

The role of relaxin family receptor 1 and 2 (RXFP1 and RXFP2) in gender- specific physiology and pathology

Waleed Fahad Alhujaili

Supervisors

Dr. Ravinder Anand-Ivell

Dr. Richard Ivell

2021

Abstract

In most female mammals the peptide hormone relaxin plays an important role in reproductive physiologies, such as decidualisation, lactation, implantation, birth, uterine vascularization, and uterine quiescence, as well as maintaining systemic osmolarity during pregnancy. Both relaxin and the structurally related hormone INSL3 (insulin-like peptide 3) are specific ligands for the large GPCR receptors RXFP1 and RXFP2, respectively. At a low physiological nanomolar range, both peptide hormones can bind and activate only their cognate receptors, though in humans, relaxin may interact with RXFP2 at the high supra-physiological concentration.

In ruminants, such as sheep or cows, the gene for relaxin has been deleted during recent evolution, though the receptors RXFP1 and RXFP2 appear to be still present. The aim of this research was to determine whether these receptors were normally expressed in the ruminant uterus, whether they were still functional in a physiological context, and therefore whether the development of relaxin analogues as a potential therapeutic agent for cows and sheep would be a plausible option.

Firstly, the expression of gene transcripts for the ruminant RXFP1 and RXFP2 receptors was assessed in different uterine cells. It was found that RXFP1 was expressed in a sheep glandular epithelial cell-line (OGE), as well as in primary cultures of bovine uterine stromal and myometrial cells. Additionally, RXFP1 appears to be weakly expressed in bovine endometrial epithelial cells. RXFP2 gene transcripts were expressed at low levels in bovine myometrial cells and ovine stromal cells.

As receptor signalling end-point, cAMP production by uterine cells was assessed. cAMP production was increased by 100ng/ml relaxin in the presence or absence of IBMX or forskolin in bovine stromal cells. However, cAMP production was inhibited by relaxin in the OGE cell-line and in primary bovine myometrial cells. Also, cAMP production was inhibited by INSL3 in bovine myometrial cells and the ovine stromal cell-line (OST).

We also tested the small molecule agonist ML290 and its analogues, which had been shown to activate human RXFP1 in an allosteric manner. None of the ML290-related compounds were found to have any effect on cAMP production at the bovine RXFP1 receptor.

As alternative end-points to test for relaxin functionality at its specific receptor RXFP1, specific gene expression was assessed in the sheep glandular epithelial (OGE) cell-line. Genes were chosen which are involved in the maternal recognition of pregnancy, using relaxin, oxytocin (OT) and interferon-t (IFN-tau) as effectors. It was found that the expression of the oxytocin receptor (OTR) at 8 hours and the progesterone receptor (PR) at 24 hours were significantly inhibited by 100nM relaxin. There were also significant effects in synergy with IFN-tau and/or OT on the expression of PDGS2, AKR1A1, and ERα. Similar indicator genes were also assessed for expression in response to relaxin in primary cultures of myometrial smooth muscle cells. Effects were seen on both OTR (stimulatory) and ERβ (inhibitory) gene expression. No other genes tested appeared to be influenced by relaxin, though could be affected by either IFN-tau or OT.

In order to determine further relaxin-dependent end-points that might be important in the context of uterine physiology, archived RNA samples from mouse and human were utilized which derived from previous experiments in the Anand-Ivell laboratory. These samples were re-analyzed using either modern RNA-Seq technology or microarray analysis in order to obtain a global profile of transcriptome changes consequent upon relaxin stimulation. Although the species and

experimental paradigms were quite different, there were also marked similarities in the signaling pathways that were activated by relaxin. In particular, pathways associated with cytokine production, inflammation, as well as the cell cycle were significantly altered. Future research in the bovine should additionally focus on gene products typical for these pathways.

Altogether, in spite of the absence of the natural hormone relaxin from sheep and cows, my research has indicated that numerous cells and signaling pathways within the ruminant uterus can still be influenced by exogenous hormone. This opens up the possibility in the future of developing relaxin analogues as potential therapeutic agents in the treatment of uterine dysfunction in sheep and cows. Unfortunately, because of the Covid-19 epidemic, my research had to be truncated and further experimentation restricted.

Acknowledgments

This research has been a long adventure but I am grateful for the learning experience and the skills I have gained throughout the process. As I come to the completion of this research, a product of many days of research, I would like to recognize all the people who inspired and guided me through the project.

First, I would like to send my gratitude to my supervisors: Dr Ravinder Anand-Ivell and Dr Richard Ivell. Since the day they were assigned to me, they have been valuable guides throughout the research. I never forget their support to me through the encouragement I kept receiving throughout my work. From them, I witnessed and gained some incredible work ethic and laboratory skills. I cannot forget all the enjoyable moments we had as we engaged in detailed and illuminating discussions at each research stage. Their generosity, time, and help have led to my research completion, which was once a far-fetched thought.

I would like to recognize and appreciate Yanzhenzi Dai, Kiichiro Furuya, Hiroaki Soyama, Jonas Arnecke, Rieke Pohl, Valentin Wegner and Laura Blum, my colleagues. They assisted me in my research. The journey

could not have been this easy if it were not for their help and companionship.

I want to convey my special appreciation to my sponsors; The Saudi cultural bureau in London and Taibah University. The research was a lot of work, financially and resource draining. It would not have been a success if it was not for their continued financial support and well wishes.

Lastly, I would like to send my gratitude to my family; they have been extremely understanding to the extent of exempting me from most chores to concentrate on the study. They encouraged me to pursue my goals each day at whatever cost; they always prayed for me. For this reason, I dedicate this research to them.

List of Abbreviation

AC	adenylyl cyclase
AC2	adenylyl cyclase 2
AKAP79	A-kinase-anchoring protein 79
cAMP	cyclic 3’-5’ adenosine monophosphate
COX-2	cyclooxygenase-2
EPC	endometrial epithelial cells
EPIL	early placenta insulin-like
ESC	endometrial stromal cells
FSHR	follicle-stimulating hormone receptor
GPCRs	G-protein-coupled receptors
GR	glucocorticoid receptor
IFN-tau	bovine interferon-tau
IGF	insulin-like growth factor
LGR	leucine-rich repeat-containing G protein-coupled receptors
LRR	leucine-rich repeats
LH	luteinizing hormone
LHR	luteinising hormone receptor
MAPKs	protein kinases
NO	nitric oxide
NOS III	nitric oxide synthase III
OGE	ovine glandular epithelial cells
OLE	ovine luminal epithelial cells
OST	ovine endometrial stromal cells
PCOS	polycystic ovarian syndrome

PKA	protein Kinase A
PKCζ	protein Kinase C Zeta
RLF	relaxin-like factor
RXFP1	Relaxin Family Peptide Receptor 1
RXFP2	Relaxin Family Peptide Receptor 2
TAE	tris-acetate-EDTA
TSH	thyroid-Stimulating Hormone Receptor
OT	oxytocin
OTR	oxytocin receptor
PR	progesterone receptor
ERβ	estrogen receptor beta
ERα	estrogen receptor alpha
AR	androgen receptor
VEGF	vascular endothelial growth factor
PCOS	polycystic ovarian syndrome
MRP	maternal recognition of pregnancy
PGF2a	prostaglandin F2alpha
IBMX	isobutylmethylxanthine
PCR	polymerase chain reaction
FCS	foetal calf serum
DMEM	Dulbecco’s modified Eagle medium
Pen/strep	penicillin-streptomycin
DPBS	Dulbecco's phosphate-buffered saline
EtOH	ethanol
HBSS	Hanks’ balanced salt solution
DMSO	dimethyl sulfoxide
cDNA	complementary DNA
TRFIA	time-resolved fluorescence immunoassay

NIPA	Nottingham integrated pathway analysis
Myo	myometrial cell
hRLN	human relaxin
pRLN	porcine relaxin
rhRLN	recombinant human H2 relaxin
hINSL3	human insulin-like 3
bINSL3	bovine insulin-like 3
AKR1A1	aldoketo-reductase 1A1
AKR1B1	aldoketo-reductase 1B1
IFNAR	interferon A receptor
OPN	osteopontin
P4	progesterone
PGE2	prostaglandin E2
8Br-cAMP	8bromo-adenosine 3’,5’-cyclic monophosphate

Table of content

Abstract ..... *I*

Acknowledgments ..... *V*

List of Abbreviation ..... *VII*

Chapter1: Introduction *14*

1. Introduction *15*

1.1 Signalling pathways of RXFP1 18

1.2 Relaxin and the endometrium 23

1.3 Effect of Relaxin on ovarian function 24

1.4 Effect of Relaxin on the early embryo 25

1.5 Effect of Relaxin on the cardiovascular system 26

1.6 Effect of Relaxin on the immune system 27

1.7 INSL3 28

1.7.1 The Structure of INSL3 30

1.8 The signaling mechanism of INSL3 and RXFP2 31

1.9 Maternal recognition of pregnancy (MRP) 33

1.10. Hypothesis and Objectives *36*

1.10.1 Hypothesis 38

1.10.2 Objectives 38

Chapter2: Materials and Methods *41*

2.1 Primary bovine tissue and cell culture 42

2.2 Primary bovine endometrial stromal cell isolation and culturing 43

2.3 Primary bovine myometrial cell isolation and culturing 45

3.4.1 Starting a new culture from frozen cells. 46

2.4.2 Secondary cells and media used 47

2.4.3 HEK293T cells 47

2.4.3.1 Transfection of HEK 293T cells 48

2.5 Stimulations 49

2.6 Total cAMP production: 50

2.7 Gene expression studies 52

2.7.1 RNA extraction 52

2.7.2 RNA quantitation 54

2. 7.3 RNA quality check 54

2. 7.4 Synthesis of complementary DNA (cDNA) 55

2.7.5 Primer selection and preparation 55

2.7.6 Agarose gel electrophoresis for PCR products 59

2.7.7 Normalization of real-time RT-PCR gene expression 60

2.8 Time-resovled fluorescent immunoassay (TRFIA).....61

2.9 RNA Seq experiments.....65
2.9.1 In vivo mouse uterus subject to relaxin- or vehicle treatment 65
2.9.2 NIPA analysis of unpublished microarray results available for human primary endometrial stromal cells treated or not with relaxin.....68

