Evidence for expression of relaxin hormone-receptor system in the boar testis

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Abstract

Although the physiological role of relaxin (RLN) in males remains largely unknown, there is limited evidence that the testis might be a candidate source and target of RLN in boars, as *RLN* transcripts are detected in the boar testis and it contains RLN-binding sites. To determine whether the boar testis acts as a source and target tissue of RLN, we characterised the expression pattern and cellular localisation of both RLN and its own receptor LGR7 (RXFP1) in boar testes during postnatal development by molecular and immunological approaches. Testes were collected from Duroc boars, and partial cDNA sequences of the boar homologue of human *RXFP1* were identified. *RLN* expression increased through puberty onwards, while *RXFP1* expression changed little during development.

RLN mRNA and protein expression were restricted to the Leydig cells, whereas both Leydig cells and seminiferous epithelial cells expressed *RXFP1* mRNA and protein. Interestingly, RLN was expressed in the testis as an 18 kDa form (the expected size of proRLN), but not as the 6 kDa mature form, during development because of a lack of the enzyme required for proRLN processing. In contrast, RXFP1 was detected at all stages as specific bands of 75 and 91–95 kDa (likely non-glycosylated and glycosylated RXFP1 respectively). Thus, we provide evidence for expression of RLN–RXFP1 ligand–receptor system in the boar testis, suggesting that the testis act as a source and possible target tissue of RLN.

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Introduction

Testicular function is regulated by the complex interplay of many different molecules that include both endocrine and paracrine signalling, and proper regulation is critical for optimum reproductive capacity. Relaxin (RLN) was initially described as a hormone of pregnancy that is best known for its role during parturition in various species, but it has also been implicated in regulation of testicular function, although testicular source of the hormone in mammalian males still remains largely unknown (Sherwood 1994, 2004, Kohsaka *et al.* 2003). Recent studies in adult male mice with knockout of the gene encoding either RLN or its distinct receptor, now known to be RLN family peptide receptor 1 (RXFP1; originally called LGR7, leucine-rich repeat-containing G-protein-coupled receptor 7) (Hsu *et al.* 2002), have demonstrated a decrease in sperm maturation, an increase of apoptosis in the testis and a reduction in the fertility of such mice (Samuel *et al.* 2003, Krajnc-Franken *et al.* 2004), although another study indicated normal male fertility in *Rxyp1* knockout mice (Kamat *et al.* 2004).

Only in the boar, there is limited evidence that the testis might be a candidate source and target of RLN, although it is presently unknown whether RLN actually has any effect on the testis. An earlier study indicated the presence of immunoreactive RLN in the interstitial cells and Sertoli cells of the boar testis by indirect immunofluorescence with antiserum for crude porcine RLN (Dubois & Dacheux 1978), although subsequent studies could not confirm this finding when repeated with antiserum for purified porcine RLN (Arakaki *et al.* 1980, Kohsaka *et al.* 1992). Nevertheless, a study using the reverse transcription and PCR (RT-PCR) method demonstrated the presence of *RLN* transcripts in the testis (Lobb *et al.* 1995). Furthermore, a ligand-binding study has revealed that biotinylated porcine RLN binds to the boar testis (Min & Sherwood 1998). However, no studies have yet been carried out to identify whether *RLN* mRNA is translated into RLN protein in the boar testis, and if so, which cell type(s) actually produce RLN. Furthermore, its specific receptor *RXFP1* and protein have not been identified in the boar testis. In addition, the possible site(s) of action of

RLN within the testis, i.e. the cellular expression and distribution of RXFP1, is still unclear. Interestingly, our preliminary study suggested the possibility for the presence of RLN and its receptor gene in the testis (Kohsaka *et al.* 2009).

As a major step towards elucidating the potential role of RLN in boar testicular function, this study was performed to determine whether the testis acts as a source and target tissue of RLN in boars by characterising the expression and cellular localisation of both RLN and its own receptor RXFP1 and their developmental changes in the boar testis. We used both molecular and immunological approaches, such as RT-PCR, semi-quantitative RT-PCR, cell fractionation, western blotting and immunohistochemistry, to characterise the mRNA and protein levels. For these immunological approaches, rabbit polyclonal antisera against highly purified RLN from pregnant sow corpora lutea (CL) and the recombinant C-terminal intracellular domain (endodomain) of porcine RXFP1 expressed in *Escherichia coli* were generated.

Materials and Methods

Animals and tissue sampling

Duroc boars from the Shizuoka Swine and Poultry Experimental Station were used in this study. Testes were obtained by castration or just after slaughter of 1-week-old (postnatal, $n=3$), 5-, 7- and 9-week-old (prepubertal, $n=3$ for each group), 18- and 30-week-old (pubertal, $n=3$ for each group), and 38-week-old (postpubertal, $n=2$) boars. After removal of the epididymis, each testis was cut in half longitudinally through the midline, and the capsule was partially removed. Small cubes ($0.5\text{--}2\text{ cm}^3$) of tissue were cut from the parenchyma in the medial part of the testis with a razor blade or scalpel. In addition, various tissues, including the cerebrum, were harvested from pubertal Duroc boars at 18 weeks of age. Furthermore, the CL, uterus and placenta were collected from two Duroc sows on days 50 and 100 of pregnancy. Pregnant CL is well known as the primary source of RLN and was used as a control for RLN production. Pieces of the harvested tissues were rapidly frozen and stored in liquid nitrogen until RNA extraction, protein extraction or testicular cell fractionation, while other specimens were fixed in Bouin's solution and embedded in paraffin for immunohistochemistry. For *in vitro* incubation study of Leydig cells, fresh testes harvested from prepubertal Duroc boars at 7 weeks of age were used. All procedures were performed in accordance with Shizuoka University of Health Guidelines for Care and Use of Experimental Animals.

Fractionation of testicular cells

Testicular cells were fractionated by a modification of the methods described by Raeside & Renaud (1983) and Koga *et al.* (1998). Small tissue cubes taken from the pubertal testis

