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Relaxin in the male reproductive system

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Abstract

Relaxin (RLN) belongs to a family of hormones structurally related to insulin and presents a broad spectrum of actions. Humans have three forms of RLN, encoded by three different genes (*RLN1*, *RLN2* and *RLN3*), but nonprimate vertebrates have only two forms of relaxin (RLN1 and RLN3). RLN1 of these animals is encoded by *Rln1*, orthologous to the human *RLN2* gene, and both genes, *Rln1* and human *RLN2*, encode the major form of relaxin found in the male reproductive system. In the reproductive tract of human males, RLN is mainly produced by the prostate and secreted into the seminal fluid, where it seems to play a role in sperm function. RLN may also play a role in prostate cancer progression. A lack of RLN in animal models impairs male fertility, and RLN knockout mice display decreased sperm maturation. The precise role of RLN in the male reproductive system, however, is still far from clear. RLN action is due to its interaction with the G-protein coupled receptor RXFP1. Studies from our laboratory have shown that RLN and RXFP1 are expressed in rat Sertoli cells, and exogenous RLN stimulates Sertoli cell proliferation. RLN receptors can also be detected in rat germ cells at different stages of development, suggesting that RLN may play a direct role in spermatogenesis. The distribution of RLN/RXFP1, however, appears to be species-dependent, because in the boar testis RLN production seems restricted to the Leydig cells, whereas RXFP1 is found in Leydig, Sertoli and germ cells. The co-expression of RLN and RXFP1 in several regions of the male reproductive system suggests that the peptide may act in an autocrine/paracrine fashion.

Keywords: germ cells, male reproductive system, relaxin, RXFP1, Sertoli cells, testis.

Introduction

Relaxin (RLN) was discovered in 1926 by Frederick Hisaw (Hisaw, 1926), who observed that an injection of serum from pregnant guinea pigs or rabbits in non-pregnant female guinea pigs caused relaxation of the interpubic ligament. In 1930, Hisaw and coworkers verified that this effect could be mimicked by a crude extract from the corpus luteum of sows (Fevold *et al*., 1930). During the next 15-20 y, RLN was found to promote the growth of the mammary gland, inhibit uterine contractile activity and soften the uterine cervix (Hamolsky and Sparrow, 1945; Krantz *et al*., 1950; Graham and Dracy, 1953), and RLN therefore became considered a hormone of pregnancy and parturition. Since then, several other actions have been attributed to RLN, including actions in vascular and renal systems, the brain, cancer metastasis and neoangiogenesis, and others (Sherwood, 2004; Bathgate *et al*., 2006). The antifibrotic action of RLN, which results from inhibition of collagen biosynthesis and promotion of collagen breakdown in several tissues, is among the most well established functions of RLN.

Knowledge regarding RLN function greatly improved after the discovery of the RLN receptor (Hsu *et al*., 2002). Although RLN is structurally similar to insulin, RLN interacts with RXFP1, a member of the G-protein coupled receptor (GPCR) family, and stimulates several intracellular pathways. The co-expression of RLN and its receptor in several tissues suggests that RLN may be more important as an autocrine or paracrine mediator than as an endocrine factor, especially in the male, where levels of circulating relaxin are very low. This review intends to present an overview of structural and molecular aspects of the RLN-RXFP1 system, with special emphasis on the RLN role in the male reproductive system.

Relaxin structure and biosynthesis

The structure of RLN was described in the late 1970's: a peptide structurally similar to insulin, with two chains (A and B) linked by disulfide bonds (Schwabe *et al*., 1978). The receptor binding site is located in the middle of the B-chain (Bullesbach and Schwabe, 2005a). The A-chain ensures the correct three-dimensional conformation of the B-chain and permits it to interact with and activate the receptor (Bathgate *et al*., 2006).

