Leucine-rich repeat-containing G-protein-coupled receptor 8 in mature glomeruli of developing and adult rat kidney and inhibition by insulin-like peptide-3 of glomerular cell proliferation

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Abstract

Leucine-rich repeat-containing G-protein-coupled receptor 8 (LGR8, or RXFP2) is a member of the type C leucine-rich repeat-containing G protein-coupled receptor family, and its endogenous ligand is insulin-like peptide-3 (INSL3). Although *LGR8* expression has been demonstrated in various human tissues, including testis, ovary, brain and kidney, the precise roles of this receptor in many of these tissues are unknown. In an effort to better understand INSL3–LGR8 systems in the rat, we cloned the full-length *Lgr8* cDNA and investigated the presence and cellular localization of *Lgr8* mRNA expression in adult and developing rat kidney. On the basis of these findings, we investigated the presence and distribution of renal ¹²⁵I-labelled human INSL3-binding sites and the nature of INSL3–LGR8 signalling in cultured renal cells. Thus, using *in situ* hybridization histochemistry, cells expressing *Lgr8* mRNA were observed in glomeruli of renal cortex from adult rats and were tentatively identified as mesangial cells. Quantitative,

real-time PCR analysis of the developmental profile of *Lgr8* mRNA expression in kidney revealed highest relative levels at late stage gestation (embryonic day 18), with a sharp decrease after birth and lowest levels in the adult. During development, silver grains associated with *Lgr8* mRNA hybridization were observed overlying putative mesangial cells in mature glomeruli, with little or no signal associated with less-mature glomeruli. In adult and developing kidney, specific ¹²⁵I-INSL3-binding sites were associated with glomeruli throughout the renal cortex. In primary cultures of glomerular cells, synthetic human INSL3 specifically and dose-dependently inhibited cell proliferation over a 48 h period, further suggesting the presence of functional LGR8 (receptors) on these cells (mesangial and others). These findings suggest INSL3–LGR8 signalling may be involved in the genesis and/or developmental maturation of renal glomeruli and possibly in regulating mesangial cell density in adult rat kidney.

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Introduction

Insulin-like peptide-3 (INSL3), also known as relaxin-like factor, belongs to the insulin/relaxin superfamily that now comprises at least ten members in mammals (Ivell 1997, Bathgate *et al.* 2003, Hsu 2003). INSL3 is a developmentally regulated hormone with sexually dimorphic expression (Balvers *et al.* 1998, Zimmermann *et al.* 1999). In male rats, *Insl3* expression is high before birth at day 19 of gestation in Leydig cells of fetal testes and declines after birth (Boockfor *et al.* 2001). *Insl3* mRNA levels remain low in mesenchymal Leydig cell precursors until sexual maturation and initiation of spermatogenesis (Spiess *et al.* 1999).

In female mice, *Insl3* transcripts are first detected at day 6 after birth in ovarian theca and luteal cells, with a likely involvement in follicle development (Balvers *et al.* 1998). In adult women, INSL3 is detected in ovarian theca cells of large antral follicles and in the corpora lutea during the reproductive cycle and in pregnancy, and INSL3 serum levels are higher in adult men than in children or post-pubertal women (Büllesbach *et al.* 1999).

A genetic lack of INSL3 causes cryptorchidism – a developmental disorder involving failure of testis descent associated with defective spermatogenesis and infertility, and a high risk of testicular malignancy (Hutson *et al.* 1997). INSL3 normally induces growth and

differentiation of gubernacula during the initial trans-abdominal movement of the testes (Hutson *et al.* 1997, Adham *et al.* 2000). Male mice homozygous for a disruption of the *Insl3* gene exhibit bilateral cryptorchidism (Nef & Parada 1999, Zimmermann *et al.* 1999), while female homozygotes display impaired fertility with abnormal oestrous cycles (Nef & Parada 1999). In contrast, transgenic overexpression of *Insl3* causes ovaries to descend into the inguinal region due to an overdeveloped gubernaculum (Adham *et al.* 2002).

Recent data have demonstrated that the native receptor for INSL3 is the type C leucine-rich repeat-containing G-protein-coupled receptor 8 (LGR8) (Hsu *et al.* 2002, Kumagai *et al.* 2002) (known also as RXFP2; see Bathgate *et al.* 2005 for review). INSL3 bound with high affinity to LGR8 and receptor activation by INSL3 was associated with cAMP signalling. *LGR8* mRNA was detected in human gubernaculum and treatment of gubernacular cells with INSL3 stimulated cAMP production and thymidine incorporation (Kumagai *et al.* 2002). Transgenic mice with a deletion of *Lgr8* (also known as *Great*) exhibit cryptorchidism (Overbeek *et al.* 2001), a phenotype identical to *Insl3*-deficient mice (see Bogatcheva *et al.* 2003). Furthermore, analysis of human *INSL3* and *LGR8* mutations confirms cryptorchidism has a genetic aetiology related to these genes and INSL3–LGR8 signalling (Ferlin *et al.* 2003).