at 18 weeks of age were cut thinly by hand to obtain tissue slices measuring $1\text{ cm}^2 \times 3\text{--}5\text{ mm}$ thick. Testicular slices were placed in cold 10 mmol/l PBS (pH 7.2) in plastic Petri dishes (Becton Dickinson, Franklin Lakes, NJ, USA), shaken vigorously to remove some germ cells including spermatozoa that had begun to leak from the lumen of the seminiferous tubules and washed with PBS. The testicular slices were then placed in DMEM/Ham's F12 (DMEM/F12; Gibco) containing 0.05% w/v collagenase (Type 1; Gibco) in Petri dishes (Becton Dickinson), and the seminiferous tubules were gently teased apart with fine forceps, after which incubation was done at 33°C for 15 min. After the addition of PBS containing 1 mM EDTA (EDTA-PBS), the tubular suspension was transferred into a conical test tube and left to stand for 2 min at room temperature to precipitate fragments of the tubules. The supernatant containing separated cells was filtered through $70\text{ }\mu\text{m}$ steel mesh and centrifuged at 800 g for 5 min, after which the precipitate was used as the Leydig cell fraction. The remaining tubular suspension was incubated in DMEM/F12 containing 0.05% w/v collagenase at 33°C for 15 min and then dispersed by gentle pipetting several times in EDTA-PBS to remove residual Leydig cells. Next, the tubules were placed in Petri dishes, cut into small segments with a razor blade and transferred to a 15 ml conical test tube. After the addition of EDTA-PBS, the test tubes were kept standing for 5 min, and the supernatant was filtered through steel mesh to obtain the germ cell fraction. The remaining tubular sediment was vigorously pipetted several times and then left to stand for 5 min. Finally, the supernatant (mainly containing residual germ cells) was discarded, and the sediment was used as the tubular cell fraction, which appeared to comprise both peritubular tissue constituting tubular wall and mostly Sertoli cells with only some germ cells. Thus, this tubular cell fraction was used as the Sertoli cell fraction. The fractionated testicular cells were snap frozen and stored in liquid nitrogen for extraction of RNA. The quality of cellular fractionation was verified by detecting $3\text{-}\beta$ hydroxysteroid dehydrogenase (*HSD3B*), protamine 1 (*PRM1*) and inhibin α (*INH4*) gene expression, which are specifically expressed by Leydig cells (Raeside & Renaud 1983, Chemes *et al.* 1992), germ cells (Domenjoud *et al.* 1991) and both Sertoli and Leydig cells (Jin *et al.* 2001) respectively.

RNA extraction and RT-PCR

Total RNA was extracted by a modification of the method of Chomczynski & Sacchi (1987) using ISOGEN reagent (Nippon Gene, Tokyo, Japan). The amount and purity of the RNA were determined by spectrophotometry (Biophotometer; Eppendorf, Hamburg, Germany) at 260 and 280 nm, whereas its quality was assessed by u.v. visualisation of rRNA bands stained with ethidium bromide after agarose gel electrophoresis. Aliquots of $10\text{ }\mu\text{g}$ of total RNA were used for first-strand cDNA synthesis in $20\text{ }\mu\text{l}$ reaction mixtures using oligo(dT)₁₈ primer with a RT kit (Invitrogen Life Technologies).

Table 1 List of primers employed for boar target genes

Gene	Forward primer (5'→3')	Reverse primer (5'→3')	Annealing (°C)	Product (bp)	GenBank accession no.
<i>RLN</i>	gtctggctgctcctgagccaact	agcaatatctttctgatacaacc	50	506	NM213872
<i>RXFP1</i> (outer)	acagcaactgaaatacggaaatca	oligo(dT) ₁₈	50	517	NM021634
<i>RXFP1</i> (inner)	gaaatgatccatcagtttgg	tcatgaataggaattgagtc	50	210	–
<i>GAPDH</i>	gtgaaggtcggagtcacacggat	caaattcattgtcataccagga	53	947	AF017079
<i>PRM1</i>	actgagtcacatccatcacca	tagcaggctcctgtcagtc	53	283	NM214253
<i>INHA</i>	cctccagtttcatcttcca	agtgctgggtgagaaggttg	55	273	NM214189
<i>HSD3B</i>	accagaagttcgggaggaat	ctctatgctgctggtgtgga	56	263	AB529538
<i>PC1/3</i>	attacaccgaccagcgaatc	ccagagcittggcatttagc	55	273	NM214038

The primers for target genes are summarised in Table 1. The primer pair for amplification of porcine *RLN* was prepared according to the sequence designed by Lobb *et al.* (1995) to yield a product of 506 bp, which encompassed sequences beginning at the signal peptide and spanning the B- and C-chains to reach the mid-point of the A-chain. On the other hand, for amplification of the coding sequence of the endodomain of porcine *RXFP1*, nested RT-PCR was performed using outer and inner primer pairs (Table 1). The outer forward primer was designed according to the human *RXFP1* sequence, whereas the inner primer pair was specific for porcine *RXFP1*.

PCR was carried out in a 50 µl reaction mixture containing 1 µl of cDNA template, 5 µl of 10× PCR buffer, 10 mmol/l of dNTP mix, 20 pmol of each primer and 2.5 U of Taq DNA polymerase (New England Biolabs, Beverly, MA, USA). Amplification was performed using a DICE PCR Thermal Cycler (Takara Bio, Shiga, Japan) with the following programme: initial denaturation for 2 min at 94 °C, followed by 30 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 50–56 °C and extension for 1 min at 72 °C, followed by a final extension step for 10 min at 72 °C.

The PCR products were electrophoresed on 2% w/v DNA agarose gels (LO3 agarose; Takara Bio), and were visualised under u.v. light after staining with ethidium bromide. In addition, the 506 bp *RLN* product and 210 bp *RXFP1* endodomain product were cloned into the pGEM-T EASY vector (Promega) and sequenced with a cycle sequencing kit containing BigDye fluorescent terminators on a model 3730XL sequencer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

Semi-quantitative RT-PCR

Optimised semi-quantitative PCR was employed to analyse the overall levels of *RLN* and *RXFP1* expression in the testis at different stages. Extraction of total RNA and first-strand cDNA synthesis for each sample were performed as described above. Total RNA was treated with DNase (Nippon Gene) before RT to ensure the absence of genomic DNA contamination, and reactions without RT were performed to rule out the possibility of spurious amplification of the signals.

The primer pairs are shown in Table 1. Amplification of *RXFP1* was carried out with the inner primer pair (Table 1). To enable appropriate exponential phase amplification of each target, PCR amplification of *RLN*, *RXFP1* and *GAPDH* transcripts was carried out by separate reactions with a different number of cycles, but using a similar amount of cDNA as the template, in a final reaction volume of 25 µl in the presence of 1.25 U of Taq DNA polymerase (New England Biolabs) and the relevant specific primers. After initial denaturation at 94 °C for 2 min, a variable number of amplification cycles were performed at 94, 50 and 72 °C (45 s each) for *RLN*; 94, 50 and 72 °C (30 s each) for *RXFP1*; and 94, 52 and 72 °C (1 min each) for *GAPDH*. Amplification was followed by a final extension step at 72 °C for 5 min. The optimal numbers of PCR cycles for exponential amplification were determined for each gene. Plotting the intensity of PCR signals expressed as log optical density (OD) values against the number of amplification cycles revealed a strong linear relationship from cycles 28 to 32 for *RLN* (correlation coefficient $r^2=0.9756$), cycles 37–44 for *RXFP1* ($r^2=0.9764$) and cycles 23–27 for *GAPDH* ($r^2=0.9631$). Thus, PCR amplification of *RLN*, *RXFP1* and *GAPDH* transcripts was carried out using 30, 40 and 25 amplification cycles respectively.

The cDNA fragments generated were subjected to electrophoresis on 2% w/v agarose gels (LO3 agarose; Takara Bio) containing ethidium bromide (0.1 µg/ml) and visualised under u.v. light. The molecular sizes were determined by comparison with size markers running together with the PCR products, and the specificity of the products was confirmed by direct sequencing (data not shown). For quantitative evaluation, the OD of the RT-PCR products was measured by densitometric scanning using NIH Image J software (<http://rsb.info.nih.gov/ij/>). The value obtained for each product was normalised to that of *GAPDH* to express the relative abundance of each message.

