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Transcriptional Regulation of the Prolactin Receptor in Pigs

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TABLE OF CONTENTS

Acknowledgements ..... *iv*

Abstract ..... *v*

Chapter 1. Heterogeneity of the prolactin receptor and its expression: A Comprehensive Review *1*

Introduction 2

Expression of PRLR across tissues 4
- **Brain** 4
- **Liver** 8
- **Female Reproductive Tissues** 9
- **Male reproductive tissues** 14
- **Adipose tissue** 15
- **Cardiovascular System** 16
- **Adrenals** 16
- **Pancreas** 17
- **Immune system** 18
- **Mammary glands** 19
- **Intestine, kidney, and bone** 21
- **Avian** 23
- **Rodents** 24
- **Humans** 26
- **Ungulates** 28

Transcriptional regulation of the PRLR 29

Genetic variation of the PRLR 32
- **Reproductive Function** 32
- **Lactation Phenotypes** 34
- **Growth & Development** 35
- **Pathology** 38

Conclusion 41

References 42

Chapter 2. Prolactin Receptor Regulation in the Kidneys of Swine *75*

ABSTRACT 76

INTRODUCTION 77

MATERIALS & METHODS 78

RESULTS 82

DISCUSSION 86

REFERENCES 89

TABLES 96

FIGURE LEGENDS 98

`

FIGURES 101
FIGURE 1. 101
FIGURE 2. 102
FIGURE 3. 103
FIGURE 4. 104
FIGURE 5. 105
FIGURE 6. 105
FIGURE 7. 106
FIGURE 8. 108
SUPPLEMENTARY FIGURE 1. 109
SUPPLEMENTARY FIGURE 2. 110
Appendix A. Optimization of Chromatin immunoprecipitation ***111***
ChIP METHOD 114
MATERIALS 117

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Abstract

The cell-specific actions for prolactin (PRL) are dependent on the coordinated transcriptional regulation of the prolactin receptor (PRLR) gene across tissues. Several lines of evidence indicate that the use of multiple promoters underlies the tissue specific regulation for PRLR expression in states such as pregnancy and lactation. However, the role and regulation of PRL/PRLR action in the kidneys is less clear. We previously identified that one of several first exons of the porcine PRLR is expressed exclusively in the kidneys, small intestine, and liver. We found the 5' non-coding region of this first exon (E1.3) is conserved across mammalian PRLR, including ungulates and humans, and its expression increased throughout gestation. We hypothesized E1.3 expression in the kidney was regulated by tissue-specific factors acting on the E1.3 promoter (promE1.3**)**, and its expression increased in support of osmoregulation. Our objectives were to 1) resolve the factor(s) regulating E1.3 expression in the kidneys of pigs and 2) develop an *ex vivo* model to study E1.3 regulation in the kidneys. We found promE1.3 activation was tissue and cell line specific, whereby the proximal promoter was only active in kidney (LLC-PK1) and colorectal (Caco-2) cells. We also found an element downstream of the transcription start site (TSS) that acts to repress promoter activation in all cell lines tested. In silico transcription factor binding analysis of the proximal promoter revealed multiple consensus sequences for tissue-specific transcription factors, including hepatocyte nuclear factor 1 (HNF1). Mutation of one of the binding sites for HNF1 within the E1.3 promoter resulted in a significant ($p<0.05$) downregulation in promoter activity in Caco-2 cells, and a reduction in promoter activity in LLC-PK1 cells. Using our kidney explant model, we found E1.3 was more highly expressed than the ubiquitous first exon of the PRLR (exon 1A, E1A) *ex vivo*. Interestingly E1.3 expression significantly ($p<0.05$) declined with time in culture, and E1A became the

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predominant transcript expressed after 48 hours (h). There was no significant difference in PRLR long form or short form expression over time in culture. However, mRNA expression for HNF1 and HNF4 variants declined ($p<0.05$) over 48h in culture. Together, these data demonstrate that elements within the proximal promoter region of promE1.3 are vital for E1.3 expression, and tissue-specific elements may be required for its sustained expression. It remains to be determined if HNF transcription factors are directly or indirectly responsible for the regulation of PRLR expression in the kidneys of pigs.

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Chapter 1. Heterogeneity of the prolactin receptor and its expression: A Comprehensive Review

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Introduction

The importance for the pleiotropic hormone prolactin (PRL) was first demonstrated in the early 1900s via its role in stimulating milk secretion in rabbits and crop sac growth in pigeons (Stricker & Grueter 1928; Riddle et al., 1933). Sixty years later over 300 physiological functions have been identified for PRL across tissues, many of which are vital to the survival of mammalian and avian species (Bole-Feysot et al., 1998). In mammals, the most noteworthy functions for PRL are regulating reproduction and lactation. Thus, PRL has become a potential target to alter phenotypic outcomes in the agricultural industry. In addition, the contribution of PRL to numerous pathologies has become of increasing interest in the biomedical field.

The multifaceted roles for PRL are initiated by its profound ability to direct the function of multiple tissues in a coordinated, spatiotemporal manner. One mechanism that supports the diverse outcomes of PRL is the varied distribution and activation of its *trans*-membrane bound receptor (PRLR). The PRLR sequence is relatively well-conserved across mammalian and avian species at both the genetic and protein level, sharing at least 75% and 50% homology in their mRNA or amino acid sequences, respectively (https://www.ncbi.nlm.nih.gov). Alongside sequence homology, there are a range of clues suggesting that the gene regulation of PRLR expression is also conserved. More specifically, multiple conserved mechanisms may allow for tissue-specific responses to PRL during distinct physiological states. An intricate system of tissue-specific promoter utilization across mammals and birds directs coordinated gene transcription in support of cellular functions (Zheng & Baum 2008). At the same time, diversity in gene and protein structure may support species-specific PRL activity in distinct tissues. This review will summarize our current understanding of the intrinsic and extrinsic elements

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controlling differential PRLR expression and will highlight the genetic diversity of the PRLR gene that confers species-specific phenotypes.

PRLR structure and signaling

The PRLR belongs to the class 1 cytokine receptor superfamily that also includes the growth hormone and erythropoietin receptors (Liongue and Ward, 2007). Mammals have one copy of the PRLR gene that is thought to have arisen from a PRLR ancestor during early vertebrate evolution (Ocampo Daza and Larhammar, 2018). The PRLR gene in mammals comprise at least 10 exons that are alternatively spliced to create short (PRLR-SF), intermediate (PRLR-IF), and long isoforms (PRLR-LF). The PRLR-LF includes a conserved extracellular domain (ECD) that binds PRL in a 2:1 stoichiometry of receptor to ligand, a transmembrane domain, and an intracellular domain (ICD) containing two relatively conserved regions, box 1 and box 2, that are required for signal transduction (Bole-Feysot et al., 1998). In most cases, PRLR-SF, -IF and -LF share an identical ECD but differ in the length and composition of the ICD. Interestingly, the chicken PRLR gene contains exons encoding two ligand binding domains, giving rising to a "double antenna" structure (Tanaka et al., 1992; Bu et al., 2013a).

In most species there are several ligands that can differentially bind and activate mammalian PRLR, including PRL, growth hormone, and placental lactogen. Upon ligand binding, or in some cases as the result of ligand-independent receptor dimerization, downstream signal transduction is activated (Kossiakoff, 2004; Gadd and Clevenger, 2006; Brooks, 2012). This leads to activation of the constitutively associated tyrosine kinase, Janus kinase 2 (JAK2), that transphosphorylates itself and phosphorylates other tyrosine residues on the PRLR (Rui et al., 1994; Lebrun et al., 1995a; Lebrun et al., 1995b; Ferrag et al., 1997). Activation of transduction pathways including the JAK-Signal Transducer and Activator of Transcription

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(STAT), Ras/Raf-MAP kinase and PI3K-Akt cascades, can then initiate transcription of genes that mediate cell proliferation and survival (Clevenger and Kline, 2001a).

Expression of PRLR across tissues

The actions of PRL have been classified into 7 key categories: 1) water and electrolyte balance 2) growth and development 3) endocrinology and metabolism 4) brain and behavior 5) reproduction 6) immunoregulation and protection and 7) pathological disease (Bole-Feysot et al., 1998). Accordingly, PRLR have been identified in organ systems corresponding with each of these categories. The distribution of PRLR expression amongst each organ vastly varies with respect to sex, cell type, and physiological state in support of distinct cellular functions. This spatiotemporal regulation of PRLR expression across tissues relies on intrinsic and extrinsic factors that support vital biological functions across species.

Brain

There is a wide range of PRLR expression across different regions of the brain, where its expression varies by sex, age, location, reproductive state, and endocrine status (Reviewed by Cabrera-Reyes et al., 2017). Compared to other regions, the hypothalamus (HPT) has the greatest expression for PRLR-LF which localizes to the ventromedial, supraoptic, paraventricular, arcuate suprachiasmatic nucleus, medial septum, and preoptic areas (Shamgochian et al., 1995; Bakowska and Morrell, 1997; Pi and Grattan, 1999). Prolactin receptors are also widespread in HPT neurons, including tuberoinfundibular dopamine (TIDA) neurons, coinciding with the established role of PRL in the negative feedback loop on hypothalamic dopamine neurons (Fitzgerald and Dinan, 2008; Grattan and Kokay, 2008).

Differences in PRLR expression within the HPT of females and males aligns with the sex-specific functions of PRL across physiological states. In chickens, roosters have more

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expression of PRLR mRNA in the basal HPT and anterior pituitary than laying hens (Ohkubo et al., 1998b). Interestingly, this gender-dependent difference in PRLR expression is restricted to the basal HPT given expression levels were not different in the preoptic HPT, optic lobe, cerebellum, or forebrain of male and female chickens (Ohkubo et al., 1998a; Ohkubo et al., 1998b). In contrast, overall hypothalamic expression of PRLR in female rats is greater than males (Muccioli et al., 1991; Pi and Voogt, 2002). In females, PRLR expression has been identified in hypothalamic nuclei associated with maternal care, milk ejection, osmoregulation, and increased food intake (Cabrera-Reyes et al., 2017). Accordingly, hypothalamic expression of the PRLR in females varies by reproductive state, where mRNA abundance for both the PRLR-LF and -SF isoforms in rats was lowest at estrus and highest at proestrus (Pi and Voogt, 2002). Gestation and lactation also directs substantial changes in hypothalamic PRLR expression that varies across nuclei. Our lab has shown there is an overall increase in PRLR mRNA expression across gestation in the hypothalamus of pigs (Trott et al., 2009). In contrast, changes within specific hypothalamic nuclei have been identified in rats. In the medial preoptic area/anterior hypothalamus (MPOA) and arcuate nucleus, parity increases PRLR mRNA expression in rats whereby primiparous rats expressed 3-fold more PRLR mRNA compared to virgin rats (Anderson et al., 2006). While not statistically significant, the paraventricular and ventromedial hypothalamic nuclei of female rats showed an increase in PRLR mRNA expression from diestrus to day 7 of gestation (G7). No significant changes were found across gestation to lactation (Augustine et al., 2003). Interestingly, PRLR expression could not be detected in the ventro-dorsomedial nucleus of sheep during gestation (Szczesna et al., 2020).

In rats, the choroid plexus (CP) expresses high level of PRLR-LF and -SF expression compared to other regions, and the abundance of both isoforms is greater in female mice and rats

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compared to males (Pi and Voogt, 2002; Brown et al., 2010; Cabrera-Reyes et al., 2017). Correspondingly, the level of PRLR-LF and -SF mRNA in the CP of rats increased across gestation and lactation, then declined 7 days after weaning, supporting the increase in PRL levels typically observed during these states (Augustine et al., 2003; Grattan and Kokay, 2008). Hypothalamic PRLR expression in mice is also hormonally regulated by estradiol and PRL (Pi et al., 2003; Tabata et al., 2012; Mangurian et al., 1992). As a case in point, administering PRL to *prl*-deficient female mice upregulated the expression of the first exon mE1$_4$-PRLR mRNA in their CP (Tabata et al., 2012). Similarly, administering 17β-Estradiol (E_2) to ovariectomized mice also increased PRLR expression in the CP (Pi et al., 2003). Aside from studies in rats and mice, to our knowledge the relative expression of PRLR isoforms in the CP has not been examined in other species.

Expression of the PRLR in the pituitary has unique endocrine sensitivity. In the anterior pituitary of mice, PRLR-LF is the predominant isoform and PRLR-LF and -SF-S3 were expressed at significantly higher levels at proestrus in comparison to diestrus (Ferraris et al., 2014). While expression of PRLR in the pituitary was upregulated during gestation and lactation, treatment with E_2 or progesterone (P) did not alter PRLR expression in female mice (Shao et al., 2008). In ovariectomized (OVX) rats, however, estradiol valerate or 17-hydroxyprogesterone caproate increased PRLR-LF mRNA to levels similar to those recorded during late gestation (Sugiyama et al., 1994). Interestingly, P alone or in combination with E_2 reduced the number of cells that express PRLR-LF mRNA in the MPOA of nulliparous OVX rats, indicating a suppressive effect of progestins on PRLR-LF expression in this region of the brain (Bridges and Hays, 2005). In addition to rodents, significant changes in PRLR expression have been noted in

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sheep during gestation whereby Szczesna et al. (2020) report PRLR expression in the anterior pituitary and median eminence decreases during gestation.

Outside of the HPT, CP, and pituitary, expression of the PRLR has been recorded in other regions of the brain including the olfactory bulb of fetal, neonatal, and adult rats, and the medial amygdala of mice and rats (Freemark et al., 1996; Bakowska and Morrell, 2003; Bakowska and Morrell, 2003; Brown et al., 2010; de Moura et al., 2015; Salais-Lopez et al., 2017). The thalamus, the bed nucleus of the stria terminalis, and the lateral septum are additional sites of PRLR-SF expression within the brain (Bakowska and Morrell, 2003; Brown et al., 2010). Like findings for other regions, the level of PRLR mRNA was higher in the hippocampus of female rats compared to males (de Moura et al., 2015; Vergara-Castañeda et al., 2016; Cabrera-Reyes et al., 2017). Besides neural tissue, both the PRLR-LF and -SF are expressed in the trigeminal ganglion, a sensory ganglion located in the dura mater. In female rats, PRLR-LF predominates in glial cells of the trigeminal ganglion with little expression in nerves and neurons, while PRLR-SF is expressed in the nerves and neurons of the trigeminal ganglion (Diogenes et al., 2006). No differences in mRNA expression for PRLR-LF and -SF were recorded in the dorsal root ganglia of male and female mice. However, PRLR expressing subsets of DRG varied between male and female mice (Patil et al., 2019).

Within the forebrain of young and adult rats, the cingulate cortex, neocortex, prefrontal cortex and parietal cortex all express the PRLR (Pi and Grattan, 1999; Pi and Grattan, 1999; Bakowska and Morrell, 2003). Among the different PRLR isoforms, the PRLR-LF predominates in the cerebral cortex of female rats, where its expression is higher in proestrus compared to diestrus (Nagano and Kelly, 1994; Bakowska and Morrell, 1997; Bakowska and Morrell, 2003).

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Liver

Expression of the PRLR in rodents is unique in that the liver has some of the highest levels of PRLR mRNA in the body (Nagamo & Kelley 1994). In fact, hepatic PRLR expression exceeds or is equal to levels found in the mammary glands (Jahn et al., 1991; Yue et al., 2014). Both PRLR-LF and -SF isoforms are expressed by hepatocytes and cholangiocytes, where some studies indicate that the -SF is the predominant isoform in rodent and human hepatocytes (Nagano and Kelly, 1994; Hartwell et al., 2014; Smirnova et al., 1998; Orlova et al., 1999). Nagano and Kelly (1994) reported that the PRLR-SF mRNA represented 80-90% of total PRLR mRNA in rat hepatocytes, where the ratio of SF:LF mRNA in female rats was approximately 10:1. Hepatic PRLR expression during the estrous cycle is also unique in that there is 12 times more PRLR-SF mRNA at proestrus, and 4-fold more at diestrus I, relative to that for the PRLR-LF (Nagano and Kelly, 1994). Others have suggested that the PRLR-LF is the predominant isoform in the liver of rats and mice, perhaps as a function of the limitations with earlier methods (Corbacho et al., 2004; Bridges et al., 2011). Amongst the various PRLR-SF isoforms, the PRLR-S1a is one of the most abundant in the human liver whereas PRLR-SF3 is the predominant isoform in adult mice (Corbacho et al., 2004; Hartwell et al., 2014). The PRLR-LF and -SF are also expressed in the liver of fetal and adult sheep, and the liver of female pigs (Bispham et al., 1999; Phillips et al., 2001; Schennink et al., 2013a).

During gestation, the liver undergoes several biochemical changes in response to changes in plasma volume and placental development (Ryan & Heneghan, 2014). There is a parallel increase in hepatic PRLR expression alongside these changes in mice and rats, which supports the suggestion for a functional role of PRL in the liver during these states (Buck et al., 1992; Moldrup et al., 1996; Varas and Jahn, 2005). To this end, PRL has been implicated in the

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cholestasis of gestation, whereby both PRLR-SF and -LF expression are altered in models of this disease **(**Abramicheva and Smirnova, 2019**)**. In fetal sheep, the abundance of PRLR mRNA also increased during gestation, but levels significantly decreased at 6 months of age (Hyatt et al., 2007). The abundance of PRLR transcripts in the liver of rodents also changes during the initiation and transition to lactation. Prior to the onset of lactation, PRLR expression in rats peaks at gestation day 21 (G21) and falls abruptly during the postpartum period (Varas and Jahn, 2005). In rats, hepatic PRLR-SF expression increased on L1, then fell after 3 days, whereas PRLR-LF levels remained elevated up to 4 days after parturition.

Hepatic PRLR expression is also subject to endocrine regulation. In the fetal liver of sheep, PRLR-LF and -SF expression was stimulated by fetal cortisol (Phillips et al., 1997). Interestingly, thyroxine treatment decreased PRLR-LF expression by 50% in G21 pregnant rats. In pigs, PRLR-LF was unaltered by any combination of exogenous E, P, or haloperidol, a dopamine D2 receptor antagonist in the liver of pigs.