Importantly, *LGR8* mRNA has been detected in a number of human tissues besides the gubernaculum, including ovary, testis, uterus, brain and kidney (Hsu *et al.* 2002). Until recently though, little has been published on the regional and cellular distribution of *LGR8* expression or the functional role of INSL3 in these tissues of humans or other species (Kawamura *et al.* 2004). In an attempt to further elucidate the biology of the INSL3–LGR8 system in the rat, we cloned and sequenced rat *Lgr8* cDNA and screened various tissues for *Lgr8* mRNA expression using RT-PCR (Scott *et al.* 2005). Based on these initial findings, we employed *in situ* hybridization histochemistry to localize *Lgr8* expression in adult rat kidney and subsequently examined the temporal and spatial expression profile during development, using *in situ* hybridization histochemistry and quantitative real-time PCR. Results revealed *Lgr8* mRNA associated with mature glomeruli during development and in adulthood, and subsequent autoradiographic studies with ¹²⁵I-labelled human INSL3 revealed specific binding sites associated with glomeruli in renal cortex. Therefore, using primary cultures of renal glomerular cells isolated from rat kidney (containing mesangial and other cell types), we identified *Lgr8* mRNA expression in these cells by RT-PCR analysis and examined the effect of INSL3 treatment on their viability. (A preliminary account of some of these findings appeared in a monograph on the proceedings of the *Relaxin 2004* Conference (Fu *et al.* 2005)).

Materials and Methods

Animals

All procedures were approved by the Howard Florey Institute Animal Welfare Committee and were performed in strict accordance with guidelines of the National Health and Medical Research Council of Australia. Adult and postnatal Sprague–Dawley rats were deeply anaesthetized with the inhalation anaesthetic, isoflurane (Isoflo; Abbott Australia, Sydney, NSW, Australia) and then decapitated. Kidneys and other tissues were rapidly removed, frozen over liquid nitrogen and stored at -80°C (see details below).

Database search, rat *Lgr8* sequence amplification and alignment

As described briefly elsewhere, the protein sequence of human LGR8 (Accession No. NP_570718) was used to screen the National Center for Biotechnological Information (NCBI) high-throughput genome sequences (HTGSS) and fragmented hits were assembled through homology comparison with human LGR8 (Scott *et al.* 2005). Oligonucleotide primers based on the identified rat sequences were used to generate the full-length coding region via RT-PCR. Total RNA was extracted from dissected, snap-frozen rat tissues (testis, ovary, brain, kidney and embryonic day (E) 16 fetal gubernaculum) by homogenization in RNeasy (Ambion Inc, Austin, TX, USA) and cDNA was synthesized from 2.5 µg of total RNA using an RT-kit (Promega) and random hexamer primers. Putative LGR8-coding regions were amplified from cDNA, using a Taq polymerase PCR kit (Eppendorf, Sydney, NSW, Australia), cloned into the pGEM-T vector (Promega) and confirmed using Big Dye Terminator sequencing (Applied Biosystems, Foster City, CA, USA). The full coding sequence was assembled from overlapping fragments and confirmed by RT-PCR, using primers from outside the coding region. Alignment of human, mouse and rat sequences was done using ClustalW (<http://www.ebi.ac.uk/clustalw/>) and Boxshade 3.21 (http://www.ch.embnet.org/software/BOX_form.html) (Scott *et al.* 2005).

Detection of *Lgr8* mRNA in rat tissues and glomerular cells by RT-PCR

Rat tissues and samples of cultured rat glomerular cells were screened for *Lgr8* mRNA expression using RT-PCR, as described (Kubota *et al.* 2002). Briefly, total RNA was extracted with RNeasy reagent (Ambion Inc) according to the manufacturer's instructions. Primers equivalent to nucleotides 1338–1358 and 2121–2141 of the final rat *Lgr8* cDNA sequence were used for RT-PCR reactions. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as a control

for the quality of the cDNA synthesis and was amplified using primers equivalent to nucleotides 789–808 and 1017–1036 of rat GAPDH cDNA (Accession No. NM_017008). All the primers were designed to cross intron–exon boundaries to control for genomic DNA contamination.