Preparation of porcine *RLN*

The porcine *RLN* used here was highly purified R-II 1 *RLN* obtained from pregnant sow CL, as described by Kohsaka *et al.* (1993). This *RLN* has 22 amino acid (aa) residues in the

A-chain and 29 aa residues in the B-chain, with a total molecular mass of 5815 kDa. It is equivalent to CM-a' RLN, as designated by Sherwood & O'Byrne (1974).

Preparation of recombinant porcine RXFP1 endodomain

The recombinant endodomain of porcine RXFP1 (RXFP1 endodomain) was expressed in the pMAL-c2 prokaryotic expression vector (New England Biolabs) as a fusion protein with maltose-binding protein (MBP). Briefly, the cDNA of the boar *RXFP1* endodomain cloned into the pGEM-T EASY vector was subjected to PCR amplification using primers incorporating HincII and HindIII restriction sites. The PCR product was then cleaved with HincII and HindIII and inserted into the pMAL-c2 expression vector, which had previously been cut with XmnI and HindIII. Plasmid construction was confirmed by DNA sequencing, and the pMAL-c2 plasmid with *RXFP1* endodomain insert was used to transform competent *E. coli* JM109 cells (Toyobo, Osaka, Japan).

After overnight culture at 37 °C in Luria Broth (LB) containing 50 µg/ml ampicillin and 0.2% w/v glucose (LB-AG), cells were diluted 1:90 in fresh LB-AG, incubated at 37 °C to an A_{600} of 0.8 and induced for 120 min with 0.3 mmol/l isopropyl-β-D-thiogalactopyranoside (IPTG; Wako Pure Chemicals, Osaka, Japan). The cultures were then centrifuged at 4000 g for 20 min, and the cell pellets were stored at -80 °C. The pellets were thawed, resuspended in 20 mmol/l Tris-HCl (pH 7.4) containing 200 mmol/l NaCl, 1 mmol/l EDTA, 10 mmol/l β-mercaptoethanol and 1 mmol/l Na₂ (column buffer), and sonicated ten times for 1 min each time in an ice water bath. After centrifugation at 14 000 g for 20 min at 4 °C, the supernatant was applied to an amylose resin (New England Biolabs) affinity column (2.5×2.5 cm) preequilibrated with column buffer at a flow rate of 1 ml/min. After washing with a sufficient volume of the column buffer, the absorbed protein (fusion protein) was eluted with column buffer containing 10 mmol/l maltose. The eluted protein was further purified by HPLC with a model L-6300 unit (Hitachi) and a Wakosil ODS-5C18T column (4×150 mm; Wako Pure Chemicals).

Production of antisera for RLN and RXFP1

Rabbit polyclonal antisera for porcine RLN and the RXFP1 endodomain were generated with highly purified R-II 1 RLN obtained from pregnant sow CL and the recombinant MBP-RXFP1 endodomain expressed in *E. coli* respectively, being produced by a modification of the method of Vaitukaitis *et al.* (1971). Briefly, purified porcine RLN or recombinant MBP-RXFP1 endodomain (3 mg/ml in PBS) was emulsified in an equal volume of Freund's complete adjuvant (Difco, Detroit, MI, USA) containing 5 mg of desiccated Bacto M. Butyricum (Difco) and immunised adult male New Zealand white rabbits ($n=2$ for porcine RLN and $n=3$ for MBP-RXFP1 endodomain) by intradermal

injection at around 100 sites. A suspension of inactive *Bordetella pertussis* (4×10^{10} cells/ml; Wako Pure Chemicals) was also administered by intradermal injection at the time of primary immunisation. From 40 days later, booster injections consisting of the same quantity of antigen in Freund's incomplete adjuvant (Difco) were given twice with an interval of 2–3 weeks, and blood was collected from the ear vein 3–5 weeks after the booster injections. Blood of sufficiently high titre was collected from an ear vein and by heart puncture, after which serum was separated and stored at -80 °C. The titre of the antiserum was monitored by ELISA.

Specificity of antisera for RLN and RXFP1

The specificity of anti-RLN antiserum was verified by western blotting with immunising antigen (purified porcine RLN) and recombinant RLN-like factor (RLF)/insulin-like factor 3 (INSL3), which was expressed as proform of ~16 kDa in *E. coli* using the expression construct having proRLF/INSL3 sequence inserted into pCold I vector as described by us (Sigin *et al.* 2010). On the other hand, the specificity of anti-RXFP1 antiserum was also verified by western blotting with immunising antigen (recombinant MBP-RXFP1 endodomain) and recombinant MBP-RXFP2 endodomain. RXFP2 is the receptor for RLF/INSL3, and endodomain of human RXFP1 and RXFP2 is considerably divergent in amino acid sequence homology (Hsu *et al.* 2002). Thus, recombinant porcine RXFP2 endodomain was prepared by the same method as the recombinant RXFP1 endodomain: based on predicted cDNA sequence (GenBank accession no. XM001927967) of porcine homologue of human *RXFP2*, the sequence encoded a reduced peptide sequence of 55 aa (nucleotides 1681–1848), which corresponded to endodomain of porcine *RXFP2* (aa 561–615), was generated by RT-PCR and expressed as a fusion protein with MBP. The specificity of both antibodies was also validated by running preadsorption controls in which each antibody was incubated overnight in antibody diluent with an excess of immunising antigen (1 mg/ml porcine RLN for RLN antibody and 400 µg/ml MBP-RXFP1 endodomain for RXFP1 antibody).

Western blotting

To detect RLN, testicular extracts were prepared by a procedure that minimised proteolysis (Walsh & Niall 1980, Kohsaka *et al.* 1993). For detection of RXFP1, testicular and uterine tissue samples were extracted in lysis buffer (100 mmol/l potassium phosphate buffer, pH 7.4, 1 mmol/l dithiothreitol, 1 mmol/l EDTA, 20% v/v glycerol and 0.6% w/v cholic acid) containing a protease inhibitor cocktail (Boehringer Mannheim). The protein concentration of the homogenates was determined by the method of Lowry and Folin with BSA as the standard (Hess *et al.* 1978).

Samples (35 µg of protein/lane for tissue extracts and 2 µg/lane for purified proteins) were separated by

SDS-PAGE under reducing condition on 9% w/v gels for RXFP1 and under non-reducing condition on 14% w/v gels for RLN. Next, the proteins were transferred onto nitrocellulose membranes (Bio-Rad) using a semi-dry transfer cell (Bio-Rad), and stained with Ponceau to verify equivalent protein loading. The membranes were blocked for 1 h in TBST (20 mmol/l Tris-HCl, pH 7.4, 150 mmol/l NaCl and 0.05% v/v Tween-20) and then incubated for 2 h at room temperature with a 1:20 000 dilution of rabbit anti-RLN serum or anti-RXFP1 serum in TBST. As negative controls, some membranes were incubated with primary antisera after absorption with immunising antigens. After washing three times for 5 min each in PBST, the membranes were incubated with peroxidase-conjugated goat anti-rabbit IgG (ICN/Cappel, Aurora, OH, USA) diluted 1:40 000 in TBST. Specific signals were detected using an ECL system (Amersham Biosciences). As the loading control, ACTB (also known as β -actin) was detected by sequential incubation with monoclonal antibody to ACTB (Sigma), peroxidase-conjugated goat anti-mouse IgG (Zymed Laboratories, San Francisco, CA, USA) and ECL (Amersham Biosciences).

