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Regucalcin is broadly expressed in male reproductive tissues and is a new androgen-target gene in mammalian testis

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Abstract

Regucalcin (RGN) is a calcium (Ca^{2+})-binding protein which regulates intracellular Ca^{2+} homeostasis by modulating the activity of enzymes regulating Ca^{2+} concentration and enhancing Ca^{2+}-pumping activity. Several studies have described the pivotal role of proper Ca^{2+} homeostasis regulation to spermatogenesis and male fertility. Recently, *RGN* was identified as a sex steroid-regulated gene in prostate and breast; however, a possible role of RGN in spermatogenesis has not been examined. In this study, the expression and localization of RGN in rat and human testis, and other rat reproductive tissues was analyzed. Moreover, we studied whether RGN protein was present in seminiferous tubule fluid (STF). Finally, we examined the effect of 5α-dihydrotestosterone (DHT) on the expression of *Rgn* mRNA in rat seminiferous tubules (SeT) cultured *ex vivo*. The results presented in this study show that RGN is expressed in Leydig and Sertoli cells, as well as in all types of germ cells of both rat and human testis. RGN is also expressed in rat prostate, epididymis, and seminal vesicles. Moreover, RGN protein is present in rat STF. The results also demonstrate that *Rgn* expression is age dependent in rat testis, and is upregulated by the non-aromatizable androgen DHT in rat SeT cultured *ex vivo*. Taken together, these findings indicate that *Rgn* is a novel androgen-target gene in rat testis and that it may have a role in male reproductive function, particularly in the control of spermatogenesis.

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Introduction

Regucalcin (RGN) was first identified in the late 1970s by the Yamaguchi group in Japan as a calcium (Ca^{2+})-binding protein that does not contain EF-hand motif (Yamaguchi & Yamamoto 1978). Independently, another research group identified and named it senescence marker protein 30, based on its characteristic down-regulated expression with aging in rat liver (Fujita *et al.* 1992). RGN plays an important role in intracellular Ca^{2+} homeostasis by modulating the activity of enzymes regulating Ca^{2+} concentration, and enhancing Ca^{2+}-pumping activity through the plasma membrane, endoplasmic reticulum and mitochondria of several cell types (Yamaguchi 2005). In turn, *Rgn* expression is upregulated by increased Ca^{2+} concentration in liver and kidney cells (Shimokawa & Yamaguchi 1992, 1993, Yamaguchi & Kurota 1995).

Although there are no studies characterizing expression of RGN in testis, several evidences have highlighted the importance of Ca^{2+} to spermatogenesis. Calcium is essential for the maintenance of Sertoli cell (SC) tight junctions forming the blood–testis barrier (Grima *et al.* 1998) and modulates the activity of enzymes interfering in SC architecture (Franchi & Camatini 1985). The tight regulation of Ca^{2+} influx and outflux maintaining intracellular Ca^{2+} homeostasis also seems to be essential for Leydig cells (LC) steroidogenesis, for example by controlling the expression of STAR protein (Manna *et al.* 1999, Pandey *et al.* 2010). Moreover, it has been shown that administration of Ca^{2+} channel blockers has deleterious effects on mammalian spermatogenesis, being associated with reversible infertility (Juneja *et al.* 1990, Benoff *et al.* 1994, Almeida *et al.* 2000, Lee *et al.* 2006, 2011).

Recently, we have identified *Rgn* as a sex steroid-regulated gene in rat reproductive organs such as breast and prostate (Maia *et al.* 2008, 2009). Also, we have shown that 5α-dihydrotestosterone (DHT) treatment

decreases *RGN* expression in human prostate cancer cells (LnCaP; Maia *et al*. (2009)). However, the effects of sex steroids controlling RGN testicular expression are unknown.

The first aim of the present work was to study the expression and cellular localization of RGN in rat and human testis and other male reproductive tissues, such as prostate, epididymis, and seminal vesicles. RGN has been shown to be secreted to biological fluids, namely saliva (Carolan *et al*. 2009) and plasma (Isogai *et al*. 1994*a*, 1994*b*, Lv *et al*. 2007, 2008). Thus, secondly, we investigated whether RGN is present in seminiferous tubule fluid (STF). Our third aim was to determine the effect of DHT on *Rgn* expression in rat seminiferous tubules (SeT) cultured *ex vivo*.

Results

### RGN expression and localization in rat and human cell types of the testis

RGN mRNA and protein expression was analyzed in rat and human whole testis and isolated SC by RT-PCR, *in situ* hybridization (ISH), western blot (WB), immuno-histochemistry (IHC), and/or immunocytochemistry (ICC).

Analysis by RT-PCR demonstrated *RGN* mRNA expression in rat and human testis (Fig. 1A). Through real-time quantitative PCR (qPCR) analysis at different postnatal ages it was shown that *Rgn* mRNA expression in rat testis reaches a maximum at 120 days, and decreases during aging process (Fig. 1C).

The localization of *Rgn* mRNA in rat testis was assessed using a specific digoxigenin-labeled probe (Fig. 1D). We were able to localize *Rgn* hybridization signal in SC and several germ cells, namely spermato-gonia (SG), spermatocytes (ST), and round spermatids (RS). Specificity of ISH staining was evaluated by hybridization with sense probe, which resulted in absence of signal (Fig. 1D insert).

