Comparative genomics reveals tissue-specific regulation of prolactin receptor gene expression

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Abstract

Prolactin (PRL), acting via the PRL receptor (*PRLR*), controls hundreds of biological processes across a range of species. Endocrine PRL elicits well-documented effects on target tissues such as the mammary glands and reproductive organs in addition to coordinating whole-body homeostasis during states such as lactation or adaptive responses to the environment. While changes in *PRLR* expression likely facilitates these tissue-specific responses to circulating PRL, the mechanisms regulating this regulation in non-rodent species has received limited attention. We performed a wide-scale analysis of *PRLR* 5' transcriptional regulation in pig tissues. Apart from the abundantly expressed and widely conserved exon 1, we identified alternative splicing of transcripts from an additional nine first exons of the porcine *PRLR* (*pPRLR*) gene. Notably, exon 1.5 transcripts were expressed most abundantly in the heart, while expression of exon 1.3-containing transcripts was greatest in the kidneys and small intestine. Expression of exon 1.3 mRNAs within the kidneys was most abundant in the renal cortex, and increased during gestation. A comparative analysis revealed a human homologue to exon 1.3, hE1_{N2}, which was also principally transcribed in the kidneys and small intestines, and an exon hE1_{N3} was only expressed in the kidneys of humans. Promoter alignment revealed conserved motifs within the proximal promoter upstream of exon 1.3, including putative binding sites for hepatocyte nuclear factor-1 and Sp1. Together, these results highlight the diverse, conserved and tissue-specific regulation of *PRLR* expression in the targets for PRL, which may function to coordinate complex physiological states such as lactation and osmoregulation.

Key Words

- prolactin
- receptor
- kidney
- transcription factors
- gene regulation

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Introduction

A successful lactation requires mammary epithelial cells to proliferate and then differentiate in preparation for the synthesis and secretion of milk (Hovey & Trott 2004), while peripheral organs coordinately absorb, metabolise

and/or mobilise a massive amount of various nutrients including water to support lactation. As has been established for lactating dairy animals, the systemic coordination of nutrient uptake and mobilisation occurs

in liver, muscle and adipose tissues during homeorhetic and homeostatic processes in support of lactation (Bauman & Currie 1980, Loor 2010). However, the mechanisms that coordinate and regulate these processes as well as the uptake and/or redistribution of other components in milk, such as water and ions, such as sodium and calcium, are not well understood in any species.

One hormone that can participate in the regulation of these processes and that fulfils >300 physiological functions across a broad range of species (Bole-Feysot *et al.* 1998) is the polypeptide hormone prolactin (PRL), which is secreted by lactotrophs in the anterior pituitary (Freeman *et al.* 2000). PRL stimulates the uptake of calcium, which is a major ion in milk, in the small intestine (Jantarajit *et al.* 2007, Ryska *et al.* 2012) and interacts with the renal dopamine system to effect natriuresis in the kidneys (Ibarra *et al.* 2005). The pre-partum surge of PRL that is essential for the subsequent lactation also likely increases blood flow to the mammary gland (MG) at the onset of lactation (Hanwell & Linzell 1973, Peaker 1976, Ota & Peaker 1979), given that PRL increases various measures of cardiac output (Nassar *et al.* 1974, Karmazyn *et al.* 1982). Both of these functions are peripheral to the major role of PRL in the development and differentiation of the MG and the stimulation of lactation (Trott *et al.* 2012). Ironically, the regulation of PRL receptor (PRLR) expression in tissues peripheral to the MG is almost completely unknown in most species, including humans.

Information regarding the physiological and genomic regulation of PRLR expression *in vivo* has mostly stemmed from data for rodents (Hu *et al.* 1996, 1998, Tabata *et al.* 2012, Hirai *et al.* 2013), or has specifically focused on PRLR expression in cancer cells *in vitro* and the response to stimulation by ovarian hormones (Hu *et al.* 2002, Dong *et al.* 2006). More than one promoter has been identified within the *PRLR* gene of mice (Ormandy *et al.* 1998), rats (Hu *et al.* 1998) and humans (Hu *et al.* 2002), although limited data exist regarding the activity of these known first exon promoters in different tissues from these species. Furthermore, while there are commonalities between these species, as to which tissues express PRLR (Bole-Feysot *et al.* 1998), there also are differences such as low expression of PRLR in the liver and ovaries of humans vs notably higher levels in rodents (Nagano & Kelly 1994, Peirce & Chen 2001). Thus, studies of how PRLR expression is regulated during various physiologic states in humans may not be closely recapitulated in rodents.

Pigs have become an increasingly important biomedical model, given that they are genetically more similar

to humans than mice (Wernersson *et al.* 2005) and are physiologically similar to humans (Swindle *et al.* 2012). Expression of the long form of the porcine PRLR (pPRLR) is differentially expressed in the pig in a tissue and cell-type specific manner (Trott *et al.* 2009), which is similar to that described for humans (Peirce & Chen 2001). Furthermore, we previously identified a homologous first exon promoter for the pPRLR (Schennink *et al.* 2013) that is expressed most abundantly in primary PRL targets such as the MG, adrenal glands, placenta, endometrium and pituitary, as well as the regulation of PRLR expression by estrogen. Given that various tissues differentially modulate their PRLR expression during states such as lactation (Jahn *et al.* 1991, Ling *et al.* 2000) and in response to hormonal stimulation (Trott *et al.* 2009), we hypothesised that multiple promoters direct pPRLR expression in a tissue-specific manner.

Using 5' RACE, we performed the most comprehensive screen to date of 5'*PRLR* gene regulation across tissues for any species. Among the nine new first-*PRLR* exons we identified, we found that expression of one first exon, pE1.3, was primarily expressed in the kidney cortex and small intestine, where a homologous first exon, hE1_{N2}, is uniquely expressed in these same tissues in humans. Furthermore, we show that certain transcripts encoding pE1.5 are restricted to expression in the heart, which further emphasises a potential role for PRL in the regulation of cardiac function.