2.10 Statistical analysis 69

Chapter 3: Expression of the ruminant RXFP1 and RXFP2 receptors in uterine cells. 70

3.1 Introduction 71

3.2 Results 76
3.2.1Expression of RXFP1, RXFP2 in Secondary cell-lines 76
3.2.2Expression of RXFP1, RXFP2 in bovine primary cell cultures 80
3.2.3Expression of RXFP1, RXFP2 in bovine primary myometrial cell cultures.....84

3.3 Discussion 87

Chapter 4: cAMP production in diverse uterine cells treated with Relaxin or INSL3 91

4.1 Introduction.....92
4.1.1 Signaling pathways of RXFP1.....92

4.2 Results.....94
4.2.1 cAMP production in bovine endometrial stromal cells (ESC).....95
4.2.2cAMP production in the ovine glandular epithelial (OGE) cell-line 97
4.2.3Effect of INSL3 on cAMP production by the ovine stromal (OST) cell-line.....99
4.2.4cAMP production in bovine primary myometrial cells.....101

4.3 Discussion 103

Chapter 5: Effect of the small molecule RXFP1 agonists, ML290 and analogues, on transfected human and bovine receptors 106

5.1 Introduction 107

5.2 Results 112

5.2.1 Dose-response experiments for human recombinant relaxin in HEK293T cells permanently transfected with a human RXFP1 expression construct or transiently transfected with a bovine RXFP1 expression construct.....112

5.2.2. Dose-response experiments for ML 290 in HEK293T cells permanently transfected with a human RXFP1 expression construct or transiently transfected with a bovine RXFP1 expression construct. 114

5.3 Discussion 116

Chapter 6: Effect of relaxin and relaxin-like peptides on gene expression by the Ovine Glandular Epithelial (OGE) cell-line and primary bovine myometrial cells. 117

6.1 Introduction.....118

6.1.1 The intracellular signal pathways in ruminant endometrial epithelial cells (and their surrogate OGE cells) involved in the maternal recognition of pregnancy 118

6.1.2 Intracellular signaling pathways in ruminant myometrial cells.....122

6.2 Results 126

__6.2.1 Effect of relaxin on gene expression by the Ovine Glandular Epithelial (OGE) cell-line. 126

__6.2.2 Time course study of specific gene expression in OGE cells under the influence of relaxin and other effectors. 128

__6.2.3 Assessment in cultures of OGE cells of the functionality of Relaxin at its specific receptor RXFP1..... 131

__6.2.4 Effect of relaxin-like peptides and other effectors on myometrial cell gene expression..... 134

6.3 Discussion 140

Chapter 7: Application of global transcript analysis to determine novel relaxin targets in the uterus. 144

7.1 Introduction 145

7.2 Results 149

7.2.1Significantly altered transcript pathways in mouse uterus subjected *in vivo* to relaxin. 149

7.2.2Significantly altered transcript pathways in human endometrial stromal cells in response to relaxin. 152

7.3 Discussion 154

Chapter 8: Discussion. 156

8. Discussion..... 157

8.1 Expression of RXFP1 and RXFP2 in uterine cells and tissues 158

8.2 cAMP production in diverse uterine cells 161

8.3 ML290 and analogues effects on transfected human and bovine receptors 163

8.4 The search for alternative relaxin end-points, applying specific gene expression analysis. 165

8.5 Application of global transcript analysis to determine novel relaxin target genes in the uterus. 168

References 177

Chapter1: Introduction

1 Introduction

Relaxin was first discovered by Hisaw as a small peptide hormone in 1926 (Bathgate et al., 2013). Derived from the corpus luteum of pregnant guinea pigs, it was able to induce the widening of the pubic symphysis and cervical softening associated with birth in non-pregnant animals. In 1983, the first human relaxin gene transcript sequence was revealed, since referred to as H1-relaxin (Hudson et al., 1983). In the following year, a second, H2-relaxin gene was discovered. Most mammals only have one relaxin gene, equivalent to the human H2-relaxin, but all higher primates (including humans) have two separate genes: RLN1 encodes H1-relaxin and the RLN2 gene encodes H2-relaxin. It is believed that the second gene in higher primates is the result of a recent gene duplication event. H2-relaxin performs the same functions as the main relaxin product in mammals other than higher primates, whereas H1-relaxin appears to be restricted to the placenta and may have local growth promoting effects. Relaxin-3 was recently discovered in humans and other animals by genome mining (Bathgate et al., 2002) and later shown to be expressed mostly in the brain.

For a long time there has been a discussion encompassing the presence or not of relaxin in ruminants, including dairy cattle, sheep, and goats. It was found that a gene corresponding to RLN2 is expressed at the

mRNA and/or protein levels in related ungulates, such as pigs, camels, llamas and horses (James et al., 1977; Bravo et al., 1996; Stewart et al., 1991; Hombach-Klonisch et al., 2000). However, molecular techniques were used to search extensively but could not identify related transcripts in ruminants, except for a partially deleted sequence representing part of the A-chain in sheep (Roche et al., 1993). Demonstrating the absence of a gene within a large genomic sequence is difficult, although there now seems to be unanimity, because chromosomal synteny studies have found a substantial deletion encompassing the RLN2 locus in the bovine genome on chromosome 8 (Dai et al., 2017a). In ruminants, protein studies had been confused and it was later found that some antibodies that were raised against porcine relaxin and thought to be highly specific could be cross-reacting with some other possibly related peptide (Ivell et al., 2010).

The insulin/relaxin superfamily comprises ten peptide hormones, among which are insulin and the insulin-like growth factors (IGF) 1 and 2. Within this superfamily of structurally related peptides, the relaxin family of peptide hormones comprises seven members: H1-relaxin (Hudson et al., 1983), H2-relaxin (Hudson et al., 1984), relaxin-3 (Bathgate et al., 2002), insulin-like peptide 3 (INSL3), earlier called Leydig insulin-like peptide or relaxin-like factor (RLF) (Adham et al.,

1993), INSL4, also referred to as placentin or early placenta insulin-like (EPIL) (Chassin et al., 1995), INSL5 (Conklin et al., 1999), and INSL6 (Lok et al., 2000). These seven members are found on three separate chromosomes (Wilkinson et al., 2005). H1- and H2-relaxin are found close together along with INSL4 and INSL6 on human chromosome 9 at 9p24, suggesting their origin through local gene duplication. Meanwhile, relaxin-3 is found on human chromosome 19 at 19p13.3, and INSL3 is also located on human chromosome 19 at 19p13.2. INSL5 is found on human chromosome 1 at 1p31.1.

Peptides in the relaxin family are structurally alike and this is attributable to the existence of six cysteine residues that form three disulphide cross-links. There is one intra-chain disulphide bond in the A-chain and two inter-chain disulphide bonds between the A- and B-chains (Fig 1.1). Despite this structural similarity it has been now shown that, unlike insulin, IGF1 and IGF2, which act via specific tyrosine kinase receptors with single transmembrane domains, the remaining members of the family all act via G-protein-coupled receptors (GPCRs) with 7 transmembrane domains. Within this group of insulin/relaxin-like peptides RLN3 and INSL5 interact with GPCRs called RXFP3 and RXFP4 which have only a very short extracellular domain, whereas RLN1, RLN2 and INSL3 interact very specifically with two quite different GPCRs with

very large extracellular domains. These are named RXFP1 and RXFP2 and are the cognate receptors for relaxin and INSL3, respectively (Bathgate et al., 2006).

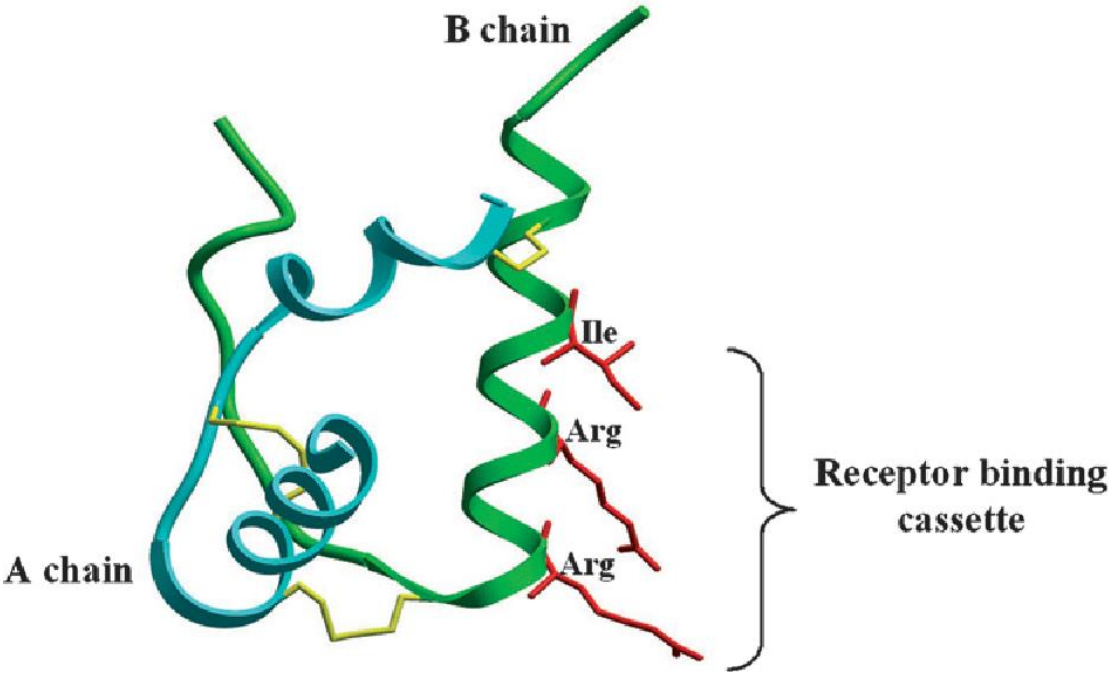


Figure 1.1 Human relaxin structure. A chain (blue) and B chain (green) linked by disulphide bonds (yellow) (Samuel et al., 2007).