In rat testis RGN protein was localized to LC and SC and also in a variety of germ cells, more specifically SG, ST, RS, elongating spermatids (ES), and spermatozoa (Sz; Fig. 2A and C). IHC in human testicular tissue showed a similar expression pattern for RGN, as observed by the staining of the same cell types as in rat testis (Fig. 2B and D). Although cell localization was essentially cytoplasmic, some nuclear staining is visible particularly in rat sections (Fig. 2A and C). Specificity of the IHC staining was assessed by omission of primary antibody, resulting in complete absence of immunological staining (inserts in corresponding panels, Fig. 2).

The expression of RGN mRNA and protein in SC was confirmed using rat and human primary SC cultures. PCR amplification of SC-specific markers vimentin (*Vim*) and anti-Müllerian hormone (*Amh*; Fig. 1A), and ICC detection of VIM (Fig. 2G and H) were used to confirm the isolation of SC. After 96 h of culture contaminant cells
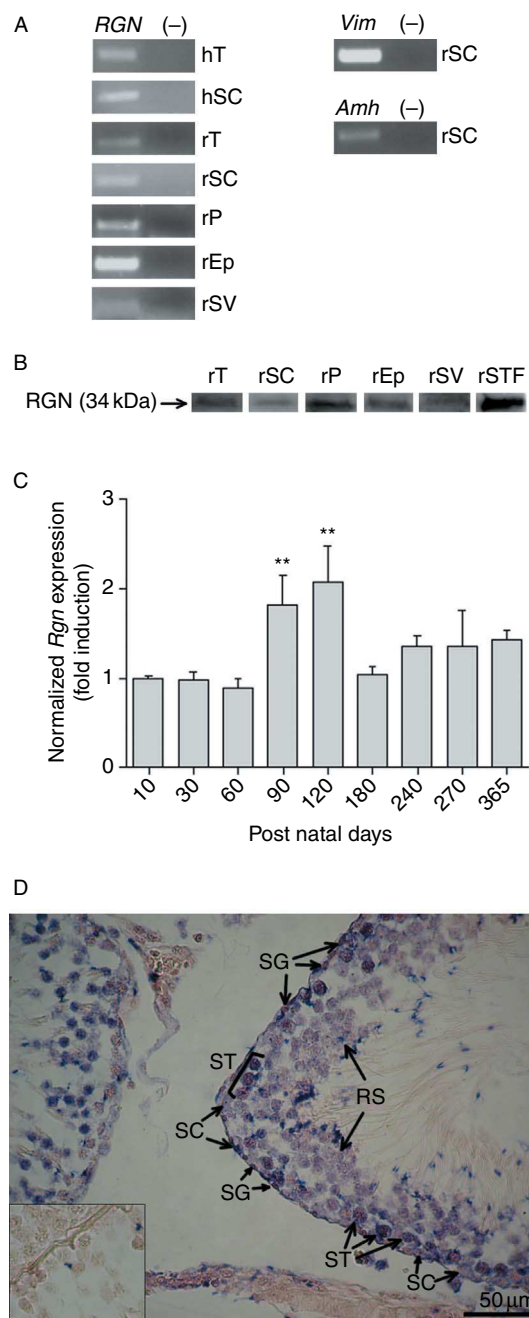
Figure 1 Expression of regucalcin (RGN) in reproductive tract tissues and seminiferous tubule fluid (STF). (A) RT-PCR of RGN, Sertoli cells (SC)-specific markers vimentin (*Vim*), and anti-Müllerian hormone (*Amh*). (−), Negative control (no cDNA template). (B) Western blot detection of RGN in protein extracts. hT, human testis; rT, rat testis; hSC, human Sertoli cells; rSC, rat Sertoli cells; rP, rat prostate; rEp, rat epididymis; rSV, rat seminal vesicles; rSTF, rat seminiferous tubule fluid. (C) Expression of *Rgn* in rat testis at different postnatal ages, determined by quantitative PCR, normalized with cyclophilin A and β2-micro-globulin as internal reference genes. $n \geq 5$ in each group. **$P < 0.005$ relative to ten postnatal days. Error bars represent s.e.m. (D) Localization of *Rgn* mRNA transcript in adult rat testis by *in situ* hybridization using a digoxigenin-labeled antisense probe. Insert – hybridization with sense probe resulting in no staining. SC, Sertoli cell; SG, spermatogonia; ST, spermatocyte; RS, round spermatid. Magnification indicated as scale bar.
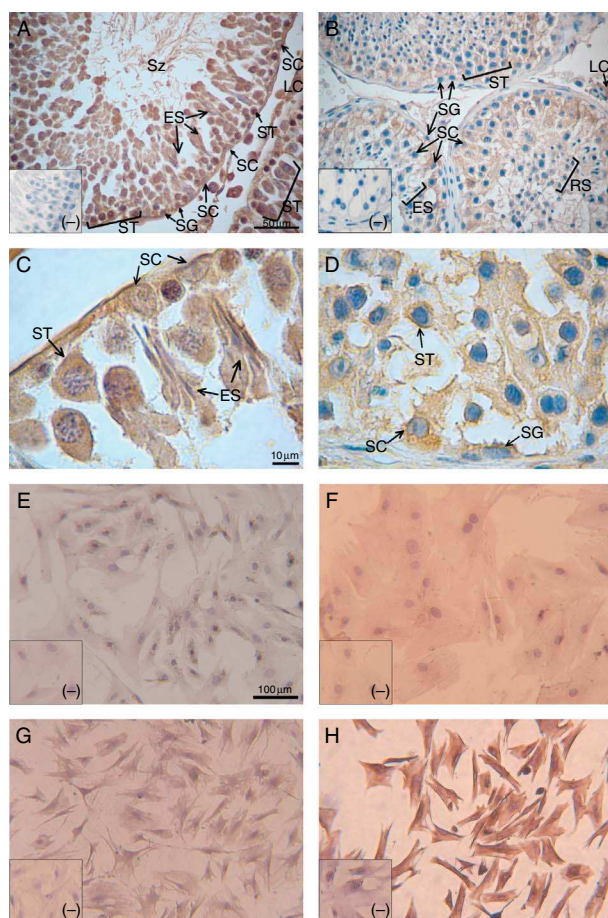
Figure 2 Immunochemical localization of regucalcin in testis paraffin-sections and Sertoli cell (SC) cultures. (A and C) Rat testis, (B and D) human testis, (E) rat SC, and (F) human SC. Purity of the rat and human SC cultures was assessed by staining with anti-vimentin antibody (G and H respectively). (−), Negative control obtained by omission of the primary antibody (inserts in corresponding panels). SC, Sertoli cell; LC, Leydig cell; SG, spermatogonia; ST, spermatocyte; RS, round spermatid; ES, elongated spermatid; Sz, spermatozoa. Magnification indicated as scale bar (B similar to A; D similar to C; F, G, and H similar to E).