In situ hybridization histochemistry

Frozen kidney sections (14 µm) from both developing (E16, E18, postnatal day (P) 2, 5, 10, 20, 30 and 40, $n \geq 3$ for each age group) and adult rats were cut on a cryostat at -16°C and thaw-mounted onto microscope slides coated with 0.2% poly-L-lysine (M_r 300 000). Slides were stored at -80°C until use. *Lgr8* mRNA was detected with three synthetic oligonucleotide probes corresponding to nucleotides 181–219, 1371–1409 and 2018–2056 of rat *Lgr8* cDNA sequence. Probes were labelled with [α - ^{35}S]dATP (≥ 1200 Ci/mmol; Perkin-Elmer Life Sciences, Melbourne, VIC, Australia) using terminal deoxynucleotidyl transferase (Roche Diagnostics) to a specific activity of $1\text{--}2 \times 10^9$ d.p.m./µg. Sections were hybridized as described (Shen & Gundlach 1996, Wisden & Morris 2002) and then exposed to Kodak Biomax film (Integrated Sciences, Sydney, NSW, Australia) for 4–5 weeks, or coated with nuclear emulsion (Ilford K5, diluted 1:1 with dH₂O; Ilford Imaging, Melbourne, VIC, Australia) and exposed for 20–25 weeks, prior to development, counterstaining with 0.01% thionin and analysis using bright- and dark-field microscopy. The specificity of the hybridization signal was assessed by confirming the similarity of hybridization patterns obtained with individual probes (data not shown) and hybridization in the presence of a 100-fold excess of unlabelled oligonucleotides.

Photography and image production

Digital images of film autoradiograms were produced using a Sony XC-77CE CCD video camera coupled to a Nikon 1:2.8, 55 mm lens and a light box, with MCID M2 software (Imaging Research Inc, St Catharines, ON, Canada). Digital micrographs of nuclear-emulsion-dipped, counterstained sections were produced under bright- and dark-field illumination on an Olympus IX70 microscope (Olympus Imaging, Melbourne, VIC, Australia) equipped with a SPOT real-time digital camera with Image-pro 4.5 software (Diagnostics Instruments, Sterling Heights, MI, USA). All micrographs were archived as high-resolution images in Adobe Photoshop (Version 5.0; Adobe Systems Inc, San Jose, CA, USA) and after any required cropping, adjustment for contrast or removal of obvious artefacts, plates were compiled and labelled in PowerPoint (Microsoft Corporation).

Real time-PCR analysis of Lgr8 mRNA in rat kidney

Kidney tissue from embryonic, developing and adult rats was finely diced under liquid nitrogen and immediately homogenized with RNeasyWiz reagent (Ambion Inc.). Total RNA was extracted and real-time PCR was used to quantify *Lgr8* mRNA levels in each sample. Real-time PCR primers and probes were designed using Primer Express (Applied Biosystems). The primer sequences are located at nucleotides 1898–1917 and 1998–1977 of the *Lgr8* cDNA respectively and span a 9.0 kb intron. Total RNA (1 µg) from each sample was used for the RT, in a 30 µl reaction (Siebel *et al.* 2002). The probe sequence, corresponding to nucleotides 1925–1961, was labelled at the 5'-end with 6-carboxy fluorescein (Qiagen) and included with the primers in each reaction tube. The relative C_T standard curve method was adopted and ribosomal RNA (18S) was used as an internal, endogenous control, with primers and probe(s) as described (Siebel *et al.* 2002).

^{125}I -INSL3 autoradiography

^{125}I -Human INSL3 binding autoradiography was performed essentially as described in earlier studies with ^{33}P -labelled human relaxin (Burazin *et al.* 2002). Slide-mounted cryostat sections (14 µm) of kidney were pre-incubated in 25 mM Hepes, 5 mM MgCl₂, pH 7.2 for 30 min. Slides were then incubated in the same buffer plus 0.1% BSA (w/v), 0.015% Pefabloc (w/v) and 100 pM ^{125}I -human INSL3 (Perkin-Elmer Life Sciences) at 21°C for 1 h. Non-specific binding was determined by the addition of 100 nM unlabelled human INSL3 (Fu *et al.* 2004) to the incubation buffer. Following incubation, slides were washed twice for 5 min each in ice-cold incubation buffer and rinsed in ice-cold dH₂O. Sections were allowed to air-dry overnight and were apposed to Kodak Biomax film for 5 days.

Rat glomerular cell culture

Male Sprague–Dawley rats ($n=12$ per study) were anaesthetized using Isoflo and kidneys were rapidly removed and placed on ice. Renal cortical tissue was excised and minced with a razor blade into millimetre-sized pieces. Glomeruli were isolated by passing the minced cortex through a 150 µm steel sieve and collecting strained material on a 75 µm steel sieve. Intact glomeruli were removed from the sieve, rinsed twice in PBS and centrifuged at 400 g for 5 min. Glomeruli were resuspended and incubated with 0.1% collagenase (190 U/ml) at 37°C for 30 min. After incubation, cells were centrifuged and the pellet was washed twice with PBS. Cells were then plated onto two 60 mm dishes in DMEM medium supplemented with 20% fetal calf serum,

2 µM glutamine, 5 µg/ml insulin, 100 U/ml penicillin, 100 µg/ml streptomycin (culture medium) in a 95% air–5% CO₂ humidified atmosphere at 37 °C (Gruden *et al.* 1997).