Immunohistochemistry

Paraffin sections (3 μ m) were cut and then mounted on microscope slides coated with 0.02% v/v poly-L-lysine (MW. 3 000 000). Deparaffinised sections were heated at 80 °C for 30 min in 10 mmol/l sodium citrate buffer (pH 9.0) to perform antigen retrieval, as described by Jiao *et al.* (1999). After cooling to room temperature, the sections were rinsed in PBS, treated with 3% v/v hydrogen peroxidase in PBS for 20 min to block endogenous peroxidase activity and then incubated for 20 min with 10% v/v normal goat serum to saturate non-specific binding.

The sections were next incubated for 2 h with rabbit anti-RLN serum diluted 1:1500 in PBS or with rabbit anti-RXFP1 serum diluted 1:1000 in PBS, followed by incubation with goat anti-rabbit IgG polymer conjugated to peroxidase (DakoCytomation, Carpinteria, CA, USA) for 30 min at room temperature. After each step, the sections were washed three times for 5 min each in PBS. As negative controls, some sections were incubated with primary antisera after absorption with immunising antigen.

Visualisation of peroxidase activity was performed using 3,3'-diaminobenzidine (DakoCytomation) as the substrate. After washing in running water, the sections were counterstained with haematoxylin (Wako Pure Chemicals), dehydrated in a graded ethanol series, mounted and examined under a BX50 Olympus microscope equipped with a CCD camera (DP50; Olympus, Tokyo, Japan).

Statistical analysis

Values were presented as the means $\pm$ S.E.M. of three animals in each group, except at 38 weeks ($n=2$) for semi-quantitative RT-PCR data. Data were analysed by one-way ANOVA,

together with Fisher's Protected Least Significant Difference multiple range test to compare means of different groups. $P<0.05$ was considered statistically significant.

Results

Gene expression and tissue distribution of RLN and its distinct receptor RXFP1 in the boar testis

To examine the expression of RLN and its receptor RXFP1, RT-PCR analysis was performed with total RNA prepared from the testes of 18-week-old pubertal boars. RT-PCR revealed a single amplification product of 506 bp encoding RLN (Fig. 1A). The RLN transcript encompassed sequences from the signal peptide through the B- and C-chains to reach the mid-point of the A-chain of known porcine RLN cDNA

Figure 1 Gene expression and tissue distribution of RLN and its receptor RXFP1 genes in the boar. RT-PCR analysis was performed with total RNA from various tissues of 18-week-old pubertal boar. (A) Gene expression of RLN and its receptor RXFP1 in the testis. RT-PCR revealed a single amplification product of 506 bp encoding RLN mRNA and an amplicon of 210 bp encoding RXFP1 mRNA. (B) Tissue distribution of RLN and RXFP1 gene expression. RLN was strongly expressed in the testis, while there was weak expression in the brain, lungs and prostate gland. No expression was detected in other organs or tissues, such as the heart, spleen and kidneys. In contrast, RXFP1 was expressed in a wide range of organs and tissues in addition to the testis.

(data not shown). On the other hand, RT-PCR found an amplicon of 210 bp encoding RXFP1 (Fig. 1A); the sequence is shown in Fig. 2. The partial *RXFP1* cDNA sequence of 210 bp encoded a deduced peptide sequence of 69 aa, which corresponded to the endodomain of human RXFP1 (Hsu *et al.* 2002). Boar RXFP1 peptide displayed 82.6% aa homology with human RXFP1. In contrast, boar RXFP1 peptide displayed only 11% aa homology with the endodomain of predicted porcine RXFP2 (GenBank accession no. XM001927967).

The tissue distribution of *RLN* and *RXFP1* was also examined by RT-PCR analysis from various tissues of pubertal boars (Fig. 1B). *RLN* transcripts were mainly expressed in the testis, but weak expression was also detected in the brain, lungs and prostate gland. No expression was detected in other tissues, such as the heart, spleen, liver and kidneys. *RLN* transcripts were also expressed in pregnant CL, which is the primary source of *RLN*, while weak expression was noted in the uterus and placenta (secondary sources of *RLN*). In contrast, the *RXFP1* transcripts were expressed in a wide range of organs and tissues in addition to the testis.

Cellular expression of *RLN* and *RXFP1* genes in the boar testis

To examine the cellular expression of *RLN* and *RXFP1* transcripts within the testis, fractionated testicular cells were prepared from the pubertal testes of 18-week-old pubertal boars and examined by RT-PCR analysis. When fractionation was assessed by histological staining, the germ cell fraction was observed as a mixture of spermatids and spermatozoa (Fig. 3A). The Sertoli cell fraction appeared to comprise both peritubular tissue constituting the tubular wall and mostly Sertoli cells, with only some germ cells that appeared to be spermatogonia (Fig. 3A). The Leydig cell fraction mainly contained Leydig cells with purity of 85–90% (Fig. 3A). The purity of these fractionated cells was also verified by determining the expression of *HSD3B*, *PRM1* and *INHA*, which are expressed specifically by Leydig cells (Chemes *et al.* 1992), germ cells (Domenjoud *et al.* 1991) and both Sertoli and Leydig cells (Jin *et al.* 2001) respectively. Fractionation was shown to be quite satisfactory, i.e. *HSD3B* and *PRM1* expression were limited to the Leydig cell and germ cell fractions respectively, while expression of *INHA* was found in both the Sertoli and Leydig cell fractions

	1	GAA	ATG	ATC	CAT	CAG	TTT	TGG	TAT	AAC	TAC	AGA	CAA	AGA	CGA	TCC	45
Pig	RXFP1	E	M	I	H	Q	F	W	Y	N	Y	R	Q	R	R	S	15
Human	RXFP1	*	*	*	*	R	*	*	*	*	*	*	*	*	K	*	704
Predicted pig	RXFP2	D	T	L	K	*	L	L	H	*	-	Q	R	*	K	*	574
	46	ATT	GAC	AGC	AAA	GGA	AGT	CAG	AAA	GCC	TAT	GCA	CCA	TCA	TTC	ATC	90
Pig	RXFP1	I	D	S	K	G	S	Q	K	A	Y	A	P	S	F	I	30
Human	RXFP1	M	*	*	*	*	-	*	*	T	*	*	*	*	*	*	718
Predicted pig	RXFP2	*	F	K	T	K	K	K	S	L	S	T	S	I	V	W	589
	91	TGG	GTA	GAA	ATG	TGG	CCC	CTG	CAG	GAG	ATG	ACT	GCT	GAC	TTA	ATG	135
Pig	RXFP1	W	V	E	M	W	P	L	Q	E	M	T	A	D	L	M	45
Human	RXFP1	*	*	*	*	*	*	*	*	*	*	P	P	E	*	*	733
Predicted pig	RXFP2	T	D	D	S	S	S	*	K	L	G	V	L	N	K	I	604
	136	AAG	CCG	GCT	CTT	TTC	AAA	GAC	CCC	TGT	GAA	TTA	TCA	CTA	ATT	TCT	180
Pig	RXFP1	K	P	A	L	F	K	D	P	C	E	L	S	L	I	S	60
Human	RXFP1	*	*	D	*	*	T	Y	*	*	*	M	*	*	*	*	748
Predicted pig	RXFP2	P	L	G	D	N	I	V	K	P	L	S					615
	181	CAA	TCA	ACG	AGA	CTC	AAT	TCC	TAT	TCC	TGA						210
Pig	RXFP1	Q	S	T	R	L	N	S	Y	S	stop						69
Human	RXFP1	*	*	*	*	*	*	*	*	*	*						757