The RLN family of peptides structurally related to insulin also includes the insulin-like growth factors IGF1 and 2, the insulin-like peptide of Leydig cell (INSL3), the placental insulin-like peptide (INSL4) and the insulin-like peptides INSL5 and INSL6 (Sherwood, 2004; Ivell *et al*., 2011). Insulin is considered the ancestor of the family, and a RLN3-like ancestor seems

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to have originated the entire RLN peptide family (Wilkinson *et al*., 2005; Ivell *et al*., 2011). The human RLN family encompasses three members, H1-RLN, H2-RLN and H3-RLN, encoded by the *H1*, *H2* and *H3* genes, respectively (Hudson *et al*., 1983, 1984). The *H1* gene is the result of ancestral duplication of the *H2* gene. The *H3* gene encodes the highly conserved neuropeptide H3-RLN (Hsu, 2003; Wilkinson *et al*., 2005; Bathgate *et al*., 2006; Kong *et al*., 2010). The *H1* and *H2* genes are expressed in decidua, placental trophoblasts, the prostate, atria, ventricles, arteries and veins. Only the *H2* gene is expressed in the corpus luteum, endometrium and mammary gland (Hansell *et al*., 1991; Dschietzig *et al*., 2003, 2006). The *H3* gene is expressed in the spleen, the thymus, leukocytes, lymph nodes, testes, and the brain, where H3-RLN plays a role as a neuropeptide (Bathgate *et al*., 2002). Only H2-RLN seems to be secreted into the bloodstream and semen (Bathgate *et al*., 2006). A gene orthologous to human *H1* has been described in monkeys, but is not expressed in rats and mice (Hansell *et al*., 1991, Sherwood, 2004). Rats and mice have a gene orthologous to the human *H2* gene, which encodes the RLN1 of these animals. A gene orthologous to the human *H3* gene has also been described in the rat, mouse and pig, and it encodes the RLN3 of these animals (Bathgate *et al*., 2006).

Similar to insulin, RLN is synthesized as a 21 kDa pre-pro-hormone, with an aminoterminal signal sequence, and B, C and A domains, respectively. The signal peptide is cleaved in the interior of the endoplasmic reticulum to originate pro-RLN, which is transported via Golgi to the secretory granules (Ivell *et al*., 2011). The mature peptide (6 kDa) is produced after a double cleavage that releases the C peptide from the pro-RLN: a prohormone convertase-1 cleaves between the B and C domains, and subsequently furin-like convertases cleave between the C and A domains, releasing the C domain (Marriot *et al*., 1992). Upon specific stimulation, equal amounts of RLN and pro-RLN are released, and pro-RLN, unlike pro-insulin, seems to be biologically active (Vu *et al*., 1993; Zarreh-Hoshyari-Khah *et al*., 2001). Several agents are able to stimulate RLN secretion, including luteinizing hormone, chorionic gonadotropin, basic fibroblast growth factor, progesterone and glucocorticoids (Dschietzig *et al*., 2006).

Relaxin receptors

The RLN receptor remained unknown until 2002, when Hsu and colleagues found that RLN was able to bind and activate the still orphan receptors LGR7 and LGR8, which belong to the LGR (leucine-rich repeat containing GPCRs) family of GPCRs. All members of the LGR family present a large extracellular aminoterminal domain rich in leucine repeats (LRRs; Hsu *et al*., 2002). The LGR family originated during the early evolution of metazoans and comprises three subfamilies (A, B and C), each one presenting a unique LRR domain (Hsu, 2003). Type A LGRs include the follicle-stimulating hormone receptor (FSHR), the luteinizing hormone receptor (LHR) and the thyroid-stimulating hormone receptor (TSHR). In mammals, the type B LGRs (LGRs 4-6) remain orphans. There are only two type C LGRs: LGR7 and LGR8 (Hsu *et al*., 2002). According to the recommendation of the nomenclature committee of IUPHAR (International Union of Pharmacology) LGR7 and LGR8 are now called RXFP1 and RXFP2 (RLN family peptide receptors 1 and 2; Bathgate *et al*., 2006). RXFP1 and RXFP2 contain 10 leucine-rich repeats in the aminoterminal region and are the only LGRs to present a low density lipoprotein (LDL) receptor class A module at the end of the N-terminal region. The LDL-A module is essential for hormone binding, cAMP production, receptor maturation and cell surface delivery (Hsu, 2003; Halls *et al*., 2007; Kern *et al*., 2007).

