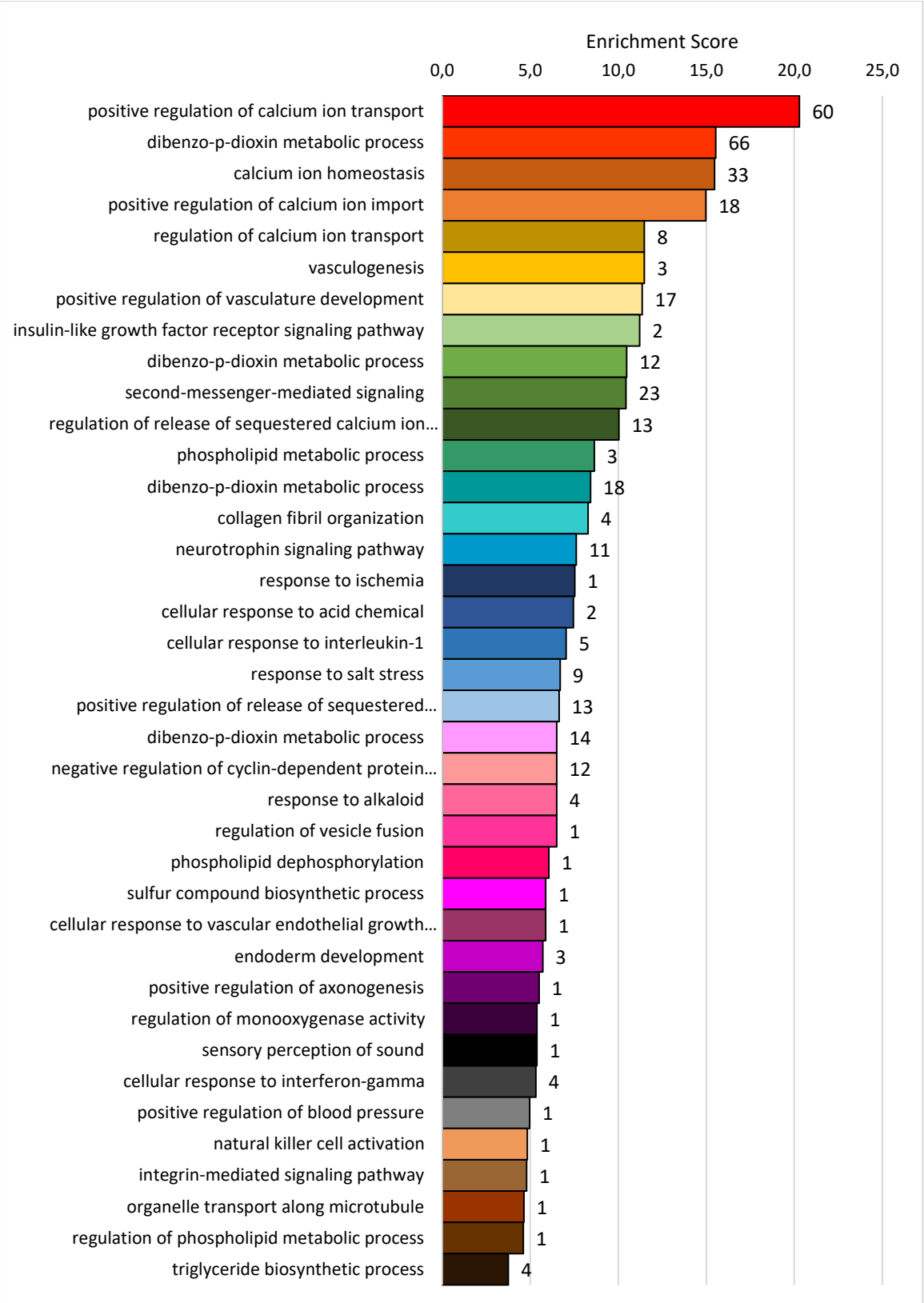

Figure 8 - Main functionally related networks (groups) of Gene Ontology - Biological Processes (GO-BP) enriched among the list of the 350 up-regulated genes in Tg-RGN testis, using ClueGO/Cytoscape. GO enriched groups are sorted by enrichment score (calculated as - Log2 (group FDR)) with the most significant enrichments (lower p-value) being shown on top. Each GO-BP group identified in the left panel summarizes a higher number of significant GO-BP terms (number of terms indicated at the end of each respective bar) and was named after its most significant term.

In agreement with the RGN well-established role in Ca^{2+} homeostasis and as a modulator of Ca^{2+}-handling proteins (124,135), the differential expression of Ca^{2+}-related genes in the testis of Tg-RGN rats was somehow expected. The importance of Ca^{2+} homeostasis for mammalian spermatogenesis and sperm function (43–46), as well as the involvement of Ca^{2+} as a second messenger in the somatic and germ testis cells (35–37), is well-known. Ca^{2+} tight regulation is necessary in the maintenance of LC steroidogenesis (190,191), SC function (192,193), tight-junctions structure (194) and remodeling (195), as well as in the regulation of sperm motility, hyperactivation (196,197), chemotaxis (198), capacitation (199,200) and acrosome reaction (201–205). Thus, RGN action up-regulating the expression of genes involved in Ca^{2+} transport, homeostasis and signaling in the testis can be a probable molecular mechanism by which it influences male fertility. Furthermore, considering that dibenzo-p-metabolic process GO-BP leading term is associated with genes such as *Star* (steroidogenic acute regulatory protein) and *Cyp11a1* (cytochrome P450 family 11 subfamily A Member 1), the enrichment of this term in the up-regulated genes also indicates that RGN may be involved in the regulation of steroid hormone synthesis. However, it may also reflect RGN modulation of response to noxious stimuli.

Regarding the KEGG pathway analysis, the nine significantly enriched pathways included “renin secretion”, “sulfur metabolism”, “aldosterone synthesis and secretion”, among others (Figure 9).

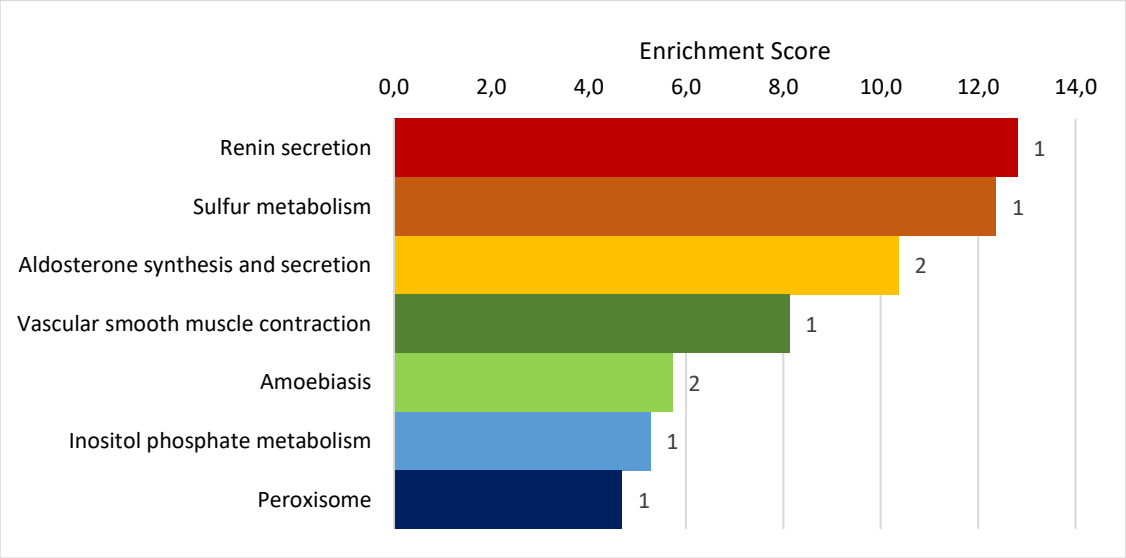

Figure 9 - Main functionally related networks (groups) of Kyoto Encyclopedia of Gene and Genome (KEGG) pathways enriched among the list of the 350 up-regulated genes in Tg-RGN testis, using ClueGO/Cytoscape. KEGG enriched groups are sorted by enrichment score (calculated as - Log2 (group FDR)) with the most significant enrichments (lower p-value) being shown on top. Each KEGG group identified in the left panel summarizes a higher number of significant KEGG terms (number of terms indicated at the end of each respective bar) and was named after its most significant term.

The modulation of Renin and Aldosterone associated pathways implicates RGN on the activity of renin-angiotensin-aldosterone system (RAAS). This system is best known for its role in the maintenance of blood pressure and electrolyte and fluid homeostasis, but some reports also

speculate for a role of the RAAS in male reproduction. Tubular contractility, spermatogenesis, sperm maturation, capacitation, acrosomal exocytosis and fertilization, due to the local synthesis of components of the RAAS, as well as the presence of angiotensin receptors, have been relating the RAAS with male reproduction (reviewed in (249)). Therefore, the importance of RGN modulation of genes involved in renin and aldosterone secretion in the testis should be clarified in future studies.

The list of down-regulated genes was found to be significantly enriched (FDR ≤ 0.05) in 35 GO-BP terms (Figure 10 and Supplementary table S5) and 6 KEGG pathways (Figure 11 and Supplementary table S6), that were group into seven or three main groups of terms respectively. Most overrepresented GO-BP groups among the down-regulated genes included "meiotic cell cycle" and "regulation of integrin activation", among others (Figure 10) and were linked with "homologous recombination", "type I diabetes mellitus", "alanine, aspartate and glutamate metabolism" enriched KEGG pathways (Figure 11).

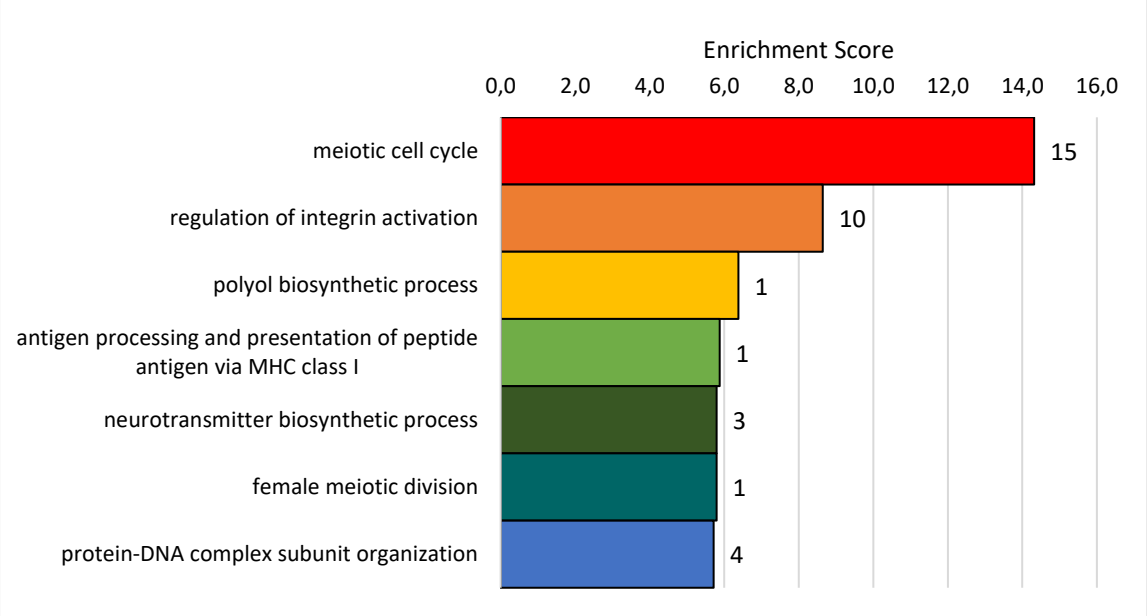

Figure 10 - Main functionally related networks (groups) of Gene Ontology - Biological Processes (GO-BP) enriched among the list of the 714 down-regulated genes in Tg-RGN testis, using ClueGO/Cytoscape. GO enriched groups are sorted by enrichment score (calculated as - Log2 (group FDR)) with the most significant enrichments (lower p-value) being shown on top. Each GO-BP group identified in the left panel summarizes a higher number of significant GO-BP terms (number of terms indicated at the end of each respective bar) and was named after its most significant term.

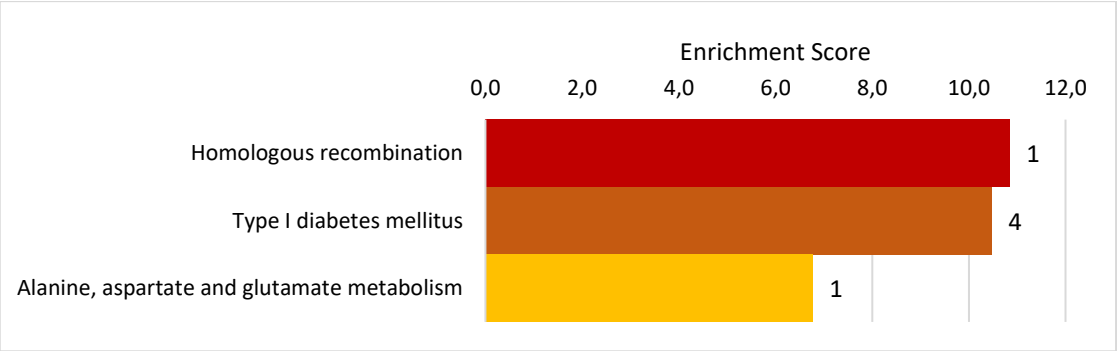

Figure 11 - Main functionally related networks (groups) of Kyoto Encyclopedia of Gene and Genome (KEGG) pathways enriched among the list of the 714 down-regulated genes in Tg-RGN testis, using ClueGO/Cytoscape. KEGG enriched groups are sorted by enrichment score (calculated as - Log2 (group FDR)) with the most significant enrichments (lower p-value) being shown on top. Each KEGG group identified in the left panel summarizes a higher number of significant KEGG terms (number of terms indicated at the end of each respective bar) and was named after its most significant term.

Meiosis and homologous recombination are critical events in the spermatogenic process, from those depends the development of normal sperm (3,8). Failures in meiosis are one of the known causes of male fertility. Interestingly, a down-regulation of meiosis-associated genes was found in consequence of RGN overexpression. This finding strengthens the hypothesis that RGN has a relevant role in a basic biological process such as meiosis, which is fundamental for spermatogenesis.

In addition, integrins are were other enriched group among the down regulated transcripts in the testis of Tg-RGN. These are well known proteins associated to cytoskeleton elements that confer cell adhesion between SCs and germ cells in the SeT, as well as between SCs and the basement membrane at the hemidesmosome, regulating junction restructuring events during spermatogenesis (250,251). There are no reports linking RGN with the architecture of cell adhesions and integrins, but the enrichment in transcripts encoding this class of proteins in the transcriptome of Tg-RGN animals deserves further investigation to disclose its relevance in the spermatogenic process.

Overall, the discovery of biological processes and pathways differentially activated in response to RGN overexpression provided a valuable basis for investigating and understanding the specific processes, pathways and functions in which RGN is involved in the testis, and to clarify its role in male reproduction. In addition, new avenues of research were open to further explore the general biological function of RGN in different cell types and tissues. Details on enrichment analysis results, namely the GO-BP and KEGG enriched terms grouped under each GO-BP and KEGG group leading term among the lists of global, up- and down-regulated genes, are provided in the supplementary tables in the Appendixes (Appendix I).

4.3 qPCR analysis and validation of the potential RGN molecular partners

To validate the obtained RNA-seq results using a parallel independent technique and to investigate possible individual variation among the groups, a qPCR analysis was performed. Several differentially expressed genes in the Tg-RGN rat testis comparatively with Wt were selected (Table 5), based on differential expression levels, results of enrichment analyses and relevance to spermatogenesis. Genes with high fold-change values, related to several GO and KEGG-enriched groups or linked in the literature with relevant processes associated with spermatogenesis were given priority. Gene selection also took into consideration the coverage of a wide range of transcript abundance. The final 10 selected genes, including 8 up-regulated and 2 down-regulated genes (Table 5), represented a broad range of fold-change values (~ 3 to 30-fold), allowing the examination of RNA-seq validity considering distinct patterns of differential expression.

A thorough literature search was done to identify genes with biological functions potentially relevant to spermatogenesis. The majority of the selected genes was mainly associated with processes thought to be important to spermatogenesis, such as Ca^{2+} homeostasis, meiosis, proliferation, apoptosis, intracellular signaling and steroid hormone synthesis. However, other genes also were selected despite not being associated to spermatogenesis yet, but were here included because of the very high differential expression in the testis of Tg-RGN compared to Wt.

Table 5 – List of differentially expressed (DE) genes selected for qPCR analysis and validation of RNA-seq results. Transcripts per million (TPM) in control (Wt) and transgenic (Tg-RGN) cDNA pools and fold-change (FC) variation were indicated.

Gene symbol	Gene name	DE	Pool Wt (TPM)	Pool Tg-RGN (TPM)	FC
Atp10b	ATPase phospholipid transporting 10B (putative)	Up	0,06	1,03	17,96
Eng	endoglin	Up	5,40	14,81	2,74
Fign	fidgetin, microtubule severing factor	Down	1,82	0,07	-27,29
Lum	lumican	Up	0,27	1,08	3,98
Orai1	Ca^{2+} release-activated Ca^{2+} modulator 1	Up	17,84	101,56	5,69
Plcb1	phospholipase C beta 1	Up	1,41	8,01	5,69
Sfrp2	secreted frizzled-related protein 2	Up	0,03	0,87	29,68
Star	steroidogenic acute regulatory protein	Up	28,33	80,60	2,85
Sycp1	synaptonemal complex protein 1	Down	56,86	18,22	-3,12
Tnni1	troponin I1, slow skeletal type	Up	0,43	6,61	15,53

Legend: Up: up-regulated gene; Down: down-regulated gene.

Statistical analysis of the qPCR data, allowed confirming that 6 of the 10 selected genes, were significantly differentially expressed in Tg-RGN rats compared with Wt testis controls, namely *Atp10b*, *Fign*, *Orai1*, *Sfrp2*, *Sycp1* and *Tnni1*. This finding validated the RNA-seq results even after taking into account the individual variability. The *Eng*, *Lum*, *Plcb1* and *Star* genes did not show significant differential expression in qPCR, probably due to individual variability or because their detected changes by RNA-seq were only moderate. However, it was possible to see a similar tendency to what was described in RNA-seq data for *Eng*, *Lum* and *Star*.