Materials and methods

Animals

Experiments were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals, as approved by the Institutional Animal Care and Use Committee at UC Davis. Nulliparous Hampshire×Yorkshire female pigs ($n=11$) were housed, killed and tissues sampled as described previously (Trott *et al.* 2011). The pregnant pigs were unilaterally hysterectomised–ovariectomised and then killed at different stages of gestation, as described previously (Freking *et al.* 2007, Trott *et al.* 2009).

RNA extraction, RT and 5' RACE

Extraction of porcine RNA and generation of cDNA were performed as described previously (Schennink *et al.* 2013). Human RNA (0.5 µg) from the Human total RNA master panel I (Clontech) was subjected to RT as described

previously (Schennink *et al.* 2013). We performed 5' RACE as described previously (Schennink *et al.* 2013) using poly A+ RNA from pig MG, placenta, liver, kidney, adrenal, ovary and testis using the SMARTer RACE cDNA Amplification Kit (Clontech) and the primers GSP1a (5'-CG-GAGGTGACTGTCCATTTCAGAAAGGCTG-3'; exons 3/4) and NGSP1a (5'-TTGGCTCCCTTCTCTTCCACAGG-CAG-3'; exon 3) designed from the pPRLR (GenBank NM_001001868).

Cloning pig exon 1 transcripts

The primer pairs were designed to span either from exon 1 of pPRLR to the junction of exons 3/4, or from exon 1 to the junction of exons 2.1/3 (Table 1) in an attempt to clone three splice variants for each exon 1. The transcripts of pPRLR were generated by PCR of cDNA prepared from multiple pig tissues (Table 1), gel purified, cloned into pCR2.1 TOPO (Life Technologies) and sequenced.

Quantitative PCR

The primer sequences used to amplify pPRLR first exon splice variants are presented in Table 2. Quantitative PCRs were performed as described previously (Schennink *et al.* 2013), using the T_m in Table 2 for the combined annealing and extension step. Standard curves were prepared as tenfold serial dilutions (10^{-10} – 10^{-7} copies/well) of each pPRLR cDNA transcript that had been cloned into pCR2.1-TOPO

and then spiked into either mouse liver cDNA or tRNA (10 ng/ml, Ambion, Carlsbad, CA, USA). The standards were assayed in duplicate or triplicate. The expression levels of each transcript were calculated using an absolute quantification standard curve of cycles to threshold vs plasmid copy number ($\log_{10}$ transformed). A relative standard curve for 18S rRNA was also prepared using fivefold serial dilutions of pooled cDNA that were amplified in duplicate using previously published primers (Trott *et al.* 2011). All standard curves had a linear regression coefficient of determination of at least 99.1%. The average transcript copy number, determined from duplicates, was divided by the corresponding relative amount of 18S rRNA (Trott *et al.* 2011).

Promoter and first exon sequence homology

The NCBI BLAST programme (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) was used to search genomic sequences for homologues to the porcine first exon sequences. The promoter sequences (2000 bp upstream) for *Sus scrofa* (pig), *Canis lupus familiaris* (dog), *Bos taurus* (cow), *Papio anubio* (olive baboon), *Macaca mulatta* (Rhesus monkey) and *Homo sapiens* (human) that were homologous to the pE1.3 first exon were downloaded from GenBank and aligned using Dalign to identify the regions of homology (Brudno *et al.* 2003). We used AliBaba2.1 (<http://www.gene-regulation.com/pub/programs/alibaba2/index.html>) to predict transcription factor-binding sites from the

Table 1 Forward (F) and reverse (R) PCR primers used for cloning the transcripts of multiple splice variants of nine first exons of the pig PRLR

Plasmid insert	F (5'–3')	R (5'–3')
1.1b/3/4	GTGCCTGTGAGCCTGAAAGTACT	GGAGGTGACTGTCCATTTCAGA
1.1b/2/3/4		
1.2/3/4	AGGTGTGGCTCTTCCAAAAAG	GGAGGTGACTGTCCATTTCAGA
1.2/2/3/4		
1.2/2.1/3/4		
1.3/3/4	GTGACGCACGGCAGGAA	GGAGGTGACTGTCCATTTCAGA
1.3/2/3/4		
1.3/2.1/3	GTGACGCACGGCAGGAA	GGTTCATTCAAAGCGTGCTC
1.4/3/4	CCTGGGAGATGCTCTTTGGA	GGAGGTGACTGTCCATTTCAGA
1.4/2/3/4		
1.5/3/4	GCTCTTTCTAGCAATCAGAACTGTCTAC	GGAGGTGACTGTCCATTTCAGA
1.5/2/3/4		
1.5/2.1/3	GCTCTTTCTAGCAATCAGAACTGTCTAC	GGTTCATTCAAAGCGTGCTC
1.6/3/4	TTCTGGTTTCTATTTTCATCTTCGT	GGAGGTGACTGTCCATTTCAGA
1.6/2/3/4		
1.6/2.1/3	TTCTGGTTTCTATTTTCATCTTCGT	GGTTCATTCAAAGCGTGCTC
1.7b/3/4	GGAATTGCCAGCGTGTTACTG	GGAGGTGACTGTCCATTTCAGA
1.7b/2/3/4		
1.8/2/3/4	TTCCAATTACGTGACCAGAGATCA	GGAGGTGACTGTCCATTTCAGA
1.9/3/4	GGGAGGCGCACTTACGAAA	GGAGGTGACTGTCCATTTCAGA

Table 2 Sequences of forward (F) and reverse (R) primers used for qPCR of pig PRLR first exon splice variants and PCR of human PRLR first exon transcripts across tissues