1.1 Signalling pathways of RXFP1

When relaxin binds to the GPCR RXFP1, this brings about cell-type specific activation of multiple pathways (Kong et al., 2010). The different types of signalling achieved by these pathways include phosphorylation of mitogen activated protein kinases (MAPKs), activation of peroxisome proliferator-activated receptor gamma (PPARgamma), production of cyclic 3'-5' adenosine monophosphate (cAMP) and/or nitric oxide (NO), and engagement with the

glucocorticoid receptor (GR).

The area that has been analysed in greatest detail in the empirical literature is the elevation of intracellular and extracellular cAMP. The main signalling pathways triggered in cells expressing RXFP1 have also received considerable attention by researchers (Bartsch et al., 2001; Fei et al., 1990; Dai et al., 2017b). It has been established that heightened levels of cAMP can be biphasic. This requires the receptor to engage in as many as three different forms of G-protein coupling: $G_{\alpha s}$, $G_{\alpha oB}$ and G_{i_3} (Halls et al., 2006).

When RXFP1 interacts with G-proteins, this can occur in numerous different ways depending on the type of cell and the result could be any one of a wide selection of cellular responses. For instance, RXFP1 receptors artificially expressed in HEK293 cells, or naturally expressed in primary cultures of rat cardiac fibroblasts and the THP-1 cell line are able to combine with any one of three different G-protein pathways (Halls et al., 2009).

It is distinctly probable that cAMP may have a role to play in several different pathways because of the biphasic nature of the way in which it accumulates. The first stage of the activation process for RXFP1 takes between one and two minutes and starts when the ligand binds with the

receptor; the resulting cAMP increase is the result of Gαs being activated and GαoB being inhibited. After a period of between 10 and 20 minutes there is a delayed cAMP increase that is mediated by the βγ subunits of Gi3. In turn, this activates phosphatidyl inositol 3 kinase (PI3K) and protein kinase C zeta (PKCζ). The second stage of cAMP accumulation begins when the activated PKCζ then transfers to the cell membrane and itself stimulates adenylyl cyclase (AC) (Fig 1.2).

Protein kinase A (PKA) can also be activated by cAMP. PKA can then phosphorylate numerous signalling proteins. The ability of RXFP1 to trigger the Gi3-Gβγ- PI3K - PKCζ cAMP pathway is dependent on the final 10 amino acids of the receptor C terminus, and Arg(752) in particular. Furthermore, the ability of the receptor to link to a particular pathway is dictated by the localisation in the lipid raft microdomains (Halls et al., 2009) . For Gαs and GαoB (which are also downstream targets of cAMP), the cAMP-response element (CRE) that determines gene transcription is influenced by the pathway. However, nuclear factor kappaB (NF-κB) mediated gene transcription is only influenced by the Gi3 pathway and none of the other pathways (Halls et al., 2007) .

The signalling response of cAMP is also influenced by the concentration of relaxin that engages with RXFP1. RXFP1 combines with Gαs, GαoB and Gi3 and produces physiological actions. It is the nanomolar

concentration of relaxin that causes this to occur. Additional complexity of the relaxin-triggered cAMP signalling pathway can take place within signalosomes that only react to sub-picomolar concentrations of relaxin (Halls and Cooper, 2010).

A-kinase-anchoring protein 79 (AKAP79) is responsible for combining RXFP1 to adenylyl cyclase 2 (AC2). When the ultra-low concentration of relaxin binds to RXFP1, AC2 becomes activated, thereby creating cAMP. Crucially, when concentrations reach nanomolar levels or greater, relaxin triggers the classical signalling pathway which, in turn, results in cAMP production through Gαs, GαoB and Gi3.

Binding of relaxin to RXFP1 may also activate NO production, which can induce vasodilation via two different pathways. The first pathway occurs when relaxin binds to RXFP1 and activates PI3K. Activated PI3K stimulates Akt which results in the activation of endothelial nitric oxide synthase III (NOS III) via phosphorylation of Ser1179 (Fig 1.2). Another pathway involves increased cAMP levels in cells, which then activate PKA. Activated PKA can phosphorylate and inactivate the inhibitor subunit of transcription factor NF-κB, IkB-α, which leads to the translocation of NF-κB into the nucleus, and increases nitric oxide

synthase II (NOSII) gene expression (Nistri and Bani, 2003).

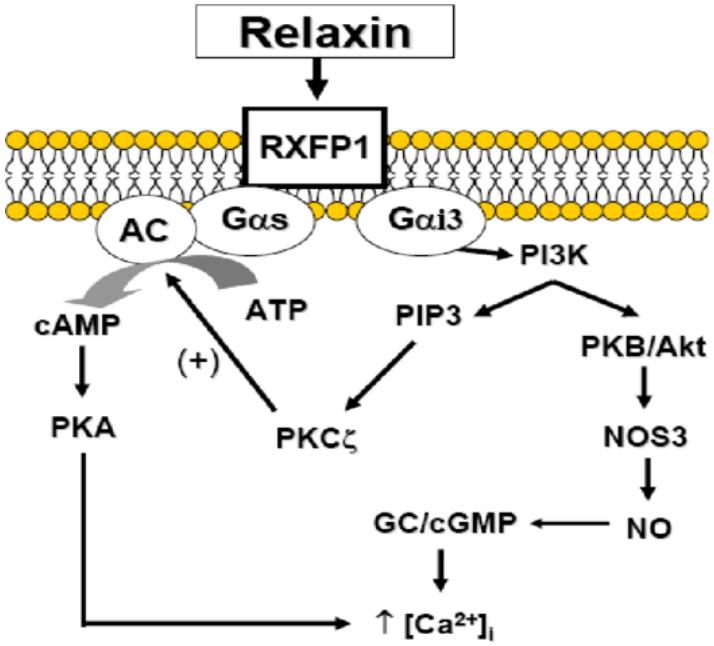


Figure 1.2 Signaling pathways activated by relaxin following the stimulation of the RXFP1 receptor (Tamargo et al., 2010).

1.2 Relaxin and the endometrium

During pregnancy in most mammals relaxin is produced by the corpus luteum of the ovary. Later in some species, relaxin is also made by the placenta itself and sometimes the endometrium. In rodents and pigs, relaxin production increases gradually from the first days of pregnancy until term, with circulating levels of relaxin reaching 100–200 ng/ml. On the other hand, in humans and primates the relaxin concentrations are very low, and reach maximally about 1 ng/ml (Anand-Ivell and Ivell, 2014), and that in the first half of pregnancy rather than at term.

In pregnancy, relaxin appears to target the uterus, where it interacts with RXFP1 receptors on endometrial epithelial cells, on endometrial stromal cells and in the myometrium (Ivell et al., 2007; Parry and Vodstrcil, 2007; Heng et al., 2008; Dai et al., 2017b). The human uterine endometrium needs to undergo the process of decidualization to be receptive and able to receive an implanting blastocyst. Decidualization takes about 10 days and normally occurs under progesterone and estradiol influence (Gellersen et al., 2007). In vitro decidualization of primary endometrial stromal cell cultures can also be induced by relaxin alone during 2–3 days (Ivell et al., 2007). In the monkey, independent studies have indicated that supplementary relaxin leads to a thickening of the endometrium, with increased vascularization

and reduced spontaneous abortion (Goldsmith et al., 2004a; Hayes et al., 2004; Einspanier et al., 2009). Other studies have shown in the human that there is a significant positive relationship between mid-pregnancy levels of relaxin in the blood, decreased pregnancy loss, and raised blood flow (Jauniaux et al., 1994; Anumba et al., 2009). In some species, such as the rat and pig, relaxin also affects the smooth muscle cells (myometrium) to cause quiescence (Anumba et al., 2009), thus limiting fetal loss due to unexpected spontaneous contractions. And in multiparous species such as the rat, it was found that exogenous relaxin, may be involved in the spacing of embryos within the uterus (Rogers et al., 1983).

1.3 Effect of Relaxin on ovarian function

In most mammals relaxin is produced by the corpus luteum and is necessary for pregnancy and parturition (Guico-Lamm and Sherwood, 1988) and throughout pregnancy relaxin has a vital role in the growing female tract (see section 1.2). The cells of the corpus luteum derive from follicle cells which undergo an epithelial-mesenchymal-transition (EMT) after ovulation. In cultured luteinizing human granulosa cells relaxin begins to be secreted after about 4-6 days of cell culture (Stewart and VandeVoort, 1997). In vivo, luteal relaxin production ceases at luteolysis and begins again with the new cycle. In the event

of pregnancy, when the corpus luteum is maintained, relaxin production persists. In the human and pig, the theca interna cells of antral follicles before the LH surge may also express the relaxin gene (Ohleth and Bagnell, 1999).

1.4 Effect of Relaxin on the early embryo

Under normal circumstances, when oocytes, cumulus-oocyte complexes, or the early embryo are exposed to relaxin, the blastocyst and the inner cell mass increase in their rate of development. Studies on the effects of relaxin on porcine and primate embryos, as well as on cryopreserved cat oocytes confirm this (VandeVoort et al., 2011; Luu et al., 2013). It is thought that these effects could be due to release of relaxin either by rupture of follicles or reduced circulating levels from the corpus luteum or both. It is also suggested that another possible source could be the oviduct (Tang and Chegini, 1995). It is not clear where exactly between the embryo or the trophoblast relaxin binds. However, there is evidence that relaxin influences the receptive endometrium (see section 1.2). Further, relaxin may facilitate the process of fertilization through positive effects on the spermatozoa (Ferlin et al., 2012) .

1.5 Effect of Relaxin on the cardiovascular system

Pregnancy is associated with numerous physiological changes in almost all systems of the body. One such change concerns the regulation of fluid volume and osmolality. As a result, there is an increase in secretion of vasopressin, heart stroke volume with marked vasodilation and readjustment of arterial compliance. Studies on animals, mainly rats, reveal that these physiological changes are due to the effects of relaxin in the body (Conrad, 2011). Studies now show that relaxin has a direct effect on the arterial smooth muscle cells and endothelial cells. In arterial smooth muscle cells, there is consequent production of endothelin, and metalloproteinases. The endothelin acts on the endothelin receptors of vessels with RXFPI coupling to P13-kinase and activation of eNOS (McGuane et al., 2011). In addition, relaxin has also been shown to induce the process of blood vessel formation that helps in wound healing, and in the regeneration of the endometrium (Unemori et al., 2000; Segal et al., 2012; Goldsmith et al., 2004b).The angiogenesis properties of relaxin are thought to be due to its inductive role on vascular endothelial growth factor (VEGF) as seen in endometrial stromal cells (Unemori et al., 1999). Whether relaxin has a negative or positive effect on the invasion of blood vessels into tumors as in prostate cancer is still being debated because of its role in encouraging extracellular matrix turnover (Klonisch et al., 2007).