Glomerular cell viability/proliferation assay

Glomerular cell samples, likely to be enriched in mesangial cells but also containing endothelial and epithelial cells, were seeded into 96-well plates at a density of 1×10^4 cells/well in culture medium in a 95% air–5% CO₂ humidified atmosphere at 37 °C. After a 24 h incubation, the medium was replaced by serum-free medium containing 0.1% BSA and INSL3 or other peptide. Cells were incubated for a further 48 h and then cell viability/proliferation was assessed by an MTT assay (Hansen *et al.* 1989, Loveland *et al.* 1992). Briefly, MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; final concentration 0.5 µg/ml) was added, the cells were incubated for 3 h and then the insoluble reaction product was dissolved by the addition of 100 µl of cell lysis buffer (20% w/v SDS, 50% N,N'-dimethyl-formamide). In several wells, cells were lysed by the addition of cell-lysis buffer immediately prior the addition of MTT, to control for background absorbance. Absorbance of each well was measured at 595 nm using a Microplate Reader Model 3550-UV Spectrophotometer (Bio-Rad Laboratories). Data are expressed as a percentage of the maximum control response $\pm$ S.D. from three to five independent experiments. Statistical differences were assessed using one-way ANOVA.

Results

Cloning, sequencing and tissue distribution of rat Lgr8

In preliminary studies, rat *Lgr8* gene was cloned and sequenced (see Scott *et al.* 2005) and subsequently its distribution in different rat tissues was explored by RT-PCR. Briefly, sequences homologous to regions of the human *LGR8* cDNA were identified in bacterial artificial chromosome clones in the NCBI HTGS database. Using derived, specific RT-PCR primers the full rat *Lgr8* cDNA was assembled by sequencing multiple, independent overlapping clones from E16 fetal rat gubernaculum (Genebank Accession No. AY906861). Cells transfected with full-length rat *LGR8* demonstrate high-affinity binding for ¹²⁵I-INSL3 and respond to ligand stimulation with cAMP accumulation (Scott *et al.* 2005). RT-PCR analysis of rat tissues revealed strong *Lgr8* expression in gubernaculum, testis and ovary (data not shown; Kawamura *et al.* 2004). Notably, *Lgr8* mRNA was also found in rat brain (Shen *et al.* 2005) and kidney (Fu *et al.* 2005; this study).

Detection of regional and cellular Lgr8 mRNA expression in adult and developing kidney

In an attempt to discover the regional and cellular distribution of these receptors in rat kidney, frozen longitudinal sections from adult (8- to 10-week-old) male rats were subjected to *in situ* hybridization histochemistry for *Lgr8* mRNA using ³⁵S-labelled oligonucleotides. Relatively low-resolution film autoradiograms revealed the expression of *Lgr8* mRNA restricted to the renal cortex, in a pattern consistent with labelling of cells within the glomeruli (Fig. 1A). The specificity of the *Lgr8* mRNA signal detected was confirmed by several methods. In competition experiments, the signal was abolished when a 100-fold excess of unlabelled oligonucleotides was added to the incubation (Fig. 1A'). Secondly, sections hybridized with the three individual oligonucleotide probes displayed the same distribution of labelling (data not shown), and finally the oligonucleotide probes used produced strong, specific labelling of sections of gubernaculum from a 17-day rat embryo and adult testis (data not shown; Scott *et al.* 2005). Microscopic analysis of high-resolution nuclear emulsion autoradiograms from counterstained kidney sections indicated that *Lgr8* mRNA expression was restricted to putative mesangial cells within the adult glomeruli (Fig. 1B and C), with no obvious specific hybridization observed in glomerular podocyte or epithelial cells (Hoffmann *et al.* 2004). Notably, parallel *in situ* hybridization studies with *Lgr7*-directed oligonucleotides (Burazin *et al.* 2005) failed to detect *Lgr7* mRNA-positive cells in rat kidney (data not shown).

Profile of Lgr8 mRNA expression during embryonic and postnatal development

Subsequent to the identification of cell-specific expression in adult kidney, we assessed the possible renal expression of *Lgr8* mRNA in samples of developing kidney to provide further possible insights into the functional involvement of *LGR8*. In these studies the profile of *Lgr8* expression during fetal and postnatal kidney development was examined by *in situ* hybridization and quantitative real-time RT-PCR. Although expression of *Lgr8* was detectable in frozen sections from E16 through to adulthood, highest relative levels were observed in the embryonic stages, around E18 (Fig. 2). Shortly after birth at P2–5, a sharp decrease in *Lgr8* mRNA levels was observed and the reduction continued until *Lgr8* transcripts reached their lowest level in adult kidney (Fig. 2). Kidney sections from P2 rats, in which glomerulogenesis is still occurring, displayed strong *Lgr8* expression in mature glomeruli, whereas little or no distinct signal was observed in immature glomeruli or the nephrogenic zone, located at the edge of the renal cortex (Fig. 3A–A"). In P10 kidney, the nephrogenic zone is absent as glomerulogenesis has

