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Estrogens and regucalcin in testicular apoptosis and sperm function: “a matter of life and death”

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Resumo

A espermatogénese é um processo complexo e rigorosamente coordenado, através do qual milhares de espermatozóides são produzidos diariamente nas gónadas masculinas. Para além de realizarem esta função, os testículos dos mamíferos são um órgão endócrino, e produzem as hormonas esteróides sexuais necessárias para a estimulação do processo espermatogénico e para o normal desenvolvimento do fenótipo masculino. Nos últimos anos, os estrogénios têm sido identificados como importantes reguladores da função reproductiva masculina, no entanto, a sua função na espermatogénese não tem encontrado unanimidade entre os investigadores. Alguns estudos sugerem que os estrogénios actuam como factores de sobrevivência para as células germinativas, enquanto outros, associam a acção destas hormonas à apoptose testicular e à diminuição do número de células germinativas. Para além disso, os alvos moleculares subjacentes aos efeitos dos estrogénios no testículo dos mamíferos, levando à sobrevivência ou à morte celular, permanecem por esclarecer. A regucalcina (RGN) é uma proteína de ligação ao cálcio (Ca^{2+}) que tem um papel importante na homeostase da concentração intracelular deste ião, e para a qual foi sugerida uma função na espermatogénese. A RGN foi identificada em diversos tecidos do tracto reproductivo masculino, sendo descrita a sua regulação pelos androgénios no testículo, bem como, a sua regulação pelos estrogénios em células de próstata de rato e humana. No entanto, são totalmente desconhecidos os efeitos dos estrogénios nos níveis de expressão da RGN no testículo. É de salientar ainda a função que tem vindo a ser atribuída à RGN ao nível do controlo da sobrevivência celular e apoptose, assim como, as suas propriedades antioxidantes. Apesar de se reconhecer que o controlo rígido da apoptose e do stress oxidativo são de importância fulcral para a função testicular, a função da RGN no testículo permanece desconhecida. É durante a passagem pelo epidídimo que os espermatozóides sofrem um processo de maturação que lhes permite adquirir motilidade progressiva e a capacidade de fertilizar o oócito. Apesar de ter sido descrita a existência de um gradiente de concentração de Ca^{2+} ao longo do epidídimo, os efeitos deste ião no funcionamento do epidídimo encontram-se insuficientemente explorados.

A presente tese tem como objectivo principal elucidar a relação existente entre os efeitos estrogénicos e a apoptose das células germinativas, incluindo igualmente os efeitos reguladores destas hormonas esteróides na expressão testicular da RGN. Em segundo lugar, será explorado o papel da RGN na regulação da apoptose e do stress oxidativo no testículo, assim como, na maturação dos espermatozóides no epidídimo. Uma dose de 100 nM de 17ß-estradiol (E_2), mimetizando as concentrações de estrogénios encontradas no testículo de pacientes inférteis, culminou na apoptose das células germinativas e na diminuição da proliferação, o que aconteceu simultaneamente com alterações na expressão do sistema SCF/c-kit. O tratamento com E_2 foi ainda acompanhado por um aumento da expressão da RGN, o que sugere que os níveis desta proteína podem estar aumentados como um mecanismo

de prevenção da apoptose induzida pelo E_2. Relativamente ao papel da RGN na modulação da apoptose testicular, observou-se que esta tem um papel importante na prevenção da morte celular induzida por estímulos nocivos. Mais ainda, verificou-se que a sobreexpressão da RGN conduz a um aumento da protecção contra o stress oxidativo no testículo, o que confirma o papel citoprotector desta proteína. Os resultados obtidos demonstraram também a relevância da manutenção dos níveis de Ca^{2+} no lúmen do epidídimo e suportam o papel da RGN na maturação dos espermatozóides.
Em suma, a presente tese demonstrou que níveis elevados de estrogénios podem destabilizar a expressão do sistema SCF/c-kit, o que leva a uma deplecção das células germinativas devido ao aumento da apoptose e diminuição da proliferação celular. O trabalho aqui desenvolvido contribuiu para o conhecimento da base molecular de casos de infertilidade masculina associados a hiperestrogenismo. Estes resultados forneceram ainda uma base racional para explicar o sucesso do uso de inibidores da aromatase no tratamento da infertilidade masculina, sugerindo também que a manipulação dos níveis de c-kit e da RGN poderá funcionar como um possível mecanismo de preservação da integridade das células germinativas e, consequentemente, da fertilidade. Assim sendo, a acção dos estrogénios e da RGN na fisiologia testicular e na função dos espermatozóides estabelece-se como "uma questão de vida ou de morte".

Palavras-chave

Testículo, Epidídimo, Espermatogénese, Espermatozóides, Estrogénios, Regucalcina, Apoptose, Stress oxidativo, Cálcio, Infertilidade masculina

Resumo Alargado

A espermatogénese é o processo complexo e rigorosamente coordenado através do qual os gametas masculinos ou espermatozóides são produzidos, e que se inicia com a diferenciação das espermatogónias estaminais. Este processo compreende três fases principais: mitose, meiose e espermiógenese. Na fase proliferativa as espermatogónias sofrem uma série de divisões mitóticas diferenciando-se posteriormente em espermatócitos primários. Estes iniciam a divisão meiótica, para originar espermatócitos secundários, que por sua vez sofrem a segunda divisão meiótica transformando-se em espermátides. A terceira fase, espermiogénese, envolve o rearranjo da estrutura das células e reorganização do citoplasma deferenciando as espermátides em espermatozóides. Para além de serem responsáveis pela produção dos espermatozóides, os testículos dos mamíferos são um órgão endócrino, produzindo as hormonas esteróides sexuais necessárias para a estimulação do processo espermatogénico e para o normal desenvolvimento do fenótipo masculino. As propiedades funcionais dos testículos dos mamíferos dependem de múltiplos mensageiros hormonais que agem através de vias endócrina, parácrina ou autócrina. No âmbito da regulação hormonal, destaca-se a ação do eixo hipotálamo-pituitária-gónada, que uma vez activado culmina na produção de androgénios pelas células testiculares e na estimulação da espermatogénese. De facto, os androgénios são reconhecidos como os principais reguladores da função reproductiva masculina, apesar de nos últimos anos terem surgido várias evidências de que também os estrogénios têm um papel importante na regulação da espermatogénese. No entanto, este é um assunto para o qual não tem existido unanimidade entre os investigadores, havendo estudos que sugerem que os estrogénios actuam como factores de sobrevivência para as células germinativas, enquanto outros, associam a acção destas hormonas à apoptose testicular e à diminuição do número de células germinativas. Para além disso, os alvos moleculares subjacentes aos efeitos dos estrogénios no testículo dos mamíferos, levando à sobrevivência ou à morte celular, permanecem por esclarecer.

A regucalcina (RGN) é uma proteína de ligação ao cálcio (Ca^{2+}) que tem um papel importante na homeostase da concentração intracelular deste ião actuando através da modulação da actividade de canais e transportadores de Ca^{2+} na membrana celular, retículo endoplasmático e mitocôndria. Recentemente, foi demonstrado que a RGN é expressa em diversos tecidos do tracto reprodutor masculino, e que esta apresenta um padrão de expressão alterado em casos de desordens da espermatogénese, o que sugere uma função para esta proteína no desenvolvimento das células germinativas. Para além disso, foi descrita a sua regulação pelos androgénios no testículo, bem como, a sua regulação pelos estrogénios em células de próstata de rato e humana. No entanto, são totalmente desconhecidos os efeitos dos estrogénios na regulação dos níveis de expressão da RGN no testículo. É de salientar ainda a função que tem vindo a ser atribuída à RGN ao nível do controlo da sobrevivência celular e apoptose, assim como, as suas propriedades antioxidantes reduzindo os níveis intracelulares de stress

oxidativo. Apesar de se reconhecer que o controlo rígido da apoptose e do stress oxidativo são de importância fulcral para a função testicular, a função da RGN no testículo permanece desconhecida.

O epidídimo é um órgão tubular altamente enrolado que se encontra em íntima associação com o testículo recebendo os produtos da secreção testicular, fluído e espermatozóides. É durante a passagem pelo epidídimo que os espermatozóides sofrem um processo de maturação que lhes permite adquirir motilidade progressiva e a capacidade de fertilizar o oócito. Este microambiente único do lúmen do epidídimo é gerado pela actividade específica de secreção e absorção das células epiteliais, sendo o fluído do epidídimo uma mistura complexa de iões, proteínas e outras moléculas orgânicas. No entanto, a função concreta de cada componente do fluído ainda permanece por esclarecer e apesar de ter sido descrita a existência de um gradiente de concentração de Ca^{2+} ao longo do epidídimo, os efeitos deste ião no funcionamento do epidídimo encontram-se insuficientemente explorados.

A presente tese tem como objectivo principal elucidar a relação existente entre os efeitos estrogénicos e a apoptose das células germinativas, incluindo igualmente os efeitos reguladores destas hormonas esteróides na expressão testicular da RGN. Em segundo lugar, será explorado o papel da RGN na regulação da apoptose e do stress oxidativo no testículo, assim como, na maturação dos espermatozóides no epidídimo. E, será ainda averiguada a importância da manutenção dos níveis de Ca^{2+} para o processo de maturação dos espermatozóides.

Uma dose de 100 nM de 17β-estradiol (E_2), mimetizando as concentrações de estrogénios encontradas no testículo de pacientes inférteis, culminou na apoptose das células germinativas e na diminuição da proliferação, o que aconteceu simultaneamente com alterações na expressão do sistema SCF/c-kit. O tratamento com E_2 foi ainda acompanhado por um aumento da expressão da RGN, o que sugere que os níveis desta proteína podem estar aumentados como um mecanismo de prevenção da apoptose induzida pelo E_2. Relativamente ao papel da RGN na modulação da apoptose testicular, observou-se que esta tem um papel importante na prevenção da morte celular induzida por estímulos nocivos. Podendo este efeito ser mediado pela prevenção do aumento do Ca^{2+} intracelular. Mais ainda, verificou-se que a sobreexpressão da RGN conduz a um aumento da protecção contra o stress oxidativo no testículo, o que confirma o papel citoprotector desta proteína. Os resultados obtidos demonstraram também a relevância da manutenção dos níveis de Ca^{2+} no lúmen do epidídimo e suportam o papel da RGN na maturação dos espermatozóides.

Em suma, a presente tese demonstrou que níveis elevados de estrogénios podem destabilizar a expressão do sistema SCF/c-kit, o que culmina na deplecção das células germinativas devido ao aumento da apoptose e diminuição da proliferação celular. O trabalho aqui desenvolvido contribuiu para o conhecimento da base molecular de casos de infertilidade masculina associados a hiperestrogenismo. Estes resultados forneceram ainda uma base racional para explicar o sucesso do uso de inibidores da aromatase no tratamento da infertilidade masculina, sugerindo também que a manipulação dos níveis de c-kit e da RGN poderá

funcionar como um possível mecanismo de preservação da integridade das células germinativas e, consequentemente, da fertilidade. A manipulação dos níveis de expressão da RGN poderá ainda ter um impacto clínico relevante no tratamento de doenças oncológicas.
Assim, a acção dos estrogénios e da RGN na fisiologia testicular e na função dos espermatozóides estabelece-se como “uma questão de vida ou de morte” que contribuiu para aprofundar o conhecimento das bases moleculares da infertilidade masculina.

Abstract

Spermatogenesis is the intricate and coordinated process by which thousands of spermatozoa are produced daily within the male gonad. In addition, mammalian testis also serves as an endocrine organ, producing the sex steroid hormones needed for normal spermatogenesis and development of male phenotype. Over the years, estrogens have emerged as important regulators of male reproductive function, but their role in spermatogenesis has remained a matter of controversy. There are reports indicating that estrogens are survival factors for germ cells, while other strong evidences associated their actions with testicular apoptosis and diminution of germ cell number. Furthermore, the molecular targets underpinning the survival or apoptotic effects of estrogens in mammalian testis remain to be fully elucidated. Regucalcin (RGN) is a calcium (Ca^{2+})-binding protein playing an important role in the maintenance of intracellular Ca^{2+} homeostasis, for which a role in spermatogenesis has been suggested. RGN was identified in male reproductive tract tissues, being described as an estrogen-target gene in rat and human prostate cells, and as an androgen-target gene in the testis. However, the effect of estrogens controlling the expression levels of RGN in the testis is entirely unknown. Noteworthy, it has been indicated the role of RGN in the control of cell survival and apoptosis, and its antioxidant properties also have been reported. Although a tight control of apoptosis and oxidative stress are of the paramount importance for proper testis function, the role of RGN in testicular physiology has not deserved attention yet. Sperm undergo maturation acquiring progressive motility and the capacity to fertilize oocyte only during passage through the epididymis. Although a gradient of Ca^{2+} along the epididymis has been described, its effects on epididymal function remain poorly explored.

The main objective of this thesis is to disclose the relationship between estrogens and apoptosis of germ cells, including the regulatory effects of these sex steroid hormones on the testicular expression of RGN. Secondly, the role of RGN in regulating apoptosis and oxidative stress in the testis, as well as, in sperm maturation in epididymis will be explored.

A 100 nM dose of 17ß-estradiol (E_2), mimicking the elevated concentrations of estrogens found in the testis of infertile patients, induced apoptosis of germ cells and decreased cell proliferation, which was accompanied by disrupted expression of the SCF/c-kit system. E_2-stimulation also increased RGN expression, suggesting that the augmented expression of this protein may be a mechanism to counteract E_2-induced apoptosis. Concerning the function of RGN in modulation of apoptosis in the testis, it was shown that RGN plays a pivotal role rescuing cells from apoptosis induced by noxious stimuli. Moreover, RGN overexpression led to increased protection against oxidative stress in the testis, which further confirmed the cytoprotective role of RGN. The results presented herein also demonstrated the importance of maintaining Ca^{2+} levels in the epididymal lumen and supported a role for RGN in sperm maturation.

In conclusion, the present thesis demonstrated that elevated concentrations of estrogens may unbalance the expression of SCF/c-kit system leading to depletion of germ cells due to augmented apoptosis and decreased proliferation, which has contributed to understand the molecular basis of hyperestrogenism related male infertility. These findings also provided a rationale to explain the successful use of aromatase inhibitors to treat male infertility, and suggested that manipulation of c-kit and RGN levels may be a possible mechanism to preserve germ cell integrity and fertility. Thus, the action of estrogens and RGN on testis physiology and sperm function has emerged as “a matter of life and death”.

Keywords

Apoptosis, Calcium, Estrogens, Epididymis, Male infertility, Oxidative stress, Sperm, Spermatogenesis, Regucalcin, Testis

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List of Abbreviations

$[Ca^{2+}]_i$	Intracellular calcium concentration
17β-HSD	17β-hydroxysteroid dehydrogenase
Act D	Actinomycin D
Apaf-1	Apoptotic protease activating factor 1
ArKO	Aromatase knockout mice
BSA	Bovine serum albumin
c-kit	Tyrosine kinase receptor
Ca^{2+}	Calcium
CaM	Calmodulin
CaM-PDEs	Ca^{2+}/CaM-dependent phosphodiesterases
CaMBP	CaM-binding protein complex
CamK4	Ca^{2+}/Cam-dependent protein kinase IV
cAMP	Cyclic AMP
CaNBP75	Calcineurin-binding protein
CatSper	Sperm-specific Ca^{2+}-channel
CBPs	Ca^{2+}-binding proteins
Cd	Cadmium chloride
CDNB	1-Chloro-2,4-dinitrobenzene
cetn1	Centrin 1
DBD	DNA-binding domain
DES	Diethylstibestrol
DHT	5α-dihidrotestosterone
DNase	Deoxyribonuclease
E	Estrogens
E_2	17β-estradiol
EF	Epididymal fluid
ER	Estrogen receptor
FasL	Fas ligand
FRAP	Ferric Reducing Antioxidant Power
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin releasing hormone
GPR30/GPER	G-protein-coupled-receptor 30
GST	Glutathione-S-transferase
HBSS	Hank´s Buffered Salt Solution
HP	Hypospermatogenesis
hpg	Hypogonadal
HSP	Heat-shock protein

IP_3R	Inositol 1,4,5-trisphosphate receptors
KRb	Krebs Ringer-bicarbonate
LBD	Ligand binding domain
LC	Leydig cell
LH	Luteinizing hormone
MOR23	Mouse OR
NF1-A1	Nuclear factor 1- A1
OR	Olfactory receptor
PBA	PBS containing 1% (w/v) BSA
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol 3-kinase
pNA	p-nitro-aniline
qPCR	Quantitative real-time PCR
Ran	Ras-related small G protein
RGN	Regucalcin
ROS	Reactive oxygen species
RyR	Ryanodine receptors
SC	Sertoli cell
SCF	Stem cell factor
SCOS	Sertoli cell only syndrome
SeT	Seminiferous tubules
SMP30	Senescence marker protein-30
SOD	Superoxide dismutase
SSCs	Spermatogonial stem cells
T	Testosterone
TBHP	tert-butyl hydroperoxide
Tg-RGN	Transgenic rats overexpressing RGN
TGF-β	Transforming growth factor β
Thap	Thapsigargin
TNF	Tumor necrosis factor
TNFR1	Tumor necrosis factor receptor 1
TRP	Transient receptor potential
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
VDCC	Voltage-dependent Ca^{2+}-channels
WB	Western Blot
Wt	Wild-type
αERKO	α estrogen receptor knockout

Chapter I

General Introduction

Brief Introduction to Mammalian Spermatogenesis

Estrogens as apoptosis regulators in mammalian testis: angels or devils?

Regucalcin, a calcium-binding protein with a role in male reproduction?

Calcium homeostasis in the regulation of spermatogenesis and sperm maturation

Brief Introduction to Mammalian Spermatogenesis

Brief Introduction to Mammalian Spermatogenesis

Cellular players and developmental stages

Mammalian spermatogenesis is a complex process involving cell division and maturation of spermatogonial stem cells (SSCs) that culminates with the production of male gametes, the spermatozoa. It is a continuous and highly regulated process that takes place in the seminiferous tubules (SeT, Figure I.1.1), the functional unit of the testis [1]. The seminiferous epithelium (Figure I.1.1) is composed by germ cells that form numerous concentric layers penetrated by a single type of somatic cell, the Sertoli cell (SC). SSCs are localized on the basal membrane of the SeTs as single cells and upon division originate daughter cells, the spermatogonia [2]. The cytoplasm of SCs extends as thin arms around all the germ cells nursing and maintaining their cellular associations throughout the several steps of spermatogenesis [3]. The presence of tight junctions between neighbouring SC forms the so-called blood-testis barrier (Figure I.1.1), which divides the SeT in basal and adluminal compartments [3].

Each spermatogenic cycle in the SeT encompasses three main phases: mitosis, meiosis, and the final stage of cell differentiation, spermiogenesis [1]. Spermatogenesis (Figure I.1.1) starts with the proliferation of spermatogonia and after a species-specific fixed number of mitotic divisions spermatogonia differentiate into primary spermatocytes [4]. These proceed to the first division of meiosis originating secondary spermatocytes, which undergo the second meiotic division and become haploid spermatids. The cellular restructure of spermiogenesis transforms round-spermatids in elongated-spermatids and then elongated-spermatids into spermatozoa [1]. The output of spermatogenesis and the number of produced spermatozoa also depends of programmed cell death processes. High rates of apoptosis have been associated with the first waves of spermatogenesis [5], and in adult testis not all germ cells achieve maturity being prone to die in response to a variety of factors. It is believed that apoptosis represents a mechanism to discard excess and unfit cells maintaining the appropriate ratio of germ cells to SCs [6].

The interstitial space between SeT is mainly occupied by blood vessels and the somatic Leydig cells (LC), which are the testosterone (T) producing cells within the testis and play a crucial role in the regulation of spermatogenic process [7].

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 the capacity to fertilize [8]. The epididymis is a highly compartmentalized organ with three distinct regions (Figure I.1.1), the *caput*, *corpus* and *cauda,* which sustain different functions to achieve the final goal, sperm maturation and fertilizing ability [9].

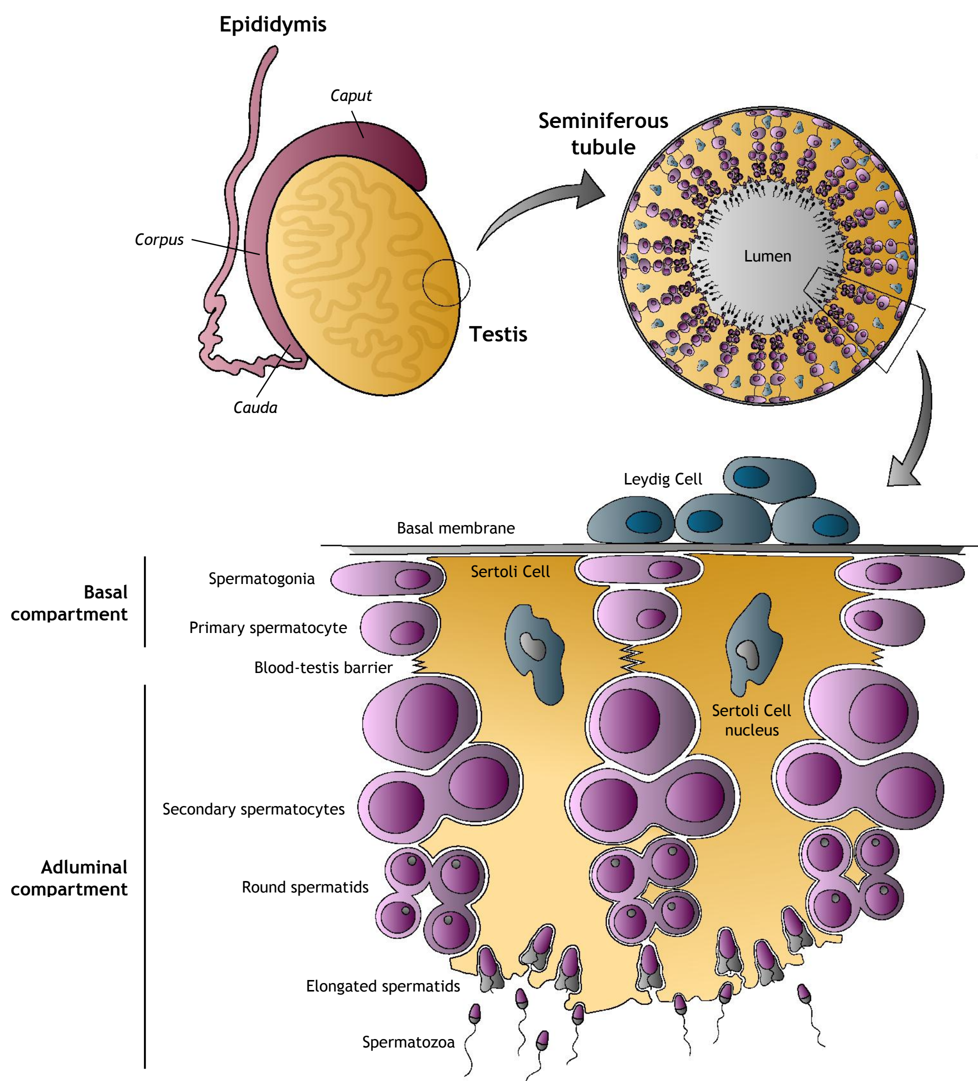

Figure I.1.1. Schematic representation of the testicular histology and mammalian spermatogenesis. Also, the anatomic relationship between testis and epididymis, as well as, the distinct functional regions of the epididymis are shown.

Hormonal regulation

Development and maintenance of successful spermatogenesis depends on assortment of a set of hormonal messengers, which exert their actions by endocrine, paracrine, juxtacrine and autocrine signaling mechanisms. The major player in the hormonal control of spermatogenesis is the hypothalamic-pituitary-gonadal axis [10] (Figure I.1.2). The hypothalamus releases gonadotropin releasing hormone (GnRH), which acts on the pituitary inducing the release of gonadotropins, namely, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). In the testis, LH acts on LCs stimulating the synthesis of T, while FSH acts on SCs inducing the production of several growth factors and other stimulatory factors of spermatogenesis. T diffuses into the SeT where together with FSH exerts stimulatory effects on the activity of SCs activity, which is determinant for germ cells maturation and sperm production [11, 12].

Moreover, T regulates the spermatogenic process by a negative feedback mechanism on the hypothalamus and pituitary inhibiting, respectively, the release of GnRH and LH [10].

Other negative feedback regulatory mechanism is driven by inhibin, a member of the transforming growth factor β (TGF-β) superfamily produced by SCs in response to FSH (Figure I.1.2). Inibin blocks the production and release of FSH by the pituitary [13, 14] controlling the output of spermatogenesis.

Although androgens and FSH are perfectly recognized as the main regulators of spermatogenesis, the last decades have witnessed the emergence of estrogens as important regulators of male reproductive function with their roles starting to be intensively discussed by the scientific community [15, 16].

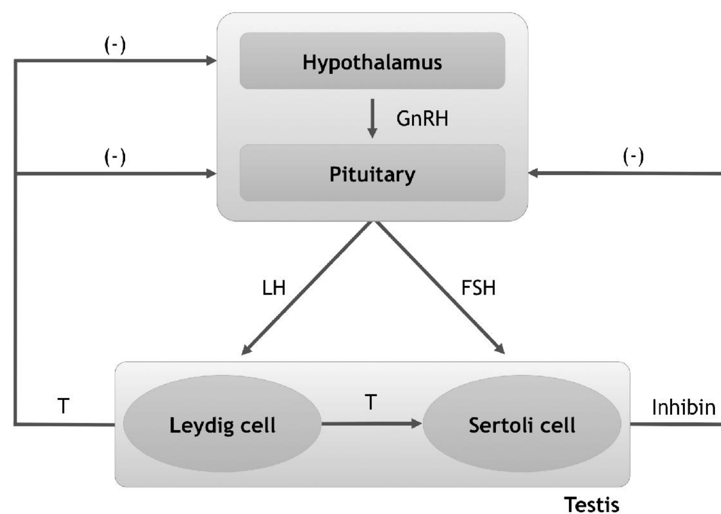

Figure I.1.2. Hormonal regulation of spermatogenesis. Release of gonadotropin-releasing hormone (GnRH) from the hypothalamus stimulates the pituitary to secrete two gonadotropins, the follicle-stimulating hormone (FSH) and the luteinizing hormone (LH). FSH stimulates the activity of Sertoli cells and LH acts on Leydig cells, 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 of a negative feedback (-) by inhibin secreted by Sertoli cells.

Non-hormonal factors in the regulation of spermatogenesis

Besides sex steroids, pituitary and hypothalamic hormones, a panoply of other factors play a role in the regulation of spermatogenesis. The germ cell cycle and movement along the SeT is a process under tight control involving distinct mechanisms that include several families of kinases and phosphatases activated, for example, in response to growth factors and cytokines [17]. Many of these molecules are secreted by SCs, namely, the glial cell line-derived neurotrophic factor, a member of the TGF-β superfamily and the first molecule identified in the regulation of self-renewal and differentiation of SSCs [18]. The stem cell factor (SCF) is a cytokine, also produced and secreted by SCs, which plays a crucial role controlling survival and proliferation of both SSCs and spermatogonia [19, 20]. The SCF, by interaction with its tyrosine kinase receptor the c-KIT also seems to control the differentiation of spermatogonia

and progression into meiosis [21, 22]. Moreover, SCs produce transport or bioprotective proteins that are secreted in relative high abundance and include proteases and protease inhibitors, and metal ion transport proteins such as transferrin and ceruloplasmin [3]. Transferrin transports iron to the adluminal compartment of SeT ensuring that the developing germ cells have access to adequate and tightly regulated levels of this ion [23]. Although germ cells require considerable amounts of iron for proliferation and differentiation [23], other inorganic molecules have been implicated in spermatogenesis. This is, for example, the case of calcium (Ca^{2+}), zinc, selenium, and copper [24].

In the context of this thesis, Ca^{2+} deserves particular attention and several evidences have highlighted for its importance for spermatogenesis. A tight control of intracellular Ca^{2+} homeostasis has been shown to be of uttermost importance for SC function [25, 26], maintenance of SCs tight junctions and integrity of the blood-testis barrier [27]. It also modulates the activity of enzymes that interfere in the structure of SCs [28]. A strict regulation of Ca^{2+} fluxes maintaining intracellular Ca^{2+} homeostasis also seems to be related with the expression of the steroidogenic acute regulatory protein and LC steroidogenesis [29]. Moreover, it has been shown that administration of Ca^{2+} channels blockers for treatment of hypertension causes reversible male infertility [30], which demonstrates the physiological importance of Ca^{2+}.

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Estrogens as apoptosis regulators in mammalian testis: angels or devils?

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Estrogens as apoptosis regulators in mammalian testis: angels or devils?

Abstract

In the mammalian testis, spermatogenesis is the highly coordinated process of germ cell development, which ends with the release of "mature" spermatozoa. The fine regulation of spermatogenesis is strictly dependent of sex steroid hormones, which orchestrate the cellular and molecular events underlying normal development of germ cells. Sex steroids actions also rely on the control of germ cell survival, and the programmed cell death by apoptosis has been indicated as a critical process in regulating the size and quality of the germ line. Recently, estrogens have emerged as important regulators of germ cell fate. However, the beneficial or detrimental effects of estrogens in spermatogenesis are controversial, with independent reports arguing for their role as cell survival factors or as apoptosis-inducers. The dual behavior of estrogens, shifting from "angels to devils" is supported by the clinical findings of increased estrogens levels in serum and intratesticular milieu of idiopathic infertile men. This review aims to discuss the available information concerning the role of estrogens in the control of germ cell death and summarises the signaling mechanisms driven estrogen-induced apoptosis. The present data represent a valuable basis for clinical management of hyperestrogenism-related infertility and provide a rationale for the use of estrogen-target therapies in male infertility.

Keywords: 17ß-estradiol; apoptosis; aromatase; estrogens; estrogen receptor; germ cells; male infertility; sex steroids; spermatogenesis; testis

Introduction

The mammalian testis fulfills two essential functions in male reproduction: steroidogenesis and spermatogenesis. Steroidogenesis corresponds to the biosynthesis of sex steroids hormones whereas spermatogenesis is the complex cellular process that transforms spermatogonial cells into spermatozoa. Progression of spermatogenesis takes place in the seminiferous tubules, the functional units of the testis, encompassing tightly coordinated mitosis, meiosis and cell differentiation events [1]. Mitotic divisions maintain the population of spermatogonial cells and differentiation of spermatogonia originates primary spermatocytes. The first meiotic division produced secondary spermatocytes, which undergo the second meiosis originating spermatids. Spermiogenesis is the cellular restructure process that transforms round spermatids in elongated spermatids and finally in spermatozoa [2]. Nonetheless, not all germ cells achieve maturity, and the spontaneous cell death by apoptosis is a common event, particularly, in the first waves of spermatogenesis [3]. On the other hand,

deregulated apoptosis may be indicated in the etiology of male infertility, since augmented rates of apoptosis were observed in the testes of subfertile and infertile men [4-6].

Development and maintenance of spermatogenesis depends of an assortment of hormonal messengers, which exert their actions by endocrine, paracrine, and autocrine signaling mechanisms. The sex steroids androgens are widely recognized as the main regulators of male reproductive function, and the androgenic actions are absolutely required for successful spermatogenesis [7]. Over the years, the dogma that spermatogenesis depends solely of androgens and gonadotropin actions has changed, and cumulative evidences have demonstrated the importance of estrogens on the regulation of spermatogenic process. However, discordant reports about the role of estrogens in male reproduction have been produced including those ascribing their role as survival or apoptosis-inducer agents in male germ cells. The present review will discuss the current knowledge of estrogen-induced apoptosis in the testis and summarize the molecular mechanisms driven apoptotic effects in male germ cells. This issue is of paramount importance for clinical management of male infertility because hiperestrogenism has been a condition associated with cases of idiopathic infertility.

Apoptosis in spermatogenesis

Signaling mechanisms of apoptotic cell death

The apoptotic cell death, characterized by several hallmarks, such as, cell shrinkage, DNA fragmentation, and externalization of phosphatidylserine at cell membrane, may be triggered by distinct pathways [8]. The receptor-mediated (or extrinsic) and the mitochondrial (or intrinsic) are the two major pathways governing apoptosis (Figure I.2.1) [9]. In both cases, cell-death depends of specific proteases, the so-called caspases, which are the executioners of apoptosis. The caspases enzymes cleave serine residues and are synthesized as inactive zymogens (procaspases) becoming active in response to death stimuli [10]. Initiator caspases (caspase-8 and -9) are directly activated by dimerization and, in turn, cleave the effector caspases (caspase-3, -6 and -7) promoting their activation [10]. The extrinsic pathway (Figure I.2.1) is initiated by ligand-binding activation of death receptors at cell membrane, namely the Fas (CD95/Apo-1) and the tumor necrosis factor receptor 1 (TNFR1), which induce the activation of procaspase-8 [9]. The intrinsic pathway of apoptosis (Figure I.2.1) could be activated by different stimuli, such as DNA damage, starvation, oxidative stress and autophagy. It is characterized by a decrease in the membrane potential of mitochondria and release of cytochrome c, which interact with dATP, cytosolic apoptotic protease activating factor 1 (Apaf-1) and procaspase-9, assembling the apoptosome complex [11]. Both extrinsic and intrinsic pathways of apoptosis converge at the activation of effector caspase-3 (Figure I.2.1), which has been considered an end-point of apoptotic process [8].

The apoptosis of germ cells has been shown to play an important role in controlling sperm output in many species, and massive germ cell death occurs under physiological conditions

during the earlier stages of spermatogenic process (constitutive apoptosis). The specificities of apoptosis in the first waves of spermatogenesis have been revised in recent reports [3, 12] and will not be further explored here.

In adult testis, the fine control of apoptosis is critical for maintenance of spermatogenesis and male fertility, since germ cells are very sensitive to damaging conditions, such as, heat shock, ionizing radiation, growth factor deprivation and chemotherapeutic agents. Therefore, apoptosis is a relevant mechanism for elimination of damaged germ cells avoiding passage of defects to the future generations [12].

On the other hand, male germ cells strictly depend on the physical and biochemical support of Sertoli cells (SCs), the somatic cells within the seminiferous tubules, which nourish and sustain the developing germ cells [13]. However, SCs have a limited capacity for the number of germ cells they can support [14], and it has been accepted that they play a crucial role determining germ cell fate. SCs secrete paracrine factors and establish direct cell-to-cell membrane contacts with adjacent germ cells, which promote germ cell survival or death maintaining the appropriate ratio of germ cell to SCs [15]. The stem cell factor (SCF), a membrane-bound cytokine at the surface of SCs [16], and its tyrosine kinase receptor the c-kit, present on the surface of adjacent germ cells [17], are the main mediators of survival communication between SCs and germ cells protecting germ cells from apoptosis [18]. Perturbations in the SCF/c-kit system by abolishment of SCF production [19], impairment of SCF binding to c-kit [20] or disruption of c-kit signaling mechanisms [21] have been linked to increased apoptosis and reduced proliferation of germ cells. The death communication between SCs and germ cells is established by the Fas system being accepted that SCs express the Fas ligand (FasL) while germ cells mostly express the death receptor Fas [22-24].