1.6 Effect of Relaxin on the immune system

The human monocyte cell-line THP1 is one of the best cell models used to study relaxin signaling in cells which naturally express the RXFPI receptor (Anand-Ivell et al., 2007). The cells have been shown to have a biphasic relaxin-driven signaling mechanism. In the first few minutes, relaxin engages the Gs-dependent pathway with adenylyl cyclase being induced (Anand-Ivell et al., 2007; Ivell et al., 2007). After some few minutes, relaxin shifts to engage the PI3-kinase pathway and PKC-zeta translocation (Nguyen and Dessauer, 2005; Halls et al., 2007). In THP1 cells, as in human endometrial stromal cells, adenylyl cyclase activity is also dependent on a specific tyrosine kinase activity (Bartsch et al., 2001; Anand-Ivell et al., 2007). Not only the human THP1 monocyte phenotype is relaxin-responsive, but also the final macrophage phenotype, obtained after in vitro differentiation, is also relaxin-responsive. It is therefore very likely that relaxin is able to stimulate diverse immune cell types, including those in the endometrium (Piccinni et al., 1999; Piccinni, 2006). Studies have also demonstrated that relaxin plays a role in the activation of resident T cells into Th1-like effectors that ultimately produce interferon gamma (Piccinni et al., 1999). In the human, interferon gamma has a role in remodeling of the spiral arteries which temporarily carry blood in the endometrium within

the luteal phase (Monk et al., 2005). In experimental studies, relaxin has been shown to induce the production of pro-inflammatory cytokines such as CXCL1 and CXCL10 into the uterine lumen of mice (Anand-Ivell and Ivell, 2014). It can therefore be concluded that relaxin is also able to modulate pro-inflammatory cytokines, a process that is key to the implantation and placentation processes (Granot et al., 2012) .

1.7 INSL3

INSL3 and relaxin are structurally closely related, and this is why it was originally referred to as the relaxin-like factor (Büllesbach et al., 1999; Ivell, 1997). It is mainly secreted from the testicular Leydig cells in the adult male (Adham et al., 1993; Pusch et al., 1996) as well as in the fetus. Here its role is by activation of RXFP2 to cause the gubernacular bulb connecting the testis to the lower abdominal wall to thicken. This retains the testes in the lower abdomen at a time when the fetus is growing rapidly and the kidney and neighboring organs move relatively in an antero-dorsal direction. Thus the testes appear to change position from being perirenal to the inguinal region and to what is referred to as the transabdominal phase of testicular descent (Ivell et al., 2014). Expression of INSL3 in the female is much lower than in the male. In cows, monkeys and humans it has been shown to be predominantly secreted by the theca interna cells of ovarian antral follicles (Bamberger

et al., 1999; Hanna et al., 2010), and mRNA has also been detected in the corpus luteum (Bathgate et al., 1996; Balvers et al., 1998). In the bovine, low concentrations of LH stimulate theca cell primary cultures to secrete INSL3; however, high luteinizing concentrations of the gonadotropin are inhibitory (Bathgate et al., 1996; Bathgate et al., 1999).

Studies with mice in which the INSL3 gene had been disrupted indicated that INSL3 promotes the number of developing follicles and hence resulting ovulations and litter size (Spanel-Borowski et al., 2001). Immunohistochemical studies on bovine follicles suggest that INSL3 has an anti-apoptotic/pro-survival role during the development of antral follicles (Irving-Rodgers et al., 2002).

Even though the INSL3 concentration in the blood is low in women compared to men, it is quantifiable (ca. 0–100 pg/ml) and reflects the growth of waves of antral follicles (Anand-Ivell et al., 2013). In cows, the concentration of INSL3 in the blood is somewhat greater than in humans (Anand-Ivell et al., 2011; Satchell et al., 2013).

Circulating INSL3 concentration appears to be greater in women with diagnosed PCOS (polycystic ovarian syndrome), probably as a result of

the increased number of such follicles in affected women (Havelock et al., 2005; Gambineri et al., 2007; Anand-Ivell et al., 2013). Conversely, the INSL3 concentration in blood is reduced in women who have low ovarian reserve (Anand-Ivell et al., 2013).

1.7.1 The Structure of INSL3

This insulin-like molecule was first recognized using differential cloning techniques. It was identified as the product of a frequent testis-specific gene transcript which originated from rodents and pigs. It was also known as the Leydig-insulin or relaxin-like factor. A human sequence was identified by screening cDNA or genomic libraries where similar insulin-like sequences were found, as reported by Ivell and Anand-Ivell in 2009. There is a very short gene, in fact, less than one kilobyte in length, that encodes INSL3, located near or just within the adjacent JAK3 gene. The INSL3 promoter region also appears to be very short at around 100-1000bp. There is a single open reading frame which resembles the structure of the precursor proteins for relaxin and insulin with a B-domain region, a C-domain, and an A-domain region, altogether comprising approximately 180 amino acids. INSL3 was also suggested to be highly expressed in tissues like the prostate and in adult Leydig cells. It has also been found to exist in the female, mostly in the ovary, as well as in the hypothalamus of the cow.

1.8 The signaling mechanism of INSL3 and RXFP2

According to Halls and colleagues, INSL3, like relaxin, is responsible for inducing the production of cAMP through its activation of adenylyl cyclase in human embryonic kidney cells expressing the transfected RXFP2 receptor (Halls et al., 2006). Further, accumulation of cAMP also takes place after INSL3 stimulates rat gubernacular cells, which also express RXFP2, as also in human osteoblasts (Ferlin et al., 2009) (Fig 1.3). In contrast, RXFP2 appears to couple to Gi/Go proteins in oocytes or in primary cultures of testicular germ cells, resulting in a decrease of cAMP. Thus the cAMP response can be influenced by the type of cell and the pattern of expression of the relevant G proteins (Kong et al., 2010).

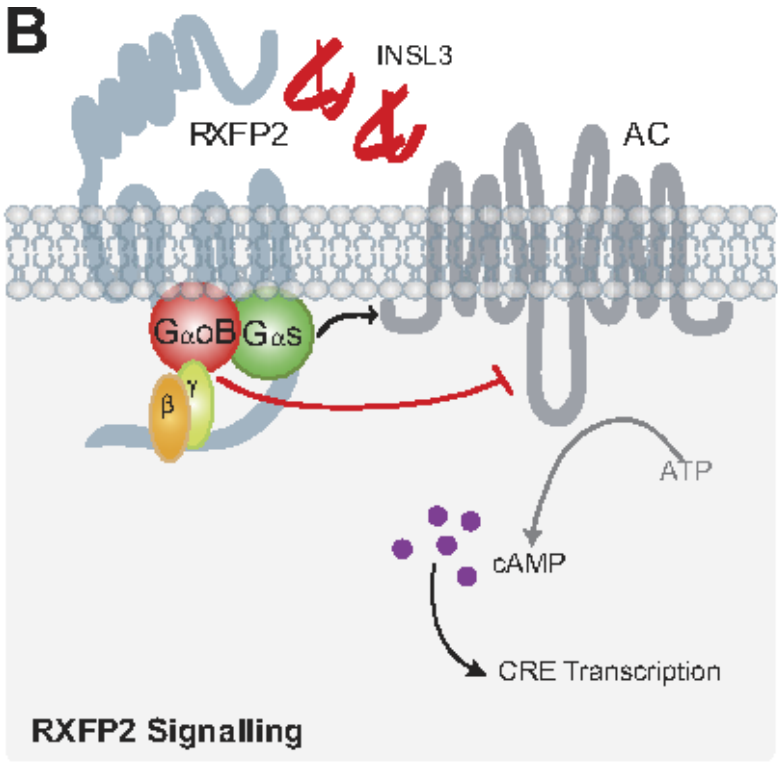


Figure 1.3 signalling pathway activated INSL3 following the stimulation of the RXFP2 receptor (Bathgate et al., 2012). AC, adenylyl cyclase; CRE, cAMP-responsive element.

1.9 Maternal recognition of pregnancy (MRP)

In all mammals a process has evolved by which maternal physiology is informed that pregnancy has occurred, and the maternal body must adapt to the changing conditions of carrying one or more embryos. This process is referred to as the maternal recognition of pregnancy. In ruminants such as cows or sheep this involves the conceptus releasing proteins, amongst which interferon-tau (IFN- tau) is the most prominent and essential for the establishment of pregnancy (Bowen and Burghardt, 2000). In the non-pregnant situation in cows, as the end of the natural estrous cycle approaches, the endometrium starts to produce PGF2α. In turn, this triggers the luteal cells to release oxytocin (OT). This in turn interacts with oxytocin receptors (OTR) on the endometrial epithelium leading to further release of PGF2α (Fuchs, 1987). In turn, increasing pulsatile PGF2α released from the endometrium causes the lysis of the corpus luteum (luteolysis) and thus the end of the estrous cycle, allowing the commencement of a new cycle, and causing the death of any embryos through a lack of luteal progesterone (Bowen and Burghardt, 2000). It is necessary for the new conceptus to release interferon-tau from about 14-20 days after fertilization. This causes a down-regulation of the endometrial OTR, and hence interrupts the OT-PGF2a luteolytic positive feedback loop (Bowen and Burghardt, 2000). The oxytocin receptors are relatively suppressed and pregnant cows are

less responsive to OT-induced PGF2α (Raheem, 2017). It is not fully understood precisely how IFN- tau functions. In sheep and possibly beef cows, though not in dairy cows, it has been suggested that IFN- tau suppresses the number of estradiol receptors and this has the effect of lowering estrogen-induced OTR numbers. Whether the estrogen receptors are also suppressed in all breeds of ruminant is less clear. For dairy cows, the Ivell group showed that IFN-tau induces inhibitory transcription factors in the endometrial epithelium which directly suppress OTR gene transcription (Telgmann et al., 2003). It has furthermore been shown in a number of research papers that IFN-τ also inhibits cyclooxygenase-2 (COX-2) which precedes the production of PGF2α. It is thought that the reduction in COX-2 occurs independently of any regulation of OTR (Xiao et al., 1999). There are fewer OTRs expressed in the endometrium as a result of IFN- tau but it is highly probable that there are other ways in which the embryo limits PGF2α. For instance, it has been shown that PGF2α synthesis in cows can be reduced by linoleic acid (Thatcher et al., 1994). It is the endometrium that releases this inhibitor. Arachidonic acid and linolenic acid compete for active sites on the cyclooxygenase enzyme. It is this enzyme that is primarily responsible for transforming arachidonic acid into precursors of PGF2α production (Xiao et al., 1999). The adverse effects caused by

PGF2α can also be restricted if interferon-tau can act on the oxytocin receptor gene.