Figure 1 Regional and cellular distribution of *Lgr8* mRNA in frozen sections of adult kidney from male rats. (A) *In situ* hybridization of ^{35}S -labelled oligonucleotide probes revealed that *Lgr8* mRNA was present in renal cortex ('C') in a specific pattern consistent with an association with glomeruli, while no hybridization signal was detected in the renal medulla ('Me'). (A') In a competition experiment, the hybridization signal was eliminated by a 100-fold excess of unlabelled oligonucleotides added to the hybridization mixture containing labelled probes, leaving only 'non-specific hybridization' ('NSH'). (B) Nuclear-emulsion autoradiograms confirmed that *Lgr8* mRNA was associated with cortical glomeruli ('G', *). (C) At high magnification, *Lgr8* mRNA-associated grains were observed over individual cells putatively identified as mesangial cells. Scale bar=2.5 mm (A, A'); 7.5 μm (B); 2.5 μm (C).

been completed by this age and *Lgr8* expression is abundant in mature glomeruli (Fig. 3B–B"). At all stages of nephron development studied, *Lgr8* expression appeared to be restricted to cells of mature glomeruli and was not detected in any other tissue regions or cell types.

Quantitative real-time PCR was also used to measure renal *Lgr8* mRNA levels at different developmental stages and confirmed that *Lgr8* expression was high in embryonic stages and decreased during postnatal kidney development. The PCR primers used cross a large intron of 9.5 kb to prevent genomic DNA contamination and 18S ribosomal mRNA was used as a control for quantification of RNA amount. Total RNA isolated from E18, P2, 5, 10, 20 and 60 (adult) kidneys was analysed ($n=4\text{--}5$ rats per time). Highest levels of *Lgr8* mRNA expression were detected in E18 kidney and relatively high levels were also observed at P2, before being significantly decreased by 4-fold at P5 (Fig. 4A). There was no significant change in *Lgr8* mRNA levels between P5 and 10, but at P20 the levels were 12-fold lower than at P2. By adulthood, there was a further reduction in *Lgr8* mRNA levels representing a 48-fold decrease in *Lgr8* mRNA expression from P2. *Lgr8* transcript levels measured at P20 and adulthood were also significantly lower than those measured at P5 and 10 (Fig. 4A). Notably, parallel RT-PCR studies failed to detect *Ins13* mRNA in embryonic, postnatal or adult kidney, despite effective detection of high levels of transcripts in adult testis, as a positive control for the primers and protocol used (data not shown).

Renal *Lgr8* expression in male and female rats

INSL3 has to date been largely considered as a male hormone and investigated for its capacity to stimulate gubernaculum growth and differentiation during the process of testicular descent. The serum concentration of INSL3 is lower in adult females than males in various species (Büllesbach *et al.* 1999, Boockfor *et al.* 2001), but INSL3 is also produced by tissues such as the ovary (Büllesbach *et al.* 1999). Comparison of levels of *Lgr8* mRNA in adult male and female kidney by quantitative real-time RT-PCR analysis revealed that *Lgr8* mRNA levels were some 6-fold higher in the female than male (Fig. 4B).

Detection of ^{125}I -INSL3-binding sites in developing and adult kidney

In studies aimed at revealing the presence and distribution of functional LGR8 in rat kidney, frozen longitudinal sections from adult and developing rats were incubated with ^{125}I -human INSL3. Specific ^{125}I -INSL3-binding sites were detected by X-ray film autoradiography in the renal cortex, in a pattern consistent with labelling of cells within the glomeruli (Fig. 5A). The specificity of the binding detected was confirmed by its inhibition in the

Figure 2 Relative abundance of *Lgr8* mRNA in rat kidney at different stages of development, determined by *in situ* hybridization. Representative dark-field images of nuclear-emulsion autoradiograms reveal the presence of *Lgr8* mRNA at all ages examined, with the strongest hybridization observed during late stages of embryonic gestation at E18 and a stronger signal in P2–P10 than in more mature (P20, P40) and adult kidney. Scale bar=500 μ m.

presence of 100 nM unlabelled human INSL3 (Fig. 5B). In addition, specific binding was also detected in renal cortex (glomeruli) of kidney from postnatal rats (Fig. 5C–H), indicating the presence of functional LGR8 throughout this period. In kidney sections from P2–5 rats, in which very strong *Lgr8* mRNA expression was detected, 125 I-INSL3-binding densities associated with glomeruli were lower than those seen in more mature rats (Fig. 5C–H).

