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ORIGINAL RESEARCH

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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ABSTRACT: Sperm undergo maturation acquiring progressive motility and the ability to fertilize oocytes 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 epididymal lumen. Sperm viability and the frequency of normal sperm, determined by the one-step eosin-nigrosin staining technique and the 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 to 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 Cornwall (2009)). 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 Guyonnet *et al.* (2011)).

The epididymal fluid (EF) is a complex mixture of ions, proteins and other organic molecules (reviewed by Cornwall (2009)). 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 Pholpramool *et al.* (2011)). 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 Da Silva *et al.* (2006)). 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 (Jenkins *et al.*, 1980).

Regucalcin (RGN) is a Ca^{2+}-binding protein (Yamaguchi and Yamamoto, 1978) 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 (Yamaguchi et al., 1988; Yamaguchi and Mori, 1989; Takahashi and Yamaguchi, 1993, 1999). Recently, RGN was identified as an androgen-target gene expressed in several tissues of male reproductive tract, namely, seminal vesicles, prostate, testis and epididymis (Maia et al., 2008, 2009; Laurentino et al., 2011b). 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 (Laurentino et al., 2011a), 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-month-old Wt and Tg-RGN Sprague-Dawley (*Rattus norvegicus*) rats were obtained, respectively, from Charles River (Barcelona, Spain) and Japan SLC (Hamamatsu, Japan). The 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 the 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 −80°C until protein extraction. Five additional Wt animals were included and epididymides collected as follows. One epididymis was subdivided into caput, corpus and cauda regions, which were immediately frozen on liquid nitrogen and stored at −80°C until protein extraction. Segments eight or nine of the contralateral epididymis (Soler et al., 2005) together with the initial portion of vas deferent were dissected for collection of 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 min 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 analyzed 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 ~0.2 mM (Weissgerber et al., 2012).

Sperm viability and morphology analysis

Sperm viability was assessed by using the one-step eosin-nigrosin staining technique (Dott and Foster, 1972). 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 (Mota and Ramalho-Santos, 2006). 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 into 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 the 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 (5 μm) of the caput region were stained with hematoxylin and eosin. The epithelial cell height and epididymal tubule 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, NJ, USA) for 60 min at RT. Sections were incubated overnight at 4°C with rabbit anti-V-ATPase (sc-28801, Santa Cruz Biotechnology, CA, 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-Aldrich, St. Louis, USA) diluted 1:20 in PBA for 60 min at RT, followed by incubation with ExtrAvidin Peroxidase (E2886, Sigma-Aldrich) 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. The specificity of the staining was assessed by the omission of primary antibody.

EF collection

EF was collected following a protocol previously described (Monclus et al., 2010). Isolated epididymal segments were kept on a petri dish cooled on ice to avoid freezing until use, in order to preserve protein 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 30 000*g* for

60 min at 4°C to completely remove spermatozoa and debris. The supernatant was concentrated by centrifugation at 3000*g* 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 $^{45}Ca^{2+}$ ($^{45}CaCl_2$; 0.1 μCi/ml, PerkinElmer, Waltham, USA) for 2.5, 7.5 and 15 min. 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 two volumes (w/v) of 70% HNO_3 and neutralized with an equivalent volume of 2 M 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 min. 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 was 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 min. 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

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). 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 4°C with rabbit anti-Na^+/H^+ exchanger (NHE3, 1:1000, sc-28757, Santa Cruz Biotechnology, CA, 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-Aldrich) 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) was used as secondary antibodies. Membranes were developed with ECF substrate (GE Healthcare) for 5 min 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 assay

Whole epididymides were dissected and weighed 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 (Benzie and Strain, 1996). 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. Six microliters of each sample was mixed with 180 μl of FRAP reagent, and absorbances (593 nm) were measured immediately and 60 min 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 the 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 (Fig. 1A) were significant lower in Tg-RGN rats compared with their Wt littermates ($1.28 \times 10^8 \pm 9.24 \times 10^6$ versus $1.72 \times 10^8 \pm 1.57 \times 10^7$, $P < 0.05$). Tg-RGN rats also displayed a statistically significant lower percentage of motile sperm (Fig. 1B; 47.88% ± 3.67 versus 64.60% ± 5.66, $P < 0.05$). In contrast, the percentage of viable sperm in the Tg-RGN rats was significantly higher than that in the Wt group (Fig. 1C; 38.75% ± 2.36 versus 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, Weissgerber *et al.*, 2012) and supra-physiological (2 mM) Ca^{2+} concentrations (Fig. 1D). As expected, sperm motility at the 0.2 mM physiological concentration was identical to that observed with standard HBSS, but exposure of sperm to 10 times higher Ca^{2+} concentrations led to a reduction in motility by 22%.