In addition, Pearson correlations between qPCR (average expression for each group) and RNA-seq data were calculated for each gene. A statistically significant linear correlation was found between qPCR and RNA-seq analysis, with $p = 5.1 \times 10^{-4}$ and r = 0,706 (Figure 12), suggesting an overall concordance between qPCR and RNA-seq techniques in detecting differential expression, and thus validating the differential expression in the testis of Tg-RGN rats comparatively with Wt rats established by the RNA-seq approach.

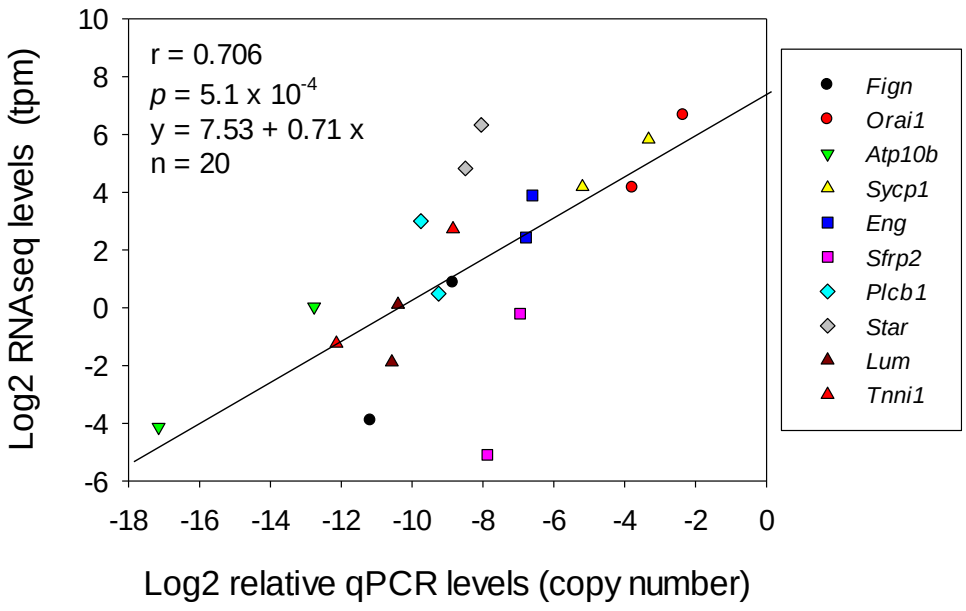

Figure 12 - Correlation analysis between gene expression data obtained from qPCR and RNA-seq results. For each gene, the control and Tg-RGN group relative expression levels detected by qPCR were plotted against those detected by RNA-seq, after log2 transformation of both ratios. Pearson correlation coefficients and the equation of the linear regression of the data are indicated.

This additional validation also demonstrates that changes detected by RNA-seq over a large range of abundance and including both up- and down-regulated transcripts are confirmed by qPCR, and that the differential expression data is credible. The differences detected in fold-change values between qPCR and RNA-seq analysis can be due to several factors, including differences in the amplification efficiency and because of the calculation algorithms employed

in RNA-seq. The existence of slightly discrepancies between these two techniques are usual and consistent with the reported in other studies following a similar approach (252).

The results of the qPCR analysis for each of the selected genes (Table 5), *Atp10b*, *Eng*, *Fign*, *Lum*, *Orai1*, *Plcb1,* Sfrp2, *Star, Sycp1* and *Tnni1*, are detailed below, as well as the discussion of their functions and putative role in spermatogenesis.

4.3.1 *Atp10b*

The *Atp10b* gene encodes an ATPase phospholipid transporter, member of the type 4 P-type ATPases family, which includes several multispan transmembrane proteins that facilitate phospholipid translocation from the exoplasmic to the cytoplasmic leaflet of membranes bilayers, at the expense of ATP. Contrary to other P-type ATPases, type 4 do not transport cations (253,254).

Phospholipid translocation ensures the maintenance of the asymmetric distribution of phospholipids between the two leaflets of biological membranes, which is crucial for many physiological processes, including signal transduction, cell morphology and movement (255,256). Phospholipid translocation also seems to be implicated in vesicle formation and in uptake of lipid signaling molecules (257).

Atp10b relative expression was detected to be approximately 18x up-regulated in the testis of Tg-RGN rats, and this gene was associated with “ion transport” GO-BP term, grouped under de GO-BP group “metal ion transport” in the enrichment analysis of all differentially expressed genes, contributing to its significant enrichment.

qPCR of *Atp10b* analysis was performed with a reaction efficiency of 85% and R^2=0.990 and a statistically significant up-regulation was detected in the Tg-RGN group, presenting a relative expression of 20.94 ± 8.61 fold change in relation to the control group ($p<0.05$; Figure 13A). The up-regulation of *Atp10b* in response to RGN overexpression was in accordance with RNA-seq data, with qPCR analysis detecting a slightly higher fold-change value between experimental groups. Individual variability was mostly detected between subjects of the Tg-RGN group, existing one identifiable outlier (Figure 13B).

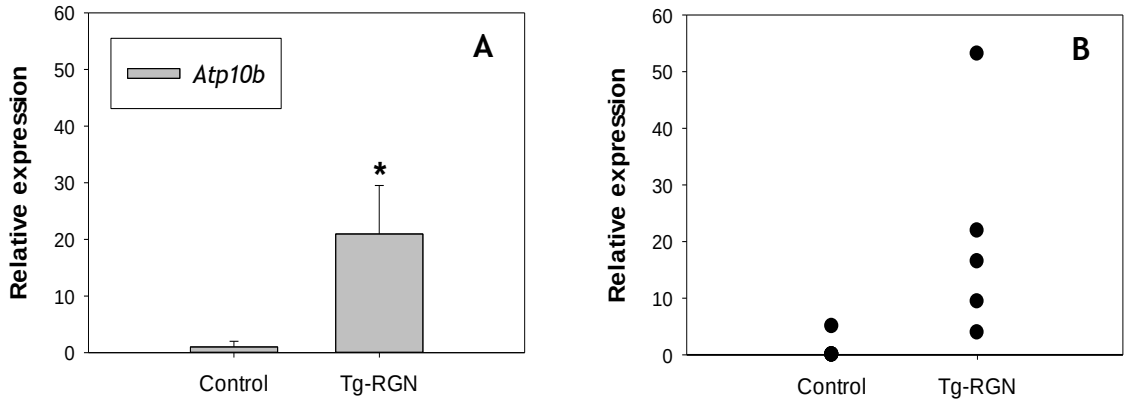

Figure 13 - Relative expression of ATPase phospholipid transporting 10B (*Atp10b*) gene in the testis of control (Wt) and Tg-RGN animals determined by quantitative RT-PCR (qPCR). *Atp10b* gene expression was normalized by the geometric mean of reference genes *Actb* and *B2M* expression and expressed as fold-change comparatively to the control. Error bars indicate mean ± S.E.M (n=5 in each group). Symbols above the bars indicate significant differences compared to the control by one-way ANOVA: * p<0.05 and ** p<0.01 (A). Individual relative expression values obtained for the samples of each group are also shown (B).

Considering that phospholipid scrambling is needed in initiating apoptosis (256) and sperm capacitation (258), a role for ATP10B may be related with these processes. Another member of the P4 ATPases family, ATP8B3, is exclusively expressed in the testis and has already been implicated in sperm acrosome formation and capacitation (259–261). Considering that capacitation and acrosome reaction both include changes in membrane composition (258,262), it is likely that more P4 ATPase family members may be involved in male reproduction. However, little is known about the specific function of ATP10B at the moment (254).

4.3.2 *Eng*

The *Eng* gene, also known as *CD105*, encodes a homodimeric transmembrane glycoprotein that is a component of the transforming growth factor beta (TGF-ß) receptor complex. Endoglin binds to several members of the TGF-ß family, including TGF-ß1 and TGF-ß3 (but not TGF-ß2) (263), activin-A, bone morphogenetic proteins (BMP) BMP-2, BMP-7, BMP-9 and BMP-10 (264,265), by interacting with type I and II related serine/threonine kinase receptors (266). In several tissues, the TGF-ß family has a critical role in the regulation of cell cycle progression and differentiation, in both normal and pathological conditions (267,268). Therefore, endoglin has been associated with cellular events such as proliferation and apoptosis (269). It is also involved in the regulation of angiogenesis (270), having a dual effect on tumor growth (265). ENG also appear to have a pivotal role in the balance of TGF-ß receptors, namely in activin receptor-like kinase 1 and activin receptor-like kinase 5 signaling, which contributes to its modulation of TGF-ß1 responses leading to cell proliferation (271).

This gene displayed approximately 3 fold-change in the testis of Tg-RGN rats detected by RNA-seq and was associated to several enriched GO-BP terms, such as “response to growth factor”, “regulation of vasculature development” and “extracellular matrix organization”, among the global list of 1064 differentially expressed genes.

qPCR analysis of *Eng* was performed with a reaction efficiency of 79% and R^2=0.994. Despite the general increase in *Eng* expression between the control and Tg-RGN group (Figure 14A), no statistically significant difference was perceived, probably due to the individual variability detected with one clear outlier in the control group (Figure 14B).

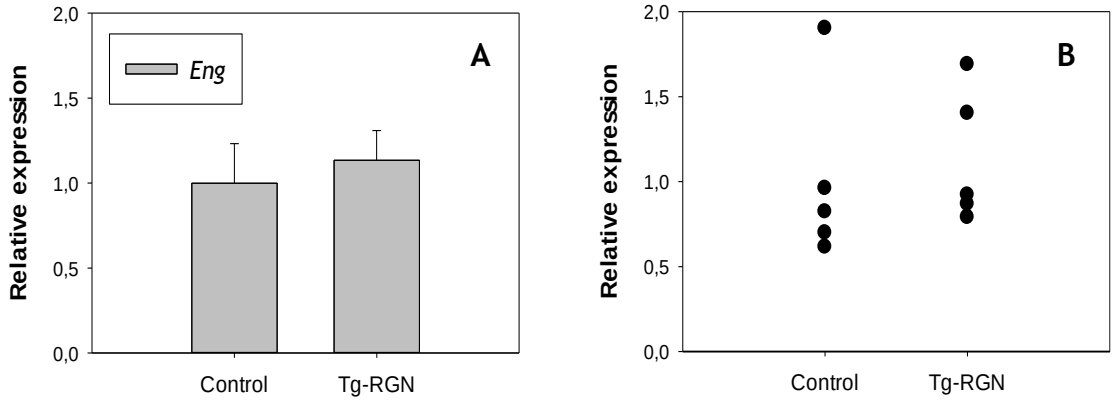

Figure 14 - Relative expression of endoglin (*Eng*) gene in the testis of control (Wt) and Tg-RGN animals determined by quantitative RT-PCR (qPCR). *Eng* gene expression was normalized by the geometric mean of reference genes *Actb* and *B2M* expression and expressed as fold-change comparatively to the control. Error bars indicate mean ± S.E.M (n=5 in each group). Symbols above the bars indicate significant differences compared to the control by one-way ANOVA: * $p<0.05$ and ** $p<0.01$ (A). Individual relative expression values obtained for the samples of each group are also shown (B).

In the literature, the TGF-ß signaling pathway has been linked with RGN expression, as *Smad3* knockout mice show significantly increased RGN levels in the liver concomitant with increased resistance to radiation-induced apoptosis (187). If *Eng* differential expression in Tg-RGN rats had been confirmed, *Eng* modulation by RGN would be a likely candidate responsible for influencing male fertility, considering that members of the TGF-ß superfamily modulate actions related to the functions of LCs and SCs (269,272,273), as well as the organization of peritubular myoid cells, testis development and testicular cell junction dynamics during spermatogenesis (268,272). In addition, the absence or overexpression of TGF-ß affects male reproductive function, supporting the hypothesis that TGF-ß signaling pathways are carefully coordinated during testicular development and spermatogenesis (268,274,275).

It has also been reported that ENG is expressed in all mouse spermatogenic cell types, with expression being higher in germ cells than in somatic testicular cells, and being differentially expressed at different stages of postnatal life, which suggests specific roles of this glycoprotein in physiological processes of mouse gonads (276). Interestingly, in testicular biopsies from normal and infertile men, it was found that endoglin expression was significantly increased in patients with LC hyperplasia compared with other groups (277).

4.3.3 *Fign*

Fidgetin (FIGN), the protein encoded by the *Fign* gene, is an ATP-dependent microtubule severing protein belonging to the superfamily of ATPases associated with diverse cellular activities (AAA superfamily). FIGN severs microtubules along their length and depolymerizes

their ends, primarily the minus-end, which leads to the suppression of microtubule growth from and attachment to centrosomes (278). Microtubule severing also promotes microtubule release from the mitotic spindle poles, by allowing the depolymerization of the microtubule end proximal to the spindle pole, leading to poleward microtubule flux and poleward motion of chromosome (278,279). Therefore, FIGN is able to regulate mitotic spindle architecture, dynamics and anaphase A chromatid-to-pole motion (278).

RNA-seq analysis showed that *Fign* was approximately 27x down-regulated and was associated with the "regulation of DNA recombination" GO-BP enriched term, grouped under the GO-BP group "meiotic cell cycle" that was significantly enriched among the list of the down-regulated genes.

qPCR analysis of *Fign* presented a reaction efficiency of 92% and R^2=0.995 and a statistically significant decrease in the relative expression levels of *Fign* was detected in the Tg-RGN group, with 0.20 ± 0.09 expression relative to the control ($p<0.01$; Figure 15A). The fold-change detected by qPCR analysis (approximately 5x) was lower than the one detected by RNA-seq but confirmed the significant down-regulation of *Fign* in Tg-RGN rat testis, despite the fact that some individual variability was detected among individuals of the control group (Figure 15B).

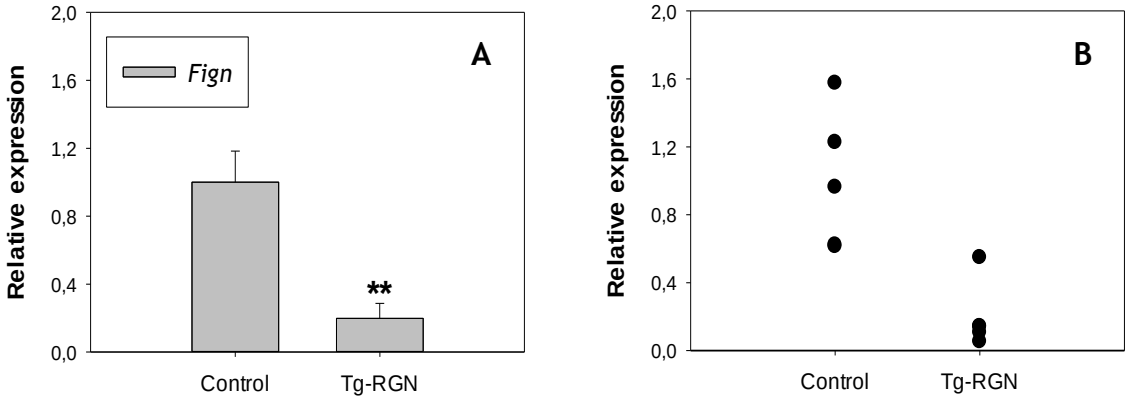

Figure 15 – Relative expression of fidgetin, microtubule severing factor (*Fign*) gene in the testis of control (Wt) and Tg-RGN animals determined by quantitative RT-PCR (qPCR). *Fign* gene expression was normalized by the geometric mean of reference genes *Actb* and *B2M* expression and expressed as fold-change comparatively to the control. Error bars indicate mean ± S.E.M (n=5 in each group). Symbols above the bars indicate significant differences compared to the control by one-way ANOVA: * $p<0.05$ and ** $p<0.01$ (A). Individual relative expression values obtained for the samples of each group are also shown (B).

Fign association with processes necessary for mitosis and meiosis, serves as a representative of RGN down-regulation of genes related to the meiotic cell cycle and homologous recombination enriched processes. The FIGN protein regulates mitosis through the suppression of microtubule growth from and attachment to centrosomes (278). Although no literature exists linking the *Fign* gene directly to spermatogenesis, there's evidence of other AAA proteins coding genes involvement in this process, namely fidgetin-like1, thought to act in mammal's male meiosis dynamics (280), and katanin, that is involved in multiple aspects of mammalian male gamete

development, including in meiotic spindle dynamics, cytokinesis, flagella development and sperm head shaping (281). Considering the similar functions of FIGN and katanin (281), it seems likely that FIGN may also have a role in mitosis and meiosis dynamics that occur during the spermatogenic process. As RGN down-regulated *Fign* expression in the testis, it is possible that chromosome segregation during meiosis and mitosis may be affected in Tg-RGN animals, delaying and/or reducing spermatozoa production, which is in agreement with fidgetin-like1 and katanin deletion results (280,281) and the lower sperm counts observed in the Tg-RGN animals (102).

4.3.4 *Lum*

Lum encodes lumican (LUM), a member of the small leucine-rich proteoglycans (SLRP) family. LUM is the major keratan sulfate proteoglycan of the cornea, but is also distributed in interstitial collagenous matrices throughout the body (282,283). It regulates collagen fibril organization and circumferential growth, corneal transparency, epithelial cell migration and tissue repair (284,285).

SLRP molecules are thought to regulate the collagenous matrix assembly in connective tissues due to their bifunctional role: protein moiety binds collagen fibrils and the highly charged hydrophilic glycosaminoglycans regulate interfibrillar spacings (286). Furthermore, they have been shown to have biological functions behind those of structural roles, such as regulation of cell proliferation, apoptosis and differentiation, acting as matrikines (active biological sequences in the extracellular matrix) by binding to cell surface receptors (e.g. integrins or growth factors receptors) and inducing intracellular signaling (284). LUM has also been associated with cell migration and proliferation during embryonic development, wound healing, inflammatory responses and tumor growth (284).