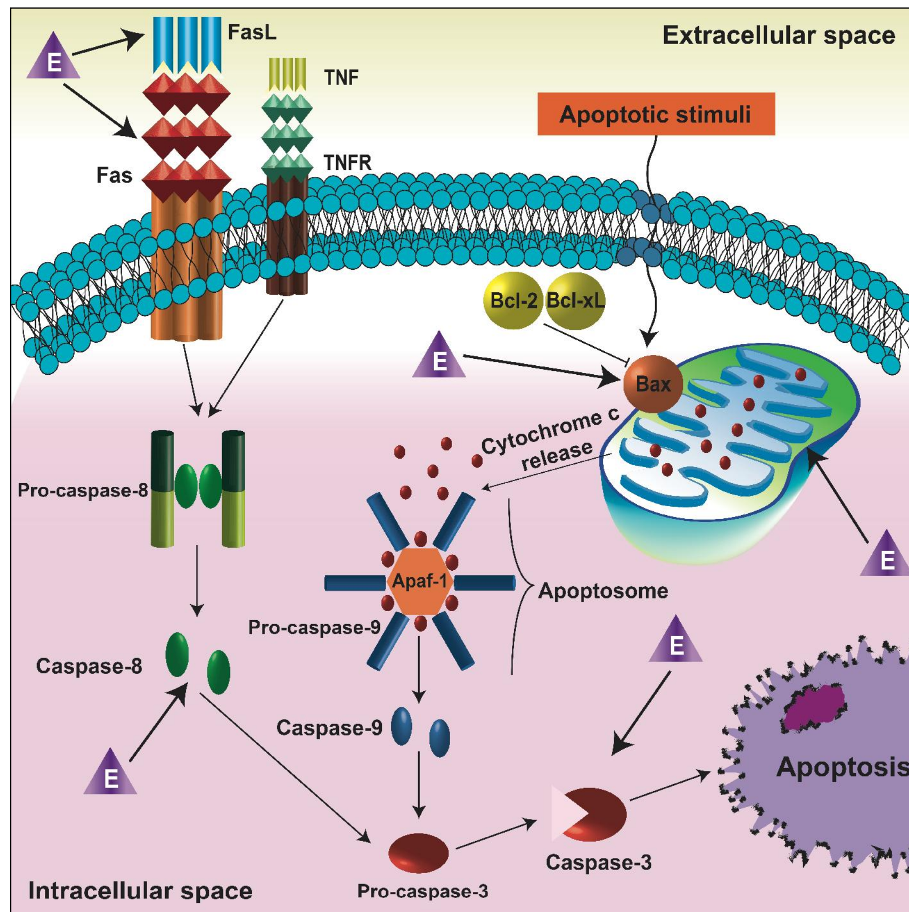

Figure I.2.1. Extrinsic and intrinsic pathways of apoptosis. Extracellular ligand binding (FasL or tumor necrosis factor, TNF) to death receptors (Fas and TNF receptor, TNFR) triggers the receptor-mediated (extrinsic) pathway resulting in the direct activation of initiator caspase-8. The mitochondrial (intrinsic) pathway is initiated in response to apoptotic stimuli leading to the activation of proapoptotic members of the Bcl-2 protein family, namely, Bax. The Bax protein is translocated to the mitochondria allowing the permeabilization of mitochondrial membrane with consequent release of cytochrome c, which in turn, together with apoptotic protease activating factor 1 (Apaf-1), forms the apoptosome and activates caspase-9. Extrinsic and intrinsic apoptosis signaling pathways converge at the activation of the executioner caspase-3. Activation and inhibition are indicated by arrows and bar-headed arrows, respectively. Target sites of estrogens (E) actions in the estrogen-induced apoptosis of germ cells are highlighted by triangles.

Clinical findings of deregulated apoptosis in the testis of infertile men

Taking into account the delicate control of apoptosis in the germ line it is not surprisingly that augmented rates of apoptosis have been identified in the testes of subfertile and infertile men [4-6, 25-31]. Accordingly, altered expression patterns of a panoply of apoptosis-related genes have been described in human testes with defective spermatogenesis. The association of death receptors and the extrinsic pathway of apoptosis with male infertility has been suggested by several studies, which showed enhanced expression of FasL in cases of maturation arrest and Sertoli cell-only syndrome, the later characterized by the absence of germ cell in the seminiferous epithelium [5, 31-35]. Cavalcanti *et al.* also found an increased expression of TNF-α family member 10 (also known as TNF-α related apoptosis inducing ligand, TRAIL) in the testes of subfertile men comparatively with potentially fertile [36].

As previously stated, caspases are the key component in the apoptotic pathway and the executioner of apoptotic cell death. Increased expression of effector caspase-6 was found in the testes of subfertile men comparatively with potentially fertile [36]. Also, the expression of effector caspase-3 was enhanced in cases of maturation arrest and Sertoli cell-only syndrome, the later characterized by the absence of germ cell in the seminiferous epithelium [31-33]. In addition, increased activity of caspase-9 and caspase-3 was also showed in isolated spermatozoa from oligozoospermic and azoospermic patients comparatively with normozoospermic [37, 38], which is indicative of active apoptosis in spermatozoa of infertile patients. In fact, the occurrence of DNA fragmentation and the externalization of phosphatidylserine, which are late apoptotic events indicating cell commitment to apoptosis, can be detected in spermatozoa from infertile men [39, 40].

The expression of novel apoptosis regulators such as Aven, survivin and regucalcin (RGN) also was found to be altered in human testis with defective spermatogenesis [41-43]. Aven is an apoptosis inhibitor that acts by binding both Bcl-xL and Apaf-1 (Figure I.2.1), enhancing the antiapoptotic function of Bcl-xL and inhibiting the assembling of a functional apoptosome complex, thus preventing caspase activation [44]. A diminished expression of Aven was found in the testes of nonobstructive azoospermic men, which was correlated with the severity of spermatogenic defect and increased rates of apoptosis [41]. In the case of survivin and RGN, both proteins with a dual role controlling apoptosis and cell cycle progression [42, 45, 46], the balance of their expression levels should be determinant for maintenance of an appropriate germ cell number and thus, for male reproductive potential. Recently, we have found increased expression of RGN in the testis of human infertile patients with hypospermatogenesis phenotype [42]. Although the causes of idiopathic hypospermatogenesis may be diverse, accelerated apoptosis, rather than proliferative dysfunction in the mitotic phase, has been implicated as responsible for the decreased number of spermatogonia [25]. In a recent report, we demonstrated that *in vivo* overexpression of RGN in the testis leads to suppression of thapsigargin- and actinomycin D- induced apoptosis through modulating the expression and activity of key regulators of apoptosis [47]. This suggests that RGN may act as a protective molecule counteracting increased rates of apoptosis associated with infertility cases such as hypospermatogenesis. On the other hand, manipulation of RGN levels may represent a suitable mechanism for fertility preservation upon treatment of oncologic conditions.

Sex steroid hormones are the crucial orchestrators of the cellular and molecular events underlying normal spermatogenesis and their roles also rely on the control of germ cell survival and apoptosis. It has been shown that androgens withdrawal induces disruption of spermatogenesis with a severe reduction in the number of spermatocytes and spermatids in consequence of increased apoptosis [48, 49]. Whereas the action of androgens has been well documented and fully accepted as an essential requirement for development and survival of testicular cells [50], in the case of estrogens a dual behaviour has been assigned with independent reports describing their actions both as germ cell survival factors and as

apoptosis inducers. The following topics of this review will focus on the role of estrogens as regulators of testicular apoptosis.

Estrogen Receptors in Testicular Cells

The classical nuclear estrogen receptor proteins

Sex steroid hormones exert their effects mainly by the interaction with specific intracellular receptor proteins, which act as nuclear transcription factors regulating the expression of target genes and, thus, modulating the cell protein network at any given time [51, 52]. The steroid receptors belong to the nuclear receptor superfamily that includes, among others, estrogen receptor (ER), androgen receptor and progesterone receptor [51]. The ER shares with other members of the nuclear receptor superfamily a common arrangement of structural-functional domains denoted A to F [53]. The A/B domain with a variable transactivation function encompasses the N-terminal region [51]. C domain or DNA-binding domain (DBD) coordinates receptor interaction with specific DNA sequences known as hormone-response–elements present in the promoter region of target genes [54]. The ligand-binding domain (LBD or E domain) is located in the C-terminal region and possesses the ability of hormone recognition ensuring both specificity and selectivity of the physiologic response [51]. The F domain at the C-terminus is characteristic of ERs and its functions are not totally known yet.

In general, estrogenic actions via nuclear ERs involve ligand-binding, dissociation of chaperones, receptor phosphorylation, receptor dimerization, nuclear translocation, DNA-binding, interaction with cofactors and, consequently, modulation of transcription activity [55]. Alternatively, ERs also can modulate transcription via interfering with other promoter-bound transcription factors or via influencing a variety of intracellular signaling pathways [56].

Two subtypes of nuclear ERs, ER alpha (ERα) and ER beta (ERβ), encoded by separate genes located on different chromosomes have been found along the evolutionary line of vertebrates [57-61]. Even so, and depending on the species considered, ERα and ERβ proteins share 41-65% of overall amino acid identity with the highly conserved DBD reaching 96% of similarity [61]. The LBD also displays high conservation (57-60%), and both receptors bind the natural ligand 17β-estradiol (E_2) [62], phytoestrogens and other natural or synthetic ligands [63]. However, selective agonists and antagonists for ERα or ERβ have been identified helping to disclose their specific actions in a broad range of tissues [64].

The biological function assigned to the ERs in a tissue or organ is not only dependent of ligand nature itself but also of the cellular localization of receptors, as well as the balance between ERα and ERβ proteins levels. ERs localization on different cell types of the testis is species-specific and associated with the developmental stage of germ cells [55]. In rodents, ERα expression is more closely associated to Leydig cells (LCs) and peritubular cells whereas ERβ is found in multiple cell types such as, LCs, peritubular cells, SCs, spermatogonia,

spermatocytes and spermatids [65]. In humans (Table I.2.1), ERβ has been located in peritubular cells, LCs, SCs, spermatogonia, spermatocytes, spermatids and spermatozoa [66-73]. However, the cellular distribution of ERα has been controversial, and reports exist stating the failure to detect this ER subtype in human testis [68, 69]. The absence of consensus on the detection of ERα by distinct independent studies [66-72, 74], together with the wide expression of ERβ in testicular cells [66, 68-72], lead some authors to assume ERβ as the main mediator of estrogenic actions in human testis. Recently, using human testicular biopsies with normal and disrupted phenotypes of spermatogenesis, our research group demonstrated that both ER subtypes are expressed in human testis [73]. ERα was detected in LCs, SCs, spermatogonia, spermatocytes, round spermatids and elongated spermatids/spermatozoa (Table I.2.1), which highlighted for its relevant role in human spermatogenesis. The importance of ERα in human testicular physiology is also supported by studies reporting associations of ERα mutations and polymorphisms with low sperm counts, reduced sperm quality and male infertility [75-80].

The G protein-coupled estrogen receptor

Apart from the classical genomic effects, mediated by ERα and ERβ, it is actually clear that estrogens elicit rapid signaling events mediated through the G-protein-coupled receptor-30 (GPR30/GPER [81]). In rodents, the testicular expression of GPER was reported to the mouse (*Mus musculus*) spermatogonia cell line and, rat (*Rattus norvegicus*) SCs, pachytene spermatocytes and round spermatids [82-85]. Although there are evidences of the functional activity of GPER in mammalian testicular cells [83, 85-87], also for this receptor a lack of consensus exists regarding its localization in human testis. Some authors exclusively identified GPER in LCs and SCs [88] while others described a more widespread distribution both in somatic and germ cells indicating its localization in SCs, spermatogonia and spermatocytes [89]. Very recently, using immunohistochemistry together with gene expression analysis in isolated cell types or cellular fractions of human testicular biopsies we demonstrated that GPER is localized in somatic cells, LCs and SCs, as well as in diploid germ cells, but it it is absent in haploid germ cells [90]. Nevertheless, further studies are needed to clarify the biological role of estrogens actions in mammalian testis upon activation of GPER mediated signaling pathways.

Table I.2.1. Localization of ERα, ERβ and GPER proteins in human testis

Receptor	Localization	References
ERα	Primary spermatocytes and elongating spermatids	[66]
	Spermatozoa midpiece	[70]
	Germ cells and spermatozoa	[71]
	Leydig cells, Sertoli cells, spermatogonia, spermatocytes, round spermatids and elongated spermatids/spermatozoa	[73]
	Spermatozoa	[74]
ERβ	Primary spermatocytes and elongating spermatids	[66]
	Sertoli cells	[67]
	Spermatogonia, spermatocytes and developing spermatids	[68]
	Leydig cells, Sertoli cells and peritubular myoid cells	[69]
	Spermatozoa tail	[70]
	Germ cells and spermatozoa	[71]
	Midpiece spermatozoa mitochondria	[72]
	Leydig cells, spermatocytes, round spermatids and elongated spermatids/spermatozoa	[73]
GPER	Leydig cells and Sertoli cells	[88]
	Sertoli cells, spermatogonia and spermatocytes	[89]
	Leydig cells, Sertoli cells and diploid germ cells	[90]

Overview of estrogenic actions in the male

Role of estrogens in sperm maturation and function

Besides being capable of responding to estrogenic stimuli, as evidenced by the expression of nuclear and membrane estrogen receptors, the mammalian testis could actively synthesize estrogens. This process occurs through the aromatization of androgenic precursors (namely, testosterone (T) and androstenedione) by the activity of the cytochrome-c P450 aromatase enzyme (Figure I.2.2). It is actually established that E_2, the most potent of estrogens, is synthesized by both rodent and human testis, which has been demonstrated by studies characterizing the presence of functional aromatase in testicular cells [65]. The responsiveness to estrogenic hormones, together with the fact that testicular cells can synthesize E_2, clearly supported the physiological role of estrogens on testicular physiology.

However, whether estrogens have beneficial or detrimental effects on spermatogenesis has been a matter of discussion over the years.

Several types of evidences strongly suggested that estrogens are needed for production of viable spermatozoa, and thus, for male fertility. Experimental data have been demonstrating that estrogens exert a negative feedback over the hypothalamus-pituitary-testis axis, suppressing production of gonadotropin releasing hormone and luteinizing hormone (LH) and thus, balancing the T levels [91]. Estrogens also can act directly on LCs downregulating the expression of steroidogenic enzymes involved in T biosynthesis and lowering T production [92-94].

There are reports describing the direct effects of estrogens inducing the onset of spermatogenesis in rodents [95, 96]. Exposure to E_2 accelerated the onset of spermatogenesis in the bank vole (*Clethrionomys glareolus*) while the antiestrogen ICI 182,780 blocked this effect [96]. It was also showed that estrogens may sustain spermatogenesis by promoting the proliferation of spermatogonia in a mechanism that seems to be mediated by the cross-talk between ERα and GPER [82, 97, 98]. However, also the ERβ selective ligand 5□-androstane-3□,17□-diol induced proliferation of spermatogonia in seminiferous tubules cultured *ex vivo* [99]. In the hypogonadal (*hpg*) mice, which are infertile due to the absence of gonadotropin production [100], estrogens were able to restore spermatogenesis [101]. Interestingly, rather than a direct effect in the testis, this stimulatory effect of E_2 in spermatogenesis happened through stimulation of pituitary and elevation of follicle-stimulating hormone (FSH) levels [101, 102].

Furthermore, in an animal rat model with depletion of all germ cell types after exposure to radiation, the administration of E_2 remarkably accelerated the spermatogenic recovery by enhancing spermatogonial differentiation [103]. In addition, it was shown that this estrogen-mediated recovery of spermatogenesis is independent of the regulation of T or gonadotropins production [103], and involves important changes in the expression of estrogen-target genes [104]. Although this study provided a valuable list of candidate genes to be considered in future research aiming at elucidate the role of E_2 in the onset of spermatogenesis, the identification of the underpinning molecular pathways triggered by estrogens still are in the infancy.

Estrogens also seem to be required for spermiogenesis and spermiation, as reported by studies in rodents and primates. Treatment with aromatase inhibitors strongly decreased the population of elongating/elongated spermatids in the testis of adult monkeys [105, 106]. These results were confirmed in rats administered subcutaneously with E_2 but the observed increase in the number of elongating and elongated spermatids was proposed to occur in consequence of a failure in spermiation [107, 108]. Recent work of Cacciola *et al.* demonstrated that estrogens play a role regulating chromatin remodeling in spermatids, particularly by preserving chromatin condensation and DNA integrity [109], and that this is involved in the nuclear size of epididymal spermatozoa [110]. Relevant information supporting the role of estrogens in spermiogenesis was also produced by the studies in mice

with disrupted aromatase *CYP19* gene. These animals displayed failure of spermiogenesis with a significant reduction in the number of round and elongated spermatids, and abnormal development of acrosome [111, 112].

Also, the function of E_2 on capacitation, loss of acrosome and increased motility of spermatozoa in human ejaculated sperm has been reported [70]. Similarly, estrogens stimulated capacitation and acrosome reaction of sperm from mice [113] and boar (*Sus scrofa*) [114].

Nevertheless, the most well know effect of estrogens in male reproductive function probably is the regulation of fluid absorption in the efferent ducts and rete testis [115], which is a crucial event for maintenance of the appropriate osmolality in the epididymis and sperm concentration as demonstrated by the elegant studies using the ERα knockout (αERKO) mice [116-118].

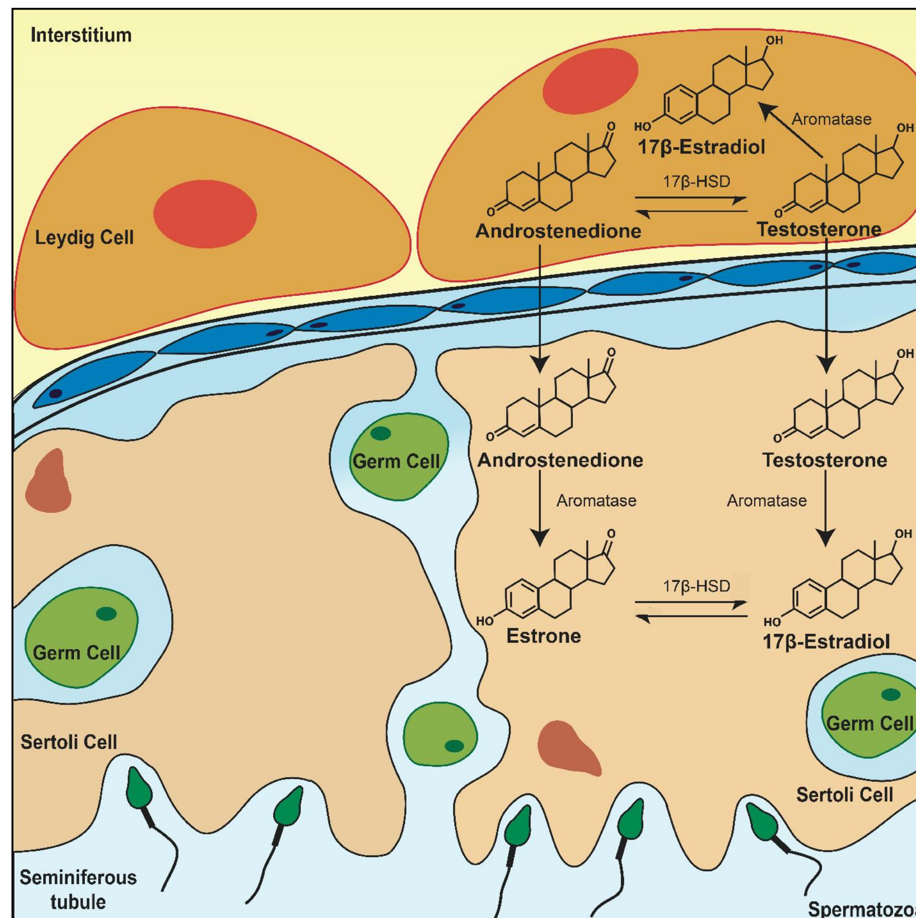

Figure I.2.2. Estrogen biosynthesis in mammalian testis. Testosterone and androstenedione are converted, respectively, to estrone and 17β-estradiol by the activity of aromatase enzyme. The 17β-hydroxysteroid dehydrogenase (17β-HSD) plays a role modulating the interconversion of androstenedione and testosterone, as well as, estrone and 17β-estradiol. In immature testis the primary source of 17β-estradiol seems to be the Sertoli cells whereas in the adult testis the main production is from Leydig cells. However, also for germs cells its capability to synthesize estrogens has been described [65].

Experimental and clinical evidences of estrogens damaging effects for spermatogenesis

Contrasting with the beneficial actions of estrogens in spermatogenesis and sperm maturation are the evidences of the deleterious effects of these hormones in male reproductive function. For example, it has been widely documented that fetal and post-natal exposure to estrogens or estrogen-like substances, known as endocrine disrupters that mimic endogenous estrogens, cause adverse effects in male sexual differentiation [119, 120] and reproductive potential of the adult [121-123]. To the same reasoning contribute the elevated concentrations of E_2 (Table I.2.2) detected in the semen [124-127], intratesticular fluid [128-130] and spermatic vein [131, 132] of idiopathic infertile men. Also, higher serum levels of E_2 together with lower levels of T were reported in men with severe impairment of spermatogenesis [124, 133]. This resulted in a decreased T/E_2 ratio, which was proposed to be the cause of male infertility since treatment with aromatase inhibitors corrected the endocrine defect and significantly improved the semen parameters in oligospermic patients [133, 134]. In this way, both experimental and clinical findings hinted for the possible involvement of estrogens in the disruption of spermatogenesis and consequently in male factor infertility.

Table I.2.2. Estrogens concentrations in serum and reproductive tract of normozoospermic and infertile patients

	E_2 Concentration		References
	Normozoospermic	**Infertile patients**	
Testicular tissue	12 ng/g tissue	175 ng/g tissue	[128]
	4.5 ng/g tissue	29.2 ng/g tissue	[129]
	53 pg/ mg protein	137 in Complete Sertoli cell only syndrome (SCOS), 133 in Focal SCOS, 143 in Maturation Arrest and 380 pg/ mg protein in Mixed atrophy	[130]
Spermatic Vein	926 pg/ml	1090 pg/ml	[132]
Seminal Plasma	235 pmol/L	260 in oligozoospermia and 275 pmol/L in oligoteratoasthenozoospermia	[124]
	162 pg/ml	258.1 pg/ml	[125]
	256.6 pmol/L	296.8 in azoospermia and 523.6 pmol/L in obstructive azoospermia	[126]
	26.0 pmol/L	38.15 and 36.4 pmol/L in in oligoasthenoteratozoospermia with and without varicocele, respectively	[127]
Serum	117 pmol/L	138 pmol/L	[124]
	43.5 ng/L	58.4 ng/L	[133]

Role of estrogens controlling survival and death of testicular cells

The adverse effects of estrogens on spermatogenesis are essentially linked to the induction of germ cell apoptosis. However, as described above estrogens seem to be able to restore spermatogenesis and play an important action promoting the proliferation of spermatogonia [97, 98, 101, 103]. In fact, estrogens are traditionally view as sex hormones that can regulate both proliferation and apoptosis in several tissues [135], and this duality of action is also evident in the context of testicular cells.

Estrogens as survival factors for male germ cells

Estrogens have been described as survival factors inhibiting the apoptosis of male germ cells (Table I.2.3). E_2 added to the culture medium of seminiferous tubules cultured *ex vivo* at 10^{-10} M and 10^{-9} M concentrations was able to inhibit the death of germ cells [66]. Moreover, it was shown that E_2 in the concentration range of 10^{-8} M to 10^{-6} M activates the phosphatidylinositol-3-OH kinase/Akt survival pathway in human ejaculated spermatozoa [70]. Findings in the aromatase knockout mice support the ability of estrogens to hold survival of germ cells [111]. These mice with a total absence of estrogen biosynthesis displayed progressive infertility by 14 weeks of age, which was consequence of impaired spermatogenesis at postmeiotic stages [111, 112]. Depletion of round and elongated spermatids in the testis of aromatase knockout mice resulted from increased rates of apoptosis demonstrating the importance of estrogens for development and survival of male germ cells [111]. Also in the case of immature SCs, it has been shown that estrogens improve their proliferation and inhibit apoptosis (Table I.2.3) [136, 137], an effect that requires the translocation of both ERα and ERβ to the plasma membrane region with the consequent activation of epidermal growth factor receptor and the mitogen activated protein kinase signaling pathway [136]. However, the *in vivo* administration of *β*-estradiol-3-benzoate (estradiol benzoate) to adult male rats inhibited SCs maturation and increased their apoptosis [98].

Estrogens as apoptosis-inducers in germ cells

Remarkably, there is another scenario in the estrogenic actions, which contrasts with the ability of estrogens to maintain germ cell survival. It has been largely reported that E_2 or estrogens analogues have a profound impact in testicular physiology by enhancing the apoptotic rate of germ cells and, thus, decreasing sperm counts and fertility (Table I.2.3). Estradiol benzoate, the synthetic ester of E_2 is one estrogen analogue widely used to study the apoptotic effects of estrogens in mammalian testis. *In vivo* administration of estradiol benzoate to adult and newborn rats in doses ranging from 12.5 to 75 µg/animal markedly increased the number of cells undergoing apoptosis for all germ cell types [98, 138-141]. The germ cell loss was further confirmed by a reduction in testis weight [98, 139] and seems to occur mainly for spermatocytes and round spermatids [138, 140].

Daily injections of E_2 (20 or 100 µg/kg/day) to adult male rats for 10 days disrupted the pituitary-gonadal axis reducing serum gonadotropins and intratesticular levels of T levels [107]. Both doses of E_2 culminated in a significant increase of apoptosis in spermatids at stages VII and VIII, which correspond to the differentiation of round spermatids to elongated spermatids [107]. Similar results were obtained through the *in vivo* administration of the synthetic estrogen diethylstilbestrol (DES). DES reduced testicular weight and spermatogenic cell loss in time- and dose-dependent manner [142]. Rat seminiferous tubules cultured *ex vivo* in the presence of 10^{-7} M E_2 displayed increased rates of germ cell apoptosis [143] while a 10^{-9} M dose had no effect (unpublished results). Also, *in vitro* treatment of spermatogenic cells isolated from rat testis with 10^{-8} and 10^{-7} M of E_2 induced the apoptotic cell death of spermatogonia, spermatocytes, and spermatids [144]. An 80% reduction in cell survival was observed 10 h after the initiation of E_2 treatment [144].

Estrogen-induced apoptosis was also observable in rat fetal testis cultured *ex vivo* in presence of E_2 or DES. It was found a deleterious effect on the gonocyte population in consequence of a dramatic increase of apoptosis [145]. The application of *in vitro* and *ex vivo* experimental approaches have demonstrated that estrogens promote apoptosis of germ cells by direct actions in the testis, which do not depend on the interference of the hypothalamus-pituitary axis.

It is worth noting that estrogenic effects inhibiting or promoting apoptosis may strictly depend on the hormonal dose used. This becomes particularly evident when comparing the same cell type and experimental approach. For example, 10^{-10} M and 10^{-9} M of E_2 promoted germ cell survival *in vitro* [66] while 10^{-8} and 10^{-7} M doses induced the apoptotic death of germ cells [143, 144]. Since 10^{-10} M and 10^{-9} M resemble the range of physiological concentrations described in male [66], it is liable to assume that the detrimental effects of estrogens depend on the E_2 concentrations increased above the normal physiological limits. Indeed, the study of Gancarczyk *et al* has demonstrated a duality of effects for low and high doses of E_2 in the testis of blank vole. A low dose of E_2 (0.1 µg/g body weight) accelerated the advance of spermatogenesis showing a positive correlation with the number of spermatids [96]. In contrast, males treated with a high dose of E_2 (10 µg /g body weight) had disrupted testicular morphology, tubular atrophy and increased frequency of apoptosis [96]. The elevated concentrations of E_2 detected in the testis and reproductive fluids of idiopathic infertile patients (Table I.2.2) [128-130] sustain the idea that estrogens could be the cause underlying oligozoospermia and azoospermia by inducing spermatogenic cellular dysfunction due to increased rates of apoptosis.

Table I.2.3. Role of estrogens controlling apoptosis of testicular cells

Cell type affected	Type of study	Agent/dose	Apoptosis	References
Germ cells	*In vivo*	17β-estradiol 20 and 100 μg/kg/day	↑	[107]
		β-estradiol-3-benzoate 75 μg/animal/day	↑	[138]
		β-estradiol-3-benzoate 75 μg/animal/day	↑	[139]
		β-estradiol-3-benzoate 50 μg/animal/day	↑	[140, 146]
		β-estradiol-3-benzoate 12.5 μg/animal/day	↑	[98]
		Diethylstilbestrol 0.01, 0.1 and 1 mg/kg	↑	[142]
	In vitro	17β-estradiol 10^{-10} M and 10^{-9} M	↓	[66]
		17β-estradiol $4x10^{-6}$ - $4x10^{-10}$ M	↑	[145]
		17β-estradiol 10^{-8} M and 10^{-7} M	↑	[144]
		Diethylstilbestrol $4x10^{-6}$ - $4x10^{-10}$ M	↑	[145]
Sertoli cells	*In vivo*	*β*-estradiol-3-benzoate 12.5 μg/animal	↑	[98]
	In vitro	17β-estradiol 10^{-7} M	↓	[137]

↑, induced; ↓, inhibited

Signaling pathways implicated in the estrogenic regulation of germ cell apoptosis

Although the mechanisms by which estrogens exert its apoptotic effects are not fully elucidated, it has been suggested the involvement of both extrinsic (death receptor) and intrinsic (mitochondrial) pathways in this regulation (Figure I.2.1). Mechanistic studies using several experimental approaches, such as, *in vivo* exposure and *in vitro* culture of seminiferous tubules or isolated spermatogenic cells have indicated that estrogens activate the receptor-mediated pathway by upregulating the expression of both Fas and FasL [142-144]. Increased expression of Fas system was responsible for initiating the apoptotic death of spermatogenic cells since it was followed by the activation of caspase-8 (Figure I.2.1) [142, 144]. Chaki and co-authors also reported increased activity of caspase-8 in the rat testis after administration of estradiol benzoate for 30 days [138].

Other works have linked the apoptotic effects of estrogens with the modulation of the intrinsic (mitochondrial) pathway of apoptosis. Bcl-2 protein family members are the key regulators of this pathway, and the interaction and relative abundance among proapoptotic and antiapoptotic members form a dynamic equilibrium, which is determinant to the threshold for apoptosis [147]. It was shown that E_2 or estradiol benzoate upregulated the

expression of Bax in testicular cells [84, 138, 143], which was accompanied by augmented protein ratio of Bax (proapoptotic)/Bcl-2 (antiapoptotic) and increased activity of caspase-3 [143]. Treatment with estrogens was also able to induce the hyperpolarization of mitochondrial membrane [144] and the translocation of Bax from the cytosol to the mitochondria, which was followed by the release of cytochrome *c* and activation of caspase-9 and caspase-3 [138, 142]. However, the use of anti-Fas antibody inhibited mitochondrial hyperpolarization and E_2-induced cell death, and the inhibition of caspase-8 prevented cell death, which indicates the major importance of the death-receptor pathway [144]. Moreover, it was demonstrated that the mitochondrial hyperpolarization is induced by the augmented generation of nitric oxide and superoxide, respectively, in consequence of increased expression of inducible nitric oxide synthase and E_2 metabolism [144]. Indeed, it has been widely accepted that oxidative stress is damaging for testicular function representing one of the main factors inducing the apoptotic cell death of male germ cells [148]. Other study also has showed that estradiol benzoate treatment causing enhanced rates of apoptosis in rat testis, concomitantly increased the lipid peroxidation and reduced the antioxidant defense due to decreased enzymatic activity of superoxide dismutase and catalase [138].

Apart from the effects on induction of apoptosis there are reports describing the anti-proliferative actions of E_2 and the underpinning signaling mechanisms. E_2 administration reduced the germ cell proliferation in rat seminiferous tubules cultured *ex vivo* by a downregulated expression of the tyrosine kinase receptor c-kit [143]. The c-kit and its ligand, the SCF, are recognized as one of the major mediators of survival communication between SCs and germ cells protecting germ cells from apoptosis [18, 149], thus, disruption of this system is expected to deeply affect the spermatogenic output. Also in spermatocytes isolated from rat testis the E_2 treatment had diminished cell proliferation [83]. This effect occurred by the activation of the EGFR/ERK/c-Jun pathway with the downstream down-regulation of the expression of cell cycle regulators cyclin A1 and B1 [83].

Conclusion

Besides the ability responding to endocrine estrogenic stimuli, the mammalian testis can also synthesize estrogens, and the role of these sex steroid hormones in male reproduction has gained increasing interest in the last decade. Our knowledge of the estrogenic actions in male reproduction highlighted for the important role of estrogens in the control of spermatogenesis. Although classically known as mitogenic agents, it was shown that estrogens can regulate both the survival and death of testicular cells. However, the literature contained controversial reports on the beneficial or detrimental effects of estrogens for spermatogenesis jumping from “heaven to hell”. Presently, a substantial amount of data has been generated pointing estrogens as apoptosis-inducers of male germ cells and consequently as deleterious agents for male fertility. However, the survival or apoptotic actions of estrogens seem to be related with the hormone dosage used in the experimental assays. At

doses above the physiological concentrations, estrogens are no longer survival agents but instead they become powerful apoptotic trigger inducing the apoptosis of germ cells. Moreover, the apoptotic effects of estrogens are consistent with the clinical findings in male infertile patients displaying decreased ratios of T to E_2. In this way, the role of estrogens as apoptosis inducers, supported by a huge amount of experimental data, provided a rationale to explain the successful use of aromatase inhibitors to treat male infertility. Finally, disclosing the complexity of estrogens signaling mechanisms in the induction of apoptosis of germ cells will provide new clues to understand the etiology of spermatogenic disorders and identify new therapeutic targets for male infertility.

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Conflicts of interest

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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Regucalcin, a calcium-binding protein with a role in male reproduction?

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Regucalcin, a calcium-binding protein with a role in male reproduction?

Abstract

Regucalcin (RGN) is a calcium (Ca^{2+})-binding protein which plays an important role in the regulation of Ca^{2+} homeostasis and has been shown to catalyze an important step in the L-ascorbic acid biosynthesis. It is encoded by an X-linked gene and differs from other Ca^{2+}-binding proteins by lacking the typical EF-hand Ca^{2+}-binding domain. RGN controls intracellular Ca^{2+} concentration by regulating the activity of membrane Ca^{2+} pumps. Moreover, RGN has been indicated to regulate the activity of numerous enzymes and to act in the regulation of cell proliferation and apoptosis. The importance of Ca^{2+} homeostasis in spermatogenesis has been demonstrated by several studies, and its disruption has been shown to cause reversible male infertility. Recently, the expression of RGN in male reproductive tissues has been described and its localization in all testicular cell types was demonstrated. In addition, RGN expression is regulated by androgens, a class of steroid hormones recognized as male germ cell survival factors and of uttermost importance for spermatogenesis. Altogether, available information allow to hypothesize that RGN might play a role in spermatogenesis, directly or as a mediator of androgen action. This review aims to discuss this hypothesis presenting novel data about RGN expression in human testis.

Key words: apoptosis / calcium / male infertility / steroid hormones / spermatogenesis

Introduction

Regucalcin (RGN) was first identified in 1978 as a calcium (Ca^{2+})-binding protein [1]. It differs from common Ca^{2+}-binding proteins such as calmodulin because it does not contain the typical EF-hand Ca^{2+}-binding motif [2]. Later it was identified by another group and named Senescence Marker Protein-30 (SMP-30), for its characteristic downregulation with aging in rat liver [3]. RGN was also shown to function as gluconolactonase, the enzyme catalyzing the penultimate step in the biosynthesis of L-ascorbic acid [4]. The ability to synthesize L-ascorbic acid has been lost during the course of evolution on a number of species including humans, becoming a vitamin; however it is still present in rats and mice [5]. The expression of RGN is stimulated by Ca^{2+} [6-8] and can be regulated by several factors which include AP-1 [9], β-catenin [10], nuclear factor I-A1 (NF1-A1) [11], and also RGN gene promoter region-related protein [12, 13]. Also, steroid and non-steroid hormones have been described to regulate RGN expression [6, 14-19].