Morphology of epididymal sperm

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

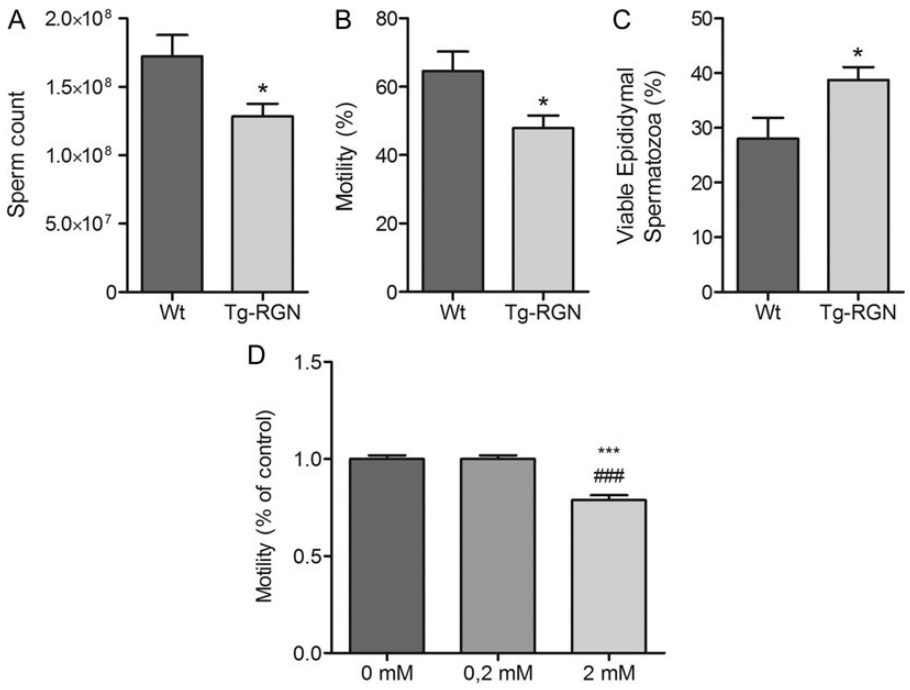

Figure 1 Epididymal sperm counts (**A**), motility (**B**) and viability (**C**) in Tg-RGN rats versus Wt; $^{*}P < 0.05$. (**D**) Sperm motility in standard HBSS medium (0 mM Ca^{2+}) and in HBSS containing Ca^{2+} at concentrations of 0.2 and 2 mM; $^{***}P < 0.001$ and $^{\#\#\#}P < 0.001$ relative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

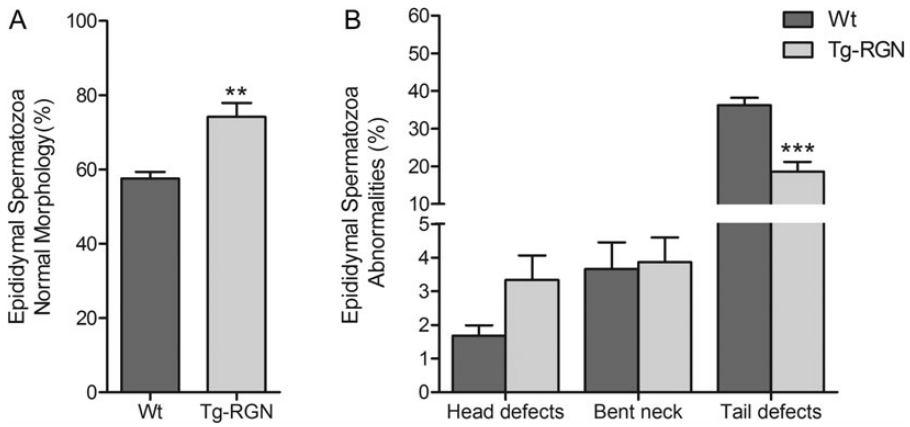

Figure 2 Normal (**A**) and abnormal (**B**) morphology of epididymal sperm in Tg-RGN rats versus Wt. Data are represented as mean ± SEM. $n \geq 5$ in each group. $^{**}P < 0.01$ and $^{***}P < 0.001$, both relative to the corresponding Wt group.