Primary cultured glomerular cells express Lgr8 mRNA

In studies aimed at examining the presence of functional renal LGR8, adult rat glomerular cells were isolated and cultured under standard conditions (e.g. Gruden *et al.* 1997; see Methods). Cells were propagated in culture and passaged a total of ten times before total RNA from these cells was isolated and subjected to RT-PCR analysis for *Lgr8* mRNA. The predicted 101 bp band specific for *Lgr8* was detected, while under the same conditions no *Lgr7* mRNA expression was detected in these cells (Fig. 6). The housekeeping gene encoding GAPDH RNA was used as a control for sample RNA content/lane loading.

Total RNAs isolated from kidney, E17 gubernaculum and cervix were used as positive controls for *Lgr8* and *Lgr7* expression. In contrast to *in situ* hybridization studies, weak expression of *Lgr7* mRNA was detected in whole kidney by RT-PCR (Fig. 6), possibly associated with expression by the renal vasculature (Danielson *et al.* 1999).

INSL3 inhibits proliferation of rat glomerular cells

Glomerular cells were incubated in serum-free medium containing 0.1% BSA to assay cell viability and proliferation and treatment with 10 nM INSL3 for 48 h resulted in a 25% decrease in cell proliferation ($P<0.01$; Fig. 7A). In contrast, treatment of these cells with another insulin/relaxin family peptide, INSL4 (10 nM), had no significant effect on cell numbers. Human relaxin, which has been shown to bind to LGR8 transfected into human fetal kidney fibroblast 293T cells and induce cellular cAMP accumulation, had no effect on cell proliferation at 10 nM, but a 100 nM concentration produced an $\sim 15\%$ decrease ($P<0.05$; Fig. 7A), consistent with a lower-affinity interaction with LGR8 than INSL3. The effect of INSL3 was also shown to be dose-dependent (Fig. 7B),

Figure 3 Regional localization of *Lgr8* mRNA in rat kidney at different ages of development, determined by *in situ* hybridization. (A–A'') In P2 kidney, high densities of *Lgr8* mRNA-associated silver grains were observed over mature glomeruli (A'', 'M', arrow) situated in the deeper part of the cortex, but transcripts were absent in the nephrogenic zone (A', 'N', short arrow). (B–B'') In P10 kidney, the nephrogenic zone has disappeared, as glomerulogenesis has been completed by this age and *Lgr8* mRNA is abundant in mature glomeruli throughout the cortex (B', B'', 'M', single/double arrows). Scale bar=7.5 µm (A, B); 2.5 µm (A', A'', B', B'').

with an approximate IC_{50} between 1 and 10 nM. There is a possibility that INSL3 and the other peptides added to the incubation may have had differential or separate effects on the different cell types present in the mixed culture that may have biased the results observed and further specific tests are required to determine whether INSL3 promotes apoptosis in this system, but otherwise these data indicate

that INSL3 is a specific and potent inhibitor of renal cell proliferation.

Discussion

In an important step towards better understanding the nature of INSL3 action in different organs, in the current

Figure 4 Quantitative real-time PCR analysis of *Lgr8* mRNA levels in rat kidney during development and in male and female rats. (A) Relative *Lgr8* mRNA levels in embryonic kidney (E18) and postnatal kidney (P2, 5, 10, 20 and 60 (adult)). Data were normalized to their 18S ribosomal RNA values, and then expressed relative to *Lgr8* expression in the adult group, which was represented as 1.0. Note the different scales used to graph embryonic, postnatal and adult data. Values represent means $\pm$ S.E.M. ($n=4-5$ rats/age group). * $P \leq 0.05$ compared with E18; † $P \leq 0.05$ compared with P2; ‡ $P < 0.05$ compared with P5 (and P10) (one-way ANOVA). (B) Comparison of *Lgr8* mRNA levels in adult male and female kidneys, with mRNA levels in the male group represented as 1.0. Values represent means $\pm$ S.E.M. ($n=5$ rats). * $P < 0.05$ (one-way ANOVA).

studies we have identified the regional and cellular expression pattern of LGR8 in adult and developing rat kidney, and conducted some preliminary *in vitro* studies of INSL3 actions on LGR8-positive renal cells. As we reported briefly elsewhere (Scott *et al.* 2005), rat *Lgr8* has a high degree of sequence and structural identity with its counterpart human and mouse receptors (Hsu *et al.* 2002, Scott *et al.* 2004). Human INSL3 is a high-affinity ligand for rat LGR8, binding and activating cAMP signalling with a similar affinity/efficacy to that at the human LGR8, and native rat relaxin is not a ligand for rat LGR8 (Scott *et al.* 2005). RT-PCR analysis identified *Lgr8* mRNA expression in rat fetal gubernaculum, brain, testis, ovary and kidney, but not in the liver. Expression in the E16 gubernaculum highlights the essential role that INSL3 plays in testis descent (Bogatcheva & AgoulNIK 2005), while expression in the ovary and testis confirms the important role that INSL3 plays in germ cell maturation in male and female rats (Kawamura *et al.* 2004). Expression of LGR8 in rat kidney and brain and studies of the putative INSL3–LGR8 systems in these tissues is also of interest (see Fu *et al.* 2005, Shen *et al.* 2005).