Figure 2 Nucleic acid sequence (210 bp) and deduced peptide sequence of the partial cDNA for the boar *RLN* receptor *RXFP1*, corresponding to the nucleic acids 2149–2356 of the human *RXFP1* cDNA. Alignment of the deduced partial peptide sequence of boar *RXFP1* (69 aa) corresponded to the C-terminal intracellular domain (endodomain) of human *RXFP1* (aa 690–757). The boar *RXFP1* peptide displayed 82.6% amino acid homology with human *RXFP1*. In contrast, boar *RXFP1* peptide displayed only 11% amino acid homology with the endodomain of predicted porcine *RXFP2* (GenBank accession no. XM001927967). aa, amino acid.

Figure 3 Cellular expression of *RLN* and *RXFP1* genes in the boar testis. The germ cell, Sertoli cell and Leydig cell fractions were prepared from the testes of 18-week-old pubertal boars. (A) Haematoxylin and eosin staining of fractionated testicular cells. Bar = 50 µm; all panels are of the same magnification. (B) Cellular expression of *RLN* and *RXFP1* genes. The quality of these fractionated cells was also verified by detecting the expression of *HSD3B*, *PRM1* and *INHA*, which are expressed specifically by Leydig cells, germ cells and both Sertoli and Leydig cells respectively. *RLN* expression was restricted to the Leydig cell fraction where *HSD3B* was detected, whereas *RXFP1* was detected in all of the Leydig cell, Sertoli cell and germ cell fractions. Full colour version of this figure available via <http://dx.doi.org/10.1677/JOE-10-0149>.

(Fig. 3B), indicating no contamination of each fraction. Expression of *RLN* was detected only in the Leydig cell fraction where *HSD3B* was detected, whereas *RXFP1* expression was detected in Leydig cell, Sertoli cell and germ cell fractions (Fig. 3B).

Changes of *RLN* and *RXFP1* gene expression within the boar testis during development

To investigate the development changes in *RLN* and *RXFP1* gene expression in the boar testis, semi-quantitative RT-PCR assay was performed using total RNA obtained from postnatal prepubertal (1, 5, 7 and 9 weeks), pubertal (18 and 30 weeks) and postpubertal (38 weeks) testes. *RLN* and *RXFP1* transcripts were detected at all of the developmental stages studied (Fig. 4A), but the expression patterns differed between *RLN* and *RXFP1* as determined by semi-quantitative RT-PCR (Fig. 4B). Relative level of *RLN* expression was high at 1 week after birth, and then decreased significantly

($P < 0.05$) at 9 weeks of age, corresponding to the prepubertal stage. Thereafter, the expression of *RLN* increased significantly ($P < 0.05$) and reached its maximum at 18 weeks, i.e. the onset of puberty, and then remained at a steady level up to 38 weeks (Fig. 4B). On the other hand, the level of *RXFP1* expression showed little change in the boar testis during development (Fig. 4B).

Expression of *RLN* protein and pro*RLN*-processing enzyme gene in the boar testis during pubertal development

To clarify whether *RLN* mRNA was translated into protein in the boar testis, rabbit polyclonal antiserum was generated against highly purified *RLN* prepared from pregnant sow CL. Two rabbits were immunised, and rabbit #1 produced the

Figure 4 Changes in *RLN* and *RXFP1* gene expression in the boar testis during postnatal development. (A) A representative semi-quantitative RT-PCR analysis. (B) Relative level of each message. The relative level of *RLN* expression was high at 1 week after birth, and then decreased significantly at 9 weeks of age (prepubertal stage). Thereafter, it increased significantly again to the maximum value at 18 weeks (onset of puberty), and then remained stable up to 38 weeks. In contrast, testicular level of *RXFP1* expression showed little change throughout development. Values are the means $\pm$ S.E.M. from three animals at each time point, except at 38 weeks ($n=2$), and values with different letters are significantly different ($P < 0.05$). —RT, negative control without reverse transcription.

antiserum with higher titre. The specificity of this antiserum was verified by western blotting with purified porcine RLN and recombinant RLF/INSL3. The anti-porcine RLN serum from rabbit #1 (anti-porcine RLN R1 antiserum) recognised the 6 kDa mature RLN purified from pregnant sow CL (Fig. 5A). This antiserum also recognised two minor components present in the purified RLN, which were a band of 18 kDa corresponding to the expected size of proRLN and a band of 10 kDa that might have been a degraded form of proRLN (Fig. 5A). These bands were not observed when the antiserum preabsorbed with purified RLN was applied (Fig. 5A). The antiserum did not cross react with recombinant RLF/INSL3 (Fig. 5A), indicating that this antiserum was specific for RLN. Similar patterns were seen when the antiserum from rabbit #2 was analysed (data not shown).

Using anti-porcine RLN R1 antiserum, western blot analysis of the testis during pubertal development (at 7, 18 and 38 weeks, corresponding to the prepubertal, pubertal and postpubertal stages respectively) found no immunoreactive band corresponding to 6 kDa RLN in any of the samples, while the 18 kDa RLN-related peptide presumed to be proRLN was always detected and the signal seemed to increase from the pubertal stage onwards (Fig. 5B). The 10 kDa band that might represent degraded proRLN was also recognised with an expression profile similar to that of the 18 kDa peptide. In marked contrast to the staining obtained

with the antiserum, there was a complete absence of immunoreactive bands when antiserum preabsorbed with purified RLN was applied (Fig. 5C).

We performed further RT-PCR analysis to assess gene expression of prohormone convertase 1/3 (*PC1/3*), which is involved in post-translational processing of proRLN by cleavage of the B-chain/C-peptide junction *in vitro*. RT-PCR of total RNA prepared from boar testes at 7, 18 and 38 weeks of age found no *PC1/3* transcripts at any of the developmental stages studied, despite it being found in pregnant sow CL (Fig. 6).