	F (5'–3')	R (5'–3')	T _m ^a
pPRLR first exon transcript			
1.1b/3	TTTGAGAAAGACAAGTGAACCTCTGA	TGTGGATGGCACATTTTCCTT	63
1.1b/2/3	TACTTTGAGAAAGACAAGCTATAGAAC	GGTCAGAGGTTCACTTCAGGGGTCAT	60
1.1b/2.1/3	GTGCTGTGAGCCTGAAAGTACT	GGATGGGAGCCTTCAGGG	63
1.2/2/3	AGGTGTGGCTCTTCCCAAAG	GGTCAGAGGTTCACTTCAGGGGTCAT	65
1.2/3	AGGTGTGGCTCTTCCCAAAG	GTCAGAGGTTCACTGACACTACCC	59
1.2/2.1/3	AGGTGTGGCTCTTCCCAAAG	GGATGGGAGCCTTCAGGG	61
1.3/2/3	GTGACGCACGGCAGGAA	GGTCAGAGGTTCACTTCAGGGGTCAT	65
1.3/3	GAAGGCCACCGTGAACCT	TGTGGATGGCACATTTTCCTT	60
1.3/2.1/3	GTGACGCACGGCAGGAA	GGATGGGAGCCTTCAGGG	60
1.5/3	GAACACTGCACTGAACCTCTGA	TGTGGATGGCACATTTTCCTT	60
1.5/2/3	GCTCTTTCTAGCAATCAGAACTGTCTAC	GGTCAGAGGTTCACTTCAGGGGTCAT	60
1.5/2.1/3	GCTCTTTCTAGCAATCAGAACTGTCTAC	GGATGGGAGCCTTCAGGG	60
1.4/3	CAAGCTGGAGGGAGTGAACCT	CTGTGGATGGCACATTTTCCTT	60
1.4/2/3	CCTGGGAGATGCTCTTTGGA	GGTCAGAGGTTCACTTCAGGGGTCAT	65
1.6/3	CATCTTCGTGGTGAACCTCTGA	TGGCACATTTTCCTTCATGTTG	60
1.6/2/3	TCATCTTCGTGGCTAAAGAACTCTT	AGTGGGTCAGAGGTTCACTTCAG	60
1.6/2.1/3	TCATCTTCGTGGCTAAAGAACTCTT	TGTGGATGGGAGCCTTCAG	60
1.7b/2/3	GGAATTGCCAGCGTGTACTG	GGTCAGAGGTTCACTTCAGGGGTCAT	60
1.7b/3	GTGTTACTGCTATGTTGAACCTCTGA	TGTGGATGGCACATTTTCCTT	60
1.8/2/3	TTCCAATTACGTGACCAGAGATCA	GGTCAGAGGTTCACTTCAGGGGTCAT	60
1.9/3	CTTACGAAAGTCAAGTGAACCTCTGA	TGTGGATGGCACATTTTCCTT	60
Total 1.3/3	GTGACGCACGGCAGGAA	TGTGGATGGCACATTTTCCTT	60
hPRLR first exon transcript			
hE1 _{N2} /2	GGCTGTACCTTTACCAG	CAGTGTTCCGCTCCATGAATA	55
hE1 _{N3} /5	ATCAGGAGGGCGGCTCAATA	GTCCACATAAAGTTCATCCGAGAA	60
hE1 ₃ /hE1 _{N2}	TTTTCTGGGCTGGGCTTTCT	GACACCTACCCACGCTTC	60

^aAnnealing temperature.

TRANSFAC 4.0 database and ClustalW2 to determine the % homology between sequences (<https://www.ebi.ac.uk/Tools/msa/clustalw2/>). The transcription factor consensus binding site motifs were obtained from the JASPAR motif database (http://fantom.gsc.riken.jp/5/sstar/Browse_JASPAR_motifs).

Heatmap

To summarise the expression of all transcripts in all target tissues, the expression profiles were clustered in R using the 'gplots' library. The expression profiles were log-transformed. Distances between expression profiles were then calculated based on euclidian distances and hierarchical clustering of expression profiles was done using the 'hclust' algorithm.

Statistical analyses

Normality of data was tested using the univariate procedure of SASv9.3 and, where possible, data were transformed to normality using the transreg procedure of SASv9.3 and a Box-Cox power transformation (SAS

Institute, Inc., Cary, NC, USA). The changes in transcript expression between tissues were assessed by two-way ANOVA using a general linear model procedure in SASv9.3 that included the effects of tissue and animal. Multiple comparisons were accounted for with Tukey adjustment (SAS Institute, Inc.). Results from quantitative PCR used to measure pPRLR expression in different regions of the kidney and small intestine were analyzed by Students' *t*-test. Differences were considered significant at $P \leq 0.05$.

Results

Identification of nine pPRLR first exons by 5' RACE

To address the coordinated and systemic regulation of PRLR expression within individuals, we searched for all potential first exons of the PRLR in seven candidate PRL-target tissues in pigs. From this analysis, we identified nine alternative first exon sequences in addition to the pE1 sequence we have recently described (Schennink *et al.* 2013). These various first exons of the pPRLR are denoted

as pE1.1–pE1.9 according to their order in the genome, and have the following GenBank accession numbers pE1.1 (KJ608380), pE1.2 (KJ608379), pE1.3 (KJ608375), pE1.4 (KJ608377), pE1.5 (KJ608373), pE1.6 (KJ608381), pE1.7 (KJ608376), pE1.8 (KJ608378) and pE1.9 (KJ608374). Alignment of pE1.1 and pE1.7 to the pig genome (Build 10.2; http://www.ncbi.nlm.nih.gov/assembly/GCF_000003025.5/) revealed that each is divided into two exons by an intron, and thus were thereafter named pE1.1a and E1.1b and pE1.7a and E1.7b respectively.

