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

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ABSTRACT: Regucalcin (RGN) is a calcium (Ca²⁺)-binding protein which plays an important role in the regulation of Ca²⁺ homeostasis and has been shown to catalyse an important step in L-ascorbic acid biosynthesis. It is encoded by an X-linked gene and differs from other Ca²⁺-binding proteins by lacking the typical EF-hand Ca²⁺-binding domain. RGN controls intracellular Ca²⁺ concentration by regulating the activity of membrane Ca²⁺ 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 Ca²⁺ 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 suggests the hypothesis that RGN might play a role in spermatogenesis, directly or as a mediator of androgen action. This review discusses 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 (Ca²⁺)-binding protein (Yamaguchi and Yamamoto, 1978). It differs from common Ca²⁺-binding proteins such as calmodulin because it does not contain the typical EF-hand Ca²⁺-binding motif (Shimokawa and Yamaguchi, 1993). Later it was identified by another group and named senescence marker protein-30 (SMP-30), for its characteristic down-regulation with ageing in rat liver (Fujita et al., 1992b). RGN was also shown to function as glucolactonase, the enzyme catalysing the penultimate step in the biosynthesis of L-ascorbic acid (Kondo et al., 2006). The ability to synthesise L-ascorbic acid has been lost during the course of evolution on a number of species including humans; however, it is still present in rats and mice (Linstead and Van Schaftingen, 2007). The expression of RGN is stimulated by Ca²⁺ (Shimokawa and Yamaguchi, 1992; Isogai and Yamaguchi, 1995; Yamaguchi and Kurota, 1995) and can be regulated by several factors which include the transcription factor, AP-1 (Murata and Yamaguchi, 1998), β-catenin (Nejak-Bowen et al., 2009), nuclear factor I-A1 (NFI-A1; Misawa and Yamaguchi, 2002) and RGN gene promoter region-related protein (Misawa and Yamaguchi, 2001; Yamaguchi, 2009). Also, steroid and non-steroid hormones have been described to regulate RGN expression (Shimokawa and Yamaguchi, 1992; Yamaguchi and Oishi, 1995; Kurota and Yamaguchi, 1996; Murata et al., 1997; Maia et al., 2008, 2009; Laurentino et al., 2011a).

The function of RGN in the regulation of intracellular Ca²⁺ concentration ([Ca²⁺]i) is achieved by controlling the activity of Ca²⁺ channels, Ca²⁺-ATPase in the membrane of mitochondria and endoplasmic reticulum (Yamaguchi and Mori, 1989; Takahashi and Yamaguchi, 1999) and (Ca²⁺–Mg²⁺)-ATPase in the plasma membrane (Yamaguchi et al., 1988; Takahashi and Yamaguchi, 1993). RGN also plays an important role in the regulation of Ca²⁺-dependent enzymes (Yamaguchi, 2011). One of these enzymes is cAMP phosphodiesterase, which degrades cAMP, providing a way by which RGN regulates cAMP levels in cells (Yamaguchi and Tai, 1991; Yamaguchi and Kurota, 1997). However, most enzymes regulated in this way are protein kinases and phosphatases, which in turn will regulate the activity of other proteins (Mori and Yamaguchi, 1990; Yamaguchi, 1999).

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and Mori, 1990; Kurota and Yamaguchi, 1997; Omura and Yamaguchi, 1999). RGN also inhibits nitric oxide synthase (Izumi et al., 2003; Ma and Yamaguchi, 2003; Yamaguchi et al., 2003) and Ca$^{2+}$-dependent endonuclease activities; this is likely related to its anti-apoptotic actions (Yamaguchi and Sakurai, 1991). RGN seems to inhibit apoptosis by up-regulating the expression of Akt-1 and Bcl-2 while down-regulating the expression of Caspase-3 (Nakagawa and Yamaguchi, 2005). RGN's antiapoptotic effect has been highlighted by the development of knockout mice, whose cells are more prone to apoptosis than their wild-type counterparts (Ishigami et al., 2002; Maruyama et al., 2004).