In situ hybridization studies with ^{35}S -labelled oligonucleotides consistently and exclusively detected *Lgr8* mRNA in mature glomeruli (possibly in mesangial cells) during development and in adulthood. Levels of renal *Lgr8* mRNA expression were also quantified at various stages of kidney development by real-time PCR,

with highest levels detected at E18 and expression observed at all times studied. Radioligand binding studies with ^{125}I -INSL3 confirmed the presence of somewhat differing levels of functional LGR8 binding sites in renal glomeruli during development and in adulthood. These anatomical findings were extended by *in vitro* studies that revealed that cultured glomerular cells isolated from adult kidney expressed *Lgr8* transcripts and that treatment of these cells with INSL3 reduced their number by inhibiting proliferation (or promoting apoptosis?). These data indicate that functional LGR8 (receptors) might be present on mesangial cells and/or on endothelial and epithelial cells, and is consistent with the abundant, specific expression of LGR8 by cells in mature glomeruli in embryonic, early postnatal and adult kidney. It suggests a potentially important role for INSL3–LGR8 signalling in regulating the development, maturation and/or normal function of these renal cells in rat and possibly other species, including mouse and human.

Glomerulogenesis involves four major cellular processes – migration, proliferation, differentiation and apoptosis. Active migration, proliferation and differentiation of mesangial cells occur largely in immature glomeruli. Glomerular mesangial cells are, however, known to have a limited capacity for proliferation and cease to proliferate when mature glomeruli are formed. In normal adult kidney, the turnover rate of mesangial cells is 1% per day (Pabst & Sterzel 1983). The *Lgr8*/LGR8 expression pattern

Figure 5 Distribution of ^{125}I -INSL3-binding sites in adult and postnatal rat kidney. (A–H) Representative X-ray film autoradiograms of ^{125}I -INSL3-binding sites in longitudinal sections of kidney from male rats reveal the presence of presumed LGR8 in the renal cortex in both adult (A) and postnatal kidney (C–H) in a pattern consistent with an association with glomeruli and with the distribution of *Lgr8* mRNA. No specific ^{125}I -INSL3 binding was observed in the renal medulla at any age. (B) Addition of 100 nM unlabelled human INSL3 to the incubation abolished all specific ^{125}I -INSL3 binding, leaving only low levels of non-specific binding (NSB). Scale bar = 3 mm (A–H).

observed is very similar to that for the angiotensin II receptor-1 (Harrison-Bernard *et al.* 1997, Norwood *et al.* 1997, Ma *et al.* 2004), with both mRNAs/proteins highly expressed in mature glomeruli (possibly by mesangial cells), but not in more immature comma- or S-shaped bodies – implying that like angiotensin II, INSL3 is more likely to be involved in the inhibition of mesangial cell proliferation and/or apoptosis during glomerulogenesis, than in differentiation and/or migration (Ma *et al.* 2004). This speculation is supported by the current *in vitro* experiments in which treatment of cultured glomerular cells (enriched in mesangial cells) with synthetic INSL3 resulted in apparent inhibition of proliferation, suggesting that like transforming growth factor- β (TGF- β) (Jaffer *et al.* 1989), INSL3 (circulating) is a growth-inhibitory factor for these renal cells.

Glomerular mesangial cells play an important role in maintaining the structure/function of the capillary ultra-filtration apparatus. Maintaining the correct number of

Figure 6 *Lgr8* mRNA expression in cultured glomerular cells from adult rat kidney. RT-PCR analysis of total RNA isolated from cultured cells of rat kidney revealed the presence of *Lgr8* mRNA. Lane 1: 100 bp DNA ladder; lane 2: glomerular cell extract; lane 3: whole kidney tissue; lane 4: positive controls (cervix – *Lgr7* (upper); gubernaculum – *Lgr8* (lower); lane 5: negative control (water as template). Expression of GAPDH mRNA was used as a control for the quality of the cDNA synthesis.

Figure 7 INSL3-induced inhibition of the proliferation of rat glomerular cells. Cells were cultured in 96-well microplates for 48 h in serum-free medium supplemented with 0.1% BSA and with various test peptides. Effects of treatment with INSL3 or other peptides were analysed using an MTT assay, as described in Materials and Methods. (A) Effect of INSL3, INSL4 and relaxin (RLX) on cell proliferation. Values (each bar) represent means $\pm$ S.D. ($n=3$). (B) Dose-dependent inhibition of cell proliferation by INSL3. Values are means $\pm$ S.D. ($n=5$). * $P=0.05$; ** $P<0.01$ (one-way ANOVA).