Female Reproductive Tissues

The generation of PRL and PRLR knockout mice highlighted the critical role for PRL in female reproduction (Horseman et al., 1997; Ormandy et al., 1997). Those models confirmed that PRL and its action on the PRLR are crucial for fertility, the production of P, and maintenance of the corpus luteum (CL) (Ormandy et al., 1997; Grosdemouge et al., 2003; Bachelot and Binart, 2007; Bachelot et al., 2010). Within the female reproductive tract across a range of species there is spatiotemporal variation several isoforms. Indeed, PRLR mRNA for PRLR-LF or SF has been reported in the ovaries of dogs, sheep, mice, rats, rabbits, bats, cows, yak, chicken, and geese (Clarke et al., 1993; Clarke and Linzer, 1993; Kermabon et al., 1994; Russell and Richards, 1999; Hu et al., 2001; Picazo et al., 2004; Kowalewski et al., 2011; Xing et al., 2011; Zi et al.,

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2012; Anuradha and Krishna, 2017). Within the ovaries of rodents, PRLR-LF and -SF mRNA is detectable in granulosa, interstitial, and luteal cells, as well as in follicles and the CL (Clarke et al., 1993; Telleria et al., 1997). Although the PRLR-LF is the most prevalent within the ovary, both the PRLR-LF and -SF are required for normal ovarian function in mice (Clarke et al., 1993; Le et al., 2012). The expression of PRLR within the ovaries is phasic over the course of the estrous cycle. In rats, PRLR were maximally expressed at proestrus, then declined at estrus prior to recovering to maximal levels by late diestrus and early proestrus. At all stages of the estrous cycle the PRLR-LF predominated (Clarke et al., 1993; Nagano and Kelly, 1994). Furthermore, the ratio of LF:SF in the murine ovary changes during ovarian cell differentiation, where PRLR-LF is most abundant in the granulosa cells prior to ovulation, whereas PRLR-SF expression increases during luteinization (Telleria et al., 1997; Russell and Richards, 1999; Stocco et al., 2007). In line with findings from rodents, expression of the PRLR-LF in ovarian tissue of sheep also changes during the estrous cycle, where PRLR-LF expression increased to a maximum at estrus and was lowest during the mid-luteal phase (Picazo et al., 2004).

Expression of the PRLR in the uterus and ovary is also regulated temporally and by endocrine signals. In the ovaries of rats, PRLR-SF_{S1}, -SF_{S2}, -SF_{S3}, and -LF mRNAs were increased at mid-gestation (Clarke and Linzer, 1993). In both rodents and dogs, the levels of PRLR-LF and -SF mRNA and protein in the ovary increase during gestation then decline just prior to parturition (Telleria et al., 1997; Russell and Richards, 1999; Stocco et al., 2000; Kowalewski et al., 2011). Reese et al. (2000) reported that PRLR expression was low to undetectable in the uterus of mice until gestation days 6-8, when expression localized to subepithelial stroma cells at the mesometrial pole, undifferentiated stromal cells at the antimesometrial pole, and in the epiblast region of the embryo. Outside of gestation, exogenous

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hormones can also alter PRLR expression in the ovary. Treatment of rats with pregnant mare's serum gonadotropin significantly increased the expression of PRLR-LF and -SF mRNA in ovarian tissue, whereas treatment with human chorionic gonadotropin caused a rapid decrease in PRLR-LF mRNA and an increase in -SF expression (Kinoshita et al., 2001). Interestingly, PGF2α suppressed expression of both the PRLR-LF and -SF in the CL of rats as well as in luteinized granulosa cells *in vitro* (Stocco et al., 2003). There have not been any reports of PRLR level changes in the ovaries of humans during the menstrual cycle. What has been profiled is PRLR expression in the endometrium of pre- and post-menopausal women, which revealed that PRLR expression is substantially downregulated in endometrial tissue from women with ongoing and miscarried pregnancies (Bersinger et al., 2008).

Expression of PRLR expression in the CL also varies with reproductive stage. In the CL of cows, both PRLR-LF and- SF mRNA abundance was lowest during the early and regressing stages and highest in the mid and late stages of the estrous cycle, with levels comparable to pregnancy (Thompson et al., 2011). In addition, PRLR-SF was identified as the predominant PRLR isoform expressed in the CL. In contrast, Ricken et al. (2007) reported that PRLR-LF expression in bovine CL was higher at the secretory and regressing stages. In the CL of pregnant dogs, the highest expression of the PRLR was recorded at pre- and post-implantation, and subsequently decreased significantly during prepartal luteolysis (Kowalewski et al., 2011). In contrast, the abundance of PRLR mRNA in the CL of menstrual primates was significantly higher in the mid and late luteal stages, whereas PRLR protein abundance was greatest during the mid to late stages of the menstrual cycle (Bogan et al., 2008).

Expression of the PRLR has been identified in the decidua of humans, baboon, and rodents, and may function to regulate the local production of PRL in this tissue (Gu et al., 1996;

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Maaskant et al., 1996; Frasor et al., 1999; Prigent-Tessier et al., 1999; Reese et al., 2000; Jabbour and Critchley, 2001). Within the decidua of pseudopregnant rats, both PRLR-LF and -SF mRNA were detected in the antimesometrial and mesometrial decidua (Gu et al., 1996). Interestingly, baboons only express the PRLR-LF in the endometrium and myometrium, with PRLR expression remaining constant during the menstrual cycle (Frasor et al., 1999). During gestation, transcription of PRLR-LF increased in the endometrium of sheep and the endometrium and placenta of pigs, respectively (Cassy et al., 2000; Trott et al., 2009; Schennink et al., 2013b). In ewes, PRLR mRNA expression within the endometrium also increased across gestation and was localized to the endometrial glandular epithelium and the intercaruncular endometrium. However, during late gestation an abundance of PRLR mRNA was restricted to the endometrial lower stratum spongiosum (Stewart et al., 2000). Martin et al. (2004) reported that IFN-τ treatment increased the abundance of PRLR-LF and -SF mRNA in the endometrium of ewes by 6-fold. In addition, the expression of both isoforms increased between gestation days (G) 7–8 and G14–15. These data also suggest IFN-τ may support an action for PRL in CL function in sheep. Interestingly, exogenous levonorgestrel significantly increased stromal PRLR immunostaining in the endometrium of women (Critchley et al., 1998). In pigs, endometrial PRLR expression increases transiently after treatment with estradiol valerate, but then was decreased 12h post-treatment (Young et al., 1990).

The PRLR are also expressed in in oocytes, preimplantation embryos, and fallopian tubes of rodents and humans (Kiapekou et al., 2005; Shao et al., 2008), as well as the glandular epithelium and myometrial smooth muscle of cyclic baboons, alongside the decidua and placental syncytiotrophoblasts throughout gestation (Frasor et al., 1999). Interestingly, Shao et al. (2008) showed that PRLR-LF and -SF were present in the fallopian tubes of humans and mice

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during the follicular, periovulatory and luteal phases. Interestingly their data also showed PRLR-LF expression in mice was higher in the fallopian tubes compared to the pituitary and liver. Unlike the response in the pituitary, exogenous PRL inhibited PRLR expression in the fallopian tubes over time (Shao et al., 2008). Conversely, bromocriptine therapy increased PRLR LF expression, thereby highlighting that PRL may negatively regulate PRLR expression in the fallopian tubes. In addition to PRL, E_2 and P treatment decreased PRLR expression in the fallopian tubes, where E_2 specifically suppressed PRLR-SF expression whereas P suppressed PRLR-LF expression (Shao et al., 2008). In addition to the fallopian tubes, a low level of immunoreactive PRLR was detected in human cervical tissue and overexpressed in cancerous cervical tissue (Lopez-Pulido et al., 2013; Ascencio-Cedillo et al., 2015). Expression of PRLR in the vagina was previously undetected in rodents (Ouhtit et al., 1993). Although the recent creation of a PRLR knock-in mouse has enabled single cell resolution of PRLR expression within the muscular layer of the vagina in mice (Aoki et al., 2019).

Prolactin receptors are also expressed in embryonic fetal tissues across mammalian species such as rodents and are regulated in a spatiotemporal manner (Freemark et al., 1995; Brown-Borg et al., 1996; Maaskant et al., 1996; Schuler et al., 1997; Tzeng and Linzer, 1997; Symonds et al., 1998). In rodents, PRLR mRNA in tissues of the fetus such as the liver, kidney, thymus, gonads, and immune cells increases across development (Royster et al., 1995; Zhang et al., 1995; Phillips et al., 1997; Phillips et al., 2001; Urtishak et al., 2001). In mice, PRLR expression in the embryo was highest at embryonic day 8 and 18 (E8, E18) and lowest at E14 (Tzeng and Linzer, 1997). Interestingly, by E18 PRLR-SF becomes the predominant receptor expressed in the fetal liver. Similarly, PRLR transcript expression in chickens is stage-dependent

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whereby type I and type II transcript expression varies across embryonic development (Bu et al., 2015).

Male reproductive tissues

While the role for PRL in female reproduction is well known, there is much to be uncovered regarding PRL and male reproduction. In rats, PRLR-LF mRNA expression was originally identified in the testis, epididymis, prostate, and seminal vesicle (Ouhtit et al., 1993), then subsequently in Leydig cells, Sertoli cells, spermatozoa, and the epithelial cells of efferent ducts in the testes (Jabbour and Lincoln, 1999; Hair et al., 2002; Klemcke et al., 1999). More recently, Aoki and colleagues (2019) revealed that the PRLR is expressed in Leydig cells, but not the seminiferous tubules, and in epithelial cells in the caput segment of the epididymis, the epididymal corpus and cauda, and the seminal vesicle in mice. In addition, few epithelial cells within the ampulla gland and only a subset of ciliated columnar epithelial cells in the vas deferens were PRLR-positive. In humans, PRLR have been identified in differentiating germ cells of the testis, Leydig cells, and in the epithelial lining of the vas deferens, epididymis, prostate, and seminal vesicles (Hair et al., 2002). Pujianto et al. (2010) have specifically identified the presence of PRLR-LF, -SF1a, and -SF1b on the post acrosomal region of the sperm head, neck, and midpiece of human sperm, raising the possibility of a role for PRL in fertilization. Furthermore, PRLR-LF and -SF have been identified in human and rat prostate tissue and were localized to the secretory epithelium and the dorsolateral prostate, respectively (Nevalainen et al., 1996; Nevalainen et al., 1997). Similar to mammals, aves also express PRLR in the male reproductive tract, where PRLR mRNA was present in the testes of Peking ducks (Wang et al., 2009) and the testes and seminal duct of geese (Xing et al., 2011). Given the presence of the PRLR throughout the male reproductive tract in a range of species, these data

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suggest a role for PRL in spermatogenesis, steroidogenesis, and/or secretory/adsorptive functions in males (Dabbous and Atkin, 2018). Indeed, studies have shown 20% of homozygous male knockout PRLR-/- mice have delayed fertility (Bole-Feysot et al., 1998) and PRL can upregulate testosterone synthesis in Leydig cells (Purvis et al., 1979). However, much is left to be uncovered regarding the exact role of PRL/PRLR action in male reproductive organs.

Adipose tissue

There has been an increased focus on the role of PRL in targeting adipose tissue via the PRLR expressed on adipocytes. Accordingly, PRLR expression has been identified in adipose tissue of a range of mammals during different physiological states. In mice, PRLR are expressed in the parametrial, retroperitoneal, and mammary fat pads of female mice, and in epididymal adipose tissue from male mice, respectively (Ling et al., 2000: 200; Hovey et al., 2001). Moreover, adipocytes from male, virgin, and pregnant mice were found to be positive for the PRLR-LF, -SF2, and -SF3 isoforms (Ling et al., 2000), or LF, -SF1, -SF2, and -SF3 in the mammary fat pad during postnatal development (Hovey et al., 2001). While Ling and colleagues (2000) found no sex differences in the expression of the PRLR-LF between male epidydimal adipose and virgin female mice parametrial and retroperitoneal adipose tissue, they did report that PRLR-LF expression increased 2.3-fold during lactation relative to the levels in virgin and pregnant mice. Similarly, expression of both the LF and SF increased between G90-125, followed by a sharp decrease after parturition in the perineal adipose tissue of sheep (Symonds et al., 1998; Pope et al., 2004). In addition, PRLR-LF and -SF were present in brown adipose tissue (BAT) from lambs (Bispham et al., 1999; Budge et al., 2000; Budge et al., 2003). In pigs, both PRLR-LF and -SF are expressed in mammary extra parenchymal (Duarte et al., 2019) and back fat adipose depots (Trott et al., 2011).

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Cardiovascular System

Prolactin can act as a pro-angiogenic factor by stimulating the expression of angiogenic factors and blood vessel formation (Grosdemouge et al., 2003; Erdmann et al., 2007; Malaguarnera et al., 2005; Ko et al., 2003; Struman et al., 1999). A truncated 16 kDa PRL isoform has anti-angiogenic effects, where mouse models found it may regulate the onset of postpartum cardiomyopathy and blood pressure (Hilfiker-Kleiner et al., 2007; Chang et al., 2016). However, many of the mechanisms underlying the effects of PRL on the cardiovascular system remain to be established. The expression of PRLR-LF and -SF mRNAs in the endothelium of the pulmonary artery, aorta, CL, and umbilical vein, alongside the presence of the PRLR protein in cow umbilical cord and aorta endothelial cells, are clear indicators for a direct action of PRL on the vascular endothelium (Merkle et al., 2000; Ricken et al., 2007). What remains to be resolved is the extent to which expression of the PRLR in endothelial cells is tissue and/or species-specific. On one hand, Ochoa et al. (2001) found no evidence for PRLR expression in rat retinal capillary endothelial cells, whereas both the PRLR-LF and -SF mRNAs are expressed in the hearts of pigs (Schennink et al., 2013b). Likewise, carotid atherosclerotic plaques from human males and females express PRLR mRNA, consistent with the immunohistochemical detection of PRLR in mononuclear cells within advanced atherosclerotic lesions (Reuwer et al., 2011).

Adrenals

The presence of PRLR in all three zones of the human adrenal glands alongside increased circulating PRL levels during stress led to a proposed role for PRL in the regulation of steroidogenesis (Armario et al., 1996; Glasow et al., 1996; Kirk et al., 2017). Although there is still a limited understanding of any role for PRL in the adrenals, it is noteworthy that the adrenals

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consistently express high levels of the PRLR across a range of species. In pigs, the adrenals had the highest level of the PRLR-LF mRNA among 17 tissues (Schennink et al., 2013b). In addition, the adrenal glands of pigs are among only a few organs that express at least 50 copies of the less-abundant PRLR-SF mRNA/ug total RNA. Similarly, the adrenals of the marmoset monkey (Dalrymple et al., 2000) and male and female rabbits (Dusanter-Fourt et al., 1991), and fetal and mature rats (Freemark et al., 1995; Royster et al., 1995; do Amaral et al., 2015) express an abundance of PRLR mRNA. Interestingly, Nagano and Kelly (1994) reported that expression of the PRLR-SF in the adrenal glands of mature rats was at a level over 1 million copies /ug of RNA. Furthermore, mRNA expression for the rat PRLR-SF increased both across gestation and in response to metoclopramide-induced hyperprolactinema. These intriguing data warrant further investigation to gain a deeper understanding of the exact role for PRL and its receptor in adrenal function.

Pancreas

A role for PRL in islet and β-cell maturation, development, and function has been discerned from PRLR-null mice that have reduced islet density and β-cell mass, decreased pancreatic insulin mRNA, impaired glucose tolerance, and a weakened insulin secretory response (Freemark et al., 2002: 200; Huang et al., 2009; Sorenson & Brelje, 2009; Newbern & Freemark, 2011). To that end, Nteeba et al. (2019) suggested that PRL acts through the PRLR to mediate adaptations by the pancreas of the dam, as supported by their finding that PRLR are essential for regulating maternal glucose homeostasis. Within the pancreas of mice, dense areas of PRLR-positive cells localized within the islets of Langerhans and a small subset of centroacinar cells (Aoki et al., 2019). This pattern of expression is in line with PRL role in maintaining gestational glucose tolerance, whereby PRLR mutant mice display decreased islet

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density, impaired glucose clearance, and decreased glucose-stimulated insulin release compared to wild-type mice (Huang et al., 2008)

In adult rats, both the PRLR-LF and -SF are expressed in the pancreas of males and females, where the PRLR-LF is the predominant isoform and undergoes increased expression in β cells during gestation (Nagano and Kelly, 1994; Ouhtit et al., 1994). Expression of the PRLR-LF in the islets of newborn rats is also sensitive to hormonal regulation, whereby growth hormone (GH), estradiol, testosterone, and PRL increased its expression (Moldrup et al., 1993). Growth hormone and PRL also increased expression of the exon 1A- and 1C-containing PRLR transcripts in rat islets, supporting the possibility for tissue-specific regulation of PRLR expression in the pancreas (Barash, 1999). Other than rodents, PRLR LF and several short forms can be found in the pancreas of humans and pigs (Trott et al., 2003; Trott et al., 2011: 20; Haglund et al., 2012). In addition, moderate to low levels of PRLR expression have been recorded in the pancreas of male and female rabbits (Dusanter-Fourt et al., 1991) and turkeys (Leclerc et al., 2007), supporting a conserved role for PRL action in the pancreas across species.

Immune system

Prolactin has emerged over the years as having immunostimulatory and immunosuppressive roles (Gala, 1991). Among immune cell types in humans, PRLR expression predominates in macrophages and to a lesser extent in T-cells and has been recorded in lymphocytes and leukocytes isolated from blood (Dohi et al., 2003; Maeda et al., 2010; Reuwer et al., 2011). In rodents, PRLR-LF and -SF are expressed in a variety of immune modulatory tissues such as the thymus, bone marrow, spleen, and lymph node. More specifically, B cells and macrophages from spleen, lymph nodes and peripheral blood, as well as T cells from the thymus express PRLR (Gagnerault et al., 1993; Koh and Phillips, 1993; Touraine et al., 1994; Ledesma-

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Soto et al., 2012). Within the splenic B-cell population of mice, transitional B-cells showed the highest relative PRLR mRNA expression compared to mature B-cells, while among mature B-cell subsets, marginal B-cells showed an approximately 2-fold higher expression of PRLR than follicular splenic B-cells (Ledesma-Soto et al., 2012). Within the spleen, B-lymphocytes expressed surface PRLR at higher levels compared to either CD4+ cells or CD8+ cells (Gunes et al., 1997). Expression of the PRLR in immunomodulatory tissues has also been reported in aves and cows, where PRLR were expressed in the bursa follicles, thymus lobules, and throughout the splenic pulp of chickens, while the PRLR-LF and -SF were found to be expressed in cow lymphocytes (Auchtung et al., 2003; Auchtung et al., 2005; Kang et al., 2007).

Mammary glands

One of the main roles of PRL across mammals is its role in regulating mammary gland (MG) function. A plethora of *in vitro*, *in vivo*, and genetic models have highlighted the ability for PRL to regulate mammary organogenesis, lobuloalveolar growth, and functional differentiation (Karayazi Atıcı et al., 2020). One of the most noteworthy mechanisms underlying the multifaceted role of PRL in the MG is the spatiotemporal regulation of the PRLR in various mammary gland compartments. In rodents, the PRLR-LF and -SF are expressed (Hovey et al., 2001) and localized to the apical and basal membranes of epithelial and stromal compartments of the MG (Bridges et al., 2011; Ueda et al., 2011). In addition, PRLR were found on myoepithelial cells and luminal cells, including the alveoli in mice (Ueda et al., 2011). Within the mammary fat pad of mice, PRLR are expressed in the nucleus, cytoplasm, and cell membrane, but is not altered by physiological state (Camarillo et al., 2001). In females, PRLR expression has been identified in both parenchymal tissue of the breast (Ueda et al., 2011) and breast preadipocytes, where its expression varies throughout adipogenesis (McFarland-Mancini et al., 2006).

