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.

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 Rxfp1 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. 1995). Furthermore, a ligand-binding study in the testis (Lobb et al. 1995). Nevertheless, a study using the reverse transcription and PCR (RT-PCR) method demonstrated the presence of RLN transcripts in the testis (Lobb et al. 1999). 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 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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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 for each group), 18- and 30-week-old (pubertal, n = 3 for each group), and 38-week-old (postpubertal, n = 2) boars.

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 cm² x 3–5 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 μm 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-β hydroxysteroid dehydrogenase (HSD3B), protamine 1 (PRM1) and inhibin α (INHA) 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 μg of total RNA were used for first-strand cDNA synthesis in 20 μl reaction mixtures using oligo(dT)18 primer with a RT kit (Invitrogen Life Technologies).
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 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) and 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 Kohnska 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 HinClI and HindIII restriction sites. The PCR product was then cleaved with HinClI 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<sub>600</sub> 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 NaN<sub>2</sub> (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 Wakoil 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<sup>10</sup> 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 antisera 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 (Siqin 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
with immunising antigen. Sections were incubated with primary antisera after absorption three times for 5 min each in PBS. As negative controls, some sections 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 μm) 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 ± 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.
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.

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**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.
Expression of relaxin and RXFP1 in the boar testis

Expression of relaxin and RXFP1 in the boar testis

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 proRLN-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

(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).

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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
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

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**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.
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 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.

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 (Fig. 7A), indicating that this antiserum was specific for RXFP1. Right panel: the band indicating MBP–RXFP1 endodomain was not observed when the antiserum 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. Ly, Leydig cells; ST, seminiferous tubules; 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 in 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. 1990, 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 hypophysecomised–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-17β 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 PXFX1 expression by oestradiol-17β 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-17β 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 LRN actually exerts on these multiple target cell types in the boar. The following suggestions would be permitted with respect to the possible significance of LRN. LRN may be involved in the autocrine regulation of Leydig cell function. Kwan et al. (1994) observed that the addition of LRN to the nucleus-free tissue homogenate from adult macaque testes inhibited testosterone production. LRN may also play a role in spermatogenesis by regulating cellular processes such as apoptosis in a paracrine manner. Rhn 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 LRN 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 tests and had normal fertility (Kamat et al. 2004).

In conclusion, we provided evidence that LRN 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 LRN, thereby providing insight into the potential role of LRN in boar testicular function.

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References


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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