Given that *S. scrofa* genome lacks complete annotation, we determined the location and orientation of all ten first exons plus exons 2 and 2.1 on *S. scrofa* chromosome 16 (Schennink *et al.* 2013), with the exception of pE1.5, which is likely located in a 50 kb gap between pE1.4 and pE1.6 that is present in the latest *S. scrofa* genome Build 10.2 (Fig. 1). The orientation and locations of exons 3 through 11 on the reverse strand of chromosome 16 were as described previously (Trott *et al.* 2011), which are in accordance with the hPRLR gene that is located on the reverse strand of *H. sapiens* chromosome 5. From our annotation (Fig. 1), it is evident that the segments including 21 608 065–21 631 107 bp (exons 1.6 through 2.1) and 21 742 631–21 787 592 bp (exons 1 through part of 1.4) of the current *S. scrofa* Build 10.2 are both incorrectly oriented (Fig. 1).

Given our previous demonstration that pE1 has multiple transcripts involving alternative splicing of exons 2 and 2.1 (Schennink *et al.* 2013), we carried out PCR to find all possible splice variants involving first exons pE1.1b through pE1.9, and exons 2, 2.1 and 3. The splice variants pE1.4/2/2.1, pE1.8/3, pE1.8/2/2.1/3, pE1.9/2/3 and pE1.9/2/2.1/3 were undetectable in either adrenal,

endometrium, kidney, liver, MG or ovary cDNA. The absence of pE1.9/2/3 and pE1.9/2/2.1/3 mRNA further confirmed that our genomic organisation of the pPRLR exons was correct (Fig. 1). The splice variant 1.7b/2/2.1/3 was undetectable in MG cDNA, leading us to conclude that none of these particular splice variants were transcribed at biologically significant levels; thus we did not pursue them further (data not shown). In Fig. 2, we have illustrated the 24 known transcripts of pPRLR first exon splice variants involving splicing of exons 1, 2, 2.1 and 3 of the pPRLR both from this manuscript in addition to those reported by Schennink *et al.* (2013).

Tissue-specific pPRLR transcript expression

We used qPCR to quantify the expression of all known pPRLR splice variants incorporating the nine new first exons that splice to exon 3, including or excluding exons 2 and 2.1. This analysis was performed on 18 tissues collected from the same individual pigs, which enabled us to assess the systemic, coordinated regulation of pPRLR transcription (Fig. 3). Nine clusters of splice variants having similar patterns of tissue-specific expression were identified (Fig. 3). Among these tissues, the mRNA for nine splice variants was most abundant in the MG, namely pE1.1b/3, pE1.1b/2/3, pE1.2/3, pE1.2/2/3, pE1.2/2/2.1/3, pE1.5/3, pE1.6/3, pE1.7b/3, pE1.9/3 (Supplementary Figures 1, 2, 3, 4 and 5, see section on supplementary data given at the end of this article), where all but two of these are in clusters 1 and 6 (Fig. 3). This abundant expression of the various pPRLR mRNAs in the MG was also previously shown for the pE1 splice variants (Schennink *et al.* 2013). When both pE1.4-containing splice variants (pE1.4/3 and pE1.4/2/3, clusters 2 and 8,

Figure 1

The pig PRLR gene is located on the reverse strand of chromosome 16 and comprises nine coding (exons 3–11) and 14 non-coding exons. Intron lengths (bp) are indicated above the line while exon lengths (bp) are indicated below each box representing an exon. The physical and numbered location on chromosome 16 according to Build 10.2 of the *Sus scrofa* genome (<http://www.ncbi.nlm.nih.gov/genome/84>) is indicated with numbers and arrows, and is currently annotated as being split between the

reverse strand (exons 1.9 through 11) and two sections of the forward strand (exons 1 through 2.1). The sections from 21 608 065 to 21 631 107 bp (exons 1.6 through 2.1) and 21 742 631 to 21 787 592 bp (exons 1 through part of 1.4) are both incorrectly oriented in the current *S. scrofa* Build 10.2 and ought to be on the reverse strand. The 290 bp polymorphic insertion in exon 11 (Trott *et al.* 2014) is indicated.

Figure 2

Diagrammatic illustration of the 24 known splice variants of the pig *PRLR* involving either exons 2/2, 1/3, 2/3 or splicing directly from exon 1 to 3 as found in this manuscript and in Schennink *et al.* (2013).

Fig. 3) were considered together, there was a tendency towards their higher expression in the endometrium, kidney and small intestine (Supplementary Figure 6). Three other splice variants (pE1.5/2/2.1/3, pE1.6/2/2.1/3 and pE1.8/2/3) did not have any specificity in their expression across tissues (Supplementary Figures 3, 4 and 5C). Two splice variants, pE1.1b/2/2.1/3 and pE1.7b/2/3, had fewer than 250 copies of mRNA/ μ g total RNA in all 18 tissues (data not shown). All tissues expressed the three transcripts in cluster 6, namely pE1.5/3 (Supplementary Figure 3), pE1.1b/3 (Supplementary Figure 1) and pE1.9/3 (Supplementary Figure 5B) as their most-highly expressed transcripts (Fig. 3), except for lymph node, heart, kidney, small intestine and liver.

While expression of *pPRLR* mRNA in the lymph node was generally low, there was enhanced expression of the

pE1.6/3, but not the pE1.6/2/3 transcript, within this tissue (Supplementary Figure 4). Interestingly, the primary transcript expressed in the heart was pE1.5/2/3 (Fig. 3), which was present at levels >20-fold higher than in any other tissue (Supplementary Figure 3; $P < 0.05$). Separately, there was a unique, elevated expression of pE1.3-containing transcripts in the kidneys, small intestine and liver (clusters 5 and 9, Fig. 3), where the pE1.3/2/3 and pE1.3/3 transcripts were the major transcripts (Supplementary Figures 7), with expression levels far-exceeding the expression levels of the three transcripts in cluster 6 that are the most highly-expressed transcripts in the other 13 tissues (Fig. 3; and Supplementary Figures 1, 3 and 5B).