These key events in the ruminant maternal recognition of pregnancy primarily involve the epithelial cells of the endometrium, which we know from other species such as rodents or humans may express the relaxin receptor RXFP1. It is logical therefore in the context of this study that I investigate whether exogenous relaxin is able to influence any of the pathways involved in ruminant maternal recognition of pregnancy and thus whether relaxin stimulation could offer a solution to problematic implantation in cows and sheep. Moreover, we know from other species that the concentration of relaxin (RLX) produced from the corpus luteum is high during diestrus and early pregnancy and that relaxin may play a role also in maintaining quiescence in the myometrium during this phase, all features suggesting an important physiological function during implantation. Furthermore, it has been highlighted that cows and sheep also produce very high amounts of INSL3 by similar cells of the corpus luteum and at a similar time to relaxin in pigs (Anand-Ivell et al., 2011; Bathgate et al., 1996) together suggesting that in ruminants INSL3 may be acting like relaxin.

1.10. Hypotheses and Objectives

It is evident that in most mammals the peptide hormone relaxin and the closely related peptide INSL3, acting through the structurally related GPCRs, RXFP1 and RXFP2, are able to exercise important physiological functions, particularly in regard to female reproduction. Especially in the context of the uterus, it is evident that relaxin plays important roles in early embryo development, implantation, angiogenesis and placental development, including endometrial differentiation and the specific immune response. And in the myometrium, relaxin is seen to act as a quiescent agent during pregnancy and to be possibly involved in embryo spacing. At term of pregnancy, relaxin is responsible for softening the cervix and allowing widening of the birth canal to facilitate labour and birth.

These functions of the relaxin-RXFP1 system are particularly prominent in ungulates, such as pigs. It was thus very surprising to discover that, relatively recently in evolutionary terms, the gene for relaxin had been deleted in ruminants such as sheep, goats and cows (Dai et al., 2017a). In this earlier study, the Anand-Ivell laboratory also showed that nevertheless the bovine RXFP1 and RXFP2 genes were still functional (Dai et al., 2017a). Compared to other species such as rodents or

humans whose functional affinities for the specific ligand, relaxin, were in the low nanomolar range (<1nM), the EC50 of porcine relaxin for the bovine RXFP1 (bRXFP1) was instead in the higher nanomolar range (~10nM). A similar EC50 was also observed for the INSL3 receptor which recognized porcine relaxin with an EC50 of ~10nM (Dai et al., 2017a). What this means is that the bovine receptor bRXFP1 could potentially be able to respond to high physiological levels of relaxin, and possibly also to INSL3.

This is important since ruminants are known to be subject to a relatively high incidence of dystocia (Mee, 2008), and dairy cows particularly show a high frequency of infertility largely due to issues relating to implantation failure (Walsh et al. 2011). Thus application exogenously of a pharmaceutical agent capable of addressing the bovine RXFP1 receptor might have considerable potential as a novel veterinary reagent to aid fertility and/or birth. Indeed, several decades ago it was shown that applying a crude porcine relaxin preparation to the cervix of pregnant cows was able to induce a significant amount of cervical softening (Perezgrovas and Anderson, 1982).

1.10.1 Hypothesis

The relaxin gene has been deleted in cattle and other ruminants, though its specific receptor appears to be functional. The hypothesis underpinning these studies is that exogenously applied relaxin or its analogues could act as new potential therapeutic agents to aid fertility particularly in the high-performance dairy cow. It is also possible that ruminant RXFP1-expressing cell systems could be making use of alternative signaling pathways and physiological endpoints, in some way compensating for the lack of endogenous relaxin.

1.10.2 Objectives

1. To confirm the expression of RXFP1 and RXFP2 in cells originating from sheep and/or bovine uterus.

2. To assess the functionality of RXFP1 and/or RXFP2 to generate cAMP in naturally expressing cells derived from ruminant uterus.

3. To test small non-peptide RXFP1 agonists on cloned and transfected bovine RXFP1 and RXFP2.

4. To assess alternative signaling pathways and physiological endpoints in ruminant RXFP1-expressing cell systems.

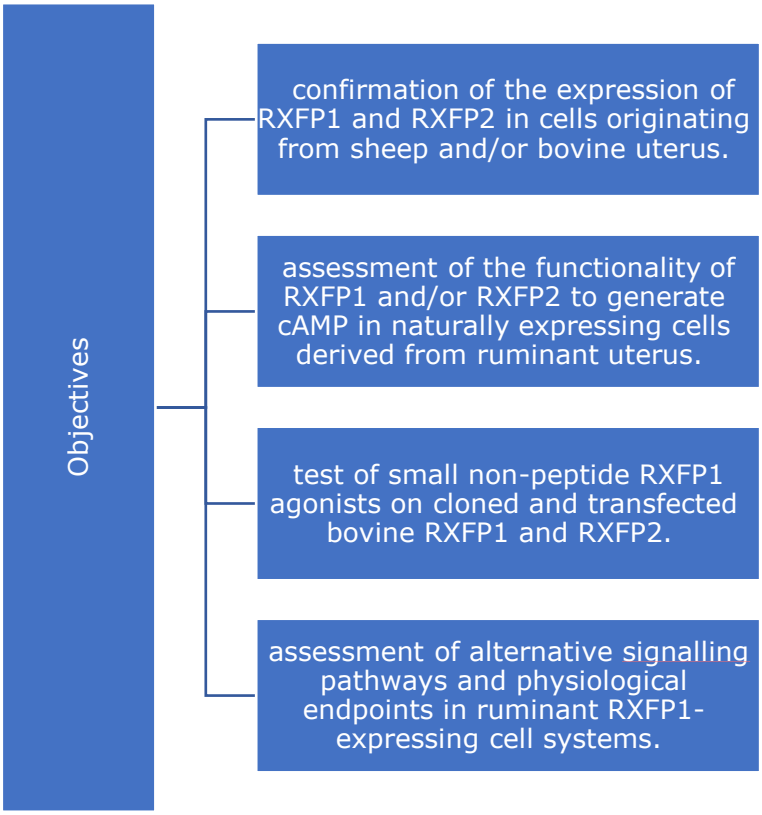


Figure 1.4 Schematic to indicate the objectives of this thesis.

Chapter 2: Materials and Methods

Chapter2: Materials and Methods

Note:
All reagents used were either from Thermo-Fisher Scientific (Paisley, UK), or from Sigma-Aldrich (Dorset, UK) unless otherwise indicated. The same batch of foetal calf serum (FCS) was used throughout, as a bulk purchase had already been tested for various cell culture systems in the laboratory, and it was shown to contain minimal cytokines, chemokines and steroids.

Plasticware/disposables: All purchased from Thermo-Fisher Scientific (Paisley, UK); these were either Nunc brand or Sarstedt.

All cell culture media and components were from Gibco (Thermo-Fisher Scientific) or Sigma-Aldrich (where indicated).

Various culture flasks or multi-well plates used and the volume of culture media per flask/well is indicated in the table below:

Table 2.1. Cell culture plasticware and culture volumes.

Type	**volume of media**	**approximate cells at seeding**
T25 flask	7ml	1 - 2 x 10^6 cells
T75 flask	13ml	2- 3 x 10^6 cells
T125 flask	20ml	3 - 6 x 10^6 cells
6-well plate	2ml	350,000/well
12-well plate	1ml	250,000/well
24-well plate	500μl	150,000/well
48-well plate	250μl	100,000/well,50,000/well or 6000/well (depending upon the cell type as indicated in later sections.)

Note: the appropriate cell number per well is determined by taking into consideration the cell type (how a cell presents its morphology), its growth rate, and the length of incubation for each experiment to avoid any overcrowding within a well.

2.1 Primary bovine tissue and cell culture

Intact bovine uteri, together with the respective ovaries, from healthy post-pubertal (22-28 month) Angus cows were collected from the local slaughterhouse. The uteri were transported to the laboratory on ice and tissue preparation was carried out within a few hours on the same day. Each uterus was briefly rinsed with 70% ethanol (EtOH; Thermo-Fisher Scientific, Loughborough, UK), followed by a rinse with wash-medium containing DPBS solution (Gibco, Thermo-Fisher Scientific) plus 1% Penicillin-Streptomycin (Pen-Strep; Invitrogen, Thermo-Fisher Scientific) and 1% amphotericin B (Sigma-Aldrich). The stage of the reproductive cycle was determined on the basis of ovarian morphology and size of the corpus luteum (Ireland et al, 1980). The horn ipsilateral to the corpus luteum was then selected and an incomplete transverse cut was made into the centre of the horn. The incision was made so as to expose the lumen of the uterus from the fundus towards the ovarian end. The last quarter of the uterus towards the ovary was not used.