The *Lum* gene presented an up-regulation of approximately 4x between Tg-RGN and the control group in RNA-seq analysis. In the enrichment analysis, this gene was associated to the “collagen fibril organization” GO-BP group and to “response to growth factor” and “organic cyclic compound” GO-BP enriched terms of the “dibenzo-p-dioxin metabolic process” group, among the list of global differentially expressed genes.

Lum qPCR analysis was performed with a reaction efficiency of 92% and R^2=0.996 and the modest up-regulation detected by RNA-seq was followed by no statistically significant difference found for *Lum* relative expression values between control and Tg-RGN group in the qPCR analysis (Figure 16A). Individual variability was detected both in the control and Tg-RGN group (Figure 16B).

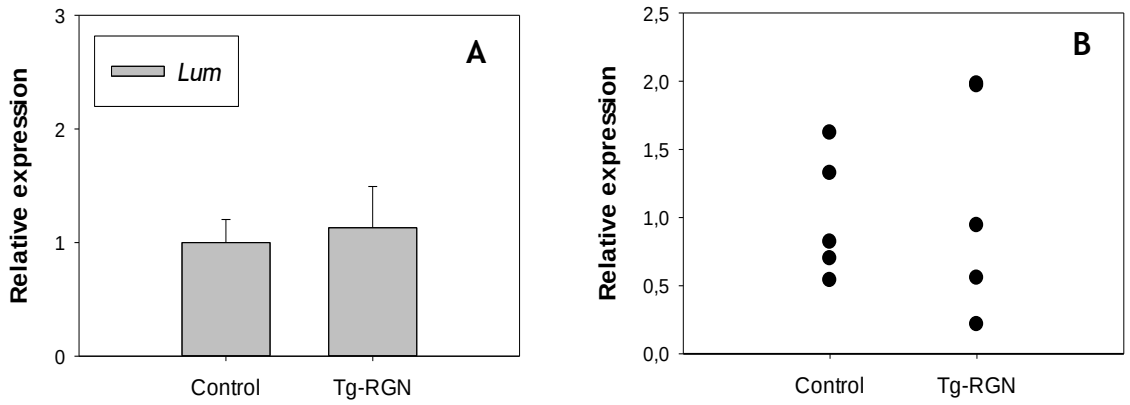

Figure 16 - Relative expression of lumican (*Lum*) gene in the testis of control (Wt) and Tg-RGN animals determined by quantitative RT-PCR (qPCR). *Lum* gene expression was normalized by the geometric mean of reference genes *Actb* and *B2M* expression and expressed as fold-change comparatively to the control. Error bars indicate mean ± S.E.M (n=5 in each group). Symbols above the bars indicate significant differences compared to the control by one-way ANOVA: * $p<0.05$ and ** $p<0.01$ (A). Individual relative expression values obtained for the samples of each group are also shown (B).

4.3.5 *Orai1*

The protein encoded by the *Orai1* gene is a pore forming subunit of membrane Ca^{2+} release-activated Ca^{2+} (CRAC) channels, that is activated by the Ca^{2+} sensor stromal interaction molecule 1 (STIM1) when Ca^{2+} stores, such as the endoplasmic reticulum, are depleted and consequently mediates Ca^{2+} influx into cells through store-operated Ca^{2+} entry (SOCE) activity (287,288). This type of channel is the primary way for Ca^{2+} influx into T-cells (289), but the channel has been detected in other cell types also.

ORAI1 channels intervene in Ca^{2+} transport and homeostasis, which are biological processes crucial to life (120–122), and has been shown to regulate cell proliferation and apoptosis (290,291).

Orai1 was approximately 6x up-regulated in the testis of Tg-RGN rats as RNA-seq analysis showed. This gene was associated to GO-BP group "metal ion transport" among the list of global differentially expressed genes and to GO-BP group "regulation of Ca^{2+} ion transport" among the list of up-regulated genes. In the enriched KEGG pathways analysis, *Orai1* was enriched for KEGG groups "renin secretion" and "aldosterone synthesis and secretion" in both the global list as well as in the list of up-regulated genes.

Orai1 qPCR analysis was performed with a reaction efficiency of 109% and R^2=0.994, with a statistically significant difference being found for the Tg-RGN group relative to the control, with 2.69 ± 0.56 of relative expression ($p<0.05$; Figure 17A), a lower fold-change than that detected by RNA-seq analysis. Individual variability was mostly observed on the Tg-RGN group (Figure 17B).

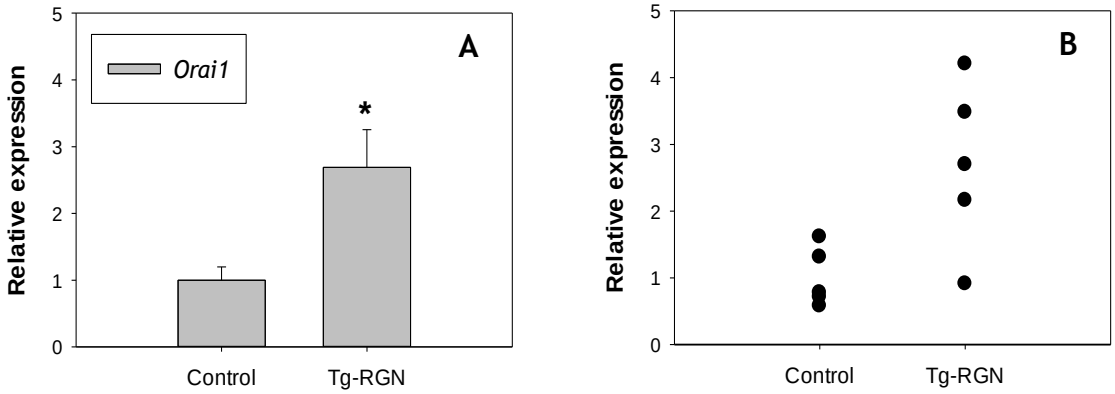

Figure 17 - Relative expression of Ca^{2+} release-activated Ca^{2+} modulator 1 (*Orai1*) gene in the testis of control (Wt) and Tg-RGN animals determined by quantitative RT-PCR (qPCR). *Orai1* gene expression was normalized by the geometric mean of reference genes *Actb* and *B2M* expression and expressed as fold-change comparatively to the control. Error bars indicate mean ± S.E.M (n=5 in each group). Symbols above the bars indicate significant differences compared to the control by one-way ANOVA: * $p<0.05$ and ** $p<0.01$ (A). Individual relative expression values obtained for the samples of each group are also shown (B).

Considering ORAI1 function, it is clear that it is a powerful regulator of cytosolic Ca^{2+} concentration, contributing to Ca^{2+} homeostasis and signaling. The importance of Ca^{2+} channels to male reproduction is emphasized, for example, by the fact that administration of certain Ca^{2+} channel blockers results in reversible infertility (38–42). RGN actions were already associated with the ability to regulate the expression and activity of CaSR (73), L-Type Ca^{2+} channels (86), Ca^{2+}-ATPase (126,127) and Ca^{2+}-Mg^{2+}-ATPase action (128,129), but this is the first time that RGN actions are linked with SOCE ORAI1 Ca^{2+} channels. Recently, the relevance of ORAI1 Ca^{2+} channels to spermatogenesis was demonstrated. SeT cells, including spermatogenic cells, have SOCE activity, which is lost in *Orai1*$^{-/-}$ male mice (292). These *Orai1* knockout mice are infertile and have severe defects in spermatogenesis, owing to a defect in spermatid maturation, most obvious at the late stages of development of elongated spermatids, and progressive degeneration and atrophy of the SeT of the testis (292). Therefore, the RGN up-regulation of *Orai1* corroborates its role as a strong candidate in the regulation of intracellular Ca^{2+} in the testis, which may have a positive impact in male fertility. Interestingly, *Orai1* also seems to be up-regulated by the activation of membrane androgen receptors (293) and was linked with apoptosis resistance in previous studies (290,291,294), although it seems to have dual pro and anti-apoptotic effects depending on the pathology. Thus, *Orai1* overexpression may also be involved in the anti-apoptotic effects observed in Tg-RGN rats (110,111), but further research on this mechanism is needed to support this hypothesis.

4.3.6 *Plcb1*

The production of the second messenger molecules inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) from phosphatidylinositol 4,5-bisphosphate (PIP2) is catalyzed by phospholipase C beta, which is activated by G-proteins (295,296). This reaction is a key step in the intracellular transduction of many extracellular signals, including neurotransmitters and hormones, considering that second messengers IP3 and DAG induce the release of Ca^{2+} from

intracellular stores and the activation of protein kinase C, respectively, eliciting several biological responses, including cell proliferation, differentiation, survival and secretion (295,296).

The *Plcb1* gene encodes the phospholipase C beta 1 isoform (PLCB1), which is of particular relevance due to its nuclear localization in addition to its presence at the plasma membrane (297). PLCB1 has been associated with an important role in cell differentiation, particularly in myogenesis, osteogenesis and hematopoiesis (298).

In the RNA-seq analysis, *Plcb1* gene exhibited approximately 6 fold-change in the testis of Tg-RGN rats compared with Wt. The gene was associated to several GO and KEGG terms, such as GO-BP groups "cellular response to cytokine stimulus", "insulin-like growth factor receptor signaling pathway" and "exocytosis", and KEGG groups "renin secretion" and "aldosterone synthesis and secretion", among the lists of global differentially expressed genes and up-regulated genes.

qPCR analysis of the *Plcb1* gene was performed with a reaction efficiency of 91% and R^2=0.998, but no statistically significant difference was found for *Plcb1* relative expression between control and Tg-RGN group in this analysis (Figure 18A). High individual variability was detected both in the control and Tg-RGN group (Figure 18B).

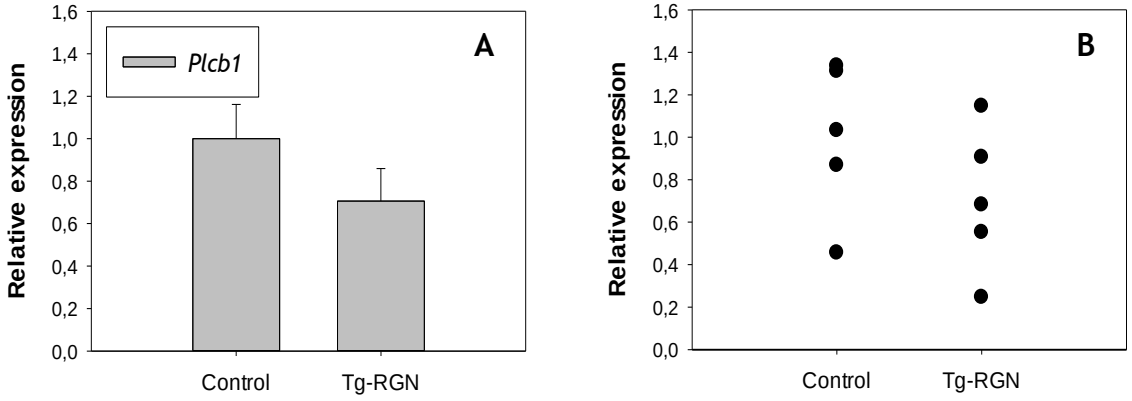

Figure 18 - Relative expression of phospholipase C beta 1 (*Plcb1*) gene in the testis of control (Wt) and Tg-RGN animals determined by quantitative RT-PCR (qPCR). *Plcb1* gene expression was normalized by the geometric mean of reference genes *Actb* and *B2M* expression and expressed as fold-change comparatively to the control. Error bars indicate mean ± S.E.M (n=5 in each group). Symbols above the bars indicate significant differences compared to the control by one-way ANOVA: * $p<0.05$ and ** $p<0.01$ (A). Individual relative expression values obtained for the samples of each group are also shown (B).

The modulation of *Plcb1* by RGN would be an interesting mechanism to explore, considering the importance of phospholipase C in several intracellular signaling pathways. Furthermore, in the case of differential expression validation, PLCB1 action might have been associated with ORAI1 action in the Tg-RGN rat, considering that upon activation of phospholipase C by various cell surface receptors, such as G-protein-coupled receptors, IP3 provokes Ca^{2+} release from the

endoplasmic reticulum, which could lead to the activation of STIM1 and consequent SOCE activity (287,288).

4.3.7 *Sfrp2*

This gene encodes a member of the secreted frizzled related proteins (SFRP) family, which are proteins that contain a cysteine-rich domain homologous to the putative wingless-related MMTV integration site protein (WNT)-binding site of Frizzled proteins (299). SFRPs are normally considered as antagonist of WNT signaling pathway, since by interacting with WNT proteins they prevent the latter from binding to Frizzled receptor, interrupting the initial step of the pathway from occurring. However, studies show that some of these proteins are also capable of activating or have synergy with the WNT signaling pathway, and as such, they appear to have a tissue specific effect (300,301).

Nevertheless, it is clear that SFRP2 can modulate WNT signaling through direct interaction with WNT proteins (299,301). The WNT signaling constitutes a complex biological pathway, which is involved in the development of a variety of cell populations and has the potential to promote or revert apoptotic injury, depending on the specific cellular influences to which it is subject (302). It regulates important aspects of cell fate determination, growth, differentiation, migration, polarity, neural patterning and organogenesis during embryonic development (303). Furthermore, WNT expression in testis somatic and germ cells has been demonstrated to be required for spermatogenesis in several recent studies (304–309). *Sfrp2* specifically has also been shown to be required for normal male embryonic sexual development in mice (310).

Sfrp2 was approximately 30x up-regulated in the testis of Tg-RGN as showed by RNA-seq analysis. This gene was associated with several GO-BP enriched groups, such as “collagen fibril organization”, “regulation of vasculature development” and “positive regulation of development process” among the list of all differentially expressed genes.

Sfrp2 qPCR analysis was performed with a reaction efficiency of 92% and R^2=0.998 and a statistically significant increase in relative expression in Tg-RGN testis, with 1.90 ± 0.25 expression relative to the control ($p<0.05$; Figure 19A) was identified. However, the fold-change values of up-regulation detected through qPCR were lower than those detected by RNA-seq, with some individual variability being observed in both groups (Figure 19B).

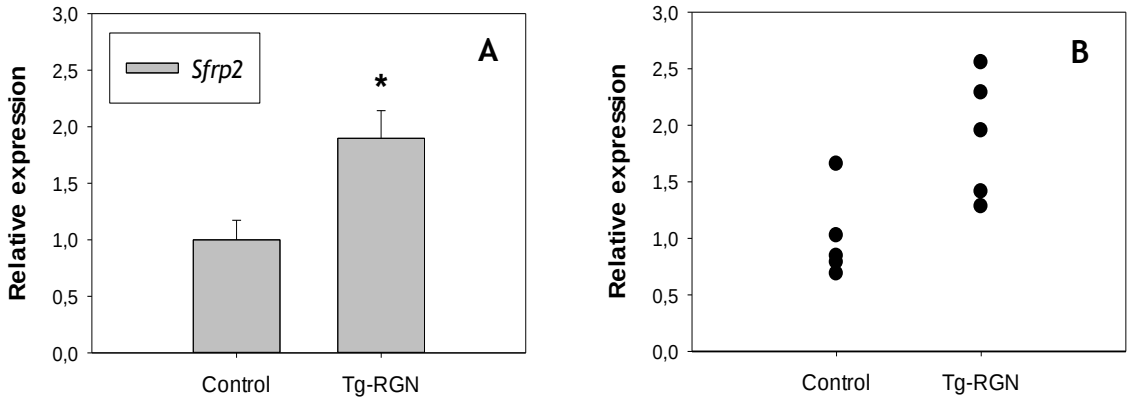

Figure 19 - Relative expression of secreted frizzled-related protein 2 (*Sfrp2*) gene in the testis of control (Wt) and Tg-RGN animals determined by quantitative RT-PCR (qPCR). *Sfrp2* gene expression was normalized by the geometric mean of reference genes *Actb* and *B2M* expression and expressed as fold-change comparatively to the control. Error bars indicate mean ± S.E.M (n=5 in each group). Symbols above the bars indicate significant differences compared to the control by one-way ANOVA: * $p<0.05$ and ** $p<0.01$ (A). Individual relative expression values obtained for the samples of each group are also shown (B).

RGN overexpression has been associated with anti-apoptotic and anti-oxidant effects (110,111), which was proposed to contribute to increased sperm viability and normal sperm morphology (102). In this context, the up-regulation of *Sfrp2* may be a relevant mechanism in the aforementioned functions of RGN, considering that SFRP2 is associated with proliferation and apoptosis signaling. A careful balance between apoptosis and proliferation is fundamental for spermatogenesis, maintaining the proper ratio between developing germ cell and somatic supporting cells (13).

Regarding SFRP2 potential action in proliferation, it was already known that the WNT signaling pathway was involved in the self-renewal of stem cells (311), but recently it has also been proven that it is essential for the development and proliferation of primordial germ cells (307,309). Overactivation of WNT signaling leads to defective spermatogenesis due to altered proliferation and differentiation of germ cells (307,309). Therefore, SFRP2 action as an antagonist may serve as a regulator that maintains a balanced WNT signaling in mammalian spermatogenesis, indirectly regulating germ cell proliferation. Interestingly, *Sfrp2* was shown to be differentially expressed in type B spermatogonia and primary spermatocytes, which also suggest a role for this gene in the regulation of the early stages of spermatogenesis, probably promoting cell differentiation (312).

WNT signaling is complex due to the existence of canonical and non-canonical pathways by which it acts, which according to specific cellular environment stimuli can either foster or restrain the processes of apoptosis (313). In this context, the fact that SFRP2 modulation also appears to cause opposite effects on apoptosis depending on the cellular environment is understandable. For instance, SFRP2 overexpression has been shown to inhibit apoptosis in rat cardiomyoblasts (314), canine mammary gland tumors (315) and human hypertrophic scars (316) but was associated with increased apoptotic injury during degenerative disease processes such

as retinitis pigmentosa (317). Moreover, SFRP2 also appears to promote cell survival in human mesenchymal stem cells under oxidative stress (318), suggesting a potential role for this protein in oxidative stress responses. Thus, RGN up-regulation of *Sfrp2* is very interesting, being a likely molecular mechanism behind the RGN' anti-proliferative, anti-apoptotic and antioxidant actions. Further research is warranted exploring the complexity of SFRP2 and WNT actions in the context of interaction with RGN.