were fewer than 5% for both cultures. By RT-PCR, *RGN* mRNA expression was detected in both rat and human SC (Fig. 1A). These cells also expressed RGN protein, which was detected mainly to the cytoplasm (Fig. 2E and F). Specificity of the immunostaining was assessed by the omission of the primary antibody, which resulted in complete absence of immunological staining (inserts in corresponding panels, Fig. 2). The presence of RGN in rat SC was further confirmed by WB analysis (Fig. 1B).

RGN expression in rat reproductive tissues and STF

The expression of RGN in other rat reproductive tissues besides testis was investigated. We detected *Rgn* mRNA expression in rat prostate, epididymis, and seminal vesicles (Fig. 1A). Subsequently, we confirmed the expression of RGN protein in these tissues by detecting

an immunoreactive band of expected size in WB (Fig. 1B). Moreover, we detected the same immunoreactive band in rat STF (Fig. 1B).

The cellular localization of RGN in rat prostate, epididymis, and seminal vesicles was determined by IHC. In all three tissues RGN was localized mainly in the epithelial cells (Fig. 3A–C); in epididymis it was also localized to smooth muscle cells and connective tissue (Fig. 3B). Specificity of staining was assessed by omission of the primary antibody, which resulted in complete absence of staining (inserts in corresponding panels, Fig. 3).

DHT regulation of Rgn expression in rat SeT

The effect of DHT (10^{-7} M) on the expression of *Rgn* mRNA in cultured rat SeT was evaluated by qPCR. First, a time-course experiment was performed showing that DHT induced a sharp increase in the expression levels of *Rgn* at 24 h (4.37 ± 0.64; $P < 0.001$) compared with control (1.00 ± 0.26), while at all other experimental times *Rgn* expression levels remained similar to control group (Fig. 4A). The 24 h experimental time was therefore selected to explore the mechanisms involved in DHT regulation of *Rgn* expression by incubating rat SeT with 10^{-7} M DHT, and with antiandrogen flutamide (Flu) and protein synthesis inhibitor cycloheximide (CHX), alone or in presence of DHT (Fig. 4B). Treatment with DHT produced an increase in *Rgn* mRNA expression (2.20 ± 0.27; $P < 0.01$) compared with the control (1.00 ± 0.08). Administration of Flu neutralized the hormone's effect (1.04 ± 0.06 vs 1.00 ± 0.08 control), while incubation with CHX (2.34 ± 0.15 vs 1.00 ± 0.08 control; $P < 0.001$) did not change the upregulating effect of DHT on *Rgn* expression. Incubation with Flu or CHX alone had no significant effect on the expression of *Rgn*.

### Rgn expression is upregulated in rat SeT cultured in presence of survival factors

Rat SeT were cultured in a hormone-free medium with or without survival factors, and the expression of *Rgn* quantified by qPCR (Fig. 4C). Under survival-promoting conditions there was an upregulation of *Rgn* expression (2.23 ± 0.22 vs 1.00 ± 0.23 without survival factors; $P < 0.005$).

Discussion

Although several studies have highlighted the importance of Ca^{2+} homeostasis and signaling for normal spermatogenic process, a possible role of RGN in testicular physiology had not been explored. In this study, we report the expression and localization of RGN in rat and human testis and the effect of DHT on its expression.