2.2 Primary bovine endometrial stromal cell isolation and culturing

Endometrial tissue strips were peeled away from the myometrium and immersed into a 100ml container containing 60ml of wash-medium (DPBS + 1% Pen/Strep + 1% amphotericin B). Tissue from each cow was kept separate in labelled containers. The tissue was washed twice by picking up the tissue and transferring it to a second and third container with the same solution using sterile forceps. The final container was then brought from the dissection room to the cell culture laminar flow bench after carefully sterilizing the container and dissection tools. The tissue was kept moist in fresh wash medium, chopped into 1.5 to 2cm pieces in a petri dish and finally transferred into a 50ml tube containing 25ml of digestion medium containing Hanks' balanced salt solution (HBSS; Sigma-Aldrich), 12.5mg Collagenase II (Sigma-Aldrich), 25mg bovine serum albumin (BSA; Sigma-Aldrich) and 63μl of 4% DNAse I from bovine pancreas (Sigma-Aldrich). The volume in the tube did not exceed 40ml. Multiple tubes were made for material from each uterus. After closing, tube was placed horizontally in a shaking water bath at 37^{o}C and 150 oscillations/min for 1h. The tube was then wiped, sterilized and taken into the laminar flow. Following unit sedimentation (tube allowed to stand for 2-3min) the supernatant was strained through a 40μm mesh into a new 50 ml tube. The digestion was

stopped by diluting the supernatant 1:1 with stop solution containing HBSS and 10% FCS (PAA Laboratories, Thermo-Fisher Scientific) and equally distributed into 50ml tubes followed by centrifugation at 700xg for 7 min at room temperature. The resultant supernatant was discarded and the pellet rapidly subjected to hypotonic shock by resuspending in 5ml sterile water (Sigma-Aldrich), vortexed briefly for 10 sec and immediately supplemented with 25ml stop solution (HBSS + 10% FCS). This effectively removed contaminating red blood cells. The tubes were centrifuged again, and the resultant yellowish-white pellet resuspended in bovine endometrial cell culture medium containing RPMI 1640, 10% FCS, 1% Pen/Strep solution (final concentration of 50 IU/ml of penicillin and 50μg/ml of streptomycin), 2.5μg/ml of amphotericin B (Sigma-Aldrich). The cells were transferred into T75 flasks and incubated at 37°C, 5% CO_2 for 16-18h so that the endometrial stromal cells can attach. The supernatant with floating epithelial cells is then removed and media replaced with fresh cell culture medium. The cells started showing good morphology within the next 48h and formed an attached monolayer. The cells were passaged on the third day by trypsinizing. Briefly, the supernatant was aspirated, adherent cells were briefly washed with 5ml of DPBS, and then 2ml of 10% Trypsin-EDTA (Sigma-Aldrich) solution was used per T75 flask. The flask was placed in the incubator at 37°C for 2min and observed under an inverted microscope

to see if all the cells had detached. 15ml of culture medium (containing 10%FCS as FCS inhibits trypsin action) was added to stop the trypsin action and the cell suspension was aspirated into a 50ml tube. This was centrifuged for 3 mins at 1300rpm and at room temp; the supernatant was removed, and the resultant pellet suspended in fresh 9ml of culture medium. The cell suspension was then equally distributed into three new T75 flasks (1:3 split). The cells from these flasks were used for further experiments carried out in multi-well plates described later.

2.3 Primary bovine myometrial cell isolation and culturing

After removing the endometrial layer as described above, the myometrial tissue was washed in wash buffer (DPBS + 1% Pen/Strep + 1% amphotericin B) and the isolated tissue was then finely chopped into 3-5mm^3 pieces in a petri dish containing 5ml of digestion solution containing DMEM-HamF12 (1:1), 0.4mg/ml collagenase type 2 (Sigma-Aldrich), 10U/ml deoxyribonuclease type I (Sigma-Aldrich) and 1% amphotericin B. The petri dish with lid was then incubated at 37°C and 5% CO_2 for 13 hours. In between 1-2 times, the contents were mixed using 1ml pipette with a slightly cut tip. In order to stop the digestion reaction, after 13h of incubation, the contents of the petri dish were transferred to a 50ml tube and topped up with 20ml of myometrial culture-medium (1% amphotericin B, 2mM L-glutamine, 1% Pen/Strep

and 10% FCS in DMEM/F-12 (1:1) without phenol red). The resultant cell suspension was subjected to unit sedimentation as described above and the cell pellet resuspended in myometrial culture medium. This was then centrifuged at 1300rpm for 5min. The pellet was resuspended in fresh culture medium and the myometrial cells cultured in a T75 flask using myometrial culture medium (see Table 3.1 above). The cells attached and started showing elongated morphology within 24-36h. Once 80% confluency was reached, usually around 3 days, the cells were trypsinized and passaged. The cells from the 2^{nd} or 3^{rd} passage were used for further experiments.

2.4 Secondary cell culture

2.4.1 Starting a new culture from frozen cells.

Cells in a cryovial (1.5ml) which had been frozen at -80^{o}C were thawed for 2-3min in warm water bath at 37^{o}C and quickly resuspended in 15ml warm cell culture medium and centrifuged at 1300rpm for 3min. The supernatant was aspirated, and the resultant pellet resuspended in 13ml of fresh culture medium. The cells were suspended homogeneously using a 10ml pipette, pipetting gently up and down for 2-3 times. This suspension was then transferred to a T75 flask and incubated at 37^{o}C, 5% CO_2. After approximately 48h, when the cells reached 70-80% confluency, the cells were trypsinized as explained earlier using 1%

Tripsin-EDTA solution (0.05% Trypsin + 0.02% EDTA) and were either passaged further into cell culture flasks, used for experimentation by seeding in multi-well plates or frozen down for future use in freezing medium containing 10% DMSO.

2.4.2 Secondary cells and media used

Ovine Glandular Epithelial (OGE), Ovine Luminal Epithelial (OLE) and Ovine Stromal (OST) cell-lines:

The Ovine Glandular Epithelial (OGE), Ovine Luminal Epithelial (OLE) and Ovine Stromal (OST) cell-lines, derived by immortalisation from sheep endometrium, were a generous gift from Dr. Jo-Ann Fleming, Texas A&M University. The cells were cultured in a T75 flask following the procedures described in Telgmann et al. (2003). The culture medium used was Dulbecco's modified Eagle medium (DMEM) supplemented with Ham's F-12 (1:1), 2mM L-glutamine, 1% Pen/Strep and 10% FCS.

2.4.3 HEK293T cells

HEK293T basic cells were obtained from Public Health England, UK. HEK293T cells permanently overexpressing human RXFP1 (LGR7) or RXFP2 (LGR8) were provided by the Anand-Ivell lab. All these various cell types were cultured and maintained using DMEM/F12 (1:1) (without phenol red) with 10% FCS, 2mM L-Glutamine and 1% Pen-Strep.

The HEK293T cells permanently transfected with human RXFP1 or human RXFP2 were as previously described (Sudo et al., 2003b) and maintained in HEK293T cells culture-medium containing DMEM/F12 (1:1) (without phenol red) with additional 10% FBS, 2mM L-Glutamine and 1% Penicillin-Streptomycin (Gibco). Before stimulation, these cells were plated at 50,000 cells/well in a 48-well plate and cultured overnight at 37°C with 5% CO_2.

2.4.3.1 Transfection of HEK 293T cells

Basic HEK293T cells were used for the transient expression of bovine RXFP1 (bRXFP1) or bovine RXFP2 (bRXFP2). The cell culture media remained the same. The full length bovine RXFP1 and bovine RXFP2 sequences were chemically synthesized and inserted into the expression vector pcDNA3.1/Zeo (+) (custom gene synthesis by GeneArt and available in Anand-Ivell laboratory). These expression plasmids had been previously validated and used by a previous PhD student, Ms Yanzhenzi Dai. Aliquots of the same plasmids were used using the previously validated and published protocol (Dai et al., 2017b) making use of the Neon transfection system (Thermo-Fisher Scientific). Briefly, the plasmids were mixed with cells to a final ratio of 2µg DNA per 500,000 cells. The appropriate number of cells were first washed with DPBS (without Ca^{++} or Mg^{++}) and the resultant pellet was resuspended

in 110µl of R buffer (Neon system buffers, Thermo-Fisher Scientific). The plasmid was added, mixed briefly, and picked up into the special transfection pipette with a 100µl Neon Tip. This was then immersed in the tube containing electroporation buffer and fitted into the Neon equipment. A single pulse of 1300 volts for 30 milliseconds was then selected (this had been previously standardized for these cells and plasmids to give maximum transfection rate and minimal cell death). After each transfection, the resultant cell suspension in Buffer R was diluted in antibiotic-free HEK293T culture-medium at 50,000cells in 500µl volume per well (48-well plate). The cells were incubated overnight at 37^{o}C and 5% CO_2. Next day, the culture media was aspirated from all wells and fresh antibiotic-free cell culture medium was added along with appropriate stimulants as discussed below.

2.5 Stimulations

The primary bovine cells or the secondary cell-lines were seeded in different multi-well plates for different endpoints to be measured. After seeding, the cells were incubated at 37^{o}C and 5% CO_2 overnight so that they could attach and start showing their appropriate morphology.

The sources of various relaxin family peptides were: porcine relaxin (pRLN, 85% pure) a generous gift from Professor David Sherwood (University of Illinois, Urbana-Champan, USA); recombinant human H2

relaxin (rhRLN), human INSL3 (hINSL3), and bovine INSL3 (bINSL3), all generous gifts from our collaborator Professor John Wade (Florey Institute of Neuroscience, University of Melbourne, Australia). The relaxin small-molecule agonist and related compounds were generously provided by Professor Jonathan Fallowfield (University of Edinburgh). Oxytocin and interferon tau were obtained from Sigma-Aldrich. The following procedures were adopted for different end-point measurements:

2.6 Total cAMP production:

6000 cells per well were seeded into a 48-well plate for the primary bovine uterine myometrial cells and primary bovine endometrial stromal cell for 24-36h, The secondary cells (The Ovine Glandular Epithelial (OGE), Ovine Stromal (OST) cell-lines) were seeded at 100,000 cells per well in a 48-well plate overnight (approximately 14-16h) and differently transfected HEK293T cells were seeded at 50,000 cells per well in a 48-well plate overnight (approximately 14-16h) prior to stimulation. The culture medium per well was 500μl. Before starting stimulations, the culture medium was aspirated from all wells and replaced with 200μl fresh culture medium. Wherever the phosphodiesterase inhibitor 1mM IBMX (isobutylmethylxanthine, Sigma-Aldrich) pre-treatment was used: 25μl of 10mM IBMX was added

and an equal amount of culture medium (as vehicle) was added to the untreated well. The plates were then placed back in the incubator for 15min. 25μl of various relaxin family peptides or other stimulants (various antagonists) were added at a ten-fold concentration to give a 1x final concentration in a total volume of 250μl; these were added as indicated in the different experimental results. The plates were again incubated for further 20min. The reaction was stopped by adding 1.75ml cold absolute ethanol (EtOH) (Fisher Scientific) to each well. The total cAMP from the conditioned culturing medium and the cells was hence extracted using 87% EtOH (Heng et al., 2008). Briefly, the alcoholic extraction of cAMP was carried out by aspirating the contents of each well carefully into labelled 5ml test tubes. The tubes were covered with aluminium foil and stored for 1h in refrigerator at 4°C. The tubes were centrifuged at 2000*xg* for 45min, the liquid phase containing cAMP was then decanted carefully into labelled 5ml glass tubes (Sarstedt) and dried at 60°C using 180mbar vacuum for 90min with a 30% speed in a RapidVap Labconco evaporator (Labconco Corporation, Kansas, USA). The dried content in each tube was re-suspended in 500μl assay matrix comprising 50% of MEM buffer in deionised water (Sigma-Aldrich). This was then used to measure total (intracellular plus secreted) cAMP using an in-house highly sensitive and previously validated cAMP TRFIA (see section 2.8).