4.3.8 *Star*

In all steroidogenic tissues, regardless of the hormones synthesized, the initial step in steroidogenesis is the conversion of cholesterol to pregnenolone. The *Star* gene encodes a key player in the acute regulation of steroid hormone synthesis, the steroidogenic acute regulatory protein (STAR), whose function is mediating the transport of cholesterol from the outer to the inner mitochondrial membrane where cholesterol is cleaved into pregnenolone. This is the rate limiting step in hormone-dependent steroidogenesis, being fundamental for the production of all steroid hormones (319). Interestingly, *Star* expression is regulated by several factors, one of which is intracellular Ca^{2+} levels (191,320).

An up regulation of approximately 3x was detected for *Star* in the testis of Tg-RGN rats, by RNA-seq analysis. This gene was associated to several GO-BP groups, namely "dibenzo-p-dioxin metabolic process", "regulation of hormone levels", "response to cytokine", and to KEGG group "aldosterone synthesis and secretion" among the lists of global differentially expressed and up-regulated genes.

Star gene qPCR analysis was performed with a reaction efficiency of 107% and R^2=0.996 and no statistically significant differences were detected between control and Tg-RGN group (Figure 20A), with high individual variability being mostly observed in the Tg-RGN group (Figure 20B).

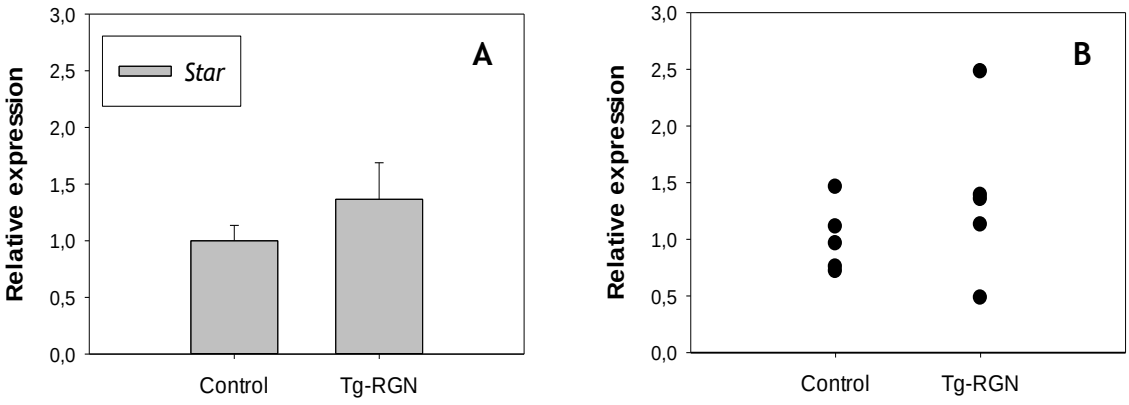

Figure 20 - Relative expression of steroidogenic acute regulatory protein (*Star*) gene in the testis of control (Wt) and Tg-RGN animals determined by quantitative RT-PCR (qPCR). *Star* gene expression was normalized by the geometric mean of reference genes *Actb* and *B2M* expression and expressed as fold-change comparatively to the control. Error bars indicate mean ± S.E.M (n=5 in each group). Symbols above the bars indicate significant differences compared to the control by one-way ANOVA: * $p<0.05$ and ** $p<0.01$ (A). Individual relative expression values obtained for the samples of each group are also shown (B).

Although no significant up-regulation was validated by qPCR, *Star* expression seems to be slightly increased in the Tg-RGN group, which could be confirmed if more animals were included. The modulation of the *Star* gene by RGN overexpression indicates the potential influence of RGN in LCs steroidogenesis, which is fundamental for the hormonal regulation of spermatogenesis and germ cell survival (14).

4.3.9 *Sycp1*

Sycp1 encodes the major component of the transverse filaments of synaptonemal complexes, which are formed between homologous chromosomes during meiotic prophase, being required for chromosome pairing, synapsis and recombination (321–323). The encoded protein ensures the normal assembly of the central element of synaptonemal complexes, preventing cell death, impaired meiotic recombination and aneuploidy (324).

A 3 fold-change decrease was detected in the testis of Tg-RGN rats comparatively to control, by RNA-seq. In the enrichment analysis, *Sycp1* gene was associated to "meiotic cell cycle" GO-BP group among both the lists of global differentially expressed genes and of down-regulated genes.

The qPCR analysis for the *Sycp1* gene was performed with a reaction efficiency of 88% and R^2=0.998 and a statistically significant down-regulation was detected in the Tg-RGN group, with 0.28 ± 0.12 of expression relative to the control ($p<0.05$; Figure 21A). The detected fold-change value of approximately 3-fold was similar to RNA-seq results, being in accordance with this data. Individual variability was mostly observed in the control group (Figure 21B).

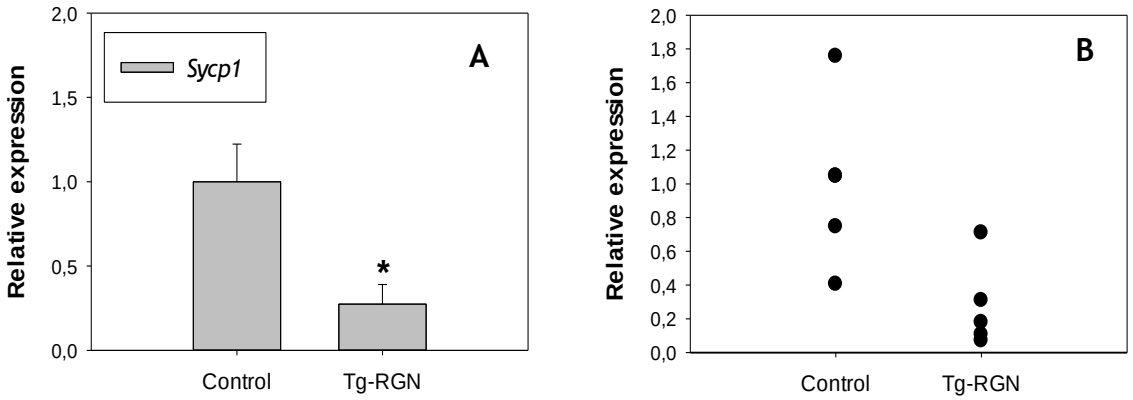

Figure 21 - Relative expression of synaptonemal complex protein 1 (*Sycp1*) gene in the testis of control (Wt) and Tg-RGN animals determined by quantitative RT-PCR (qPCR). *Sycp1* gene expression was normalized by the geometric mean of reference genes *Actb* and *B2M* expression and expressed as fold-change comparatively to the control. Error bars indicate mean ± S.E.M (n=5 in each group). Symbols above the bars indicate significant differences compared to the control by one-way ANOVA: * $p<0.05$ and ** $p<0.01$ (A). Individual relative expression values obtained for the samples of each group are also shown (B).

Knockout studies show that *Sycp1*$^{-/-}$ animals are sterile (322–324), presenting testis where spermatocyte stages beyond diplonema are rare and post-meiotic spermatogenic cells

(spermatids and spermatozoa) are completely lacking (322). Therefore, *Sycp1* expression is fundamental for fertility, allowing the development of normal gametes through successful meiosis (322–324). Considering the importance of SYCP1 to meiosis and consequently to spermatogenesis, the observed significant down-regulation of *Sycp1* in Tg-RGN animals agrees with the previously mentioned down-regulation of *Fign* (section 4.3.3). This further supports the theory that the mitotic and meiotic processes are affected in Tg-RGN rats, which may delay spermatozoa production and result in lower sperm counts, as already shown in these animals (102). However, since the Tg-RGN rats are fertile (102), RGN cytoprotective mechanisms appear to be able to compensate the down-regulation of meiotic cell cycle-associated genes and the reduced sperm counts.

4.3.10 *Tnni1*

The *Tnni1* gene is responsible for encoding troponin I1, a slow skeletal type protein and one of the 3 subunits of the troponin complex (along with troponin T and troponin C). This is a Ca^{2+}-sensitive molecular switch that regulates striated muscle contraction in response to alterations in intracellular Ca^{2+} levels (325). Troponin I is the inhibitory subunit of the troponin complex, blocking actin-myosin interaction by binding to the actin filament at low Ca^{2+} concentration, and thus preventing muscle contraction and promoting relaxation. At high Ca^{2+} concentrations, binding of Ca^{2+} to the troponin C subunit induces dissociation of troponin I from actin and allows actomyosin contraction and myosin ATPase activation (325,326).

Tnni1 was associated to GO-BP enriched terms related with cardiac and striated muscle contraction among the list of up-regulated genes and was identified as approximately 16x up-regulated in the Tg-RGN group in the RNA-seq analysis.

qPCR analysis for *Tnni1* was performed with a reaction efficiency of 90% and R^2=0.994. A statistically significant up-regulation was found between the control and Tg-RGN group with 9.81 ± 2.51 of fold change in the testis of the transgenic group ($p<0.01$; Figure 22A). This up-regulation was in accordance with RNA-seq data, although qPCR analysis detected a slightly lower fold-change value between groups. Individual variability was observed in the Tg-RGN group, with one clear outlier (Figure 22B).

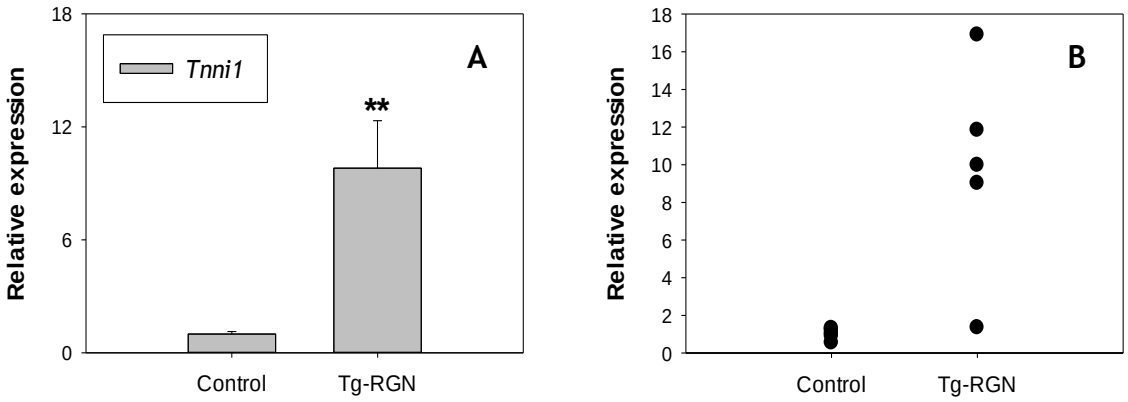

Figure 22 - Relative expression of troponin I1, slow skeletal type (*Tnni1*) gene in the testis of control (Wt) and Tg-RGN animals determined by quantitative RT-PCR (qPCR). *Tnni1* gene expression was normalized by the geometric mean of reference genes *Actb* and *B2M* expression and expressed as fold-change comparatively to the control. Error bars indicate mean ± S.E.M (n=5 in each group). Symbols above the bars indicate significant differences compared to the control by one-way ANOVA: * $p<0.05$ and ** $p<0.01$ (A). Individual relative expression values obtained for the samples of each group are also shown (B).

No available literature has linked the *Tnni1* gene with spermatogenesis. However, TNNI1 (encoded by *Tnni1*) is known to be expressed in the testis (327) and its altered expression has been associated with testicular atrophy (328), but further insights into its role in the male gonad still are needed. Considering the Ca^{2+}-modulation of TNNI1 activity, RGN overexpression in the Tg-RGN rat testis may be stimulating the activity of troponin I due to lower intracellular Ca^{2+} concentrations, thus preventing muscle contraction by inhibiting Ca^{2+}-mediated conformational changes in actin-myosin complexes (325). *Tnni1* relevance to spermatogenesis is not immediately apparent, but troponin I was shown to control ovulatory contraction of non-striated actomyosin networks in the *C. elegans* somatic gonad (329). It would be interesting to investigate the possibility of TNNI1 involvement, and consequently of RGN, in the control of the SeT peristaltic contractions, which are thought to facilitate spermiation, the transport of non-motile spermatozoa to the epididymis after spermatogenesis (11).

4.4 Potential RGN molecular partners are expressed in GC-6spg cells and SCs

The differential gene expression in the testis of Tg-RGN rats and enrichment analysis allowed to associate the selected/validated genes with the possible RGN roles in testicular physiology and in spermatogenesis. However, their precise individual contributions are still unclear, considering that spermatogenesis is a complex process depending on the action and regulation of diverse cell populations (8). In the SeT, the local where spermatogenesis occurs, different cell types, including both somatic and germ cells, namely SCs, spermatogonia, spermatocytes, spermatids and spermatozoa, interact in a cooperative way that establishes the spermatogenic output (8). Therefore, due to its cellular diversity the testis exhibits a relatively complex transcriptome in comparison with other organs (330). This transcriptomic complexity was shown to decrease as spermatogenesis proceeds, with spermatogonia showing the highest

complexity (4). Changes in gene expression in the testis of Tg-RGN rats may reflect changes in transcription in a specific cell type of the testis.

To further explore the hypothesis of the involvement of the selected genes in spermatogenesis, their expression in rat GC-6spg cells (cell line with SSCs characteristics) and SCs was determined by RT-PCR analysis. These two cell types were chosen as representatives of germ and somatic cells and because both are fundamental for normal spermatogenesis development.

RT-PCR analysis showed that *Eng, Fign, Orai1, Star, Sycp1* and *Tnni1* genes were expressed in both GC-6spg and SCs, whereas *Lum, Plcb1* and *Sfrp2* were only detectable in SCs. *Atp10b* was not detected in any of these cell populations (Table 6). Electrophoresis agarose gel images displaying these findings are included in the Appendixes (Appendix II, Supplemental figure S1 to S3).

Table 6 – Expression of the selected genes from the RNA-seq analysis in GC-6spg cells and SCs determined by RT-PCR analysis.

Gene symbol	GC-6spg	SCs
Atp10b	–	–
Eng	+	+
Fign	+	+
Lum	–	+
Orai1	+	+
Plcb1	–	+
Sfrp2	–	+
Star	+	+
Sycp1	+	+
Tnni1	+	+

Legend: + or − indicate, respectively, presence or absence of signal in RT-PCR.

Previous studies had already demonstrated the expression of the proteins encoded by the 10 selected genes in the testis (information available in The Human Protein Atlas (327)), but this is the first time the presence of *Atp10b*, *Eng*, *Fign*, *Lum*, *Orai1*, *Plcb1*, *Sfrp2*, *Star*, *Sycp1* and *Tnni1* was analyzed in the GC-6spg cell line.

Interestingly, with the exception of *Atp10b* and *Fign* (for which less information was available), all selected genes had been previously shown to be expressed in SCs and in all germ cells (information available from project (331) in the Genevestigator software). Our results showed that *Fign* is also expressed in the SCs and corroborated the SCS results for the remaining genes.

However, *Atp10b* was not found in SCs, nor in GC-6spg, which may suggest that this gene is expressed in other testis cell types, but the negative results may also be due to low sensibility of the RT-PCR method in detecting low expression levels, considering that *Atp10b* showed low expression in the qPCR analysis, especially in the Wt group.

The demonstrated expression of some of the 10 selected genes in GC-6spg and SCs, as well as previous information of expression in other testis cell types, further supports their involvement in the spermatogenic process and the hypothesis that they are potential molecular partners of RGN cytoprotective action in spermatogenesis. However, additional research of each individual gene is needed to validate this hypothesis.

5. Conclusions and Future Perspectives

RGN had been previously identified as an androgen-target that is broadly expressed in the male reproductive tract, including in both somatic and germ testicular cells. Making use of the Tg-RGN rat model it was shown that RGN can be associated with increased sperm viability with lower incidence of tail defects, as well as with resistance to oxidative stress, and chemical- or radiation-induced apoptosis of testicular cells. Thus, it was hypothesized that RGN is involved in spermatogenesis, protecting cells against noxious stimuli. Spermatogenesis is a complex biological process that is the basis of male fertility, and highly susceptible to endogenous or exogenous damage. The understanding of its molecular control and the discovery of potential cytoprotective agents against damaging factors is fundamental for the development of new and more efficient fertility-preserving strategies and treatments.

The present dissertation first characterized the testicular transcriptome of Tg-RGN rats, evidencing the broad range of molecular players and biological pathways that may underlie the RGN actions in the testis. Herein, it was demonstrated that the testicular transcriptome of Tg-RGN rats differs greatly from that of Wt *R. norvegicus* with 1064 genes showing significant changes at transcription level with at least 2 fold-variation. The multiplicity of different transcripts abundance found in the Tg-RGN testis highlights to the complexity of its response and supports the notion of RGN involvement in the testicular dynamics. The enrichment analysis also showed that the differentially expressed genes are involved in several biological processes and pathways of testis physiology, some of them known to be linked with the spermatogenic process, such as Ca^{2+} transport, homeostasis and signaling, dibenzo-p-dioxin metabolism, hormone regulation, meiotic cell cycle, homologous recombination, integrin activation, and others.

From ten selected genes with relevance to spermatogenesis, six (namely *Atp10b*, *Fign*, *Orai1*, *Sfrp2, Sycp1* and *Tnni1*) were confirmed to have significant differential expression in the Tg-RGN testis by qPCR, which indicates them as potential molecular partners of RGN actions in spermatogenesis. Furthermore, these genes, except for *Atp10b and Sfrp2,* were also confirmed to be expressed both in SCs in GC-6spg, which further supports their involvement in male reproduction, considering that these cell types are crucial and at the basis of normal spermatogenesis development.

However, more research is warranted, considering that transcriptomic studies are based on altered levels of mRNA and assume parallel, but not confirmed, changes in protein abundance, and because the testis is an heterogenous organ with several cellular types, any of which can produce the observed expression profiles. Therefore, the next steps should be focused at the proteomics level, and disclose which testis cell types are accounting for the differential expression profile observed. Moreover, other mandatory future research goal should explore the association of each differentially expressed gene with the RGN cytoprotective actions in the testis.