Expression of *RGN* mRNA was detected in rat and human testicular tissue (Fig. 1A) and transcripts were
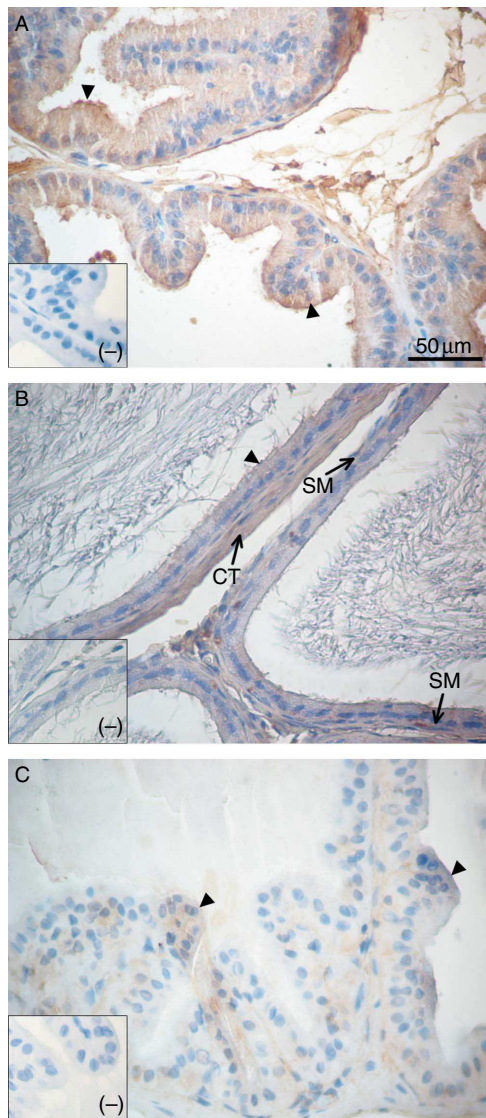
Figure 3 Immunohistochemical localization of regucalcin in rat prostate (A), epididymis (B), and seminal vesicles (C). (−), Negative control obtained by omission of the primary antibody (inserts in corresponding panels). Arrowhead, epithelium; CT, connective tissue; SM, smooth muscle cells. Magnification indicated as scale bar (similar in all panels).

localized both to somatic and germ cells in adult rat testis (Fig. 1D). A developmentally regulated expression pattern, where a peak is reached after which levels decrease with aging, has been described for *Rgn* in rat kidney and liver tissues. In rat kidney the expression of *Rgn* mRNA starts to increase at 21 postnatal days and reaches a peak at 35 days, levels are maintained high until 3 months when it starts to decrease, returning to the low levels observed before 21 days (Fujita *et al.* 1996). In a comparable manner, *Rgn* expression in liver increases until 10-day-old, reaching a plateau that is maintained until 6.5 months, decreasing in senescent rats (Fujita *et al.* 1996). The authors hypothesized that the

age-dependent increase of *Rgn* expression in liver and kidney was coupled with periods of maturation and differentiation for both tissues and suggested it as a senescence marker (Fujita *et al.* 1996). Developmental analysis shows that in rat testis the characteristic downregulation of *Rgn* expression during aging is also observed. The expression of *Rgn* mRNA increases until it reaches a maximum at 120 days of age, a period which corresponds to rat adulthood, decreasing afterwards with rat aging (Fig. 1C).

The results presented in this study demonstrate that RGN protein is broadly expressed in rat (Fig. 2A) and human (Fig. 2B) testis, being localized to all cell types of the SeT epithelium, somatic as well as germ cells. Relative intensity of RGN staining appears to be weaker in human sections, a pattern we have consistently observed when analyzing the localization of other proteins in human and rat testis sections (data not shown). We think that the use of different fixation protocols may be causing this histological artifact. RGN immunostaining is visible in cytoplasm as well as in nucleus, which is in accordance with reports showing that RGN is able to translocate to the nucleus regulating DNA synthesis and gene expression (Inagaki & Yamaguchi 2001, Tsurusaki & Yamaguchi 2004, Maia *et al.* 2009). This is the first report describing RGN expression and localization in testis of any vertebrate.

Tight control of intracellular Ca^{2+} homeostasis has been shown to be of uttermost importance to LC steroidogenesis (Manna *et al.* 1999, Pandey *et al.* 2010) and maintenance of SC function (Franchi & Camatini 1985, Grima *et al.* 1998, Gorczynska-Fjalling 2004). The deleterious effects of Ca^{2+} channel blockers on male fertility emphasize even more the importance of tight Ca^{2+} regulation to spermatogenesis (Juneja *et al.* 1990, Benoff *et al.* 1994, Almeida *et al.* 2000, Lee *et al.* 2006, 2011). The common cellular localization pattern observed in rat and human testis, together with the wide cellular distribution of RGN indicate a relevant role in testicular physiology suggesting that RGN may play a role in spermatogenesis as a Ca^{2+} homeostasis regulator in both somatic and germ cells.