The function of RGN in the regulation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), is achieved by controlling the activity of Ca^{2+} channels, Ca^{2+}-ATPase in the membrane of mitochondria and endoplasmic reticulum [20, 21] and (Ca^{2+}-Mg^{2+})-ATPase in the plasma membrane [22, 23].

RGN also plays an important role in the regulation of Ca^{2+}-dependent enzymes [24]. One of these enzymes is cyclic AMP (cAMP) phosphodiesterase, which degrades cAMP, providing a way by which RGN regulates cAMP levels in cells [25, 26]. However, most enzymes regulated this way are protein kinases and phosphatases, which in turn will regulate the activity of other proteins [27-30]. RGN also inhibits nitric oxide synthase [31-33] and Ca^{2+}-dependent endonuclease activities; this is likely related with its anti-apoptotic actions [34]. RGN seems to inhibit apoptosis by upregulating the expression of Akt-1 and Bcl-2 while downregulating the expression of Caspase-3 [35]. RGN's antiapoptotic effect has been highlighted by the development of a knockout mice, whose cells are more prone to apoptosis than their wild-type counterparts [36, 37].

The importance of androgens, Ca^{2+} homeostasis, and apoptosis control to spermatogenesis has been indicated by a number of studies [38-46], and recently RGN was identified as an androgen-target gene, being broadly expressed in the testis and other tissues of male reproductive tract [16, 18]. This led us to hypothesize that RGN might play a role in the regulation of the complex biological process of spermatogenesis which is the base of male fertility. The purpose of this review is to discuss this hypothesis presenting also novel data of RGN expression in distinct phenotypes of human spermatogenesis.

RGN is a highly conserved X-linked gene

The importance of the X-chromosome to mammalian spermatogenesis has been suggested by its enrichment in genes highly expressed in testis, which play important roles in regulation of male fertility [47, 48]. RGN is an X-linked gene localized in the p11.3-q11.2 and q11.1-12 segments of the human [49] and rat [50] X chromosome, respectively. Both in human and rodents the RGN gene consists of seven exons and the cDNA contains an open reading frame encoding 299 amino acids with an estimated molecular weight of 33 kDa [2, 51, 52] Figure I.3.1). Two alternatively spliced mRNA variants, originated by exon skipping mechanisms, have been described for RGN in breast and prostate tissues and cell lines [18], RGNΔ4 with deletion of exon 4, and RGNΔ4,5 missing exons 4 and 5. Using the strategy described in [18] these transcripts were also detected in human testis (Figure I.3.1). The *in vitro* and *in vivo* existence of two forms of RGN protein with lower molecular weights (28 and 24kDa) has been recently suggested [53]. However, it is unknown whether they represent the translated RGNΔ4 and RGNΔ4,5 transcripts or are generated by post-translational processing. The precise function of these variants remains unexplored.

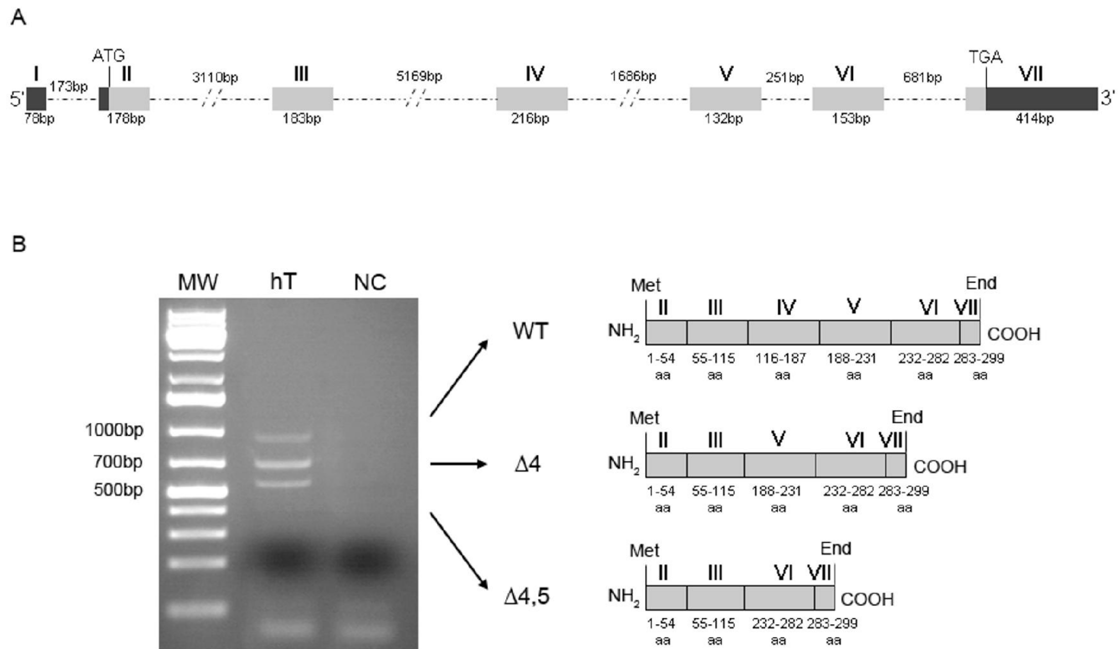

Figure I.3.1 RGN gene organization, testicular mRNA transcripts and hypothetical proteins. (A) Human RGN gene organization. Grey and black boxes indicate coding and noncoding regions, respectively. Dotted lines correspond to introns. Roman numerals indicate the exons and Arabic numerals the number of base pairs. (B) Using primers spanning the entire RGN coding region and the strategy described in [18], two hypothetical alternatively spliced mRNA variants (Δ4 and Δ4,5) were found. On the right panel are represented the proteins encoded by alternative transcripts. MW, DNA molecular weight marker; hT, human testis; NC, negative control (no cDNA template); WT, Wild type RGN; Δ4, RGN exon 4 deleted variant; Δ4,5, RGN exon 4 and 5 deleted variant. Arabic numerals indicate the number of amino acids (aa) encoded by each exon.

RGN protein is highly conserved from eukaryotes to prokaryotes, with 18% of residues absolutely conserved in all species (Figure I.3.2). The amino acid sequence of human RGN shows 98% of similarity with primates, 93-96% with other mammalian species and 79-85% with non-mammalian vertebrates. Considering invertebrates, bacteria and fungi the percentage of similarity ranges from 43 to 47% which still is considerably high. The high conservation of RGN protein throughout evolutive line, from invertebrates to vertebrates, indicates its involvement in basic and important biological functions.

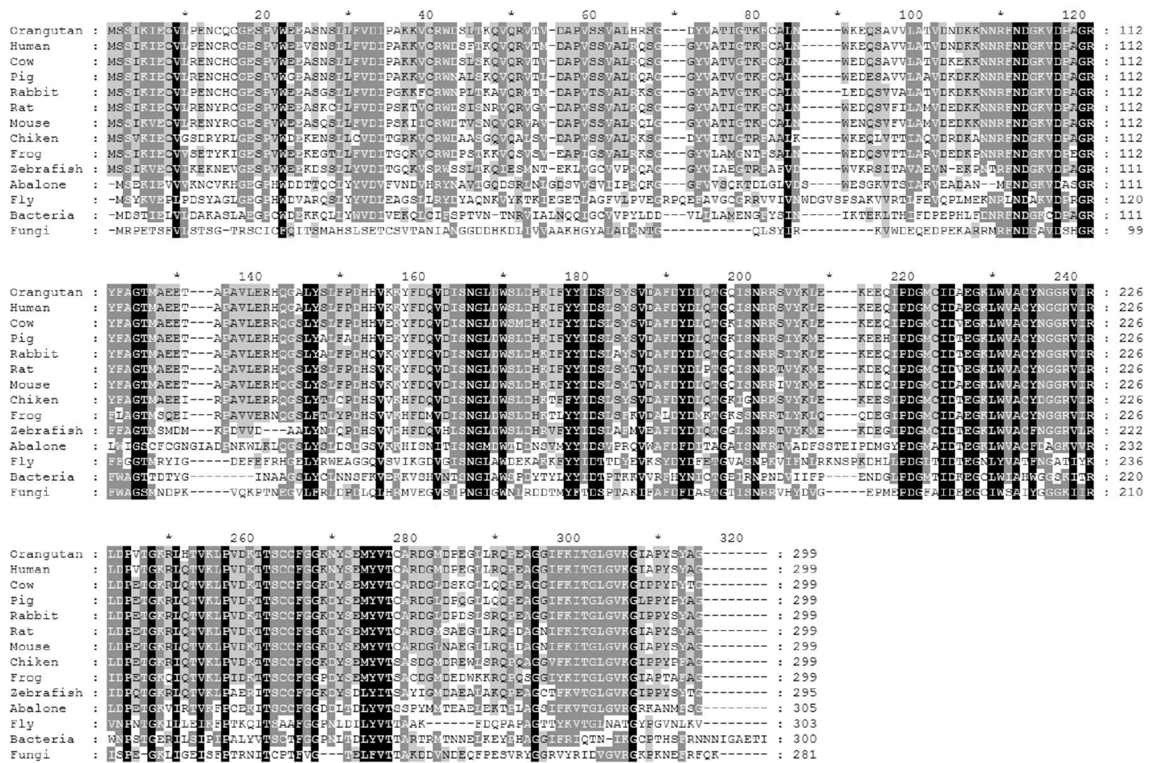

Figure I.3.2 Multiple sequence alignment of RGN proteins and homologues in eukaryotes and prokaryotes. Alignment was produced with Clustal X alignment tool [54] displaying the conservation shade mode. Three shading levels from black to light grey indicate residues 100%, 80% or 60% conserved, Numbers indicate residue's position and total number of residues in each sequence. 18% of residues are 100% conserved (black shading) in all species. Latin names and Genbank accession numbers for corresponding sequences were: orangutan, *Pongo abelii* (NP_001127502.1); human, *Homo sapiens* (NP_004674.1); cow *Bos taurus* (NP_776382.1); pig, *Sus scrofa* (NP_001070688.1); rabbit *Oryctolagus cuniculus* (NP_001075472.1); rat, *Rattus norvegicus* (NP_113734.1); mouse, *Mus musculus* (NP_033086.1); chiken, *Gallus gallus* (NP_990060.1); frog, *Xenopus laevis* (NP_001079124.1); zebrafish, *Danio rerio* (NP_991309.1); disk abalone, *Haliotis discus discus* (ABO26616.1); fruit fly, *Drosophila melanogaster (*NP_727586.1); bacteria, *Bacillus cereus* (NP_978918.1); fungi,(XP_751966).

RGN Expression in Male Reproductive Tract

For a long time the study of RGN expression was focused mainly on non-reproductive tissues. It was shown to be expressed mainly in liver and kidney cortex [6, 55], but also in brain [56], heart [57], bone [58], lung [59], and submandibular gland [60]. However, RGN was also shown to be expressed in reproductive tissues such as the ovary [61], breast and prostate [17, 18].

More recently, RGN expression was studied in male reproductive tract [16] (Table I.3.1). In seminal vesicles RGN immunoreactivity was confined to epithelial cells while on epididymis, besides epithelium, RGN was also localized to smooth muscle and connective tissue [16]. In prostate, RGN mRNA and protein are localized to epithelial cells [16-18] and it seems to be associated with cancer development, since loss of RGN expression was detected in human prostate cancer cases [18]. Both in rat and human testis, a broad expression of RGN protein was confirmed by immunohistochemistry, showing that all testicular cell types, somatic and germ line, express RGN (Table I.3.1, [16]). This common interspecies testicular localization of RGN suggests that it may play an important role in testicular physiology.

RGN has been shown to be secreted into pea aphid saliva [62] and plasma of several species [63-66]. In a recent report, RGN protein was also detected in seminiferous tubule fluid [16], which is mainly a product of Sertoli cells (SC) secretory activity [67]. This complex fluid creates the perfect environment for germ cell development and maturation [67, 68]. It is known that RGN protein can enter cells and modulate the activity of several enzymes, including protein kinases and phosphatases. RGN is also known to regulate Ca^{2+}-ATPases, which are known to play an important role in the mechanisms of sperm capacitation and motility [69, 70]. Whether or not the presence of RGN in seminiferous tubule fluid is related to the control of testicular sperm production and maturation through the regulation of enzyme activities and $[Ca^{2+}]_i$ is unknown at this point, however it would be interesting to explore RGN possible actions in sperm physiology.

Table I.3.1. Localization of RGN in male reproductive organs.

Tissue	Localization	Reference
Epididymis	Epithelium, smooth muscle, connective tissue,	[16]
Prostate	Epithelium	[16, 17]
Seminal vesicles	Epithelium	[16]
Testis	Leydig cells, Sertoli cells, spermatogonia, spermatocytes, spermatids	[16]

RGN Expression in Distinct Spermatogenic Phenotypes

Successful spermatogenesis demands a delicately regulated equilibrium between germ cell apoptosis and proliferation [71]. Hypospermatogenesis (HP) has been linked to a deregulation in germ cell proliferation and apoptosis [72]. Also, the expression of several apoptosis-related genes has been shown to be altered in testis with defective spermatogenesis [73-78]. This led us to analyse RGN expression in abnormal phenotypes of human spermatogenesis, namely HP and Sertoli cell-only syndrome in comparison with cases of oligoazoospermia (OAZ) with conserved spermatogenesis (Figure I.3.3). Interestingly, testis from men with HP show higher expression of RGN mRNA (1.6 fold; $P<0.001$) relative to testis with conserved spermatogenesis (Figure I.3.3). Considering RGN's role suppressing cellular proliferation [79, 80], the presented results raise the question whether the higher expression of RGN in testis of patients with HP may be causing a blockage in cell proliferation in this phenotype. Moreover, these data corroborate the importance that RGN may have to the mammalian spermatogenic process.

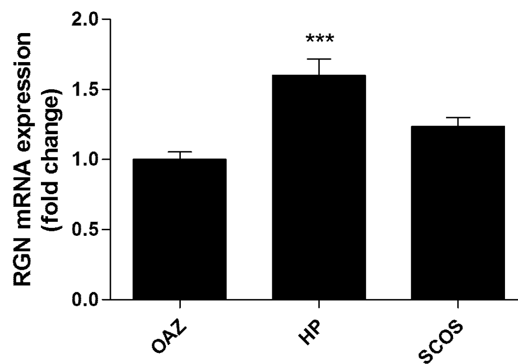

Figure I.3.3 Expression levels of RGN in testicular biopsies from men with obstructive azoospermia with conserved spermatogenesis (OAZ; n=6), hypospermatogenesis (HP; n=5) and Sertoli cell-only syndrome (SCOS; n=6). Human testis samples were obtained by testicular biopsy from men undergoing fertility treatment under informed consent according to the local ethics committee and complying with the Declaration of Helsinki for Medical Research involving Human Subjects [81]. Upon RNA extraction and cDNA synthesis RGN mRNA expression in different groups was determined by quantitative PCR, using specific primers for amplification of RGN (sense: gcaagtacagcgagtgacc; antisense: ttcccatcattgaagcgattg). Normalization was made using β2-microglobulin (sense: atgagtatgcctgccgtgtg; antisense: caaacctccatgatgctgcttac) and GAPDH (sense: cgccagccgagccacatc; antisense: cgcccaatacgaccaaatccg) as internal reference genes. ***P<0.001. Statistically significant differences between the three groups was evaluated by one-way ANOVA followed by Bonferroni's multiple comparison test, using GraphPad Prism v5.00 (GraphPad Software, San Diego, CA, USA) [16].

Effects of Sex Steroids on RGN Expression

RGN expression is regulated by numerous factors, including Ca^{2+} [6], insulin [82], aldosterone [15], sex steroid hormones [15-19], amongst others (Table I.3.2). The regulation of RGN expression by sex steroid hormones was first described in 1995 [19]. Subcutaneous administration of 17β-estradiol (E_2) to rats leads to a sharp increase in the expression of RGN mRNA in liver, which was suggested to be related to estrogen regulation of liver metabolism [19]. However, this estrogenic control of RGN expression is not limited to the liver, as shortly after it was reported the regulation of RGN expression by E_2 in rat kidney cortex [15]. It was shown that administration of E_2 to rats caused a reduction of RGN expression in the rat kidney cortex, an effect opposite to the one observed in liver [15]. More recently, the effect of sex steroid hormones regulating RGN expression in breast and prostate has been reported [17, 18]. Administration of E_2 to rats induces a downregulation of RGN expression in prostate and mammary gland [17]. On the other hand stimulation of MCF-7 breast cancer cells with E_2 causes an upregulation of RGN expression by a mechanism that is likely to involve a membrane estrogen receptor (ER), [18]. In LNCaP prostate cancer cells 5α-dihidrotestosterone (DHT) downregulates RGN expression by a mechanism that seems to involve the AR and *de novo* protein synthesis [18]. In rat seminiferous tubules cultured *ex vivo* the expression of RGN has been shown to be upregulated by DHT administration (10^{-7}M), in a mechanism that involves the AR but does not require *de novo* synthesis of protein [16]. This allowed the identification of RGN as a new androgen-target gene in the testis.

Table I.3.2. Hormonal factors regulating RGN expression in reproductive and non-reproductive tissues.

Hormone	Tissue	Effect	References
Aldosterone	Kidney	↓	[15]
17β-estradiol	Liver	↑	[19]
	Kidney	↓	[15]
	Breast	↓ (↑ MCF-7 cells)	[17, 18]
	Prostate	↓	[17]
Calcitonin	Liver	↑	[83]
5α-dihydrotestosterone	Testis Prostate	↑ ↓ (↓ LnCaP cells)	[16, 18]
Dexamethasone	Kidney	↑	[15, 84]
Insulin	Liver	↑	[82]

↑ up-regulation; ↓ down-regulation

RGN Actions in Testis Physiology

Although RGN has been shown to be an important regulator of cellular Ca^{2+} homeostasis in several tissues, its role in spermatogenesis is only beginning to be studied. The testicular localization of Ca^{2+} shows that this ion is quite abundant in all testicular cell types, which indicates that it may play a role in cell growth and differentiation [85], [86]. Tight control of $[Ca^{2+}]_i$ homeostasis has also been shown to be of critical importance for the maintenance of SC function [40, 41, 87, 88] and to Leydig cells steroidogenesis [89, 90]. On the other hand Ca^{2+} channel blockers are known for causing negative effects on mammalian spermatogenesis, being associated with reversible infertility [42-46, 91, 92].

Ca^{2+} serves important biological functions, acting as a second messenger in several transduction pathways or regulating apoptotic cell death, among others [93, 94]. Apoptosis is vital for the occurrence of normal spermatogenesis [71] since about 75% of testicular germ cells undergo apoptosis [95]. Deregulation in the cell death/survival balance is thought to cause disruption of spermatogenesis [96] and altered expression of apoptosis-related genes has been shown to be related to male infertility [74, 75, 77, 78]. It has been demonstrated that RGN is able to regulate apoptosis both *in vivo* and *in vitro* [37, 97]. Its presence in all testicular cell types, and also in seminiferous tubule fluid, indicates a potentially important role for this protein in testicular physiology, probably through the control of proliferation and apoptosis [16]. Androgens are regulators of testicular cell death and are recognized as germ

cell survival factors [98-102]. The upregulation of RGN expression by androgens in mammalian testis [16] may be part of the mechanism by which these hormones protect germ cells from apoptosis. In figure I.3.4 a hypothetical pathway for the regulation of RGN gene expression by androgens is depicted, as well as the intracellular signalling pathways in which RGN is expected to be involved, based on the available knowledge of its functions in other cells and tissues. It includes the classical action through the AR as well as other pathways triggered by androgens which might also be involved. It is known that RGN expression is regulated by NF1-A1 through PI3K [11, 103], AP-1 [9] and β-catenin [10], transcription factors which in turn can be activated by AR ([104-106] and references therein). RGN acts in the control of $[Ca^{2+}]_i$ by regulating the activity of Ca^{2+} pumps and channels [20-23] and regulates the activity of protein kinases and phosphatases [27-30], which in turn can regulate the activity of numerous phospho-proteins [107]. Moreover, RGN can regulate the expression of genes and the activity of proteins involved in apoptosis, favouring cell survival [34, 35] which could be of crucial relevance in the spermatogenic process.

RGN has also been shown to play a role suppressing oxidative stress [108-110]. The study of RGN deficient mice has demonstrated that RGN acts as gluconolactonase, a key enzyme in the biosynthesis of L-ascorbic acid [4]. Ascorbic acid is a known powerful antioxidant [111], and a role in the balance between cell survival and death has been indicated for this molecule [112-114]. Indeed, RGN knockout mice were shown to display higher levels of oxidative stress than their wild type counterparts [108], and RGN was shown to increase the activity of anti-oxidant enzyme superoxide dismutase [110, 115, 116] and to suppress the generation of reactive species [109, 110], indicating that it might have antioxidant activity. It is well established that oxidative stress contributes to defective spermatogenesis by damaging spermatogenic cells and sperm function, being one of the major causes of male infertility (reviewed by [117]. Therefore, protection against oxidative stress is other of potential roles of RGN in spermatogenesis.

Altogether, the available information is suggestive of a role for RGN in germ cells survival and development, likely associated with control of apoptosis and oxidative stress. Moreover, RGN has been linked to development of prostate and liver cancers [18, 118-120] but a possible connection between RGN and testicular cancer is yet to be explored.

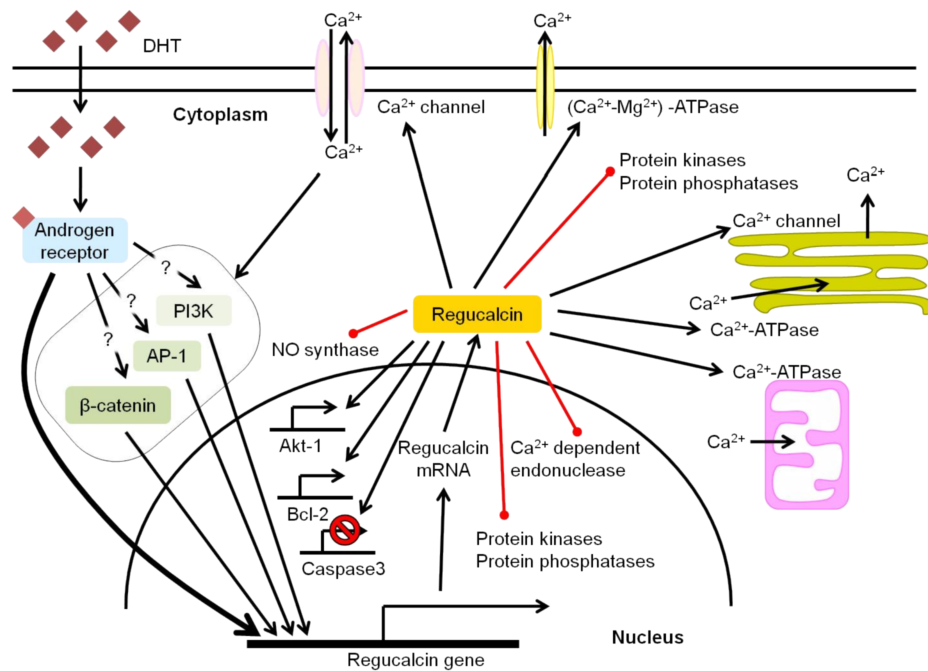

Figure I.3.4. Schematic representation of the potential signalling pathways involved in the androgenic control of RGN expression in testis, and the possible roles of RGN protein in testicular cells. Ball-headed arrows represent inhibition. RGN expression can be regulated by an increase in the intracellular concentration of calcium ($[Ca^{2+}]_i$) which activates multiple transcription factors, including PI3K, AP-1 and ß-catenin. These transcription factors are also known to be activated by androgen (such as 5α-dihidrotestosterone, DHT)-bound androgen receptor, which can also activate RGN transcription independently. In turn, RGN can increase the expression of apoptosis inhibitors Akt-1 and Bcl2, while repressing the expression of Caspase 3. It can also inhibit the activity of nitric oxide (NO) synthase and Ca^{2+} dependent endonucleases, thereby inhibiting apoptosis. RGN regulates $[Ca^{2+}]_i$ by regulating the activity of Ca^{2+} channels, Ca^{2+}-ATPase in mitochondria and endoplasmic reticulum and (Ca^{2+}-Mg^{2+})-ATPase in the plasma membrane. It can also control the activity of numerous proteins by inhibiting the activity of protein kinases and phosphatases.

Reproductive phenotype of RGN knockin and knockout models

A knockout mouse for RGN has been developed by introducing a germ line null mutation [37]. These mice were indistinguishable from their wild-type litter mates but have decreased body weight and life span [121], as well as an increased susceptibility to hepatocyte apoptosis and liver injury [37], impaired pancreatic ß-cell function [122], and scurvy [4]. However, no reproductive abnormalities have been reported for these mice which display a fertilization capability indistinguishable from their wild-type littermates [37]. On the other hand RGN-overexpressing transgenic rats (knockin) have been generated by pronuclear microinjection of a transgene containing cDNA encoding RGN [123]. These rats appear normal, although females display decreased body weight when compared to their wild-type counterparts [123]. Knockin males and females seem to be fertile and able to breed normally [123]. Nevertheless, the reproductive phenotype of both RGN knockin and knockout animals has never been described in detail and therefore mild or later age alterations in spermatogenesis might have passed unsuspected. For example, mice lacking L-gulono-γ-lactone oxidase, another key enzyme in ascorbate biosynthesis, have been shown to have abnormal spermatogenesis due to increased

spermatocyte apoptosis [124], however they were able to breed normally [125]. Aromatase knockout mice (ArKO) come as an interesting example in the importance of evaluating the fertility over extended periods of time. Male ArKO mice were initially described as phenotypically normal, fertile and able to breed normally [126]. However, detailed studies of their reproductive phenotype have revealed that these animals progressively develop a disruption of spermatogenesis, despite no decreases in gonadotrophins or androgens [127]. A thorough study of the spermatogenic status of RGN KO mice will be helpful to indicate the precise function for this protein in mammalian spermatogenesis.

Conclusions and Perspectives

RGN is a Ca^{2+} binding protein that plays an important role in the control of Ca^{2+} homeostasis by regulating the activity of membrane Ca^{2+} pumps and transporters. Ca^{2+} is recognized to play a role in the regulation of testicular functions and inappropriate Ca^{2+} homeostasis in testis is known to disrupt spermatogenesis. RGN is widely expressed along the male reproductive tract, most notably in several cells in the testis, and its testicular expression is controlled by androgens, the main regulators of spermatogenesis. Therefore it is predicted it might play a role in the regulation of spermatogenesis, directly or as a mediator in androgen's response.

In addition to its role as a regulator of $[Ca^{2+}]_i$, RGN is able to regulate cell proliferation and apoptosis, and to suppress oxidative stress, a set of processes known to influence successful spermatogenesis. Further studies will help determine if RGN is involved in the regulation of germ cell proliferation and apoptosis, as well as to decipher its role in the pathophysiology of testicular function, such as unbalance in germ cell survival which leads to disruption of spermatogenesis.

In conclusion, RGN is a protein with potential importance in the regulation of mammalian spermatogenesis. The study of its precise functions can improve the knowledge about the androgenic regulation of spermatogenesis, as well as the control of cell survival and proliferation in testis and regulation of spermatozoa production and maturation. In addition, new perspectives of research providing novel clues to understand the etiology of idiopathic male infertility and testicular cancer are already open.

Authors' roles

S.L. performed the experimental work, analysed the data and wrote the paper. S.C. participated in the research and data analysis. J.E.C. and P.F.O. have participated in manuscript discussion. M.S. participated in the selection and testicle tissue analysis. A.B. participated in patient recruitment. S.S. was responsible for overall study design, critical discussion and approval of the manuscript.

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Calcium homeostasis in the regulation of spermatogenesis and sperm maturation

Chapter in preparation for submission **Correia S**, Vaz CV, Marques R, Cavaco JE and Socorro S. Calcium homeostasis in the regulation of spermatogenesis and sperm maturation.

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Abstract

Spermatogenesis is a highly coordinated process that takes place inside the seminiferous tubules and that transforms spermatogonial stem cells into spermatozoa. Successful production of male germ cells and proper function of spermatozoa depend on an accurate supply of hormonal and non-hormonal factors. In the last years, the importance of Ca^{2+} homeostasis in spermatogenesis has been demonstrated, and its disruption has been associated to reversible male infertility. Moreover, the role of Ca^{2+} homeostasis in the biochemical and molecular mechanisms underlying the development of germ cells and its maturation has started to be disclosed. This review systematically addresses the different types of evidence confirming the involvement of Ca^{2+} in the regulation of spermatogenesis and sperm maturation.

Introduction

Spermatogenesis is the complex biological process that transforms spermatogonial stem cells into spermatozoa over an extended period of time within the boundaries of seminiferous tubules in the testis [1]. Normal development and maintenance of spermatogenesis depends on an accurate regulation of cell division and differentiation orchestrated by a specific assortment of hypothalamic, pituitary and gonadal hormonal messengers [2]. Besides the well-defined hormonal events, other non-hormonal factors have been identified to play a role in the regulation of spermatogenesis. Among other, rises in intracellular calcium concentration ($[Ca^{2+}]_i$) are key signals triggering cell division, differentiation, and maturation [3]. Therefore, Ca^{2+} handling mechanisms are likely important for the unique processes of carried out by male germ cells. In fact, several clinical and experimental evidences pointed to a pivotal role of Ca^{2+} homeostasis in the regulation of spermatogenesis and male fertility. The references indicating that Ca^{2+}-channel blockers cause negative effects on mammalian spermatogenesis, being associated with male reversible infertility, were of paramount importance [4-10]. Also, tight control of $[Ca^{2+}]_i$ homeostasis has been shown to be critical for maintenance of Sertoli cells (SCs) function [11] and to Leydig cells steroidogenesis [12].
This review will discuss the roles of Ca^{2+} in spermatogenesis and sperm maturation contributing to further elucidate the mechanisms regulating male reproduction and its implications in infertility.

Ca^{2+} homeostasis in spermatogenesis

The first evidence that Ca^{2+} dependent events are crucial for developing germ cells emerged in the 80s [13, 14]. It was shown at that time that Ca^{2+} currents are generated by germ cells

and that the density of these currents increase during the development from spermatogonia to early spermatids [15]. More recently, it has been suggested that survival and differentiation of spermatogenic cells also involves changes in the concentration of $[Ca^{2+}]_i$ [16-20], which is not properly surprising considering the ubiquitous well known roles of Ca^{2+} controlling processes such as, proliferation, differentiation, secretion, contraction, transcription, phosphorylation and also apoptosis [21].

In a resting cell $[Ca^{2+}]_i$ is kept very low (≈100 nM) by an orchestrated action of several proteins (Figure I.4.1): Ca^{2+} pumps, that either extrude Ca^{2+} across the plasma membrane to the extracellular environment or uptake Ca^{2+} into intracellular organelles; Ca^{2+} channels that allow entry of extracellular Ca^{2+}, or release Ca^{2+} from intracellular organelles; and a diverse array of Ca^{2+}-binding proteins (CBPs) [3]. Male germ cells possess many mechanisms to regulate cytoplasmic Ca^{2+}, some of them specific for this cell type, but, the role of Ca^{2+} in spermatogenesis and the precise mechanisms that maintain $[Ca^{2+}]_i$ in testicular cells have remained elusive. In the next topics, data on the developmental gene expression and detection of Ca^{2+} modulators in the testis, as well as, the characterization of the phenotype of knockout animals with disruption of Ca^{2+} regulators, and clinical findings will be summarized in order to systematically address these questions.

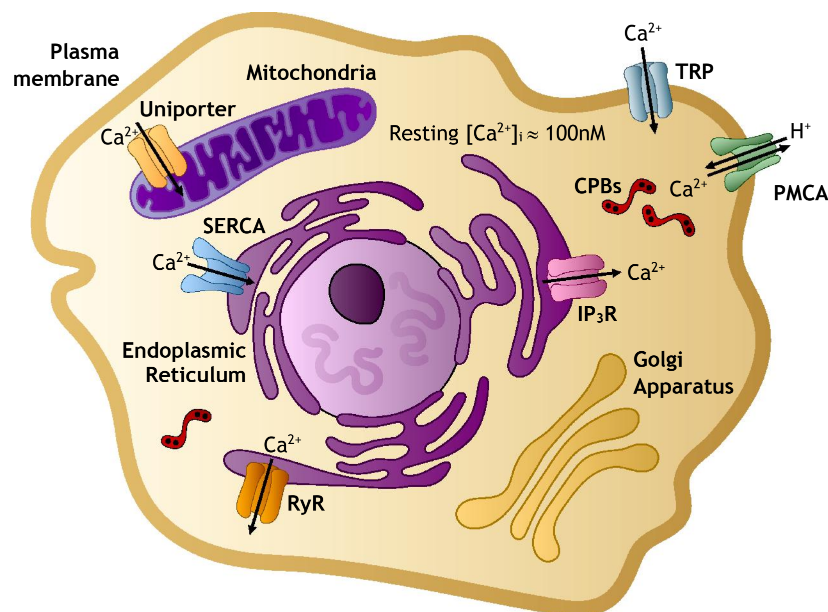

Figure I.4.1. Molecular players in intracellular Ca^{2+} homeostasis. Resting intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is kept low (≈100 nM) by an orchestrated action of Ca^{2+} transporters, Ca^{2+}pumps, ion channels and Ca^{2+}-binding proteins (CPBs). Inositol 1,4,5-trisphosphate receptors (IP_3R) and ryanodine receptors (RyR) are responsible for the release of Ca^{2+} stored at organelles, like endoplasmic reticulum. Also in the endoplasmic reticulum, a Ca^{2+}-ATPase (SERCA) actively transports Ca^{2+} across the membrane. Ca^{2+} entrance to the mitochondria occurs by an uniporter mechanism. At cell membrane, transient receptor potential (TRP) ion channels are responsible for Ca^{2+} entry into the cells while the Ca^{2+}-ATPase (PMCA) pumps Ca^{2+} from the cytosol.

Developmental gene expression studies

A distinct and developmental appearance of CBPs, such as, calmodulin (CaM), calbindin D-28K, S-100 and parvalbulmin in mammalian and non-mammalian testis suggested a possibly role for these proteins in hormone production and, consequently, in the regulation of spermatogenesis [22]. In fact, a mobility shift assay in extracts of adult mouse testis showed 29 spots of different CBPs. Moreover, drastic changes in the expression of high-molecular weight CBPs were observed in the testis 3 weeks after birth whereas a large mass of other CBPs were expressed constitutively throughout postnatal development [23].

Three CaM genes that encode an identical protein were detected in the adult testis of a variety of mammalian species supporting a unique developmental pattern for CaM during spermatogenesis [24]. In mouse testis, three well known Ca^{2+}/CaM-dependent phosphodiesterases (CaM-PDEs) genes, PDE1A, PDE1B, and PDE1C are highly expressed in developing germ cells exhibiting a distinct spatial and temporal expression profile [25]. These CaM-PDEs are one of the ways by which Ca^{2+} regulates cellular cyclic nucleotide content and their differential expression pattern suggests their involvement during spermatogenesis [25]. Moreover, a major CaM-binding protein complex (CaMBP) was identified in rat testes and found to be similar to the members of the heat-shock protein (HSP) family, namely the testis-specific HSP70 [26]. Immunohistochemistry analysis demonstrated that CaMBP was co-localized with CaM in the cytoplasm of pachytene spermatocytes and nuclei of round spermatids. In addition, CaMBP, but not CaM, was localized at a high level in the residual bodies of elongated spermatids highlighting for the importance of CaMBP on the regulation of cell cycle progression and spermiogenesis [26]. CaM was also identified during the spermiogenesis of a phytophagous bug (*Platyscytus decempunctatus*) and found to be expressed virtually in the same stages were Ca^{2+} was observed, which demonstrated that Ca^{2+} and CaM contribute together for spermiogenesis in these insects [27].