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Ruminants also express both the PRLR-LF and -SF in the MG, though the specifics of cell type and location for its expression has not yet been identified (Auchtung et al., 2005; Bernier-Dodier et al., 2010; Morammazi et al., 2016). Interestingly PRLR are also present in the inguinal sinus of sheep, a skin pouch that secretes a waxy substance located bilaterally near the MG (Alexandre-Pires et al., 2017). In pigs the PRLR-LF is expressed in both the adipose and parenchymal regions of the MG (Sakai et al., 1984; Duarte et al., 2019) and was expressed at levels 2.5x higher than the -SF in membrane preparations isolated from the MG parenchyma in late gestation (Schennink et al., 2013b).

The expression of the PRLR in the MG is also subject to hormonal and temporal regulation. That said, the localization of PRLR expression in the MG of rodents changes with reproductive state. In the epithelium of virgin rats PRLR were mainly associated with the cytosol and nucleus while pregnant rats expressed PRLR in the perinuclear and cell membrane (Camarillo et al., 2001). Contrarily, epithelial PRLR expression in lactating rats was localized in the cytosol. Aoki and colleagues (2019) found that mammary ducts in mice expressed PRLR, although the adipose tissue surrounding the ducts was negative for PRLR expression. Regarding expression, parity alters PRLR levels in rats. The baseline expression of PRLR-LF in the MG of multiparous female rats was significantly higher than in age-matched nulliparous females and was also expressed at a higher level in the first parity relative to the second (Bridges et al., 2011). Likewise, PRLR-SF mRNA levels were higher in the MG of primiparous, lactating rats compared to in multiparous dams. Expression of the PRLR LF increased across lactation in primiparous and multiparous rats whereas no significant changes were found with PRLR-SF expression (Bridges et al., 2011). Contrary to data in rats, the level of PRLR mRNA in mammary epithelial cells of ewes increased during the second half of gestation, followed by a decrease near

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parturition, and then remained relatively stable during lactation (Cassy et al., 2000). Trott et al. (2009) reported that the level of *PRLR*-LF mRNA and protein expression in the MG of pigs also increased across gestation. Furthermore, relative to a range of other PRL target tissues, the MG had some of the highest expression levels for the pig PRLR-LF during gestation, alongside the adrenal glands and endometrium (Trott et al., 2009).

The level of PRLR in the MG alters in response to a changing endocrine environment in a species-specific manner. Treatment of mouse MG epithelial cells with insulin, hydrocortisone, and PRL increased PRLR mRNA expression, which was then attenuated by P in a dose-dependent manner (Nishikawa et al., 1994). They also found that EGF inhibited the induction of PRLR-LF mRNA in mouse mammary epithelial cells and this inhibitory effect was exacerbated by P. In rats, ovariectomy and hysterectomy of virgin females increased only PRLR-SF mRNA in mammary tissues while exogenous PRL suppressed PRLR gene expression suggesting that steroid hormones can suppress PRLR expression in the MG (Jahn et al., 1997). Conversely culturing MG tissue from mice in late gestation with corticosterone increased PRLR-LF mRNA (Mizoguchi et al., 1997). Contrary to rodents, when nulliparous pigs treated with E_2 or P and haloperidol, a dopamine receptor antagonist that increased PRL secretion, there was a positive main effect of haloperidol on pPRLR-LF mRNA expression MG (Trott et al., 2009). However, treating nulliparous pigs with bromocriptine, a dopamine agonist, decreased PRL and PRLR numbers (Farmer et al., 2000).

Intestine, kidney, and bone

Parathyroid hormone, $1,25(OH)_2D_3$, and calcitonin are widely recognized as the principal hormones that regulate calcium metabolism. However, several lines of evidence support the notion that PRL, acting through its receptors, may also regulate calcium metabolism across a

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range of species (reviewed by Charoenphandhu et al., 2010). Indeed, PRLR are widely distributed across a range of calcium-regulating organs including the small intestine (SI), kidneys, and bone. Much of the data gathered regarding the role for PRL as a calciotropic hormone was derived from rodent studies. As reviewed by Charoenphandhu et al. (2010), PRL stimulates intestinal calcium absorption, enhances bone turnover and calcium release, and modulates renal calcium flux.

In the intestines of rodents, both the PRLR-LF and -SF mRNA were identified in the duodenum, proximal jejunum, distal jejunum, ileum, cecum, proximal colon, and distal colon of rats, and in the epithelial cells of villi in the duodenum, jejunum, and ileum of adult mice (Jantarajit et al., 2007; Aoki et al., 2019). Expression of the PRLR in the villi within the SI of rodents can be detected during fetal development and between birth and 96 days of age; thereafter, intraepithelial lymphocytes within the SI of rats express the PRLR (Royster et al., 1995; Urtishak et al., 2001). The kidneys of rodents also express both PRLR-LF and -SF, where its expression is upregulated in several physiological states (Shirota et al., 1990; Al-Trad, 2015). Both gestation and stage of estrous cycle alter PRLR expression in the kidneys, where in rats the expression of the PRLR in the kidneys was maximal during proestrus and diestrus (Buck et al., 1992; Nagano and Kelly, 1994). Interestingly, the kidneys have among the highest levels of PRLR expression across various tissues in adult mice, further supporting a role for PRL in renal function (Yue et al., 2014). In the same way, the kidneys in adult humans were described as having the third-highest level of PRLR mRNA expression among various organs. Expression of the PRLR can also be detected in the kidneys and SI of fetal humans (Fagerberg et al., 2013; Freemark et al., 1997; Freemark, 2001). Likewise, the PRLR-LF and -SF isoforms have been detected in the kidneys of fetal sheep (Phillips et al., 2008).

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As mentioned previously, research has suggested a role for PRL in calcium turnover from bone. Both PRLR-LF and -SF or PRLR mRNA is expressed on osteoblasts or osteoclast-like cells within rodent skull bones, tibiae, femora and vertebrae, consistent with a role for PRL in modulating calcium flux in bone (Biller et al., 1992; Clement-Lacroix et al., 1999; Coss et al., 2000; Charoenphandhu et al., 2007; Charoenphandhu et al., 2008; Seriwatanachai et al., 2008; Ledesma-Colunga et al., 2017). The patterns and distribution of PRLR in bone in other species does not appear to have been described. However, many bone-derived cell lines such as MG-63 and Saos-2 human osteocarcinoma cells, SV-HFO human pre-osteoblasts, and UMR106 rat osteoblast-like cells, all have stable PRLR expression and have been successfully utilized to investigate the effects of PRL action on osteoblasts (Bataille-Simoneau et al., 1996; Seriwatanachai et al., 2009; Wongdee et al., 2011).

Gene Structure & Isoforms of the Prolactin Receptor

Avian

The genetic structure of the PRLR gene has been described for many avian species including chickens (Tanaka et al., 1992), turkeys (Zhou et al., 1996), pigeons (Chen and Horseman, 1994), ducks (Wang et al., 2009) and geese (Xing et al., 2011). Despite having similar genetic organization and protein structure to mammals (Xing et al., 2011), avian PRLR contain 2 ligand binding domains (Tanaka et al., 1992; Xing et al., 2011) distinguishing them as the only group of species aside from the leopard gecko with this feature (Kato et al., 2005).

The chicken Prlr (cPRLR) maps to chromosome Z and consists of at least 25 exons, comprising 10 non-coding exons (exons 1A-1J) upstream of the transcription start site, and 15 coding exons. A partially duplicated *Prlr* gene in chickens (*dPRLR*) with an incomplete last exon is also present on chromosome Z at the K locus and is linked to the partially

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duplicated *SPEF2* gene (Elferink et al., 2008; Bu et al., 2013a). The dPRLR has a nucleotide and amino acid sequence that is nearly identical to the cPRLR except for absence of the C-terminal tail lacking 149 amino acids (Elferink et al., 2008). Alternative first exons and mRNA splicing gives rise to various cPRLR mRNA transcripts comprised of multiple 5′-UTR sequences. Type I transcripts utilize the exon 1G promoter (P1) and include 3 variants; T1a, T1b and T1c. Type II transcripts utilize the exon 1A promoter (P2) and splice to create 8 variants spanning from exons 1A to 1L: T2a, T2b, T2c, T2d, T2e, T2f, T2g, and T2h (Bu et al., 2013a). Interestingly the nucleotide sequence and transcription factor binding sites within P2 are like those for the PIII promoter reported in humans, rats, and mice (Bu et al., 2013a). In addition to promoters 1A and 1G, promoters for exon 13 (TSE-1) and exon 15 (TSE-2) were identified in testes mRNA transcripts (Bu et al., 2013; Mao et al., 1999, Tanaka et al., 2000). Transcripts of cPRLR can be spliced to create two isoforms; The cPRLR-v1 isoform is created via deletion of exon 3, while the truncated isoform cPRLR-v2 is generated via insertion of exon 8 between exons 7 and 9, which causes a frameshift mutation that introduces a premature stop codon ay exon 9 (Bu et al., 2013a).

Rodents

The rat Prlr (rPRLR) maps to chromosome 2 and comprises 14 exons and 5 alternative first exons that direct its tissue-specific expression. Transcripts including first exons $E1_1$, $E1_2$, or $E1_3$ are expressed in the gonads, liver, or ubiquitously across a range of tissues, respectively (Hu et al., 1996; Moldrup et al., 1996). Tanaka and colleagues (2005) have identified two additional first exons; $E1_4$, that was most abundantly expressed in transcripts from the brain, and $E1_5$ that was expressed transcripts from the brain, liver, and kidney.

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In rats there are three promoters that direct tissue-specific rPRLR transcription; a gonad-specific PI, a liver-specific PII, and a ubiquitously active PIII (Hu et al., 1996). The PI promoter is activated by steroidogenic factor-1 (SF-1) (Hu et al., 1998), PII is activated by hepatocyte nuclear factor-4 (Moldrup et al., 1996), while PIII is activated by C/EBPβ and Sp1/Sp3 (Boutin et al., 1988; Hu et al., 1998). As in most other mammals, multiple isoforms of the rPRLR are generated via alternative splicing. The rPRLR can be spliced to create a PRLR-LF-encoding transcript via splicing exon 9 to exon 10, or a PRLR-SF encoding transcript via splicing from exon 9 to exon 11. Rats, but not mice, express an intermediate form (rPRLR-IF) that is generated by a 198-amino acid deletion in the intracellular domain (ICD).

The mouse PRLR (mPRLR) gene is similar to the rPRLR in both structure and sequence. The mPRLR gene contains 13 exons consisting of 2 non-coding and 11 coding exons, as well as five promoters and alternative first exons. Three of the first exons ($mE1_1$, $mE1_2$, and $mE1_3$) are homologous to the rat $E1_1$, $E1_2$, and $E1_3$, while $mE1_4$, and $mE1_5$, are 69.4 and 91.8% identical to the rat $E1_4$ and $rE1_5$, respectively (Davis and Linzer, 1989; Hu et al., 1998; Hu et al., 1998; Tabata et al., 2012). The last four coding exons of the mPRLR are utilized by alternative splicing to generate one long (mPRLR-LF) and three short (mPRLR-SF_{1-3}) PRLR isoforms.

Notably, the cytoplasmic region for each isoform is encoded by a different exon; exon 10 encodes the cytoplasmic region for mPRLR-LF, exon 12 the cytoplasmic region for mPRLR-SF_1, exon 11 for mPRLR-SF_2, and exon 13 for mPRLR-SF_3, a homologue to the rPRLR-SF (Boutin et al., 1988; Davis and Linzer, 1989; Clarke and Linzer, 1993; Moore and Oka, 1993; Ormandy et al., 1997; Ben-Jonathan et al., 2008; Tabata et al., 2012).

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Humans

The human PRLR gene (hPRLR) is located on chromosome 5 and contains 16 coding and non-coding exons (E1-E11) that give rise to 11 isoforms. The 5' UTR of the hPRLR can include 6 non-coding first exons ($hE1_{N1-5}$ and $hE1_3$), exon 2, and part of exon 3, while the coding region consists of 9 coding exons (E3-E11), with coding starting part way through exon 3. The 6 non-coding first exons all splice to a conserved exon 2 and are transcribed in a tissue-specific manner. The first exon $hE1_{N1}$ utilizes the hP_{N1} promoter, while the first exon $hE1_3$ (the human ortholog to the rodent $E1_3$ utilizes hPIII (Hu et al., 2002). The promoters upstream of hE_{N2-5} have not been characterized. Like the rPRLR PIII, the human PIII is activated by both SP1/SP3 and C/EBPβ transcription factors. However, hPRLR PIII requires both SP1/SP3 and C/EBPβ to induce transcription whereas the rPRLR PIII can sustain activation w just C/EBPβ (Dong et al., 2006).

The hPRLR is spliced and translated into long (hPRLR-LF), intermediate (hPRLR-IF), or one of 9 short isoforms (hPRLRBP, hPRLR-ΔS1, hPRLR-$S1_a$, hPRLR-Δ7/11, hPRLR-Δ4-Δ7/11, hPRLR-$S1_b$, hPRLR-Δ4 $S1_b$, hPRLR-Δ4/6 $S1_a$, hPRLR-$S1_c$) (Ben-Jonathan et al., 2008). The hPRLR-IF is generated via a frameshift mutation and contains only three of the nine tyrosines in Box 1. Despite having fewer tyrosine residues, the hPRLR-IF can still activate Jak2, but is unable to signal PRL-induced cell proliferation (Kline et al., 1999). Of the PRLR-SF isoforms generated via alternative splicing, 6 are transmembrane PRLR, while the other three are classified as soluble PRLR due to the absence of encoding for a transmembrane region. Five alternatively spliced PRLR-SF variants containing exon 11 sequence have been reported (Schennink et al., 2013b). Among these, PRLR $S1_a$ and $S1_b$ are generated from splicing from exon 9 to exons 10 or 11 (Hu et al., 2001). The hPRLR-$S1_a$ isoform is derived via alternative

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splicing of exons 10 and exon 11 whereby part of exon 10 is spliced to exon 11. In contrast hPRLR-$S1_b$ is devoid of exon 10 and contains a part of exon 11(Hu et al., 2001). These two hPRLR-SF are similar to hPRLR-LF in both their binding affinities for PRL and their ECD, TMD, and proximal ICD regions. Both -$S1_a$ and -$S1_b$ contain the proline rich Box1 domain, but only $S1_a$ contains Box 2. In addition, only -$S1_a$ and -$S1_b$ contain the unique, truncated ICD generated from exon 11 at their C terminus. Interestingly both isoforms act as dominant negatives when expressed together, highlighting the important implications for PRLR genetic variation on PRLR function (Hu et al 2001). Both -$S1_a$ and -$S1_b$ are expressed in the human breast, kidney placenta, kidney, liver, and pancreases.

The hPRLR-ΔS1 gene transcript lacks exons 4 and 5 that encodes for the S1 N-terminal motif of the hPRLR ECD. Despite its truncated ECD, the hPRLR-ΔS1 is capable of binding PRL, albeit at a lower affinity compared to hPRLR-LF and can activate Jak2. However, hPRLR-ΔS1 cannot heterodimerize with hPRLR-LF (Kline et al., 2002)). The novel hPRLR-Δ4 $S1_b$ identified by Trott et al. (2003) lacks the signal peptide and first 47 aa of the ECD. While little is known about its functionality, they predicted that the lack of a signal peptide and part of ECD would significantly alter, if not abolish, its ability to bind PRL. Lastly, the transcript variant hPRLR-$S1_c$ omits exon 10 and splices to a site in the middle of exon 11 downstream of the end of the reading frames of hPRLR-$S1_a$ and hPRLR-$S1_b$ (Pujianto et al., 2010). Little is known about this variant including whether it can initiate signal transduction.

Unlike membrane-spanning PRLR, putative soluble PRLR such as hPRLR-Δ7/11 lack the transmembrane domain while hPRLR-Δ4-Δ7/11 also lacks both Box 1 and Box 2 (Trott et al., 2003). The hPRLR-Δ7/11 transcript comprises exons 3–7 and splices to exon 11 in the same reading frame as the hPRLR-SF1a. The transcript for -Δ4-Δ7/11 contains exons 3, 5-7 and

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splices to exon 11, while the transcript for Δ7/11 contains exons 3-7 and splices to exon 11. The Δ4-Δ7/11 PRLR is devoid of a signal peptide, a complete ECD, and was expressed at low levels in breast tissue, but not in the ovary among 9 other tissues. An additional PRLR binding protein (PRLRBP) was first identified in human sera and was observed to have the same molecular mass as the ECD of the hPRLR-LF (Clevenger and Kline, 2001b). Based on its ability to bind both PRL and GH, Kline & Clevenger (2001) proposed that the PRLBP may function to maintain circulating PRL levels. To this end, others have proposed that soluble PRLR may aid in regulating biological activities of PRL and act as a carrier for PRL.

Ungulates

The porcine Prlr (pPRLR) gene is located on the reverse strand of chromosome 16. The longest isoform of the pPRLR has an amino acid sequence that is 80% and 74% identical to the human and mouse PRLR-LF, respectively (Trott et al., 2007). The pPRLR gene consists of nine coding (exons 3–11) and 14 non-coding exons, including 11 alternative first exons (pE1–pE1.9) that direct tissue-specific transcription across various tissues (Schennink et al., 2013b; Schennink et al., 2015). Similar to rats, mice, and humans, the pPRLR gene gives rise to alternatively spliced transcripts that encode long or short pPRLR isoforms (Trott et al., 2003; Trott et al., 2011). The pPRLR-LF is encoded by exons 3-10 (Trott et al., 2007; Tomas et al., 2006) while the pPRLR-SF is encoded by exons 3-9 and splicing to a pPRLR-SF-specific exon 11 (Trott et al., 2011). The pPRLR-SF is a truncated PRLR that contains Box 1 and the WS motif (Trp-Ser-Xaa-Trp-Ser sequence), but not Box 2. Like the mPRLR-SF, the pPRLR-SF functions as a dominant negative by dimerizing with the pPRLR-LF to inhibit Jak2/STAT5 signaling (Trott et al., 2011).

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The genes coding for the bovine PRLR (bPRLR) and caprine PRLR (cPRLR) are located on the forward strand of chromosome 20, while the ovine PRLR (oPRLR) gene maps to forward strand chromosome 16 (Hayes et al. 1996; Scott et al 1992; Bignon et al. 1997). Both PRLR-LF and -SF isoforms can be produced by alternative splicing. Similar to the long isoforms described above, the PRLR-LF in ruminants includes both exons 9 and 10. However, the ruminant PRLR-LF lacks the last tyrosine residue (Y580) present in rPRLR-LF (Bignon et al., 1997). Additionally, splicing out exon 10 to generate a short isoform of PRLR is a conserved strategy across human, rats, mice, and pigs, but not ruminants (Ormandy et al., 1997; Trott et al., 2003; Trott et al., 2011). That said, PRLR-SF in sheep is generated by splicing from exon 9 to a 39 bp insert upstream of the start of exon 10 and lacks Box 2 (Bignon et al., 1997). While little is known about the function of PRLR-SF in ruminants, the oPRLR-SF can activate Jak2 *in vitro* and its expression increases in the liver of fetal sheep during the last 3 weeks of gestation (Phillips et al., 1997; Bignon et al., 1999)

Transcriptional regulation of the PRLR

One of PRL's most noteworthy features is its ability to regulate over 300 physiological functions across species, as described by Bole-Feysot (1998). The profound diversity of PRL function can be attributed to differential expression of its receptor. As highlighted previously, the PRLR is subject to spatiotemporal regulation amongst changes in physiological state. These changes are regulated by tissue-specific transcription factors that bind and activate specific PRLR promoters.