Nevertheless, this study showed that RGN modulates the expression of genes (and probably the activity of proteins) involved in spermatogenesis, giving a new insight into the molecular mechanisms behind RGN' actions in the testis and providing valuable transcriptomic data that can serve as a basis for future investigation in spermatogenesis and male infertility, considering the potential use of RGN as a citoprotector factor in male reproduction.

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Appendixes

Appendix I - Enrichment analysis

Supplementary table S1 - Functionally related networks (groups and terms) of Gene Ontology – Biological Processes (GO-BP) enriched among the list of all 1064 differentially expressed genes in Tg-RGN testis, using ClueGO/Cytoscape. GO-BP enriched groups are sorted by highest enrichment score (calculated as - Log2 (group FDR)), and inside each group GO-BP terms are sorted by the lowest Term FDR, representing lower p-value. The most significant term of each group is indicated in bold.

GO ID	GO Term	Term FDR	Group FDR	Enrichment Score	GO Groups	% Genes	Nr. Genes
GO:0030001	**metal ion transport**	7.5E-3	290.0E-6	11.8	24	5.85	44
GO:0006811	ion transport	10.0E-3	290.0E-6	11.8	24	4.61	66
GO:0006812	cation transport	10.0E-3	290.0E-6	11.8	24	5.13	52
GO:0019932	second-messenger-mediated signaling	11.0E-3	290.0E-6	11.8	24	8.00	16
GO:0019722	calcium-mediated signaling	11.0E-3	290.0E-6	11.8	24	9.92	12
GO:0072511	divalent inorganic cation transport	11.0E-3	290.0E-6	11.8	24	6.25	25
GO:0051928	positive regulation of calcium ion transport	11.0E-3	290.0E-6	11.8	24	9.91	11
GO:0006816	calcium ion transport	11.0E-3	290.0E-6	11.8	24	7.10	25
GO:0070838	divalent metal ion transport	12.0E-3	290.0E-6	11.8	24	6.30	25
GO:0048878	chemical homeostasis	25.0E-3	290.0E-6	11.8	24	4.69	46
GO:0098655	cation transmembrane transport	26.0E-3	290.0E-6	11.8	24	5.12	35
GO:0051924	regulation of calcium ion transport	26.0E-3	290.0E-6	11.8	24	7.18	15
GO:0090279	regulation of calcium ion import	26.0E-3	290.0E-6	11.8	24	9.47	9
GO:0050848	regulation of calcium-mediated signaling	27.0E-3	290.0E-6	11.8	24	11.67	7
GO:0051282	regulation of sequestering of calcium ion	29.0E-3	290.0E-6	11.8	24	9.18	9
GO:0051283	negative regulation of sequestering of calcium ion	29.0E-3	290.0E-6	11.8	24	9.28	9
GO:2001257	regulation of cation channel activity	29.0E-3	290.0E-6	11.8	24	9.88	8
GO:0070588	calcium ion transmembrane transport	29.0E-3	290.0E-6	11.8	24	6.58	16
GO:0051209	release of sequestered calcium ion into cytosol	29.0E-3	290.0E-6	11.8	24	9.28	9
GO:0097553	calcium ion transmembrane import into cytosol	30.0E-3	290.0E-6	11.8	24	9.09	9

GO:0051208	sequestering of calcium ion	30.0E-3	290.0E-6	11.8	24	9.09	9
GO:1902656	calcium ion import into cytosol	30.0E-3	290.0E-6	11.8	24	9.09	9
GO:0055074	calcium ion homeostasis	32.0E-3	290.0E-6	11.8	24	5.71	22
GO:0098662	inorganic cation transmembrane transport	35.0E-3	290.0E-6	11.8	24	5.06	31
GO:0051238	sequestering of metal ion	38.0E-3	290.0E-6	11.8	24	8.33	9
GO:0043270	positive regulation of ion transport	38.0E-3	290.0E-6	11.8	24	6.23	16
GO:2001259	positive regulation of cation channel activity	38.0E-3	290.0E-6	11.8	24	13.16	5
GO:0006874	cellular calcium ion homeostasis	38.0E-3	290.0E-6	11.8	24	5.61	21
GO:0051279	regulation of release of sequestered calcium ion into cytosol	39.0E-3	290.0E-6	11.8	24	10.00	7
GO:0032845	negative regulation of homeostatic process	39.0E-3	290.0E-6	11.8	24	7.14	12
GO:0090280	positive regulation of calcium ion import	40.0E-3	290.0E-6	11.8	24	10.91	6
GO:0098660	inorganic ion transmembrane transport	43.0E-3	290.0E-6	11.8	24	4.77	33
GO:0034767	positive regulation of ion transmembrane transport	44.0E-3	290.0E-6	11.8	24	7.52	10
GO:0072507	divalent inorganic cation homeostasis	44.0E-3	290.0E-6	11.8	24	5.30	22
GO:1903169	regulation of calcium ion transmembrane transport	45.0E-3	290.0E-6	11.8	24	7.83	9
GO:0034765	regulation of ion transmembrane transport	46.0E-3	290.0E-6	11.8	24	5.36	21
GO:1904062	regulation of cation transmembrane transport	47.0E-3	290.0E-6	11.8	24	6.40	13
GO:0034764	positive regulation of transmembrane transport	49.0E-3	290.0E-6	11.8	24	7.19	10
GO:0050801	ion homeostasis	49.0E-3	290.0E-6	11.8	24	4.73	31
GO:0072503	cellular divalent inorganic cation homeostasis	49.0E-3	290.0E-6	11.8	24	5.29	21
GO:0018894	**dibenzo-p-dioxin metabolic process**	9.9E-3	460.0E-6	11.1	23	41.67	5
GO:0018879	biphenyl metabolic process	10.0E-3	460.0E-6	11.1	23	36.36	4
GO:0071320	cellular response to cAMP	10.0E-3	460.0E-6	11.1	23	13.85	9

GO:0017085	response to insecticide	11.0E-3	460.0E-6	11.1	23	21.74	5
GO:0051591	response to cAMP	11.0E-3	460.0E-6	11.1	23	9.42	13
GO:0006702	androgen biosynthetic process	11.0E-3	460.0E-6	11.1	23	33.33	5
GO:0060992	response to fungicide	12.0E-3	460.0E-6	11.1	23	23.81	5
GO:0014074	response to purine-containing compound	12.0E-3	460.0E-6	11.1	23	7.84	16
GO:0046683	response to organophosphorus	14.0E-3	460.0E-6	11.1	23	8.14	14
GO:0009635	response to herbicide	22.0E-3	460.0E-6	11.1	23	23.53	4
GO:0018963	phthalate metabolic process	22.0E-3	460.0E-6	11.1	23	37.50	3
GO:0018958	phenol-containing compound metabolic process	26.0E-3	460.0E-6	11.1	23	9.57	9
GO:0071774	response to fibroblast growth factor	26.0E-3	460.0E-6	11.1	23	9.01	10
GO:0008209	androgen metabolic process	26.0E-3	460.0E-6	11.1	23	16.67	5
GO:0061370	testosterone biosynthetic process	27.0E-3	460.0E-6	11.1	23	33.33	3
GO:0044344	cellular response to fibroblast growth factor stimulus	34.0E-3	460.0E-6	11.1	23	8.82	9
GO:0043649	dicarboxylic acid catabolic process	34.0E-3	460.0E-6	11.1	23	27.27	3
GO:0019748	secondary metabolic process	37.0E-3	460.0E-6	11.1	23	11.54	6
GO:0008211	glucocorticoid metabolic process	37.0E-3	460.0E-6	11.1	23	17.39	4
GO:0071363	cellular response to growth factor stimulus	38.0E-3	460.0E-6	11.1	23	5.03	29
GO:0070848	response to growth factor	42.0E-3	460.0E-6	11.1	23	4.91	30
GO:0042446	hormone biosynthetic process	44.0E-3	460.0E-6	11.1	23	10.34	6
GO:0043648	dicarboxylic acid metabolic process	45.0E-3	460.0E-6	11.1	23	8.51	8
GO:0071222	cellular response to lipopolysaccharide	49.0E-3	460.0E-6	11.1	23	6.83	11
GO:0051321	**meiotic cell cycle**	10.0E-3	570.0E-6	10.8	21	9.14	16
GO:0007126	meiotic nuclear division	10.0E-3	570.0E-6	10.8	21	9.79	14
GO:1903046	meiotic cell cycle process	11.0E-3	570.0E-6	10.8	21	9.27	14
GO:0040020	regulation of meiotic nuclear division	19.0E-3	570.0E-6	10.8	21	18.52	5
GO:2000243	positive regulation of reproductive process	21.0E-3	570.0E-6	10.8	21	12.73	7

GO:0007127	meiosis I	26.0E-3	570.0E-6	10.8	21	10.53	8
GO:0007131	reciprocal meiotic recombination	28.0E-3	570.0E-6	10.8	21	15.63	5
GO:0035825	reciprocal DNA recombination	28.0E-3	570.0E-6	10.8	21	15.63	5
GO:0045836	positive regulation of meiotic nuclear division	34.0E-3	570.0E-6	10.8	21	27.27	3
GO:0051445	regulation of meiotic cell cycle	40.0E-3	570.0E-6	10.8	21	12.82	5
GO:0051446	positive regulation of meiotic cell cycle	40.0E-3	570.0E-6	10.8	21	23.08	3
GO:2000241	regulation of reproductive process	44.0E-3	570.0E-6	10.8	21	7.52	10
GO:0007130	synaptonemal complex assembly	48.0E-3	570.0E-6	10.8	21	20.00	3
GO:0033623	**regulation of integrin activation**	9.0E-3	610.0E-6	10.7	17	40.00	4
GO:0033622	integrin activation	11.0E-3	610.0E-6	10.7	17	29.41	5
GO:0033625	positive regulation of integrin activation	11.0E-3	610.0E-6	10.7	17	60.00	3
GO:0033634	positive regulation of cell-cell adhesion mediated by integrin	11.0E-3	610.0E-6	10.7	17	60.00	3
GO:0033630	positive regulation of cell adhesion mediated by integrin	22.0E-3	610.0E-6	10.7	17	23.53	4
GO:0033632	regulation of cell-cell adhesion mediated by integrin	27.0E-3	610.0E-6	10.7	17	33.33	3
GO:0033631	cell-cell adhesion mediated by integrin	40.0E-3	610.0E-6	10.7	17	23.08	3
GO:0018894	**dibenzo-p-dioxin metabolic process**	9.9E-3	1.9E-3	9.0	20	41.67	5
GO:0002237	response to molecule of bacterial origin	11.0E-3	1.9E-3	9.0	20	6.43	25
GO:0006702	androgen biosynthetic process	11.0E-3	1.9E-3	9.0	20	33.33	5
GO:0032496	response to lipopolysaccharide	20.0E-3	1.9E-3	9.0	20	6.15	23
GO:0018963	phthalate metabolic process	22.0E-3	1.9E-3	9.0	20	37.50	3
GO:0008209	androgen metabolic process	26.0E-3	1.9E-3	9.0	20	16.67	5
GO:0071347	cellular response to interleukin-1	34.0E-3	1.9E-3	9.0	20	8.82	9
GO:0071396	cellular response to lipid	37.0E-3	1.9E-3	9.0	20	5.09	29
GO:0042446	hormone biosynthetic process	44.0E-3	1.9E-3	9.0	20	10.34	6

GO:0070555	response to interleukin-1	44.0E-3	1.9E-3	9.0	20	7.46	10
GO:0071222	cellular response to lipopolysaccharide	49.0E-3	1.9E-3	9.0	20	6.83	11
GO:0071345	**cellular response to cytokine stimulus**	10.0E-3	1.9E-3	9.0	16	5.72	31
GO:0071347	cellular response to interleukin-1	34.0E-3	1.9E-3	9.0	16	8.82	9
GO:0070555	response to interleukin-1	44.0E-3	1.9E-3	9.0	16	7.46	10
GO:0045123	cellular extravasation	44.0E-3	1.9E-3	9.0	16	12.20	5
GO:0071222	cellular response to lipopolysaccharide	49.0E-3	1.9E-3	9.0	16	6.83	11
GO:0070813	**hydrogen sulfide metabolic process**	11.0E-3	1.9E-3	9.0	9	60.00	3
GO:0050667	homocysteine metabolic process	37.0E-3	1.9E-3	9.0	9	25.00	3
GO:0071288	**cellular response to mercury ion**	11.0E-3	2.0E-3	9.0	7	50.00	3
GO:1901342	**regulation of vasculature development**	10.0E-3	4.1E-3	7.9	18	7.52	17
GO:1904018	positive regulation of vasculature development	11.0E-3	4.1E-3	7.9	18	9.70	13
GO:0045766	positive regulation of angiogenesis	12.0E-3	4.1E-3	7.9	18	9.76	12
GO:0001944	vasculature development	24.0E-3	4.1E-3	7.9	18	5.43	30
GO:0045765	regulation of angiogenesis	24.0E-3	4.1E-3	7.9	18	7.28	15
GO:0001568	blood vessel development	26.0E-3	4.1E-3	7.9	18	5.41	29
GO:0048514	blood vessel morphogenesis	38.0E-3	4.1E-3	7.9	18	5.36	24
GO:0048009	**insulin-like growth factor receptor signaling pathway**	26.0E-3	5.1E-3	7.6	5	16.13	5
GO:0010759	**positive regulation of macrophage chemotaxis**	29.0E-3	5.5E-3	7.5	19	30.00	3
GO:0002687	positive regulation of leukocyte migration	43.0E-3	5.5E-3	7.5	19	8.04	9
GO:0050920	regulation of chemotaxis	44.0E-3	5.5E-3	7.5	19	6.78	12
GO:0071675	regulation of mononuclear cell migration	44.0E-3	5.5E-3	7.5	19	21.43	3
GO:0002690	positive regulation of leukocyte chemotaxis	44.0E-3	5.5E-3	7.5	19	9.21	7
GO:0010758	regulation of macrophage chemotaxis	48.0E-3	5.5E-3	7.5	19	20.00	3
GO:0050921	positive regulation of chemotaxis	49.0E-3	5.5E-3	7.5	19	7.63	9
GO:0019221	cytokine-mediated signaling pathway	49.0E-3	5.5E-3	7.5	19	5.57	18

GO:0006887	**exocytosis**	11.0E-3	5.5E-3	7.5	11	6.77	21
GO:0032940	secretion by cell	28.0E-3	5.5E-3	7.5	11	4.80	41
GO:0017157	regulation of exocytosis	36.0E-3	5.5E-3	7.5	11	6.95	13
GO:0034097	**response to cytokine**	10.0E-3	5.7E-3	7.5	22	5.50	37
GO:0071345	cellular response to cytokine stimulus	10.0E-3	5.7E-3	7.5	22	5.72	31
GO:0033993	response to lipid	11.0E-3	5.7E-3	7.5	22	4.84	56
GO:0002237	response to molecule of bacterial origin	11.0E-3	5.7E-3	7.5	22	6.43	25
GO:0032496	response to lipopolysaccharide	20.0E-3	5.7E-3	7.5	22	6.15	23
GO:0006954	inflammatory response	26.0E-3	5.7E-3	7.5	22	5.38	29
GO:0001101	response to acid chemical	29.0E-3	5.7E-3	7.5	22	5.63	24
GO:0071347	cellular response to interleukin-1	34.0E-3	5.7E-3	7.5	22	8.82	9
GO:0014070	response to organic cyclic compound	36.0E-3	5.7E-3	7.5	22	4.38	54
GO:0032526	response to retinoic acid	37.0E-3	5.7E-3	7.5	22	8.13	10
GO:0071396	cellular response to lipid	37.0E-3	5.7E-3	7.5	22	5.09	29
GO:1901700	response to oxygen-containing compound	40.0E-3	5.7E-3	7.5	22	4.09	69
GO:0002687	positive regulation of leukocyte migration	43.0E-3	5.7E-3	7.5	22	8.04	9
GO:0019221	cytokine-mediated signaling pathway	49.0E-3	5.7E-3	7.5	22	5.57	18
GO:0071222	cellular response to lipopolysaccharide	49.0E-3	5.7E-3	7.5	22	6.83	11
GO:0030199	**collagen fibril organization**	26.0E-3	5.7E-3	7.5	12	16.13	5
GO:0043062	extracellular structure organization	29.0E-3	5.7E-3	7.5	12	7.34	13
GO:0030198	extracellular matrix organization	29.0E-3	5.7E-3	7.5	12	7.39	13
GO:0044242	**cellular lipid catabolic process**	29.0E-3	5.7E-3	7.5	3	7.34	13
GO:0010817	**regulation of hormone levels**	37.0E-3	6.4E-3	7.3	8	5.34	25
GO:0051094	**positive regulation of developmental process**	37.0E-3	6.6E-3	7.2	6	4.42	51
GO:0060840	**artery development**	39.0E-3	6.7E-3	7.2	1	10.00	7
GO:0042136	**neurotransmitter biosynthetic process**	27.0E-3	6.9E-3	7.2	15	33.33	3
GO:0001505	regulation of neurotransmitter levels	38.0E-3	6.9E-3	7.2	15	6.81	13

GO:0046928	regulation of neurotransmitter secretion	42.0E-3	6.9E-3	7.2	15	10.71	6
GO:0042133	neurotransmitter metabolic process	44.0E-3	6.9E-3	7.2	15	14.81	4
GO:0034284	**response to monosaccharide**	37.0E-3	7.0E-3	7.2	2	6.35	16
GO:0046173	**polyol biosynthetic process**	44.0E-3	8.9E-3	6.8	4	11.90	5
GO:0050954	**sensory perception of mechanical stimulus**	44.0E-3	9.0E-3	6.8	10	7.41	10
GO:0007605	sensory perception of sound	45.0E-3	9.0E-3	6.8	10	7.83	9
GO:0030816	**positive regulation of cAMP metabolic process**	40.0E-3	9.2E-3	6.8	14	9.72	7
GO:0030801	positive regulation of cyclic nucleotide metabolic process	42.0E-3	9.2E-3	6.8	14	8.70	8
GO:0045981	positive regulation of nucleotide metabolic process	44.0E-3	9.2E-3	6.8	14	7.89	9
GO:1900544	positive regulation of purine nucleotide metabolic process	44.0E-3	9.2E-3	6.8	14	7.96	9
GO:0045661	**regulation of myoblast differentiation**	36.0E-3	9.4E-3	6.7	13	11.76	6
GO:0045663	positive regulation of myoblast differentiation	38.0E-3	9.4E-3	6.7	13	16.67	4
GO:0051155	positive regulation of striated muscle cell differentiation	48.0E-3	9.4E-3	6.7	13	10.00	6

Supplementary table S2 - Functionally related networks (groups and terms) of Kyoto Encyclopedia of Gene and Genome (KEGG) pathways enriched among the list of all 1064 differentially expressed genes in Tg-RGN testis, using ClueGO/Cytoscape. KEGG enriched groups are sorted by highest enrichment score (calculated as - Log2 (group FDR)), and inside each group KEGG terms are sorted by the lowest Term FDR, representing lower p-value. The most significant term of each group is indicated in bold.