In chickens, the comparison of the time-course appearance and increase of calbindin D-28K expression in spermatogonias and spermatocytes support its involvement in the mitotic phase of spermatogenesis [28].

Beyond its identification in mammalian testis, a parvalbumin-like protein has been identified as a major protein of common carp (*Cyprinus carpio*) spermatozoa appearing during the final stage of spermatogenesis [29]. The presence of parvalbumin-like protein in high amounts in fish semen suggests that parvalbumin may be an important piece of the Ca^{2+}-mediated mechanisms of sperm activation [29].

Calpains constitute a superfamily of intracellular Ca^{2+}-dependent neutral cysteine proteases whose members are widely expressed in a variety of tissues and cells [30]. A role in mediation of specific Ca^{2+}-dependent processes including cell fusion, mitosis and meiosis has been demonstrated for these proteases [30]. Both RNA and protein studies revealed that calpain isoform 1 and 2 are expressed in all spermatogenic cells, and both isoforms are expressed in mouse spermatozoa and localized in the acrosomal region [31]. However, the expression levels of calpain 1 are slightly higher in spermatocytes entering the meiotic phase. Also,

calpain 11 has been identified in the acrosomal region of mouse spermatozoa [32]. In addition, it has been shown that a number of known calpain substrates that are present in spermatogenic cells are potential calpain 11 targets, and that this calpain is present in spermatogenic cells during the meiotic divisions and spermiogenesis [32].

Other CBPs have been described to be differentially expressed throughout spermatogenesis. This includes calreticulin, a resident protein of the endoplasmic reticulum, which was identified in both pre- and post-meiotic germ cells, and calmegin, a testis specific CBP detectd in meiotic and post-meiotic germ cells [33-35]. The testis-specific calcineurin B subunit isoform beta 1 was specifically identified in meiotic spermatocytes and shows to be almost inexistent in other spermatogenic cells types, such as spermatogonia, spermatids and sperm [36]. Moreover, a calcineurin-binding protein, CaNBP75, has been identified in scallop testis *(Mizuhopecten yessoensis)* [37] and a coordinated action between calcineurin, CaNBP75 and Ran (Ras-related small G protein) in the regulation of spermatogenesis has been suggested [38]. Another differentially expressed protein, calgizzarin that belongs to the family of S-100 CBPs shows a decreased expression in SC-germ cell cocultures in comparison with SCs cultured alone. This downregulation seems to be directly or indirectly originated by factors produced by germ cells [39]. Another CBP identified as essential for mouse spermatogenesis was CIB1, a protein that shares approximately 50% homology with CaM and calcineurin [40].

Developmental analysis of CBP regucalcin (RGN) mRNA in rat testis showed that its expression increases until it reaches a maximum at 90-120 days of age, a period which corresponds to adulthood and spermatogenesis peak, decreasing afterwards with rat aging [41].

The aforementioned studies suggested that CBPs have the appropriate temporal and spatial distribution to be involved in the regulation of key signal transduction events in processes like meiosis, spermiogenesis, and sperm function.

Specificities of Ca^{2+} modulators in the testis

The idea that spermatogenic cells possess the machinery necessary to generate controlled intracellular Ca^{2+} oscillations emerged with a report showing that rat round spermatids can generate intracellular Ca^{2+} signals upon activation of Ca^{2+}-channels or release of Ca^{2+} from intracellular stores [19]. To extend the characterization of the elements involved in Ca^{2+} signaling in male germ cells, Treviño and collaborators characterized the subcellular distribution of intracellular Ca^{2+} release channels in different stages of spermatogenic cell differentiation [42]. Inositol 1,4,5-trisphosphate receptors (IP_3R) and ryanodine receptors (RyR), the two principal intracellular Ca^{2+} channels responsible for the release of Ca^{2+} stored at organelles were analyzed. IP_3R types I, II and III were expressed from spermatogonia up to the last stages of spermatogenesis, while, RyR-1 and RyR-3 were detected in all spermatogenic cells; RyR-3 was the only RyR expressed in spermatozoa [42]. In addition, an homogeneous distribution of RyR was observed in the cytoplasm of spermatogenic cells during differentiation [42]. Later on, evidences that spermatogonia express RyR1, but not RyR2, that

RyR3 mRNA is present in mixed populations of germ cells, and that the receptor is indeed a functional ryanodine sensitive Ca^{2+}-channel appear with the experiments carried out by Stefaninnis´s group [43]. Moreover, blockage of RyR channels with high concentrations of ryanodine *in vitro* resulted in reduced proliferation of spermatogonia and accelerated maturation of early meiotic cells, suggesting that Ca^{2+}mobilization through RyR channels participate in the regulation of male germ cell maturation [43].The gene expression of Ca^{2+}-channels in testis and the nature of voltage-dependent Ca^{2+}-channels (VDCC) have been a matter of considerable speculation. Expression of the of VDCC mRNA and its co-ordinated translation is initiated early in the development of rat male germ line and continues throughout spermatogenesis [44]. Patch clamp analysis allowed to record T-type currents in mouse and rat immature spermatogenic cells [44, 45], while atypical L-type Ca^{2+}currents were detected in rat mature spermatozoa [44]. Son et al. [46] also detected L-type (α1D) Ca^{2+}-channels in the mouse testis, specifically in round spermatids. A sensitive non-radioactive *in situ* hybridization demonstrated specific localization of mRNA transcripts of T-type (α1H) Ca^{2+}-channel in spermatocytes, spermatogonia and SCs. Moreover, since T-type (α1H) Ca^{2+}-channel mRNA starts to be expressed 2 weeks after birth and increases thereafter, the involvement of this Ca^{2+}-channel in differentiation and maturation of germ cells was suggested [46].

The G-protein-coupled olfactory receptors (ORs) are members of a large multigene family that includes approximately 1000 members in mice [47]. *In situ* hybridization techniques showed that MOR23 (a mouse OR expressed in the testis) is present in round spermatids during stages VI-VIII of spermatogenesis [48]. MOR23 activation leads to Ca^{2+} increases and to modulation of flagellar configuration, resulting in chemotaxis [48]. A thorough study of the ORs expression during spermatogenesis showed that OR transcripts are expressed in three developmental stages of spermatogenic cells, namely, late pachyten spermatocytes, early round spermatids and late round spermatids [49]. In addition, it has been suggested that each spermatogenic cell-type could express more than one OR [49]. Nevertheless, the role of ORs in spermatogenic process remains largely unknown.

Successful spermatogenesis depends on a delicate and tightly regulated equilibrium between germ cell apoptosis and proliferation, being accepted that SCs play a crucial role in germ cell fate. [50]. It has been shown that one of the mechanism by which SCs modulate apoptosis of germ cells is through the activity of VDCC, more precisely the P/Q type [51, 52]. Independently of the direct action of SCs, the elegant study of Mishra et al. has shown that enhancement of $[Ca^{2+}]_i$ through the activity of T-type Ca^{2+} channels determines the apoptotic cell-death of spermatogenic cells [17]. This occurs by the effect of Ca^{2+} regulating the expression of apoptotic regulators of the Bcl family. The expression of Bcl-xS (proapoptotic) increases and Bcl-xL (anti-apoptotic) decreases, which triggers apoptosis [17].

On the other hand, SCs are pivotal elements in maintenance of spermatogenesis, since they provide nourishment and sustain the developing germ cells [53]. The tight control of intracellular Ca^{2+} homeostasis has been shown to be of uttermost importance for the

maintenance of SCs function [11, 54], and in turn for the success of spermatogenic process. A purinergic Ca^{2+} mobilization mediated by both P2X2 and P2Y2 receptors was identified as an effective route for situational control of SC Ca^{2+} homeostasis and developmental stage, being the SC mitochondrial network a key component of its Ca^{2+} signaling [55]. N and P/Q type Ca^{2+}-channels seem to be involved in the regulation of SC secretion into the lumen of seminiferous tubules [56]. The tight regulation of Ca^{2+} homeostasis also seems to be essential for LCs steroidogenesis, by controlling the expression of the steroidogenic acute regulatory protein [12], which plays an indispensable role in steroid biosynthesis, namely, in the delivery of cholesterol from the outer to the inner mitochondrial membrane [57].

Recently, a sperm-specific Ca^{2+}-channel was identified, the CatSper, which is a cation channel controlling the $[Ca^{2+}]_i$ [58]. CatSper is developmentally regulated in mice testis and its expression is directly correlated with sexual maturation [59]. CatSper expression coincides with the appearance of round spermatids in the developing mouse testis [59, 60]. CatSper3, and CatSper4 protein expression was also found within the acrosome of late spermatids and spermatozoa [60]. Moreover, it was described that progesterone stimulation of the Ca^{2+} increase required for sperm hyperactivation and acrosomal exocytosis is dependent of the CatSper activation [61]. Very recently, the use of inhibitors that abolish the CatSper currents allowed characterizing the specific human sperm behaviour dependent on CatSper. Alasmari and co-authors demonstrated that activation of CatSper by pH or progesterone caused sustained increase of $[Ca^{2+}]_i$ but did not induce hyperactivation [62]. However, CatSper activity was related with sperm capacity to penetrate cervical mucus or cumulus matrix [62].

Perspectives from knockout animals

Development of knockout animals for some Ca^{2+} regulators has given further evidence for the role of this ion in spermatogenesis.

Mice with targeted deletion of the gene Ca^{2+}/Cam-dependent protein kinase IV (CamK4) are infertile, exhibiting impairment of spermiogenesis at the stage of late elongating spermatids [63], which links Ca^{2+}/Cam with the spermiogenesis events. Also, Yuan et al., [40] showed the importance of the CBP CIB1 for mouse spermatogenesis by means of Cib(-/-) mice generation. These animals showed reduced testis size, decreased number of germ cells within seminiferous tubules, increased germ cell apoptosis and loss of elongated spermatids and sperm. In addition, an increased expression of the cell cycle regulators Cdc2/Cdk1 was observed, suggesting a role for CIB1 in the regulation of cell cycle and differentiation of germ cells as well of SCs differentiation [40].

Corroborating all these data is the information that germline deletion of the centrin 1 (cetn1), a CaM-like CBP, causes infertility in male mice [64]. The observation of spermatids without tail and consequent sperm morphologic abnormalities suggests severe defects at the late maturation phase of spermiogenesis in the Cetn1 (-/-) mice [64]. Nevertheless, further studies are warranted to clarify the role of these and other Ca^{2+} handling molecules in spermatogenesis.

Clinical findings

The importance of Ca^{2+} in spermatogenesis has been highlighted by information driven from the clinical setting.

Interestingly, it was found that men undergoing treatment for hypertension Ca^{2+}-channels blockers had reversible infertility [4-10]. This observation was corroborated by a report in which the toxicity of this therapeutic application was analysed using mice as study model [9]. To start elucidating the underlying mechanism for Ca^{2+} channel blockage, Lee et al. [10] used specific L- and T-type channels blockers. A premature arrest of developing spermatids and diminishing LC abundance by abrogating StAR protein expression was observed with a consequent diminution of testosterone (T) production [10].

Although there are no reports establishing a relationship between the administration of anti-inflammatory drugs and Ca^{2+} deregulation in human testis, the inhibition of T-type Ca^{2+} currents in mouse spermatogenic cells by the usage of *celecoxib* was reported [65], which also highlights for the important effects of Ca^{2+} for male fertility. On the other hand, blockade of Ca^{2+} channels by means of pharmacological inhibitors seems to be a promising mechanism for male contraception [66].

Also, altered gene expression of important Ca^{2+} modulators has been described in the testis of infertile men. For example, a significant reduction in the level of CatSper gene expression was observed among patients lacking sperm motility in comparison with control group [59].

Considering the Camk4 gene, encoding a Ca^{2+}/Cam-dependent protein kinase, it was found that 9 of the 25 variable sites identified were exclusively observed in infertile men. Moreover, these 9 variants were not found in any of the men with unknown fertility status, supporting that mutations in Camk4 could be related with infertility and are not neutral variants [67].

Analyses of biopsies from infertile men with varicocele indicated that L-type channel α1 subunit variants occur in testicular tissue in more than 60% of the cases. The presence of these deletions alters Ca^{2+} homeostasis in germ cells being associated with increased testicular cadmium levels and with elevated apoptosis [68].

The role of Ca^{2+} in sperm maturation

Sperm released from the testis are inactive and are only modifications that occur throughout the passage in the epididymis that render them functional gametes [69].

The epididymal lumen has a unique microenvironment, which is created by the specific secretory and absorptive activities of the epididymis epithelial cells [70]. This environment is rich in inorganic ions and organic molecules that create the appropriate ionic, oxidative and pH environment for sperm maturation throughout epididymis transit [69]. It has been established that acidification of the epididymal fluid [71] and water reabsorption by epididymal epithelial cells [72] are crucial events. Also, a decreased concentration of Ca^{2+} along the epididymis generating a luminal Ca^{2+} gradient has been described [73], but the importance of this has remained obscure. Curiously, around or on the plasma membrane of

the three different subcellular regions of spermatozoa (head, midpiece and tail) Ca^{2+} concentration decreases gradually during sperm passage throughout the male reproductive tract [74, 75]. Ca^{2+} concentration on head region of rat sperm in the vas deferens is about one-third of that found in the testis [74]. A more thorough experiment measuring Ca^{2+} levels in bovine *caput* and *cauda* epididymal spermatozoa revealed that Ca^{2+} uptake and Ca^{2+}-Ca^{2+} exchange in *caput* spermatozoa were about 2- to 3-fold higher than in *cauda*, and that intracellular free Ca^{2+} was 6-fold higher in *caput* [76]. In addition, authors observed that total cellular Ca^{2+} levels were 626 +/- 30 and 304 +/- 19 ng/10(8) sperm in *caput* and *cauda* epididymal sperm, respectively, arguing for the higher rate and extent of mitochondrial Ca^{2+} accumulation in *caput* compared to *cauda* sperm as one of the reasons for high Ca^{2+} content of *caput* region [76].

Besides the efforts for the quantification of Ca^{2+} along epididymis, changes in its distribution in cell compartments were also analyzed. For mouse sperm in the *caput* region, it was described that Ca^{2+} in sperm head was mainly localized on the inner surface of the outer acrosomal membrane, while Ca^{2+} in sperm tail was mainly distributed in the mitochondria [77], but the importance of this compartmentalization needs to be further explored.

The main role of Ca^{2+} in sperm functionality has been associated with the attainment of a capacitated stated that occurs in the female reproductive tract [78]. The capacitation process depends on the progressive activation of a cAMP-PKA-dependent signaling pathway mediating protein tyrosine phosphorylation [79]. The sperm capability to respond to high levels of intracellular cAMP leading to tyrosine residues phosphorylation is acquired during epididymal maturation [80], a process negatively regulated by Ca^{2+} [81]. All these data point for the critical role of the mechanisms that control intracellular Ca^{2+} concentration in sperm maturation process. It has been shown that the ion pumping (Ca^{2+}-ATPase) activity in bovine spermatozoa is higher in spermatozoa isolated from the epididymal *cauda* relative to spermatozoa isolated from the epididymal *caput* [82]. In mouse spermatozoa, the plasma membrane Ca^{2+}-ATPase (PMCA) has been shown to be the essential pump for achieving a low resting Ca^{2+} concentration [83]. Moreover, variable quantities of the two splice variants of the PMCA isoform 4 (4a and 4b) are present in epididymal bovine [84] and murine [85] spermatozoa, with a clear shift of the 4b to the 4a calcium pump from *caput* to *cauda* spermatozoa. Martin-DeLeon´s group not only confirmed its presence but also showed that PMCA 4a and PMCA 4b are secreted into the epididymal luminal fluid [85], being consistent with its transfer from the epididymal luminal fluid through epididymosomes [85, 86]. Beyond the increased expression of PMCA 4a on mature caudal sperm compared with *caput* [84, 85] the relevance of epididymal PMCA4 for spermatozoa function was evidenced by reports showing that homozygous deletion of PMCA 4 results in male infertility and strikingly elevated Ca^{2+} levels [87, 88].

In 2001, the aforementioned CatSper channel was identified in sperm as the first member of a completely new family of Ca^{2+}-selective ion channels subunits [58]. Later, it was considered as one of the major Ca^{2+}-conducting channel in spermatozoa [89]. Immunostaining assays have

found that the CatSper proteins are localized in the sperm tail [90, 91]. In mice, all four CatSper genes (Catsper1, Catsper2, Catsper3,and Catsper4) have been knocked out, and each of these knockout models shared the phenotype of male infertility [91, 92]. The presented defects affect sperm physiology rather than spermatogenesis [91]. CatSper mutations have also been found in infertile humans, being the mutations in CatSper1 and CatSper2 associated with asthenoteratozoospermia and male infertility [93-95]. The presence of other ion channels in mature sperm is being questioned.

There is a large amount of evidence for the involvement of transient receptor potential (TRP) channels in sperm development and function [96]. Reliable evidences were obtained from functional studies comparing spermatozoa from wild-type and TRP null mice. Specifically, knockout mice for TRPV6 Ca^{2+}-channels displayed a pronounced decrease of Ca^{2+} absorption through the epididymal epithelium, and Ca^{2+} concentrations in the epididymal fluid 10 times higher than their Wild-type littermates [97, 98]. Although presenting intact spermatogenesis, these animals had severely diminished fertility as a result of decreased sperm motility and viability [97, 98], which undoubtedly demonstrated the importance of appropriate regulation of intraluminal Ca^{2+} concentration in the epididymal duct for the production of fertilization-ready spermatozoa.

Navarro et al. [99] screened a number of neurotransmitters and biomolecules examining their ability to induce ion channel currents in the whole spermatozoa. These authors found a cation-nonselective, Ca^{2+}-permeable current originating from the midpiece of mouse epididymal spermatozoa that is activated by external ATP and have concluded that this ATP-induced current is mediated by the P2X2 purinergic receptor/channel [99]. It was also postulated that sperm counts and/or motility of P2rx2 (−/−) males declines with frequent matting within a short period, resulting in reduced fertility [99].

Reports using a $^{45}Ca^{2+}$ overlay technique identified CBPs on boar spermatozoa and boar seminal plasma and showed that the majority of these proteins are not integral to the membrane, appearing to develop an association with the plasma membrane during epididymal maturation [100]. In mouse epididymal spermatozoa, the presence of CBPs was analyzed by a motility shift assay. Relatively high molecular weight CBPs (>20 kDa) showed a differential expression between *caput* and *cauda* epididymal spermatozoa [101].This allowed authors to suggest that changes in CBPs of spermatozoa are important features of sperm maturation during epididymal transit [101]. One of the CBPs that binds Ca^{2+} during the maturation in the epididymis is CABS1. This protein was localized in the principal piece of flagellum of mouse mature sperm in the *cauda* epididymis [102]. Recently, the CBP regucalcin was identified in rat epididymis [41], and its putative role in sperm maturation will be discussed in the following section.

The calcium-binding protein regucalcin in spermatogenesis and sperm maturation

RGN is a Ca^{2+}-binding protein that differs from common Ca^{2+}-binding proteins by the absence of the typical EF-hand Ca^{2+}-binding motif [103]. RGN plays an important role in the regulation of Ca^{2+} homeostasis controlling the activity of Ca^{2+}-ATPase in the membrane of mitochondria and endoplasmic reticulum and (Ca^{2+}-Mg^{2+})-ATPase in the plasma membrane [104-107]. Beyond its ability in the regulation of Ca^{2+}-pumping activity, an association with intracellular signalling pathways has been suggested, since it regulates several Ca^{2+}-dependent enzymes, such as, protein kinases, tyrosine kinases, phosphatases, phosphodiesterase, nitric oxide synthase and proteases [108-112].

Recently, the expression of RGN was characterized in male reproductive tract [41]. Both in rat and human testis, immunohistochemistry analysis revealed a broad expression of RGN protein in testicular cells, from the germ line cells to somatic cells. In addition, the presence of RGN was confirmed in epididymis, prostate, seminal vesicles, seminiferous tubule fluid [41] and epididymal fluid [113]. It was also shown, that RGN expression in reproductive tissues is under the control of sex steroids. *In vitro* and *in vivo* studies have demonstrated that both androgens and estrogens can regulate RGN expression in the testis and prostate [41, 114-116]. Altogether, the available information on RGN expression supports the hypothesis that it might play a role in the regulation of spermatozoa production and maturation. Moreover, it will be crucial characterizing the reproductive phenotype of RGN knock-in and knock-out animals.

Conclusion

The presented data on the developmental gene expression and activity of Ca^{2+} modulators in the testis, as well as, on the characterization of the phenotype of knockout animals with disruption of Ca^{2+} regulators, and clinical findings in patients using Ca^{2+} channels blockers has provided new insights into the molecular mechanisms driving Ca^{2+} homeostasis in spermatogenesis. Moreover, the identification of different Ca^{2+}-permeable channels and their specific localization in distinct regions of spermatozoa started elucidating how sperm ion channels participate in sperm maturation. Nevertheless, much has to be done before we can have completed the picture of all Ca^{2+}-dependent actions in human reproduction.

Learning more about Ca^{2+} handling in multiple signaling processes that are needed for spermatogenesis and sperm maturation is a matter of capital importance and will help societies face growing pressure to counteract rising male infertility rates, providing also safe male gamete-based contraceptives.

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Chapter II

Aim and outline of the thesis

Aim and outline of the thesis

Development and maintenance of spermatogenesis depends on an assortment of hormonal messengers, as well as a panoply of other organic and inorganic molecules. In the hormonal context, androgens are widely recognized as the pivotal agents for the occurrence of successful spermatogenesis. Also, several kinds of evidence have suggested estrogens as important regulators of male reproductive function. However, discordant reports about their role in spermatogenesis have been presented. If there are studies describing estrogens as survival factors for germ cells, others have established their function as apoptosis-inducers. Nevertheless, the molecular mechanisms underpinning the survival/apoptotic effects of estrogens in mammalian spermatogenesis are yet to be completely elucidated.

In the last years, the calcium (Ca^{2+})-binding protein Regucalcin (RGN), which is also related with the control of apoptosis and oxidative stress, has been identified as a sex steroid target gene in male reproductive tissues. Notwithstanding, the effect of estrogens controlling the expression levels of RGN in the testis is entirely unknown. Furthermore, deepening the knowledge on the RGN actions in modulating apoptotic pathways and oxidative stress levels in the testis will help to define the role of this protein on testicular physiology and spermatogenesis. In addition, understanding the importance of RGN and Ca^{2+} homeostasis for sperm function may provide new insights to treat cases of infertility associated with disorders of sperm maturation.

The first aim of this thesis was to disclose the relationship between estrogens and apoptosis of germ cells, including the regulatory effects of these sex steroid hormones on the testicular expression of RGN. Secondly, the role of RGN in regulating apoptosis and oxidative stress in the testis, as well as, in sperm maturation in epididymis will be explored.

After the general introduction (Chapter 1) and the establishment of the main objectives of the thesis, chapters 3-7 were organized as follows:

Chapter 3 describes the effect of a supra-physiological dose of 17β-estradiol on the survival of male germ cells, as well as, on the expression of RGN. Also, the estrogenic regulation of the testicular expression of stem cell factor (SCF) and its tyrosine kinase receptor c-kit are evaluated.

In Chapter 4 the role of RGN in regulating testicular apoptosis is investigated. The apoptotic index and the expression of key regulators of apoptotic cell death is compared between seminiferous tubules (SeT) of transgenic rats overexpressing RGN (Tg-RGN) and their wild-type (Wt) counterparts after *ex vivo* culture in the presence and absence of the apoptosis inducers thapsigargin and actinomycin D.

Chapter 5 explores the role of RGN in the modulation of oxidative stress in the testis. The activity of antioxidant enzymes and the total antioxidant capacity is compared between SeT of Tg-RGN animals and their Wt counterparts after *ex vivo* culture in the presence and absence of the pro-oxidant inducers tert-butyl hydroperoxide and cadmium chloride.

In Chapter 6 the sperm parameters of Tg-RGN rats are firstly characterized. This chapter also compares the morphology and function of epididymis, as well as the antioxidant potential and

Ca^{2+} fluxes between Tg-RGN and Wt animals. The expression of RGN across the three distinct regions of epididymis and in epididymal fluid is also evaluated.
Finally, Chapter 7 contains an integrative view of the results presented in the thesis and discusses the potential impact of the findings in the context of infertility of idiopathic origin. In addition, this chapter states the final conclusions and the future perspectives on treatment of male infertility by targeting estrogens and RGN signaling mechanisms.

Chapter III

Estrogenic regulation of testicular expression of stem cell factor, c-kit and regucalcin: implications in germ cell survival and male fertility

Chapter published in Correia S, Alves MR, Cavaco JE, Oliveira PF and Socorro S. Estrogenic regulation of testicular expression of SCF and c-kit: implications in germ cell survival and male fertility. *Fertility and Sterility* (2014) 102(1): 299-306.

Estrogenic regulation of testicular expression of stem cell factor, c-kit and regucalcin: implications in germ cell survival and male fertility

Abstract

Objective: To study the effect of estrogens regulating the testicular expression of stem cell factor (SCF), c-kit and regucalcin (RGN).

Design: Experimental study.

Setting: University research center.

Animal(s): Male Wistar rats.

Intervention(s): Rat seminiferous tubules (SeT) cultured in the presence or absence of 17ß-estradiol (E_2).

Main Outcome Measure(s): Expression of SCF, c-kit and RGN as well as apoptotic factors, FasL, FasR, Bcl-2, and Bax analyzed via quantitative reverse transcription-polymerase and Western blot; enzymatic activity of apoptosis effector caspase-3 assessed by colorimetric assay; proliferation index in SeT epithelium determined via fluorescent immunohistochemistry of nuclear proliferation marker Ki67.

Result(s): E_2 (100 nM) induced a decrease in c-kit expression while increasing expression of SCF and RGN. Altered expression of the SCF/c-kit system relied on apoptosis of germ cells, as evidenced by the up-regulated expression of FasL/FasR, the increased ratio of proapoptotic/ antiapoptotic proteins (Bax/Bcl-2), and the augmented activity of caspase-3. Decreased proliferation was also found in SeT in response to E_2.

Conclusion(s): A 100 nM dose of E_2 unbalance the SCF/c-kit system, with a crucial impact on germ cell survival and thus male fertility. These findings contribute to our knowledge of the mechanisms underlying male idiopathic infertility associated with hyperestrogenism and open new perspectives on treatment targeting estrogen-signaling mechanisms.

Key Words: Apoptosis, c-kit, estrogens, male infertility, regucalcin, SCF

Introduction

Spermatogenesis is a complex cellular process involving mitosis, differentiation, and meiosis, which gives rise to the male haploid germ cells [1]. Successful spermatogenesis also depends on the strict control of the balance between germ cell proliferation and apoptosis [2], which is of the uttermost relevance for male fertility. In fact, increased rates of apoptosis [3-5] together with altered expression of apoptosis-related genes [6-9] have been described in the testis of infertile men.

Experimental evidence has associated estrogens with testicular apoptosis and diminution of germ cell numbers [6, 10-13]. Moreover, it has been documented that prenatal exposure to estrogens or estrogen-like substances has deleterious effects on spermatogenic output [14].

Clinical studies also have suggested that augmented intratesticular production of estrogens is linked to spermatogenic failure and thus to male infertility because increased levels of 17ß-estradiol (E_2) have been detected in the testis of idiopathic infertile patients [15, 16]. However, the molecular targets underpinning the estrogenic effects in mammalian testis are not fully elucidated.

Stem cell factor (SCF), a membrane-bound cytokine in Sertoli cells (SCs) [17], and its tyrosine kinase receptor c-kit, present on the surface of adjacent germ cells [18, 19], have been indicated as a powerful mechanism for the control of germ cell proliferation and apoptosis. Multiple experimental approaches have shown that blocking SCF/c-kit interaction results in increased apoptosis and reduced proliferation of germ cells, demonstrating the importance of this system for successful spermatogenesis [20-22].

Although it has been demonstrated that E_2 regulates the expression of SCF and c-kit in distinct cell types [23-26], the effect of estrogens in controlling the testicular expression of these factors has been unknown. We analyzed the effect of E_2 stimulation on SCF and c-kit expression in rat seminiferous tubules (SeT) cultured ex vivo. The effect of E_2 on the expression of regucalcin (RGN), a calcium (Ca^{2+})-binding protein with apoptosis related roles [27], also was investigated. We used a 100 nM dose of E_2 to mimic the elevated concentration of estrogens found in the testis of infertile patients and determined the expression and/or activity of proliferation and apoptosis markers.

Materials and Methods

Chemicals

All chemicals, culture media, and antibodies unless otherwise stated were purchased from Sigma-Aldrich.

Animals

We housed 90-day-old Wistar (*Rattus norvegicus*) male rats under a 12-hour light/dark cycle, with food and water available ad libitum, and all animals were handled in compliance with the guidelines established by the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (NIH Publication No. 85–23, revised 1996) and the European Union rules for the care and handling of laboratory animals (Directive no. 86\609\EEC). In addition, the animal protocol was approved by the local institutional animal care and use committee. All rats were euthanized under anesthesia (Clorketam 1000; Vetoquinol).

Ex Vivo Culture of SeT

Rat SeT were cultured as previously described elsewhere [6, 28]. Briefly, testes (n=5) were removed, trimmed free of fat, washed in cold phosphate-buffered saline (PBS) and placed in

Dulbecco's modified Eagle's medium/Ham's F-12 culture medium supplemented with 20 mg/L of gentamicin sulfate, 0.1 mM 3-isobutyl-1-methylxanthine, and 1 µg/L of bovine serum albumin (BSA) at 33ºC. Tunicae were cut and peeled back to expose tubules. Ten fragments of SeT (≈1 cm each) were cultured with or without 100 nM of E_2 for 24 hours and 48 hours at 33ºC in an atmosphere of 5% CO_2. The 100 nM dose of E_2 was intended to mimic the elevated concentrations of estrogens found in the intratesticular milieu of infertile patients, as normal physiologic concentrations range from 0.5 to 57 nM [29-31]. At the end of the experiment, SeT were recovered from the culture medium, snap-frozen in liquid nitrogen, and stored at -80ºC until RNA or protein isolation. The SeT were also collected at 0, 24, and 48 hours and fixed in 4% paraformaldehyde for paraffin embedding and histologic processing.

RNA Isolation and cDNA Synthesis

Total RNA was isolated from rat SeT using TRI reagent according to the manufacturer's instructions. To assess the quantity and integrity of total RNA, its optical density was determined (NanoPhotometer; Implen), and an agarose gel electrophoresis analysis was performed. We synthesized cDNA in a final volume of 20 µL using 1 µg of each RNA sample, 160 IU Moloney Murine Leukemia Virus reverse transcriptase (Promega), 0.5 µg random primers (Invitrogen), and 10 mM each deoxynucleotide triphosphates (dNTP) (GE Healthcare) according to the protocol supplied by the manufacturer. Synthesized cDNA was stored at -20ºC until further use.

Real-time Quantitative Polymerase Chain Reaction

Expression analysis of *SCF*, *c-kit and RGN* RNA in rat SeT was performed by quantitative polymerase chain reaction (qPCR). The *SCF*, *c-kit and RGN* specific primers sets were, respectively, [1] sense: ATGGCTTGGGAAATGTCTG; antisense: GCTGATGCTACGGAGTTAC; [2] sense: CCGTCTCCACCATCCATCC; antisense: TTCGCTCTGCTTATTCTCAATCC; [3] sense: GGAGGAGGCATCAAAGTG; antisense: CAATGGTGGCAACATAGC. As an internal reference for normalization of expression of interest target genes, we used *β-actin* (sense: ATGGTGGGTATGGGTCAG; antisense: CAATGCCGTGTTCAATGG) and *GAPDH* (sense: GTTCAACGGCACAGTCAAG; antisense: CTCAGCACCAGCATCACC). Reactions were performed in an iQ5 system (Bio-Rad Laboratories), and the efficiency of the amplifications was determined for all primer sets using serial dilutions of cDNA (1, 1:5, and 1:25). The primer concentration and annealing temperature were optimized before the assay, and the specificity of the amplicons was determined by melting curve analysis. The annealing temperature was 60ºC for all primer sets except *SCF* (58ºC). Each reaction consisted of Maxima SYBR Green/Fluorescein qPCR Master Mix (Bio-Rad Laboratories), sense and anti-sense primers (200 nM for all primer pairs), and 1 µL of cDNA in a final volume of 20 µL. Also, a no-template control was included for each reaction, and all reactions were performed in

triplicate. Normalized expression values were calculated according to a published mathematical model proposed by Hellemans et al. [32].

Western Blot

Total protein was isolated from rat SeT using radioimmunoprecipitation assay buffer (150 mM NaCl, 1% Nonidet-P40 substitute, 0.5% Na-deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0, and 1 mM EDTA) supplemented with protease inhibitors cocktail. Protein concentration was determined by the Bradford assay (Bio-Rad Laboratories), and proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12.5% gels and electrotransferred to a polyvinylidene difluoride membrane (GE Healthcare).
Membranes were incubated overnight at 4ºC with rabbit anti-SCF (1:500, H-189: sc-9132; Santa Cruz Biotechnology), rabbit anti-c-kit (1:500, C-19: sc-168; Santa Cruz Biotechnology), rabbit anti-RGN (1:1000, COSMO BIO CO, Tokyo, Japan), rabbit anti-FasL (1:500, C-178: sc-6237; Santa Cruz Biotechnology), rabbit anti-FasR (1:500, A-20: sc-1023; Santa Cruz Biotechnology), rabbit anti-Bcl-2 (1:5000, no. 2876; Cell Signaling Technology), or rabbit anti-Bax (1:1000, no. 2772; Cell Signaling Technology). A mouse anti-α-tubulin monoclonal antibody (1:5000, T9026) was used for protein loading control in all Western blot analyses. Goat anti-rabbit IgG-AP (1:5000, NIF1317; GE Healthcare) or goat anti-mouse IgG+IgM-AP (1:5000, NIF1316, GE Healthcare) were used as secondary antibodies. Membranes were developed with ECF substrate (GE Healthcare) for 5 minutes and scanned with Molecular Imager FX Pro plus MultiImager (Bio-Rad Laboratories). Band densities were obtained according to standard methods using the Quantity One Software (Bio-Rad Laboratories) and normalized by division with the respective α-tubulin band density.

Ki67 and c-Kit Fluorescent Immunohistochemistry

Formalin-fixed paraffin sections (5 µm) of SeT were deparaffinized in xylene and rehydrated in graded alcohols. After heat-induced antigen retrieval (citrate buffer bath, 10 mM, pH 6.0), the sections were permeabilized with 0.1% Triton X-100 for 15 minutes at room temperature. Unspecific staining was blocked by incubation with PBS containing 1% (w/v) BSA (PBA) and 0.3 M glycine (Fisher Scientific) for 30 minutes at room temperature. Sections were incubated overnight at 4ºC with rabbit anti-Ki67 (ab16667; Abcam) or rabbit anti-c-kit (C-19: sc-168; Santa Cruz Biotechnology) primary antibodies diluted 1:50 in PBA. Sections were then incubated with Alexa Fluor 546 goat anti-rabbit IgG or Alexa Fluor 488 goat anti-rabbit IgG secondary antibodies (Invitrogen) diluted 1:500 in PBA for 1 hour at room temperature. Cell nuclei were stained by incubation with Hoechst 33342 (10 µg/mL, Invitrogen) for 5 minutes. Sections were then washed with PBS for 10 minutes and mounted in Dako fluorescent mounting medium. Specificity of the staining was assessed by the omission of primary antibody. Images were acquired using a Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss). The proliferation index was determined by the percentage of Ki67-positive cells

out of the total number of Hoechst stained nuclei in 20 randomly selected X40 magnification fields in each section.