The importance of androgens, Ca$^{2+}$ homeostasis and apoptosis control to spermatogenesis has been indicated by a number of studies (Franchi and Camatini, 1985; Juneja et al., 1990; Benoff et al., 1994; Hershlag et al., 1995; Grima et al., 1998; Lee et al., 2006; Xu et al., 2007; Walker, 2009; Lee et al., 2011), and recently RGN was identified as an androgen-target gene, being broadly expressed in the testis and other tissues of male reproductive tract (Maia et al., 2009; Laurentino et al., 2011a). 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 indicated by a number of studies (Stouffs et al., 2009; Zheng et al., 2010). RGN is an X-linked gene localized in the p11.3–q11.2 and q11.1–12 segments of the human (Fujita et al., 1995) and rat (Shimokawa et al., 1995) X chromosome, respectively. Both in human and rodents, the RGN gene consists of seven exons and the cDNA contains an open reading frame encoding a protein of 299 amino acids long with an estimated molecular weight of 33 kDa (Fujita et al., 1992a; Shimokawa and Yamaguchi, 1993; Fujita et al., 1996, Fig. 1). Two alternatively spliced mRNA variants, originated by exon skipping mechanisms, have been described for RGN in breast and prostate tissues and cell lines (Maia et al., 2009), RGN$\Delta 4$ with the deletion of exon 4, and RGN$\Delta 4,5$ missing exons 4 and 5. Using the strategy described in Maia et al. (2009), these transcripts were also detected in human testis (Fig. 1). The in vitro and in vivo existence of two forms of RGN protein with lower molecular weights (28 and 24 kDa) has been recently suggested (Arun et al., 2011). However, it is unknown whether they represent the translated RGN$\Delta 4$ and RGN$\Delta 4,5$ transcripts or are generated by post-translational processing. The precise function of these variants remains unexplored.

RGN protein is highly conserved from eukaryotes to prokaryotes, with 18% of residues absolutely conserved in all species (Fig. 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 evolution, from invertebrates to vertebrates, indicates its involvement in basic and important biological functions.
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 (Shimokawa and Yamaguchi, 1992; Yamaguchi and Isogai, 1993), but also in brain (Yamaguchi et al., 2000), heart (Yamaguchi and Nakajima, 2002), bone (Yamaguchi et al., 2002a), lung (Mori et al., 2004) and submandibular gland (Ishii et al., 2005). However, RGN was also shown to be expressed in reproductive tissues such as the ovary (Fayad et al., 2004), breast and prostate (Maia et al., 2008, 2009).

More recently, RGN expression was studied in male reproductive tract (Laurentino et al., 2011a, Table I). 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 (Laurentino et al., 2011a). In prostate, RGN mRNA and protein are localized to epithelial cells (Maia et al., 2008, 2009; Laurentino et al., 2011a) and it seems to be associated with cancer development, since loss of RGN expression was detected in human prostate cancer cases (Maia et al., 2009). 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, Laurentino et al., 2011a). 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 in pea aphid saliva (Carolan et al., 2009) and plasma of several species (Isogai et al., 1994a, b; Lv et al., 2007, 2008). In a recent report, RGN protein was also detected in seminiferous tubule fluid (Laurentino et al., 2011a), which is mainly a product of Sertoli cells (SCs) secretory activity (Fisher, 2002). This complex fluid creates the perfect environment for germ cell development and maturation (Griswold, 1988; Fisher, 2002). 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 play an important role in the mechanisms of sperm capacitation and motility (Triphan et al., 2007; Sengupta et al., 2008). Whether or not the presence of RGN in seminiferous tubule fluid is related to the testicular sperm production and maturation through the regulation of enzyme activity and [Ca\(^{2+}\)], is unknown at this point; however, it would be interesting to explore RGN possible actions in sperm physiology.

### RGN expression in distinct spermatogenic phenotypes

Successful spermatogenesis demands a delicately regulated equilibrium between germ cell apoptosis and proliferation (Print and Loveland, 2000). Hypospermatogenesis (HP) has been linked to a deregulation in germ cell proliferation and apoptosis (Takagi et al., 2001). Also, the expression of several apoptosis-related genes has been shown to be altered in testis with defective spermatogenesis (Feng et al., 1999; Weikert et al., 2004; Weikert et al., 2005; Kim et al., 2007; Weikert et al., 2008; Laurentino et al., 2011b). This led us to analyse RGN expression in abnormal phenotypes of human spermatogenesis, namely HP and SC-only syndrome in comparison with cases of oligozoospermia (OAZ) with conserved spermatogenesis (Fig. 3).

Interestingly, testis from men with HP shows higher expression of RGN mRNA (1.6 fold; \(P < 0.001\)) relative to testis with conserved spermatogenesis (Fig. 3). Considering RGN’s role suppressing cellular proliferation (Nakagawa et al., 2005; Yamaguchi and Daimon, 2005), 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.