In rodents, PRLR expression is controlled by three principal promoters, PI, PII, and PIII. The gonad-specific promoter, PI, is controlled by steroidogenic factor-1 (SF-1) that activates PRLR transcription in mouse Leydig tumor cells and primary cultures of rat ovarian granulosa

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and Leydig cells (Hu et al., 1996). Despite sharing structural and functional similarities, PI in mice contains a non-functional SF-1 element and is only expressed at low levels in mice as compared to ovarian tissue from pseudopregnant rats (Hu et al., 1996). Hu et al. (1996) also found PI activity was quiescent in the MG and liver, corresponding to a lack of SF-1 protein expression in those tissues. These data highlight the tissue-specific transcriptional mechanisms used to confer PRLR expression. Similarly, the liver of rats contains a specific promoter (rPII) that directs PRLR expression. The rPII promoter is activated by hepatocyte nuclear factor 4 (HNF4) binding to the HNF4 element in liver cells (Hu et al., 1996; Moldrup et al., 1996). The PIII promoter directs ubiquitous PRLR expression in rats (Dong et al. 2006; Ormandy et al. 1998) and it is primarily regulated by C/EBPβ and Sp1 (Hu et al., 1997; Goldhar et al., 2011). In mice, P induced cooperative action of C/EBPβ and Sp1 enhanced PRLR transcription in human and mouse mammary cells, as shown by promoter deletion and site directed mutagenesis analyses (Goldhar et al., 2011). Contrarily, C/EBPβ and Sp1 require a downstream sequence element or two Sp1 elements to sustain basal PIII activity in primary rat granulosa cells (Hu et al., 1998). In addition to the aforementioned factors, mouse PI and PIII are also activated by the widely expressed transcription factor, Nectin-4. Nectin-4 serves as a stimulatory co-receptor for the PRLR in the mouse MG whereby the extracellular regions of nectin-4 and the PRLR interact to stimulate PRL-induced tyrosine phosphorylation of the PRLR and STAT5a in EpH4 cells (Maruoka et al., 2017; Kitayama et al., 2016).

One noteworthy aspect of PRLR transcriptional regulation is its conservation across species. As in rats and mice, the hPIII promoter also contains SP1 and C/EBPβ binding sites (Hu et al., 1998; Hu et al., 2002). However, hPIII activity in human breast cancer cells can also be stimulated by epidermal growth factor (EGF/ERBB1) (Kavarthapu and Dufau, 2016). More

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specifically, EGF binding to its receptor activates signal transduction pathways that lead to the phosphorylation of estrogen receptor alpha (ERα) and STAT5b. Once phosphorylated and bound to its site on hPIII promoter, STAT5b interacts with phosphorylated ERα. The ERα protein then complexes with DNA bound SP1 and C/EBPβ to activate PRLR gene transcription (Kavarthapu & Dufau 2016). The ERα can also regulate PRLR expression independent of EGFR activation. In MCF-7 cells, activation of ERα by E increased recruitment of C/EBPβ to hPIII and induced the association of ERα with C/EBPβ. At the hPIII promoter, Sp1, C/EBPβ, and ERα form a complex to induce transcriptional activity of the hPRLR (Dong et al., 2006).

The hPIII promoter does not contain canonical ER elements or functional half sites, thereby requiring SP1 to serve as the primary transactivation factor that recruits ERα to the complex and facilitates C/EBPβ association to the PRLR promoter (Kang et al., 2011). In addition to activating hPIII, E also upregulates expression of PRLR- hE13 and hE_1N_1 transcripts, suggesting a role for E in activating the corresponding promoters for hE13 and hE1N1 (Leondires et al., 2002). In both normal and cancerous breast cells, locally produced PRL can upregulate PRLR expression via phosphorylation of STAT5 and the parallel phosphorylation, nuclear translocation, and complex formation of ERα with DNA bound Sp1 and C/EBPβ (Kavarthapu et al., 2014).

In addition to rodents and human, data from our lab suggest PRLR regulation is also controlled by tissue-specific mechanisms in pigs (Schennick et al., 2015). As highlighted by Schennink et al. (2015), 9 first exons of the pig PRLR have unique expression patterns across tissues, suggesting multiple promoters direct tissue specific PRLR expression in pigs. The kidney and small intestine are dominated by a tissue specific first exon, E1.3. Consistent with expression of PRLR in the kidneys of mice, E1.3 expression increased across gestation, and the nucleotide

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sequence and the transcription regulatory sites of its promoter (promE1.3) are conserved across ungulates and humans. In addition, promE1.3 is homologous to the human first exon, hE1N2 (Hu et al., 2002), which is also uniquely expressed in these same tissues. Not only do these data provide evidence for conserved tissue-specific regulation of PRLR expression across species, but also lend strong support for a role for PRL/PRLR action in regulating calcium homeostasis.

Genetic variation of the PRLR

In addition to transcriptional and translational variations, the large degree of genetic variants within the *prlr* gene contribute to the diverse PRLR expression across the tissues of many species. Likewise, conserved expression patterns and variations of the PRLR may support the unique functions of PRL across tissues in mammalian and avian species. To this end, genetic variation identified within the *prlr* gene of mammals and birds are universally associated with reproductive function, milk production, growth and development, and pathological traits.

Reproductive Function

The *prlr* gene was first identified as a genetic marker for reproductive function in 1997 when an *AluI* PCR-Restriction Fragment-Length Polymorphism was identified at the porcine *prlr* locus (Vincent et al., 1997). Since then, numerous studies have identified associations of *prlr* AluI polymorphisms (genotypes AA, Ab, BB) with total number of piglets born or number of piglets born alive in a breed- and parity-dependent manner (Rothschild et al., 1998; Vincent et al.,1998, Mihaiov et al 2014; Drogemuller et al 2001; van Rens BT & van der Lende, 2002; Isler et al. 2000; Southwood et al 1999; van Rens & van der Lende, 2003; Tempfli et al 2011; Rempel et al., 2012; Putnova et al 2002). Data collected by Van rens and colleagues (2003) suggest the effects of *AluI* genotypes may act by altering individual components of litter size. Accordingly, first estrus, number of CL, placental weight and total weight of ovaries were all significantly

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associated with *AluI* genotypes. Phenotypes associated with porcine *prlr* AluI polymorphisms are not limited to female reproduction. Male reproductive traits, such as volume of ejaculate, percentage of live sperm, sperm per ejaculate, or concentration of sperm were all significantly associated with *AluI* genotypes (Kmiec &Terman, 2006). Alongside *AluI*, the polymorphism, *HpaII,* is also associated with litter size. To this end, the *HpaII* A allele was significantly correlated with increased total number born, number born alive, and number weaned by approximately 1.12, 0.45 and 0.56 pigs per litter, respectively (Putnova et al., 2002). Contrary to the data above, one study found no relationship between *prlr* genotypes and litter size (Tomas et al., 2006). They suggest inconsistencies in reproductive phenotypes associated with *prlr* SNP could be due to a failure to account for *prlr* haplotypes. Correspondingly, data observing the effects of *prlr* haplotypes revealed significant effects on number of CL, confirming previous data, but not litter size (Tomas et al., 2006).

Genetic variations within the *prlr* gene have also been associated with reproductive phenotypes in sheep, goat, and chickens. In ewes, *prlr* SNP are significantly associated with enhanced prolificacy (Chu et al., 2007) and maternal behavior (Wang et al., 2015). Thirteen mutations within the exon, intron, or 3′ UTR regions have been identified in the *prlr* gene of goats and are associated with components of litter size (Xiong et al., 2016). The most notable of the mutations, g.184822G/A locus, was associated with ~1kg heavier average litter weights and average birth weights (Xiong et al., 2016).

Genetic variants in the *prlr* gene are also associated with reproductive traits in aves. Chen et al. (2012) identified a SNP in exon 10 of Wijian white geese that was associated with higher annual egg production. Similarly, haplotypes based on 6 SNP discovered by Zhang et al. (2012) were associated with egg production traits including, total number of eggs laid at 300 days of age

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and age at first lay. By contrast, SNP located within exon 3 and exon 6 of the chicken *prlr* gene had no significant effect on reproductive behaviors such as broodiness and early egg productivity in Blue-Shell hens (Jiang et al., 2005). These studies suggest the effects of PRLR SNP may be breed and location dependent.

It is still unclear if SNP in the *prlr* gene are responsible for the many phenotypic differences observed in the studies cited above or if it was a marker for a closely linked causative gene. However, these data do strongly suggest that the *prlr* gene is a candidate for selection of reproductive phenotypes across species.

Lactation Phenotypes

Identification of a segregating quantitative trait locus for milk yield traits on bovine chromosome 20 first highlighted the associations between SNP in the *prlr* gene and milk production (Arranz et al., 1998; Scotti et al., 2007; Fontanesi et al., 2007). Subsequently, Lu et al. (2011) detected two novel SNP in Chinese Holstein cows within exon 10 (g.9206G>A and g.9681C>T) of the *prlr* gene that were associated with milk yield, fat percentage, and protein percentage. *In silico* analysis revealed the g.9206G>A SNP may alter the structure of the PRLR, as indicated by a putative phosphorylation site at the amino acid variation. In addition to the SNP in exon 10, Zhang et al. (2008) identified two polymorphisms in exon 3 and four in exon 7 that were significantly associated with milk yield or fat percentage. In addition, the combined genotypes of both SNP showed significant effects on milk yield and the percentages of milk fat and protein. Given these exons encode the signal peptide and the ECD of the PRLR, it is possible mutations in these regions could also alter PRLR function.

Alongside Holsteins, an association between milk production or composition have also been reported in Brown Swiss, Finnish Ayrshire, and buffalo (Cecchinato et al., 2014; Zang et al.,

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2008; Conzensa et al., 2018). Conzensa et al. (2018) reported two SNP at the buffalo *prlr* locus were associated with milk fatty acid composition and higher contents of odd branched-chain fatty acids. Although none of the 24 polymorphisms annotated in the study mapped to regulatory regions of the PRLR, SNP g.12100A>G is predicted to regulate PRLR expression via modulating miRNA binding. Interestingly, two polymorphisms in the buffalo *prlr* are similar to those found in goat (SNP g.11188A>G) and cows (g.11936G>A) (Conzensa et al., 2018; Iso-Touru et al., 2009), offering evidence for conserved PRLR regulation across species.

Aside from bovids, SNP within the *prlr* gene have been identified in pigs, goats, and sheep. Within exon 10 of the porcine *prlr* gene, a SNP was associated with higher dry matter content during the second lactation and had higher protein and fat in the colostrum and milk (Skrzypczak et al., 2015). However, these results must be interpreted with caution as the study did not account for feed intake, litter size, milk yield, or maternal genetics. Several *prlr* SNPs can be found within the *prlr* coding region of goats. Zidi et al. (2010) identified 4 SNP within exon 3 and exon 9 of the caprine *prlr*, however there were no associations with milk yield and milk fat, or lactose, protein, and dry matter content. Contrarily, a SNP located in the 3' UTR of the pPRLR gene and four additional SNP in intron 2 and exon 9 in the goat genome were found to be positively correlated with milk yield in a breed-dependent manner (Hou et al., 2014; Hou et al., 2013). In sheep, a SNP located in intron 2 of the *prlr* gene was significantly associated with lactose content, somatic cell score, coagulation time, and curd firming time (Detorri et al., 2020).

Growth & Development

Poultry

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In chickens, a partially duplicated PRLR gene (dPRLR) on chromosome Z that is expressed on the K locus is responsible for feather development (Elferink et al., 2008, Wang et al., 2010a, Luo et al., 2012). As described previously, the dPRLR gene encodes a truncated receptor lacking 149 amino acids of exon 16 that is linked to the partially duplicated SPEF2 gene (Bu et al., 2013; Elferink et al., 2008). Chicks expressing the K allele display a late feathering phenotype as shown by delayed flight feather emergence, while individuals with the k+ allele display early-feathering (Serebrovsky 1922; Elferink et al., 2008; Bu et al., 2013). Previous reports suggested the late feathering (LF) phenotype associated with dPRLR was due either to an increase in PRLR mRNA or dPRLR encoding a PRLR protein with increased function (Luo et al., 2012; Bu et al., 2013). However, Okamura et al. (2018) proposed the LF phenotype may be due to alterations in PRLR signaling via mechanisms that involve inhibiting or enhancing PRLR dimerization via both dPRLR and dSPEF2. They suggest dSPEF2 transcripts may interfere with splicing of the PRLR transcripts to favor expression of the 5′UTR splice variant over wild type. However, more research is needed to elucidate the exact mechanism underlying feathering phenotypes in chickens. In addition to feathering phenotypes, PRLR polymorphisms are also associated with growth and carcass traits in chickens. Liang et al. (2019) identified an 80-bp insertion/deletion (indel) polymorphism in the 3′-UTR of the c*PRLR* gene. Animals homozygous for the deletion allele were significantly associated with lighter carcass weight, semi-evisceration weight, evisceration weight, head weight, claw weight, wing weight and leg weight while heterozygous individuals had the highest values for these traits. The deletion genotype also contributed negatively to carcass weight qualities such as lower body weight, shank length, shank girth, sternal length, and body slanting length compared to chickens homozygous or heterozygous for the insertion allele.

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In line with mutations identified in chickens, a truncated PRLR is also responsible for the LF phenotype in turkeys (Derks et al., 2018). A hemizygous 5-bp deletion within the terminal exon of the *prlr,* which introduces a premature stop codon, generates a truncated PRLR with a 98-aa deletion at the C-terminus. Interestingly turkeys with the slow feathering (SF) phenotype were hemizygous for the allele containing the PRLR truncation while fast feathering (FF) turkeys were hemizygous or homozygous (Derks et al., 2018). Together, these data highlight the impact of PRLR mutations involving losses of a part of the C-terminal tail to feather growth phenotypes in poultry.

Goats

In goats, a 16-bp and a 5-bp indel detected in Shaanbei white cashmere goats is significantly associated with growth traits (Liu et al., 2019). Goats carrying the deletion allele of the 16-bp indel had larger chest depth, body length, body height, but lower body length index, heart girth index, and cannon circumference index compared to homozygous individuals. Interestingly, the 5-bp indel was only associated with lower chest depth and body length index percentage in homozygous individuals of a specific population of goats tested (Liu et al., 2019). Prolactin receptor polymorphisms also associate with hair phenotypes in goats whereby a polymorphism in the 5' flanking region of Liaoning cashmere goats was associated with a 33g heavier cashmere fiber weight (Zhou et al., 2011).

Cows

In line with traits observed in goats and sheep, two SNP within the signal peptide of the bPRLR associated with superior carcass traits such as hucklebone width, body weight, average daily gain, body height, body length and heart girth (Lu et al., 2011). However, one of the most noteworthy phenotypes associated with *prlr* SNP is coat length. The slick hair phenotype

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commonly found in tropically adapted cattle breeds is closely associated with thermo-tolerance. The slick locus is dominantly inherited and has been mapped to the same location for the bPRLR on chromosome 20 (Olson et al., 2003; Mariasegaram et al., 2007). Given the proximity of the slick locus to the bPRLR, the bPRLR has been investigated as a candidate gene for slick coats in cows. To this end, Littlejohn et al. (2014) identified a single homozygous frameshift mutation in exon 10 of bPRLR that introduces a premature stop codon in Senepol cows (p.Leu462 mutation) that was highly associated with the slick-coat phenotype. However, as Huson et al. (2014) highlight, it is possible that more than one mutation is responsible for the slick phenotype in different breeds of cows. In support of this hypothesis, Port-Neto et al. (2018) found the p. Leu462 mutation was absent in Carora and Limonero slick haired cattle, suggesting a different causative mutation. Whole-genome sequencing of Limonero cows revealed stop codons in the cytoplasmic region of the PRLR that generate truncated PRLR (Port-Neto et al., 2018). Similar to the truncation caused by p.Leu462, the truncation mutations identified occurred after box 1, but before a conserved amino acid (tyrosine Y512) found in both the human and bovine GHR and PRLR (Port-Neto et al., 2018). In addition, both p.Leu462 and the mutations identified by Port-Neto and colleagues (2018) lack tyrosines Y512 and Y543. Collectively, these data suggest multiple PRLR mutations can produce identical phenotypes, and the possibility of convergent evolution for smooth coats in tropical bovine breeds.

Pathology

Alongside its role in modulating physiological processes such as reproduction and lactation, an array of epidemiological and experimental data supports the involvement of PRLR genetic variants in clinical pathologies. To this end, Bogorad and colleagues (2008) have identified a SNP in exon 6 ($PrlR_{I146L}$) within the ligand-binding domain of the hPRLR in benign

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and malignant human breast tumors (Bogorad et al., 2008; Courtillot et al., 2010; Canbay et al., 2004) that exhibits a gain-of-function phenotype. The $PrlR_{I146L}$ mutant displayed constitutive receptor activation, as shown by PRL-independent tyrosine phosphorylation, STAT5 activation, transcriptional activity, as well as enhanced cell proliferation and protection from cell death. Further investigations of $PrlR_{I146L}$ revealed its location is crucial to ECD folding, PRL-responsiveness, and ligand-independent activity of the PRLR, which may underlie the effects observed by Bogorad and colleagues (Zhang et al., 2015). In addition to $PrlR_{I146L}$, a PRLR variant in exon 5 ($PrlR_{I76V}$) also displays constitutive activity in breast fibroadenomas patients via enhanced basal activity in breast cancer cells (Courtillot et al., 2010). While the location of SNP clearly alters PRLR function, the exact role of $PrlR_{I76V}$ and $PrlR_{I146L}$ in the onset or progression in breast carcinoma and its mechanism for constitutive activation remains unclear. Unlike $PrlR_{I76V}$ and $PrlR_{I146L}$, several heterozygous mutations of the PRLR gene within exon 10 can produce a truncated PRLR protein lacking most of the cytoplasmic tail in Stat1 null mouse mammary tumors (Griffith et al., 2016). The truncated mutant PRLR was found to enhance the progression of breast cancer via promoting phosphorylation and activation of STAT3 and STAT5, anchorage-independent growth of mouse embryonic fibroblasts, and tumor formation in nude mice.

Other studies have shown associations between *prlr* variants and the occurrence of breast cancer. Mong et al. (2011) have identified a SNP that was highly associated with the occurrence of breast cancer and with a serum biomarker of breast cancer, cancer antigen 15-3. Similarly, three SNPs in intron 3 of the human *prlr* gene were associated with breast cancer risk in premenopausal women and postmenopausal women (Nayante et al., 2011). In contrast with positive associations found between *prlr* gene variants and breast cancer risk, Vaclavicek et al.