RLNs from some species appear to bind and activate both RXFP1 and RXFP2. This has allowed the establishment of a rank order of affinity (Halls *et al*., 2007). With human RXFP1, the rank order is H2-RLN = rhesus monkey RLN > porcine RLN > H1-RLN > H3-RLN > rat RLN >> INSL3 (no binding). With RXFP2, the order is INSL3 > H1-RLN = H2-RLN > porcine RLN = rhesus monkey RLN >> H3-RLN = rat RLN (no binding). Therefore, in contrast to human RLN, rat RLN does not activate RXFP2.

Although human RLN can bind to both RXFP1 and RXFP2, the phenotype of RXFP1 knockout mice (Kamat *et al*., 2004; Krajnc-Franken *et al*., 2004) is very similar to that of RLN knockout mice (Zhao *et al*., 1999, Samuel *et al*., 2003a,b), while the phenotype of RXFP2 knockouts (Overbeek *et al*., 2001; Gorlov *et al*., 2002) is similar to INSL3 knockouts (Zimmermann *et al*., 1999), suggesting that RLN is the endogenous ligand of RXFP1 and INSL3 is the endogenous ligand of RXFP2. Furthermore, in vivo pharmacological studies confirmed that INSL3 and RXFP2 represent an exclusive hormone-receptor pair (Bogatcheva *et al*., 2003).

RXFP1 and RXFP2 have two RLN binding sites: a high affinity site, present in the LRR region of the amino-terminal ectodomain, and a secondary low affinity binding site, located in the transmembrane loops (Sudo *et al*., 2003; Halls *et al*., 2005). The presence of the unique LDL-A module at the N-terminus of these receptors is essential for activation of the cAMP signaling pathway. Its absence allows normal ligand-receptor binding, but signaling is absent (Scott *et al*., 2006). In fact, the LDL-A module competitively inhibits RXFP1 function and might be used to suppress RLN signaling. RXFP1-LDL-A expression in prostate cancer cell line PC3 cells inhibited AKT phosphorylation and metalloproteinase 2 activation, and led to the down-regulation of several genes previously implicated in tumorigenesis (Feng and Agoulnik, 2011).

Both the A- and B-domains of RLN are required for RXFP1 and RXFP2 activation. The B-domain is

responsible for primary ligand binding, while the A-domain is responsible for secondary ligand binding. Arginine residues at positions 13 and 17 and an isoleucine or valine at position 20 within the B-domain of RLN are required to bind to specific residues in the LRR domain of the RXFP1 receptor, forming a "RLN binding cassette" (Arg-X-X-X-Arg-X-X-Ile/Val; Bullesbach and Schwabe, 2000, 2005b). The first eight amino acids at the N-terminus of the A-domain of RLN are important for RXFP1 binding affinity and cAMP signaling activation (Hossain *et al*., 2008).

Splice variants of RXFP1 and other members of the LGR family have been described, which can be retained in the endoplasmic reticulum or secreted, and may function as dominant-negative counterparts of the wild-type receptor (Scott *et al*., 2005, 2006; Halls *et al*., 2007; Kern *et al*., 2008).

Relaxin-activated signaling pathways

RLN binding to RXFP1 induces the activation of multiple intracellular effectors (Kong *et al*., 2010). In the human embrionary kidney cell line HEK293T overexpressing RXFP1, RLN induces a biphasic increase of cAMP, caused by an initial activation of G_s and inhibition of G_{0B}, followed by an increase in cAMP that involves βγ subunits of G_{i3} (Halls *et al*., 2006, 2007). βγ subunits of G_{i3} activate the phosphatidyl inositol 3 kinase (PI3K), which, in turn, activates the atypical isoform of protein kinase C, PKC ζ, that activates adenylyl cyclase V/VI (Halls *et al*., 2006, 2007a,b). The last 10 amino acids of the RXFP1 C-terminus, specifically Arg752 residue and partly Ser755, represent an absolute requirement for coupling to the G_{i3}-$G_{\beta\gamma}$-PI3K-PKCζ pathway, and the coupling of the receptor to this pathway is dependent upon localization in the membrane raft microdomains (Halls *et al*., 2009). In other cell lines, such as the human breast cell line T-47D, the increase in cAMP levels is monophasic and is completely due to the activation of $G_{\alpha s}$ (Halls *et al*., 2009).