Caspase-3 Activity Assay

Caspase-3 activity assay was performed as previously described elsewhere [33]. Briefly, 25 µg of total protein extracted from SeT were incubated with reaction buffer (25 mM HEPES, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 10% sucrose, and 10 mM dithiothreitol, pH 7.5) and 200 µM of caspase-3 substrate (Ac-DEVD-pNA) for 2 hours at 37ºC. Upon caspase cleavage, p-nitro-aniline (pNA) is released producing a yellow color, which is measured spectrophotometrically at 405 nm. The amount of generated product was calculated by extrapolation of the standard curve of free pNA.

Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling

We performed the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) analysis using the In Situ Cell Death Detection Kit, TMR red (Roche) following the manufacturer's instructions. Briefly, SeT sections (5 µm) were deparaffinized in xylene, rehydrated in graded alcohols, rinsed in 0.15 M PBS (pH 7.4), and permeabilized with 0.25% Triton X-100 (Sigma-Aldrich) for 30 minutes at room temperature. Thereafter, sections were incubated in a humidified atmosphere for 1 hour at 37ºC in the dark with TUNEL reaction mixture (enzyme solution equilibrated in label solution). After an additional rinse in PBS, the cell nuclei were stained by incubation with Hoechst 33342 (10 µg/mL; Invitrogen) for 5 minutes. Sections were then washed with PBS for 10 minutes and mounted in Dako fluorescent mounting medium. Images were acquired using a Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss).

Statistical Analysis

Statistical significance of differences between controls and hormonal treatment were evaluated by unpaired *t*-test with Welch's correction, using GraphPad Prism v5.00 (GraphPad Software). $P<0.05$ was considered statistically significant.

Results

E_2 Treatment Decreases c-Kit Expression while Increasing Expression of Its Ligand SCF

Because SCF and c-kit have been identified as estrogen-target genes in several tissues, we investigated the effect of E_2 on their expression in testicular cells. When SeT was cultured ex vivo for 24 hours in the presence of 100 nM E_2, it showed decreased expression of c-kit receptor. An approximately 0.4-fold-reduction ($P<0.01$) was observed both at the mRNA (Figure III.1A) and protein (see Figure III.1B) level.

Regarding SCF, both RNA (see Figure III.1A) and protein (see Figure III.1B) expressions were increased in response to E_2-treatment. However, only the protein levels displayed statistically significant differences (1.34-fold variation relative to control; *P*<0.05).

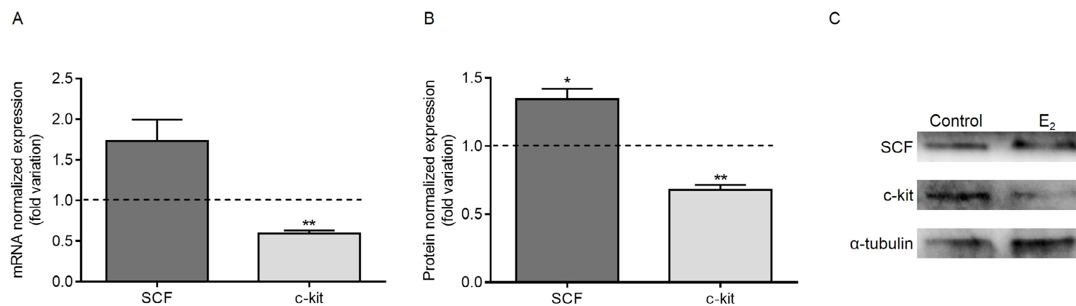

Figure III.1. Effect of 100 nM E_2 on mRNA (A) and protein (B) expression of SCF and c-kit in rat SeT cultured ex vivo for 24 hours. The mRNA expression was determined by qPCR after normalization with β-actin and GAPDH housekeeping genes. Protein expression was determined by Western blot analysis after normalization with α-tubulin. Results are expressed as the fold variation relative to 0 nM E_2 (dashed line). *P<0.05; **P<0.01. Error bars indicate mean ± standard error of the mean (n=5). (C) Representative immunoblots.

Apoptosis is Favored and Proliferation Index is Decreased in Response to E_2 Stimulation

Germ cell death in human, rat, and mouse testis has been associated with the activation of the extrinsic pathway of apoptosis involving the death factors FasL (ligand) and FasR (receptor) [8, 34]. Western blot analysis showed that protein levels of both FasL and FasR (Figure III.2A) were statistically significantly increased in response to 100 nM of E_2 with a fold induction of approximately 1.5 relative to the control group in both cases (*P*<0.01).

The Bax and Bcl-2 proteins are, respectively, proapoptotic and antiapoptotic members of the Bcl-2 family of apoptosis regulators [35], and the Bax/Bcl-2 protein ratio has been considered a powerful indicator of cell commitment to undergo apoptosis [36, 37]. In the stimulation experiments of SeT with 100 nM of E_2 (48 hours), the calculation of the Bax/Bcl-2 protein ratio demonstrated a 1.8-fold variation in the E_2-treated animals compared with the control group (*P*<0.01) (see Figure III.2B).

Activation of cell death systems invariably leads to stimulation of caspase activity, culminating in the activation of caspase-3 [35]. Thus, we determined caspase-3 activity as a measurement of apoptosis. An increase of almost 40% in caspase-3 activity was observed in cultured SeT in response to 100 nM E_2 stimulation for 48 hours (*P*<0.05) (see Figure III.2D).

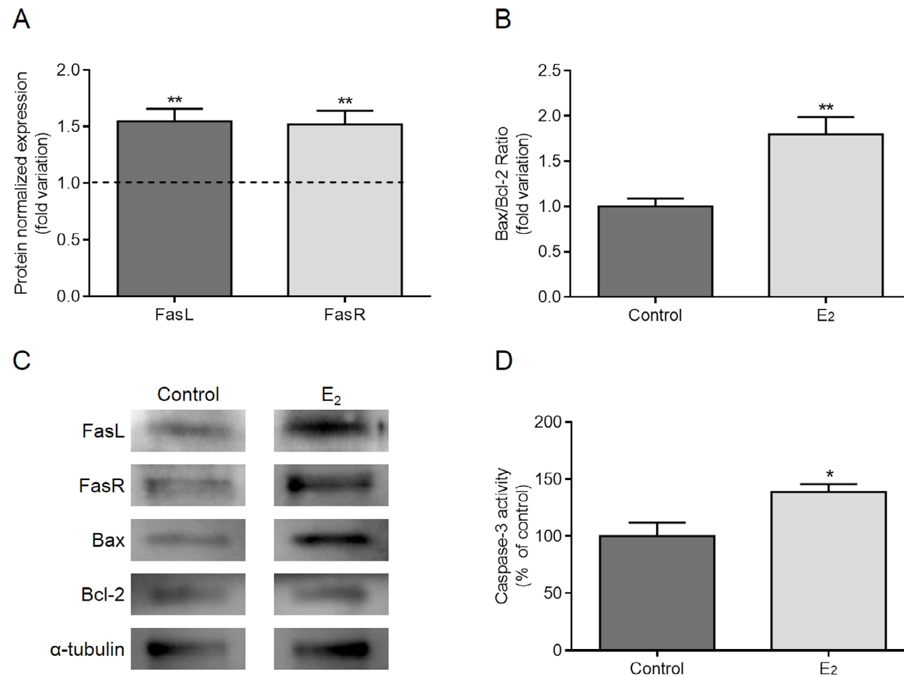

Figure III.2. Apoptosis in rat SeT cultured ex vivo for 48 hours in presence (E_2) or absence (control) of 100 nM of E_2. (A) Expression of death factors FasL and FasR (24 hours). (B) Ratio of proapoptotic (Bax) and antiapoptotic (Bcl-2) proteins. Protein expression was determined by Western blot analysis after normalization with α-tubulin. (C) Representative immunoblots. (D) Caspase-3 activity. Results are represented as mean ± standard error of the mean (n=5). *$P<0.05$; **$P<0.01$.

The presence of apoptotic cells in the SeT with and without E_2 treatment (100 nM) was analyzed by detection of DNA fragmentation using the TUNEL assay. Representative photomicrographs showing TUNEL-positive cells in the control and E_2-treated groups during the time-course of the experiment are provided in Figure III.3A.

The c-kit has been widely recognized as a germ cell marker highly expressed in spermatogonia and early spermatocytes of adult testis [19, 38-40]. Therefore, c-kit immunofluorescent labeling was used as an indicator of the germ cell population in control and E_2-treated groups (see Figure III.3B).

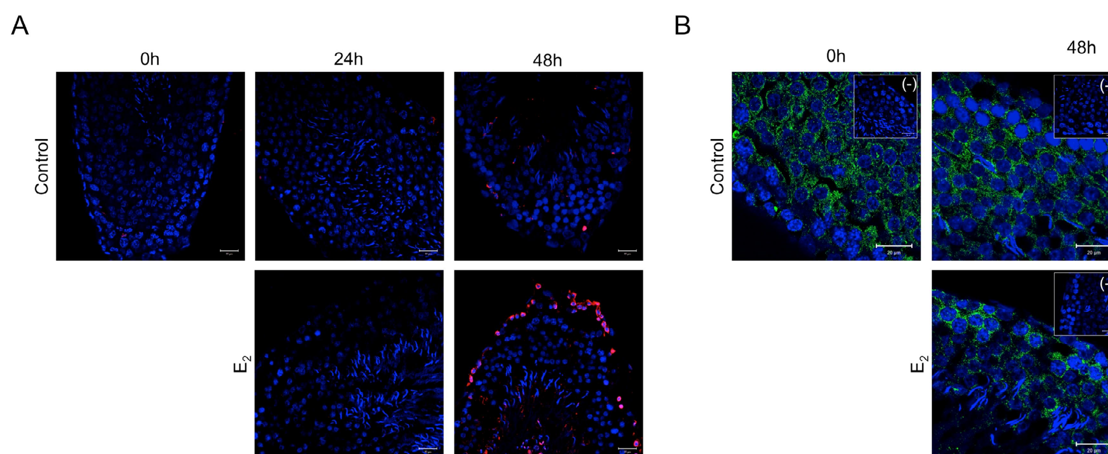

Figure III.3. Representative confocal microscopy images showing TUNEL (A) and c-kit (B) positive cells in rat SeT cultured ex vivo in presence (E_2) or absence (control) of 100 nM of E_2 at different experimental time-points (0, 24, and 48 hours). Nuclei are stained with Hoechst 33342 (*blue*), and fluorescence for TUNEL- and c-kit-positive cells is red and green, respectively. Negative controls for c-kit obtained by omission of the primary antibody are provided as insert panels (−).

We also sought to determine the effect of E_2 on proliferation of testicular cells, which was assessed by means of Ki67 immunofluorescence analysis. Ki67 is detected in the nucleus of proliferating cells in all active phases of the cell division cycle but is absent in nonproliferating cells [41]. The cell proliferation index, determined by the number of Ki67-positive cells relative to the total cell number, was reduced by approximately half in SeT treated with 100 nM of E_2 for 48 hours in comparison with the control group ($P<0.001$) (Figure III.4).

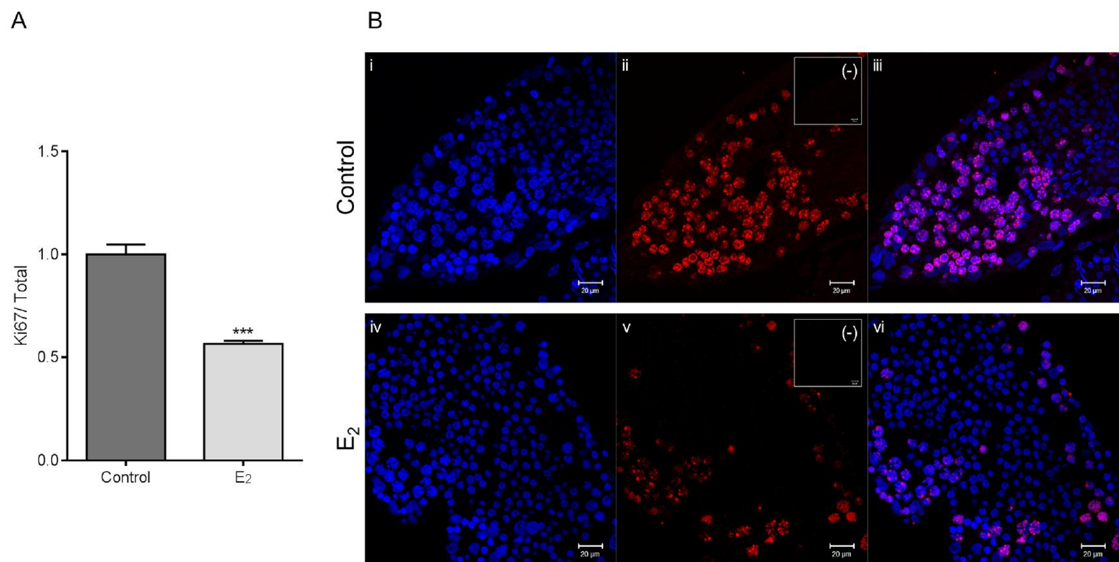

Figure III.4. Proliferation index in rat SeT cultured ex vivo for 48 hours in presence (E_2) or absence (control) of 100 nM of E_2. Proliferation was determined by Ki67 immunofluorescence analysis. (A) Percentage of Ki67-positive cells relative to the total cell number. Results are expressed as the fold variation compared with control. Error bars indicate mean ± standard error of the mean (n=5 in each group). ****P*<0.001. (B) Representative images of Hoechst stained nuclei (i, iv), Ki67 immunofluorescence (ii, v), and corresponding merged images (iii, vi) in control and E_2-treated groups. Negative controls for Ki67 obtained by omission of the primary antibody are provided as insert panels (−).

Enhanced apoptosis in response to E_2 is concomitant with increased expression of RGN

RGN is a Ca^{2+}-binding protein playing an important role in maintenance of intracellular Ca^{2+} homeostasis [42], which also has been associated with the control of cell proliferation and apoptosis [27, 43]. Our previous studies have indicated RGN both as protein expressed in male reproductive tract tissues [7, 28, 44] and as an estrogen-target gene in rat and human prostate cells [45, 46]. Therefore, we decided to explore the effect of E_2 controlling RGN expression in cultured SeT. Both mRNA (Figure III.5A) and protein levels (Figure III.5B) of RGN were strongly enhanced in response to E_2-treatment, respectively, 3.5- (*P* <0 .001) and 1.4-fold (*P* <0 .05) relatively to control group.

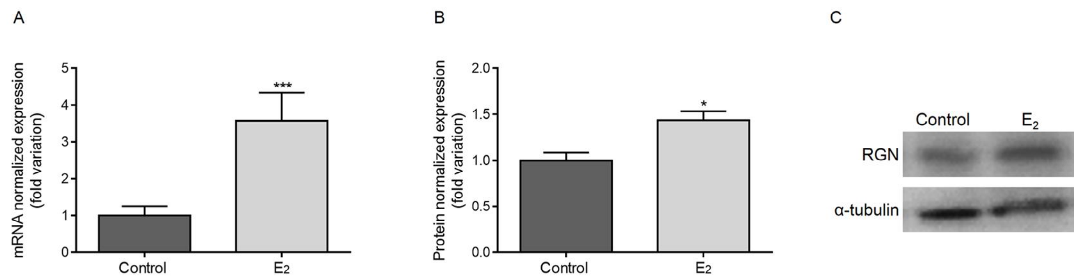

Figure III.5. Effect of 100 nM E_2 on mRNA (A) and protein (B) expression of RGN in rat SeT cultured ex vivo for 24 hours. The mRNA expression was determined by qPCR after normalization with ß-actin and GAPDH housekeeping genes. Protein expression was determined by Western blot analysis after normalization with α-tubulin. Results are expressed as the fold variation relative to 0 nM E_2 (control). **P*<0.05; ****P*<0.001. Error bars indicate mean ± standard error of the mean (n=5). (C) Representative immunoblots.

Discussion

Achievement of successful spermatogenesis relies on an accurate regulation of germ cell proliferation and apoptosis, which has been associated with the activity of SCF/c-kit system. The SCF, present on the membrane of SCs [47], has been regarded as an important germ cell survival regulator [48], acting through the c-kit receptor, which is present on the surface of adjacent germ cells [18]. Evidence also exists to demonstrate that loss of c-kit signaling causes increased apoptosis of germ cells [5, 20]. Thus, enhanced or diminished expression of c-kit should disturb germ cell and SC communication, with a profound impact on germ cell survival.

Several studies have indicated that estrogens are apoptosis inducers in the testis [10, 11, 13, 49], and reports exist demonstrating its capacity in regulating the expression of SCF and c-kit in several cell types [23-26]. This led to our hypothesis that E_2 action toward an increase in germ cell apoptosis depends on the control of the testicular expression of SCF and c-kit.

In our present study, we investigated the effect of 100 nM E_2 on the expression of SCF and c-kit in ex vivo cultures of rat SeT. A statistically significant decrease of c-kit expression (see Figure III.1A and B) was observed in SeT in response to E_2 treatment for 24 hours. However, 100 nM of E_2 increased SCF expression in SeT (see Figure III.1B). To the best of our knowledge, this is the first study to report on the regulation of SCF/c-kit in response to estrogenic stimuli in testicular cells, which could have a relevant impact on germ cell numbers. As a growth factor receptor that transduces growth regulatory signals [50] to the germ cells, c-kit ensures the maintenance of the self-renewal and differentiation ratio of spermatogonia [51]. It has been shown that mice with a mutant form of c-kit [52] or allele inactivation [20] are sterile due to reduced proliferation and increased apoptosis of spermatogonia. Therefore, despite the up-regulation of SCF levels in response to E_2, a decreased expression of c-kit suggests lower proliferation and increased apoptosis of germ cells.

Proliferation analysis by means of fluorescent immunohistochemistry using the proliferation marker Ki67 confirmed the decreased proliferation index in SeT treated with 100 nM E_2 (see Figure III.4), as suggested by the decreased expression of c-kit (see Figure III.1). Although they did not study the engagement of c-kit signaling, Chimento et al. [49] also found that 100 nM E_2 has a detrimental effect on germ cell proliferation, drastically down-regulating the expression of cell cycle regulators cyclins A1 and B1. Curiously, promotion of spermatogonia proliferation through SCF/c-kit action seems to involve the phosphatidylinositol 3-kinase (PI3K) survival pathway, including up-regulation and nuclear accumulation of cyclin D3 [53]. Further studies are needed to determine whether cyclins A1 and B1 might be the targets of c-kit signaling within the testis.

As previously noted, besides governing germ cell proliferation, the SCF/c-kit system also influences germ cell apoptosis. Protease inactivation of c-kit [54] or blocking of SCF/c-kit interaction [55] has been shown to induce apoptosis of spermatogonia. Therefore, attenuated c-kit signaling due to diminished expression levels of c-kit protein in consequence of E_2 stimulation could be responsible for increased apoptosis of germ cells.

The interplay between apoptotic death factors FasL and FasR has been identified as a crucial mechanism for determining germ cell death through the activation of the extrinsic pathway of apoptosis [34]. Our results have demonstrated that the expression of both FasL and FasR is increased in cultured SeT in presence of 100 nM E_2 (see Figure III.2A).

The protein ratio of mitochondria-related apoptosis regulators, namely, Bax (proapoptotic) and Bcl-2 (antiapoptotic), is a recognized indicator of the activation of apoptotic pathways and was included as a measurement of apoptosis. An increased Bax/Bcl-2 protein ratio was found in the E_2-treated group (see Figure III.2B). The imbalance toward proapoptotic proteins is due to increased expression of Bax, but the Bcl-2 levels remained virtually unchanged. This finding is concordant with the up-regulated expression of Bax observed when the SCF/c-kit interaction is blocked [55]. The use of an anti-c-kit antibody diminishing c-kit activity, as could happen when c-kit levels are reduced as we reported herein, was associated with increased protein ratio of proapoptotic/antiapoptotic proteins due to elevated expression of Bax [55].

The process of apoptosis independent of the triggered pathway converges on the activation of caspase-3, an effector caspase, which is considered a remarkable end point of apoptosis [56]. Determination of caspase-3 enzymatic activity in SeT cultured in the presence or absence of 100 nM E_2 demonstrated an increase of almost 40% in caspase-3 activity (see Figure III.2D) in response to E_2. The up-regulated expression of FasL and FasR, the increased ratio of proapoptotic/antiapoptotic proteins, and the augmented activity of caspase-3 suggest augmented apoptosis in response to E_2, which was confirmed by the TUNEL-labeling assay (see Figure III.3A).

A considerable amount of data have established that germ cells essentially express the death receptor FasR whereas SCs mainly present the ligand FasL [57, 58]. Therefore, the increased expression of FasL (see Figure III.2A) concomitant with an increased response of germ cells to

death signals, because FasR expression is augmented (see Figure III.2A), indicates that an augmentation of apoptosis is occurring in the germ cell population. Because c-kit is a known germ cell marker [19, 38-40], the reduced labeling of c-kit in SeT treated with E_2 for 48 hours (see Figure III.3B) demonstrated the loss of germ cells. This is also strongly supported by our recent report on isolated SCs that showed that 100 nM of E_2 diminishes SCs apoptosis by reduction of Bax/Bcl-2 ratio and caspase-3 activity [12]. In addition, the increased expression of Bax, as reported herein, has been considered a feature of germ cells undergoing apoptosis in response to E_2 [10, 49].

The question of E_2 induction of apoptosis in testicular cells has remained a matter of controversy for in vivo and in vitro studies, with the use of natural or synthetic estrogens and/or different doses and distinct routes of administration producing not always concordant results. In fact, there are reports pointing to E_2 as a germ cell survival factor that inhibits apoptosis and restores spermatogenesis upon a harmful stimulus [29, 59]. However, these studies have used low doses of E_2 within the range of physiologic concentrations (0.5–57 nM) [29-31].

In our study, using ex vivo cultures of SeT, we demonstrated that elevated concentrations of E_2 (100 nM), mimicking those found in the intratesticular milieu of infertile patients, unbalanced the expression of the SCF/c-kit system, disrupting the survival and death communication between germ cells and SCs toward germ cell apoptosis. In sum, our results showed that E_2 induced a decrease in c-kit expression, which was coupled with augmented apoptosis as evidenced by the increased expression of FasL, FasR, and Bax/Bcl-2 ratio as well as caspase-3 activity. A decreased proliferation was also found in response to E_2 downregulation of c-kit expression, indicating diminished survival and increased apoptosis of germ cells in the epithelium of SeT. Moreover, E_2 administration increased both the mRNA and protein levels of RGN (Figure III.5). Since RGN seems to play a pivotal role rescuing cells from apoptosis induced by noxious stimuli [60-62] the augmented expression of RGN in SeT treated with E_2 may be a mechanism to counteract estrogens-induced apoptosis. These data are of paramount importance, considering hyperestrogenism-related cases of idiopathic infertility [15, 16] and the observed diminished expression of c-kit in subfertile testicular tissues [5].

Although the causes are not totally known, it has been reported that an increase in the intratesticular concentration of E_2 in infertile men is accompanied by an augmented ratio of estradiol/testosterone [15, 16]. It seems likely that this hormone deregulation may occur as a result of the increased expression and/or activity of the aromatase enzyme. Lardone et al. [16] found increased levels of aromatase mRNA in the testis of infertile men with mixed atrophy and Sertoli cell-only syndrome. More recently, it was shown that the use of aromatase inhibitors for treatment of infertile men with high estradiol/testosterone ratios improved both hormone and semen parameters [63], which supports that the cause of the disorder is on the abnormal activity of aromatase. Because elevated levels of E_2 [15, 16] and increased rates of apoptosis have been found in the testis of infertile men [5-9], we may

extrapolate that the increased estrogenic response underlies the impairment of spermatogenesis and infertility by causing depletion of germ cells due to augmented apoptosis and reduced proliferation in consequence of diminished expression of c-kit.

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Chapter IV

Transgenic overexpression of regucalcin leads to suppression of thapsigargin- and actinomycin D-induced apoptosis in the testis by modulation of apoptotic pathways

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Transgenic overexpression of regucalcin leads to suppression of thapsigargin- and actinomycin D-induced apoptosis in the testis by modulation of apoptotic pathways

Summary

Recent evidence suggested the involvement of calcium-binding protein regucalcin (RGN) in testicular apoptosis. Herein we investigated the role of RGN controlling apoptotic pathways in the testis by using a transgenic rat model overexpressing RGN (Tg-RGN). Seminiferous tubules (SeT) from Tg-RGN and their wild-type (Wt) counterparts were cultured ex vivo in presence or absence of apoptosis-inducers thapsigargin (Thap, 10^{-7} and 10^{-6} M) and actinomycin D (0.5 and 1 µg/ml). Expression levels of key regulators of apoptosis in SeT of Tg-RGN and Wt animals were determined by quantitative real-time PCR and Western Blot analysis. Measurement of caspase-3 enzymatic activity was included as an end-point of apoptosis. Tg-RGN SeT treated with 10^{-6} M of Thap or 1 µg/ml of Act D showed a diminished enzymatic activity and gene transcription of caspase-3, along with increased mRNA and protein expression of antiapoptotic Bcl-2. Bcl-2/Bax (antiapoptotic/proapoptotic) protein ratio was also enhanced in these SeT. Although caspase-9 mRNA was increased in the SeT of Tg-RGN treated with Thap, no differences were observed at protein level, and no differences were also found on protein levels of apoptosis-inducing factor. mRNA expression of proapoptotic p53 and p21 was strongly decreased in Tg-RGN SeT treated with Thap (10^{-6} M) or Act D (1 µg/ml). These findings demonstrated that RGN suppresses Thap- and Act D-induced apoptosis in SeT by modulating the expression and activity of key apoptotic and antiapoptotic factors. Moreover, results indicate that RGN overexpression protects germ cell from apoptosis induced by noxious stimuli, which could be a relevant mechanism for fertility preservation in situations of oncological treatments.

Keywords: rat, apoptosis, calcium, regucalcin, testis, seminiferous tubules, spermatogenesis, germ cells, thapsigargin, actinomycin D

Introduction

Regucalcin (RGN) is a calcium (Ca^{2+})-binding protein playing an important role in intracellular Ca^{2+} homeostasis [1] and, among other functions, it has also been suggested its role suppressing cell death and apoptosis in liver and kidney cells [2-4]. In the testis, the tight control of germ cells survival and apoptosis maintains the proper ratio between developing germ cell and somatic supporting cells [5], which is of paramount importance for a successful spermatogenesis [6]. The significance of the accurate regulation of testicular apoptosis became also evident by the generation of knockout mouse models for genes encoding apoptosis regulators, which are infertile or exhibit spermatogenesis defects [7-9]. It has been

shown that testosterone withdrawal stimulates Sertoli [10] and germ cells apoptosis [11] and, besides their action as the essential regulators of spermatogenesis [12, 13], androgens are also recognized as germ cell survival factors [14]. Recently, our research group has demonstrated that androgens up-regulate RGN expression in the testis [15], which allowed to link this protein with the control of testicular apoptosis.

In this study, we investigated the role of RGN in regulating apoptosis in the testis by comparing the expression of key regulators of this process between seminiferous tubules (SeT) from transgenic rats overexpressing RGN (Tg-RGN) and their wild-type (Wt) counterparts after ex vivo culture in the presence or absence of apoptosis-inducers thapsigargin (Thap) and actinomycin (Act D). We observed a diminished activity of caspase-3, which was accompanied by decreased transcription of caspase-3 gene, and increased mRNA and protein expression of anti-apoptotic Bcl-2 protein in SeT of Tg-RGN rats. Also, the Bcl-2/Bax protein ratio was augmented in these SeT relatively to their Wt counterparts. Moreover, mRNA expression of proapoptotic proteins p53 and p21 was also strongly decreased in Tg-RGN treated with Thap or Act D. Our results indicate that RGN overexpression suppresses Thap- and Act D-induced apoptosis, pointing RGN as an important target in the control of testicular apoptosis by acting as a germ cell survival factor, alone or as a mediator in androgen signaling pathways.

Materials and Methods

Animals

Three months old Wt and Tg-RGN Sprague Dawley (*Rattus norvegicus*) rats were obtained, from Charles River (Barcelona, Spain) and Japan SLC (Hamamatsu, Japan), respectively. Tg-RGN rats were originally generated by Yamaguchi M by oocyte transgene pronuclear injection and have been a useful model to explore the RGN roles in vivo [16, 17]. Animals were housed under a 12 h light:12 h darkness cycle, with food and water available ad libitum during the course of the experiment, and handled in compliance with the guidelines established by the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and the European Union rules for the care and handling of laboratory animals (Directive number 86\609\EEC). All rats were euthanized under anesthesia (Clorketam 1000, Vetoquinol, Lure, France).

Ex vivo culture of rat SeT

Testes from Wt and Tg-RGN animals (n=6 in each group) were removed, trimmed free of fat, washed in cold PBS and placed in DMEM F-12 culture medium (Sigma-Aldrich, St.Louis, USA) supplemented with 20 mg/L gentamicin sulfate, 0.1 mM 3-isobutyl-1-methylxanthine, and 1 µg/L BSA at 33°C. Tunica albuginea was cut and peeled back to expose tubules. Ten fragments of SeT of about 1 cm were placed in culture plate (Nunclon D 12 well multidishes; Nunc, Roskilde, Denmark) wells containing 2 ml of pre-warmed culture medium with or without apoptosis-inducers, Thap (10^{-7} and 10^{-6} M, Sigma-Aldrich) or Act D (0.5 and 1 µg/ml).

These concentrations were found to be effective in inducing apoptosis in liver and kidney cells [2, 4, 18]. The SeT from Wt and Tg-RGN rats were incubated for 48 h at 33°C in an atmosphere of 5% CO_2. At the beginning and end of the experiment SeT tubules were recovered from medium, snap-frozen in liquid nitrogen and stored at -80°C until RNA or protein isolation. Alternatively, SeT were fixed in 4% PFA for histological processing and apoptotic cell-labeling.

RNA isolation and cDNA synthesis

Total RNA was isolated from rat SeT using TRI reagent (Sigma-Aldrich) according to the manufacturer's instructions and decontaminated from genomic DNA by digestion with deoxyribonuclease I (amplification grade DNase I, 1 U/µg RNA). In order to assess the quantity and integrity of total RNA, its optical density was determined (NanoPhotometer, Implen, Munich, Germany) and an agarose gel electrophoresis analysis was performed. cDNA was synthesized in a final volume of 20 µL using 1 µg of each RNA sample, 160 IU M-MLV reverse transcriptase (Promega, Madison, USA), 0.5 µg random primers (Invitrogen, Karlsruhe LMA, Germany) and 10 mM of each dNTP (GE Healthcare, Buckinghamshire, UK) according to the protocol supplied by the manufacturer. Synthesized cDNA was stored at -20°C until further use.

Quantitative Real-time PCR (qPCR)

Quantification of expression of apoptosis-related genes, namely, Bcl-2, Bax, p53, p21, caspase-9 and caspase-3 in SeT from Wt and Tg-RGN rat was performed by qPCR. Gene specific primers details and cycling conditions are summarized in Table IV.1. ß-actin and GAPDH were used for normalization of expression of interest target genes as it have been demonstrated to be suitable internal reference genes in SeT samples [15, 19]. Reactions were carried out in an iQ5 system (Bio-Rad, Hercules, USA) and efficiency of the amplifications was determined for all primer sets using serial dilutions of cDNA (1, 1:5 and 1:25). Primer concentration and annealing temperature were optimized before the assay and specificity of the amplicons was determined by melting curve analysis. Each reaction consisted of Maxima SYBR Green/Fluorescein qPCR Master Mix (Bio-Rad), sense and anti-sense primers (200 nM for all primer pairs), and 1 µL of cDNA in a final volume of 20 µl. Also, a no-template control was included for each reaction and all reactions were carried out in triplicate. Normalized expression values were calculated using the geometric mean of ß-actin and GAPDH housekeeping genes according to the mathematical model proposed by Vandesompele and collaborators [20].

Table IV.1. qPCR primer sequences, cycling conditions, and amplicon size.

Gene	Primer Sequences (5'- 3')	AT[a] (°C)	Amplicon Size (bp)
Bcl-2	Sense: GGG CTA CGA GTG GGA TAC Antisense: AGG CTG GAA GGA GAA GAT G	53	63
Caspase- 3	Sense: AGG CCT GCC GAG GTA CAG AGC Antisense: CCG TGG CCA CCT TCC GCT TA	60	255
Caspase- 9	Sense:TGC AGG GTA CGC CTT GTG CG Antisense: CCT GAT CCC GCC GAG ACC CA	60	130
Bax	Sense: CGC GTG GTT GCC CTC TTC TAC TTT Antisense: CAA GCA GCC GCT CAC GGA GGA	60	124
p21	Sense: GTT CCT TGC CAC TTC TTA C Antisense: ACT GCT TCA CTG TCA TCC	53	103
p53	Sense: CTG CCC ACC ACA GCG ACA GG Antisense: AGG AGC CAG GCC GTC ACC AT	60	471
β-actin	Sense: ATG GTG GGT ATG GGT CAG Antisense CAA TGC CGT GTT CAA TGG	60	79
GAPDH	Sense: GTT CAA CGG CAC AGT CAA G Antisense: CTC AGC ACC AGC ATC ACC	60	177

[a]annealing temperature.

Western Blot (WB)

Total protein was isolated from rat SeT using RIPA buffer (150 mM NaCl, 1% Nonidet-P40 substitute, 0.5% Na-deoxycholate, 0.1% SDS, 50 mM Tris pH 8, and 1 mM EDTA) supplemented with protease inhibitors cocktail (Sigma-Aldrich). Protein concentration in tissue extracts was determined by the Bradford assay (Bio-Rad). Proteins were resolved by SDS-PAGE on 12.5% gels and electrotransferred to a PVDF membrane (GE Healthcare). Membranes were incubated overnight at 4ºC with rabbit anti-Bcl-2 (1:5000, no.2876, Cell Signaling Technology), mouse anti-apoptosis-inducing factor (AIF, 1:300, (E-1):sc-13116, Santa Cruz Biotechnology), rabbit anti-cleaved caspase-9 (1:1000, no.9507, Cell Signaling Technology), and rabbit anti-Bax (1:1000, no.2772, Cell Signaling Technology). A mouse anti-actin antibody (1:1000, A5441, Sigma- Aldrich) was used for protein loading control in all WB analysis. Goat anti-rabbit IgG-AP (1:5000, NIF1317, GE Healthcare) or goat anti-mouse IgG+IgM-AP (1:5000, NIF1316, GE Healthcare) were used as secondary antibodies. Membranes were developed with ECF substrate (GE Healthcare) for 5 minutes and scanned with Molecular Imager FX Pro plus MultiImager (Bio-Rad). Band densities were obtained according to standard methods using the Quantity One Software (Bio-Rad) and normalized by division with the respective actin band density.

Caspase-3 like colorimetric activity assay

Caspase-3 activity assay was performed as previously described [21]. Briefly, 25 µg of total protein extract were incubated with reaction buffer (25 mM HEPES, pH 7.5, 0.1% CHAPS, 10% sucrose and 10 mM DTT) and 100 µM of caspase-3 substrate (Ac-DEVD-pNA) for 2 h at 37ºC. Upon caspase cleavage p-nitro-aniline (pNA) is released producing a yellow color, which is measured spectrophotometrically at 405 nm. The amount of generated product was calculated by extrapolation of a standard curve of free pNA.

Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling (TUNEL)

TUNEL was performed as previously described by [22] with some modifications. Briefly, SeT sections (5 µm) were deparaffinized in xylene, rehydrated in graded alcohols, rinsed in 0.15 M PBS (pH 7.4) and permeabilized with 0.25% Triton X-100 (Sigma-Aldrich) for 30 minutes at room temperature. Thereafter, sections were incubated for 10 minutes in 3% H_2O_2 and reacted for terminal transferase (0.25 U/µl) biotinylated dUTP (6 µM) nick-end labeling of fragmented DNA in TdT buffer (pH 7.5, Roche, Basel, Switzerland) for 1 h at 37°C in a humidified chamber. The enzymatic reaction was stopped by 15 minutes of incubation in 300 mM NaCl (Sigma-Aldrich) and 30 mM sodium citrate (Sigma-Aldrich) buffer. Following an additional rinse in PBS, SeT sections were then incubated for 1h at room temperature with rhodamine avidin D (1:200, Vector Laboratories, Inc., Burlingame, CA). Cell nuclei were stained by incubation with Hoechst 33342 (10 µg/mL, Invitrogen) for 5 minutes. Sections were then washed with PBS for 10 minutes and mounted in Dako fluorescent mounting medium (Dako, Carpenteria, CA, USA). Images were acquired using a Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss SMT Inc., Jena, Germany).

Statistical analysis

Statistical significance of differences between Wt and Tg-RGN rats was evaluated by unpaired t-test with Welch´s correction or by one-way ANOVA followed by Bonferroni's multiple comparison test as applicable, using GraphPad Prism v5.00 (GraphPad Software, San Diego, CA, USA). Statistically significant differences were considered for $P<0.05$.

Results

SeT from rats with transgenic overexpression of RGN and Wt were cultured ex vivo in presence or absence of Thap (10^{-7} and 10^{-6} M) or Act D (0.5 and 1 µg/ml), well known apoptosis inducing agents [18, 23]. The expression and activity of target regulators of apoptosis in Tg-RGN comparatively to their Wt littermates, in control conditions and in response to Thap or Act D treatment, were studied by means of qPCR, WB and enzymatic activity assays.

The process of apoptosis involves two major pathways (Figure IV.1A), the receptor-mediated or extrinsic pathway, occurring in response to activated death receptors present in the plasma membrane of the cell, and the mitochondrial or intrinsic pathway, activated by a variety of extra- and intracellular signals (reviewed by [24]). In both cases, signalling mechanisms converge to the activation of caspases, the executioners of apoptosis, (reviewed by [25]). The death receptor pathway leads to activation of procaspase-8, whereas the mitochondrial pathway involves procaspase-9. Once activated, either of these initiator caspases can cleave and activate effector caspases namely, caspase-3, caspase-6 and caspase-7 [26]. Once caspases are initially activated, cell death cannot be reversed (reviewed by [25]), and the activation of effector caspase-3 has been considered a remarkable end-point of apoptosis (Figure IV.1A) [27].

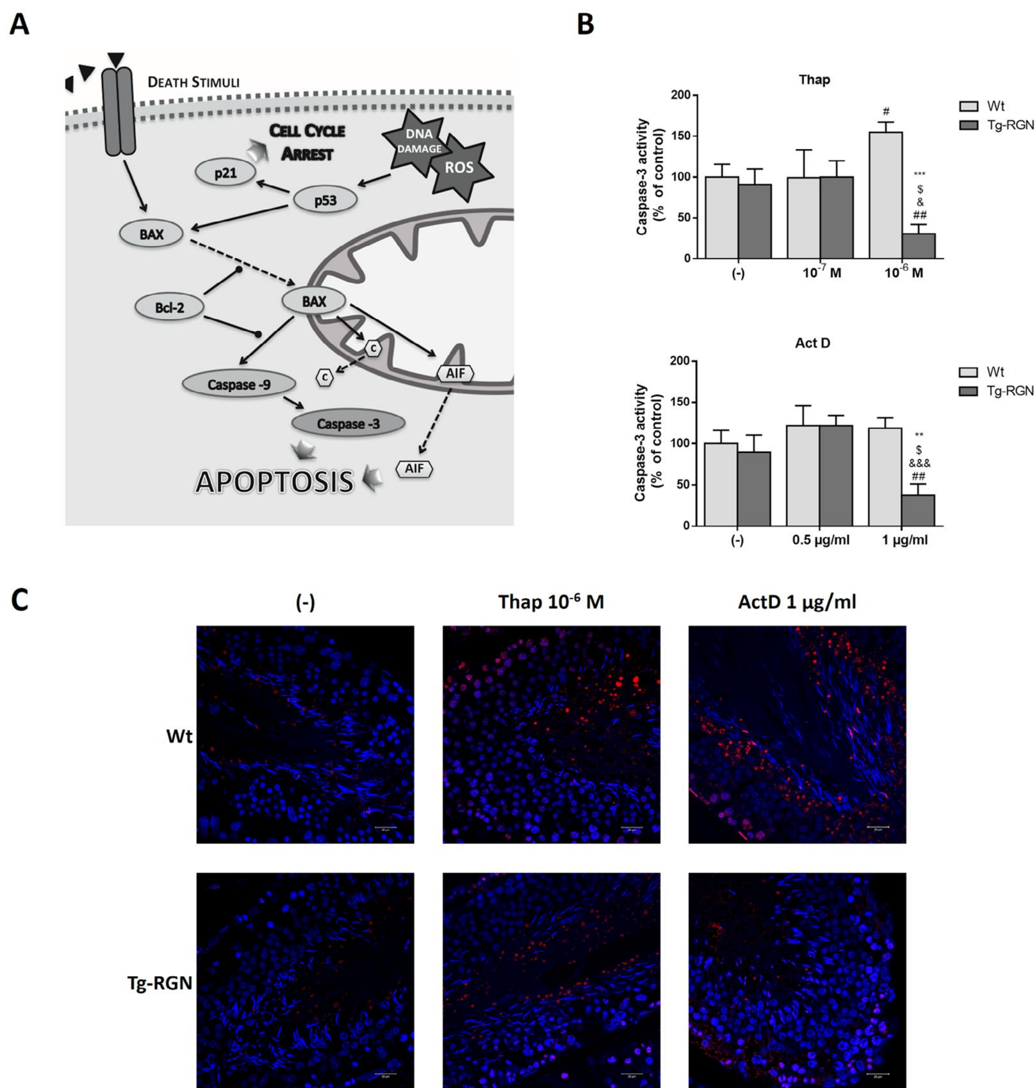

Figure IV.1. Caspase-3 activity and TUNEL-positive nuclei in SeT of Tg-RGN rats vs. Wt cultured in the presence or absence of apoptosis-inducers Thap (10^{-7} M and 10^{-6} M) and Act D (0.5 and 1 µg/ml). (A) Schematic representation showing caspase-3 as an end point of apoptosis. Death stimuli result in the activation of proapoptotic Bax, which leads to its translocation to the mitochondria with consequent release of cytochrome c and AIF and caspase-9 activation. Alternatively Bax is activated in response to a variety of stress stimuli via p53 which also activates p21, arresting cell cycle. The pathways converge at caspase-3 activation and apoptosis. Bcl-2 regulates cell survival inhibiting Bax translocation and caspase-9 activation. Arrows and ball ended arrows indicate activation and inhibition, respectively. Dashed arrows indicate translocation. (B) Caspase-3 activity in % relatively to Wt group in absence of Thap or Act D (-). Data are represented as mean ± S.E.M. with n≥ 4 in each group. $^{\#}$ statistically significant different when compared to Wt (-) group; $^{\&}$ statistically significant different when compared to Tg-RGN Thap 10^{-7} M or Tg-RGN Act D 0.5 µg/ml groups; $^{\$}$ statistically significant different when compared to Tg-RGN (-) group; * statistically significant different when compared to Wt Thap 10^{-6} M or Act D 1 µg/ml groups. (C) Representative imunofluorescent confocal microscopy images showing merged images of TUNEL-positive (red) and Hoechst 33342-stained nuclei (blue) in Wt and Tg-RGN SeT after Thap 10^{-6} M or Act D 1 µg/ml treatment.

Thus, we have started by measuring caspase-3 enzymatic activity in the SeT of Tg-RGN and Wt animals (Figure IV.1B). In Wt group the highest dose of Thap treatment (10^{-6} M) induced a pronounced increase in caspase-3 activity (154.8% ± 12.86 vs. 100.0% ± 15.79 in non-treated

group, *P*<0.05). On the contrary, a diminished activity of caspase-3 was observed in Tg-RGN SeT treated with Thap 10^{-6} M (30.95% ± 11.04) or Act D 1 µg/ml (37.50 ± 13.62) comparatively with their Wt counterparts in the same experimental condition (respectively, *P*<0.001 and *P*<0.01). Moreover, the diminished activity of caspase-3 in Tg-RGN rats SeT treated with Thap 10^{-6} M or Act D 1 µg/ml was also significantly different from that measured after treatment with Thap 10^{-7} M, Act D 0.5 µg/ml or under control conditions without apoptosis-inducers. No significant differences were observed in caspase-3 activity between Tg-RGN and Wt groups without Thap or Act D stimulation (Figure IV.1B). The reduction of caspase-3 activity in Tg-RGN in response to Thap and Act D treatment (Figure IV.1B) was accompanied by a decrease in the transcription of caspase-3 gene (Figure IV.2 and Figure IV.4), which was evident upon comparison of non-treated Tg-RGN SeT with Thap or Act D groups (in both cases *P*<0.001). Moreover, caspase-3 transcript levels in Tg-RGN SeT were already lower than that observed in Wt SeT in the absence of apoptosis-inducers (*P*<0.05).

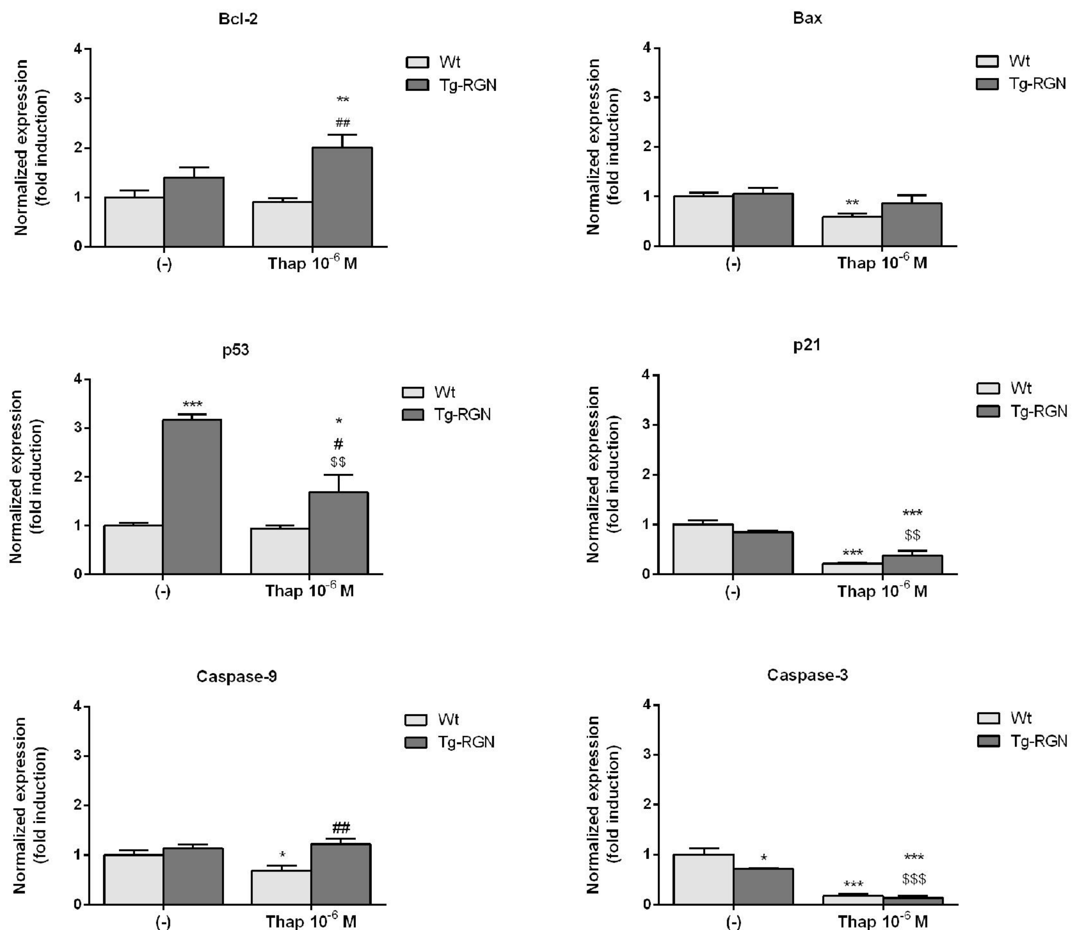

Figure IV.2. Transcript levels of apoptosis-related genes in SeT of Tg-RGN rats *vs.* Wt cultured in presence (10^{-6}M) or absence of Thap (-). Data are represented as mean ± S.E.M. with n≥4 in each group. Results are expressed as fold-variation relatively to Wt SeT in absence of Thap (-) * statistically significant different when compared with Wt (-) group; # statistically significant different when compared with Wt Thap 10^{-6}M group; $ statistically significant different when compared with Tg-RGN (-) group (* *P<0.05;* ** *P<0.01;* *** *P<0.001;* # *P<0.05;* ## *P<0.01;* $$ *P<0.01;* $$$ *P<0.001).*

The presence of apoptotic cells in the SeT of Wt and Tg-RGN in response to apoptotic-stimuli was analyzed by detection of DNA fragmentation using the TUNEL assay. Representative photomicrographs showing TUNEL-positive cells are provided in Figure IV.1C.

The Bcl-2 family includes a set of mitochondrial membrane proteins that regulate cell survival. Bcl-2 and Bax are anti- and pro- apoptotic members, respectively, with the ratio of Bcl-2/Bax protein dictating cell susceptibility to apoptosis [28, 29]. Tg-RGN rat SeT treated with Thap (10^{-6}M) displayed an approximately two-fold statistically significant higher expression of anti-apoptotic Bcl-2 mRNA (Figure IV.2) in comparison with Wt treated SeT, as well with non-treated group (*P*<0.01 in both cases). Also, protein levels of Bcl-2 (Figure IV.3 and Figure IV.5) were increased in Tg-RGN SeT independently of Thap or Act D treatment (*P*<0.05 and *P*<0.01, respectively).

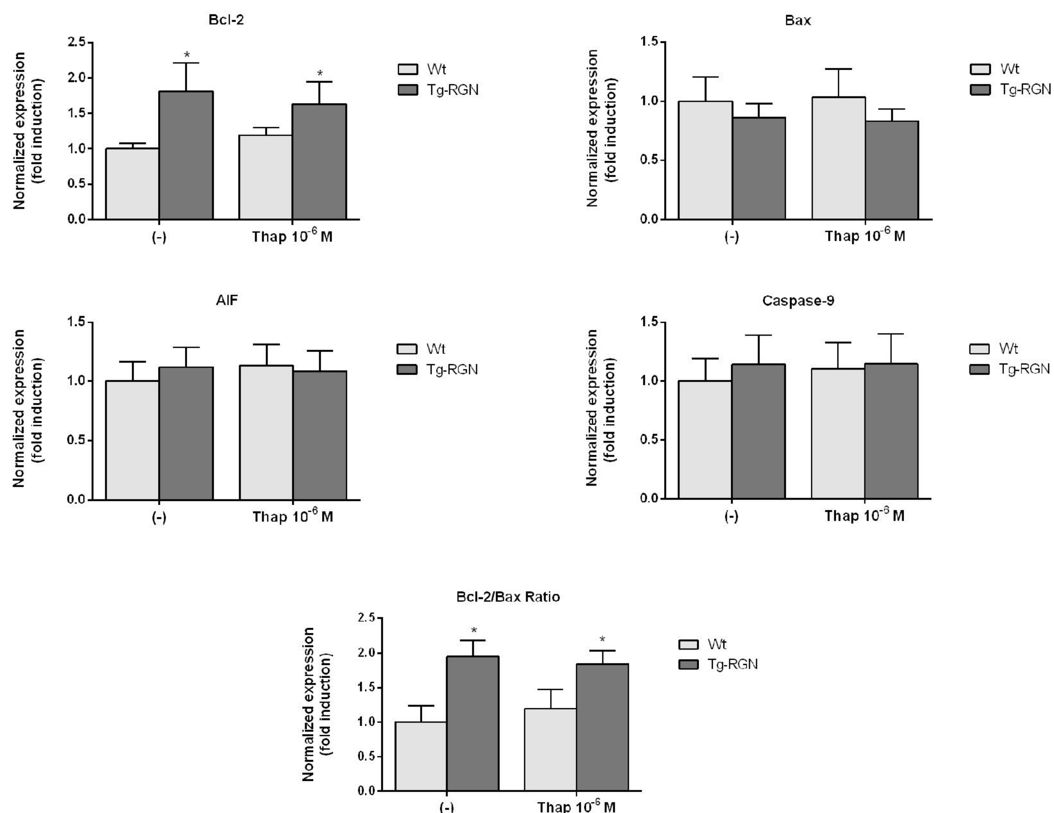

Figure IV.3. Protein expression of apoptosis-related genes and Bcl-2/Bax protein ratio in SeT of Tg-RGN rats *vs.* Wt cultured in presence (10^{-6}M) or absence of Thap (-). Data are represented as mean ± S.E.M. after normalization with actin. n≥4 in each group. Results are expressed as fold-variation relatively to Wt SeT in absence of Thap (-) (* *P<0.05* when compared with Wt (-) group).

No differences were detected in both mRNA (Figure IV.2) and protein (Figure IV.3) levels of Bax in Tg-RGN SeT with or without Thap induction of apoptosis. Although Act D treatment enhanced Bax transcript levels in Tg-RGN SeT (Figure IV.4), no differences were observed in protein expression (Figure IV.5). Calculation of the Bcl-2/Bax protein ratio demonstrated an

approximately 2-fold (Figure IV.3) and 1.5-fold (Figure IV.5) increase, respectively, in Tg-RGN SeT treated with Thap or Act D (*P*<0.05).

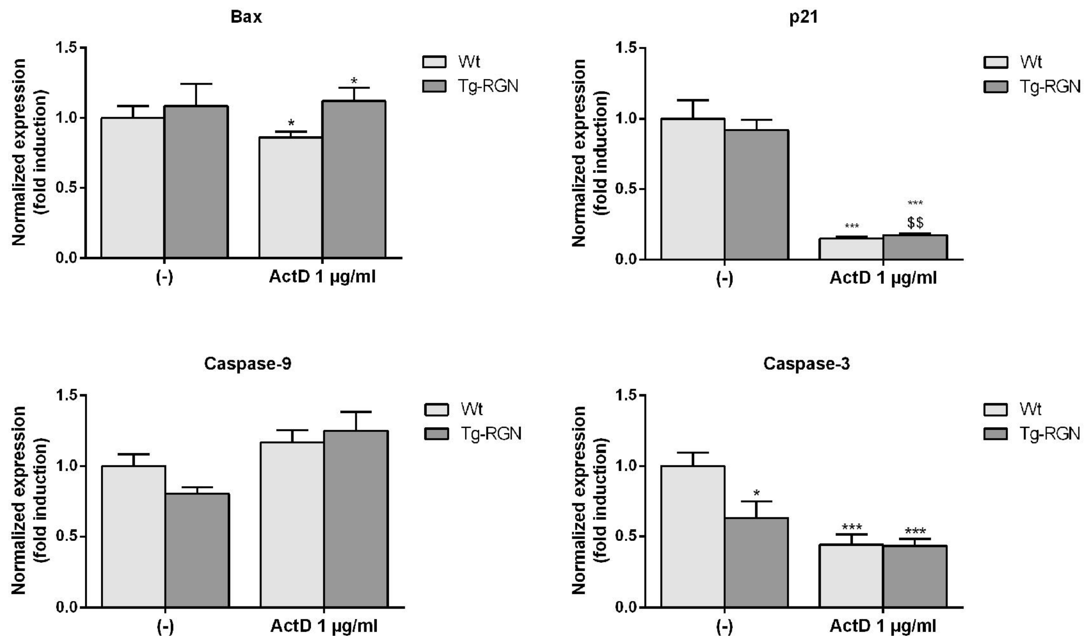

Figure IV.4. Transcript levels of apoptosis-related genes in SeT of Tg-RGN rats *vs.* Wt cultured in presence (1 µg/ml) or absence of Act D (-). Data are represented as mean ± S.E.M. with n≥4 in each group. Results are expressed as fold-variation relatively to Wt SeT in absence of Act D (-) * statistically significant different when compared with Wt (-) group; $ statistically significant different when compared with Tg-RGN (-) group (* *P<0.05;* *** *P<0.001;* $$ *P<0.01).*

The up-regulation or activation of Bax leads to its translocation and consequent permeabilization of the outer membrane of mitochondria (Figure IV.1A), which is followed by the release of many apoptosis promoting proteins that reside in the mitochondrial intermembrane space, including cytochrome c, AIF and pro-caspase 9 [29]. Although caspase-9 mRNA was increased in Tg-RGN treated with Thap (Figure IV.2) in comparison to Wt (*P*<0.01), no differences were observed at protein level (Figure IV.3). Also, no differences on protein levels of AIF (Figure IV.3) were found between Tg-RGN and Wt groups.

The tumor suppressor protein p53 has a critical role in regulation of the Bcl-2 family members and also upregulates the expression of cyclin-dependent kinase inhibitor, p21, thus arresting cell cycle at G1 phase and promoting apoptosis in response to a variety of stress stimuli (reviewed by [30, 31]). Non-treated Tg-RGN SeT showed p53 levels 3-fold higher than those found in their Wt counterparts (*P*<0.001). However, Thap treatment induced a pronounced decrease of approximately 50% in mRNA expression levels of both p53 and p21 (Figure IV.2). It was observed the same response pattern in p21 mRNA when the apoptosis inducer was Act D (Figure IV.4).

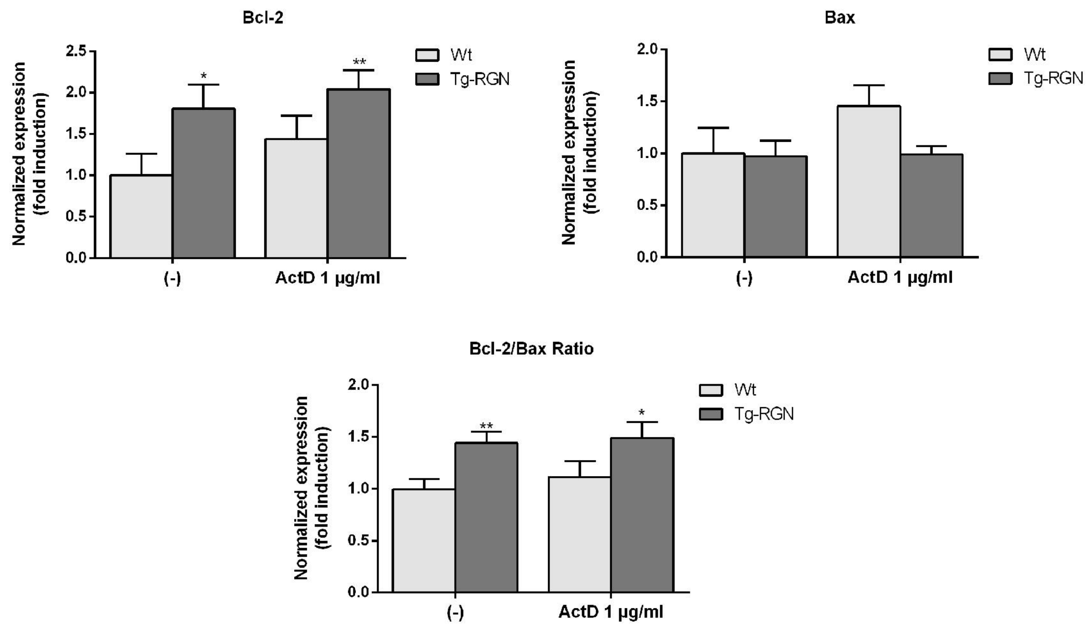

Figure IV.5. Protein expression of apoptosis-related genes and Bcl-2/Bax protein ratio in SeT of Tg-RGN rats *vs.* Wt cultured in presence (1 µg/ml) or absence of Act D (-). Data are represented as mean ± S.E.M. after normalization with actin. n≥4 in each group. Results are expressed as fold-variation relatively to Wt SeT in absence of Act D (-) * statistically significant different when compared with Wt (-) group; (* *P<0.05*; ** *P<0.01*).

Discussion

The objective of this study was to investigate the role of RGN in modulating apoptotic pathways in the testis. For this purpose we stimulated apoptosis in the SeT of Tg-RGN and Wt using Thap or Act D, well known apoptosis inducers widely used in several cell types [18, 23, 32-34]. Act D is a cytotoxic drug acting as a transcription inhibitor [35] while Thap is a selective inhibitor of Ca^{2+}-ATPases in the membrane of endoplasmatic reticulum, causing an increase in cytoplasmatic Ca^{2+} levels and apoptosis, which is associated with the activation of the effector caspase-3 [4]. Thus, as expected, Thap treatment induced apoptosis in rat SeT as becomes evident by the observed increase in caspase-3 activity in Wt SeT (Figure IV.1B). However, this increase was completely prevented in Tg-RGN SeT that showed a significant reduction of caspase-3 activity (Figure IV.1B) in response to Thap or Act D treatment. Moreover, no significant difference was observed between caspase-3 activity in SeT of non-treated Tg-RGN and Wt animals, demonstrating that RGN overexpression has a suppressive effect on Thap- and Act D-induced apoptosis, as suggested also by TUNEL-labelling in the SeT of Tg-RGN and their Wt counterparts in response to apoptotic stimuli (Figure IV.1C). The reduction in caspase-3 activity in Tg-RGN was accompanied by decreased transcription of the caspase-3 gene (Figure IV.2 and Figure IV.4) and increased levels of Bcl-2, as well as the Bcl-2/Bax ratio (Figure IV.3 and Figure IV.5). As this ratio has been considered a valuable biomarker indicating cell susceptibility to apoptosis [28], the observed unbalance in this parameter towards anti-apoptotic Bcl-2 also explains the diminished apoptosis rate in Tg-RGN

SeT. No differences were found on protein levels of AIF (Figure IV.3) between Tg-RGN and Wt groups, which was not surprisingly since AIF or cytochrome c release is prevented by overexpression of Bcl-2 [36]. It has been described that in response to several stimuli, Bcl-2 inhibits the upregulation of p53 [37], which may explain the observed decrease of p53 mRNA expression in Tg-RGN SeT treated with Thap (Figure IV.2). Accordingly, also the expression of cell-cycle inhibitor p21 (Figure IV.2 and Figure IV.4), a target of p53 transcription regulation, is diminished in response to Thap or Act D treatment under RGN overexpression. A previous study in rat kidney cells also reported that RGN overexpression is linked with the modulation of expression of apoptosis-related genes, precisely increasing the expression of Bcl-2 and decreasing the expression of caspase-3 [4]. Although the mechanisms by which RGN regulate expression of apoptosis related genes remain to be disclosed, RGN has been identified as a protein translocated to the nucleus and able to interact with DNA [38, 39].

Classically RGN is a Ca^{2+}-binding protein playing a pivotal role controlling intracellular Ca^{2+} levels by enhancing the activity of Ca^{2+} pumps in plasma membrane, mitochondria and endoplasmic reticulum [1], and it has been shown that RGN inhibits Ca^{2+}-activated DNA fragmentation in the nuclei isolated from rat liver cells likely by binding of Ca^{2+} ions [40]. Therefore, it cannot be excluded that the effect of RGN suppressing Thap-induced apoptosis may occur by preventing the rise of intracellular Ca^{2+} concentrations, which would be expected to happen by inhibition of Ca^{2+}-ATPase in the membrane of endoplasmatic reticulum due to Thap action. In addition, studies exist associating modulation of cytosolic Ca^{2+} with alterations on the expression and ratios of pro- and antiapoptotic Bcl proteins [41, 42], suggesting that the increased Bcl-2 levels in Tg-RGN may also be explained by changes in Ca^{2+} homeostasis.

In rat hepatoma and kidney epithelial cells, overexpression of RGN suppresses cell death and apoptosis induced by tumor necrosis factor-α (TNF-α) or Thap [2, 4]. In addition, data from knockout animals evidenced the role of RGN protecting cells from apoptosis. Hepatocytes lacking RGN are more susceptible to TNF-α and Fas-mediated apoptosis [43]. Our results thus indicate that RGN may have a role suppressing testicular apoptosis induced in response to other noxious stimuli.

Recently, we identified RGN as a broadly expressed gene in male reproductive tract, and detailed its localization in the testis [15]. Both in human and rat testis RGN is expressed in Leydig and Sertoli cells, as well as in the entire germ cell line [15]. A characteristic of RGN is its diminished expression with aging [44], which we demonstrated that testis of aged animals display reduced levels of RGN [15]. This means that reduction of RGN expression might be an age-related event associated with impairment of spermatogenesis due to increased apoptosis. We have also demonstrated that androgens up-regulate RGN expression in the testis [15], which suggest that increasing RGN levels may be a mechanism by which androgenic stimulation sustain germ cell survival and spermatogenesis. Interestingly, in another previous study we showed an increased expression of RGN in testicular biopsies of infertile men with hypospermatogenesis [45]. Since an increased activity of caspase-3 was detected in germ cells

in hypospermatogenesis cases [46] and, as demonstrated here, transgenic RGN overexpression leads to diminished caspase-3 activity, it is reasonable to speculate that in this case of defective spermatogenesis the observed increase of RGN is a response to counteract the increased caspase-3 activity and suppress apoptosis. This assumption is supported by the fact that generation of radioresistant cell lines using fractionated irradiation is achieved with a concomitant overexpression of RGN [47]. In this way, the manipulation of RGN levels in the testis towards its overexpression could be an interesting mechanism for preserving fertility upon treatment of oncological conditions.

In conclusion, the results presented in this study demonstrated that RGN leads to suppression of Thap-or Act D-induced apoptosis in the testis by modulating the expression and activity of target regulators of apoptosis. In addition, our findings also indicate that RGN overexpression protects testicular cells from apoptosis induced by noxious stimuli, which support the role of RGN as a germ cell survival factor alone or as a mediator in androgen signaling pathways.

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Chapter V

Role of Regucalcin modulating oxidative stress in the testis

Chapter in preparation for submission Correia S, Cavaco JE and Socorro S. Role of Regucalcin modulating oxidative stress in the testis

Role of Regucalcin modulating oxidative stress in the testis

Summary

Regucalcin (RGN) is a calcium (Ca^{2+})-binding protein that has also been linked to decreased generation of reactive oxygen species and increased activity of antioxidant defense enzymes. It is widely known that oxidative stress adversely affects the spermatogenic process, disrupting the development of germ cells, and also interferes with the proper sperm function. The present study aims to analyze the role of RGN modulating oxidative stress in the testis. To address this issue, *ex vivo* cultures of seminiferous tubules (SeT) from transgenic rats overexpressing RGN (Tg-RGN) and wild-type (Wt) controls were maintained for 24 hours in the presence or absence of pro-oxidant stimuli, namely, tert-butyl hydroperoxide (TBHP, 250 and 500 μM) and cadmium chloride (Cd, 10 and 20 μM). Noteworthy, SeT from Tg-RGN animals displayed a significantly higher antioxidant capacity relatively to their Wt counterparts, both in control conditions and when treated with TBHP or Cd. Regarding the antioxidant defense systems, a significantly increase in the activity of glutathione-S-transferase was found in the SeT of Tg-RGN animals whereas no differences were observed in the activity of superoxide dismutase. The activity of caspase-3 in SeT treated with 250 μM of TBHP and 10 μM of Cd was significantly lower in Tg-RGN animals comparatively with Wt. These preliminary results showed that SeT of Tg-RGN animals display increased protection against oxidative damage and apoptosis, exhibiting lower levels of oxidative stress and increased antioxidant defenses. Moreover, the present findings support the protective role of RGN in spermatogenesis, which is an important issue to consider in the context of male infertility.

Key Words: oxidative stress, regucalcin, seminiferous tubules, spermatogenesis, tert-butyl hydroperoxide, cadmium chloride

Introduction

Regucalcin (RGN) is a calcium (Ca^{2+})-binding protein that contributes to Ca^{2+} homeostasis by the regulation of plasma membrane, mitochondria and endoplasmic reticulum Ca^{2+}-ATPases [1], for which a role in male reproductive function has been suggested [2]. Besides its function in the modulation of intracellular Ca^{2+}, the RGN protein has been linked to the control of cell survival and apoptosis, and its antioxidant properties also have been described [3-5]. The antioxidant role of RGN was effectively demonstrated by studies in the RGN-knockout mice, which display higher levels of oxidative stress than their wild-type (Wt) counterparts [6]. Accordingly, the overexpression of RGN seems to protect against oxidative stress by reducing the production of reactive oxygen species (ROS) and increasing the activity of antioxidant defense enzymes [7]. Oxidative stress is one of main harmful factors for male reproductive function, and a major cause of male infertility by its damaging effects on the development of germ cells and sperm function [8].Therefore, successful spermatogenesis and

achievement of optimal sperm function require protection from increased levels of oxidative stress. The protective mechanisms against oxidative stress depend on the activity of the antioxidant enzyme system, which includes, among others, superoxide dismutase (SOD) and glutathione-S-transferase (GST).

In the present study, we investigated the role of RGN in modulating the levels of oxidative stress in the testis by comparing the activity of antioxidant enzymes and the total antioxidant capacity between seminiferous tubules (SeT) of transgenic rats overexpressing RGN (Tg-RGN) and their Wt counterparts, after *ex vivo* culture in the presence or absence of oxidant inducers, tert-butyl hydroperoxide (TBHP) and cadmium chloride (Cd). The oxidative damage in terms of apoptotic cell-death in SeT of Tg-RGN and Wt animals was assessed by determining the enzymatic activity of the executioner of apoptose caspase-3.

Materials and Methods

Animals

Three-month-old Wt and Tg-RGN Sprague Dawley rats (*Rattus norvegicus*) were obtained from Charles River (Barcelona, Spain) and Japan SLC (Hamamatsu, Japan), respectively. Animals were housed under a 12 h light:12 h darkness cycle, with food and water available *ad libitum* during the course of the experiment, and handled in compliance with the guidelines established by the “Guide for the Care and Use of Laboratory Animals” published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and the European Union rules for the care and handling of laboratory animals (Directive number 2010/63/EU). All rats were euthanized under anesthesia (Clorketam 1000, Vetoquinol, Lure, France).

Ex vivo culture of rat SeT

Testes from Wt and Tg-RGN animals (n=6 in each group) were removed, trimmed free of fat, washed in cold phosphate buffered saline (PBS) and placed in DMEM F-12 culture medium (Sigma-Aldrich, St.Louis, USA) supplemented with 20 mg/L gentamicin sulfate, 0.1 mM 3-isobutyl-1-methylxanthine, and 1 µg/L BSA at 33°C. Tunica albuginea was cut and peeled back to expose tubules. Ten fragments of SeT of about 1 cm were placed in culture plate (Nunclon D 12 well multidishes; Nunc, Roskilde, Denmark) wells containing 2 ml of pre-warmed culture medium in the presence or absence of pro-oxidant stimuli, namely, TBHP (250 and 500 µM, Sigma-Aldrich) and Cd (10 and 20 µM, Sigma-Aldrich). The SeT from Wt and Tg-RGN rats were incubated for 24 h at 33°C in an atmosphere of 5% CO_2. At the end of the experiment, SeT were recovered from medium, snap-frozen in liquid nitrogen and stored at -80°C until protein isolation.

Total protein extraction

Total protein was isolated from SeT using RIPA buffer (1% Nonidet-P40 substitute, 0.5% Na-deoxycholate, 0.1% SDS in PBS) supplemented with protease inhibitors cocktail (Sigma-Aldrich). Protein concentration in tissue extracts was determined by the Bradford assay (Bio-Rad). 2-20 µl of total proteins were used in the biochemical enzymatic assays and results were presented /mg SeT tissue.