2.7 Gene expression studies

200,000 OGE cells, 150,000 bovine myometrial cells or 200,000 HEK293T cells were seeded in a 12-well plate, topped with 1ml culture medium and left to adhere, spread and grow for 16-24h in an incubator at 37^{o}C and 5% CO_2. The medium was then aspirated, and all stimulants were added to appropriate final concentration in fresh culture medium in each well. After the indicated times of 8, 24 or 48 hours the supernatant was aspirated. 500µl DPBS per well was used to wash each well once. 600µl Trizol (Ambion, Thermo-Fisher Scientific) was then added per well and the plate (with lid on) was placed on an orbital shaker for 10min to detach and lyse the cells. All Trizol-containing steps were carried out in a safety hood with fan. The lysed and detached cell suspension was the then aspirated carefully into labelled Eppendorf tubes and frozen down and stored at -80^{o}C until further steps for RNA extraction.

2.7.1 RNA extraction

Trizol-plus RNA isolation kit (Ambion, Thermo-Fisher Scientific) was used to extract total RNA. This method offered combining lysis capability of Trizol reagent and the convenient RNA isolation technology using the silica gel columns provided in the Purelink RNA mini kit.

Briefly, the frozen cell lysate was thawed in a refrigerator and allowed to attain room temp for 15min. The lysate was pipetted up and down several times to achieve homogeneity. 120μl chloroform was then added per 600μl of Trizol-containing lysate. The contents were mixed well and left for 5min at room temp. The tubes were then centrifuged at 12000xg for 10min. The upper aqueous phase was carefully transferred into a new labelled Eppendorf tube. An equal volume of isopropanol (Sigma-Aldrich) was added and the content mixed by vortex for 20sec. The tubes were left at room temp for 15min and then centrifuged at 12000xg at 4^{o}C for 10min.

500μl of the clear supernatant was transferred to the spin cartridge with collection tube and centrifuged at 12000xg at 4^{o}C for 10min. The flow-through in the collection tube was discarded. In order to concentrate the RNA, the content from two or three wells was pooled on to the same cartridge making sure to empty the collection tube. To the reinserted cartridge on top of the empty collection tube, 500μl of wash buffer I was then added and centrifuged at 12000xg at 4^{o}C for 5min. The flow-through was again discarded, 500μl of wash buffer II was then added and centrifugation carried out at 12000xg at 4^{o}C for 5min. This step was repeated twice to wash the silica membrane with its attached RNA. The cartridge was then placed back and the empty cartridge was centrifuged

for 20sec and left to dry in the cold centrifuge for 1min. A clean collection tube was then used and 30μl of RNAse free water (Takara-Clontech, Saint-Germain-en-Laye, France) was added. The cartridge with collection tube was centrifuged at 12000xg and 4°C for 5min. The cartridge was then discarded and the collection tube containing the eluted RNA was stored at -80°C for future use.

2.7.2 RNA quantitation

2μl of the eluted RNA sample was diluted with 98μl of 10mM sodium phosphate buffer (pH 7.0) in a tube kept on ice. The total RNA content was then measured using an Eppendorf Biophotometer. A ratio of OD_{260}/OD_{280} of between 1.8 and 2.0 indicated good quality of RNA. Alternately, 1μl of the isolated and eluted RNA sample was used directly on a Nanodrop spectrophotometer to quantify the total RNA content.

2.7.3 RNA quality check

0.5μg of the total RNA (calculated volume after quantitation) in water was mixed with RNA formaldehyde dye and loaded on to a 1% agarose gel containing 0.25% Guanidine thiocyanate and 1% Ethidium bromide (EtBr) in 1x TAE buffer (4.84g/L Tris Base, 0.12% Acetic acid, 0.2% 0.5M EDTA; pH 7.0). The RNA samples with dye were loaded and the electrophoresis was conducted using fresh 1x TAE buffer (pH 7.0). High

quality RNA should show as sharp EtBr stained 28s:18s ribosomal RNA bands in a 2:1 quantitative ratio.

2.7.4 Synthesis of complementary DNA (cDNA)

3μg of total RNA was used to synthesize cDNA using the Superscript III reverse transcriptase kit (Thermo-Fisher Scientific) and the manufacturer's protocol was followed. A sample of RNA was primed with oligo-dT (12-18) primer at 70°C for 5 minutes. It was then mixed with 0.5 volumes of 5x first strand buffer, 0.1 volume of 0.1M DTT, 0.5mM of dNTP mix (Sigma-Aldrich) and 200 units of Superscript III reverse transcriptase, to give a total volume to 25μl with RNAse-free water. The tubes were then placed at 42°C for 90min in a hybridization oven (Thermo-Fisher Scientific). After incubation, 75μl of RNAse-free water was added in each tube to raise the volume of cDNA to 100μl. The cDNA was then stored at -20°C until subsequent use for gene expression studies using semi-quantitative or quantitative real-time RT-PCR.

2.7.5 Primer selection and preparation

All primers were designed using Primer3 software and were double-checked for uniqueness with BLAST and for the exon placement (intron spanning) using the confirmed bovine gene databank (Genbank, NCBI).

They were further checked using BLAST for homology between bovine and ovine sequences. The primers were then validated with positive controls to give a single band of correct size and sequence and a final temperature and time profile for the PCR reaction (if using real-time RT-PCR) was determined. All primers were obtained from Eurofins genomics (Wolverhampton, UK) and were reconstituted to give 100nM concentration using RNAse-free water. For further use, these were further diluted to 10nM concentration with RNAse-free water.

Table 2.2. Oligonucleotide primers used for PCR reactions.

Primer number	Gene name	Gene number	Sequence (5' - 3')
503	Bovine RXFP1	XM_002694415	tgacatctcgtcctgtcttcc
504	Bovine RXFP1	XM_002694415	ggagaaccgaccaagcattc
505	Bovine RXFP1	XM_002694415	cgaggacaactgtggagacatc
506	Bovine RXFP1	XM_002694415	cagtcctcggaaagcatagaca
507	Bovine RXFP1	XM_002694415	gaagccgggtgtttttgaag
508	Bovine RXFP1	XM_002694415	gggcatgtgctgacaaagag
511	Bovine RXFP1	XM_002694415	ccgcttgcctgacaaatctc
512	Bovine RXFP1	XM_002694415	ggcttgaattgtctggattgg
513	Bovine RXFP1	XM_002694415	cccaatccagacaattcaagc
514	Bovine RXFP1	XM_002694415	ggcatgcagcttgttctcag
515	Bovine RXFP1	XM_002694415	ggagcccagatttattcagtgg
516	Bovine RXFP1	XM_002694416	gaatcgggttcaaagcactgt
517	Bovine RXFP2	NM_001304277	cttcagccgcagattgattg
518	Bovine RXFP2	NM_001304277	cagttcctttgcacacacagc
519	Bovine RXFP2	NM_001304277	gtgccagtggtttctagcaatg
520	Bovine RXFP2	NM_001304277	caaccgctgtgaaattctgc
521	Bovine RXFP2	NM_001304277	gcagaatttcacagcggttg
522	Bovine RXFP2	NM_001304277	tgaaaatgtggggtggtagc
523	Bovine RXFP2	NM_001304277	tccacctttctgtcgtgcag

Primer number	Gene name	Gene number	Sequence (5' - 3')
524	Bovine RXFP2	NM_001304277	ccgtcagtcaagggcataca
525	Bovine RXFP2	NM_001304277	gttcctggccgttgtctttc
526	Bovine RXFP2	NM_001304277	acgacccaggaggtgattgt
501	Bovine S27a	NM_174778	tcctcctgaccagcaaagactg
502	Bovine S27a	NM_174778	tgcttgttcttcttgggagtgg
541	Bovine OTR	NM_174134	gctggacgccattctttttc
542	Bovine OTR	NM_174134	atcagtcacagcgtggatgg
566	Bovine ERα	NM_001001443	agggaagctcctatttgctcc
567	Bovine ERα	NM_001001443	cggtggatgtggtccttctct
568	Bovine ERβ	NM_ 174051	gcttcgtggagctcagcctg
569	Bovine ERβ	NM_ 174051	aggatcatggccttgacacaga
570	Bovine AR	NM_001244127	cagatggcagtcattcag
571	Bovine AR	NM_001244127	cttggtgagctggtagaag
593	Bovine PR	NM_001205356	gggatgaagcatcaggctgt
594	Bovine PR	NM_001205356	ttctctggcttagggcttgg
607	Bovine PTGS2	NM_174445	cgggaacacaacagagtgtg
608	Bovine PTGS2	NM_174445	gcgtgttaaactcagcagca
609	Bovine AKR1A1	NM_001076513	acctgatgcactggccttac
610	Bovine AKR1A1	NM_001076513	tctgccgactgttgaagttg
611	Bovine - AKR1B1	NM_001012519	tcaaagcgattgcagacaag
612	Bovine - AKR1B1	NM_001012519	agaagttgctgacccaccac
639	Bovine osteopontin	NM_174187	ccgaccagttctggcagct
640	Bovine osteopontin	NM_174187	ccacactatcacctcggcca