RLN can activate the synthesis of cAMP in several systems, such as human sperm (Ferlin *et al*., 2011), the pubic symphysis of mice (Braddon, 1978), the rat uterus, where it inhibits spontaneous contractile activity (Sanborn *et al*., 1980), cultured rat myometrial cells (Hsu *et al*., 1985), human endometrial cells (Chen *et al*., 1988; Fei *et al*., 1990), breast cancer cells (Bigazzi *et al*., 1992), and the monocyte/macrophage cell line THP-1, where RLN-induced cAMP production promotes THP-1 adhesion and migration (Figueiredo *et al*., 2006). The Relaxin-stimulated cAMP signaling pathway may also occur in signalosomes (Halls and Cooper, 2010), which are macromolecular receptor-linked protein complexes that facilitate the activation of downstream targets. In this case, RXFP1 is pre-coupled to adenylyl cyclase 2 (AC2) through the A-kinase-anchoring protein (AKAP) 79. Stimulation of RXFP1 with RLN activates AC2, leading to cAMP production. This signalosome is negatively regulated by β-arrestin-2, which binds to Ser704 of RXFP1 and recruits phosphodiesterase PDE4D3 and protein kinase A (PKA) to the protein complex. This non-classic mechanism of cAMP production may explain why circulating relaxin can affect physiological targets where there is minimal production of the hormone.

The stimulation of recombinant or endogenous RXFP1 by RLN can also activate the MAPK pathway. A rapid RLN-induced ERK1/2 activation within 5 min was observed in human endometrial stromal cells, in THP-1 monocytic cells and in smooth muscle cells from coronary artery (Zhang *et al*., 2002). However, this effect may be cell type dependent, because RLN-induced ERK1/2 activation in rat myofibroblasts involved both a rapid (2 min) and a smaller but sustained (50 min) component (Mookerjee *et al*., 2009), and in endothelial cells of the umbilical vein and in HELA epithelial cells, RLN stimulated the ERK1/2 pathway only after much longer periods of stimulation (45-90 min; Dschietzig *et al*., 2003).

RLN stimulates NO production to induce vasodilation. This may involve a PI3K/AKT-mediated activation of endothelial nitric oxide synthase 3 (NOS3), or a PKA-mediated phosphorylation and inhibition of IκB, causing the activation of nuclear factor κB (NFκB) and an increase in nitric oxide synthase 2 (NOS2) gene transcription (Nistri and Bani, 2003). In addition, NO production may be involved in the RLN-mediated inhibition of the differentiation of renal myofibroblasts (Mookerjee *et al*., 2009).

Relaxin in male reproduction

The role of RLN in male reproduction is still unclear (Ivell *et al*., 2011). It was initially thought that RLN was mainly produced by the prostate and released into the seminal fluid to affect sperm motility (Sasaki *et al*., 2001; Kohsaka *et al*., 2003; Sherwood, 2004). For example, antiserum against RLN reduced sperm motility (Sarosi *et al*., 1983), RLN stimulated sperm motility and attenuated the decline in the percentage of motile spermatozoa (Essig *et al*., 1982), helped restore decreased sperm motility (Lessing *et al*., 1986), and improved the penetration of spermatozoa into cervical mucus (Brenner *et al*., 1984). Finally, Carrell *et al*. (1995) demonstrated that recombinant RLN binds to sperm with a high affinity. On the other hand, Jockenhövel *et al*. (1990) and Newinger *et al*. (1990) failed to find an effect of RLN on sperm function. However, it has been found recently that RXFP1 is expressed in human spermatozoa and that RLN stimulates sperm motility, mitochondrial function, apoptosis, capacitation and acrosome reaction (Gianesello *et al*., 2009; Ferlin *et al*., 2011; Miah *et al*., 2011), providing additional evidence that RLN is important for fertilizing ability and preservation of

sperm functionality.