### Effects of sex steroids on RGN expression

RGN expression is regulated by numerous factors, including Ca\(^{2+}\) (Shimokawa and Yamaguchi, 1992), insulin (Yamaguchi et al., 1995), aldosterone (Kurota and Yamaguchi, 1996), sex steroid hormones (Yamaguchi and Oishi, 1995; Kurota and Yamaguchi, 1996; Maia et al., 2008, 2009; Laurentino et al., 2011a), amongst others (Table II). The regulation of RGN expression by sex steroid hormones was first described in 1995 (Yamaguchi and Oishi, 1995). 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 oestrogen regulation of liver metabolism (Yamaguchi and Oishi, 1995). However, this oestrogenic control of RGN expression is not limited to the liver, as shortly after the regulation of RGN expression by E\(_2\) in rat kidney cortex was reported (Kurota and Yamaguchi, 1996). It was shown that administration of E\(_2\) to rats caused a reduction in RGN expression in the kidney cortex, an effect opposite to the one observed in liver (Kurota and Yamaguchi, 1996). More recently, the effect of sex steroid hormones regulating RGN expression in breast and prostate has been reported (Maia et al., 2008, 2009). Administration of E\(_2\) to rats induces a down-regulation of RGN expression in the prostate and mammary gland (Maia et al., 2008). On the other hand, stimulation of MCF-7 breast cancer cells with E\(_2\) causes an up-regulation of RGN expression by a mechanism that is likely to involve a membrane oestrogen receptor (Maia et al., 2009). In LNCaP prostate cancer cells, 5α-dihydrotestosteron (DHT) down-regulates RGN expression by a mechanism that seems to involve the androgen receptor (AR) and

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

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Localization</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Epididymis</td>
<td>Epithelium, smooth muscle, connective tissue</td>
<td>Laurentino et al. (2011a)</td>
</tr>
<tr>
<td>Prostate</td>
<td>Epithelium</td>
<td>Maia et al. (2008); Laurentino et al. (2011a)</td>
</tr>
<tr>
<td>Seminal vesicles</td>
<td>Epithelium</td>
<td>Laurentino et al. (2011a)</td>
</tr>
<tr>
<td>Testis</td>
<td>Leydig cells, SCs, spermatogonia, spermocytes, spermatids</td>
<td>Laurentino et al. (2011a)</td>
</tr>
</tbody>
</table>
It has been demonstrated that RGN is able to regulate apoptosis (Giampietri, 2004). 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 (Laurentino et al., 2011a). Androgens are regulators of testicular cell death and are recognized as germ cell survival factors (Tapalanen et al., 1993; Henriksen et al., 1995; Erkkila et al., 1997; Kim et al., 2001; Bakalska et al., 2004). The up-regulation of RGN expression by androgens in mammalian testis (Laurentino et al., 2011a) may be part of the mechanism by which these hormones protect germ cells from apoptosis. In Fig. 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 NFI-A1 through PI3K (Misawa and Yamaguchi, 2000, 2002). AP-1 (Murata and Yamaguchi, 1998) and β-catenin (Nejak-Bowen et al., 2009), transcription factors which in turn can be activated by AR (Pawlowski et al., 2002 and references therein; Church et al., 2005; Aquila et al., 2007). RGN acts in the control of [Ca2+]i by regulating the activity of Ca2+ pumps and channels (Yamaguchi et al., 1988; Yamaguchi and Mori, 1989; Takahashi and Yamaguchi, 1993, 1999) and regulates the activity of protein kinases and phosphatases (Mori and Yamaguchi, 1990; Yamaguchi and Mori, 1990; Kurota and Yamaguchi, 1997; Omura and Yamaguchi, 1999), which in turn can regulate the activity of numerous phospho-proteins (Johnson, 2009). Moreover, RGN can regulate the expression of genes and the activity of proteins involved in apoptosis, favouring cell survival (Yamaguchi and Sakurai, 1991; Nakagawa and Yamaguchi, 2005) which could be of crucial relevance in the spermatogenic process.

RGN has also been shown to play a role suppressing oxidative stress (Feng et al., 2004; Son et al., 2006; Handa et al., 2009). The study of RGN-deficient mice has demonstrated that RGN acts as gluconolactonase, a key enzyme in the biosynthesis of L-ascorbic acid (Kondo et al., 2006). Ascorbic acid is a known powerful antioxidant (Arrigoni and De Tullio, 2002), and a role in the balance between cell survival and death has been indicated for this molecule (Harrison et al., 2010; Bonilla-Porras et al., 2011; Jin et al., 2011). Indeed, RGN knockout mice were shown to display higher levels of oxidative stress than their wild-type counterparts (Son et al., 2006), and RGN was shown to increase the activity of antioxidant enzyme superoxide dismutase (Fukaya and Yamaguchi, 2004; Ichikawa and Yamaguchi, 2004; Handa et al., 2009) and suppress the generation of reactive species (Feng et al., 2004; Handa et al., 2009), 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 Aitken and Curry, 2011). Therefore, considering RGN antioxidant properties, another potential role for this protein in spermatogenesis may be related to protection against oxidative stress.