Glutathione S-Transferase (GST) Assay

The GST activity was determined using a commercially kit (CS0410, Sigma-Aldrich) according to the manufacturer's protocol. Briefly, the GST activity was measured by monitoring the formation of the conjugate of 1-Chloro-2,4-dinitrobenzene (CDNB) in a Dulbecco´s Phosphate Buffered Saline. The reactions occurred at 25 ºC and the conjugation of the thiol group of glutathione to the CDNB substrate was detected spectrophotometrically by an increase in the absorbance at 340 nm. Results were expressed as µmol/ml/min/mg tissue.

Superoxide dismutase (SOD) assay

Superoxide dismutase activity was measured through a competitive inhibition assay (SOD Assay Kit-WST, Sigma-Aldrich) using the WST-1 substrate (tetrazolium salt) and xanthine oxidase following manufacturer's instructions. Upon reduction with superoxide anion WST-1 produces a water soluble formazan dye, which is linear with the activity of xanthine oxidase and inhibited by SOD. Each endpoint assay was monitored by measurement of absorbance at 450 nm (the absorbance wavelength for the colored product of WST-1 reaction with superoxide) after 20 min of reaction time at 37 ºC. The percentage of reaction inhibition rate indicates SOD activity. Results were expressed as a proportion of activity (percentage of inhibition) relatively to Wt non-treated animals.

Total antioxidant capacity assay

The total antioxidant capacity was determined using a commercially kit according to the manufacturer's protocol (Cayman Chemical, Ann Arbor, MI, USA).The assay relies on the ability of antioxidants in the sample to inhibit the oxidation of 2,2′-azino-di-[3-ethylbenzthiazoline sulphonate] (ABTS®) to $ABTS^{®\cdot+}$ by metmyoglobin. The capacity of the antioxidants in the sample preventing ABTS® oxidation was compared with that of Trolox, a water-soluble tocopherol analog, and quantified as millimolar Trolox equivalents. The amount of oxidized ABTS® was monitored by reading the absorbance at 405 nm. Results were expressed as mM/mg tissue.

Caspase-3-like colorimetric activity assay

The enzymatic activity of Caspase-3 was determined as previously described [9]. In brief, 25 μg of total protein extract were incubated with reaction buffer (25 mM HEPES, pH 7.5, 0.1% CHAPS, 10% sucrose and 10 mM DTT) and 100 μM of caspase-3 substrate (Ac-DEVD-pNA) for 2 h at 37ºC. Upon caspase cleavage of Ac-DEVD-pNA the p-nitro-aniline (pNA) is released producing a yellow color, which is measured spectrophotometrically at 405 nm. The amount of generated pNA was calculated by extrapolation with a standard curve of free pNA. Results were expressed as % of Wt non-treated animals.

Statistical analysis

Statistical significance of differences between Wt and Tg-RGN rats was evaluated by unpaired t-test with Welch´s correction or by one-way ANOVA followed by Bonferroni's multiple comparison test as applicable, using GraphPad Prism v5.00 (GraphPad Software, San Diego, CA, USA). Statistically significant differences were considered for $P<0.05$.

Results

GST activity

Under control conditions a statistically significant higher GST activity was found in the SeT of Tg-RGN animals regarding their Wt littermates (Figure V.1). No statistically significant differences were found in SeT of Tg-RGN and Wt cultured with both TBHP dosages and with Cd 10 μM. Treatment with Cd at a concentration of 10 μM showed to enhance GST activity in SeT from Wt and Tg-RGN animals relatively to Set from Wt animals under control conditions ($P<0.01$ for both). In addition, SeT from Wt rats treated with the highest dose of Cd (20 μM) also exhibited a significant higher activity of this enzyme ($P<0.001$) comparatively to Wt animals under control conditions. Curiously, the highest dose of Cd induced a decrease in GST activity in Tg-RGN animals relatively to their Wt counterparts in the same experimental conditions.

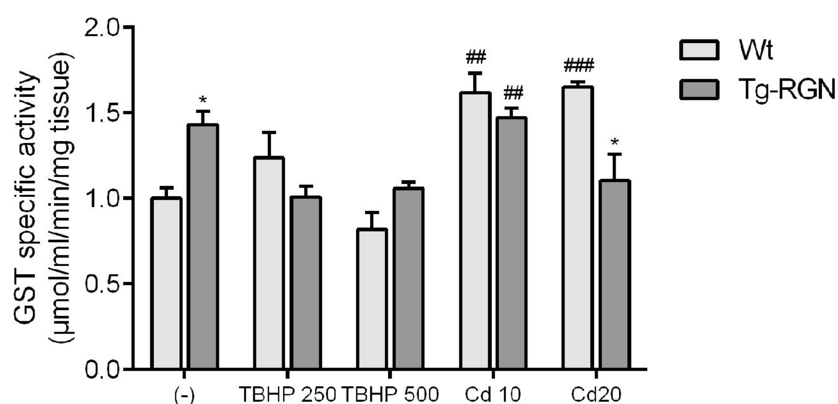

Figure V.1. Glutathione-S-transferase (GST) activity in the SeT of Tg-RGN rats and Wt control animals upon culture in the presence or absence of oxidant inducers TBHP (250 and 500 µM) and Cd (10 and 20 µM). Data are represented as mean ± SEM with n≥4 in each group. * statistically significant difference when compared to Wt group in the same experimental conditions; # statistically significant difference when compared to Wt control group (-) (*P < 0.05; ##P < 0.01; ###P < 0.001).

SOD activity

No statistically significant differences were found in the percentage inhibition of SOD between the two groups (Figure V.2).

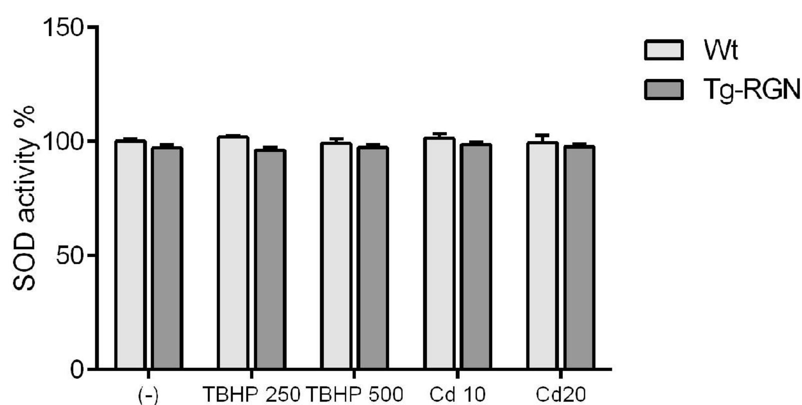

Figure V.2. Superoxide dismutase (SOD) activity in the SeT of Tg-RGN rats and Wt control animals upon culture in the presence or absence of oxidant inducers TBHP (250 and 500 µM) and Cd (10 and 20 µM). Data are represented as mean ± SEM with n=5 in each group.

Total antioxidant capacity

The overall antioxidant capacity may provide more relevant biological information compared to that obtained by the measurement of individual components, since this assay considers the cumulative effect of all antioxidants present in the samples. In the absence of pro-oxidant stimuli, Tg-RGN animals displayed a significantly higher antioxidant capacity relatively to

their Wt counterparts (1,75 fold-variation, Figure V.3, P<0.001). Also, in the presence of TBHP (250 and 500 μM), as well as, in the presence of Cd (10 and 20 μM) the amount of antioxidants is maintained significantly higher in the SeT of Tg-RGN comparatively with Wt animals in the same experimental conditions (P<0.01 and P<0.05, respectively). Therefore, SeT from Tg-RGN animals treated with TBHP (250 or 500 μM) showed a higher antioxidant capacity relatively to Set from Wt animals under control conditions (P<0.001). Also, treatment with different concentrations of Cd showed similar results (Figure V.3). SeT from Tg-RGN animals treated with Cd 10 and 20 μM exhibited a significantly higher antioxidant capacity comparatively with Set from Wt animals control animals (P<0.01 and P<0.001, respectively). However, also Wt animals treated with Cd at a 20 μM concentration showed an increase in the antioxidant capacity relatively to the Wt control group (P<0.01).

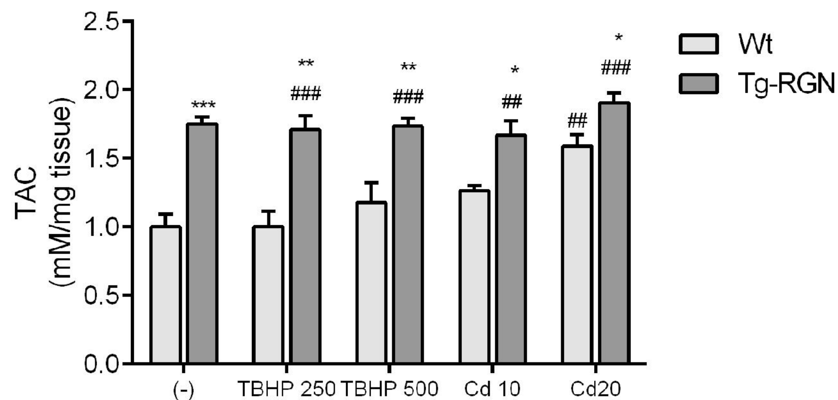

Figure V.3. Total antioxidant capacity (TAC) in the SeT of Tg-RGN rats and Wt control animals upon culture in the presence or absence of oxidant inducers TBHP (250 and 500 μM) and Cd (10 and 20 μM). Data are represented as mean ± SEM with n=5 in each group. * statistically significant difference when compared to Wt group in the same experimental conditions; # statistically significant difference when compared to Wt control group (-) (*P < 0.05; **P < 0.01; ***P < 0.001; ##P < 0.01; ###P < 0.001).

Caspase-3 activity

The oxidative stress is considered to be one of the major factors to induce germ cell apoptosis [10, 11]. Hence, we compared the rate of apoptosis in the SeT of Tg-RGN and Wt animals at different experimental conditions by measurement of caspase-3 activity. No significant differences were found in the activity of caspase-3 in SeT of Wt and Tg-RGN animals treated with the highest doses of TBHP and Cd, 500 and 20 μM, respectively. However, treatment with 250 μM of TBHP and 10 μM of Cd induced a pronounced increase in the activity of caspase-3 in the Wt group (respectively, 92 and 70 % variation relatively to control, P<0.01, Figure V.4). An effect that was not observed in the SeT of Tg-RGN rats. Thus, although no significant differences were observed in the activity of caspase-3 between Tg-RGN and Wt control groups, under expose to TBHP 250 μM and Cd 10 μM, the activity of

caspase-3 in Tg-RGN animals is significantly lower comparatively with their Wt counterparts in the same experimental conditions (P<0.05).

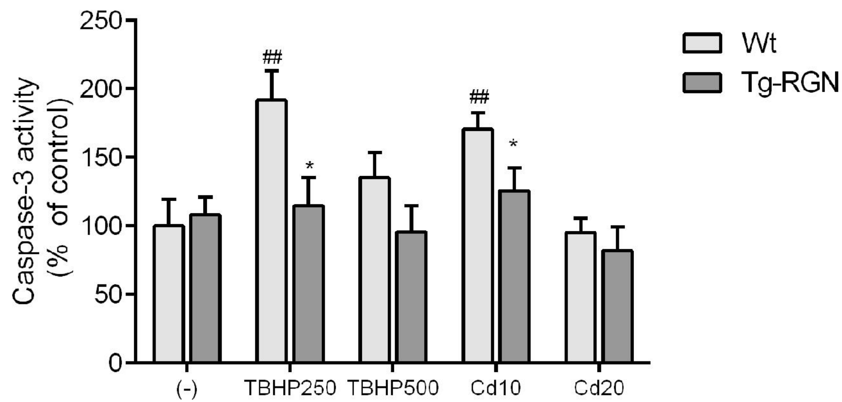

Figure V.4. Caspase-3 activity in the SeT of Tg-RGN rats and Wt control animals upon culture in the presence or absence of oxidant inducers TBHP (250 and 500 μM) and Cd (10 and 20 μM). Data are represented as mean ± SEM with n≥5 in each group. * statistically significant difference when compared to Wt group in the same experimental conditions; # statistically significant difference when compared to Wt control group (-) (*P < 0.05; ; ##P < 0.01).

Discussion

The objective of this study was to investigate the role of RGN in modulating oxidative stress in the testis. For this purpose, SeT of Tg-RGN and Wt animals were cultured *ex vivo* in presence or absence of TBHP and Cd, two well-known inducers of oxidative stress, which have been widely used in a variety of systems, including the male reproductive tract [12-15].

Reactive oxygen species may be generated by exogenous agents or via endogenous processes and have important physiological roles [16]. However, when ROS production greatly exceeds the capacity of endogenous cellular antioxidant defense system, oxidative stress occurs. Both *in vitro* and *in vivo* approaches have associated RGN actions with the protection from oxidative stress, likely by regulating the activity of enzymes involved in the antioxidant defense [4, 17, 18]. Glutathione-S-transferases are a group of enzymes that play an important role in the processes of detoxification in mammals. These enzymes protect cells against toxicants by conjugating the thiol group of the glutathione [19]. We started by measuring GST activity in SeT of Tg-RGN vs. Wt and found that under control conditions Tg-RGN animals display enhanced enzyme activity (Figure V.1). Treatment with Cd (10 and 20 μM) showed to increase the GST activity in the SeT of Wt animals (Figure V.1). This effect is in accordance with the role of glutathione acting by a Cd scavengers preventing its interaction with critical cellular targets [20]. However, the effects of Cd on antioxidative capacity are dual. It has been shown that Cd induces oxidative stress via the inhibition of antioxidant mechanisms [21]. On the other hand, Cd also activates several antioxidant components as a result of a disturbed redox balance and a consecutively induced signal transduction cascade [21]. This duality may possibly explain the distinct results observed for the activity of GST in SeT from

Tg-RGN animals treated with Cd. Although SeT from Tg-RGN animals treated with 10 μM of Cd showed increased activity of GST comparatively with the Wt control group (Figure V.1), the 20 μM concentration do not produced such effect. In addition, in this case Tg-RGN displayed a significant reduction in GST activity relatively to Wt animals in the same experimental conditions.

Glutathione-S-transferases can also reduce lipid hydroperoxides [22]. However, no differences were observed in the GST activity when SeT of both Tg-RGN and Wt animals were treated with TBHP (Figure V.1).

Superoxide dismutase belongs to the class of metallo-enzymes, which catalyze the dismutation of the superoxide anion to hydrogen peroxyde and, thus, play a relevant role in the defense against oxidative stress by reducing the levels of superoxide anions [23]. The total activity of SOD in response to Cd exposure has been intensively determined. However, the available literature includes discordant reports. There are studies showing an increase of SOD activity, while others have shown that enzyme activity decreases after exposure to Cd [24-26]. It cannot be excluded that the observed discrepancies can be attributed to different exposure conditions (time and doses), as well as, to tissue-specific features, which may lead to different outcomes. In our *ex vivo* assays, no differences were found in the SeT of Tg-RGN and Wt animals treated with Cd (Figure V.2). Also, no differences were found when SeT were exposed to TBHP. Nevertheless, Tg-RGN display enhanced total antioxidant capacity relatively to their Wt counterparts (Figure V.3), which suggests that other antioxidant defense enzymes are maintaining this phenotype. This idea is supported by the fact that mice with disruption of RGN gene show unchanged activity of SOD, while, for example, the activity of glutathione peroxidase activity was reduced [6, 27]. The measurement of thiobarbituric acid reactive substances as a method for screening and monitoring lipid peroxidation could be an elucidative option. Moreover, the measurement of ROS will be of uttermost importance to understand, which species are being produced. The higher antioxidant capacity in the SeT of Tg-RGN is maintained upon exposure to the oxidant inducers, Cd and TBHP (Figure V.3), which reinforces the importance of RGN protecting cells from oxidative stress. In accordance with our findings it was shown that RGN overexpression in the mouse embryonic carcinoma P19 cell line increased cell viability and protected cells from oxidative stress induced by TBHP [4]. The influence of RGN maintaining physiological levels of ROS and oxidative stress was undoubtedly demonstrated by studies using the RGN-knockout mice. The generation of ROS and the NADPH oxidase activity were significantly increased in the brain of RGN-knockout mice [6]. Moreover, lungs of RGN-knockout mice exposed to cigarette smoke showed elevated levels of protein carbonyls, an oxidative biomarker, in comparison with Wt animals, and were the only group in which oxidase glutathione levels were sufficiently elevated to be measured [28].

It is perfectly established that a relationship exists between damaging effects of oxidative stress and apoptosis. Therefore, we examined the enzymatic activity of caspase-3, an executioner of apoptosis activated by the mitochondrial pathway of apoptosis, which is

associated with apoptotic cell-death induced in response to cell damage [29]. Our results showed that TBHP 250 µM and 10 µM of Cd induced apoptosis in SeT of Wt animals as indicated by the enhanced activity of caspase-3 observed in these groups (Figure V.4). This increase was prevented in the Tg-RGN rats, which displayed a significantly lower activity of caspase-3 comparatively with their Wt counterparts. We and other reports have showed that overexpression of regucalcin leads to suppression of apoptosis induced by noxious stimuli in rat testis, kidney and liver [9, 30, 31].

In conclusion, increased activity of GST, enhanced total antioxidant capacity and the suppression of apoptosis observed in the SeT of Tg-RGN support the cytoprotective role of RGN. Moreover, the preliminary results presented herein pointed for the antioxidant properties of RGN, and linked its actions to the protection against oxidative stress during the spermatogenic process, which could be of the uttermost importance to better understand and resolve certain cases of male infertility.

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Chapter VI

Sperm parameters and epididymis function in transgenic rats overexpressing the Ca^{2+}-binding protein regucalcin: a hidden role for Ca^{2+} in sperm maturation?

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Sperm parameters and epididymis function in transgenic rats overexpressing the Ca^{2+}-binding protein regucalcin: a hidden role for Ca^{2+} in sperm maturation?

Abstract

Sperm undergo maturation acquiring progressive motility and the ability to fertilize oocyte through exposure to the components of the epididymal fluid (EF). Although the establishment of a calcium (Ca^{2+}) gradient along the epididymis has been described, its direct effects on epididymal function remain poorly explored. Regucalcin (RGN) is a Ca^{2+}-binding protein, regulating the activity of Ca^{2+}-channels and Ca^{2+}-ATPase, for which a role in male reproductive function has been suggested. This study aimed at comparing the morphology, assessed by histological analysis, and function of epididymis, by analysis of sperm parameters, antioxidant potential and Ca^{2+} fluxes, between transgenic rats overexpressing RGN (Tg-RGN) and their wild-type littermates. Tg-RGN animals displayed an altered morphology of epididymis and lower sperm counts and motility. Tissue incubation with $^{45}Ca^{2+}$ showed also that epididymis of Tg-RGN displayed a diminished rate of Ca^{2+}-influx, indicating unbalanced Ca^{2+} concentrations in the epydymal lumen. Sperm viability and the frequency of normal sperm, determined by the one-step eosin-nigrosin staining technique and Diff-Quik staining method, respectively, were higher in Tg-RGN. Moreover, sperm of Tg-RGN rats showed a diminished incidence of tail defects. Western blot analysis demonstrated the presence of RGN in EF as well as its higher expression in the *corpus* region. The results presented herein demonstrated the importance of maintaining Ca^{2+}-levels in the epididymal lumen and suggest a role for RGN in sperm maturation. Overall, a new insight into the molecular mechanisms driving epididymal sperm maturation was obtained, which could be relevant for development of better approaches in male infertility treatment and contraception.

Key Words: Regucalcin / Epididymis / Sperm / Calcium / Male Infertility

Introduction

Sperm leave the testis as non-functional gametes and it is only during passage through the epididymis that they acquire the ability to move progressively, and to capacitate, eventually gaining the ability to fertilize (reviewed by [1]). This occurs in the unique microenvironment of the epididymal lumen, which is created by the specific secretory and absorptive activities of the epididymis epithelial cells (reviewed by [2]).

The epididymal fluid (EF) is a complex mixture of ions, proteins and other organic molecules (reviewed by [1]). Although the precise role of each component of the EF needs to be deciphered, it has been established that acidification is essential for the alterations on sperm surface proteins required for sperm maturation and storage (reviewed by [3]). Also, water

movement across the epididymis epithelium is crucial for proper sperm function. Sperm capacity for maturation is enhanced by sperm concentration in the epididymal duct, achieved by water removal from the luminal fluid (reviewed by [4]). However, very little is known about the role of calcium ions (Ca^{2+}) in epididymal function, although a decrease of concentrations along the epididymal duct generating a luminal Ca^{2+} gradient has been described [5].

Regucalcin (RGN) is a Ca^{2+}-binding protein [6] playing an important role in the maintenance of Ca^{2+} homeostasis by controlling the activity of Ca^{2+}-channels and Ca^{2+}-ATPase in plasmatic, mitochondrial and endoplasmic reticulum membranes [7-10]. Recently, RGN was identified as an androgen-target gene expressed in several tissues of male reproductive tract, namely, seminal vesicles, prostate, testis and epididymis [11-13]. In addition, it is relevant to mention that an altered expression of RGN was found in the testis of human infertile patients with abnormal phenotypes of spermatogenesis [14] which evidences a role in male reproductive function.

In the present study quantitative and qualitative sperm parameters, as well as the morphology and function of epididymis tubules were compared between transgenic animals overexpressing RGN (Tg-RGN) and their wild-type (Wt) littermates. Our results demonstrate the importance of RGN and Ca^{2+} on epididymis function contributing to detail the molecular mechanisms associated with sperm maturation, which could be a fundamental step to treat male infertility and develop new targets for male contraception.

Materials and Methods

Animals and Tissue Collection

Three-months old Wt and Tg-RGN Sprague Dawley (*Rattus norvegicus*) rats were obtained, respectively, from Charles River (Barcelona, Spain) and Japan SLC (Hamamatsu, Japan). Animals were handled in compliance with the NIH guidelines and the European Union rules for the care and handling of laboratory animals (Directive number 86\609\EEC). They were housed under a 12 h light:12 h darkness cycle, with food and water available *ad libitum* during the course of the experiment, and all rats were euthanized under anesthesia (Clorketam 1000, Vetoquinol, Lure, France).

Epididymides from Wt and Tg-RGN animals (n=8, from each group) were removed and dissected free from fat. One epididymis from each animal was subdivided into three segments, i.e., *caput*, *corpus*, and *cauda*. *Cauda* segments were used for the determination of sperm parameters while *caput* regions were fixed in 4% paraformaldehyde and embedded in paraffin for histological analysis. The contralateral epididymides of both Wt and Tg-RGN animals were immediately frozen on liquid nitrogen and stored at -80ºC until protein extraction. Five additional Wt animals were included and epididymides collected as follows. One epididymis was subdivided in *caput*, *corpus*, and *cauda* regions which were immediately frozen on liquid nitrogen and stored at -80ºC until protein extraction. Segments eight or nine

of the contralateral epididymis [15] together with the initial portion of vas deferent were dissected for collection of epididymal fluid (EF). Likewise, epididymides from Wt and Tg-RGN rats (n=5, from each group) were dissected free from fat, weighted, cut longitudinally in two halves and placed in ice-cold phosphate buffer saline (PBS) and used for $^{45}Ca^{2+}$ influx and efflux assays.

Epididymal Sperm Count and Sperm Motility

The epididymis *cauda* was removed and a sperm suspension was prepared by mincing the tissue with a scissor in 3 ml of Hank´s Buffered Salt Solution (HBSS) at 37°C. The suspension was filtered to remove tissue fragments and incubated for 5 minutes at 37°C. An aliquot of sperm suspension was diluted 1:100 with HBSS, and introduced into a Neubauer´s counting chamber (Tiefe Depth Profondeur, Optik Labor, Switzerland) for sperm counting. Sperm motility was determined by placing a drop of 100 µl of the sperm suspension in a 37°C pre-warmed slide and covered with a cover slip. At least 10 fields were assessed for each semen sample using a bright-field microscope with closed diaphragm (Primo Star, Zeiss, Germany) and the percentage of motile sperm was calculated. Alternatively, the effect of increasing Ca^{2+} concentrations on in vitro sperm motility was analysed comparing the percentage of motile sperm in standard HBSS medium or in HBSS containing 0.2 or 2 mM Ca^{2+}. The reported physiological Ca^{2+} concentration in EF is approximately 0.2 mM [16].

Sperm Viability and Morphology Analysis

Sperm viability was assessed by using the one-step eosin-nigrosin staining technique [17]. A sample of 10 µl of sperm suspension was mixed with 10 µl of 0.5% eosin/nigrosin stain and placed on a pre-warmed slide. Morphology was evaluated using the Diff-Quik staining kit (Baxter Dale Diagnostic AG,Dubinger, Switzerland) using standard protocols [18]. The smears were done using 10 µl of sperm suspension dragged with a cover slip and allowed to dry on air. The slide was immersed in each solution of the staining kit for at least 1 min and dipped rapidly in water, air dried and observed in a bright-field microscope (Primo Star, Zeiss). The sperm was classified as normal or abnormal, and abnormalities divided in head, neck/midpiece or tail defects. Some abnormalities were exclusive but some sperm showed more than one type of defect. In this case, if one of the defects was on the head, it accounted exclusively for number of head-defects. Sperm viability and morphology were assessed for a total of 333 sperm in each semen sample.

Measurement of Epithelial Cell Height and Lumen Diameter of Epididymal Tubules

Paraffin sections (5µm) of the *caput* region were stained with hematoxylin and eosin. The epithelial cell height, and epididymal tubules diameter, area, and perimeter were measured using the AxioVision v4.8.2 software and the Axio Imager A1 microscope with an AxioCam MRc (Zeiss). Measurements were performed in at least 20 epididymis tubules per animal. Epithelial

cell height was measured from the basement membrane to the surface of epithelium considering always the highest thickness. For diameter calculation, two perpendicular measurements were made in each tubule.

Immunohistochemistry

Parafin sections (5 µm) of *caput* region were deparaffinized in xylene and rehydrated in graded alcohols. After heat-induced antigen retrieval, endogenous peroxidase was blocked by incubating samples in 3% (v/v) H_2O_2 (Panreac, Barcelona, Spain) for 10 min at room temperature (RT). Unspecific staining was blocked by incubation with PBS containing 1% (w/v) BSA (PBA) and 0.3 M glycine (Fisher Scientific, New Jersey, USA) for 60 min at RT. Sections were incubated overnight at 4 °C with rabbit anti-V-ATPase A1 (H-140) (sc-28801, Santa Cruz Biotechnology, California, USA) or rabbit anti-aquaporin 9 (AQP9, Alpha Diagnostic International, San Antonio, USA) primary antibodies diluted 1:50 in PBA. Sections were then incubated with secondary goat anti-rabbit biotinylated antibody (B6648, Sigma) diluted 1:20 in PBA for 60 min at RT, followed by incubation with ExtrAvidin Peroxidase (E2886, Sigma) diluted 1:400 in PBA. Immunological reaction was detected using HRP substrate solution (Dako, Glostrup, Denmark). Sections were slightly counterstained with Harris' hematoxylin (Merck, Darmstadt, Germany), dehydrated, cleared, and mounted. Specificity of the staining was assessed by the omission of primary antibody.

EF Collection

EF was collected following a protocol previously described [19]. Isolated epididymal segments were kept on a petri dish cooled on ice to avoid freezing until use, in order to preserve proteins integrity. A -30G1/2" needle (Sterican, B|Braun, Melsungen, Germany) attached to a syringe was inserted into the end of the vas deferens and the content of the *cauda* epididymis was displaced in a retrograde perfusion by gentle pressing with 1 ml of PBS, pH 7.4. The fluid obtained was centrifuged at 30.000g for 60 min at 4°C to completely remove spermatozoa and debris. The supernatant was concentrated by centrifugation at 3.000g using a Centricon 10 YM concentrator (cut-off 10.000 Da, Vivaspin, Sartorius stedium biotech, Goettingen, Germany) and the EF stored at -20°C until use.

$^{45}Ca^{2+}$ influx and efflux experiments

Dissected epididymides were pre-incubated in Krebs Ringer-bicarbonate (KRb) buffer (122 mM NaCl; 3 mM KCl; 1.2 mM $MgSO_4$; 1.3 mM $CaCl_2$; 0.4 mM KH_2PO_4; 25 mM $NaHCO_3$; pH=7.4) for 20 min at 4°C. Tissues were transferred to fresh KRb at 34°C and allowed to stand for another 20 min. For extracellular $^{45}Ca^{2+}$-uptake studies, tissues were incubated at 34°C in KRb containing $^{45}Ca^{2+}$ ($^{45}CaCl_2$; 0.1µCi/mL, PerkinElmer, Waltham, USA) for 2.5, 7.5 and 15 minutes. After the incubation period tissues were washed twice in a solution (pH=7.4) containing 127.5 mM NaCl, 4.6 mM KCl, 1.2 mM $MgSO_4$, 10 mM HEPES, 11 mM glucose, and 10 mM $LaCl_3$. After washing,

tissues were incubated for 20 min in the same solution to prevent Ca^{2+} release from the tissue while removing Ca^{2+} adhering to the exterior. Tissues were then digested with 2 volumes (w/v) of 70% HNO_3 and neutralized with an equivalent volume of 2M NaOH solution. Two 100 µl replicates of medium were collected for determination of radioactive decay. Tissue efflux assays were performed by incubating the tissues in $^{45}Ca^{2+}$ -containing medium as described above for 60 minutes. The tissues were briefly rinsed in fresh KRb without $^{45}Ca^{2+}$ and placed in a microwell plate containing 2 ml of fresh KRb. Two replicates of 100 µl were collected at different time points, and the volume immediately replaced with new KRb. For both influx and efflux assays 3 ml of scintillation liquid were added to each digest/medium replicate and the decay was counted in a Perkin Elmer 1450 Wallac MicroBeta Trilux Liquid Scintilation Counter (American Laboratory Trading, East Lyme, USA) for 3 minutes. In both assays incubations were carried out under an atmosphere of O_2:CO_2 (95:5;v/v). Preliminary assays were performed to determine the incubation time required to equilibrate intra- and extracellular $^{45}Ca^{2+}$ levels.

Western Blot (WB)

Total proteins were isolated from whole epididymis and from *caput*, *corpus* and *cauda* epididymal regions using RIPA buffer supplemented with a cocktail of protease inhibitors (Sigma-Aldrich, St.Louis, USA). Protein concentration in tissue extracts and EF was determined by the Bradford assay (Biorad, Hercules, USA). Proteins were resolved by SDS-PAGE on 12,5% polyacrylamide gels and electrotransferred to a PVDF membrane (GE Healthcare, Buckinghamshire, UK). Membranes with whole epididymis protein samples were incubated overnight at 4ºC with rabbit anti-Na^+/H^+ exchanger (NHE3, 1:1000, sc-28757, Santa Cruz Biotechnology, California, USA) or mouse anti-aquaporin 1 (AQP1, 1:200, sc-25287, Santa Cruz Biotechnology) primary antibodies. Membranes with protein samples from *caput*, *corpus* and *cauda* regions of epididymis, and EF were incubated with rabbit anti-RGN (1:1000, SML-ROI001-EX, COSMOBIO CO., LTD.,Tokyo, Japan) primary antibody. A mouse anti-α-tubulin monoclonal antibody (1:5000, T9026, Sigma) was used for protein loading control. Goat anti-rabbit IgG-AP (1:5000, NIF1317, GE Healthcare) or goat anti-mouse IgG+IgM-AP (1:5000, NIF1316, GE Healthcare) were used as secondary antibodies. Membranes were developed with ECF substrate (GE Healthcare) for 5 minutes and scanned with Molecular Imager FX Pro plus MultiImager (Biorad). Band densities were obtained according to standard methods using the Quantity One Software (Biorad) and normalized by division with the respective α-tubulin band density.

Ferric Reducing Antioxidant Power (FRAP) assay

Whole epididymides were dissected, weighted and total proteins extracted using PBS. Protein concentration was determined by the Bradford method (Biorad) and the ferric reducing antioxidant power (FRAP) assay was performed following a protocol previously described [20].

Ferric (III) to ferrous (II) ion reduction at acidic pH causes a colored ferrous-tripyridyltriazine complex to form, which can be monitored spectrophotometrically indicating the potential antioxidant activity of a tissue. In brief, working FRAP reagent was prepared by mixing acetate buffer (300 mM, pH 3.6), 2,4,6-Tripyridyl-s-Triazine (TPTZ, 10 mM in 40 mM HCl) and $FeCl_3$ (20 mM) in a 10:1:1 (v:v:v) proportion. 6 µl of each sample were mixed with 180 µl of FRAP reagent and absorbances (593 nm) were measured immediately, and 60 minutes later using an Anthos 2010 microplate reader (Biochrom Cambridge, England). Sample antioxidant potential was determined against standards of ascorbic acid which were handled following the same procedures. The changes in absorbance values of tested reaction mixtures were used to calculate the FRAP value of epididymal tissues (µM/mg tissue).

Statistical Analysis

Statistical significance of differences in sperm parameters, epithelial cell height and lumen diameter of *caput* epididymal tubules, FRAP value and protein expression levels between Wt and Tg-RGN rats was evaluated by unpaired t-test with Welch´s correction. Differences in sperm motility after incubation with different Ca^{2+} concentrations and RGN expression in the different epididymal regions were evaluated by one-way ANOVA followed by Bonferroni's multiple comparison test. Nonlinear regression analyses were performed to fit Wt and Tg-RGN Ca^{2+}-uptake and release curves, using GraphPad Prism v5.00 (GraphPad Software, San Diego, CA, USA). Statistically significant differences were considered for $P<0.05$.

Results

Epididymal Sperm Counts, Motility and Viability

Epididymal sperm counts (Figure VI.1A) were significant lower in Tg-RGN rats compared with their Wt littermates ($1.28\times10^8 \pm 9.24\times10^6$ *vs*. $1.72\times10^8 \pm 1.57\times10^7$, $P<0.05$). Tg-RGN rats also displayed a statistical significant lower percentage of motile sperm (Figure VI.1B; 47.88% ± 3.67 *vs*. 64.60% ± 5.66, $P<0.05$). In contrast, the percentage of viable sperm in the Tg-RGN rats was significantly higher than that of the Wt group (Figure VI.1C; 38.75% ± 2.36 vs. 28.00% ± 3.84, $P<0.05$).

In addition, the effect of increased Ca^{2+} levels on sperm motility was tested *in vitro* by incubating sperm isolated from epididymis *cauda* in physiological (0.2 mM, [16]) and supra-physiological (2 mM) Ca^{2+} concentrations (Figure VI.1D). As expected, sperm motility in the 0.2 mM physiological concentration was identical to that observed with standard HBSS, but exposure of sperm to ten-times higher Ca^{2+} concentrations led to a reduction in motility by 22%.