Expression of RXFP1 protein in the boar testis during pubertal development

To determine whether *RXFP1* mRNA was translated into protein in the boar testis, rabbit polyclonal antiserum was generated against MBP fusion protein of the porcine *RXFP1* endodomain (MBP-*RXFP1* endodomain) expressed in *E. coli*. Among three rabbits immunised, rabbit #2 produced antiserum with the highest titre. The specificity of this antiserum was verified by western blotting with the immunising antigen (MBP-*RXFP1* endodomain) and MBP fusion protein of porcine *RXFP2* endodomain (MBP-*RXFP2* endodomain) expressed in *E. coli*. The anti-porcine *RXFP1* antiserum from rabbit #2 (anti-porcine

Figure 5 Specificity of antiserum for RLN and expression of RLN protein in the boar testis during pubertal development. (A) Specificity of antiserum for RLN. Left panel: purified porcine RLN (CL RLN) and recombinant RLF/INSL3 (rRLF/INSL3) separated on SDS-PAGE under non-reducing conditions and stained with Coomassie brilliant blue. Centre panel: verification of specificity of anti-porcine RLN antiserum by western blot analysis. The anti-porcine RLN serum from rabbit #1 (anti-porcine RLN R1 antiserum) strongly recognised 6 kDa RLN purified from pregnant corpora lutea (CL RLN). This antiserum also recognised two minor components of the purified RLN preparation; a band of 18 kDa (expected to be proRLN) and a band of 10 kDa (possibly a degraded form of proRLN). However, this antiserum did not cross react with recombinant RLF/INSL3. Right panel: three bands of 6, 10 and 18 kDa detected in purified RLN preparation were not observed when the antiserum preabsorbed with purified RLN was applied. (B) Expression of RLN proteins in the boar testis during pubertal development. Using anti-porcine RLN R1 antiserum, western blot analysis of RLN in boar testes at the ages of 7 weeks (prepubertal stage), 18 weeks (puberty) and 38 weeks (postpubertal stage) indicated that 6 kDa mature form was not detected at any stage, although both the 18 kDa proRLN and the 10 kDa degraded form were recognised. Both bands seemed to increase in intensity from puberty onwards. (C) Verification by preabsorbed antiserum. Immunoreactive bands detected in the testis or purified RLN preparation was effectively blocked when the antiserum was preabsorbed with purified porcine RLN. ACTB (β -actin) was used as the loading control during all western blotting experiments. CBB, Coomassie brilliant blue-stained SDS-PAGE; CL RLN, RLN purified from pregnant sow corpora lutea.

Figure 6 *PC1/3* gene expression in the boar testis during pubertal development and in the sow pregnant corpora lutea (CL). RT-PCR of total RNA prepared from CL on days 50 and 100 of pregnancy found *PC1/3* transcripts. However, *PC1/3* expression was not detected in the testes of 17-, 18- and 38-week-old boars.

RXFP1 RR2 antiserum) was confirmed to specifically recognise a single protein of ~51 kDa, corresponding to the molecular size of MBP–RXFP1 endodomain (Fig. 7A), but did not detect MBP alone (data not shown). This band was not observed when the antiserum preabsorbed with immunising antigen was applied (Fig. 7A). The antiserum did not cross react with recombinant MBP–RXFP2 endodomain of ~49 kDa (Fig. 7A), indicating that this antiserum was specific for RXFP1. Similar patterns were seen when the antiserum from rabbit #3, but not that from rabbit #1, was analysed (data not shown).

Using anti-porcine RXFP1 RR2 antiserum, western blot analysis of the boar testis during pubertal development (at 7, 18 and 38 weeks, corresponding to the prepubertal, pubertal and postpubertal stages respectively) revealed that immunoreactive RXFP1 was detected at all ages as three protein species with apparent bands of 75, 91 and 95 kDa (Fig. 7B). These bands seemed to change little with age (Fig. 7B) and were also observed in the uterus of pregnant sows in which *RXFP1* mRNA was expressed (Fig. 7B). These bands were effectively blocked when the antiserum was preabsorbed with immunising antigen (Fig. 7C). Similar patterns were seen using the anti-RXFP1 antiserum from rabbit #3, but not that from rabbit #1 (data not shown). The antiserum from rabbit #1 recognised only a band of 75 kDa.

Immunolocalisation of RLN and RXFP1 proteins in the testis during pubertal development

To examine the localisation of RLN and RXFP1, immunohistochemical analysis was performed using anti-porcine RLN R1 antiserum (1:1500 dilution) and anti-porcine

RXFP1 RR2 antiserum (1:1000 dilution) with sections of boar testis harvested during pubertal development (at 7, 18 and 38 weeks, corresponding to the prepubertal, pubertal and postpubertal stages respectively).

Figure 7 Specificity of antiserum for RXFP1 and expression of RLN protein in the boar testis during pubertal development. (A) Specificity of antiserum for RXFP1. Left panel: recombinant MBP–RXFP1 and MBP–RXFP2 endodomains separated on SDS-PAGE under reducing conditions and stained with Coomassie brilliant blue. Centre panel: verification of specificity of anti-porcine RXFP1 antiserum by western blot analysis. Anti-porcine RXFP1 antiserum from rabbit #2 (anti-porcine RXFP1 RR2 antiserum) recognised 51 kDa MBP–RXFP1 endodomain. However, this antiserum did not cross react with recombinant MBP–RXFP2 endodomain of ~49 kDa. Right panel: the band indicating MBP–RXFP1 endodomain was not observed when the antiserum preabsorbed with MBP–RXFP1 endodomain was applied. (B) Expression of RXFP1 protein in the boar testis during pubertal development. Using anti-porcine RXFP1 RR2 antiserum, RXFP1 proteins were detected at all ages as specific bands of 75, 91 and 95 kDa. The 91 and 95 kDa bands are likely to be glycosylated RXFP1 forms, whereas the 75 kDa band may represent a non-glycosylated form. These bands seemed to change little with age. These bands were also observed in the uterus on days 50 and 100 of pregnancy. (C) Verification by preabsorbed antiserum. Immunoreactive bands detected in the testis and uterus were effectively blocked when the antiserum was preabsorbed with recombinant MBP–RXFP1. ACTB (β -actin) was used as the loading control during all western blotting experiments. CBB, Coomassie brilliant blue-stained SDS-PAGE.

Like the distribution of *RLN* transcripts detected in fractionated testicular cells (Fig. 3B), immunostaining for *RLN* was only positive in Leydig cells at all stages of development studied, and the positive signal seemed to

become more intense as development progressed (Fig. 8A). Under the same conditions, staining of the testis was less intense in comparison with that of pregnant sow CL (data not shown). Staining of Leydig cells did not occur when the

Figure 8 Immunolocalisation of *RLN* and *RXFP1* proteins in the boar testis during pubertal development. Sections were taken from testes obtained at the age of 7, 18 and 38 weeks, corresponding to the prepubertal, pubertal and postpubertal stages respectively, and also from the uterus on days 50 and 100 of pregnancy. (A) Testis. Using anti-porcine *RLN* R1 antiserum, immunostaining for *RLN* was only visualised in Leydig cells at all developmental stages studied, and the signals seemed to become more intense as development progressed. Staining of Leydig cells was abolished by preabsorbing the antiserum with porcine *RLN*. With anti-porcine *RXFP1* RR2 antiserum, immunostaining for *RXFP1* was clearly observed in both Leydig cells and seminiferous tubule epithelial cells within the testis, although the staining of seminiferous epithelial cells was less intense than that of Leydig cells. (B) Pregnant uterus. Immunostaining for *RXFP1* was observed in luminal and glandular epithelial cells and myometrial smooth muscle. Le, luminal epithelial cells; Ge, glandular epithelial cells; Ms, myometrial smooth muscle. Bars = 100 µm (all panels in A) and 1000 µm (all panels in B).

antiserum was preabsorbed by incubation with porcine RLN (Fig. 8A).