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(2006) identified three SNP between the hPN promoter and exon 2 that were not individually associated with breast cancer risk. However, one haplotype combining all three SNPs was associated with a reduced risk of breast cancer.

Genetic variants of *prlr* are associated with clinical pathologies outside of the mammary glands. A PRLR ECD mutant ($His_{188}Arg$) was first reported to result in a loss of function in association with familial hyperprolactinaemia, which was later shown to abolish pSTAT5 expression, transcription of cytokine-inducible SH2-containing protein, and to impair the pAkt response, which may underlie its loss of function phenotype (Newey et al., 2013; Gorvin et al., 2018). In addition, six other *prlr* variants, 3 ECD variants and 3 PRLR ICD variants have been identified in tissues derived from prolactinomas (Gorvin et al., 2018). Interestingly cells expressing the ECD variants $Gly_{57}Ser$ or $Ile_{146}Leu$ showed decreased transcription of cytokine inducible SH2 containing protein (CISH) but increased pSTAT5, albeit at supraphysiological concentrations of PRL. In contrast, the ICD variant $Asn_{492}Ile$ displayed enhanced functional activity as shown by increased cell proliferation via the Akt pathway (Gorvin et al., 2018). However, not all *prlr* variants found in prolactinomas alter PRLR function. Bernard et al (2016) found 4 *prlr* variants that did not alter PRLR expression, localization and signaling with PRL stimulation. Similarly, SNP in the hPN promoter region and in intron 3 identified in patients with multiple sclerosis and Systemic Lupus Erythematosus were predicted to not have any functional relevance (Mellai et al., 2003). The inconsistencies in function and phenotypes associated with identical *prlr* variants indicate other biological factors may contribute to disease risk, onset, and progression of pathologies.

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Conclusion

One important reason for tissue specific PRLR expression is to generate differentiated phenotypes among organ systems. For example, PRLR expression in the MG is required for its development and function, while PRLR in the gonads are needed for female fertility. However, successful PRLR expression requires coordinated distribution and expression of the PRLR. At the phenotypic level, heterogeneity in PRLR mRNA and protein expression is controlled by multiple spatiotemporal factors. Moreover, multiple promoters within the *prlr* gene are utilized to coordinate expression in a location and time-dependent manner. Our review highlighted the genetic heterogeneity within the *prlr* gene, which has been implicated in phenotypes that significantly impact agricultural species and human pathology.

In depth analyses of 5' untranslated region (UTR) of the *prlr* gene have uncovered potential alternative first exons that may coordinate tissue-specific PRLR expression. However, not much is known regarding the genetic and protein elements that control expression in each organ system and in various physiological states across species. In addition, the function of *prlr* genetic variation is not clear. Several studies highlight correlations between *prlr* SNP and phenotypic outcomes but lack evidence demonstrating causative links and mechanisms. Prolactin coordinates many life-sustaining processes, such as pregnancy, lactation, and ionic flux in the intestines. Therefore, it is important to understand the regulation of PRLR expression, including *cis*- and *trans* acting elements, genomic organization, conserved mechanisms, and crosstalk between signaling pathways, to utilize PRL as a strong biological marker for phenotypic traits and pathologies in mammals.

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Chapter 2. Prolactin Receptor Regulation in the Kidneys of Swine

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ABSTRACT

The heterogeneity of PRLR expression across different tissues suggests its expression is dependent on spatiotemporal transcriptional regulation. However, many of the mechanisms coordinating tissue specific PRLR expression have not been established. Our objective was to resolve the factor(s) that direct PRLR expression in the kidneys of pigs. We found the promoter (promE1.3) for the kidney specific PRLR first exon, E1.3, has tissue-specific transcriptional activity, whereby the proximal promoter is activated in kidney and colorectal cells, but not in ovarian, fibroblast, or breast cell lines. We also found elements within the proximal promoter direct promE1.3 activity while factors between +1 and +140 bp downstream of the transcription start site repress transcriptional activation by 1.7 and 6.8-fold in kidney and colorectal cells, respectively. Mutating binding sites for hepatocyte nuclear factor 1 (HNF1) within the -150bp proximal promoter repressed transcriptional activity. Using a kidney cortical explant model, we found the expression of HNF1 and HNF4 variants declined significantly ($p<0.05$) *ex vivo*. In parallel, we found E1.3 expression declined. Together these data offer evidence for tissue-specific regulation of PRLR gene expression in the kidneys of pigs.

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INTRODUCTION

To fulfill the needs of fetal and neonatal development, maternal organ systems must undergo functional and structural changes to accommodate the cardiovascular, metabolic, and mineral demands of pregnancy and lactation. At the level of the kidney, renal blood flow, tubular function, and glomerular filtration rate shift in support of the nutrient and oxygen needs of rapid fetal growth (Cheung and Lafayette, 2013; Conrad & Davison 2014). Changes in gene expression and cell activity within renal cell populations coordinate the physiological changes observed, such as increased sodium retention and calcium excretion. The pituitary hormone prolactin (PRL) has gained recognition as a hormone that assists in regulating water and electrolyte balance. In aquatic species, PRL regulates water uptake and ion conservation via its direct actions on the gills, kidney, and intestine (Reviewed by Manzon, 2002). Similarly, PRL acts directly on mammalian small intestine (Charoenphandhu and Krishnamra, 2007), bone (Karayazi Atıcı et al., 2020), and kidney (Ibarra et al., 2005) to regulate sodium and calcium reabsorption. The presence of, and increase in, prolactin receptor (PRLR) gene expression during pregnancy and lactation in the kidneys suggest PRL acting through its receptors may regulate changes in renal function to support pregnancy and lactation (Buck et al., 1992; Schennink et al., 2015).

The actions of PRL depend on transcriptional regulation and downstream protein expression of the membrane bound PRLR. The use of multiple promoters within the 5'-untranslated region (UTR) is one major mechanism used to control PRLR gene expression in a tissue-specific and temporal fashion. As an example, PRLR gene expression in rats is controlled by three promoters, PI, PII, and PIII, that are activated by tissue specific transcription factors. In fact, the rat PII promoter is activated by the kidney and liver enriched transcription factor,

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hepatocyte nuclear factor 4 (HNF4), coinciding with tissue-specific regulation of PRLR expression to support the osmoregulatory roles of PRL (Hu et al., 1996; Moldrup et al., 1996). Aside from rodents and humans, data identifying the trans-acting factors that coordinate cell specific PRLR expression in agricultural species is limited.

Our lab discovered a first exon of the porcine PRLR (E1.3) with exclusive expression in the kidneys, liver, and small intestine (Schennick et al., 2015). Its promoter contains consensus sequences for tissue enriched transcription factors and is highly conserved in ungulates and humans. In this study we aimed to identify the cis- and trans-acting factors regulating PRLR expression in the kidneys of pigs. We hypothesized E1.3 expression is directed by tissue-specific factor(s) that activate its promoter (promE1.3). Our data revealed a -150bp region within the proximal promoter that conferred tissue and cell line specific activation. *In silico* binding analysis revealed sites for hepatocyte nuclear factor 1 (HNF1). Point mutations of the HNF1 binding site repressed promoter activation in both kidney and colorectal cell lines. Using a kidney explant model, we also found that mRNA expression for multiple HNF variants declined in parallel with E1.3 mRNA expression *ex vivo.*

MATERIALS & METHODS

Cell culture

The LLC-PK1 cell line (porcine kidney; epithelial) was cultured in Medium 199 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 3% (v/v) fetal bovine serum (FBS, GeminiBio, Sacramento, CA, USA), 1% penicillin/streptomycin (v/v; 100 U penicillin and 100 µg streptomycin/ml; P/S), and 2.2 g/L sodium bicarbonate (Sigma-Aldrich, St. Louis, MO, USA). The Caco-2 cell line (human large intestine; epithelial) was cultured in Eagle's minimum essential medium (Sigma-Aldrich) supplemented with 20% FBS, 1% P/S, and 2.2 g/L sodium

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bicarbonate. The CHO cell line (hamster ovary; epithelial) was cultured in Alpha minimum essential medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% FBS, 1% P/S, and 2.2 g/L sodium bicarbonate. The NIH/3T3 cell line (mouse embryonic; fibroblast) was cultured in Dulbecco's modified eagle's medium high glucose (Corning, Glendale, AZ, USA) supplemented with 10% calf serum (Cytiva, Malborough, MA, USA), 4 mM L-glutamine (GeminiBio), 1 mM sodium pyruvate (Corning), 1% P/S, 2.38 g/L HEPES (Sigma-Aldrich) and 1.5 g/L sodium bicarbonate (Sigma-Aldrich). The T47D (human breast; epithelial) cell line was cultured in RPMI 1640 supplemented with 5% FBS, 1%P/S, 1 μg/ml insulin, 2 mg/ml sodium bicarbonate, and 2.42 mg/ml HEPES. All cell lines were purchased from ATCC (Manassas, VA, USA) and cultured in a humidified incubator at 37° C with 5% CO_2.

Plasmids

A pPRLR promoter construct containing 1973 bp of promE1.3 and 140 bp of E1.3 was created as described previously (Schennink et al., 2015). Promoter constructs bearing deletions (Figure 1) were synthesized (GeneWiz, South Plainfield, NJ, USA) or generated by PCR. Promoter deletion fragments amplified by PCR were generated using Phusion® High-Fidelity PCR Master Mix (New England Biosystems, Ipswich, MA, USA), gel purified (Wizard SV Gel Clean-Up System, Promega) and subcloned into the pGL3-Basic vector (Promega) via Gibson Assembly (New England BioLabs, Ipswich, MA, USA). Oligonucleotides bearing mutations in putative HNF binding sites within promE1.3 were synthesized by GeneWiz (Figure 4) and ligated into the pGL3-Basic vector using the Quick Ligation Kit (New England BioLabs). All promoter fragment sequences were verified by Sanger sequencing prior to preparation of endotoxin-free DNA preparations using the GenElute™Endotoxin-free Plasmid Midiprep Kit (Sigma-Aldrich).

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Transient Transfections

To examine promoter activity, cells were plated at 80,000-120,000 cells/well in 12 well plates in growth medium 24h before transfection. The PK1, Caco-2, and NIH/3T3 cell lines were transfected with 0.35-0.75 μg pPRLR promoter or empty vector plasmid (PGL3 Basic) DNA and 0.15-0.25 μg pCMV–eGFP DNA using 1.5-4 μl Lipofectamine 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The CHO cell line was transfected with 0.75 μg pPRLR promoter vector or PGL3 Basic and 0.25 μg pCMV–eGFP DNA using 3 μl Lipofectamine LTX with Plus transfection reagent (Invitrogen). The T47D cell line was transfected with 0.75 μg pPRLR promoter vector or PGL3 Basic and 0.25 μg pCMV–eGFP DNA using 1 μl X-tremeGENE HP transfection reagent (Roche). Transfection with pCMV–eGFP (gifted by David Kerr, University of Vermont) was used to assess transfection efficiency. All cell lines were transfected in fresh growth medium without antibiotics. Medium was replaced with fresh growth medium containing 3% FBS and 1% P/S either 4h (PK1) or 16h (Caco-2, CHO, NIH/3T3, T47D) after transfection. Forty-eight hours after transfection, cells were lysed in 200 μl of Glo Lysis buffer (Promega), scraped, and assayed for eGFP and luciferase activity (Bright-Glo™ Luciferase Assay System, Promega) using a Synergy HT plate reader (BioTek, Winooski, VT, USA).

Kidney Explant Culture

Kidneys from nulliparous Yorkshire x Hampshire gilts were dissected under sterile conditions and cortical explants generated using a Stadie-Riggs Microtome (Thomas Scientific, Swedesboro, NJ, USA). Cortical slices (100 mg) were cultured on siliconized lens paper (Plaut et al., 1993) in 6-well plates containing medium 199 supplemented with 1% (v/v) insulin-transferrin-selenium (ITS, Corning, Glendale, AZ, USA) and 2% antibiotic-antimycotic (Gibco,

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Waltham, MA, USA) in a 37°C incubator with 80% O_2 and 5% CO_2 for 48h. Medium was changed daily. Cortical slices were flash frozen in liquid nitrogen after 0, 12, 24, and 48h in culture.

RNA preparation

Kidney explants were homogenized in TriReagent (Molecular Research Center, Inc., Cincinnati, OH, USA) and total RNA extracted according to the manufacturer's instructions. Total RNA (5 µg) was treated with DNaseI (5U; Roche, Indianapolis, IN, USA) and purified using the DNA-Free RNA kit (Zymo Research Corporation, Orange, CA, USA). Total RNA integrity was confirmed by formaldehyde–agarose gel electrophoresis. A 500 ng aliquot of DNase-treated RNA was denatured at 70 °C for 5 min and reverse transcribed in the presence of oligo-dT (500 ng, New England BioLabs) and random hexamers (50 ng, Amersham Pharmacia Biotech) using 1X RT buffer (Promega), dNTP mixture (10 mM, Promega), Moloney's murine leukemia virus reverse transcriptase (100 U, Promega), and RNase inhibitor (15 U, Promega) at 25°C for 5min, 37 °C for 60 min, and 95 °C for 5 min.

Quantitative PCR (qPCR)

The cDNA synthesized from kidney explants was diluted 1:4 and 2 µl were used as a template for qPCR using Fast SYBR Green Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) on a QuantStudio 3 Real-Time PCR System (ThermoFisher Scientific). Samples were assayed in duplicate. Primer sequences and their associated T_m are presented in Table 1. Relative mRNA expression was quantified using a standard curve prepared using fourfold serial dilutions of cDNA synthesized from porcine kidney tissue. To quantify pPRLR long form (LF) and pPRLR short form (SF) copy number in unknowns, cDNA from

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kidney explants was diluted 1:2 and 4 µl was used as a template for qPCR using TaqMan Fast Advanced Master Mix (Applied Biosystems) on a QuantStudio 3 Real-Time PCR System (ThermoFisher Scientific). Samples were assayed in duplicate. Absolute quantification of PRLR-LF and -SF copy number was determined from a standard curve prepared as tenfold serial dilutions (10–10^7copies/well) as previously described (Trott et al., 2011; Schennink et al., 2013a). Primer and probe sequences used for absolute quantification are presented in Table 2. Relative and absolute standard curves were assayed in triplicate. All qPCR data was normalized to 18s rRNA by dividing the average transcript copy number by the corresponding amount of 18S rRNA for each unknown (Trott et al., 2011).

Statistical Analysis

Promoter activation analysis and qPCR data were analyzed in GraphPad Prism using a one-way ANOVA and Tukey's multiple comparisons test was used to compare treatment means. Comparisons of PRLR transcripts in kidney explants were analyzed using a two-way ANOVA and Sidak's multiple comparisons test was used to compare treatment means. Differences were considered significant at $p \leq 0.05$.

RESULTS

Exon 1.3 promoter directs transcriptional activity in a cell line specific manner

We previously found E1.3 was abundantly expressed in the porcine kidney cortex, and its promoter (promE1.3) sequence and transcription factor binding sites were highly conserved across ungulates and humans (Schennink et al., 2015). Given these findings, we sought to investigate the transcriptional regulation of promE1.3. We generated pPRLR-E1.3 promoter deletion constructs between positions -1973 and -65bp (Figure 1), which were transiently

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transfected into porcine kidney **(**LLC-PK1; PK1), human colorectal (Caco-2), hamster ovarian (CHO), human breast (T47D), and mouse embryonic fibroblast (NIH/3T3) cell lines. As shown in Figure 2, promE1.3 deletion constructs between -65 and -884bp upstream of the transcription start site (TSS) demonstrated significant ($p<0.05$) transcriptional activity in PK1 cells when compared against the promoterless pGL3 Basic construct. Similarly, transcription from constructs between -142 and -280bp was significantly ($p<0.05$) induced in Caco-2 cells. In contrast, all promoter constructs transfected into CHO, T47D, and NIH/3T3 cell lines failed to induce transcriptional activity above pGL3 Basic (Figure 2). These data show cell line specific activation of promE1.3 in PK1 and Caco-2 cells and suggest elements within the proximal promoter (-65bp to -187bp) are required for promoter activation in these cell lines. Given our deletion constructs also contained 140bp of E1.3 downstream of the TSS, we sought to determine if elements in E1.3 influenced promoter activation. As seen in Figure 3, deletion of the +140bp E1.3 sequence stimulated a 3, 6.8, and 110-fold induction of expression in PK1, CHO and NIH/3T3 cells, respectively. Collectively, these data indicate elements within E1.3 may work cooperatively with the proximal promoter to regulate transcriptional activity of this promoter.

Mutation of the putative HNF1 site on the promE1.3 proximal promoter decreases promoter activity

In rats, PRLR expression is controlled by tissue-specific factors that activate its promoters (Hu et al., 1996; Møldrup et al., 1996; Hu et al., 2002). To test the hypothesis that promE1.3 is also activated by tissue enriched transcription factors (TF), we first identified putative transcription factor-binding sites within promE1.3 using AliBaba2.1 (http://www.gene-regulation.com/pub/programs/alibaba2/index.html). The analysis revealed two binding sites for HNF1α between -20/-3 bp upstream of the TSS. To determine if HNF1α is required for

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promE1.3 activation, we mutated these HNF1α binding sites in a -65bp promoter construct (Figure 4a). As seen in Figure 4, mutating the HNF-1α binding site proximal to the TSS (HNF 3p mut) reduced transcriptional activity by 1.7 and 6.8-fold in PK1 and Caco2 cells, respectively. Disruption of the distal (HNF 5p mut) or both (HNF 2x mut) HNF-binding sites had no significant effect on promoter induction in PK1 cells (Figure 4b), but significantly ($p<0.05$) reduced promoter activity in Caco2 cells when compared to the wild-type promoter (Figure 4c).

Reductions in HNF transcription factor mRNA expression corresponds with a decrease in PRLR transcript expression *ex vivo*

We developed an explant model to study E1.3 regulation in a system that maintains organ architecture and cellular diversity. Kidney cortical explants from pigs were cultured for 48h and gene expression quantified by qRT-PCR. We first compared expression of the kidney predominant transcript, E1.3, with the porcine PRLR (pPRLR) E1A transcript that was found to be hormone sensitive and abundant in PRL target tissues (Schennink et al., 2013a). While not statistically significant, the relative expression of E1.3 was numerically higher than E1A at the time of slaughter (Figure 5). However, E1.3 expression declined over time in culture, and by 12h, E1A was the dominant transcript expressed (Figure 6). After 48h in culture E1A expression increased by 3-fold and was significantly ($p<0.05$) higher than for E1.3. To assess if changes in PRLR transcripts altered PRLR isoform expression, we also quantified PRLR-LF and -SF mRNA expression. There was no significant difference in either PRLR-LF or PRLR-SF expression over 48h (Figure 7). Multiple lines of evidence show PRLR gene expression is sensitive to PRL (Galsgaard et al., 1999; Shao et al., 2008; Schennink et al., 2013a). Thus, we examined the effect of porcine PRL (pPRL) on E1.3 expression. Unlike other PRLR transcripts,

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our preliminary experiment indicated pPRL does not alter the expression of E1.3 (Supplementary Figure 1).