Morphology of caput epididymal tubules

The epididymal caput tubule area, boundwidth, boundheight and perimeter in Wt and Tg-RGN animals are presented in Table I. Overall, measurements did not differ between Tg-RGN and Wt groups, except for the epithelial cell height (Fig. 3A), which significantly decreased from 28.35 μm ± 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 (Cornwall, 2009). Clear cells are also of the uttermost importance, having endocytic activity and being responsible for clearing proteins from the lumen (Hermo *et al.*, 1988). 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 (Da Silva *et al.*, 2006). Intense staining for V-ATPase was seen in plasma membrane of clear cells in Tg-RGN as well as in their Wt counterparts (Fig. 3B, upper panels). As reported by others (Oliveira *et al.*, 2005; Da Silva *et al.*, 2006), labeling for AQP9 was detected along the microvillus border of the principal cells, which was common to both animal groups (Fig. 3B, lower panels). Immunolocalization of V-ATPase and AQP9 (Fig. 3B) showed no difference in shape and proportion of clear and principal cells between Tg-RGN and Wt animals.

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 Yamaguchi (2005)). 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 high in this tissue and the time-course curve for epididymis Ca^{2+} influx was obtained at 2.5, 7.5 and 15 min (Fig. 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 with Wt animals. However, $^{45}Ca^{2+}$ release rates were not significantly different between the two experimental groups (Fig. 4B).

Table I Epididymal caput tubule area (μm^2), boundwidth, boundheight and perimeter (μm) in Tg-RGN rats versus Wt.

	Wt	Tg-RGN
Area	73 832.9 ± 3211.0	74 503.6 ± 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.

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 EF by the activity of Na^+/H^+ exchangers, particularly the NHE3 isoform, has been considered to be of the uttermost importance in this process (Kaunisto *et al.*, 2001; Pholpramool *et al.*, 2011). Also, water reabsorption along male excurrent ducts is needed to significantly increase sperm concentration, being a fundamental step for the establishment of male fertility (Da Silva *et al.*, 2006). The water transport process is controlled by aquaporins, and AQP-1 seems to be the isoform associated with epididymal function (Oliveira *et al.*, 2005; Danyu *et al.*, 2008). 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 (Fig. 5) between experimental groups.

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 (Fig. 6A and B). An ~2-fold statistically significant higher expression was found in the corpus (1.06 ± 0.08) compared with 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 (Carolan *et al.*, 2009), murine (Lv *et al.*, 2007, 2008), rat (Isogai *et al.*, 1994a, b) and human plasma (Lv *et al.*, 2008) and rat seminiferous tubule fluid (Laurentino