Immunostaining for RXFP1 was demonstrated not only in Leydig cells, but also in seminiferous tubule epithelial cells (possibly Sertoli cells and germ cells) within the testis (Fig. 8A), confirming the expression of *RXFP1* by fractionated cells (Fig. 3B). However, the staining of seminiferous tubule epithelial cells seemed to be less intense in comparison with that of Leydig cells (Fig. 8A). Using the uterus of pregnant sows in which *RXFP1* expression was observed as a positive control, the optimal staining intensity of the uterus was effective at a dilution of 1:3000. Immunostaining was mainly observed in luminal and glandular epithelial cells and myometrial smooth muscle (Fig. 8B). Staining also appeared to be associated with individual cells located within the stroma. These are probably, at least in part, fibroblasts. At the same antiserum dilution, staining of the testis was still evident, but was very weak (data not shown). In marked contrast to the staining obtained with the antiserum, there was a complete absence of immunostaining when antiserum preabsorbed with MBP-RXFP1 endodomain was applied (Fig. 8A and B).

Discussion

The present study characterised the expression profiles and cellular localisation of both ligand and receptor of the RLN signalling system within the testis.

Previously, there has been limited evidence regarding RLN ligand that the testis might be a candidate source of RLN in boars, as *RLN* transcripts are detected therein (Lobb *et al.* 1995), although no RLN-expressing cells were identified (Arakaki *et al.* 1980, Kohsaka *et al.* 1992). In the present study, we substantiated previous observations and demonstrated for the first time that RLN protein was produced in the Leydig cells of the boar testis based on a number of findings as follows: first, *RLN* was strongly expressed in the testis, as described by Lobb *et al.* (1995), and was always detected in the testis from birth to adulthood with changes in transcriptional activity. Second, *RLN* transcripts were expressed exclusively in the Leydig cells. Third, *RLN* mRNA was translated into the protein in the testis during development with an elevation from the pubertal stage onwards, and the protein was restricted to the Leydig cells. Leydig cells in a number of species are known to express RLF/INSL3, which is closely related to RLN (Ivell & Bathgate 2002). It is evident, however, that the anti-RLN antiserum used here does not detect RLF/INSL3, since it did not cross react with recombinant RLF/INSL3 as revealed by western blotting.

It appears that the boar may differ from other mammalian species in having a large amount of testicular RLN, which may be linked to the fact that the ratio of Leydig cells in the boar testis is much higher than those in the mouse or rat testis (60 vs 2–4%; Van Straaten & Wensing 1978, Christensen & Peacock 1980, Mori *et al.* 1982), and that the promoter of

RLN is very strong due to the need for very high RLN expression in the ovary (Haley *et al.* 1987). With the help of semi-quantitative RT-PCR analysis, we found relatively high levels of *RLN* expression at 1 week after birth, decreasing significantly at 9 weeks of age (prepubertal stage) followed by a marked increase again to a maximum level at 18 weeks (onset of puberty), subsequently remaining at a similar level up to 38 weeks (postpubertal stage). The changes of *RLN* expression seemed to correspond fundamentally to the developmental status in Leydig cells (Van Straaten & Wensing 1978, Peyrat *et al.* 1981, Lunstra *et al.* 1986, Franca *et al.* 2000). However, it is still unclear what factor(s) regulate the expression of *RLN* mRNA in the boar testis. Treatment of intact boars with hCG has been reported to have little effect on circulating levels of RLN detectable by RIA, although it induces testosterone secretion (Juang *et al.* 1996), but there is evidence that LH and prolactin increase RLN production by porcine CL *in vitro* (Huang *et al.* 1992), while prolactin maintains RLN secretion for 10 days in hypophysectomised-hysterectomised gilts (Li *et al.* 1989). Thus, there may be marked differences in the factors regulating *RLN* gene expression between Leydig cells and CL cells.

The present histochemical study revealed the localisation of immunoreactive RLN within the Leydig cells during testicular development, although previous histochemical studies performed in our (Kohsaka *et al.* 1992) and other (Arakaki *et al.* 1980) laboratories were not successful in detecting immunoreactive RLN in the testis. The differing results seem to be related to the use of an antigen retrieval step using water bath heating method (Jiao *et al.* 1999), because previous studies did not include such treatment (Arakaki *et al.* 1980, Kohsaka *et al.* 1992). Recent immunohistochemical studies with various antigen retrieval methods have highlighted the necessity for re-evaluation of the results of many previous studies (Shi *et al.* 1997). Although the mechanism underlying antigen retrieval remains unclear, it seems likely that the retrieval process reverses the masking of antigens by aldehyde fixation (Shi *et al.* 1997), thereby making it possible to successfully detect RLN in boar Leydig cells in the present study.

It is important to determine what form immunoreactive RLN actually takes in the testis. RLN is known to be synthesised as a large precursor molecule of 18 kDa named proRLN, which is then processed to the 6 kDa mature form (Sherwood 1994). It has been reported that antiserum for rat RLN can bind with high affinity to RLN-related peptides with a molecular weight higher than that of RLN itself (Sherwood *et al.* 1984). As is also the case for rat RLN antisera reported previously (Sherwood *et al.* 1984), our RLN antisera were shown by western blotting to recognise the 6 kDa mature form and two minor components, which were an 18 kDa band of the size expected for proRLN and a smaller band of 10 kDa that may be a degraded form of proRLN, both of which were present at extremely low levels in the purified porcine RLN from pregnant CL. The boar testis during pubertal development, however, had

no immunoreactive band corresponding to the 6 kDa mature form of RLN detected by western blot analysis, although the RLN-related peptides of 18 and 10 kDa were detected. Taking these results into consideration, it is possible that the immunoreactive RLN visualised histochemically in the testis represented both 18 and 10 kDa RLN-related peptides, but not the 6 kDa mature form of RLN. Therefore, it is necessary to determine why the 6 kDa mature RLN was not detectable in the testis, despite the expression of RLN mRNA in this tissue. There are at least two possible explanations. First, the concentration of mature RLN within the testis may be too low to be detected by our technique. This is based on the finding of a previous RIA study indicating that the peripheral blood level of RLN in boars is very low (<500 pg/ml; Juang *et al.* 1996). Second, there may be differences in post-translational regulation between the testis and the CL. This was emphasised by the results of RT-PCR analysis of *PC1/3*, which can cleave proRLN *in vitro* (Marriott *et al.* 1992, Renegar *et al.* 2000), although it has not been confirmed that *PC1/3* is involved in cleaving proRLN *in vivo*. We found that *PC1/3* mRNA was not expressed in the boar testis at any of the developmental stages studied, while its presence was detected in the pregnant CL, as reported by Renegar *et al.* (2000). Therefore, it is possible that the 6 kDa mature RLN is not produced in the boar testis due to a lack of the enzyme required for processing proRLN, unlike the pregnant CL. Besides, the present findings also suggest the need to study whether the 18 kDa band is actually proRLN and is biologically active.