As outlined above, point mutations of HNF1 binding sites within promE1.3 suggested a role for this factor in regulating promE1.3 expression. Thus, we measured the relative mRNA expression for HNF1A, HNF1B, HNF4A, and HNF4G in kidney explants. Among the HNF1 and HNF4 variants analyzed, HNF4G was the most highly expressed variant expressed in the porcine kidney at the time of necropsy (data not shown). After 48h in culture we found HNF1A, HNF1B, HNF4A, and HNF4G expression significantly declined ($p<0.05$) in parallel with E1.3 *ex vivo* (Figure 8). The HNF proteins are pivotal to metabolic function whereby genetic variations in HNF1α, HNF1β and HNF4α are associated with osmoregulatory pathologies such as, maturity onset diabetes of the young3 (MODY3), MODY5, and renal Fanconi syndrome (reviewed by Lau et al., 2018). Moreover specifically, HNF proteins are shown to regulate genes that control calcium reabsorption in the kidney and intestine. The calcium-sensing receptor in the thick ascending limb is regulated by HNF1B (Kompatscher et al., 2018) while Calbindin D9k, a gene vital to intestinal calcium absorption, is regulated by HNF1A (Wang et al., 2004). We examined the expression of calcium modulatory genes in kidney explants. While pPRL had no effect on expression, time in culture tended to negatively affect the expression of genes regulating calcium. Expression of genes that encode for Na-Cl Symporter (SLC12A3), Claudin 2 (CLDN2,) and Sodium/Calcium Exchanger 1 (SLC8A1) declined after just 12h in culture. Interestingly, transient receptor potential vanilloid 5 (TRPV5), ATPase Plasma Membrane Ca2+ Transporting 1 (ATP2B1), and Calbindin 1 (CALB1) expression increased at 12h but declined or remained unchanged at later time points (Supplementary Figure 2).

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DISCUSSION

Differential activation of promoters within the PRLR gene is vital to the diverse actions of PRL across tissues. As a case in point, PRLR expression increases in tissues that support pregnancy and lactation such as the mammary glands, kidney, and intestine. We previously identified 11 alternative first exons within the porcine PRLR gene, some of which displayed tissue-specific expression. The E1.3 transcript was exclusively expressed in tissues that support osmoregulation, whereby its expression was most abundant in the kidney, small intestine, and liver of pigs. We also found E1.3 promoter sequence was conserved across ungulates and humans (Schennink et al., 2015). In line with those data, our experiments show E1.3 promoter activation was cell line specific, where the activation of promE1.3 was only recorded in porcine kidney and human colorectal cell lines. We also found a 150bp region within the proximal promoter that was vital to promoter activity in these cells. On the contrary, elements within a 140bp region downstream of the TSS repressed transcription in all cell lines tested. These findings suggest elements within the proximal promoter may work cooperatively with those located downstream of the transcription start site to regulate PRLR transcription.

Expression of PRLR in osmoregulatory tissues is observed in various species. In chickens, PRLR transcripts containing exon 1G were predominately expressed in the kidney and small intestine (Bu et al., 2013b). Additionally, the promoter for exon 1A in rats was most highly expressed in the liver and is activated by tissue specific transcription factor, HNF4 (Moldrup et al., 1996). Interestingly the chicken 1G, rat 1A, and porcine E1.3 promoters share putative transcription factor-binding sites for tissue enriched HNF proteins within their proximal promoter regions (Schennink et al., 2015). Our site-directed mutagenesis experiments support a candidate function for HNF as regulators of PRLR expression in the kidneys of pigs. When one

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or all the consensus sequences for HNF1 were mutated, transcriptional activity was significantly ($P<0.05$) repressed in Caco-2 cells. While not statistically significant, activity in PK1 cells displayed a numerical reduction in transcriptional activity when the HNF binding site on the 3P location was mutated.

Hepatocyte nuclear factors are categorized into 4 major families, HNF1, HNF3, HNF4, and HNF6, and are expressed in a tissue specific fashion (Reviewed by Lau et al., 2018). The HNF1 family comprises HNF1-α/A and HNF-1β/B while HNF4 belongs to the orphan nuclear receptor family and consists of HNF4-α/A and HNF4-γ/G. While they are most abundant in the liver, HNF proteins are also highly expressed in the kidney and are well known for regulating renal development and osmoregulatory functions (Lau et al., 2018). In addition, the liver-enriched HNF4 was found to bind and activate the rodent PRLR-1A promoter (Moldrup et al., 1996). Given the possible relationship between HNF1A and PRLR transcription found in our site-directed mutagenesis studies, we examined the expression of HNF transcription factors in an explant model. Our data showed transcription factors HNF1A, HNF1B, HNF4A, and HNF4G decreased in parallel with expression from the E1.3 promoter. The HNF transcription factors are known to participate in an autoregulatory loop whereby the expression of HNF4A expression is upregulated by HNF1A and HNF1B (Hatzis and Talianidis, 2001), while HNF4A can positively regulate HNF1A and HNF1B expression (Kanazawa et al., 2010). Thus, it is possible in our model that the decline in one HNF protein downregulated the expression of the others in its family. Since disruption of the HNF1 binding site within promE1.3 also reduced promoter activation, it is possible that the decline in E1.3 expression *ex vivo* could be driven by a decline in HNF1 and HNF4 expression. Taken together, these results suggest a network of factors, including the HNF network, may cooperatively regulate PRLR gene expression in the kidney.

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Alongside tissue specific transcription factors, endocrine factors also regulate PRLR expression. A ubiquitously expressed first exon of the pPRLR, E1A, is positively regulated by 17β-estradiol and PRL, and is highly expressed in tissues that support lactation, including the kidney (Schennick et al., 2013). Given the unique expression and regulation of E1A, we used a kidney cortical explant model to examine the comparative mRNA expression of E1.3 against E1A. In line with previous work (Schennink et al., 2015), E1.3 was the predominant PRLR transcript expressed in the kidney cortex whereby relative mRNA expression of E1.3 was 3.5x higher than E1A. However, time in culture induced a decline in E1.3 expression over 48h whereas E1A was preferentially expressed by 48h.

Differential gene expression and modulation in activity of ion transporters in osmoregulatory organs are a few ways mammals meet the ionic demands of physiological states such as pregnancy and lactation. During these states serum PRL increases and acts directly on the intestine to increase calcium absorption in support of neonatal development (Ajibade et al., 2010). While evidence suggests a larger role for PRL as a calcitropic hormone (Charoenphandhu et al., 2010), not much is known regarding its role in regulating calcium in the kidneys. We analyzed the relative mRNA expression of genes that regulate calcium (Ca^{2+}) flux in a kidney explant model. Our preliminary data revealed exogenous PRL treatment had no effect on the expression of genes that support Ca^{2+} flux. However, our study presents some limitations, namely sample size, and the absence of medullar tissue. Ionic regulation in the kidney requires the concerted action of specialized channels and transporters located in the medulla and the cortex. Our explants were limited to the kidney cortex, given 60-70% of calcium reabsorption occurs in the proximal convoluted tubules by passive transport mechanisms, and 10% in the distal convoluted tubules by active transport (Blaine et al., 2015). However, it is possible

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elements within the medulla are required for the sustained expression or function of the genes examined in our study. As an example, tissue kallikrein, a protease produced in the renal collecting duct, enhances plasma membrane expression of TRPV5 in the kidneys (Gkika et al., 2006). Thus, ion transport studies and explants containing fully intact nephrons are needed to determine a potential role and mechanism for PRL in regulating calcium homeostasis.

Together our findings highlight the potential role for HNF1 and HNF4 in the differential regulation of PRLR expression in cells derived from osmoregulatory tissues, like the kidneys and large intestine. Our data suggest transcriptional activation of the PRLR gene in the kidney of pigs is a result of the putative action of HNF1 binding the E1.3 proximal promoter, alongside synergistic cooperativity with elements within the +140bp region to regulate cell specific expression. In line with our speculations, HNF1 and HNF4 mRNA expression declined in tandem with E1.3 expression *ex vivo*. Our site-directed mutagenesis experiments in Caco-2 cells suggest this mechanism may be conserved in other osmoregulatory tissues such as, the large intestine. However, more conclusive methods, such as Chromatin Immunoprecipitation, must be performed to confirm the recruitment and binding of HNF proteins to the PRLR promoter and elucidate the exact mechanism underlying PRLR expression in the kidneys.

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TABLES

Table 1. Sequences of forward (F) and reverse (R) primers used for relative mRNA expression via qPCR

Gene Name	F (5'-3')	R (3'-5')	T_m
pPRLR E1.3	GTGACGCACGGCAGGAA	GGTCAGAGGTTCACTTCAGGGGTCAT	64
pPRLR E1A	GACTGATACTGCAGACTGACTTTGCT	GGTCAGAGGTTCACTTCAGGGGTCAT	64
HNF1A	CTCAACCAGTCCCACCTGTC	CTGCGTGAGTGAACTGCTGG	62
HNF1B	GAGGACGGGGACGACTATGA	TGCTGCATGTAGCCCTTGAT	62
HNF4A	AGATTTAGTCGGCAGTGCGT	CAGGCTGCTATCCTCGTAGC	62
HNF4G	AGGTTCAGTCGGCAATGTGT	TCACGCTCATTTTGCACAGC	62
18s rRNA	ACGGCTACCACATCCAAGGA	CCAATTACAGGGCCTCGAAA	60

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Table 2. Sequences of primers (F and R) and probes (P) used for absolute mRNA expression via qRT-PCR

Oligo	**Primer and probe sequence (5′–3′)**	**T_m**
p*PRLR*-LF F	CGCCGCTTTGCTGGAA	60
p*PRLR*-LF R	GCCAGTCTCGGTGGTTTTTG	60
p*PRLR*-LF P	AACGGACCGACATGCTTTCAACCCT	60
p*PRLR*-SF F	AAGGATTTGACACCCATCTGTTG	60
p*PRLR*-SF R	TTGGAGATGATTCTTGGGCTGTA	60
p*PRLR*-SF P	CCTCATGCTGTTGGATGCCTCTTACCATAAC	60

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FIGURE LEGENDS

Figure 1. A representation of the pPRLR E1.3 promoter fragments used to analyze promoter activity. Deletion constructs contained varying lengths of the promE1.3 promoter and 140bp of E1.3 sequence downstream of the transcription start site (TSS). Constructs were ligated into a pGL3 Basic vector containing a luciferase reporter gene (Luc).

Figure 2. Promoter analysis for promE1.3 in (A) PK1 (B) Caco-2 (C) NIH/3T3 (D) CHO and (E) T47D cells transiently transfected with plasmids containing varying lengths promE1.3 and 140bp E1.3 upstream of a luciferase reporter. Cells were transfected in complete growth medium and harvested 48h later. Luciferase output was normalized to total cells transfected as determined by GFP expression. Data are shown as fold induction over basal activity observed in the promoterless construct pGL3-Basic. Data are normalized means ± S.E.M. (n=3; two independent experiments). *$p<0.05$ versus pGL3-Basic.

Figure 3. Promoter analysis for promE1.3 in PK1, Caco-2, NIH/3T3, and CHO cells transiently transfected with plasmids containing 65bp of promE1.3 with or without 140bp of E1.3, upstream of a luciferase reporter. Promoter activity is expressed relative to the -65/+140 bp construct which was set as 1. Luciferase output was normalized to total cells transfected as determined by GFP expression. Cells were transfected in complete growth medium and harvested 48h later. Data are normalized means ± S.E.M. (n=3-4; 3 independent experiments). A significant difference between the truncated (-65) and wild type (-65/+140) construct in each cell line is indicated by ***$p<0.0001$.

Figure 4. (A) A representation of the pPRLR E1.3 promoter fragments used for site mutagenesis assay. Deletion constructs contained -65bp of promE1.3 promoter and 140bp of E1.3 sequence downstream of the transcription start site (TSS). The -65/+140 construct (WT) was mutated at

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the 5' HNF1 binding site (HNF 5P mut), the 3' HNF1 binding site (HNF 3Pmut), or both HNF1 binding sites (HNF 2x mut). * represents mutations in the HNF1 consensus sequence. Constructs were ligated into a pGL3 Basic vector containing a luciferase reporter gene (Luc). Mutant promoter constructs were transiently transfected into (B) PK1 and (C) Caco-2 cells. Cells were transfected in complete growth medium and harvested 48h later. Promoter activity is expressed relative to the -65/+140 bp construct which was set as 1. Data are normalized means ± S.E.M. (*n*=3-4; three independent experiments). A significant difference between mutant constructs and wild type (-65/+140) is indicated by *p<0.05; **p<0.01; ***p<0.001; ****p<0.00001.

Figure 5. Analysis of PRLR E1.3 and E1A mRNA expression in kidney cortex slices immediately after slaughter. Data are means normalized to the corresponding level of 18s rRNA ± S.E.M. (n=3-4 independent animals).

Figure 6. Analysis of pPRLR E1.3 and E1A mRNA expression in kidney explants. Explants were cultured in Medium 199 supplemented with 1x ITS and harvested 12, 24, and 48h later. Relative mRNA expression at 12, 24, and 48h is relative to 0h which was set as 1. Data are means normalized to the corresponding level of 18s rRNA ± S.E.M. (n=3; 3-4 independent experiment). A significant difference between E1.3 and E1A is indicated by ****p<0.00001.

Figure 7. Analysis of (A) PRLR long form and (B) PRLR short form mRNA copy number in kidney explants. Explants were cultured in M199 supplemented with 1x ITS and harvested 12, 24, and 48h later. Data are means normalized to the corresponding level of 18s rRNA ± S.E.M. (n=3-4 independent animals).

Figure 8. Analysis of (A) HNF1A (B) HNF1B (C) HNF4A (D) HNF4G mRNA expression in kidney explants. Explants were cultured in M199 supplemented with 1x ITS and harvested 12,

`

24, and 48h later. Relative mRNA expression at 12, 24, and 48h is relative to 0h which was set as 1. Data are means normalized to the corresponding level of 18s rRNA ± S.E.M. (*n*=3; 3-4 independent experiments). Significant differences between timepoints are indicated by *p<0.05; **p<0.01; ***p<0.001; ****p<0.00001.

Supplementary Figure 1. Analysis of E1.3 relative mRNA expression in kidney explants. Explants were cultured in M199 supplemented with 0-1000 ng porcine PRL (pPRL) and harvested 12, 24, and 48h later. Relative mRNA expression at 12, 24, and 48h is relative to 0h which was set as 1. Data are means normalized to the corresponding level of 18s rRNA ± S.E.M. (*n*=1 animal).

Supplementary Figure 1. Analysis of (A) TRPV5 (B) ATP2B1 (C) SLC12A3 (D) CALB1 (E) CLDN2 and (F) SLC8A1 relative mRNA expression in kidney explants. Explants were cultured in M199 supplemented with 0-1000 ng porcine PRL (pPRL) and harvested 12, 24, and 48h later. Relative mRNA expression at 12, 24, and 48h is relative to 0h which was set as 1. Data are means normalized to the corresponding level of 18s rRNA ± S.E.M. (*n*=1 animal).

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FIGURES

FIGURE 1.

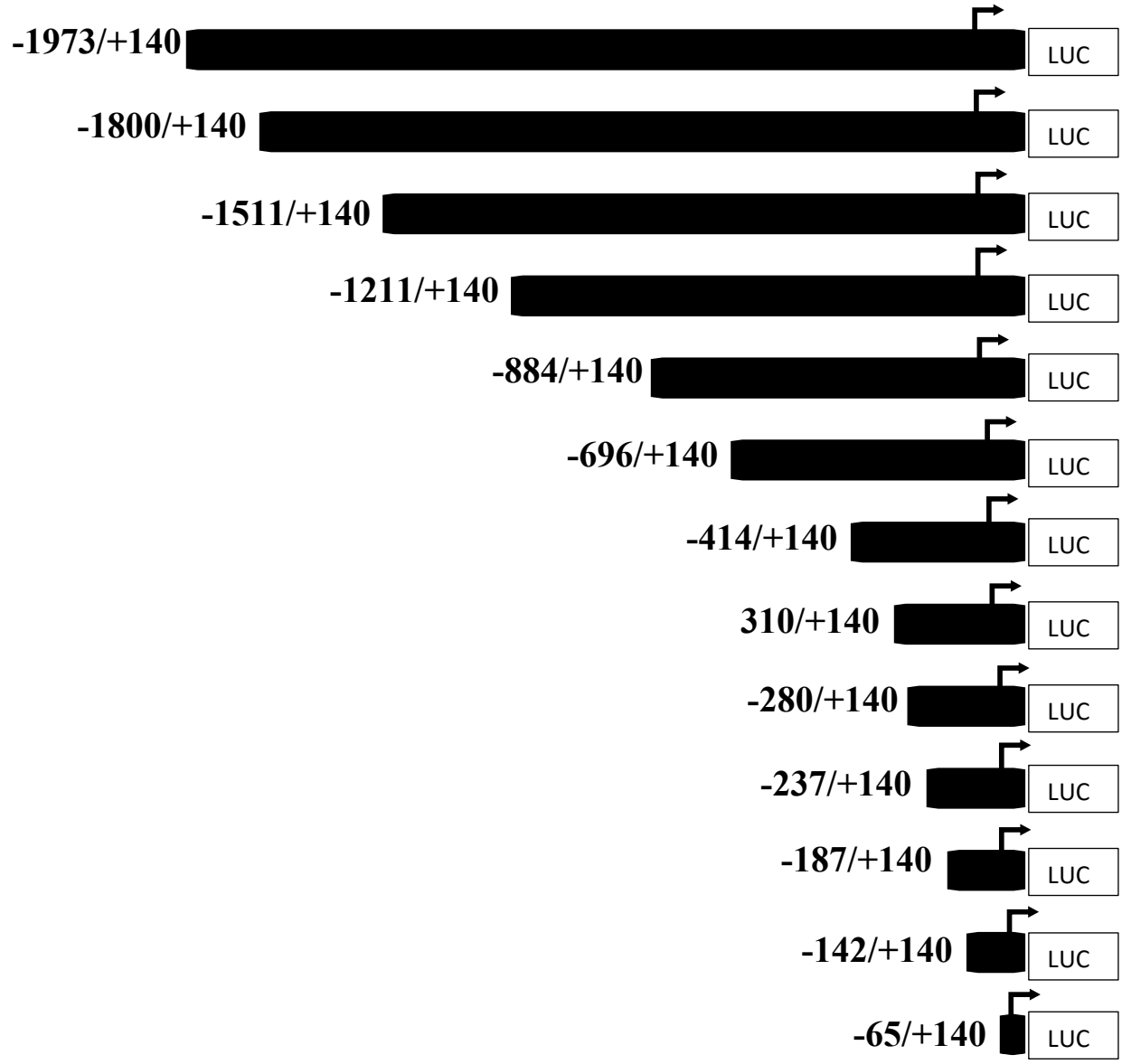

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FIGURE 2.