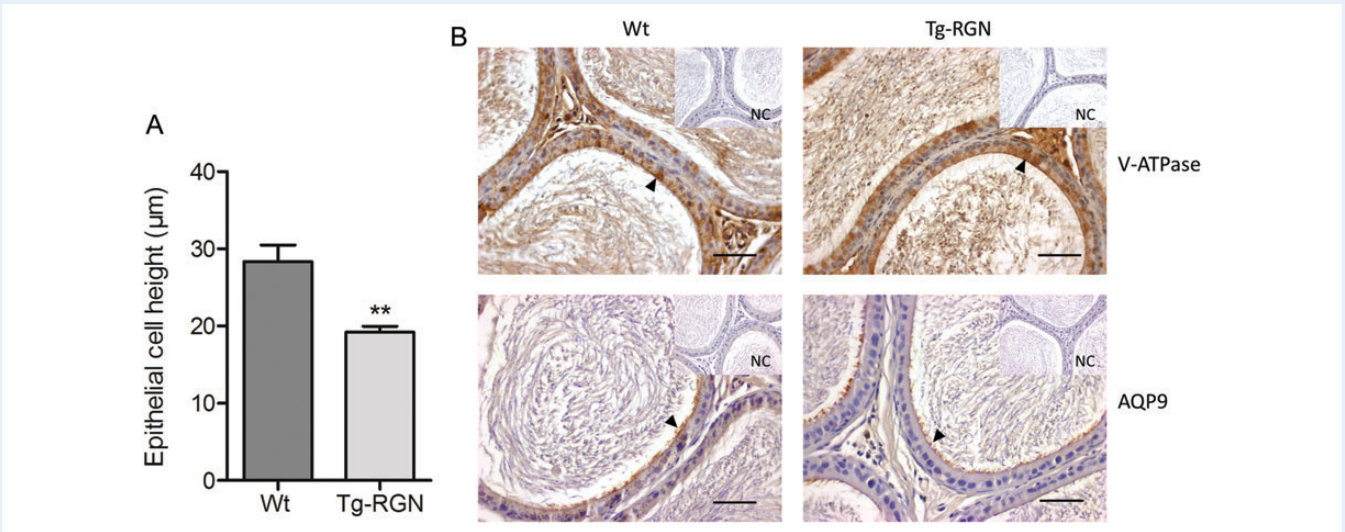

Figure 3 Epithelial cell height of the caput epididymis (**A**) and immunolocalization of V-ATPase and AQP9 (**B**) in Tg-RGN rats versus Wt. (A) Data are represented as mean ± SEM. $n = 5$ in each group. $^{**}P < 0.01$. (B) Arrows exemplificate regions of positive immunostaining. Scale bar indicates 50 μm. Insets in each panel are representative negative controls (NC) obtained by omission of the primary antibody.

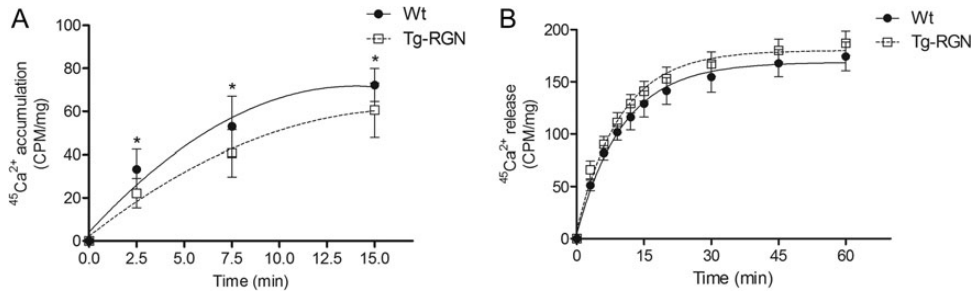

Figure 4 Time-course of Ca^{2+} influx (**A**) and efflux (**B**) in the epididymis of Tg-RGN rats versus Wt. Data are represented as mean ± SEM. $n = 5$ for both groups, at each time point.*$P < 0.05$.

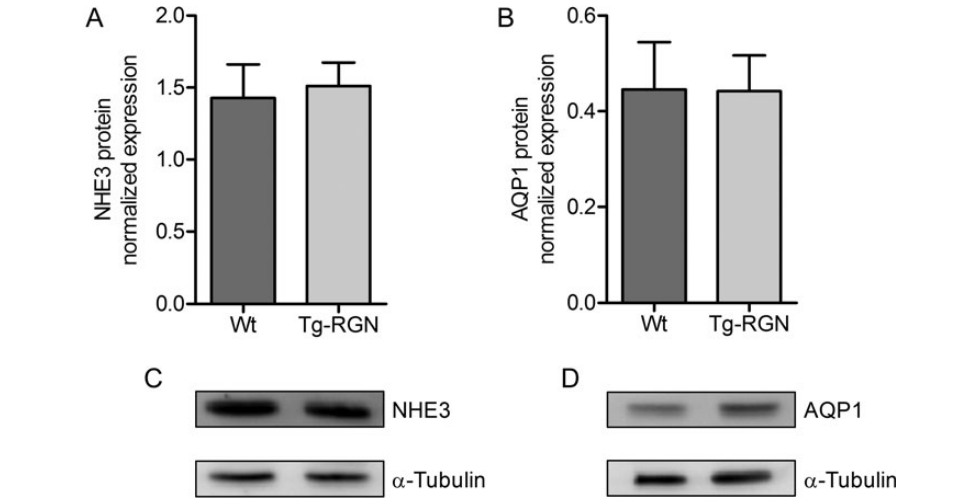

Figure 5 Expression of NHE3 (**A**) and AQP1 (**B**) in the epididymis of Tg-RGN rats versus Wt. Data are represented as mean ± SEM after normalization with α-tubulin. $n \geq 5$ in each group. Representative images of immunoblots for NHE3, AQP1 and α-tubulin are provided in (**C**) and (**D**).