With the recent availability of knockout animals for RLN or its receptors, it has been possible to establish the physiological importance of this hormone. RLN has an antifibrotic effect in several tissues, and the RLN knockout mouse is a model of fibrosis (Samuel *et al*., 2003a,b, 2005). RLN interferes with collagen metabolism and increases the expression and activity of metalloproteinases (MMPs) in uterine, cardiac, vascular and renal tissues (Lenhart *et al*., 2001; Jeyabalan *et al*., 2003, 2007; Mookerjee *et al*., 2005).

In the reproductive tract of female mice, the disruption of the *Rln* or the *Rxfp1* gene causes the same abnormalities: an absence of the relaxation and elongation of the interpubic ligament and impaired nipple development (Zimmermann *et al*., 1999; Kamat *et al*., 2004). In the male reproductive system, however, studies with knockouts of *Rln* (*Rln*$^{-/-}$) or *Rxfp1* (*Rxfp1*$^{-/-}$) genes are controversial (Ivell *et al*., 2011). Studies from Samuel *et al*. (2003a,b, 2005) with *Rln*$^{-/-}$ suggest that RLN plays a major role in the growth and development of the male reproductive system. These authors showed that *Rln*$^{-/-}$ mice have smaller testes, epididymides, prostates, seminal vesicles, decreased sperm maturation and decreased epithelial proliferation in the prostate (Samuel *et al*., 2003a, b, 2005). They also report notable changes in the extracellular matrix of the testis and prostate in *Rln*$^{-/-}$ mice, and increased apoptosis. Another *Rln* knockout developed by Ganesan *et al*. (2009), however, failed to show similar effects. The consequences of the disruption of the *Rxfp1* gene are not clear either. Kamat *et al*. (2004) did not find abnormalities in the testes and prostate of *Rxfp1*$^{-/-}$ mice, whereas Krajnc-Franken *et al*. (2004), using a different strain of knockout mice, observed impaired spermatogenesis, leading to azoospermia and reduced fertility in animals from the first generations, even though the following generations and older animals had normal fertility.

Although most RLN production in the male reproductive system occurs in the prostate, studies from ours and other laboratories have demonstrated that the testes are also a source of RLN (Gunnersen *et al*., 1995; Kohsaka *et al*., 2009; Cardoso *et al*., 2010; Kato *et al*., 2010). It seems that the main site of relaxin expression in the testes varies depending on the species. While RLN in the rat is mainly found in the seminiferous epithelium and is absent in the interstitium (Cardoso *et al*., 2010; Fig. 1A and 1B), in the boar testis RLN expression is only found in Leydig cells (Kato *et al*., 2010).