Analysis of pE1.3 expression in pig kidney and small intestine

Given our novel finding that transcription of pE1.3 was tightly restricted to high levels of expression in the kidneys and small intestine, we investigated its distribution within these tissues given the important role for PRL in both these organs (Ryszka *et al.* 2012, Wongdee & Charoenphandhu 2013). Within the kidneys most pE1.3 mRNA was expressed in the cortex (Fig. 4), with similar amounts ($P = 0.7$) in the outer and inner regions ($126 \times 10^3 \pm 7 \times 10^3$ copies pE1.3 mRNA/ μ g total RNA, and $131 \times 10^3 \pm 13 \times 10^3$ copies pE1.3 mRNA/ μ g total RNA respectively). By contrast, much lower levels ($P < 0.001$) were present in the medulla ($3.0 \times 10^3 \pm 1.2 \times 10^3$ copies pE1.3 mRNA/ μ g total RNA) and pelvis ($1.7 \times 10^3 \pm 0.7 \times 10^3$ copies pE1.3 mRNA/ μ g total RNA).

We next examined whether the expression of pE1.3 in the kidney changed during the transition from gestation to lactation given that renal function is dramatically increased during this window. Indeed, expression of pE1.3 mRNA in the kidney increased linearly during this period (Fig. 5; $P = 0.04$). Finally, we assessed whether pE1.3 expression differed throughout the small intestine. In contrast to the kidney, we found no evidence for differential expression of pE1.3 mRNA between its three regions (Fig. 6; $P > 0.18$).

Homologous PRLR first exons are transcribed in the human

We compared the ten first exons of the *pPRLR* (the nine described herein plus pE1 (Schennink *et al.* 2013)) with first exon PRLR sequences from the four species reported to have more than one first-exon, namely mouse, rat, human and chicken (Fig. 7). The pE1 closely aligned to

Figure 3

Heatmap of 19 pig *PRLR* first exon splice variants expressed in 18 different tissues. Each coloured square depicts the intensity of log-transformed transcript expression levels according to the colour key (cream=lowest expression, red=highest expression). The dendrogram on the left side shows the distances between the expression profiles of different

transcripts, while the dendrogram on the top shows the distances between the expression profiles of different tissues. Short branch lengths indicate smaller differences. Individual clusters one to nine are manually separated by white rows to highlight specific clusters of interest. A full colour version of this figure is available via <http://dx.doi.org/10.1530/JME-14-0212>.

rat E1₃ (rE1₃), mouse E1₃ (mE1₃) and human E1₃ (hE1₃) as previously described (Schennink *et al.* 2013). Not surprisingly, for mice and rats, there was conservation of the first *PRLR* exons rE1₄ and mE1₄, rE1₂ and mE1₂, rE1₅ and mE1₅, but these had no homology with any known human or pig first exons. Separately, we found that pE1.3 aligned with a *PRLR* first exon that was previously identified in humans, namely hE1_{N2} (Hu *et al.* 2002). Given the high level of genomic sequence identity between pigs and humans (Wernersson *et al.* 2005), we hypothesised that sequence information for these ten *pPRLR* first exons would allow us to identify additional first exons that are transcribed from the human genome. Seven of the pig first exons shared some identity with sequences upstream of h*PRLR* exon 2. The similarity between these putative homologue pairs was 66% (pE1.6), 69% (pE1.3), 69% (pE1.4), 71% (pE1.2), 71% (pE1.5), 71% (pE1.8) and 74% (pE1). Two of these human sequences, namely hE1_{N2} (homologous to pE1.3) and hE1₃ (homologous

to pE1), were previously found to be expressed as first exons (Hu *et al.* 2002). We also investigated whether the remaining putative homologues were expressed in humans by performing RT-PCR on 20 human tissues using primers in putative human exons 1.2, 1.4, 1.6 and 1.8 and exon 6, but did not record any evidence for the transcription of these exons (data not shown). Furthermore, primers positioned in the putative human exon 1.5 and exon 5 of the h*PRLR* did not amplify a PCR product from human heart cDNA (not shown). Finally, we found no evidence in GenBank for the expressed sequences of the candidate human first exon homologues 1.2, 1.4, 1.5, 1.6 or 1.8. From these collective findings, we presently deduce that only the homologous exons pE1.3/hE1_{N2} and pE1/hE1₃ are expressed in both pigs and humans.

Given the homology between pE1.3 and hE1_{N2} and the pronounced tissue specificity of pE1.3 expression, we sought to determine whether hE1_{N2} expression was also tissue-restricted in humans. Using PCR primers in hE1_{N2}

Figure 4

Expression of pig *PRLR* exon 1.3 (pE1.3) transcripts in different regions of the porcine kidney. The kidneys were dissected from three nulliparous female pigs into outer and inner cortex, medulla and pelvis and the RNA was extracted and subjected to RT-PCR using the total pE1.3/3 and 18S rRNA primer pairs. Products were separated using electrophoresis on 2% agarose gels.

and exon 6, we identified three splice variants expressed in T47D cells, which included either hE1_{N2} or hE1_{N3} (GenBank KJ651922), hE1_{N2} and a novel first exon we called hE1_{N6} (GenBank KJ651923), or splicing directly from hE1_{N2} to exon 2 and then exons 3–6 (GenBank KJ651924). According to Repbase Update (<http://www.girinst.org/censor/index.php>; 2 September 2014), the hE1_{N6} sequence is a human-specific MSTA repeat sequence (a member of the ERVL-mammalian apparent long terminal repeat retrotransposon (MaLR) family; Kohany *et al.* 2006) with 26 566 occurrences in the human genome, Annotation Release 106 (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&BLAST_SPEC=OGP_9606_9558; 2 September 2014). This hE1_{N6} sequence is located between hE1_{N3} and hE1_{N4} in the h*PRLR* gene.