Altogether, the available information is suggestive of a role for RGN in germ cells survival and development, probably associated with the control of apoptosis and oxidative stress. Moreover, RGN has been linked to the development of prostate and liver cancers (Tsurusaki and Yamaguchi, 2003; Maia et al., 2009; Xu et al., 2011; Zhou et al., 2011) but a possible connection between RGN and testicular cancer is yet to be explored.

### Table II Hormonal factors regulating RGN expression in reproductive and non-reproductive tissues.

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Tissue</th>
<th>Effect</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Aldosterone</td>
<td>Kidney</td>
<td>↓</td>
<td>Kurota and Yamaguchi (1996)</td>
</tr>
<tr>
<td>E2</td>
<td>Liver</td>
<td>↑</td>
<td>Yamaguchi and Oishi (1995)</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>↓</td>
<td>Kurota and Yamaguchi (1996)</td>
</tr>
<tr>
<td>Breast</td>
<td>↓ (MCF-7 cells)</td>
<td>↑</td>
<td>Maia et al. (2008, 2009)</td>
</tr>
<tr>
<td>Prostate</td>
<td>↓</td>
<td></td>
<td>Maia et al. (2008)</td>
</tr>
<tr>
<td>Calcitonin</td>
<td>Liver</td>
<td>↑</td>
<td>Yamaguchi et al. (1994)</td>
</tr>
<tr>
<td>DHT</td>
<td>Testis</td>
<td>↑</td>
<td>Laurentino et al. (2011a)</td>
</tr>
<tr>
<td>Prostate</td>
<td>↓ (LnCaP cells)</td>
<td>↑</td>
<td>Maia et al. (2009)</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>Kidney</td>
<td>↑</td>
<td>Kurota and Yamaguchi (1996)</td>
</tr>
<tr>
<td>Insulin</td>
<td>Liver</td>
<td>↑</td>
<td>Yamaguchi et al. (1995)</td>
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↑, up-regulation; ↓, down-regulation.

de novo protein synthesis (Maia et al., 2009). In rat seminiferous tubules cultured ex vivo, the expression of RGN has been shown to be up-regulated by DHT administration (10^-7 M), in a mechanism that involves the AR but does not require de novo synthesis of protein (Laurentino et al., 2011a). This identified RGN as a new androgen-target gene in the tests.
Reproductive phenotype of RGN knockin and knockout models

A knockout mouse for RGN has been developed by introducing a germ line null mutation (Ishigami et al., 2002). These mice were indistinguishable from their wild-type litter mates but have decreased body weight and life span (Ishigami et al., 2004), as well as an increased susceptibility to hepatocyte apoptosis and liver injury (Ishigami et al., 2002), impaired pancreatic β-cell function (Hasegawa, 2010) and scurvy (Kondo et al., 2006). However, no reproductive abnormalities have been reported for RGN knockout mice which display a fertilization capability indistinguishable from their wild-type littermates (Ishigami et al., 2002). On the other hand, RGN-overexpressing transgenic rats (knockin) have been generated by pronuclear microinjection of a transgene containing cDNA-encoding RGN (Yamaguchi et al., 2002b). These rats appear normal, although females display decreased body weight when compared with their wild-type counterparts (Yamaguchi et al., 2002b). Knockin males and females seem to be fertile and able to breed normally (Yamaguchi et al., 2002b). Nevertheless, the reproductive phenotype of both RGN knockin and knockout animals has never been described. No studies have been directed to assess reproductive competence of RGN knockout mice, and nothing is known about litter size, testicular histology and sperm production and morphology or quality in these animals. 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 (Yazama et al., 2006); however, they were able to breed normally (Maeda et al., 2000). 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 (Fisher et al., 1998). However, detailed studies of their reproductive phenotype have revealed that these animals progressively develop a disruption of spermatogenesis, despite no decrease in gonadotrophins or androgens levels (Robertson et al., 1999). A thorough study of the spermatogenic status of RGN knockout 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 that RGN 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 $[\text{Ca}^{2+}]$, RGN is able to regulate cell proliferation and apoptosis and 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 imbalance 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 tests and regulation of spermatozoa production and maturation. In addition, new perspectives of research providing novel clues to understand the aetiology 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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