Knowing that RGN was identified as a secreted protein in a pea aphid saliva (Carolan *et al.* 2009), and murine (Lv *et al.* 2007, 2008), rat (Isogai *et al.* 1994a, 1994b), and human plasma (Lv *et al.* 2008) we decided to investigate its presence in STF, which could be confirmed by WB analysis (Fig. 1B). The STF is produced essentially due to the secretory activity of SC (Fisher 2002), which we demonstrated, by several approaches, to express RGN. Therefore, it is highly expected that RGN present in STF may be a secretion product of SC. Exogenous RGN has been shown to translocate to the nucleus being capable of altering gene expression and modulating enzyme activity in osteoblasts (Otomo & Yamaguchi 2006) and liver cells (Omura & Yamaguchi 1999). Nevertheless, to
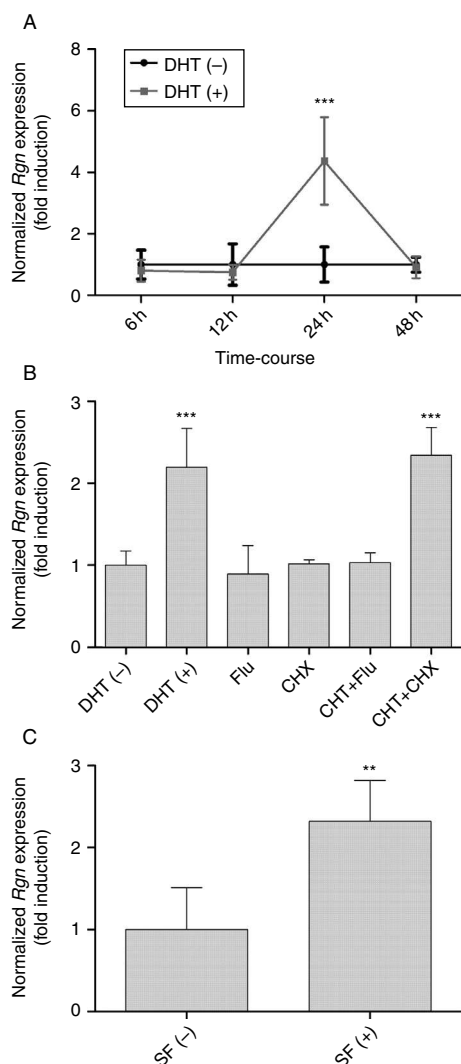
Figure 4 Effect of DHT and survival factors on regucalcin (*Rgn*) expression in rat seminiferous tubules (SeT) cultured *ex vivo* determined by quantitative PCR. *Rgn* expression was normalized with β-actin and *Gapdh* as internal reference genes. $n=5$ in each experimental condition. Error bars represent s.e.m. (A) Time-course experiment in which rat SeT were cultured in the absence [DHT (−)] or presence of 10^{-7} M DHT [DHT (+)] for 6, 12, 24, or 48 h. (B) Rat SeT cultured for 24 h with 10^{-7} M DHT, 10^{-7} M flutamide (Flu), 10 μg/ml cycloheximide (CHX), 10^{-7} M DHT plus 10^{-7} M Flu, and 10^{-7} M DHT plus 10 μg/ml CHX. **$P<0.005$ and ***$P<0.001$ compared with DHT (−). (C) Rat SeT cultured for 24 h in the absence [SF (−)] or presence of [SF (+)] survival factors **$P<0.005$.

this point, the role of SeT secreted RGN remains to be determined.

In addition to testis we also analyzed the expression and localization of RGN in other rat reproductive tissues: prostate, epididymis, and seminal vesicles (Fig. 3). This is the first time RGN expression and immunolocalization have been reported in rat epididymis and seminal vesicles. In seminal vesicles, RGN immunostaining is confined to epithelial cells, while in epididymis it is also present in connective tissue and smooth muscle cells.

In rat prostate RGN protein was detected in epithelial cells, which is in accordance with published results in rat and human prostatic tissue (Maia *et al.* 2008, 2009). RGN has been proposed to have a physiological function in prostate, as its expression is downregulated in prostate cancer tissues, and RGN immunoreactivity correlates with the grade of adenocarcinoma cellular differentiation (Maia *et al.* 2009). However, further studies are required to detail RGN function in these tissues.

Administration of 17β-estradiol (E$_2$) to rats causes an increase in the expression of *Rgn* mRNA in the liver (Yamaguchi & Oishi 1995). The same effect is observed in cultured rat hepatoma cells (Nakajima *et al.* 1999). Also, in osteoblastic cells, incubation with E$_2$ causes an upregulation of *Rgn* expression, while treatment with 1,25-dihydroxyvitamin D$_3$ causes downregulation of *Rgn* expression (Yamaguchi *et al.* 2008). Contrarily, administration of E$_2$ to rats decreases the expression of *Rgn* in renal cortex (Kurota & Yamaguchi 1996). The hormonal regulation of *Rgn* expression has also been described in sex hormone target organs. Our group has described the downregulation of *Rgn* expression in rat prostate and mammary gland by E$_2$ (Maia *et al.* 2008). Moreover, *RGN* is underexpressed in breast and prostate cancer cases and E$_2$ upregulated while DHT downregulated *RGN* mRNA expression in MCF-7 and LNCaP cell lines, respectively (Maia *et al.* 2009). DHT, a non-aromatizable androgen, has been shown to stimulate spermatogenesis in a similar way to testosterone (Singh *et al.* 1995, Meachem *et al.* 2007, O'Shaughnessy *et al.* 2010), therefore it was used to analyze the effect of androgens on *Rgn* expression in rat SeT cultured *ex vivo*. qPCR analysis showed that DHT upregulates *Rgn* mRNA expression in rat cultured SeT at 24 h of exposure (Fig. 4A). DHT upregulation of *Rgn* expression is completely reversed by incubation with antiandrogen Flu, but not with CHX, an inhibitor of protein synthesis (Fig. 4B). These data suggest that the involvement of a classical genomic mechanism of gene expression regulation through androgen receptor, which seems not to depend on *de novo* protein synthesis. *In silico* analysis of the *RGN* promoter region has in fact enabled the identification of androgen response elements upstream from transcription initiation site at positions −906, −915, −4126, and −5822 bp (Maia *et al.* 2009). Nevertheless, androgens are known to increase intracellular [Ca^{2+}] in a wide array of cells, namely SC (Gorczynska & Handelsman 1995), human prostatic stromal cells (Oliver *et al.* 2010), rat thoracic aorta (Montano *et al.* 2008), and human lymphocytes (Popova *et al.* 2007). It is also known that *Rgn* expression is upregulated by increased [Ca^{2+}] (Shimokawa & Yamaguchi 1992, 1993). Therefore, we do not exclude that the DHT-induced rise in *Rgn* expression may be partly due to a secondary increase in intracellular Ca^{2+}.