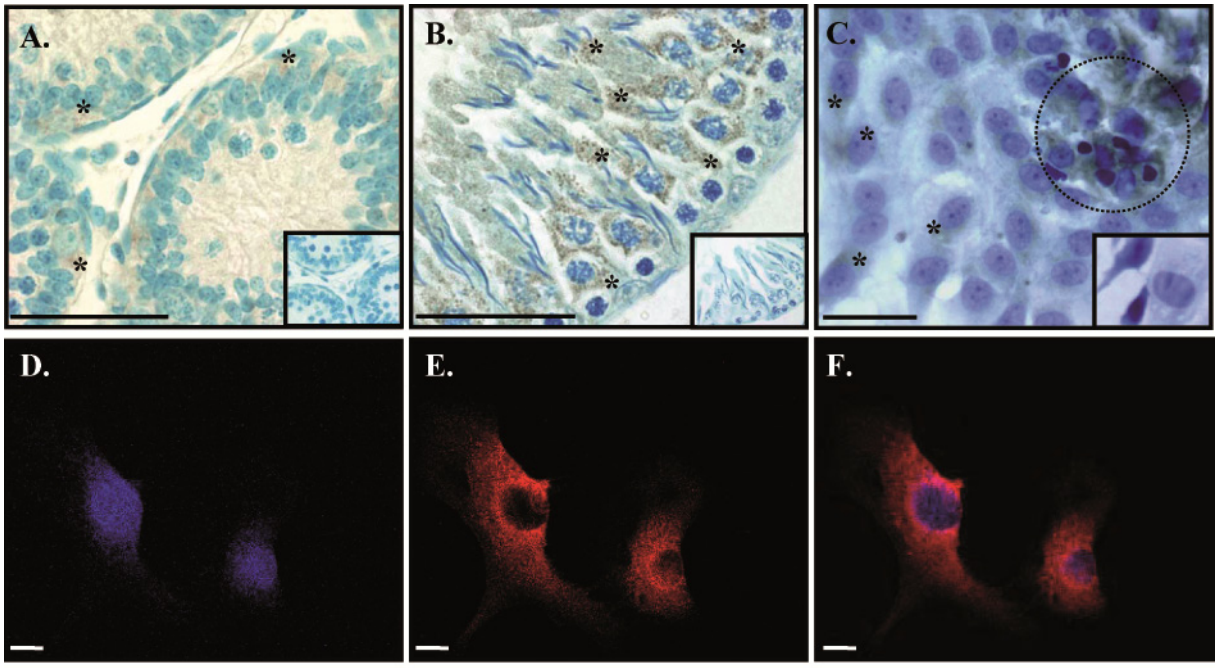

Figure 1. Immunolocalization of relaxin in testicular cells using anti-rat relaxin antibody (ab70803, Abcam; 1:100 dilution). **(A)** Testis section from immature, 15-day old rats. **(B)** Testis section from adult, 120-day old rats. RLN immunoreactivity (asterisk) was observed in the seminiferous epithelium but not in the interstitial compartment. **(C)** Co-culture of Sertoli and germ cells from 7-day old animals after 7 days in culture. RLN immunoreactivity was observed in Sertoli cells (asterisk) and in germ cells (circle). **(D) to (F)** Primary culture of Sertoli cells from 15-day old animals after 4 days in culture. **(D)** Nuclei were stained with DAPI (blue). **(E)** Relaxin immunofluorescence (red) was detected with Alexa Fluor 594-labeled IgG. **(F)** Merged image. Bars = 10 µm. Insets show negative controls incubated in the absence of the primary antibody. **(A)** and **(B)** were reproduced from Cardoso *et al*. (2010).

RLN mRNA levels are higher in the testis of immature than adult rats (Cardoso *et al*., 2010). Sertoli cells of immature 15 day-old rats represent an important source of RLN mRNA, and a relaxin precursor is expressed in cultured Sertoli cells from 15-day old rats (Fig. 1D-1F). Furthermore, RLN induces the proliferation of cultured rat Sertoli cells, suggesting an autocrine/ paracrine role for RLN in the testis (Filonzi *et al*., 2007; Cardoso *et al*., 2010). We have recently investigated the signaling pathways involved in the proliferative effect of RLN in a primary culture of Sertoli cells from immature rats. In this system, the mitogenic effect of RLN involved the activation of both the MEK/ERK1/2 and PI3K/AKT pathways (unpublished data). Furthermore, since RLN stimulates NO production in several systems by PKA-mediated phosphorylation and inhibition of IκB, causing the activation of nuclear factor κB (NFκB) and an increase in gene transcription of inducible NOS (*Nos2*; Nistri and Bani, 2003), we investigated the effect of RLN on the mRNA levels of *Nos2* in the primary culture of rat Sertoli cells. Preliminary experiments of real time RT-PCR have shown that RLN tended to increase the expression of *Nos2* (Fig. 2). This result is intriguing because studies with *Nos2*$^{-/-}$ mice have demonstrated that NOS2 plays an important role in the regulation of somatic cell number in the testis, with an impact on the survival of germ cells (Auharek *et al*., 2011).