mesangial cells is important for renal physiology and abnormalities are associated with renal pathology. Thus, uncontrolled mesangial cell proliferation is a primary event leading to the pathogenesis of progressive renal diseases including diabetic nephropathy, membrane-proliferative glomerulonephritis and lupus nephritis (Haas *et al.* 1999). Increased mesangial cell proliferation may also be essential for glomerular repair. Several growth factors and mediators can regulate mesangial cell proliferation, including platelet-derived growth factor (Blom *et al.* 2001), basic fibroblast growth factor (Floege *et al.* 1993), insulin-like growth factors (Feld *et al.* 1995) and TGF- β

(Jaffer *et al.* 1989). Among these, TGF- β is the only peptide with anti-mitogenic and anti-inflammatory actions (Schoecklmann *et al.* 1997, 1999), but TGF- β also plays an important role in the pathogenesis of glomerular disease and mediates extracellular matrix deposition leading to glomerulosclerosis (Shankland & Johnson 1998). Nonetheless, the possibility that INSL3 inhibits mesangial cell proliferation (and/or promotes apoptosis) predicts a potentially beneficial application of the INSL3–LGR8 system in glomerular diseases, especially those involving excessive mesangial cell proliferation.

A characteristic action of INSL3 is to stimulate gubernacular cell proliferation and differentiation during testicular descent (see Introduction). *LGR8* transcripts are present in human gubernaculum and INSL3 stimulates cAMP production and thymidine incorporation in cultured E17 gubernaculum (Kubota *et al.* 2002, Kumagai *et al.* 2002). In rat testis, however, INSL3 inhibits germ cell apoptosis (Kawamura *et al.* 2004). Thus, in rat kidney INSL3 may have a different function to that in gubernacular or germ cells – inhibiting cell proliferation. Such differential, cell-type specific effects have been observed for other peptide ligand–receptor systems, including members of the insulin/relaxin superfamily (Saile *et al.* 2004).

Expression of *Lgr8* mRNA was 6-fold higher in female than male rat adult kidney. The serum INSL3 concentration is reportedly higher in adult men than in post-pubertal women (Büllesbach *et al.* 1999) and higher in adult male rats than females (Boockfor *et al.* 2001). Whether the higher level of *Lgr8* in female rat kidney reflects a biological compensation for the low level of INSL3 in female serum and is reflected by a correspondingly higher expression of LGR8 protein in female kidney requires further investigation, along with studies of the precise relationship between gene and protein expression in renal glomeruli during development (in males and females). In this respect it is possible that levels of 125 I-INSL3 binding observed accurately reflect the densities of LGR8 protein present in developing kidney or that binding levels are influenced by the level of endogenous ligand present in the tissue prior to its collection. The ratio of transcript to protein and the exact nature of the glomerular cells expressing LGR8 (mesangial or other?) might be better understood following the eventual development and application of specific antisera to rat LGR8. Nevertheless our current findings, along with the apparent absence of endogenous renal *Ins13* expression, suggest that actions of circulating INSL3 at LGR8 may play a role in regulating both male and female kidney function.

Ins13- and *Lgr8*-deficient mice have been generated by several independent groups (Nef & Parada 1999, Zimmermann *et al.* 1999, Overbeek *et al.* 2001, Kamat *et al.* 2004), but their investigations have not reported any phenotypic kidney abnormalities. This suggests that

INSL3 may not be essential for mouse nephrogenesis, but at this stage some role for INSL3 in embryonic kidney development cannot be precluded. It is perhaps likely that other mediators can compensate for the lack of INSL3 and/or LGR8 activity, but alternatively there may be some minor phenotypic changes in these knockout mice which have not been discovered. Interestingly, Nef & Parada (1999) reported that mice lacking *Ins13* produced a somewhat smaller average litter size, but it remains to be determined whether this is due to some germ cell abnormalities (Kawamura *et al.* 2004). Future studies in these *insl3* – / – mice should allow clarification of the physiological role of the INSL3–LGR8 system in renal development and normal renal function. In this context, the distribution of LGR8 should also be assessed in wild-type mouse kidney and compared with that in the rat.

Conclusion

These studies have demonstrated that functional receptors for INSL3 – LGR8 (RXFP2) – are expressed by mature glomeruli in developing and adult rat kidney *in vivo* and by glomerular cells *in vitro*, which suggests a potential role for INSL3–LGR8 signalling in the regulation of renal physiology. In particular, the presence of *Lgr8* mRNA and specific INSL3-binding sites in mature glomeruli in embryonic kidney suggests that INSL3 is involved in glomerulogenesis and/or mesangial cell development, possibly by regulation of cell proliferation and/or apoptosis. Further studies are warranted to examine the effects of INSL3, and newly developed, high-affinity LGR8 antagonists (Büllesbach & Schwabe 2005), on kidney function *in vivo* and on various renal glomerular cell types *in vitro*, including studies of characterized mesangial cells, to provide a better understanding of INSL3 action on cell proliferation/survival.

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