Maintenance of spermatogenic epithelium homeostasis requires a fine-tuning between germ cell

proliferation and death. Apoptosis is an essential mechanism that enables the elimination of abnormal and exceeding germ cells and therefore its regulation is vital for normal spermatogenesis (Print & Loveland 2000). Up to 75% of germ cells undergo death by apoptosis in testis during the pubertal maturation process (Giampietri et al. 2005). On the other hand, androgens are known to inhibit apoptosis of male germ cells (Erkkila et al. 1997, Pentikainen et al. 2000) and testosterone withdrawal stimulates their apoptosis (Nandi et al. 1999, Tesarik et al. 2002). Germ cell apoptosis induced by androgen deprivation seems to be associated with caspases activation (Vera et al. 2006, Johnson et al. 2008). The role of RGN controlling apoptotic cell death has been established. RGN overexpression inhibits apoptosis induced by several factors, namely tumor necrosis factor-α and thapsigargin, dibucaine, and Bay K (Izumi & Yamaguchi 2004a, 2004b), and it was suggested that it may regulate AKT activity (Matsuyama et al. 2004). In addition, hepatocytes of RGN knockout mice are more susceptible to apoptotic cell death than their wild-type counterparts (Ishigami et al. 2002). We hypothesize that the upregulated expression of Rgn in SeT in response to DHT might be a mechanism by which androgens regulate apoptosis in testis. This is further supported by the observation that culture of SeT under hormone-free conditions although in presence of survival factors induces an upregulation of Rgn expression similar to the one caused by treatment with DHT (Fig. 4C).

In conclusion, we demonstrate that RGN is expressed in rat and human testis and other tissues of male reproductive tract, namely prostate, epididymis, and seminal vesicles. In addition, the presence of RGN in STF was identified. Our results also indicate Rgn as a novel androgen-target gene in rat testis that may have an important role in the control of spermatogenesis. This opens new lines of research to explore the role of RGN and Ca^{2+} homeostasis in male spermatogenic process and fertility.

Materials and Methods

Animals and tissues

Wistar male rats (*Rattus norvegicus*) were obtained from Charles River (Barcelona, Spain) and housed under a 12 h light:12 h darkness cycle, with food and water available *ad libitum* during the course of all experiments. Housing, maintenance and handling of animals was in compliance with the NIH guidelines and the rules for the care and handling of laboratory animals (Directive 86/609/EEC). All rats were killed under anesthesia (Clorketam1000, Vetoquinol, Lure, France).

Human testicular samples with conserved spermatogenesis were obtained by testicular sperm extraction from men undergoing treatment testicular biopsy due to obstructive azoospermia (aged 35–38, with normal karyotypes). Samples were obtained under informed consent according to the local ethics committee guidelines, and treated as described previously (Sousa et al. 2002a).

Reagents

All chemical reagents were purchased from Sigma–Aldrich and all antibodies were obtained from AbCam (Cambridge, UK) unless stated otherwise.

Primary SC culture

Testes were removed from 22-day-old rats and washed in ice-cold Hanks balanced salt solution without Ca^{2+} and Mg^{2+} (HBSSf). Testes were decapsulated and washed in HBSSf. Rat SC were then isolated by a method adapted by Oliveira et al. (2009a). Briefly, decapsulated testicular tissue was placed in a glycine-containing medium (HBSSf, 1 M glycine (Merck), 0.005% (w/v) DNase, 2 mM EDTA and 0.002% (w/v) soybean trypsin inhibitor, pH 7.2) for 10 min at room temperature (RT) to remove peritubular cells. The dispersed tubules were forced through a large-bore Pasteur pipette and digested with 0.015% (w/v) type I collagenase and 0.005% (w/v) DNase in HBSSf for 20 min at RT. The SC suspension was collected by centrifugation at 300 *g* for 3 min, washed three times in HBSSf and resuspended in SC culture medium (1:1 mix of Ham's F12 and DMEM supplemented with 15 mM HEPES, 50 IU/ml penicillin, 50 mg/ml streptomycin sulfate, 0.5 mg/ml Fungizone, 50 µg/ml gentamicin, and 5% (v/v) heat-inactivated FBS (Biochrom, Berlin, Germany)). The cell suspension was forced through a 20G needle, plated in culture flasks (Cell+; Sarstedt, Nümbrecht, Germany) at a concentration of 5000 clusters/ml and incubated at 37 °C in an atmosphere of 5% CO_2:95% O_2.

To obtain a culture of human SC, testicular biopsies were treated according to a method described by Sousa et al. (2002b). Isolation of SC was made by a method described elsewhere (Oliveira et al. 2009b) with some modifications. The tubules were centrifuged at 500 *g* for 5 min and washed in HBSSf followed by another centrifugation. The pellet was redissolved in SC culture medium with a composition similar to the one used for rat SC culture except for the addition of 10% (v/v) heat-inactivated FBS, and cultured in a similar way.