GO ID	GO Term	Term FDR	Group FDR	Enrichment Score	GO Groups	% Genes	Nr. Genes
GO:0000920	**Sulfur metabolism**	1.0E-3	59.0E-6	14.0	2	38.46	5
GO:0004924	**Renin secretion**	1.9E-3	110.0E-6	13.2	3	13.85	9
GO:0004925	**Aldosterone synthesis and secretion**	42.0E-3	2.4E-3	8.7	4	9.52	8
GO:0000430	**Taurine and hypotaurine metabolism**	46.0E-3	2.7E-3	8.5	1	27.27	3

Supplementary table S3 - Functionally related networks (groups and terms) of Gene Ontology – Biological Processes (GO-BP) enriched among the list of the 350 up-regulated genes in Tg-RGN testis, using ClueGO/Cytoscape. GO-BP enriched groups are sorted by highest enrichment score (calculated as -Log2 (group FDR)), and inside each group GO-BP terms are sorted by the lowest Term FDR, representing lower p-value. The most significant term of each group is indicated in bold.

GO ID	GO Term	Term FDR	Group FDR	Enrichment Score	GO Groups	% Genes	Nr. Genes
GO:0051928	**positive regulation of calcium ion transport**	230.0E-6	780.0E-9	20.3	37	9.01	10
GO:0006816	calcium ion transport	270.0E-6	780.0E-9	20.3	37	5.11	18
GO:0072511	divalent inorganic cation transport	400.0E-6	780.0E-9	20.3	37	4.50	18
GO:0070838	divalent metal ion transport	480.0E-6	780.0E-9	20.3	37	4.53	18
GO:0051924	regulation of calcium ion transport	730.0E-6	780.0E-9	20.3	37	5.74	12
GO:0090279	regulation of calcium ion import	880.0E-6	780.0E-9	20.3	37	8.42	8
GO:0055074	calcium ion homeostasis	950.0E-6	780.0E-9	20.3	37	4.16	16
GO:0090280	positive regulation of calcium ion import	1.0E-3	780.0E-9	20.3	37	10.91	6
GO:0043270	positive regulation of ion transport	1.8E-3	780.0E-9	20.3	37	4.67	12
GO:0050921	positive regulation of chemotaxis	1.9E-3	780.0E-9	20.3	37	6.78	8
GO:0019722	calcium-mediated signaling	1.9E-3	780.0E-9	20.3	37	6.61	8
GO:0006874	cellular calcium ion homeostasis	1.9E-3	780.0E-9	20.3	37	4.01	15
GO:0097553	calcium ion transmembrane import into cytosol	2.0E-3	780.0E-9	20.3	37	7.07	7
GO:0051282	regulation of sequestering of calcium ion	2.0E-3	780.0E-9	20.3	37	7.14	7
GO:0051208	sequestering of calcium ion	2.0E-3	780.0E-9	20.3	37	7.07	7
GO:0051283	negative regulation of sequestering of calcium ion	2.0E-3	780.0E-9	20.3	37	7.22	7
GO:0051209	release of sequestered calcium ion into cytosol	2.0E-3	780.0E-9	20.3	37	7.22	7
GO:1902656	calcium ion import into cytosol	2.0E-3	780.0E-9	20.3	37	7.07	7
GO:0051279	regulation of release of sequestered calcium ion into cytosol	2.1E-3	780.0E-9	20.3	37	8.57	6
GO:0060326	cell chemotaxis	2.1E-3	780.0E-9	20.3	37	4.98	10
GO:0019932	second-messenger-mediated signaling	2.1E-3	780.0E-9	20.3	37	5.00	10
GO:0070588	calcium ion transmembrane transport	2.3E-3	780.0E-9	20.3	37	4.53	11

GO:0002690	positive regulation of leukocyte chemotaxis	2.5E-3	780.0E-9	20.3	37	7.89	6
GO:0051238	sequestering of metal ion	2.9E-3	780.0E-9	20.3	37	6.48	7
GO:0048520	positive regulation of behavior	3.4E-3	780.0E-9	20.3	37	5.41	8
GO:0030595	leukocyte chemotaxis	3.4E-3	780.0E-9	20.3	37	5.41	8
GO:2000021	regulation of ion homeostasis	3.7E-3	780.0E-9	20.3	37	4.81	9
GO:0010522	regulation of calcium ion transport into cytosol	3.9E-3	780.0E-9	20.3	37	6.98	6
GO:0002688	regulation of leukocyte chemotaxis	4.3E-3	780.0E-9	20.3	37	6.74	6
GO:0050848	regulation of calcium-mediated signaling	4.3E-3	780.0E-9	20.3	37	8.33	5
GO:0050900	leukocyte migration	4.5E-3	780.0E-9	20.3	37	4.26	10
GO:0060402	calcium ion transport into cytosol	4.5E-3	780.0E-9	20.3	37	5.69	7
GO:0014074	response to purine-containing compound	5.2E-3	780.0E-9	20.3	37	4.41	9
GO:0051281	positive regulation of release of sequestered calcium ion into cytosol	5.3E-3	780.0E-9	20.3	37	10.26	4
GO:0034767	positive regulation of ion transmembrane transport	6.0E-3	780.0E-9	20.3	37	5.26	7
GO:0071804	cellular potassium ion transport	6.0E-3	780.0E-9	20.3	37	4.68	8
GO:0071805	potassium ion transmembrane transport	6.0E-3	780.0E-9	20.3	37	4.68	8
GO:0050920	regulation of chemotaxis	6.7E-3	780.0E-9	20.3	37	4.52	8
GO:0034764	positive regulation of transmembrane transport	6.8E-3	780.0E-9	20.3	37	5.04	7
GO:0002685	regulation of leukocyte migration	8.3E-3	780.0E-9	20.3	37	4.79	7
GO:0050918	positive chemotaxis	8.7E-3	780.0E-9	20.3	37	8.33	4
GO:0002687	positive regulation of leukocyte migration	8.8E-3	780.0E-9	20.3	37	5.36	6
GO:0051149	positive regulation of muscle cell differentiation	9.5E-3	780.0E-9	20.3	37	6.25	5
GO:1903169	regulation of calcium ion transmembrane transport	9.6E-3	780.0E-9	20.3	37	5.22	6
GO:0060306	regulation of membrane repolarization	10.0E-3	780.0E-9	20.3	37	11.11	3

GO:0010524	positive regulation of calcium ion transport into cytosol	10.0E-3	780.0E-9	20.3	37	7.69	4
GO:0032845	negative regulation of homeostatic process	14.0E-3	780.0E-9	20.3	37	4.17	7
GO:0086009	membrane repolarization	15.0E-3	780.0E-9	20.3	37	9.38	3
GO:0051155	positive regulation of striated muscle cell differentiation	15.0E-3	780.0E-9	20.3	37	6.67	4
GO:0050850	positive regulation of calcium-mediated signaling	16.0E-3	780.0E-9	20.3	37	9.09	3
GO:1904427	positive regulation of calcium ion transmembrane transport	16.0E-3	780.0E-9	20.3	37	6.45	4
GO:0060048	cardiac muscle contraction	17.0E-3	780.0E-9	20.3	37	5.05	5
GO:1904064	positive regulation of cation transmembrane transport	17.0E-3	780.0E-9	20.3	37	5.05	5
GO:0046058	cAMP metabolic process	17.0E-3	780.0E-9	20.3	37	4.38	6
GO:2001259	positive regulation of cation channel activity	20.0E-3	780.0E-9	20.3	37	7.89	3
GO:0030814	regulation of cAMP metabolic process	23.0E-3	780.0E-9	20.3	37	4.55	5
GO:0086003	cardiac muscle cell contraction	28.0E-3	780.0E-9	20.3	37	6.67	3
GO:2001257	regulation of cation channel activity	30.0E-3	780.0E-9	20.3	37	4.94	4
GO:0051153	regulation of striated muscle cell differentiation	35.0E-3	780.0E-9	20.3	37	4.60	4
GO:0055117	regulation of cardiac muscle contraction	47.0E-3	780.0E-9	20.3	37	5.17	3
GO:0018894	**dibenzo-p-dioxin metabolic process**	530.0E-6	21.0E-6	15.5	38	33.33	4
GO:0071774	response to fibroblast growth factor	600.0E-6	21.0E-6	15.5	38	8.11	9
GO:0071288	cellular response to mercury ion	860.0E-6	21.0E-6	15.5	38	50.00	3
GO:0006702	androgen biosynthetic process	890.0E-6	21.0E-6	15.5	38	26.67	4
GO:0019748	secondary metabolic process	1.0E-3	21.0E-6	15.5	38	11.54	6
GO:0044344	cellular response to fibroblast growth factor stimulus	1.0E-3	21.0E-6	15.5	38	7.84	8
GO:0018958	phenol-containing compound metabolic process	1.9E-3	21.0E-6	15.5	38	7.45	7
GO:0071320	cellular response to cAMP	1.9E-3	21.0E-6	15.5	38	9.23	6
GO:0061370	testosterone biosynthetic process	1.9E-3	21.0E-6	15.5	38	33.33	3

GO:0017085	response to insecticide	2.0E-3	21.0E-6	15.5	38	17.39	4
GO:0018879	biphenyl metabolic process	2.0E-3	21.0E-6	15.5	38	27.27	3
GO:0051412	response to corticosterone	2.1E-3	21.0E-6	15.5	38	11.11	5
GO:0008209	androgen metabolic process	2.8E-3	21.0E-6	15.5	38	13.33	4
GO:0009651	response to salt stress	3.1E-3	21.0E-6	15.5	38	12.90	4
GO:0097306	cellular response to alcohol	3.8E-3	21.0E-6	15.5	38	4.76	9
GO:1990776	response to angiotensin	4.1E-3	21.0E-6	15.5	38	18.75	3
GO:0009635	response to herbicide	4.5E-3	21.0E-6	15.5	38	17.65	3
GO:0016101	diterpenoid metabolic process	4.9E-3	21.0E-6	15.5	38	7.94	5
GO:0014074	response to purine-containing compound	5.2E-3	21.0E-6	15.5	38	4.41	9
GO:0051385	response to mineralocorticoid	5.2E-3	21.0E-6	15.5	38	7.69	5
GO:0042445	hormone metabolic process	5.3E-3	21.0E-6	15.5	38	4.88	8
GO:0043279	response to alkaloid	5.3E-3	21.0E-6	15.5	38	4.43	9
GO:0034754	cellular hormone metabolic process	5.9E-3	21.0E-6	15.5	38	6.12	6
GO:0046689	response to mercury ion	6.0E-3	21.0E-6	15.5	38	15.00	3
GO:0071804	cellular potassium ion transport	6.0E-3	21.0E-6	15.5	38	4.68	8
GO:0071805	potassium ion transmembrane transport	6.0E-3	21.0E-6	15.5	38	4.68	8
GO:0006721	terpenoid metabolic process	6.3E-3	21.0E-6	15.5	38	7.14	5
GO:0060992	response to fungicide	6.6E-3	21.0E-6	15.5	38	14.29	3
GO:0051591	response to cAMP	6.6E-3	21.0E-6	15.5	38	5.07	7
GO:0071560	cellular response to transforming growth factor beta stimulus	7.5E-3	21.0E-6	15.5	38	4.42	8
GO:1901655	cellular response to ketone	7.5E-3	21.0E-6	15.5	38	5.61	6
GO:0071372	cellular response to follicle-stimulating hormone stimulus	7.9E-3	21.0E-6	15.5	38	13.04	3
GO:0071559	response to transforming growth factor beta	8.6E-3	21.0E-6	15.5	38	4.28	8
GO:0071549	cellular response to dexamethasone stimulus	9.5E-3	21.0E-6	15.5	38	8.00	4
GO:0071236	cellular response to antibiotic	12.0E-3	21.0E-6	15.5	38	10.34	3
GO:0032354	response to follicle-stimulating hormone	12.0E-3	21.0E-6	15.5	38	10.34	3

GO:0071222	cellular response to lipopolysaccharide	12.0E-3	21.0E-6	15.5	38	4.35	7
GO:0042446	hormone biosynthetic process	14.0E-3	21.0E-6	15.5	38	6.90	4
GO:0006720	isoprenoid metabolic process	14.0E-3	21.0E-6	15.5	38	5.49	5
GO:0071219	cellular response to molecule of bacterial origin	14.0E-3	21.0E-6	15.5	38	4.12	7
GO:0071385	cellular response to glucocorticoid stimulus	14.0E-3	21.0E-6	15.5	38	5.43	5
GO:0046683	response to organophosphorus	15.0E-3	21.0E-6	15.5	38	4.07	7
GO:0007422	peripheral nervous system development	15.0E-3	21.0E-6	15.5	38	6.56	4
GO:0042537	benzene-containing compound metabolic process	16.0E-3	21.0E-6	15.5	38	8.82	3
GO:0071371	cellular response to gonadotropin stimulus	16.0E-3	21.0E-6	15.5	38	8.82	3
GO:0071384	cellular response to corticosteroid stimulus	16.0E-3	21.0E-6	15.5	38	5.10	5
GO:0042181	ketone biosynthetic process	17.0E-3	21.0E-6	15.5	38	8.57	3
GO:0071869	response to catecholamine	18.0E-3	21.0E-6	15.5	38	8.33	3
GO:0097237	cellular response to toxic substance	19.0E-3	21.0E-6	15.5	38	8.11	3
GO:0071346	cellular response to interferon-gamma	19.0E-3	21.0E-6	15.5	38	5.88	4
GO:0071867	response to monoamine	20.0E-3	21.0E-6	15.5	38	7.89	3
GO:2001259	positive regulation of cation channel activity	20.0E-3	21.0E-6	15.5	38	7.89	3
GO:0071548	response to dexamethasone	24.0E-3	21.0E-6	15.5	38	5.41	4
GO:0051851	modification by host of symbiont morphology or physiology	25.0E-3	21.0E-6	15.5	38	7.14	3
GO:0051702	interaction with symbiont	27.0E-3	21.0E-6	15.5	38	6.82	3
GO:0071312	cellular response to alkaloid	29.0E-3	21.0E-6	15.5	38	6.52	3
GO:0006970	response to osmotic stress	30.0E-3	21.0E-6	15.5	38	4.94	4
GO:2001257	regulation of cation channel activity	30.0E-3	21.0E-6	15.5	38	4.94	4
GO:0034341	response to interferon-gamma	36.0E-3	21.0E-6	15.5	38	4.55	4
GO:0046686	response to cadmium ion	38.0E-3	21.0E-6	15.5	38	5.77	3
GO:0043648	dicarboxylic acid metabolic process	43.0E-3	21.0E-6	15.5	38	4.26	4
GO:0070301	cellular response to hydrogen peroxide	46.0E-3	21.0E-6	15.5	38	4.12	4
GO:0008203	cholesterol metabolic process	46.0E-3	21.0E-6	15.5	38	4.12	4

GO:0034698	response to gonadotropin	48.0E-3	21.0E-6	15.5	38	5.08	3
GO:1902652	secondary alcohol metabolic process	48.0E-3	21.0E-6	15.5	38	4.04	4
GO:0021766	hippocampus development	48.0E-3	21.0E-6	15.5	38	4.04	4
GO:0055074	**calcium ion homeostasis**	950.0E-6	22.0E-6	15.5	36	4.16	16
GO:1904018	positive regulation of vasculature development	1.0E-3	22.0E-6	15.5	36	6.72	9
GO:0050921	positive regulation of chemotaxis	1.9E-3	22.0E-6	15.5	36	6.78	8
GO:0010759	positive regulation of macrophage chemotaxis	1.9E-3	22.0E-6	15.5	36	30.00	3
GO:0045766	positive regulation of angiogenesis	1.9E-3	22.0E-6	15.5	36	6.50	8
GO:0006874	cellular calcium ion homeostasis	1.9E-3	22.0E-6	15.5	36	4.01	15
GO:0060326	cell chemotaxis	2.1E-3	22.0E-6	15.5	36	4.98	10
GO:0071347	cellular response to interleukin-1	2.3E-3	22.0E-6	15.5	36	6.86	7
GO:0002690	positive regulation of leukocyte chemotaxis	2.5E-3	22.0E-6	15.5	36	7.89	6
GO:0071675	regulation of mononuclear cell migration	3.1E-3	22.0E-6	15.5	36	21.43	3
GO:0048520	positive regulation of behavior	3.4E-3	22.0E-6	15.5	36	5.41	8
GO:0030595	leukocyte chemotaxis	3.4E-3	22.0E-6	15.5	36	5.41	8
GO:0010758	regulation of macrophage chemotaxis	3.6E-3	22.0E-6	15.5	36	20.00	3
GO:0071674	mononuclear cell migration	4.1E-3	22.0E-6	15.5	36	18.75	3
GO:0002688	regulation of leukocyte chemotaxis	4.3E-3	22.0E-6	15.5	36	6.74	6
GO:0050900	leukocyte migration	4.5E-3	22.0E-6	15.5	36	4.26	10
GO:0071622	regulation of granulocyte chemotaxis	5.3E-3	22.0E-6	15.5	36	10.26	4
GO:0045123	cellular extravasation	5.9E-3	22.0E-6	15.5	36	9.76	4
GO:0070555	response to interleukin-1	6.0E-3	22.0E-6	15.5	36	5.22	7
GO:0050920	regulation of chemotaxis	6.7E-3	22.0E-6	15.5	36	4.52	8
GO:0048246	macrophage chemotaxis	7.2E-3	22.0E-6	15.5	36	13.64	3
GO:0002685	regulation of leukocyte migration	8.3E-3	22.0E-6	15.5	36	4.79	7
GO:0050918	positive chemotaxis	8.7E-3	22.0E-6	15.5	36	8.33	4
GO:0002687	positive regulation of leukocyte migration	8.8E-3	22.0E-6	15.5	36	5.36	6