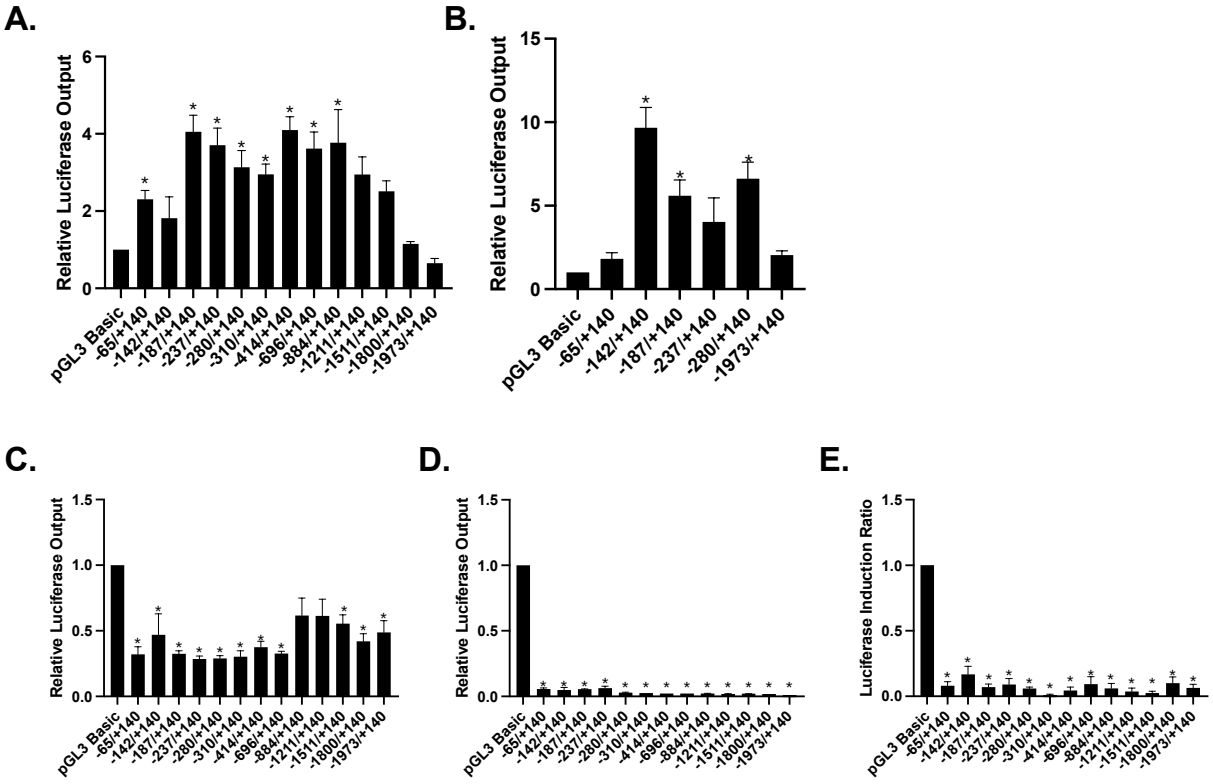

A.
Relative Luciferase Output
0
2
4
6
pGL3 Basic
-65/+140
-142/+140
-187/+140
-237/+140
-280/+140
-310/+140
-414/+140
-696/+140
-884/+140
-1211/+140
-1511/+140
-1800/+140
-1973/+140
B.
Relative Luciferase Output
0
5
10
15
pGL3 Basic
-65/+140
-142/+140
-187/+140
-237/+140
-280/+140
-1973/+140
C.
Relative Luciferase Output
0.0
0.5
1.0
1.5
D.
Relative Luciferase Output
0.0
0.5
1.0
1.5
E.
Luciferase Induction Ratio
0.0
0.5
1.0
1.5

FIGURE 3.

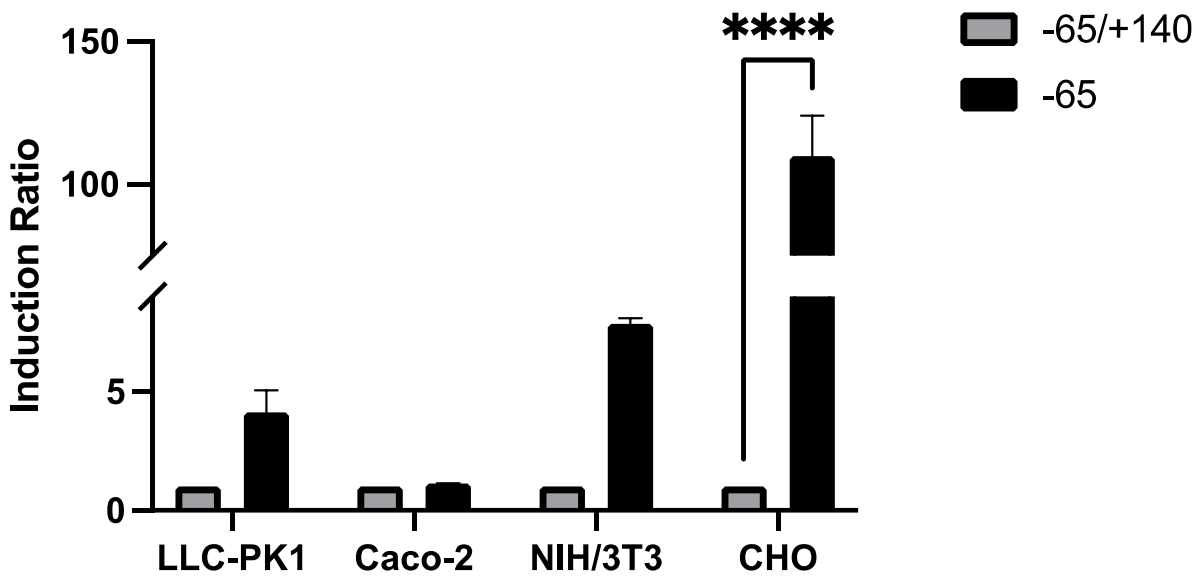
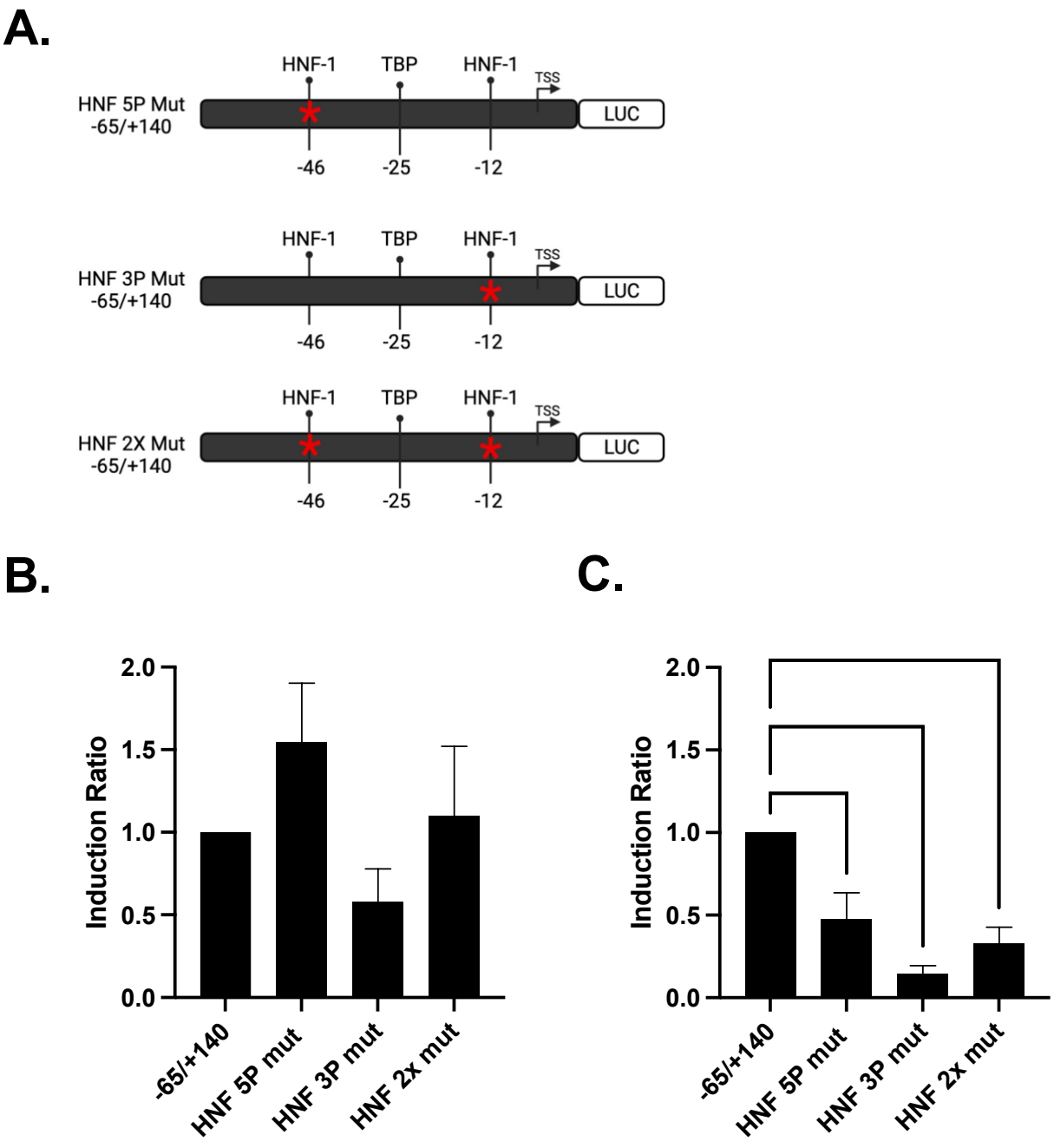


FIGURE 4.


A.
HNF-1
TBP
HNF-1
TSS
HNF 5P Mut
-65/+140
LUC
-46
-25
-12
HNF-1
TBP
HNF-1
TSS
HNF 3P Mut
-65/+140
LUC
-46
-25
-12
HNF-1
TBP
HNF-1
TSS
HNF 2X Mut
-65/+140
LUC
-46
-25
-12
B.
Induction Ratio
2.0
1.5
1.0
0.5
0.0
-65/+140
HNF 5P mut
HNF 3P mut
HNF 2x mut
C.
Induction Ratio
2.0
1.5
1.0
0.5
0.0
-65/+140
HNF 5P mut
HNF 3P mut
HNF 2x mut

`

FIGURE 5.

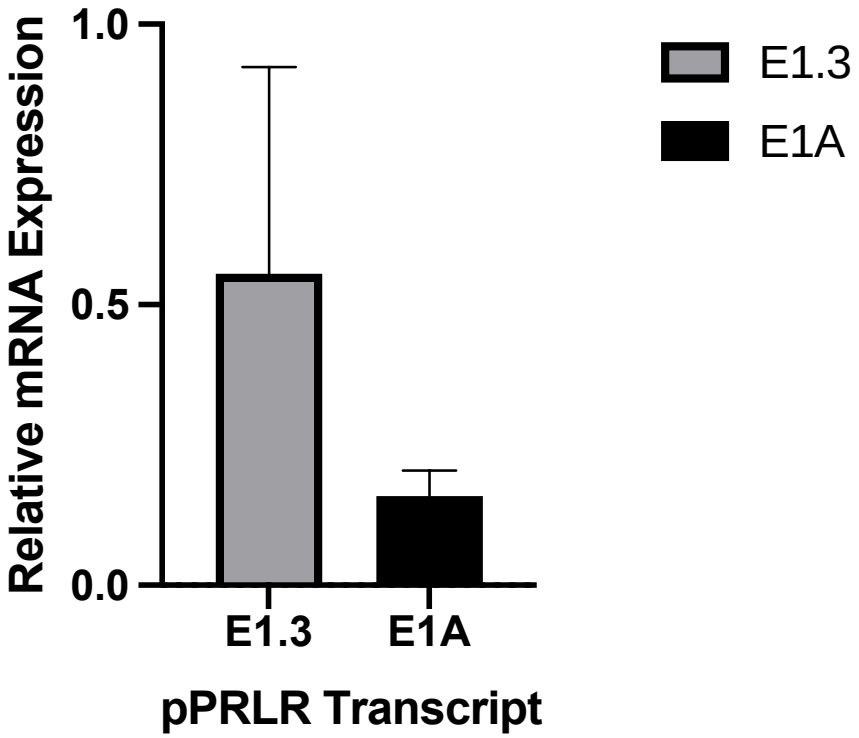
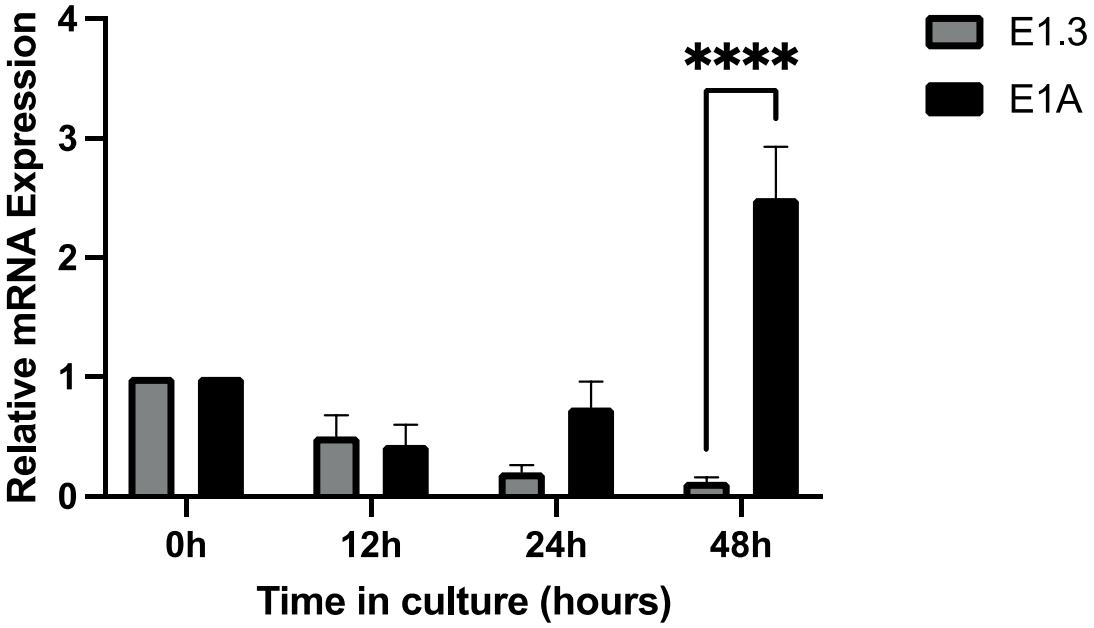

Relative mRNA Expression
1.0
0.5
0.0
E1.3
E1A
pPRLR Transcript
E1.3
E1A

FIGURE 6.


Relative mRNA Expression
4
3
2
1
0
****
E1.3
E1A
0h
12h
24h
48h
Time in culture (hours)

`

FIGURE 7.