In addition to the RLN ligand, the present study also provided the first evidence that the boar testis expresses *RXFP1* mRNA and protein and is identified as a potential target tissue of RLN based on the following findings: first, partial cDNA sequences of the boar homologue of human *RXFP1* were identified in the testis, and *RXFP1* expression showed little change during testicular development. Second, *RXFP1* transcripts were expressed in Leydig cells, Sertoli cells and germ cells. Third, *RXFP1* mRNA was translated into the protein in the testis during development, and the protein encoded by *RXFP1* was localised in the same cell types by which *RXFP1* transcripts were expressed. The antiserum used in this study was generated against recombinant MBP-RXFP1 endodomain, and its specificity was validated by demonstrating the recognition of RXFP1 endodomain, but not RXFP2 endodomain, on western blotting, and by indicating the immunolocalisation in uterine endometrium and myometrium where RLN receptor is expressed in humans, rodents and pigs (Mercado-Simmen *et al.* 1982, Weiss & Bryant-Greenwood 1982, Kohsaka *et al.* 1998, Bond *et al.* 2004). The expression of RLF/INSL3 receptor RXFP2 is also observed in the testis of humans (Anand-Ivell *et al.* 2006) and rodents (Feng *et al.* 2007), but it is clear that our generated antiserum does not detect the RXFP2 since it did not cross react with recombinant RXFP2 as mentioned above. To our knowledge, there have been no previous attempts to identify RLN receptor RXFP1 in the boar testis, although there is presently

only one study reporting that the testis has specific RLN binding. Biotinylated porcine RLN was indicated to bind to Leydig cells alone (Min & Sherwood 1998). The reason for this discrepancy in the target cells of RLN is unclear, but the lack of binding of biotinylated RLN to seminiferous tubule epithelial cells may have been due to a difference in the affinity of the RLN receptor expressed by these cells.

Unlike RLN, *RXFP1* expression changed little during postnatal development in the present semi-quantitative RT-PCT analysis. Oestrogen is considered to be one of the candidate factors regulating RLN receptor, but the effect is different between tissues and/or genders. Administration of oestradiol-17 β into female rats increases the RLN-binding sites in the uterus but has no effect in atria and cerebral cortex, whereas the identical treatment in male rats reduces the RLN-binding sites in these tissues (Osheroff *et al.* 1992, Tan *et al.* 1999). More recent studies in neonatal porcine uterus and cervix have shown an increase in *PXFP1* expression by oestradiol-17 β administration (Yan *et al.* 2008a). In the present study, however, *RXFP1* expression pattern in the testis did not correlate with the known circulating levels of oestradiol-17 β during development in boars (Allrich *et al.* 1982). In addition, there was no relevance between the expression patterns of *RXFP1* and RLN in the testis during development. In fact, RLN administration is reported to have no influence on *RXFP1* expression in the pig uterus (Yan *et al.* 2008a). Thus, putative factors that regulate expression in *RXFP1* in the boar testis are unknown at present.

The structure of RXFP1 in pigs is not yet known, as the full-length cDNA sequence of *RXFP1* has still not been determined in this species. In humans and rodents, RXFP1, like RXFP2, is characterised by a large N-terminal extracellular domain (ectodomain) with a unique low-density lipoprotein class-A module and ten leucine-rich repeats, followed by seven transmembrane domains and an endodomain (Hsu *et al.* 2002, Van der Westhuizen *et al.* 2008). Members of this family of receptors have N-glycosylation sites in the ectodomains and act through causing cAMP production (Hsu *et al.* 2002, Yan *et al.* 2008b). Expression analysis and glycosidase treatment have shown that human RXFP1 is expressed as two molecular masses of 80 and 95 kDa in human embryonic kidney cells (HEK293 cells) transfected with the full-length *RXFP1*, and that while the 95 kDa band represents the glycosylated form of the RXFP1 receptor, the 80 kDa band represents the non-glycosylated form (Kern *et al.* 2007). In the present study, the protein encoded by *RXFP1* was translated into three protein species with apparent molecular masses of 75, 91 and 95 kDa in the testis during development. Taken with previous findings (Kern *et al.* 2007), specific bands of both 91 and 95 kDa detected in the boar testis are likely to be glycosylated forms of the RXFP1 receptor, whereas the 75 kDa band may represent a non-glycosylated form, although it is somewhat smaller in size than the non-glycosylated human RXFP1.

It has been reported that *RXFP1* and *Rxfp1* are expressed in different splice variants in humans and mice, whereby often

only ~100 bp of regions encoding the ectodomain can be missing, thereby making the resulting products non-functional (Muda *et al.* 2005, Scott *et al.* 2006). In the present study, the RT-PCR data with primers amplifying the endodomain of the *RXFP1* only was not able to resolve the issue of whether splice variants involving the ectodomain-coding region are present or not. However, our western blot analysis revealed the possible existence of a splice variant in the boar testis; on the bands of 91 and 95 kDa, which were likely to be glycosylated forms of the *RXFP1* receptor, it is possible that the lower 91 kDa band was attributable to splicing, as the splice variant lacks only ~100 bp in the ectodomain as mentioned above and the predicted difference in molecular mass is only ~4 kDa (Novak *et al.* 2006). If this is the case, splicing of *RXFP1* can impact receptor functionality in the boar testis.

It remains largely unknown what effects RLN actually exerts on these multiple target cell types in the boar. The following suggestions would be permitted with respect to the possible significance of RLN. RLN may be involved in the autocrine regulation of Leydig cell function. Kwan *et al.* (1994) observed that the addition of RLN to the nucleus-free tissue homogenate from adult macaque testes inhibited testosterone production. RLN may also play a role in spermatogenesis by regulating cellular processes such as apoptosis in a paracrine manner. *Rln* knockout adult male mice have been reported to display decreased sperm maturation, although this may be due to an increase in germ cell apoptosis by marked testicular fibrosis, rather than any direct effect of the lack of RLN on germ cells (Samuel *et al.* 2003). On the other hand, *Rxfp1* knockout adult male mice showed impaired spermatogenesis along with increased germ cell apoptosis, and a reduction in fertility or even azoospermia in some animals, although these phenomena were absent in older mutant mice or in later generations (Krajnc-Franken *et al.* 2004), which might indicate the non-linked *Rxfp1* modification of genome in transgenic mice. In contrast, another study indicated that *Rxfp1* knockout male mice did not exhibit abnormalities in the testis and had normal fertility (Kamat *et al.* 2004).

In conclusion, we provided evidence that RLN is expressed as a prohormone in Leydig cells in the boar testis that is apparently linked to the absence of expression of *PC1/3* participating in processing of the mature hormone, whereas *RXFP1* mRNA and protein are expressed in Leydig cells and seminiferous epithelial cells, suggesting that the testis acts as a source and possible target tissue of RLN, thereby providing insight into the potential role of RLN in boar testicular function.

Declaration of interest

The authors declare that there are no conflicts of interest that could be perceived as prejudicing the impartiality of the research reported.

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