et al., 2011b), we decided to investigate its presence in EF. An immunoreactive band of the expected size was detected in rat EF (Fig. 6B).

Antioxidant potential of the epididymis

It has been suggested that epididymal cells, among other roles, act to protect sperm against oxidative stress (OS) (Jervis and Robaire, 2001), which crosses with the antioxidant properties described for RGN (Son *et al.*, 2006). Measurement of the antioxidant potential of epididymal tissues by means of the FRAP assay (Fig. 7) revealed that a statistically significant higher FRAP value was found in Tg-RGN relative to Wt animals ($P < 0.001$).

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 (Laurentino *et al.*, 2011a, b). 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 (Yamaguchi *et al.*, 2002), this is the first time that their sperm parameters have been characterized. The lower sperm counts and diminished sperm motility found in Tg-RGN (Fig. 1A and B) may be indicative of a subfertility phenotype. However, this seems to be at least partly compensated for by a higher viability (Fig. 1C) and higher percentage of normal morphology (Fig. 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 (Robaire *et al.*, 2006). This regional restriction is characteristically evident both in the number and the quantity of proteins secreted with the caput as the most active, while the corpus and the cauda have a lower secretory activity (Dacheux *et al.*, 2009). Thus, we looked at epididymal tubules morphology in the caput region. Although there were no differences in tubule area, boundwidth, boundheight and perimeter

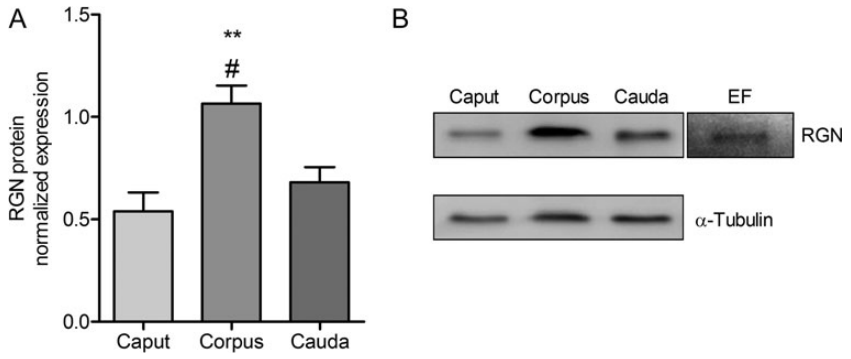

Figure 6 Expression of RGN in the caput, corpus and cauda regions of rat epididymis and EF (**A**). Data are represented as mean ± SEM after normalization with α-tubulin. $n \geq 4$ in each group. $^{**}P < 0.01$ compared with caput; $\#P < 0.05$ compared with cauda. Representative images of immunoblots for RGN and α-tubulin are provided in (**B**).

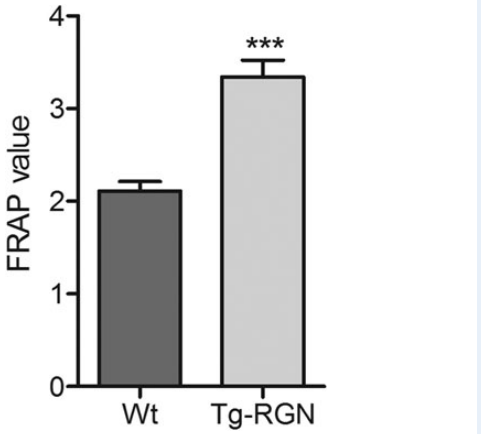

Figure 7 FRAP value (μM antioxidant potential/mg tissue) in the epididymis of Tg-RGN rats versus Wt. Data are represented as mean ± SEM. $n \geq 4$ in each group. $^{***}P < 0.001$.