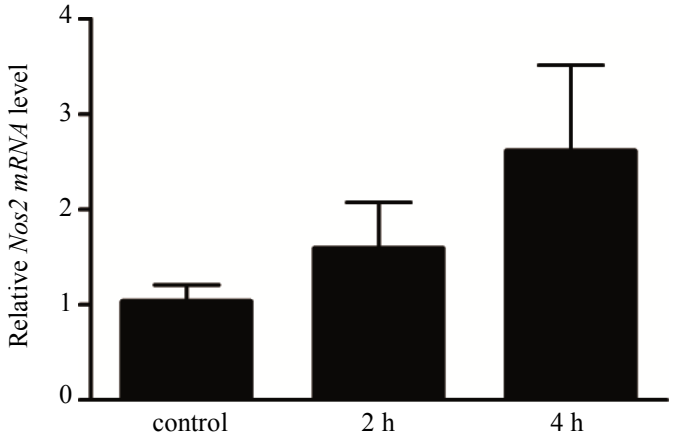

Figure 2. Relative mRNA level of *Nos2* in primary culture of Sertoli cells from 15-day old rats. Cells were treated with recombinant human relaxin (Phoenix Pharmaceuticals) for 2 and 4 h. The level of *Nos2* mRNA was analyzed by quantitative RT-PCR as previously described (Cardoso *et al*., 2010). Results are expressed as mean ± S.E.M. of 6 independent experiments (6 different cultures).

We have previously shown that mRNA for RXFP1 is widely distributed in the reproductive system of the male rat, with the testis and vas deferens having the highest levels (Filonzi *et al*., 2007). In the testis, immunohistochemistry showed the expression of RXFP1 in Sertoli and post-meiotic germ cells (elongated spermatids). In addition, Kato *et al*. (2010) characterized by semi-quantitative RT-PCR and immunohistochemistry the expression of RXFP1 in Leydig and Sertoli cells of the boar testis during postnatal development. RXFP1 transcripts have also been detected in the rhesus monkey testis (Silvertown *et al*., 2010). In the vas deferens, RXFP1 immunoreaction was detected in the smooth muscle layer and apical part of epithelial cells, suggesting a role in the secretion and composition of the seminal plasma (Filonzi *et al*., 2007). After incubation with vas deferens slices, RLN increased the tissue level of matrix metalloproteinase 7 mRNA (Filonzi *et al*., 2007), suggesting that RLN may be involved in collagen and matrix remodeling and/or apoptosis. This suggestion seems consistent with the general role of RLN as a regulator of collagen biosynthesis in several tissues.

The role of RLN receptors in the adult testis remains to be determined. Since RXFP1 receptors are expressed in germ cells during specific stages of the development, one may speculate that RLN participates in the spermatogenic process. The localization of RXFP1 receptors in Sertoli cells further supports the idea that the hormone plays a role in spermatogenesis. To investigate the role of RLN at specific stages of spermatogenesis, we developed a co-culture system of Sertoli and germ cells from 7-day old rats. After 7 days of culture, we observed the differentiation of both Sertoli and germ cells, and the expression of RLN (Fig. 1C) and RXFP1 (not shown). This co-culture provides an attractive tool to characterize the role of RLN in spermatogenesis.

RLN seems to play a role in prostate cancer progression (Thompson *et al*., 2006; Feng *et al*., 2007). H1-RLN was upregulated in tumors compared to normal prostate tissue (Welsh *et al*., 2003). The transcriptional regulation of RLN in the prostate has not been elucidated yet. The RLN promoter is positively

regulated by androgens in the PC3 prostate cancer cell line transfected with the androgen receptor, but not in LNCaP and non-prostate cells such as liver, kidney, bladder, lung, breast and ovarian cells (Brookes *et al*., 1998). In addition, castration of rats drastically inhibits RLN mRNA levels in the prostate, and this is recovered by treatment of the castrated animals with testosterone (Cardoso *et al*., 2010).

Concluding remarks

Although insight in the role of RLN and RXFP1 has improved significantly in several systems, relatively little is known about the role of RLN in male reproduction. RLN is released in the seminal plasma and affects semen function. More recent studies show that RLN is present throughout the male reproductive tract, and its co-localization with RXFP1 suggests that RLN may act as an autocrine or paracrine factor. The control of RLN expression is poorly understood, and the interplay between RLN and other local factors and hormones, such as testosterone and FSH, is not yet clear. Advances in the molecular characterization of RXFP1 structure and signaling might provide important tools for the treatment of reproductive disorders in the male. The better understanding of the role of RLN in the progression of prostate cancer may reveal its utility for the development of new therapeutic agents.

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