We also investigated whether hE1_{N2} expression was the result of splicing from an upstream exon rather than due to independent hE1_{N2} promoter activity. Given that the abundantly expressed exon hE1₃ lies upstream of hE1_{N2}, we screened for transcripts starting with hE1₃ and splicing to hE1_{N2}. Expression of the transcript including the hE1₃-hE1_{N2} sequence was highest in the adrenal gland, endometrium and placenta, and was undetectable in heart, skeletal muscle or spleen (Fig. 8). These data proved that not all hE1_{N2} expression was conferred by the hE1_{N2} promoter, which led to the identification of an internal 3' splice site marked by a consensus CAG trinucleotide (Zhang 1998). Given that transcription of first exon h*PRLR* mRNA downstream of the 3' splice site can be either from that first exon's promoter activity, or due to

splicing from an upstream first exon, we determined whether any expression of hE1_{N2} in human tissues, or for that matter hE1_{N3} and the new exon hE1_{N6}, was the result of their own promoter activity. We found that transcription of mRNA containing sequence upstream of the internal 3' splice site for hE1_{N2}, hE1_{N3} and hE1_{N6} in 20 human tissues was much less than the corresponding expression level of mRNAs containing sequence downstream of the internal 3' splice site (data not shown). This finding strongly suggested that the activity of promoters associated with hE1_{N2}, hE1_{N3} and hE1_{N6} could only be measured as the levels of transcribed mRNA sequence upstream of the internal 3' splice sites.

We used PCR primers in genomic sequence (Table 2) upstream of the 3' splice sites for exons hE1_{N2}, hE1_{N3} and hE1_{N6} to determine whether each exon's promoter was active, avoiding the complication of upstream splicing activity as a contributing source of mRNA. In T47D cells, hE1_{N2} (GenBank KJ651924), hE1_{N3} (GenBank KJ651925) and hE1_{N6} (GenBank KJ608382) first exons all contained mRNA sequence upstream of an internal 3' splice site (Fig. 9) marked by a consensus CAG trinucleotide (Zhang 1998), indicating that the three promoters are all active in T47D cells. Consistent with our findings for pE1.3 in pigs, we found that promoter activity of its homologue hE1_{N2} in human tissues was principally restricted to the kidneys and small intestine, with lower levels in the placenta, uterus, brain, lung and adrenal glands (Fig. 10A). Transcription directed by the hE1_{N3} promoter was

Figure 5

Expression of all pig *PRLR* exon 1.3 (pE1.3) transcripts in the kidneys of pregnant female pigs. Kidneys were collected on days 25, 45, 65, 85 and 105 of gestation, and RNA was DNase-treated before RT-qPCR analysis using the total pE1.3/3 primer pair with normalisation for 18S rRNA expression levels. The individual data points ($n=4-5$ /day of gestation), a best-fit linear regression curve ($R^2=0.19$; $P<0.05$) and 95% CI boundaries, are presented.

Figure 6

Expression of all pig *PRLR* exon 1.3 (pE1.3) transcripts in the different regions of the porcine small intestine. The duodenum, ileum and jejunum were dissected from four nulliparous female pigs, then the RNA was extracted and subjected to RT-qPCR analysis for pE1.3 mRNA expression using the total pE1.3/3 primer pair with normalisation for 18S rRNA expression levels.

confined to the kidneys (Fig. 10B), and hE1_{N3} spliced either directly to exon 2 or to exon hE1_{N6} and then exon 2. Separately, we found that the exon hE1_{N6} spliced either directly to exon 2 or spliced to hE1_{N4} before splicing to exon 2 in T47D cells (GenBank KJ608383). However, no expression of hE1_{N6} mRNA sequence upstream of the internal 3' splice site was recorded in the 20 human tissues analyzed (data not shown).

Comparative genomics reveals conserved exon and promoter sequences

Given that the homologous pE1/hE1₃ exons are conserved and expressed as first exons in multiple species, we hypothesised that the homologous pE1.3/hE1_{N2} exons were also conserved and expressed in mRNAs encoding the *PRLR* of other species. Although no homologous sequence was present in any rodent species, we found that hE1_{N2}/pE1.3 mRNA homologues had been sequenced for *B. taurus* (GenBank XM_005221579.1), *C. lupus familiaris* (GenBank XM_005619399.1), *Papio anubis* (GenBank FC171848.1), *M. mulatta* (GenBank XM_002804335.1) and *Macaca fascicularis* (GenBank XM_005556704). Interestingly, *M. mulatta*, *M. fascicularis* and *P. anubis* have

sequences in the RefSeq database as ESTs that start with sequence homologous to the pE1/hE1₃ exon, upstream of the exon sequence homologous to pE1.3/hE1_{N2} (data not shown). Homologues of pE1.3/hE1_{N2} were present in 14 other mammalian species across a range of taxa, including *Myotis lucifugus*, *Myotis brandtii*, *Oryctolagus cuniculus*, *Orcinus orca*, *Ceratotherium simum simum*, *Vicugna pacos*, *Equus caballus*, and seven species of primates (data not shown). Lastly, we assessed whether there was a parallel evidence for conserved

Figure 7

Phylogenetic tree of first exon sequences from the human (h), pig (p), rat (r), mouse (m) and chicken (ch) *PRLR* genes.

Figure 8

Expression of the human *PRLR* first exon hE1₃ splicing to hE1_{N2} in human tissues. Total RNA from 20 human tissues was subjected to RT then 40 cycles of PCR using primers in hE1₃ and hE1_{N2} as given in Table 1. Each sample was also subjected to 20 cycles of PCR using primers for 18S rRNA. Products were

separated by electrophoresis on a 2% agarose gel. Cerebellum, cerebellum; Fet, fetal; Saliv GI, salivary gland; Sk Musc, skeletal muscle; Small Int, small intestine.

promoter elements within ~800 bp of 5' sequences upstream of six pE1.3/hE1_{N2} first exon homologues. As shown in Fig. 11, there were seven conserved regions that ranged in homology from 57 to 100%. Conserved putative transcription binding sites within these regions include those for Sp1, Pit-1a, nuclear factor of activated T-cells, cytoplasmic 3 (NFATC3) and hepatocyte nuclear factor 1 (HNF1; Fig. 11). The conserved putative binding sites for HNF1, located just upstream of the transcription start site in the six pE1.3 homologues, matched either HNF1A (http://fantom.gsc.riken.jp/5/ssstar/JASPAR_motif:MA0046.1; 28 August 2014) or HNF1B

(http://fantom.gsc.riken.jp/5/ssstar/JASPAR_Motif:MA0153.1; 28 August 2014) consensus binding site motifs.