(Table I) between Wt and Tg-RGN groups, the epithelial cell height significantly decreased in Tg-RGN animals (Fig. 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 (Fig. 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 Cornwall (2009)). 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 Pholpramool *et al.* (2011)), water transport contributes to sperm concentration (reviewed by Da Silva *et al.* (2006)). Ca^{2+} concentrations in the EF are quite low in comparison with those of other ions such as sodium, potassium, chloride, ammonium and magnesium (Wales *et al.*, 1966), 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 (Breitbart, 2002) and depends on the progressive activation of a cAMP-PKA-dependent signaling pathway mediating protein tyrosine phosphorylation (Aitken *et al.*, 1998). It is during epididymal maturation that sperm acquire the ability to respond to high levels of intracellular cAMP leading to tyrosine residues phosphorylation (Lewis and Aitken, 2001), 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 to sperm maturation (Ecroyd *et al.*, 2004).

RGN regulates Ca^{2+}-transport in several cell types by controlling the activity of Ca^{2+} channels, transporters and pumps (reviewed by Yamaguchi (2011)). In the present study, Tg-RGN rats had a reduced rate of Ca^{2+}-influx (Fig. 4A) by epididymal tissues. This finding is supported by studies describing the role of RGN enhancing Ca^{2+}-ATPase activity (Yamaguchi and Mori, 1989; Takahashi and Yamaguchi, 1999) and suppressing the expression of the L-type Ca^{2+} channel and calcium-sensing receptor mRNA (Nakagawa and Yamaguchi, 2006) 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 in the expression of NHE3 and AQP1 proteins (Fig. 5) between Tg-RGN and Wt animals, which indicates the maintenance of proper acidification and water reabsorption in the epidydimal fluid, it is highly predictable that the decreased sperm motility observed in Tg-RGN (Fig. 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 10 times higher than those commonly found in the EF (Fig. 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 (Weissgerber *et al.*, 2011, 2012). 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 with their Wt counterparts, and despite intact spermatogenesis, they had severely diminished fertility as a result of decreased sperm motility and viability (Weissgerber *et al.*, 2011, 2012). 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 OS is one of main causes of defective sperm morphology and function (Aitken and Curry, 2011), and it was suggested that the epididymal epithelium must be able to protect sperm against oxidative damage (Jervis and Robaire, 2001). 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 (Fukaya and Yamaguchi, 2004) and decreasing the generation of reactive oxygen species (Handa *et al.*, 2009). This antioxidant role is demonstrated in RGN knockout mice which display higher levels of OS than their Wt counterparts (Son *et al.*, 2006). Accordingly, a higher antioxidant potential was found in the epididymis of Tg-RGN rats (Fig. 7). Therefore, it is appropriate to assume that the higher sperm viability (Fig. 1C), the higher percentage of normal morphology (Fig. 2A) together with the diminished incidence of tail defects (Fig. 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 understanding the functional differences between regions, which are crucial for sperm maturation (Jervis and Robaire, 2001; Belleannée *et al.*, 2012). Furthermore, analysis of genes that changed the expression at least 2-fold between adjacent segments is a way to emphasize their relative importance in distinct regions (Jervis and Robaire, 2001). Indeed, RGN expression was 2-fold higher in the corpus relative to caput and cauda regions (Fig. 6A). Other OS- (Jervis and Robaire, 2001) and Ca^{2+}-related genes (Jelinsky *et al.*, 2007) 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 Guyonnet *et al.* (2011)).

In conclusion, the results presented herein show that Tg-RGN animals display altered morphology of epididymis, which suggests important alterations in 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 contribute to a better understanding of the role of Ca^{2+} and RGN in sperm maturation and open new perspectives to detail the molecular mechanisms underlying epididymal function. This is relevant and could be of the uttermost importance to resolving certain male infertility cases associated with sperm maturation disorders, as well as to developing new targets for male contraception.

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Authors' roles

S.C. contributed in all tasks related to 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 fluxe experiments. G.L. collaborated in sperm analysis and M.G.A. in western blot analysis and FRAP assay. A.V.M.C. and J.E.C. 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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Conflict of interest

None declared.

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