GO:0071621	granulocyte chemotaxis	10.0E-3	22.0E-6	15.5	36	5.95	5
GO:0097530	granulocyte migration	15.0E-3	22.0E-6	15.5	36	5.26	5
GO:0097529	myeloid leukocyte migration	16.0E-3	22.0E-6	15.5	36	4.48	6
GO:0070098	chemokine-mediated signaling pathway	17.0E-3	22.0E-6	15.5	36	6.15	4
GO:0071346	cellular response to interferon-gamma	19.0E-3	22.0E-6	15.5	36	5.88	4
GO:0048247	lymphocyte chemotaxis	21.0E-3	22.0E-6	15.5	36	7.69	3
GO:0030593	neutrophil chemotaxis	22.0E-3	22.0E-6	15.5	36	5.56	4
GO:1990266	neutrophil migration	30.0E-3	22.0E-6	15.5	36	4.88	4
GO:0034341	response to interferon-gamma	36.0E-3	22.0E-6	15.5	36	4.55	4
GO:0090280	**positive regulation of calcium ion import**	1.0E-3	31.0E-6	15.0	34	10.91	6
GO:0071320	cellular response to cAMP	1.9E-3	31.0E-6	15.0	34	9.23	6
GO:0019722	calcium-mediated signaling	1.9E-3	31.0E-6	15.0	34	6.61	8
GO:0051279	regulation of release of sequestered calcium ion into cytosol	2.1E-3	31.0E-6	15.0	34	8.57	6
GO:0019932	second-messenger-mediated signaling	2.1E-3	31.0E-6	15.0	34	5.00	10
GO:2000021	regulation of ion homeostasis	3.7E-3	31.0E-6	15.0	34	4.81	9
GO:0010522	regulation of calcium ion transport into cytosol	3.9E-3	31.0E-6	15.0	34	6.98	6
GO:0050848	regulation of calcium-mediated signaling	4.3E-3	31.0E-6	15.0	34	8.33	5
GO:0051281	positive regulation of release of sequestered calcium ion into cytosol	5.3E-3	31.0E-6	15.0	34	10.26	4
GO:0034767	positive regulation of ion transmembrane transport	6.0E-3	31.0E-6	15.0	34	5.26	7
GO:0034764	positive regulation of transmembrane transport	6.8E-3	31.0E-6	15.0	34	5.04	7
GO:1903169	regulation of calcium ion transmembrane transport	9.6E-3	31.0E-6	15.0	34	5.22	6
GO:0010524	positive regulation of calcium ion transport into cytosol	10.0E-3	31.0E-6	15.0	34	7.69	4
GO:0050850	positive regulation of calcium-mediated signaling	16.0E-3	31.0E-6	15.0	34	9.09	3

GO:1904427	positive regulation of calcium ion transmembrane transport	16.0E-3	31.0E-6	15.0	34	6.45	4
GO:1904064	positive regulation of cation transmembrane transport	17.0E-3	31.0E-6	15.0	34	5.05	5
GO:2001259	positive regulation of cation channel activity	20.0E-3	31.0E-6	15.0	34	7.89	3
GO:2001257	regulation of cation channel activity	30.0E-3	31.0E-6	15.0	34	4.94	4
GO:0051924	**regulation of calcium ion transport**	730.0E-6	350.0E-6	11.5	24	5.74	12
GO:0090279	regulation of calcium ion import	880.0E-6	350.0E-6	11.5	24	8.42	8
GO:0050921	positive regulation of chemotaxis	1.9E-3	350.0E-6	11.5	24	6.78	8
GO:0048520	positive regulation of behavior	3.4E-3	350.0E-6	11.5	24	5.41	8
GO:0030595	leukocyte chemotaxis	3.4E-3	350.0E-6	11.5	24	5.41	8
GO:0050920	regulation of chemotaxis	6.7E-3	350.0E-6	11.5	24	4.52	8
GO:0050918	positive chemotaxis	8.7E-3	350.0E-6	11.5	24	8.33	4
GO:0070098	chemokine-mediated signaling pathway	17.0E-3	350.0E-6	11.5	24	6.15	4
GO:0001570	**vasculogenesis**	2.0E-3	350.0E-6	11.5	18	8.82	6
GO:0060840	artery development	6.3E-3	350.0E-6	11.5	18	7.14	5
GO:0048844	artery morphogenesis	29.0E-3	350.0E-6	11.5	18	6.52	3
GO:1904018	**positive regulation of vasculature development**	1.0E-3	380.0E-6	11.4	32	6.72	9
GO:1901342	regulation of vasculature development	1.9E-3	380.0E-6	11.4	32	4.87	11
GO:0045766	positive regulation of angiogenesis	1.9E-3	380.0E-6	11.4	32	6.50	8
GO:0001570	vasculogenesis	2.0E-3	380.0E-6	11.4	32	8.82	6
GO:0045765	regulation of angiogenesis	2.3E-3	380.0E-6	11.4	32	4.85	10
GO:0050900	leukocyte migration	4.5E-3	380.0E-6	11.4	32	4.26	10
GO:0045123	cellular extravasation	5.9E-3	380.0E-6	11.4	32	9.76	4
GO:0071621	granulocyte chemotaxis	10.0E-3	380.0E-6	11.4	32	5.95	5
GO:0045736	negative regulation of cyclin-dependent protein serine/threonine kinase activity	12.0E-3	380.0E-6	11.4	32	10.34	3
GO:0097530	granulocyte migration	15.0E-3	380.0E-6	11.4	32	5.26	5

GO:0097529	myeloid leukocyte migration	16.0E-3	380.0E-6	11.4	32	4.48	6
GO:1904030	negative regulation of cyclin-dependent protein kinase activity	21.0E-3	380.0E-6	11.4	32	7.69	3
GO:0030593	neutrophil chemotaxis	22.0E-3	380.0E-6	11.4	32	5.56	4
GO:0071901	negative regulation of protein serine/threonine kinase activity	24.0E-3	380.0E-6	11.4	32	4.46	5
GO:0048844	artery morphogenesis	29.0E-3	380.0E-6	11.4	32	6.52	3
GO:1990266	neutrophil migration	30.0E-3	380.0E-6	11.4	32	4.88	4
GO:0008277	regulation of G-protein coupled receptor protein signaling pathway	48.0E-3	380.0E-6	11.4	32	4.04	4
GO:0048009	**insulin-like growth factor receptor signaling pathway**	910.0E-6	420.0E-6	11.2	15	16.13	5
GO:0043567	regulation of insulin-like growth factor receptor signaling pathway	6.6E-3	420.0E-6	11.2	15	14.29	3
GO:0018894	**dibenzo-p-dioxin metabolic process**	530.0E-6	700.0E-6	10.5	27	33.33	4
GO:0071622	regulation of granulocyte chemotaxis	5.3E-3	700.0E-6	10.5	27	10.26	4
GO:0071621	granulocyte chemotaxis	10.0E-3	700.0E-6	10.5	27	5.95	5
GO:0097530	granulocyte migration	15.0E-3	700.0E-6	10.5	27	5.26	5
GO:0070098	chemokine-mediated signaling pathway	17.0E-3	700.0E-6	10.5	27	6.15	4
GO:0071346	cellular response to interferon-gamma	19.0E-3	700.0E-6	10.5	27	5.88	4
GO:0048247	lymphocyte chemotaxis	21.0E-3	700.0E-6	10.5	27	7.69	3
GO:0030593	neutrophil chemotaxis	22.0E-3	700.0E-6	10.5	27	5.56	4
GO:0071312	cellular response to alkaloid	29.0E-3	700.0E-6	10.5	27	6.52	3
GO:0051262	protein tetramerization	29.0E-3	700.0E-6	10.5	27	4.13	5
GO:1990266	neutrophil migration	30.0E-3	700.0E-6	10.5	27	4.88	4
GO:0034341	response to interferon-gamma	36.0E-3	700.0E-6	10.5	27	4.55	4
GO:0019932	**second-messenger-mediated signaling**	2.1E-3	720.0E-6	10.4	35	5.00	10
GO:0051281	positive regulation of release of sequestered calcium ion into cytosol	5.3E-3	720.0E-6	10.4	35	10.26	4
GO:0009187	cyclic nucleotide metabolic process	7.5E-3	720.0E-6	10.4	35	4.42	8
GO:0010524	positive regulation of calcium ion	10.0E-3	720.0E-6	10.4	35	7.69	4

	transport into cytosol						
GO:0030801	positive regulation of cyclic nucleotide metabolic process	14.0E-3	720.0E-6	10.4	35	5.43	5
GO:0051155	positive regulation of striated muscle cell differentiation	15.0E-3	720.0E-6	10.4	35	6.67	4
GO:0030799	regulation of cyclic nucleotide metabolic process	16.0E-3	720.0E-6	10.4	35	4.41	6
GO:1904427	positive regulation of calcium ion transmembrane transport	16.0E-3	720.0E-6	10.4	35	6.45	4
GO:0046058	cAMP metabolic process	17.0E-3	720.0E-6	10.4	35	4.38	6
GO:0052652	cyclic purine nucleotide metabolic process	18.0E-3	720.0E-6	10.4	35	4.26	6
GO:0009190	cyclic nucleotide biosynthetic process	19.0E-3	720.0E-6	10.4	35	4.20	6
GO:0030816	positive regulation of cAMP metabolic process	22.0E-3	720.0E-6	10.4	35	5.56	4
GO:0030814	regulation of cAMP metabolic process	23.0E-3	720.0E-6	10.4	35	4.55	5
GO:1900544	positive regulation of purine nucleotide metabolic process	24.0E-3	720.0E-6	10.4	35	4.42	5
GO:0045981	positive regulation of nucleotide metabolic process	25.0E-3	720.0E-6	10.4	35	4.39	5
GO:0030802	regulation of cyclic nucleotide biosynthetic process	25.0E-3	720.0E-6	10.4	35	4.35	5
GO:0030804	positive regulation of cyclic nucleotide biosynthetic process	30.0E-3	720.0E-6	10.4	35	4.94	4
GO:0030808	regulation of nucleotide biosynthetic process	31.0E-3	720.0E-6	10.4	35	4.07	5
GO:1900371	regulation of purine nucleotide biosynthetic process	31.0E-3	720.0E-6	10.4	35	4.07	5
GO:0051153	regulation of striated muscle cell differentiation	35.0E-3	720.0E-6	10.4	35	4.60	4
GO:0030810	positive regulation of nucleotide biosynthetic process	35.0E-3	720.0E-6	10.4	35	4.60	4
GO:1900373	positive regulation of purine nucleotide biosynthetic process	35.0E-3	720.0E-6	10.4	35	4.60	4
GO:0030817	regulation of cAMP biosynthetic process	45.0E-3	720.0E-6	10.4	35	4.17	4
GO:0051279	**regulation of release of sequestered calcium ion into cytosol**	2.1E-3	950.0E-6	10.0	30	8.57	6
GO:2000021	regulation of ion homeostasis	3.7E-3	950.0E-6	10.0	30	4.81	9

GO:0010522	regulation of calcium ion transport into cytosol	3.9E-3	950.0E-6	10.0	30	6.98	6
GO:0050848	regulation of calcium-mediated signaling	4.3E-3	950.0E-6	10.0	30	8.33	5
GO:1903169	regulation of calcium ion transmembrane transport	9.6E-3	950.0E-6	10.0	30	5.22	6
GO:0060306	regulation of membrane repolarization	10.0E-3	950.0E-6	10.0	30	11.11	3
GO:0006937	regulation of muscle contraction	15.0E-3	950.0E-6	10.0	30	4.55	6
GO:0086009	membrane repolarization	15.0E-3	950.0E-6	10.0	30	9.38	3
GO:0006941	striated muscle contraction	17.0E-3	950.0E-6	10.0	30	4.35	6
GO:0060048	cardiac muscle contraction	17.0E-3	950.0E-6	10.0	30	5.05	5
GO:0086003	cardiac muscle cell contraction	28.0E-3	950.0E-6	10.0	30	6.67	3
GO:2001257	regulation of cation channel activity	30.0E-3	950.0E-6	10.0	30	4.94	4
GO:0055117	regulation of cardiac muscle contraction	47.0E-3	950.0E-6	10.0	30	5.17	3
GO:0006644	**phospholipid metabolic process**	3.0E-3	2.5E-3	8.6	16	4.01	12
GO:0006650	glycerophospholipid metabolic process	9.9E-3	2.5E-3	8.6	16	4.12	8
GO:0046488	phosphatidylinositol metabolic process	26.0E-3	2.5E-3	8.6	16	4.31	5
GO:0018894	**dibenzo-p-dioxin metabolic process**	530.0E-6	2.9E-3	8.4	33	33.33	4
GO:0071288	cellular response to mercury ion	860.0E-6	2.9E-3	8.4	33	50.00	3
GO:0071320	cellular response to cAMP	1.9E-3	2.9E-3	8.4	33	9.23	6
GO:0009651	response to salt stress	3.1E-3	2.9E-3	8.4	33	12.90	4
GO:0097306	cellular response to alcohol	3.8E-3	2.9E-3	8.4	33	4.76	9
GO:0046689	response to mercury ion	6.0E-3	2.9E-3	8.4	33	15.00	3
GO:1901655	cellular response to ketone	7.5E-3	2.9E-3	8.4	33	5.61	6
GO:0071549	cellular response to dexamethasone stimulus	9.5E-3	2.9E-3	8.4	33	8.00	4
GO:0071385	cellular response to glucocorticoid stimulus	14.0E-3	2.9E-3	8.4	33	5.43	5
GO:0048146	positive regulation of fibroblast proliferation	15.0E-3	2.9E-3	8.4	33	6.56	4
GO:0071384	cellular response to corticosteroid stimulus	16.0E-3	2.9E-3	8.4	33	5.10	5
GO:0071548	response to dexamethasone	24.0E-3	2.9E-3	8.4	33	5.41	4

GO:0071392	cellular response to estradiol stimulus	28.0E-3	2.9E-3	8.4	33	6.67	3
GO:0006970	response to osmotic stress	30.0E-3	2.9E-3	8.4	33	4.94	4
GO:0048145	regulation of fibroblast proliferation	38.0E-3	2.9E-3	8.4	33	4.44	4
GO:0071391	cellular response to estrogen stimulus	39.0E-3	2.9E-3	8.4	33	5.66	3
GO:0048144	fibroblast proliferation	42.0E-3	2.9E-3	8.4	33	4.30	4
GO:0070301	cellular response to hydrogen peroxide	46.0E-3	2.9E-3	8.4	33	4.12	4
GO:0030199	**collagen fibril organization**	910.0E-6	3.2E-3	8.3	22	16.13	5
GO:0043062	extracellular structure organization	2.8E-3	3.2E-3	8.3	22	5.08	9
GO:0030198	extracellular matrix organization	2.8E-3	3.2E-3	8.3	22	5.11	9
GO:0045669	positive regulation of osteoblast differentiation	47.0E-3	3.2E-3	8.3	22	5.17	3
GO:0038179	**neurotrophin signaling pathway**	5.0E-3	5.1E-3	7.6	26	10.53	4
GO:0048011	neurotrophin TRK receptor signaling pathway	15.0E-3	5.1E-3	7.6	26	9.38	3
GO:0090288	negative regulation of cellular response to growth factor stimulus	18.0E-3	5.1E-3	7.6	26	4.95	5
GO:0071901	negative regulation of protein serine/threonine kinase activity	24.0E-3	5.1E-3	7.6	26	4.46	5
GO:0033138	positive regulation of peptidyl-serine phosphorylation	25.0E-3	5.1E-3	7.6	26	5.26	4
GO:0010518	positive regulation of phospholipase activity	29.0E-3	5.1E-3	7.6	26	6.52	3
GO:1990090	cellular response to nerve growth factor stimulus	43.0E-3	5.1E-3	7.6	26	5.45	3
GO:0010517	regulation of phospholipase activity	43.0E-3	5.1E-3	7.6	26	5.45	3
GO:0014065	phosphatidylinositol 3-kinase signaling	44.0E-3	5.1E-3	7.6	26	4.21	4
GO:0060193	positive regulation of lipase activity	45.0E-3	5.1E-3	7.6	26	5.26	3
GO:1990089	response to nerve growth factor	48.0E-3	5.1E-3	7.6	26	5.08	3
GO:0002931	**response to ischemia**	6.1E-3	5.4E-3	7.5	7	9.52	4
GO:0071229	**cellular response to acid chemical**	10.0E-3	5.7E-3	7.5	14	4.04	8
GO:0032526	response to retinoic acid	31.0E-3	5.7E-3	7.5	14	4.07	5
GO:0071347	**cellular response to interleukin-1**	2.3E-3	7.6E-3	7.0	23	6.86	7