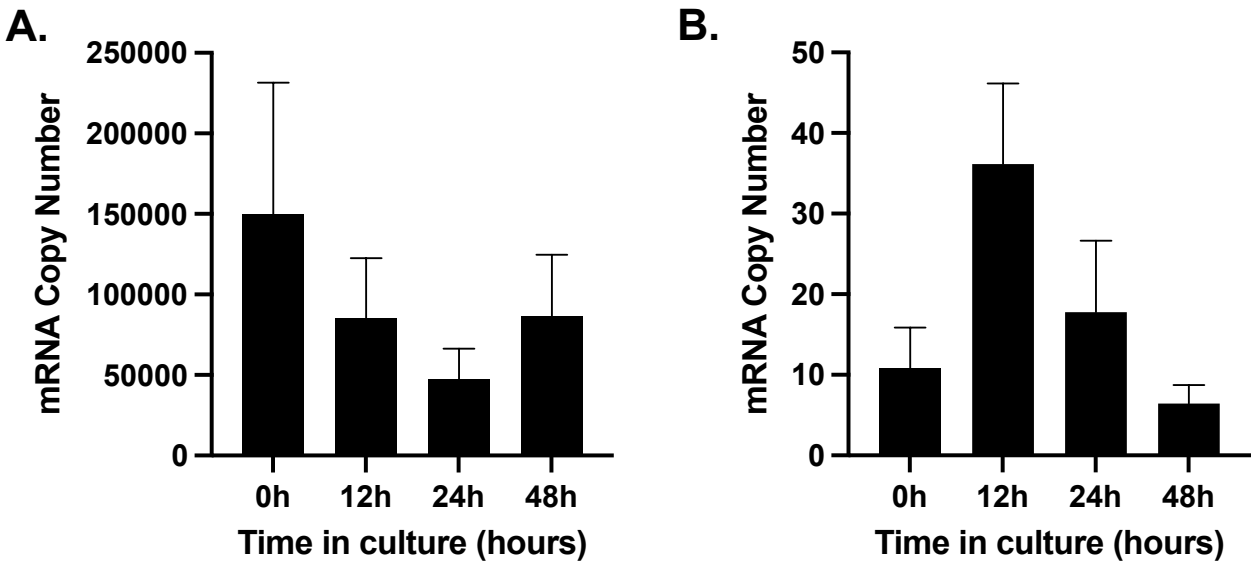
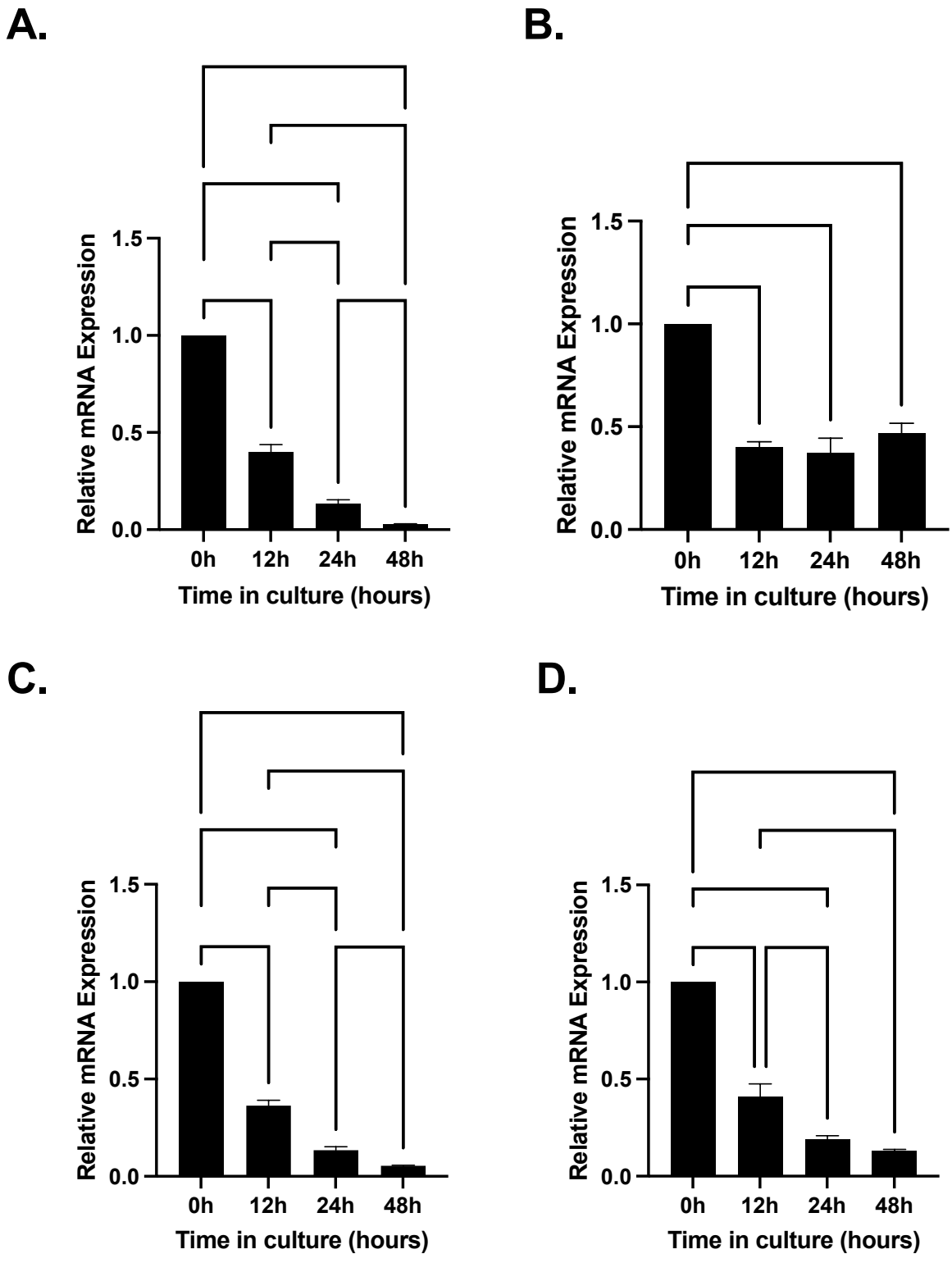
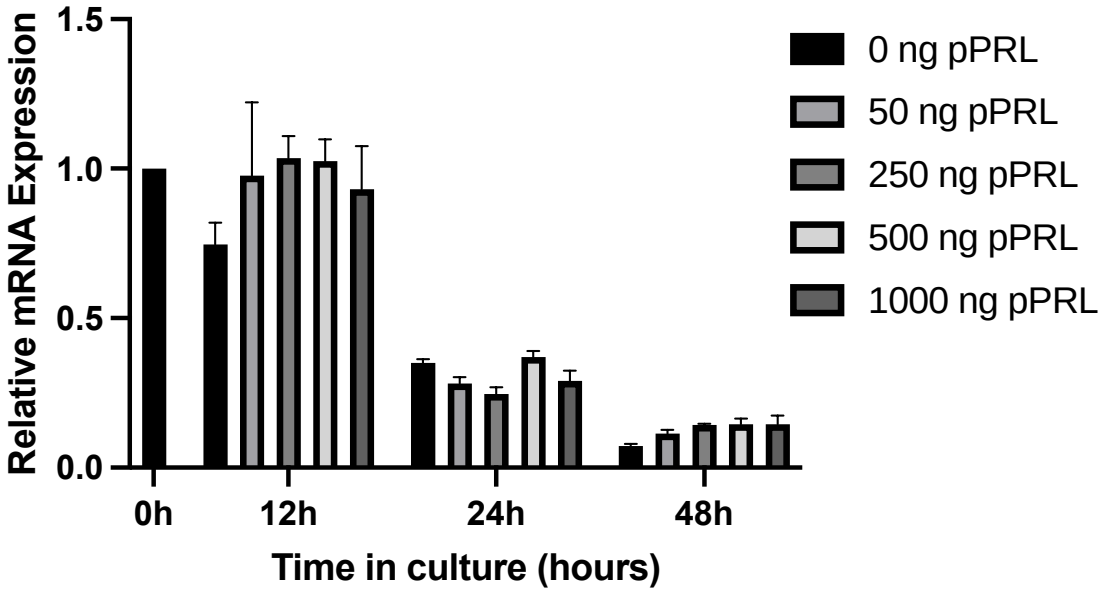

A.
mRNA Copy Number
250000
200000
150000
100000
50000
0
0h
12h
24h
48h
Time in culture (hours)
B.
mRNA Copy Number
50
40
30
20
10
0
0h
12h
24h
48h
Time in culture (hours)

FIGURE 8.

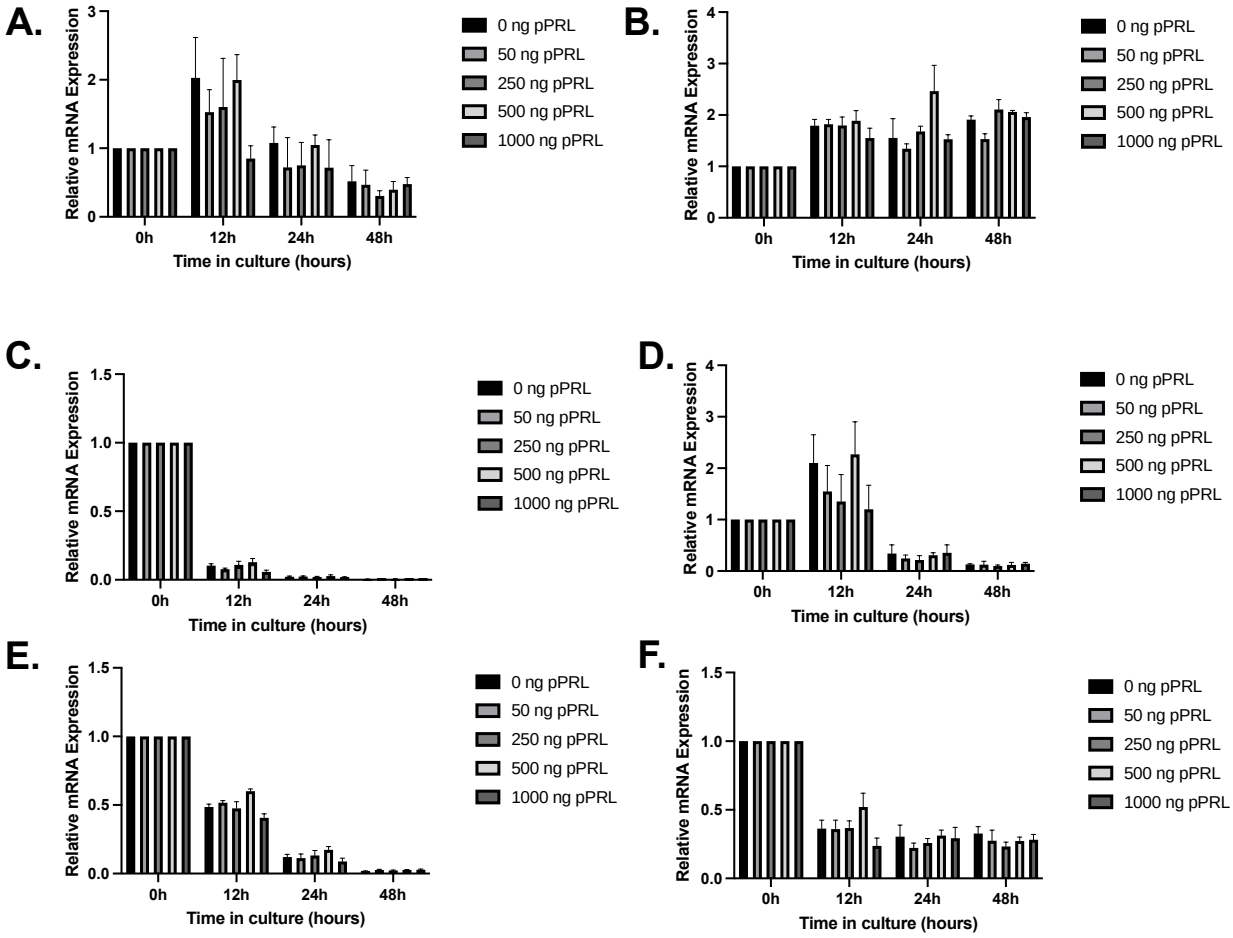

A.
B.
C.
D.
Relative mRNA Expression
1.5
1.0
0.5
0.0
0h
12h
24h
48h
Time in culture (hours)

`

SUPPLEMENTARY FIGURE 1.



SUPPLEMENTARY FIGURE 2.



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Appendix A. Optimization of Chromatin immunoprecipitation

Our *in vitro* studies revealed HNF1 as a potential trans-acting factor regulating pPRLR E1.3 expression in kidney and colorectal cell lines. We sought to confirm hepatocyte nuclear factor 1 (HNF1) recruitment to the E1.3 promoter using Chromatin Immunoprecipitation (ChIP). Chromatin Immunoprecipitation is a technique used to study protein-DNA interactions. Briefly, proteins bound to DNA are cross-linked via formaldehyde and the DNA-protein complexes are sonicated to generate small fragments. Next, an antibody specific to the protein of interest is used to immunoprecipitate it while bound to DNA. Proteinase K is used to liberate the protein from DNA, and the DNA fraction is purified. Various methods, such as qPCR, are used to amplify DNA sequences that were bound to the protein of interest. To ensure successful detection of enriched DNA fragments bound by protein(s) of interest, ChIP requires optimization at the following steps: cross linking, cell lysis, sonication, immunoprecipitation (IP), and DNA quantification. Using the LLC-PK1 (PK1) cell line, we sought to optimize ChIP and tested the variables listed in Table 1 using an antibody for Tata Binding Protein (TBP).

Using western blot analysis, we validated the TBP-XP antibody (Rabbit mAb #44059; Cell Signaling Technology, Danvers, MA, USA) in porcine kidney extract, fixed PK1 cells, and unfixed PK1 cells. Our results indicated the TBP-XP antibody successfully detected porcine TBP, as determined by the presence of a singular 40 kDa band in tissue and cell extracts. Excessive cross-linking of cells can mask the availability of epitopes and induce resistance to cell lysis. We then optimized sonication efficiency with cells that were subjected to various cross-linking times and cell lysis buffers using 3 different sonicatiors: probe sonicator, ultrasonic cleaner (Vevor), or Biorupter NGS water bath sonicator (Diagenode, Denville, NJ. Sonication

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efficiency was evaluated by agarose gel electrophoresis of purified DNA. We found the following conditions consistently generated 200-800bp DNA fragments in PK1 cells:

1. Crosslinking: formaldehyde (Methanol-free) for 3 minutes
2. Cell Lysis: 1% SDS Lysis buffer with nuclei isolation via Dounce homogenization
3. Sonication: Biorupter NGS (Diagenode) using high power output for 10 cycles (30s on/45s off)

After establishing a sonication protocol, we sought to optimize a method for immunoprecipitation (IP). Sonicated DNA (3-10 µg) was incubated with 3-5 µg of TBP-XP, Normal Rabbit IgG (Cell Signaling; #2729), or p-Histone H3 (Ser 10) (sc-8656-R; Santa Cruz Biotechnology, Dallas, TX, USA) antibodies. Protein/DNA complexes were immunoprecipitated using Magna ChIP Protein A+G Magnetic Beads **(**EMD Millipore; Burlington, MA, USA). Following immunoprecipitation chromatin crosslinks were reversed and enriched DNA was purified and analyzed via PCR. Successful immunoprecipitation was evaluated by agarose gel electrophoresis of PCR amplified DNA. We found high background in the isotype control, as indicated by PCR amplification at negative, positive, and target loci, regardless of the input mass of DNA and antibody. To reduce background, we precleared the magnetic beads before IP and added additional wash steps after IP. We also tested a range of IP incubation times and IP buffers. Ultimately, we were unable to yield consistent amplification of TBP above background. ChIP signal strength is dependent epitope accessibility, protein quantity at a locus, and antibody:epitope interaction. To enhance ChIP signal strength, we suggest increasing the mass of antibody and DNA in the IP reaction. We also suggest testing longer fixation times, as transcription factors require longer fixation times than histones. To decrease background, we suggest including a blocking reagent such as, glycogen, BSA or yeast tRNA, in the IP reaction.

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Table 1. Variables tested for ChIP Troubleshooting

Optimization Step	Variables Tested
Cross-linking	Cross linking agent • 16% Formaldehyde (Methanol-free) • 37% Formaldehyde solution (contains 10-15% Methanol as stabilizer) Cross linking time • 1-10 minutes
Cell Lysis	Lysis Buffer • 0.1% SDS Lysis buffer • 1% SDS Lysis buffer • 5% SDS Lysis buffer • 10% SDS Lysis buffer • Radioimmunoprecipitation assay buffer (RIPA) Buffer Nuclei Isolation • Dounce Homogenizer
Sonication	Sonication Device • Ultrasonic Cleaner (Vevor) • Biorupter NGS (Diagenode, Denville, NJ) • Probe Sonicator Time • 1-120 minutes Cycles • 1-30 (30s on/45s off)
Immunoprecitation	Preclearing magnetic beads Antibody mass • 3-5 µg DNA mass • 3-10 µg Washing • 1-2 washes Immunoprecipitation time • 4-24 hours Immunoprecipitation buffer • RIPA Buffer • Dilution buffer
DNA Quantification	Primer design & optimization

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ChIP METHOD

1. Preparing cells for ChIP

Seed ~10^7 cells in a 100mm dish containing 10 mL growth medium. Include one extra plate of cells for estimating cell number. Cells should be >90% confluent (or at least 20 million cells) at time of harvest for ChIP. Prior to cross linking, trypsinize and count cells on the control plate.

2. Cross-Linking & Cell harvesting

Cross-link proteins to DNA by adding formaldehyde (methanol free) dropwise directly to media to a final concentration of 1% for 5 minutes while shaking. Stop cross liking reaction by adding glycine to a final concentration of 125 mM. Incubate with shaking for 5 min at RT. Place cells on ice. Rinse cells 2x with 10 mL ice cold 1X PBS. Scrape dishes thoroughly with a cell scraper and transfer to a 15ml conical tube and centrifuge for 5 min, 4°C, 1000 x g. Discard supernatant.

3. Lyse, Nuclei Isolation & Sonication

Resuspend cell pellet in 1 mL SDS Lysis Buffer by pipetting up and down and vortexing. Incubate for 10 min on ice. Dounce homogenize 30 times. Spin at 5000 rpm for 15 min to pellet nuclei. Discard supernatant. Resuspend pellet in 0.6 mL 1% SDS Lysis Buffer and incubate on ice for 20 minutes. Sonicate lysate in an ultrasonic bath to shear DNA to a fragment size of 300-1000 bp. Pellet cell debris via centrifugation for 10 min, 4°C, 8,000 g. Transfer supernatant to a new 1.5mL tube. Remove 50 µl of each sonicated sample to determine its DNA concentration and fragment size.

4. Determination of DNA concentration and fragment size

Add 70 µl of elution buffer to the 50 µl of chromatin. Add 4.8 µL of 5 M NaCl and 2 µL RNase A (10 mg/mL) and incubate while shaking at 65°C overnight. Add 2 µL proteinase K (20 mg/mL) and incubate while shaking at 60°C for 1h. Purify DNA using a PCR purification kit.

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Determine the DNA concentration by reading the OD260. Run purified DNA on a 1.5% agarose gel to determine fragment size.

5. Immunoprecipitation using Magna ChIP™ Protein A+G Magnetic Beads

Preclear Magna ChIP beads (EMD Millipore; Burlington, MA, USA): Wash 20 uL of fully resuspended bead slurry with 1mL dilution buffer 3 times. Pellet Protein A/G magnetic beads with the magnetic separator and remove the supernatant completely. Resuspend beads in 1mL dilution buffer. Incubate overnight at 4ºC. Pellet Protein A/G magnetic beads with the magnetic separator and remove the supernatant completely. Wash beads twice with 1 mL dilution buffer. Beads are now ready to use for the IP reaction.

For each IP reaction, prepare a microcentrifuge tube containing 10 µg of sheared cross-linked chromatin. If chromatin has been previously frozen, thaw on ice. Add IP buffer into each tube to make up to 500 µL. Remove 1% of chromatin to serve as your input sample and store at -20°C until further use. Add 20 uL of resuspended protein A/G magnetic beads to chromatin, then Add 1-10 µg of antibody. Incubate O/N at 4°C with rotation. Pellet Protein A/G magnetic beads with the magnetic separator and remove the supernatant completely. Wash the Protein A/G bead-antibody/chromatin complex by resuspending the beads in 0.5 mL each of the cold buffers in the order listed below and incubating for 3-5 minutes on a rotating platform between each wash followed by magnetic separation and careful removal of the supernatant fraction: (1) Low Salt Wash Buffer (2) High Salt Wash Buffer (3) LiCl Wash Buffer (4) TE Buffer.

6. Elution of Protein/DNA Complexes and Reversal of Cross-links of Protein/DNA Complexes to Free DNA

Add final elution buffer + Proteinase K (10µL/mL) to all IP and Input tubes. Incubate at 62°C for 2 hours with shaking. Incubate at 95°C for 10 minutes. Cool the samples down to room

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temperature. Separate beads using a magnet separation device. Carefully remove and transfer the supernatant to a new tube. Purify DNA using a Wizard SV Gel & PCR clean up kit. Analyze via PCR.

`

MATERIALS

Antibody

TBP-XP (Rabbit mAb #44059; Cell Signaling Technology, Danvers, MA, USA)

1% SDS Lysis Buffer

1% SDS

10 mM EDTA

50 mM Tris pH 8.1

ChIP Lysis Buffer

50 mM HEPES-KOH pH7.5

140 mM NaCl

1 mM EDTA pH8

1% Triton X-100

0.1% Sodium Deoxycholate

0.1% SDS

Protease inhibitors (add fresh each time)

RIPA Buffer

50 mM Tris-HCl pH8

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150 mM NaCl

2 mM EDTA pH8

1% NP-40

0.5% Sodium Deoxycholate

0.1% SDS

Protease Inhibitors (add fresh each time)

Low Salt Wash Buffer

0.1% SDS

1% Triton X-100

2 mM EDTA

20 mM Tris-HCl pH 8.0

150 mM NaCl

High Salt Wash Buffer

0.1% SDS

1% Triton X-100

2 mM EDTA

`

20 mM Tris-HCl pH 8.0

500 mM NaCl

LiCl Wash Buffer

0.25 M LiCl

1% NP-40

1% Sodium Deoxycholate

1 mM EDTA

10 mM Tris-HCl pH 8.0

TE Buffer

10 mM Tris pH 8.0

1 mM EDTA

Elution Buffer

1% SDS

100mM NaHCO3

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Dilution buffer:

0.01% SDS

1.1% Triton X-100

16.7mM Tris-Hcl pH 8.0

1.2 mM EDTA

167mM NaCl

ddH2O

Protease inhibitors (add fresh each time)