STF collection

STF was collected from 90-day-old rats following a method described by Turner et al. (1984) with some modifications. Briefly, testes were removed and trimmed free of fat and connective tissue. A small incision was made at the caudal end of each testis, which was placed inside a syringe barrel within a centrifuge tube. The apparatus was centrifuged at 54 *g* for 15 min at 0 °C to remove interstitial fluid. The testis was removed from the barrel, the tunica was cut and peeled back, and tubules were washed four times in saline to remove remaining interstitial fluid and blotted onto gauze. Tubules were extruded through the hub of a syringe into a centrifuge tube and centrifuged for 30 min at 0 °C. Supernatant containing STF was collected into a fresh tube.

Ex vivo *culture of rat SeT*

Rat SeT were used for culture instead of individual cell types, since this model has been shown to be suitable to mimic the testicular cellular environment *ex vivo* by several groups (Gilleron *et al.* 2009, Kaisman-Elbaz *et al.* 2009, Geoffroy-Siraudin *et al.* 2010). Testes (90-day-old rats) were removed, trimmed free of fat, washed in cold PBS and placed in DMEM-F12 culture medium at 32 °C. Tunica was cut and peeled back to expose tubules. Ten SeT fragments of about 1 cm and 2 ml of culture medium were used per well (Nunclon D 12 well multidishes; Nunc, Roskilde, Denmark). Experimental groups according to the medium composition were ($n=5$ in each group): control, DHT (10^{-7} M), Flu (10^{-7} M), DHT+Flu (10^{-7} M DHT and 10^{-7} M Flu), CHX (10 µg/ml), and DHT+CHX (10^{-7} M DHT and 10 µg/ml of CHX). Control medium, to which DHT/Flu/CHX were added was DMEM-F12 supplemented with 20 mg/l gentamicin sulfate, 0.1 mM 3-isobutyl-1-methylxanthine, and 1 mg/l BSA. In groups with DHT plus Flu/CHX, the hormone was added 30 min after Flu/CHX. Tubules were incubated for 6, 12, 24, and 48 h in time-course experiments, and for 24 h in experiments with Flu and CHX. Tubules were also cultured in control medium supplemented with survival factors (10% (v/v) heat-inactivated FBS, 1 mM Na pyruvate, 4 mM glutamine, 100 ng/ml vitamin A, 200 ng/ml vitamin E, 50 ng/ml vitamin C, and 12 µg/ml insulin) for 24 h. SeT remained viable during the course of the experiment as assessed by morphological analysis of hematoxylin-and-eosin-stained tissue sections. At the end of each experiment tubules were recovered from medium, snap–frozen in liquid nitrogen and stored at -80 °C until RNA isolation.

RNA isolation and cDNA synthesis

RNA was isolated from rat and human testis, rat SeT, rat prostate, rat epididymis, rat seminal vesicles, and rat and human SC with TRI reagent according to the manufacturer's instructions. RNA concentration was measured in a spectrophotometer (NanoPhotometer, Implen, Munich, Germany) and integrity was assessed by agarose gel electrophoresis. Total RNAs were decontaminated from genomic DNA by digestion with DNase I (amplification grade) according to the manufacturer's instructions. cDNA was synthesized in a final volume of 20 µl using 160 IU M-MLV reverse transcriptase (Promega), 0.5 µg random primers (Invitrogen), 10 mM each dNTP (GE Healthcare, Buckinghamshire, UK) and 1 µg each RNA sample according to the protocol supplied by the manufacturer. Synthesized cDNA was stored at -20 °C until further use.

RT-PCR

For the amplification of human and rat *RGN*, rat *Vim* and rat *Amh* specific intron-spanning primer sets were designed (Table 1). cDNA (1 µl) was amplified in a final volume of 25 µl containing 1× DreamTaq buffer with 20 mM $MgCl_2$ (Fermentas, Burlington, Ontario, Canada), 0.5 IU of DreamTaq DNA polymerase (Fermentas), 10 mM each dNTP (GE Healthcare), and 0.2 µM each primer (StabVida, Oeiras, Portugal). Every set of PCR included a no-template control.

In situ *hybridization*

Detection of *Rgn* mRNA in rat testis (90-day-old) 4% PFA-fixed, paraffin-embeded sections was performed by hybridization with digoxigenin-labeled probes according to a protocol as described previously (Maia *et al.* 2008).

Western blot

Total protein was isolated from rat SC, testis, prostate, epididymis, and seminal vesicles using RIPA buffer supplemented with protease inhibitors (1 mM PMSF; 5 mM EDTA; 1× protease inhibitor cocktail). Protein content in STF was concentrated by acetone precipitation. Protein pellet was

Table 1 PCR primer sequences, amplicon size, and cycling conditions.

Gene and accession numbers	Primer sequences (5'–3')	Amplicon size (bp)	Annealing temperature (°C)
Rat vimentin NM_031140.1	S: AGATCGATGTGGACGTTTCC AS: TCCGGTATTCGTTTGACTCC	198	50
Rat anti-Müllerian hormone NM_012902.1	S: GGCTGTGTTACAGGCTGACA AS: GACTCTTGGACAGCCTCCAG	210	54
Human *RGN* NM_152869.2	S: GCCTGTCCTACTCCGTGGATGC AS: GGCCACCCAGAGCTTCCCCT	143	55
Rat *Rgn* NM_031546.1	S: TCAAAGACTGTCTGCCGATG AS: GACTGTCGAAGTGCCACTGA	93	56
Rat *Rgn*[a] NM_031546.1	S: GGAGGAGGCATCAAAGTG AS: CAATGGTGGCAACATAGC	155	60
B-actin[a] NM_031144.2	S: ATGGTGGGTATGGGTCAG AS: CAATGCCGTGTTCAATGG	97	60
Gapdh[a] NM_017008.3	S: GTTCAACGGCACAGTCAAG AS: CTCAGCACCAGCATCACC	115	60
Cyclophilin A[a] NM_017101.1	S: CAAGACTGAGTGGCTGGATGG AS: GCCCGCAAGTCAAAGAAATTAGAG	163	60
B2-microglobulin[a] NM_012512.1	S: CCGTGATCTTTCTGGTGCTTGTC AS: CTATCTGAGGTGGGTGGAACTGAG	150	60

S, sense primer; AS, antisense primer.
[a]Primer pairs used in quantitative PCR.

dissolved in RIPA buffer with inhibitors. Protein concentration was determined by Bio-Rad protein assay.