GO:0070555	response to interleukin-1	6.0E-3	7.6E-3	7.0	23	5.22	7
GO:0071222	cellular response to lipopolysaccharide	12.0E-3	7.6E-3	7.0	23	4.35	7
GO:0071219	cellular response to molecule of bacterial origin	14.0E-3	7.6E-3	7.0	23	4.12	7
GO:0097529	myeloid leukocyte migration	16.0E-3	7.6E-3	7.0	23	4.48	6
GO:0009651	**response to salt stress**	3.1E-3	9.6E-3	6.7	25	12.90	4
GO:0032897	negative regulation of viral transcription	6.0E-3	9.6E-3	6.7	25	15.00	3
GO:1901655	cellular response to ketone	7.5E-3	9.6E-3	6.7	25	5.61	6
GO:0019083	viral transcription	16.0E-3	9.6E-3	6.7	25	8.82	3
GO:0046782	regulation of viral transcription	16.0E-3	9.6E-3	6.7	25	9.09	3
GO:0019080	viral gene expression	17.0E-3	9.6E-3	6.7	25	8.57	3
GO:0051851	modification by host of symbiont morphology or physiology	25.0E-3	9.6E-3	6.7	25	7.14	3
GO:0051702	interaction with symbiont	27.0E-3	9.6E-3	6.7	25	6.82	3
GO:0006970	response to osmotic stress	30.0E-3	9.6E-3	6.7	25	4.94	4
GO:0051281	**positive regulation of release of sequestered calcium ion into cytosol**	5.3E-3	10.0E-3	6.6	29	10.26	4
GO:0045663	positive regulation of myoblast differentiation	8.5E-3	10.0E-3	6.6	29	12.50	3
GO:0051149	positive regulation of muscle cell differentiation	9.5E-3	10.0E-3	6.6	29	6.25	5
GO:0045661	regulation of myoblast differentiation	9.9E-3	10.0E-3	6.6	29	7.84	4
GO:0010524	positive regulation of calcium ion transport into cytosol	10.0E-3	10.0E-3	6.6	29	7.69	4
GO:0051155	positive regulation of striated muscle cell differentiation	15.0E-3	10.0E-3	6.6	29	6.67	4
GO:1904427	positive regulation of calcium ion transmembrane transport	16.0E-3	10.0E-3	6.6	29	6.45	4
GO:1904064	positive regulation of cation transmembrane transport	17.0E-3	10.0E-3	6.6	29	5.05	5
GO:0030814	regulation of cAMP metabolic process	23.0E-3	10.0E-3	6.6	29	4.55	5
GO:0045445	myoblast differentiation	30.0E-3	10.0E-3	6.6	29	4.94	4

GO:0051153	regulation of striated muscle cell differentiation	35.0E-3	10.0E-3	6.6	29	4.60	4
GO:0010830	regulation of myotube differentiation	38.0E-3	10.0E-3	6.6	29	5.77	3
GO:0014902	myotube differentiation	45.0E-3	10.0E-3	6.6	29	4.17	4
GO:0018894	**dibenzo-p-dioxin metabolic process**	530.0E-6	11.0E-3	6.5	31	33.33	4
GO:0006702	androgen biosynthetic process	890.0E-6	11.0E-3	6.5	31	26.67	4
GO:0071347	cellular response to interleukin-1	2.3E-3	11.0E-3	6.5	31	6.86	7
GO:0008209	androgen metabolic process	2.8E-3	11.0E-3	6.5	31	13.33	4
GO:0034754	cellular hormone metabolic process	5.9E-3	11.0E-3	6.5	31	6.12	6
GO:0070555	response to interleukin-1	6.0E-3	11.0E-3	6.5	31	5.22	7
GO:0071560	cellular response to transforming growth factor beta stimulus	7.5E-3	11.0E-3	6.5	31	4.42	8
GO:1901655	cellular response to ketone	7.5E-3	11.0E-3	6.5	31	5.61	6
GO:0071559	response to transforming growth factor beta	8.6E-3	11.0E-3	6.5	31	4.28	8
GO:0071222	cellular response to lipopolysaccharide	12.0E-3	11.0E-3	6.5	31	4.35	7
GO:0042446	hormone biosynthetic process	14.0E-3	11.0E-3	6.5	31	6.90	4
GO:0071219	cellular response to molecule of bacterial origin	14.0E-3	11.0E-3	6.5	31	4.12	7
GO:0008203	cholesterol metabolic process	46.0E-3	11.0E-3	6.5	31	4.12	4
GO:1902652	secondary alcohol metabolic process	48.0E-3	11.0E-3	6.5	31	4.04	4
GO:0045736	**negative regulation of cyclin-dependent protein serine/threonine kinase activity**	12.0E-3	11.0E-3	6.5	28	10.34	3
GO:0090288	negative regulation of cellular response to growth factor stimulus	18.0E-3	11.0E-3	6.5	28	4.95	5
GO:0010660	regulation of muscle cell apoptotic process	18.0E-3	11.0E-3	6.5	28	5.97	4
GO:0010665	regulation of cardiac muscle cell apoptotic process	18.0E-3	11.0E-3	6.5	28	8.33	3
GO:0010662	regulation of striated muscle cell apoptotic process	20.0E-3	11.0E-3	6.5	28	7.89	3
GO:1904030	negative regulation of cyclin-dependent protein kinase activity	21.0E-3	11.0E-3	6.5	28	7.69	3
GO:0010657	muscle cell apoptotic process	23.0E-3	11.0E-3	6.5	28	5.48	4

GO:0010659	cardiac muscle cell apoptotic process	24.0E-3	11.0E-3	6.5	28	7.32	3
GO:0071901	negative regulation of protein serine/threonine kinase activity	24.0E-3	11.0E-3	6.5	28	4.46	5
GO:0010658	striated muscle cell apoptotic process	25.0E-3	11.0E-3	6.5	28	6.98	3
GO:0033138	positive regulation of peptidyl-serine phosphorylation	25.0E-3	11.0E-3	6.5	28	5.26	4
GO:0010656	negative regulation of muscle cell apoptotic process	30.0E-3	11.0E-3	6.5	28	6.38	3
GO:0043279	**response to alkaloid**	5.3E-3	11.0E-3	6.5	20	4.43	9
GO:0007422	peripheral nervous system development	15.0E-3	11.0E-3	6.5	20	6.56	4
GO:0046928	regulation of neurotransmitter secretion	44.0E-3	11.0E-3	6.5	20	5.36	3
GO:0021766	hippocampus development	48.0E-3	11.0E-3	6.5	20	4.04	4
GO:0031338	**regulation of vesicle fusion**	12.0E-3	11.0E-3	6.5	8	7.14	4
GO:0046839	**phospholipid dephosphorylation**	16.0E-3	15.0E-3	6.1	12	9.09	3
GO:0044272	**sulfur compound biosynthetic process**	18.0E-3	17.0E-3	5.9	11	4.95	5
GO:0035924	**cellular response to vascular endothelial growth factor stimulus**	18.0E-3	17.0E-3	5.9	10	8.33	3
GO:0007492	**endoderm development**	20.0E-3	19.0E-3	5.7	17	5.80	4
GO:0035987	endodermal cell differentiation	24.0E-3	19.0E-3	5.7	17	7.32	3
GO:0001706	endoderm formation	32.0E-3	19.0E-3	5.7	17	6.25	3
GO:0050772	**positive regulation of axonogenesis**	23.0E-3	22.0E-3	5.5	13	5.48	4
GO:0032768	**regulation of monooxygenase activity**	25.0E-3	24.0E-3	5.4	9	7.14	3
GO:0007605	**sensory perception of sound**	25.0E-3	24.0E-3	5.4	4	4.35	5
GO:0071346	**cellular response to interferon-gamma**	19.0E-3	25.0E-3	5.3	19	5.88	4
GO:0051289	protein homotetramerization	20.0E-3	25.0E-3	5.3	19	5.80	4
GO:0051262	protein tetramerization	29.0E-3	25.0E-3	5.3	19	4.13	5
GO:0034341	response to interferon-gamma	36.0E-3	25.0E-3	5.3	19	4.55	4
GO:0045777	**positive regulation of blood pressure**	33.0E-3	32.0E-3	5.0	1	6.12	3
GO:0030101	**natural killer cell activation**	36.0E-3	35.0E-3	4.8	6	5.88	3
GO:0007229	**integrin-mediated signaling pathway**	38.0E-3	36.0E-3	4.8	2	4.44	4

GO:0072384	**organelle transport along microtubule**	43.0E-3	40.0E-3	4.6	3	5.45	3
GO:1903725	**regulation of phospholipid metabolic process**	44.0E-3	41.0E-3	4.6	5	5.36	3
GO:0019432	**triglyceride biosynthetic process**	12.0E-3	74.0E-3	3.8	21	10.34	3
GO:0046460	neutral lipid biosynthetic process	16.0E-3	74.0E-3	3.8	21	9.09	3
GO:0046463	acylglycerol biosynthetic process	16.0E-3	74.0E-3	3.8	21	9.09	3
GO:0046503	glycerolipid catabolic process	29.0E-3	74.0E-3	3.8	21	6.52	3

Supplementary table S4 - Functionally related networks (groups and terms) of Kyoto Encyclopedia of Gene and Genome (KEGG) pathways enriched among the list of the 350 up-regulated genes in Tg-RGN testis, using ClueGO/Cytoscape. KEGG enriched groups are sorted by highest enrichment score (calculated as - Log2 (group FDR)), and inside each group KEGG terms are sorted by the lowest Term FDR, representing lower p-value. The most significant term of each group is indicated in bold.

GO ID	GO Term	Term FDR	Group FDR	Enrichment Score	GO Groups	% Genes	Nr. Genes
GO:0004924	**Renin secretion**	140.0E-6	140.0E-6	12.8	5	10.77	7
GO:0000920	**Sulfur metabolism**	300.0E-6	190.0E-6	12.4	2	30.77	4
GO:0004925	**Aldosterone synthesis and secretion**	150.0E-6	760.0E-6	10.4	6	9.52	8
GO:0004913	Ovarian steroidogenesis	20.0E-3	760.0E-6	10.4	6	7.02	4
GO:0004270	**Vascular smooth muscle contraction**	5.7E-3	3.6E-3	8.1	4	5.69	7
GO:0005146	**Amoebiasis**	30.0E-3	19.0E-3	5.7	7	4.72	5
GO:0004933	AGE-RAGE signaling pathway in diabetic complications	32.0E-3	19.0E-3	5.7	7	4.81	5
GO:0000562	**Inositol phosphate metabolism**	31.0E-3	26.0E-3	5.3	1	5.41	4
GO:0004146	**Peroxisome**	48.0E-3	39.0E-3	4.7	3	4.55	4

Supplementary table S5 - Functionally related networks (groups and terms) of Gene Ontology – Biological Processes (GO-BP) enriched among the list of the 714 down-regulated genes in Tg-RGN testis, using ClueGO/Cytoscape. GO-BP enriched groups are sorted by highest enrichment score (calculated as - Log2 (group FDR)), and inside each group GO-BP terms are sorted by the lowest Term FDR, representing lower p-value. The most significant term of each group is indicated in bold.

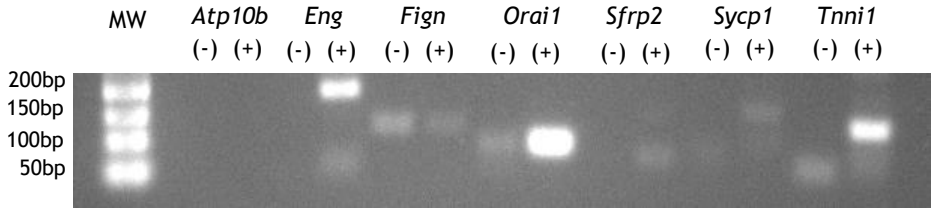
GO ID	GO Term	Term FDR	Group FDR	Enrichment Score	GO Groups	% Genes	Nr. Genes
GO:0051321	**meiotic cell cycle**	110.0E-6	49.0E-6	14.3	7	8.00	14
GO:0007126	meiotic nuclear division	190.0E-6	49.0E-6	14.3	7	8.39	12
GO:1903046	meiotic cell cycle process	220.0E-6	49.0E-6	14.3	7	7.95	12
GO:0007127	meiosis I	710.0E-6	49.0E-6	14.3	7	10.53	8
GO:0007131	reciprocal meiotic recombination	2.5E-3	49.0E-6	14.3	7	15.63	5
GO:0035825	reciprocal DNA recombination	2.5E-3	49.0E-6	14.3	7	15.63	5
GO:0070192	chromosome organization involved in meiotic cell cycle	9.8E-3	49.0E-6	14.3	7	10.64	5
GO:0007129	synapsis	13.0E-3	49.0E-6	14.3	7	12.12	4
GO:0007130	synaptonemal complex assembly	14.0E-3	49.0E-6	14.3	7	20.00	3
GO:0070193	synaptonemal complex organization	15.0E-3	49.0E-6	14.3	7	16.67	3
GO:0045132	meiotic chromosome segregation	15.0E-3	49.0E-6	14.3	7	8.33	5
GO:0006312	mitotic recombination	15.0E-3	49.0E-6	14.3	7	16.67	3
GO:0006310	DNA recombination	17.0E-3	49.0E-6	14.3	7	4.89	9
GO:0045143	homologous chromosome segregation	18.0E-3	49.0E-6	14.3	7	9.76	4
GO:0000018	regulation of DNA recombination	33.0E-3	49.0E-6	14.3	7	7.84	4
GO:0033623	**regulation of integrin activation**	5.1E-3	2.5E-3	8.6	6	30.00	3
GO:2000243	positive regulation of reproductive process	13.0E-3	2.5E-3	8.6	6	9.09	5
GO:0033622	integrin activation	14.0E-3	2.5E-3	8.6	6	17.65	3
GO:0022412	cellular process involved in reproduction in multicellular organism	15.0E-3	2.5E-3	8.6	6	4.44	12
GO:0007281	germ cell development	19.0E-3	2.5E-3	8.6	6	4.46	10
GO:2000241	regulation of reproductive process	25.0E-3	2.5E-3	8.6	6	5.26	7
GO:0040020	regulation of meiotic nuclear division	33.0E-3	2.5E-3	8.6	6	11.11	3

GO:0042516	regulation of tyrosine phosphorylation of Stat3 protein	45.0E-3	2.5E-3	8.6	6	9.09	3
GO:0034103	regulation of tissue remodeling	46.0E-3	2.5E-3	8.6	6	6.56	4
GO:0042503	tyrosine phosphorylation of Stat3 protein	47.0E-3	2.5E-3	8.6	6	8.82	3
GO:0046173	**polyol biosynthetic process**	19.0E-3	12.0E-3	6.4	3	9.52	4
GO:0002474	**antigen processing and presentation of peptide antigen via MHC class I**	46.0E-3	17.0E-3	5.9	2	6.78	4
GO:0042136	**neurotransmitter biosynthetic process**	4.2E-3	18.0E-3	5.8	4	33.33	3
GO:0042133	neurotransmitter metabolic process	33.0E-3	18.0E-3	5.8	4	11.11	3
GO:0001505	regulation of neurotransmitter levels	44.0E-3	18.0E-3	5.8	4	4.19	8
GO:0007143	**female meiotic division**	33.0E-3	18.0E-3	5.8	1	11.11	3
GO:0071824	**protein-DNA complex subunit organization**	38.0E-3	19.0E-3	5.7	5	4.73	7
GO:0006323	DNA packaging	39.0E-3	19.0E-3	5.7	5	5.17	6
GO:0065004	protein-DNA complex assembly	44.0E-3	19.0E-3	5.7	5	4.76	6
GO:0034728	nucleosome organization	45.0E-3	19.0E-3	5.7	5	5.43	5

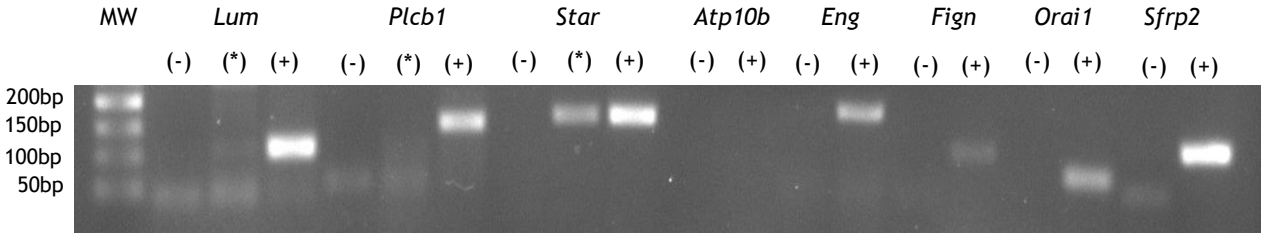
Supplementary table S6 - Functionally related networks (groups and terms) of Kyoto Encyclopedia of Gene and Genome (KEGG) pathways enriched among the list of the 714 down-regulated genes in Tg-RGN testis, using ClueGO/Cytoscape. KEGG enriched groups are sorted by highest enrichment score (calculated as - Log2 (group FDR)), and inside each group KEGG terms are sorted by the lowest Term FDR, representing lower p-value. The most significant term of each group is indicated in bold.

GO ID	GO Term	Term FDR	Group FDR	Enrichment Score	GO Groups	% Genes	Nr. Genes
GO:0003440	**Homologous recombination**	2.5E-3	540.0E-6	10.9	2	14.29	4
GO:0004940	**Type I diabetes mellitus**	1.2E-3	700.0E-6	10.5	3	7.79	6
GO:0000250	**Alanine, aspartate and glutamate metabolism**	21.0E-3	9.1E-3	6.8	1	8.57	3

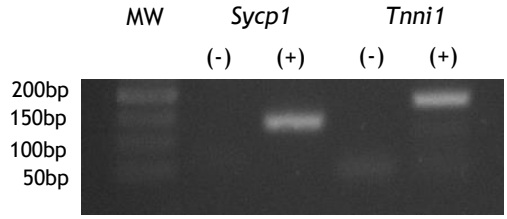
Appendix II – Selected genes expression in GC-6spg cells and SCs



Supplementary figure S1 – Detected expression of *Atp10b*, *Eng*, *Fign*, *Orai1*, *Sfrp2*, *Sycp1* and *Tnni1* genes in rat gonadal cell-6 spermatogonia (GC-6spg) cells determined by RT-PCR. Symbols above the image indicate negative (-) and positive (+) controls. Molecular Weight (MW) lane is indicated and the molecular weights of its bands of interest are shown at the left in base pairs (bp).



Supplementary figure S2 – Detected expression of *Lum*, *Plcb1* and *Star* genes in rat gonadal cell-6 spermatogonia (GC-6spg) cells and Sertoli Cells (SCs), and of *Atp10b*, *Eng*, *Fign*, *Orai1* and *Sfrp2* in rat SCs determined by RT-PCR. Symbols above the image indicate negative (-) control and positive controls for GC-6spg (*) and SCs (+). Molecular Weight (MW) lane is indicated and the molecular weights of its bands of interest are shown at the left in base pairs (bp).



Supplementary figure S3 – Detected expression of *Sycp1* and *Tnni1* genes in rat Sertoli Cells (SCs) by RT-PCR. Symbols above the image indicate negative (-) and positive (+) controls. Molecular Weight (MW) lane is indicated and the molecular weights of its bands of interest are shown at the left in base pairs (bp).