Discussion

Understanding the responsiveness to PRL during various physiological states requires an accurate depiction of the genomic structure and transcriptional mechanisms that regulate *PRLR* expression. Through a comparative genomics approach, we have identified novel and conserved promoters that facilitate tissue-specific regulation of *PRLR* expression and splicing activity in pigs and

Figure 9

Sequences of three human *PRLR* first exons, hE1_{N2}, hE1_{N3} and the novel hE1_{N6} (capital letters). Exon sequence included in transcripts as the result of both splicing machinery and promoter activity is underlined capitals and sequence transcribed only from the exon's own promoter (or included as intron sequence due to splicing from an upstream exon) is in italic capitals.

The internal 3' splice site CAG trinucleotides are indicated by ***. Additional intron and promoter sequences are in lower case. Nucleotides are numbered upstream from the ATG start codon in exon 3. The putative consensus binding site for HNF1 is indicated in bold lower case.

Figure 10

Transcription of the human *PRLR* by promoters upstream of the alternative first exons (A) hE1_{N2} and (B) hE1_{N3}. Total RNA from 20 human tissues was reverse transcribed before (A) 38 cycles or (B) 40 cycles of PCR using primers in (A) hE1_{N2} and exon 2 or (B) hE1_{N3} and exon 5, as detailed in Table 1.

Products were separated by electrophoresis on 2% agarose gels. Each sample was also subjected to 20 cycles of PCR using primers for 18S rRNA. Abbreviations are as per Fig. 8.

humans. Such a detailed analysis of how PRLR expression is regulated in different tissues has not been performed in any other species to date apart from the chicken. In that species, there are just two first exons: exon 1A, which is expressed in all 12 tissues, and, exon 1G, which is expressed only in the kidneys and small intestine (Bu *et al.* 2013). Chicken exon 1G shares no sequence homology with pE1.3 or hE1_{N2}, and its promoter shares no regions of homology with the mammalian sequences we aligned, apart from three putative HNF1A transcription factor-binding sites within the first 370 bp upstream of the transcription start site that are at different positions than the conserved HNF1A/1B binding site in the mammalian promoters (data not shown). Both HNF1A (<http://fantom.gsc.riken.jp/5/ssstar/EntrezGene:6927>; 28 August 2014) and HNF1B (<http://fantom.gsc.riken.jp/5/ssstar/EntrezGene:6928>; 28 August 2014) are associated with gene expression in the human kidney, intestinal epithelium, and liver. Taken together, these data strongly suggest that the putative HNF1A/1B transcription factor-binding site in the promoters of the pE1.3 homologues, and the HNF1A sites in the chicken exon 1G promoter, are responsible for tissue-specific PRLR expression in the kidney, small intestine and kidney.

Our data point to a key role for pE1.3/hE1_{N2} and its promoter in directing tissue-specific PRLR expression in kidney and small intestine. This tissue-restricted expression contrasts to that for other tissues having high levels of pPRLR including the MG, adrenal glands, endometrium, pituitary and placenta (Schennink *et al.*

2013) wherein the pE1 promoter is primarily active. For example, the pE1.3-containing transcripts are present in the kidneys of pigs at >530 000 copies mRNA/μg total RNA vs a lesser level of exon pE1 transcripts that are present at <130 000 copies mRNA/μg total RNA (Schennink *et al.* 2013). Similarly, the abundance of pE1.3 transcripts in the small intestine (>145 000 copies mRNA/μg) markedly exceeds the <30 000 pE1 transcripts/μg total RNA (Schennink *et al.* 2013). A similar quantitative and developmental analysis of the expression levels of the orthologous hE1_{N2} in human tissues remains unlikely given the limited availability of such samples beyond the type of pooled or unreplicated specimens we used in this study. Such a quandary highlights the importance of studying these processes in a manipulable animal model, such as the pig, from which tissues can be readily sourced.

Two primary functions of PRL in the kidneys are calcium homeostasis (Wongdee & Charoenphandhu 2013) and natriuresis, where PRL inhibits Na⁺, K⁺-ATPase activity in the cortex via the renal dopamine system (Ibarra *et al.* 2005). The kidneys are a major site of PRLR expression across species (Buck *et al.* 1992, Nagano & Kelly 1994, Peirce & Chen 2001). Kidney function changes significantly during pregnancy alongside concomitant changes in the endocrine environment and haemodynamics, where women with chronic kidney disease have increased risk of pregnancy complications and infant morbidity (reviewed by Maynard & Thadhani (2009)). In the kidneys of pigs, the expression of pE1.3 and

Promoter sequences for *PRLR* first exons with homology to pE1.3 and hE1_{N2} from *Sus scrofa* (pig; pE1.3), *Canis lupus familiaris* (dog), *Bos taurus* (cow), *Papio anubis* (olive baboon), *Macaca mulatta* (Rhesus macaque) and *Homo sapiens* (hE1_{N2}). Shaded areas are regions of homology with the % homology indicated underneath. Transcription factor binding sites predicted to be in all promoter sequences are indicated above and below

the sequence. Putative Sp1 sites were identified to be in slightly different positions in some species compared with others. Underlined bases are the consensus binding sites for HNF1. The boxed sequence is the start of the transcribed human exon hE1_{N2}. Bases are numbered upstream from the known transcription start site.

pE1 (Schennink *et al.* 2013) increased from days 25 to 105 of gestation, while in mice PRLR-long form (LF) expression increases from day 18 of pregnancy to day 5 of lactation (Buck *et al.* 1992). Interestingly, although pE1.3 expression was highest in the renal cortex where proximal tubules are located (Argenzio & Monteiro-Riviere 2001), PRLR-LF in the kidneys of rats is expressed mostly in the inner strip of the outer medulla (Ouhtit *et al.* 1993). This difference may reflect the superior ability of rodents to concentrate urine due to an increased relative length of the renal medulla (Argenzio & Monteiro-Riviere 2001).