Proteins (100 µg) were mixed with sample buffer, denatured for 10 min at 100 °C and resolved by SDS–PAGE on 12.5% gels. Proteins were blotted onto PVDF membranes (GE Healthcare) by wet transfer using 10 mM pH 11 CAPS with 20% (v/v) methanol. Blotted membranes were blocked with Tris-buffered saline with 0.05% (v/v) Tween-20 and 5% (w/v) dry skimmed milk for 1 h at RT and then incubated overnight with primary monoclonal anti-RGN antibody (1:250; ab81721). Membranes were incubated with secondary antibody conjugated with alkaline phosphatase (1:15 000; anti-mouse IgG–AP, ab7069), developed for 5 min with ECF substrate (GE Healthcare) and scanned with Molecular Imager FX (Bio-Rad).

Immunohistochemistry

Sections (5 µm) of rat (90-day-old) testis, prostate, epididymis, and seminal vesicles (4% PFA-fixed) and human testis (10% formalin-fixed) were deparaffinized in xylene and rehydrated in graded alcohols. Heat-induced antigen retrieval was performed in 10 mM citric acid pH 6.0 for 30 min, at 80–85 °C. Endogenous peroxidase was blocked by incubating samples in 3% (v/v) H_2O_2 (Panreac, Barcelona, Spain) for 10 min at RT and unspecific staining was blocked by incubation with 1:20 normal goat serum for 30 min at RT. Sections were incubated overnight at 4 °C with primary monoclonal anti-RGN antibody (ab81721) diluted 1:50 in PBS with 1% (w/v) BSA (PBA). Sections were then incubated with secondary goat anti-mouse biotinylated antibody (ab7067) diluted 1:200 in PBA for 1 h at RT, followed by incubation with ExtrAvidin Peroxidase diluted 1:20 in PBA. Antibody binding was detected using HRP substrate solution (Dako, Glostrup, Denmark). Sections were slightly counterstained with Harris' hematoxylin (Merck), dehydrated, cleared, and mounted. Specificity of the staining was assessed by the omission of primary antibody.

Immunocytochemistry

Human and rat cultured SC were washed with PBS and fixed with cold 4% PFA. Permeabilization was performed by incubation with 0.01% (w/v) digitonin for 10 min at RT. Endogenous peroxidase was blocked by incubation with 0.1% (v/v) H_2O_2 for 10 min at RT and unspecific staining was blocked with PBA for 30 min at RT. Cells were incubated overnight at 4 °C with primary monoclonal anti-RGN antibody (1:50 in PBA, ab81721) or ready-to-use polyclonal anti-Vim antibody (V9 clone, Invitrogen). Cells were then incubated with secondary horse anti-mouse biotinylated antibody (BA-200; Vector Labs, Burlingame, CA, USA) diluted 1:400 in PBA for 1 h at RT, followed by incubation with ExtrAvidin Peroxidase diluted 1:20 in PBA. Antibody binding was detected using HRP substrate solution. After color development sections were slightly counterstained with Harris' hematoxylin. Specificity of the staining was assessed by omission of primary antibody.

qPCR

Quantification of *Rgn* expression in rat cultured SeT and rat testis at different postnatal ages (10, 30, 60, 90, 120, 180, 240, 270, and 365 days, $n \geq 5$ in each group) was performed by qPCR. An intron-spanning primer set was designed for the quantification of rat *Rgn* expression (Table 1). In addition two internal reference genes (β-actin and *Gapdh* for cultured SeT; β2-microglobulin and cyclophilin A for rat testis at different postnatal ages) were selected from a panel for the normalization of the expression based on their stability in the experimental conditions used according to two methods described elsewhere (Vandesompele *et al.* 2002, Andersen *et al.* 2004; data not shown). Reactions were carried out in an iQ5 system (Bio-Rad) and efficiency of the reactions was determined for all primer sets using serial dilutions of cDNA samples (1:1, 1:10, and 1:100). Primer concentration and annealing temperature were optimized before the assay and specificity of the amplicons was determined by melting curve analysis. Reaction mixtures consisted of SYBR Green master mix (Bio-Rad), sense and antisense primers (500 nM for RGN and 200 nM for all other 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 of *Rgn* were calculated according to a published mathematical model proposed by the Vandesompele group (Hellemans *et al.* 2007).

Statistical analysis

Statistical significance of differences in *Rgn* expression between groups was evaluated by one-way ANOVA followed by Bonferroni's multiple comparison test or unpaired *t*-test with Welch's correction, using GraphPad Prism v5.00 (GraphPad Software, San Diego, CA, USA). Statistically significant differences were considered for $P < 0.05$. Experimental data are shown as mean $\pm$ S.E.M.

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