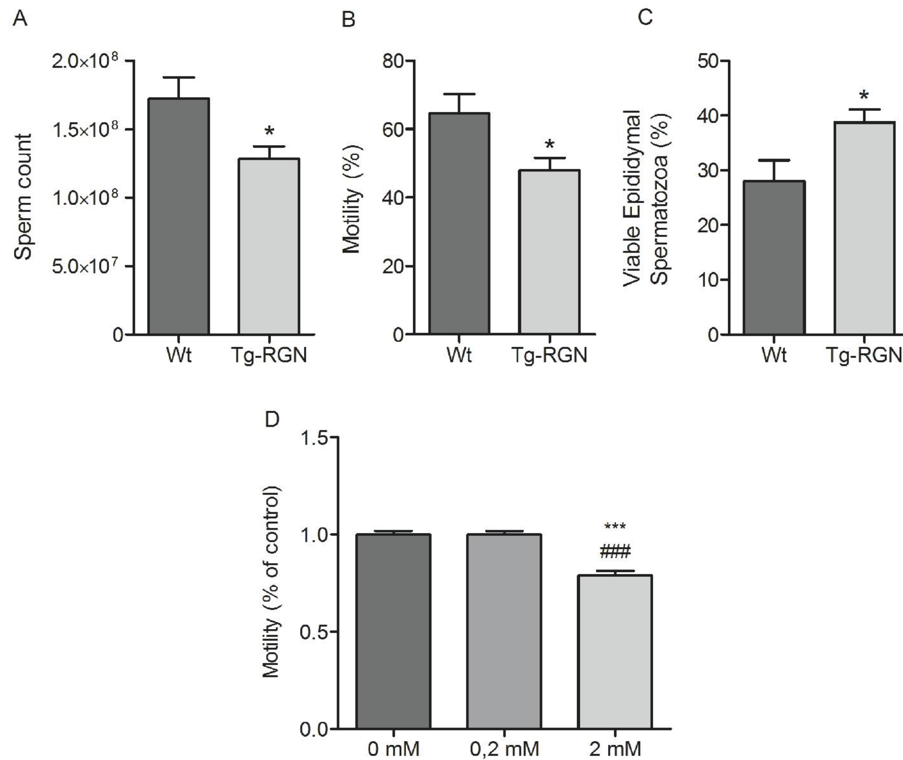

Figure VI.1. Epididymal sperm counts (A), motility (B) and viability (C) in Tg-RGN rats *vs* Wt; * *P<0.05* .(D) Sperm motility in standard HBSS medium (0 mM Ca^{2+}) and in HBSS containing Ca^{2+} at concentrations of 0.2 and 2 mM; ****P<0.001* and ###*P<0.001* relatively to 0 and 0.2 mM, respectively. All data are represented as mean ± S.E.M. n≥5 in each group.

Morphology of Epididymal Sperm

A higher percentage of normal spermatozoa (Figure VI.2A) was observed in Tg-RGN animals (74.13% ± 3.74 *vs*. 57.58 % ± 1.76 in Wt animals, P<*0.01*). Moreover, spermatozoa of Tg-RGN rats showed a diminished incidence of tail defects (Figure VI.2B) relatively to Wt animals (18.60 % ± 2.60 *vs*. 36.18 % ± 2.04, P<*0.001*). No statistical significant differences were found in head and neck defects between the two groups (Figure VI.2B).

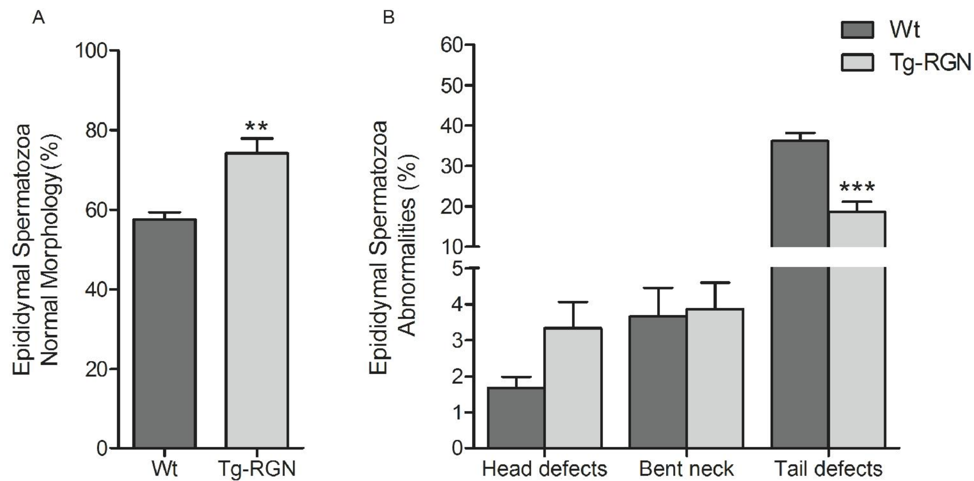

Figure VI.2. Normal (A) and abnormal (B) morphology of epididymal sperm in Tg-RGN rats *vs* Wt. Data are represented as mean ± S.E.M. n≥5 in each group. ***P<0.01* and ****P<0.001*, both relative to corresponding Wt group.

Morphology of *Caput* Epididymal Tubules

The epididymal *caput* tubule area, boundwidth, boundheight, and perimeter in Wt and Tg-RGN animals are presented in Table VI.I. Overall, measurements did not differ between Tg-RGN and Wt groups, except for the epithelial cell height (Figure VI.3A), which significantly decreased from 28.35μm ± 2.13 in Wt to 19.22 μm ± 0.76 in Tg-RGN animals (P<0.01).

The epididymis epithelium is composed predominantly of principal and clear cells, from which the principal cell-type constitutes 80% of epithelium playing a crucial role in its secretory activity [1]. Clear cells are also of the uttermost importance, having endocytic activity and being responsible for clearing proteins from the lumen [21]. The morphology of epididymal cells in Tg-RGN and Wt animals was investigated by immunohistochemistry analysis using anti-V-ATPase and anti-AQP9 antibodies, which, respectively, stain clear and principal cells [4]. Intense staining for V-ATPase was seen in plasma membrane of clear cells in Tg-RGN as well as in their Wt counterparts (Figure VI.3B, upper panels). As reported by others [4, 22] labeling for AQP9 was detected along the microvillus border of the principal cells, which was common to both animal groups (Figure VI.3B, lower panels). Immunolocalization of V-ATPase and AQP9 (Figure VI.3B) showed no difference on shape and proportion of clear and principal cells between Tg-RGN and Wt animals.

Table VI.1. Epididymal *caput* tubule area (μm^2), boundwidth, boundheight, and perimeter (μm) in Tg-RGN rats vs Wt.

	Wt	Tg-RGN
Area	73832.9 ± 3211.0	74503.6 ± 3926.6
Boundwidth	299.8 ± 5.4	312.9 ± 10.9
Boundheight	336.3 ± 13.0	331.0 ± 8.5
Perimeter	1075.6 ± 23.0	1050.1 ± 29.4

Data are represented as mean ± SEM. *n*=5 in each group.

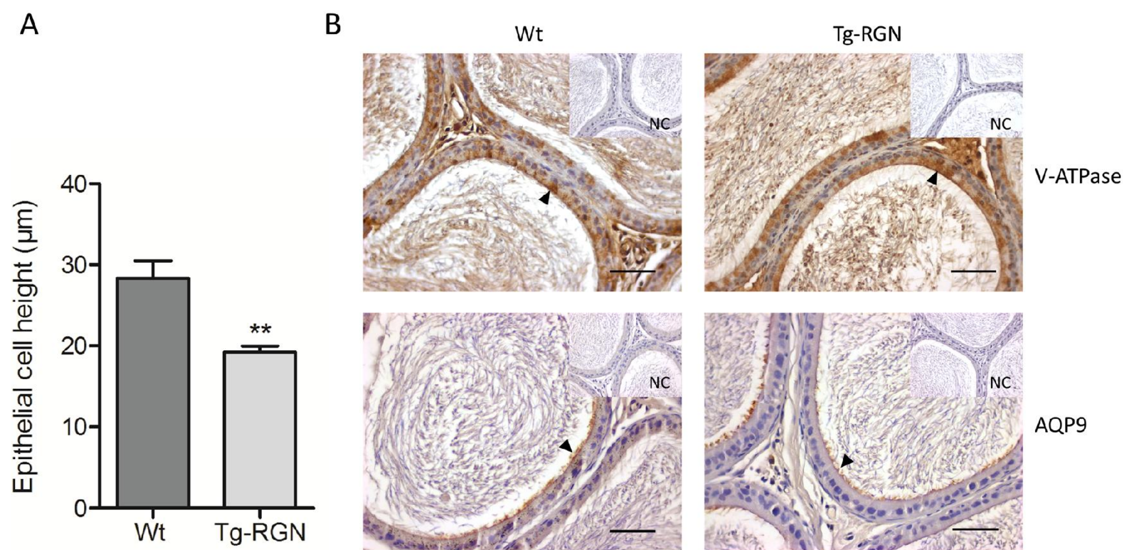

Figure VI.3. Epithelial cell height of the *caput* epididymis (A) and immunolocalization of V-ATPase and AQP9 (B) in Tg-RGN rats vs Wt. (A). Data are represented as mean ± S.E.M. n= 5 in each group. ***P<0.01*. (B). Arrows exemplificate regions of positive immunostaining. Scale bar indicate 50 μm. Inserts in each panel are representative negative controls (NC) obtained by omission of the primary antibody.

Ca^{2+} influx and efflux in the epididymis

The most widely recognized function of RGN in the context of cell physiology is the regulation of plasma membrane Ca^{2+} pumping (reviewed by [23]). Therefore, we measured the influxes and effluxes of Ca^{2+} in the epididymis of Tg-RGN and Wt animals by means of tissue incubation with $^{45}Ca^{2+}$ and radioactivity counting. Calcium uptake rates were quite fast in this tissue and the time-course curve for epididymis Ca^{2+} influx was obtained at 2.5, 7.5 and 15 min (Figure VI.4A). Fitted curves for $^{45}Ca^{2+}$ accumulation were significantly different (*P<0.05*), showing a lower rate of Ca^{2+} influx in Tg-RGN compared to Wt animals. However, $^{45}Ca^{2+}$ release rates were not significantly different between the two experimental groups (Figure VI.4B).

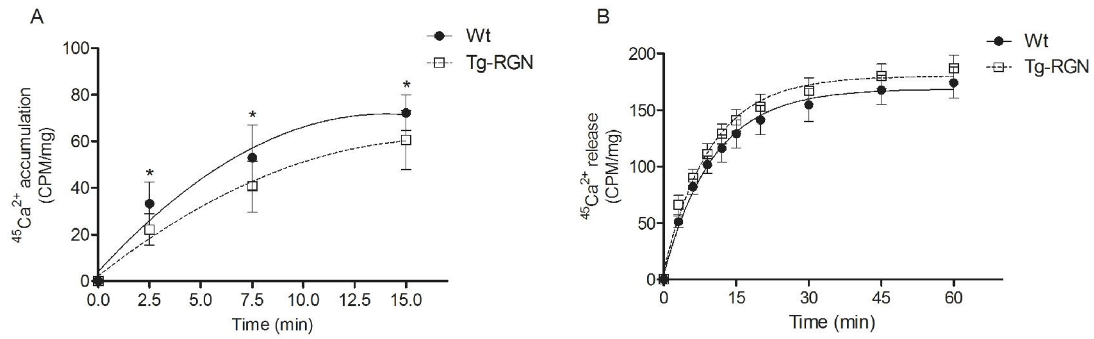

Figure VI.4. Time-course of Ca^{2+} influx (A) and efflux (B) in the epididymis of Tg-RGN rats vs Wt. Data are represented as mean ± S.E.M. n=5 for both groups, at each time point.*$P<0.05$.

Expression of Na^+/H^+ exchanger (NHE3) and water channel (AQP1)

The process of sperm maturation depends on the absorptive and secretory activities of the epididymal epithelium. Acidification of the epididymal fluid by the activity of Na^+/H^+ exchangers, particularly the NHE3 isoform, has been considered of the uttermost importance in this process [3, 24]. Also, water reabsorption along male excurrent ducts is needed to significantly increase sperm concentration, being a fundamental step for the establishment of male fertility [4]. The water transport process is controlled by aquaporins, and AQP-1 seems to be the isoform associated with epididymal function [22, 25]. Thus, we decided also to analyze the expression of NHE3 and AQP1 in the epididymis of Wt and Tg-RGN animals. No significant differences were observed in protein levels of both NHE3 and AQP1 (Figure VI.5) between experimental groups.

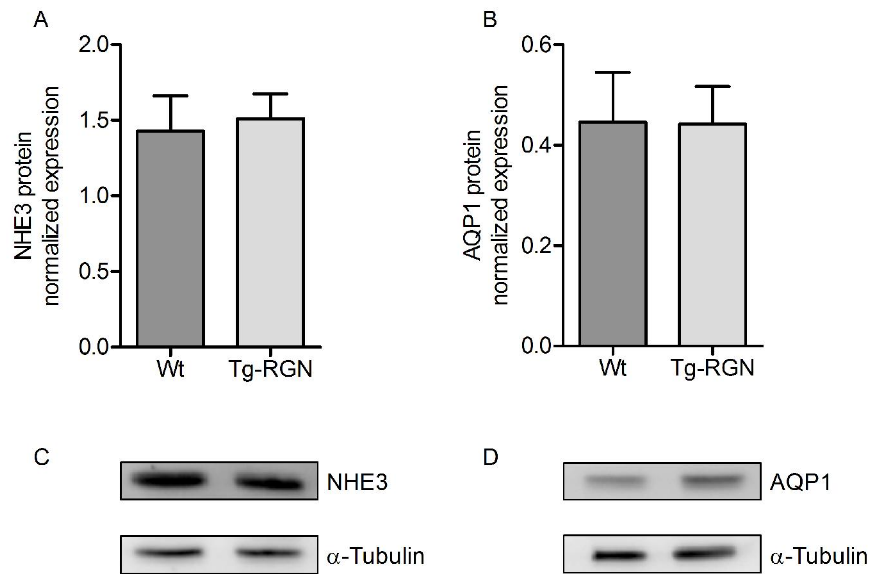

Figure VI.5. Expression of NHE3 (A) and AQP1 (B) in the epididymis of Tg-RGN rats *vs* Wt. Data are represented as mean ± S.E.M. after normalization with α-tubulin. n≥ 5 in each group. Representative images of immunoblots for NHE3, AQP1 and α-tubulin are provided in (C) and (D).

RGN expression in the distinct regions of epididymis and EF

A differential expression of RGN protein was observed in the distinct morphofunctional regions of epididymis with the highest levels detected in the *corpus* (Figure VI.6A and VI.6B). An approximately two-fold statistically significant higher expression was found in the *corpus* (1.06 ± 0.08) compared to the *caput* (0.53 ± 0.09, $P<0.01$) and *cauda* (0.68 ± 0.07, $P<0.05$) regions.

Since RGN has been described as a secreted protein detected in several biological fluids such as pea aphid saliva [26], murine [27, 28], rat [29, 30], and human plasma [28], and rat seminiferous tubule fluid [11] we decided to investigate its presence in EF. An immunoreactive band of the expected size was detected in rat EF (Figure VI.6B).

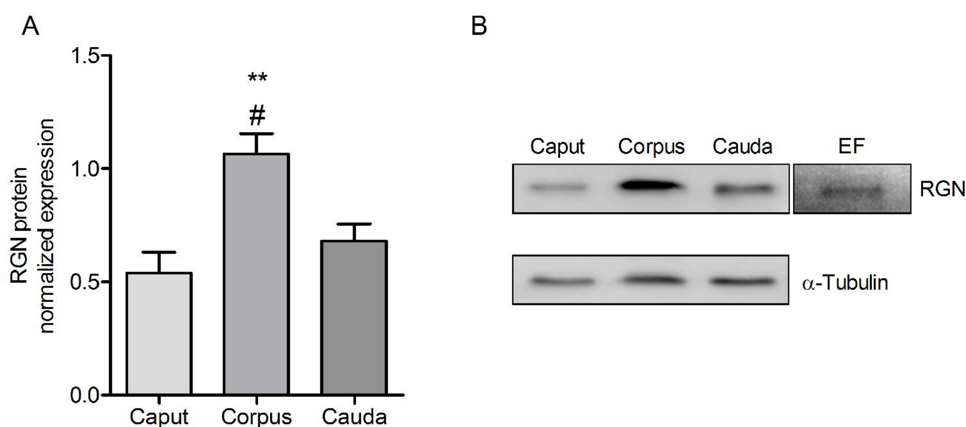

Figure VI.6. Expression of RGN in *caput*, *corpus* and *cauda* regions of rat epididymis and EF (A). Data are represented as mean ± S.E.M. after normalization with α-tubulin. n≥4 in each group. ** *P<0.01* when compared with *caput*; # *P<0.05* when compared with *cauda*. Representative images of immunoblots for RGN and α-tubulin are provided in (B).

Antioxidant potential of the epididymis

Among other roles, it has been suggested that epididymal cells act to protect sperm against oxidative stress [31], which crosses with the antioxidant properties described for RGN [32]. Measurement of the antioxidant potential of epididymal tissues by means of the FRAP assay (Figure VI.7), revealed that a statistically significant higher FRAP value was found in Tg-RGN relatively to Wt animals (*P<0.001*).

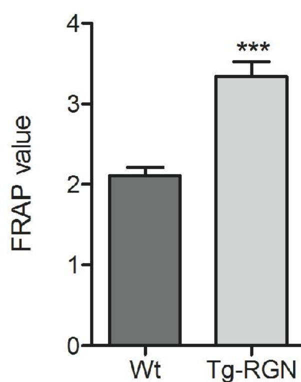

Figure VI.7. FRAP value (μM antioxidant potential/mg tissue) in the epididymis of Tg-RGN rats *vs* Wt. Data are represented as mean ± S.E.M. n≥4 in each group.****P<0.001*.

Discussion

Recent reports have demonstrated the expression of RGN protein in tissues of male reproductive tract, including epididymis, and suggested the importance of this protein in mammalian reproduction [11, 14]. In the present study, we showed significant differences in epididymal epithelium and sperm parameters between Tg-RGN rats and their Wt counterparts implicating RGN in the process of sperm maturation. Although Tg-RGN rats are able to conceive and have been described as fertile animals [33], this is the first time that their sperm parameters are characterized. The lower sperm counts and diminished sperm motility found in Tg-RGN (Figure VI.1A and VI.1B) may be indicative of a subfertility phenotype. However, this seems to be at least partly compensated by a higher viability (Figure VI.1C) and higher percentage of normal morphology (Figure VI.2A) of the sperm in these animals.

The epididymis is a highly compartmentalized organ with distinct regions (*caput*, *corpus* and *cauda*) sustaining different functions which together enable the sperm to acquire their fertilizing ability. The *caput* and *corpus* regions perform early and late sperm maturation events, respectively, while the *cauda* stores the functionally mature spermatozoa [34]. This regional restriction is characteristically evident both in the number and quantity of proteins secreted with the *caput* as the most active, while the *corpus* and *cauda* have a lower secretory activity [35]. Thus, we looked at epididymal tubules morphology in the *caput* region. Although there were no differences in tubule area, boundwidth, boundheight, and perimeter (Table VI.I) between Wt and Tg-RGN groups, the epithelial cell height significantly decreased in Tg-RGN animals (Figure VI.3A). This suggests that epididymal reabsorptive/secretory activities may be altered in Tg-RGN rats leading to changes in fluid composition. Nevertheless, no differences were observed on the morphology of principal and clear cells between the distinct experimental animal groups (Figure VI.3B), as evident by the immunohistochemistry analysis showing V-ATPase and AQP9 localization.

The epididymal lumen is rich in inorganic ions and organic molecules that create the appropriate ionic, oxidative and pH environment for sperm maturation throughout epididymis transit (reviewed by [1]). Acidification of EF and water transport along epididymis are the critical events assuring this integral and proper environment. While acidification is implicated in sperm maturation and maintenance of its quiescent state during storage (reviewed by [3]), water transport contributes to sperm concentration (reviewed by [4]). Ca^{2+} concentrations in the EF are quite low in comparison with those of other ions such as sodium, potassium, chloride, ammonium, and magnesium [36] and probably for this reason there are few studies aiming to disclose the involvement of Ca^{2+} to render sperm released from the testis functional gametes due to the modifications that occur through transit in the epididymis. The main role of Ca^{2+} in sperm functionality has been associated with the capacitation process that occurs in the female reproductive tract [37] and depends of the progressive activation of a cAMP-PKA-dependent signalling pathway mediating protein tyrosine phosphorylation [38]. It is during epididymal maturation that sperm acquire the ability to respond to high levels of intracellular

cAMP leading to tyrosine residues phosphorylation [39], a process negatively regulated by Ca^{2+}. This suggests that control mechanisms of intracellular Ca^{2+} concentration, which ultimately depend on Ca^{2+} concentrations in the epididymal lumen, are critical for sperm maturation [40].

RGN regulates Ca^{2+}-transport in several cell types by controlling the activity of Ca^{2+} channels, transporters and pumps (reviewed by [41]). In the present study, Tg-RGN rats had a reduced rate of Ca^{2+}-influx (Figure VI.4A) by epididymal tissues. This finding is supported by studies describing the role of RGN enhancing Ca^{2+}-ATPase activity [8, 10] and suppressing the expression of L-type Ca^{2+} channel and calcium-sensing receptor mRNA [42] in rat kidney and liver cells, thus, suggesting that an increase in epididymal luminal Ca^{2+} concentration can be occurring. Since no differences were found on the expression of NHE3 and AQP1 proteins (Figure VI.5) between Tg-RGN and Wt animals, which indicates the maintenance of proper acidification and water reabsorption in the epydidimal fluid, it is highly predictable that the decreased sperm motility observed in Tg-RGN (Figure VI.1B) may be due to higher luminal Ca^{2+} concentrations. This is strongly supported by the finding that motility decreases when sperm are exposed to Ca^{2+} concentrations ten-times higher than those commonly found in the EF (Figure VI.1D). Although the mechanisms associated with the diminished sperm motility in consequence of increased Ca^{2+} concentrations remain to be clarified, the importance of maintaining low concentrations of this ion in EF in order to achieve sperm function was also demonstrated in mice knockout for TRPV6 Ca^{2+} channels [16, 43]. Those animals displayed a pronounced decrease of Ca^{2+} absorption through the epididymal epithelium which led to 10 times higher Ca^{2+} concentrations in the EF compared to their Wt counterparts and despite intact spermatogenesis, they had severely diminished fertility as a result of decreased sperm motility and viability [16, 43]. Although the impaired motility is consistent with our results, in Tg-RGN rats we observed a higher viability of *cauda* sperm, and a higher percentage of sperm with normal morphology.

It has been established that oxidative stress (OS) is one of main causes of defective sperm morphology and function [44], and it was suggested that the epididymal epithelium must be able to protect sperm against oxidative damage [31]. Interestingly, besides the control of Ca^{2+} homeostasis, another function that has been assigned to RGN is the protection against OS, by increasing the activity of antioxidant superoxide dismutase enzyme [45] and decreasing the generation of reactive oxygen species [46]. This antioxidant role is demonstrated in RGN knockout mice which display higher levels of OS than their Wt counterparts [32]. Accordingly, a higher antioxidant potential was found in the epididymis of Tg-RGN rats (Figure VI.7). Therefore, it is appropriate to assume that the higher sperm viability (Figure VI.1C), the higher percentage of normal morphology (Figure VI.2A) together with the diminished incidence of tail defects (Figure VI. 2B) observed in Tg-RGN animals, may be a consequence of lower levels of OS in the epididymis epithelial cells.

Sperm maturation depends on a set of proteins synthesized and secreted by epididymis epithelial cells. Thus, the identification of genes expressed in a region-specific manner along

the epididymis is relevant to understand the functional differences between regions, which are crucial for sperm maturation [31, 47]. Furthermore, analysis of genes that changed the expression at least two-fold between adjacent segments is a way to emphasize their relative importance in distinct regions [31]. Indeed, RGN expression was two-fold higher in the *corpus* relatively to *caput* and *cauda* regions (Figure VI.6A). Other oxidative stress- [31] and Ca^{2+}-related genes [48] have also been identified in the epididymal transcriptome showing marked differences in the levels of expression along epididymis regions, which highlights the importance of these biological processes controlling sperm function. Moreover, the presence of RGN in the EF together with the higher antioxidant potential and the pattern of sperm parameters observed in Tg-RGN strongly indicate that it may play a role protecting epididymal sperm from OS. The presence of other proteins with protective roles against OS and DNA damage, such as lactoferrin and glutathione peroxidase, has also been demonstrated in the EF of several mammalian species (reviewed by [2]).

In conclusion, the results presented herein showed that Tg-RGN animals displayed altered morphology of epididymis which suggests important alterations on its secretory/absorptive activity. A diminished influx of Ca^{2+} in epididymal tissues of Tg-RGN rats could be associated with increased concentrations of this cation in EF compromising sperm motility, as indicated by the diminished motility upon exposure of sperm to elevated Ca^{2+} levels. In addition, the higher sperm viability and a diminished incidence of morphological defects exhibited by Tg-RGN animals may be linked to a possible role of RGN protecting sperm from OS.

These findings contributed to a better understanding of the role of Ca^{2+} and RGN in sperm maturation and opened new perspectives to detail the molecular mechanisms underlying epididymal function. This is relevant and could be of the uttermost importance to resolve some male infertility cases associated with sperm maturation disorders, as well as to develop new targets for male contraception.

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Author's roles

S.C. contributed in all tasks related with acquisition, analysis and interpretations of data and wrote the manuscript. P.F.O. collaborated in sperm analysis and critically revised the article. P.M.G. collaborated in calcium fluxes experiments. G.L. collaborated in sperm analysis. M.G.A in Western blot analysis and FRAP assay. A.V.M.C. and J.E.Cavaco critically revised the article. S.S. was responsible for conception and design, written, critical revision and final approval of the version to be published.

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Chapter VII

Summarizing Discussion and Conclusion

Summarizing Discussion

In the mammalian testis, spermatogenesis is the highly coordinated process of germ cell development, which ends with the release of “mature” spermatozoa. The fine regulation of spermatogenesis is strictly dependent on sex steroid hormones, which orchestrate the cellular and molecular events underlying normal development of germ cells. Sex steroids actions also rely on the control of germ cell survival, and the programmed cell death by apoptosis has been indicated as a critical process in regulating the size and quality of the germ line. Over the years, our understanding of the effects of estrogens on spermatogenesis and male fertility has undergone considerable changes. Estrogens have emerged as important regulators of the spermatogenic process with a crucial impact in germ cell fate. In fact, estrogens are traditionally view as hormones that can regulate both proliferation and apoptosis in several tissues, and this duality of action is also evident in the context of testicular cells. Despite all of the existing studies, our understanding of how estrogens regulate germ cell fate was still limited.

RGN is a calcium (Ca^{2+})-binding protein playing an important role in the maintenance of intracellular Ca^{2+} homeostasis, which also has been associated with the control of apoptosis. Previous studies have indicated RGN both as protein expressed in male reproductive tract tissues, and as an estrogen-target gene in rat and human prostate cells. The effect of 17β-estradiol (E_2) controlling RGN expression in cultured seminiferous tubules (SeT) was investigated (Chapter 3). RGN expression increased after E_2 administration, which was coincident with increased apoptosis of germ cells. Considering the role of this protein in rescuing cell from apoptosis induced by noxious stimuli one possible explanation is that the augmented expression of RGN may be a mechanism to counteract E_2-induced apoptosis (Figure VII.1).

Also, the stem cell factor (SCF), present on the membrane of Sertoli cells, has been regarded as an important germ cell survival regulator, acting through the c-kit receptor, which is present on the surface of adjacent germ cells. So, in the present thesis it was described the effect of 100 nM of E_2 on the testicular expression of SCF and c-kit (Chapter 3). This concentration intended to mimic the elevated concentrations of estrogens found in the testis of infertile patients, and it was shown that it induced a decrease in c-kit expression while increasing the expression of SCF. This unbalance of the SCF/c-kit system relied on apoptosis of germ cells as evidenced by the up-regulated expression of FasL/FasR, the increased ratio of proapoptotic/antiapoptotic proteins (Bax/Bcl-2), and the augmented activity of caspase-3 (Figure VII.1). Decreased proliferation was also found in SeT in response to E_2. This was the first study reporting the regulation of SCF/c-kit in response to estrogenic stimuli in testicular cells and the consequent impact in germ cell number.

RGN was also identified as an androgen-target gene in the testis being up-regulated by androgens in this tissue. Considering that androgens are recognized as germ cell survival factors and the association of RGN with apoptotic cell death, this raised the question about

the role of RGN in the control of testicular apoptosis. In Chapter 4 of this thesis the role of RGN in regulating apoptosis in the testis was investigated by using a transgenic rat model overexpressing RGN (Tg-RGN). The obtained results demonstrated that overexpression of RGN suppresses thapsigargin (Thap)- and actinomycin D- induced apoptosis by modulating the expression and activity of key apoptotic and antiapoptotic factors. It is possible that the effect of RGN suppressing Thap-induced apoptosis occurs by preventing the rise of intracellular Ca^{2+} concentrations, which would be expected to happen by inhibition of Ca^{2+}-ATPase in the membrane of endoplasmatic reticulum. In addition, there are studies associating modulation of cytosolic Ca^{2+} with alterations on the expression and ratios of pro and antiapoptotic Bcl proteins, suggesting that the increased Bcl-2 levels in Tg-RGN may also be explained by changes in Ca^{2+} homeostasis (Figure VII.1). It is liable to assume that increasing RGN levels may be a mechanism by which androgenic stimulation sustain germ cell survival and spermatogenesis. This is further supported by recent findings identifying RGN in chicken primordial germ cells and associating its increased expression with the protection of germ cells.

The protective mechanisms for germ cell include keeping them away from oxidative stress, which is widely accepted as damaging for testicular function and represents one of the main factors inducing apoptotic cell death of male germ cells in the testis. It has been shown that E_2 causes enhanced rates of apoptosis in rat testis, with concomitant increase in lipid peroxidation and reduced antioxidant defense, due to decreased enzymatic activity of superoxide dismutase and catalase. Moreover, RGN also has been linked with the decreased generation of reactive oxygen species and increased activity of antioxidant defense systems. In Chapter 5 of this thesis, the role of RGN in the modulation of oxidative stress in the testis was investigated. Tg-RGN animals displayed increased protection against oxidative damage by exhibiting lower levels of oxidative stress and increased antioxidant defenses.

Oxidative stress is also considered as one of the main causes of defective sperm morphology and function, and it was suggested that the epididymal epithelium must be able to protect sperm against oxidative damage. The antioxidant role of RGN in epididymal function was demonstrated by using Tg-RGN animals, which displayed a higher antioxidant potential in the epididymis comparatively with their wild type counterparts (Chapter 6).

The epididymis is a highly compartmentalized organ with different regions, the *caput*, *corpus* and *cauda* sustaining different functions. The *caput* and *corpus* regions perform early and late sperm maturation events, while the storage of functionally mature sperm occurs on the *cauda* region. Interestingly, RGN expression was 2-fold higher in the *corpus* relative to *caput* and *cauda* regions (Chapter 6), emphasizing its importance for sperm maturation. In addition, RGN protein was also identified in epididymal fluid. The role of RGN in the molecular mechanisms underlying sperm maturation also started to be elucidated. Quantitative and qualitative sperm parameters, as well as the morphology and function of epididymis tubules, were compared between Tg-RGN and their Wt littermates. Tg-RGN animals displayed altered morphology of epididymis, which suggested important alterations in its secretory/absorptive

activity. A higher sperm viability and diminished incidence of morphological defects exhibited by these animals may be linked to the referred role of RGN protecting sperm from oxidative stress (Chapter 6). However, the role of Ca^{2+} ion in this process has received little attention to date. The diminished influx of Ca^{2+} observed in the epididymal tissues of Tg-RGN rats could be associated with increased concentrations of this cation in epididymal fluid compromising sperm motility, as indicated by the diminished motility upon exposure of sperm to elevated Ca^{2+} levels (Chapter 6).

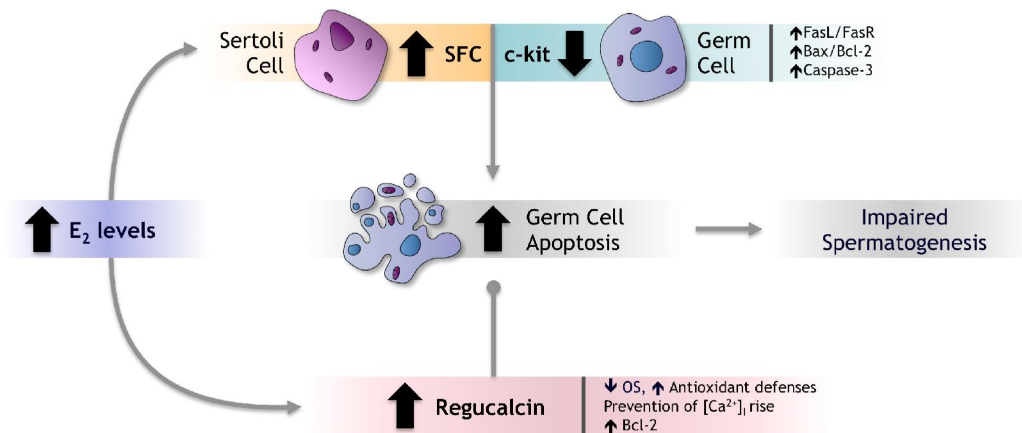

Figure VII.1 Integrative view of the potential actions of estrogens and regucalcin in testicular apoptosis. Elevated concentrations of 17β-estradiol (E_2) enhance the expression of stem cell factor (SCF) on the membrane of Sertoli cells, and decrease the c-kit levels on the surface of adjacent germ cells, leading to increased apoptosis. Germ cell apoptosis occur in consequence of: i) increased expression of the Fas system (FasL/FasR), ii) augmented ratio of Bax (proapoptotic)/Bcl-2 (antiapoptotic) proteins and iii) enhanced activity of caspase-3. E_2 also stimulates the expression of regucalcin, which may be a protective mechanism to counteract germ cell apoptosis. Regucalcin also decreases oxidative stress (OS) by increasing antioxidant defenses, and may also act by preventing the rise of intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) and increasing Bcl-2 levels. Arrows and ball-ended arrows indicate activation and inhibition, respectively.

Conclusion

Considering the dual behavior of estrogens, shifting from “angels to devils” the “Goldilocks” concept seems to be the key. Too much is bad, but too little is also bad – it needs to be just right. A list of candidate genes to be considered in future research aiming at elucidate the role of E_2 in the onset of spermatogenesis have already emerged in literature. However, the identification of the underpinning molecular pathways triggered by estrogens still are in the infancy. Addressing this question would not only help to gain a better understanding of how estrogen signaling contributes to pathophysiological conditions, but also allow for targeted therapeutic strategies to manipulate estrogens-signaling and improve the prognosis of male idiopathic infertility associated with hyperestrogenism. It is now known that elevated concentrations of E_2, mimicking those found in the intratesticular milieu of infertile patients, unbalance the expression of SCF/c-kit system and up-regulate RGN leading to a depletion of germ cells due to augmented apoptosis and decreased proliferation (Figure VII.1).

It has been reported that an increase in the intratesticular concentration of E_2 in infertile men is accompanied by an augmented ratio of estradiol/testosterone and that this hormone deregulation may occur as result of the increased expression and/or activity of the aromatase enzyme. Moreover, the use of aromatase inhibitors for treatment of infertile men has been efficient in cases of high estradiol/testosterone ratios improving both hormone and semen parameters, but its application lacked strong scientific evidence and has been mainly empirical. The present findings provided a rationale to explain the successful use of aromatase inhibitors to treat male infertility and suggested that manipulation of c-kit may be a possible mechanism to preserve germ cell integrity in cases of infertility associated with hyperestrogenism. Moreover, the altered expression of RGN by estrogens in association with its role rescuing cells from induced apoptosis and oxidative stress pointed this protein also as a target. The manipulation of RGN levels in the testis towards its overexpression could be a possible mechanism to preserve fertility, which could have a relevant clinical impact upon treatment of oncological conditions.

The present findings also contributed to a better understanding of the role of RGN and Ca^{2+} in sperm maturation, highlighting for the molecular mechanisms underlying epididymal function. This could be of the uttermost importance resolving certain cases of male infertility associated with sperm maturation and, on the other hand, helping to develop new strategies for male contraception.

Even more, the present thesis opened new challenges for the next future in order to further explore the aetiology of idiopathic male infertility and perhaps testicular cancer. In conclusion, the action of estrogens and RGN on testis physiology and sperm function is “a matter of life and death" that it is far from finished at the present time.