Expression of pPRLR in the small intestine is dominated by the first exon pE1.3, indicating that the pE1.3 promoter is likely the main determinant of pPRLR-LF expression for this tissue in pigs. The small intestine plays crucial roles during lactation when the duodenum surface area increases along with its absorptive and digestive capacity. These changes occur alongside the secretion of acid and pepsin to meet the increased nutrient requirements and energy demand for milk synthesis (Lichtenberger & Trier 1979, Hammond 1997). Recent research has demonstrated multiple roles for PRL in the small intestine of mammals. In rodents, PRL mediates water uptake and retention during lactation in addition to the uptake of glucose, sodium, chloride and amino acids to support fluid, solute and energy requirements (Ramsey & Bern 1972, Mainoya 1975, Teerapornpuntakit *et al.* 2012). Both active and passive transport mechanisms of calcium uptake are rapidly stimulated by PRL via the phosphoinositide 3-kinase pathway (Jantarajit *et al.* 2007, Ryszka *et al.* 2012). In addition, PRL increases the expression of genes such as *CLDN19*, which modulates paracellular permeability, *FGFR2C*, which is required for differentiation and the sodium pump *ATP1B2*, which is essential for intestinal transport of glucose and calcium (Teerapornpuntakit *et al.* 2014). Despite these clear effects of PRL on the intestine, there are few data regarding the action of PRL via PRLR in the small intestines of livestock or humans.

A unique finding from our studies is the restricted expression of one specific pPRLR first exon transcript in the heart of pigs, namely pE1.5/2/3. Lactating females have elevated cardiac output (Hanwell & Linzell 1973, Peaker 1976, Ota & Peaker 1979), which increases nutrient and water delivery to the MG, liver and gastrointestinal tract (Chatwin *et al.* 1969), where PRL increases the force and rate of contractions in heart tissue (Nassar *et al.* 1974, Karmazyn *et al.* 1982). In humans, peripartum cardiomyopathy occurs in late pregnancy/early lactation and leads to a 6–10% mortality rate (Bhattacharyya *et al.* 2012). One proposed cause of this idiopathic heart failure is local

cleavage of PRL to its 16 kDa form, where pharmacologic inhibition of PRL secretion successfully treats the condition (Bachelier-Walenta *et al.* 2013). Data from previous studies indicate that expression of PRLR in random adult human hearts is relatively low (Kline *et al.* 1999, Fagerberg *et al.* 2014), while the factors that regulate PRLR expression in the human heart are unknown. The pE1.5 promoter is the principal determinant of total pPRLR expression in the porcine heart. For example, there were >65 000 copies of pE1.5 mRNA/μg total RNA in the heart and <12 500 copies of pE1 mRNA/μg total RNA, the next most abundant first exon. The factors that regulate the cardiac-specific splicing of pE1.5/2/3 remain to be resolved; however, our data demonstrate that PRLR expression from the pE1.5 promoter is not restricted to the heart, unlike the pE1.3 promoter that is active only in the kidney/small intestine/liver.

Our findings also emphasise that the mRNA splicing pattern for pPRLR first exons is tissue-specific. For example, abundance of the pE1.5/3 transcript was highest in the MG and lowest in the skeletal muscle, a pattern present in nearly 50% of the transcripts, whereas the pE1.5/2/3 transcript (and to some extent the pE1.5/2/2.1/3 transcript) was enriched in the heart but not in any other tissue. Along similar lines, the pE1.6/2/3 transcript, but not pE1.6/3 or pE1.6/2/2.1/3, was enriched in the kidney. Likewise, the pE1.3/3 and pE1.3/2/3 transcripts were most highly expressed in the kidney followed by the small intestine and then the liver, whereas the pE1.3/2/2.1/3 transcript was most highly expressed in the kidney followed by the liver, and was absent from the small intestine. Tissue-specific splicing, which incorporates up to eight different types of alternative splicing events (Wang *et al.* 2008), has been extensively analyzed using microarrays in budding yeast and metazoans and using RNA-seq in human cell lines and tissues (Calarco *et al.* 2011). Our data indicate an even more complicated level of mRNA splicing for the pPRLR gene, where the final mRNA composition not only depends on which tissue the transcript is expressed in but also on which first exon is transcribed. An example is the inclusion or absence of exon 2, where the pE1.8/3 transcript was not detected in any tissues while the pE1.8/2/3 transcript was present at 4–60 copies mRNA/μg total RNA in all 18 tissues. Conversely, the pE1.7b/3 transcript was expressed at 100–1000 copies mRNA/μg total RNA in all tissues, whereas pE1.7b/2/3 was present at only 0–250 copies mRNA/μg total RNA. These data support the presence of an interaction between the splicing and transcriptional machineries, which occurs by two main mechanisms: i) transcription can be coupled with splicing by the

recruitment of splicing factors to the promoter or ii) changes in the rate of transcription can alter splicing due to, for example, changes in the pre-mRNA chromatin structure as a result of different first exon sequences, or by different rates of transcription from alternate promoters (Kornblihtt 2007). Further experiments are required to establish precisely how this tissue-specific and first exon-dependent alternative splicing of the *pPRLR* is being regulated.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JME-14-0212>.

Declaration of interest

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

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