Table 2.3. PCR condition

gene	Primer pair	RT-PCR (semi-quantatitive (sq) or Real-Time quantitative (RTq)	Denaturation at 95°C	Annealing temperature (°C) or Tm and time	Elongation at 72°C	Number of cycles
bov S27a	#501/ #502	sq and RTq	30s	61 / 30s	40s	30
bov RXFP1	#503 / #504	sq	30s	62 / 30s	45s	40
	#505 / #506	sq	30s	60 / 30s	45s	40
	#507 / #508	sq	30s	62 / 30s	45s	40
	#511 / #512	sq	30s	60 / 30s	45s	40
	#513 / #514	sq	30s	62 / 30s	45s	40
	#515 / #516	sq	30s	62 / 30s	45s	40
	#503 / #508	sq	30s	60 / 30s	45s	40
	#517 / #518	sq	30s	60-56 / 30s	45s	40
	#519 / #520	sq	30s	60-56 / 30s	45s	40
	#521 / #522	sq	30s	62-60/ 30s	45s	40
	#523 / #524	sq	30s	60-56 / 30s	45s	40
	#525 / #526	sq	30s	60-56 / 30s	45s	40
bov ER alpha	#566 / #567	RTq	20s	62 / 20s	25s	35
bov ER Beta	#568 / #569	RTq	20s	62 / 20s	25s	35
bov AR	#570 / #571	RTq	30s	60-54 / 30s	45s	35
bov PR	#593 / #594	RTq	30s	61 / 30s	40s	35
bov AKR1A1	#609/ #610	RTq	30s	60 / 30s	40s	30
bov AKR1B1	#611 / #612	RTq	30s	60 / 30s	40s	35
bov OTR	#541 / #542	RTq	20s	62 / 20s	25s	35
bov COX2	#607 / #608	RTq	30s	60 / 30s	40s	30
bov Osteopontin	#639 / #640	RTq	30s	61 / 45s	72s	30

The primers used in carrying out RT-PCR are listed in table 2.2, together with their database sources. PCR conditions are indicated in Table 2.3. DNA was denatured at 95^{o}C. Ribosomal protein S27a, that has been shown to be expressed relatively constantly by mammalian cells and tissues (Lee et al.,2002) was used as the housekeeping gene in the RT-PCR analysis. For each reaction, 0.25μM of oligonucleotide primer was used in the amplification of 1μl of the cDNA, using Emerald AMP MAX HS PCR master mix (Takara-Clontech) for semi-quantitative RT-PCR in a MJ Research PTC-200 DNA Engine Thermal Cycler (Bio-Rad, Hercules, USA), or using Takara Sybrgreen Premix (Takara-Clontech) for real-time qPCR in a Rotor-Gene 3000 machine (Corbett Research, Sydney, Australia). Total volume of the reaction mixture was adjusted to 20μl with PCR-grade water (Takara-Clontech)

2.7.6 Agarose gel electrophoresis for PCR products

2μl of 1x Track-it cyan/orange Loading Buffer (Invitrogen, Thermo-Fisher Scientific) was mixed with 8μl of RT-PCR product. This was loaded on to a 2% agarose gel containing 1% EtBr in 1xTAE buffer. A 100bp DNA-ladder (Thermo-Fisher Scientific) was run in the first well of the gel alongside the samples as a marker lane. The running buffer was 1xTAE (pH 7.0). The electrophoresis was stopped after the dye had migrated approximately 3/4th of the gel and was visualized in a closed Bio-Rad

GelDoc XR System (Bio-Rad, Gladesville, Australia). In the case of semi-quantitative RT-PCR analysis, the PCR mastermix already contained the loading dye (Emerald mix, Takara-Clontech) and hence no additional loading dye was needed.

2.7.7 Normalization of real-time RT-PCR gene expression

The bovine S27a gene was used as a house-keeping gene for all analyses and was checked not to vary under different conditions and treatments of the cells used during experiments. The use of this gene alone had been suggested, as a result of numerous microarray analyses, by Lee et al. (2002) as the one least likely to change in diverse mouse tissues under a range of different developmental and other conditions, a conclusion confirmed by our own studies in the bovine. To normalize the gene expression, the following equation was used as described by Dai et al (2018)

Normalized expression = $\frac{(E\ Reference)^{CT\ Refence}}{(E\ Target)^{CT\ Target}}$

Where *E Reference* and *E target* stand for the PCR amplification efficiencies of the target and the housekeeping gene (bovine S27a), respectively. CT *reference* and CT *target* represents the threshold cycles of the PCR amplification of the target gene and the housekeeping gene,

2.8 Time-resolved fluorescent immunoassay (TRFIA)

For a TRFIA reaction a 96 well plate (Nunc, Thermo-Fisher Scientific) was pre-coated by the secondary antibodies (Rockland, Limerick, USA) . The cyclic adenosine monophosphate (cAMP) TRFIA assay used is an in-house well established and validated assay and the standard protocol was strictly followed. (Anand-Ivell et al., 2007). Fig. 2.1 shows a representative cAMP TRFIA standard curve ranging from 1 to 243 pmol/ml with less than 2% within and less than 8% between-plate CV. The cross-reactivity of this assay had been previously determined (Schumacher et al., 1984) and is given in the following table:

Table 2.4: Cross-reactivity of compounds with structural relationship to cAMP

Cross-reactant		Cross-reactivity %
cAMP		100.00
cGMP		0.00028
Adenosine		0.00020
2´3´cAMP		0.00013
AMP	<	0.00001
ADP	<	0.00001
ATP	<	0.00001
Guanosine	<	0.00001
2´3´cGMP	<	0.00001
GMP	<	0.00001
GDP	<	0.00001
GTP	<	0.00001
Isobutylmethylxanthine	<	0.00001

Briefly, a range of standards or sample containing the required antigen was added per well alongside appropriate antibody and tracer controls, and positive control samples. The primary antibody at an appropriate concentration was then added. The plates were incubated at 4^{o}C for 20h. After aspiration of the contents and one wash with 1x wash buffer, an appropriate volume and concentration (count per sec/well) of Europium (Eu^{3+})-labelled tracer was added followed by further incubation at 4^{o}C for 3h. The contents were aspirated, and each well was washed four times with 1x wash buffer. 200μl of enhancement solution (Perkin Elmer, UK) added per well and plate kept in a dark chamber for 1h to dissociate

the Eu^{3+} from the bound tracer. The time-resolved fluorescence was then observed by exciting the sample with pulsed light at 340nm and measuring the emission of intense fluorescent light at 614nm using a Victor3^{TM} 1420 multilabel reader in delayed-response mode. The intensity of fluorescence generated from the bound tracer inversely reflected the amount of antigen present in a sample.

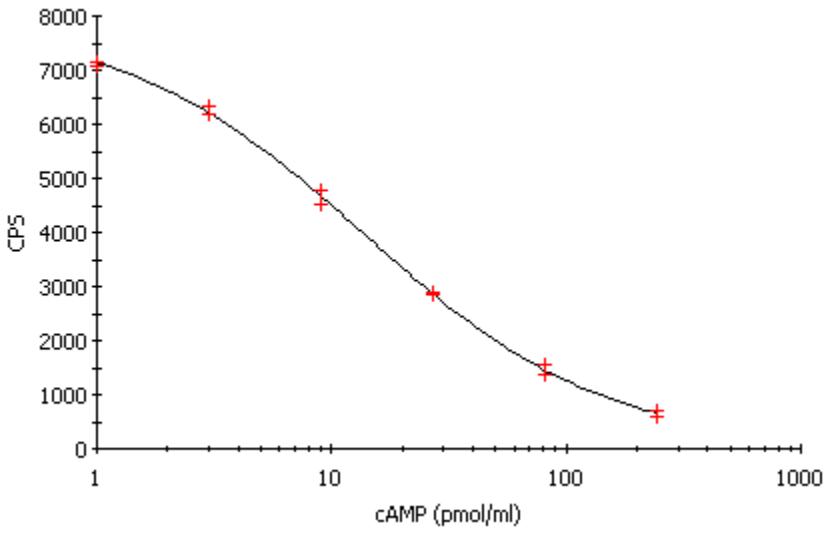


Figure 2.1 A representative standard curve for cAMP TRFIA.

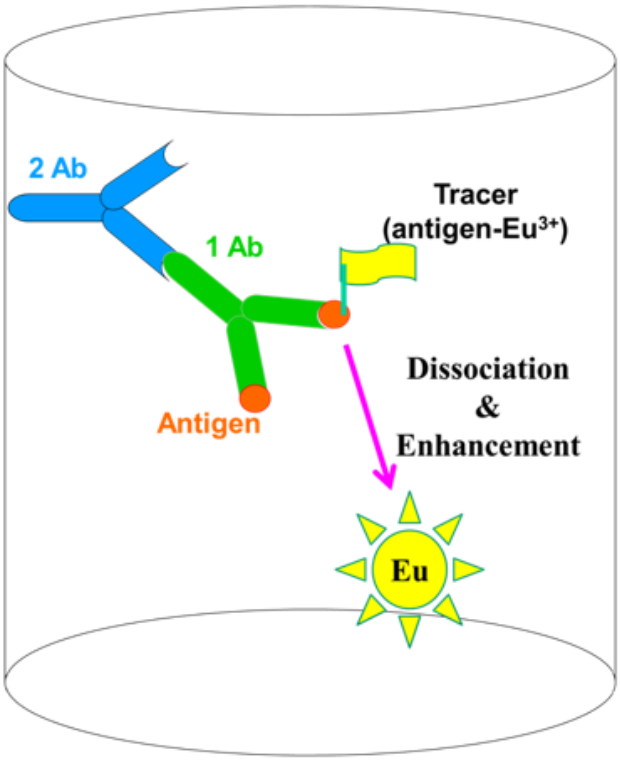


Figure 2.2. Principle of Time-resolved fluorescence immunoassay (TRFIA**)**. Pre-coated secondary antibodies (2. Ab) can bind to the primary antibody (1. Ab) that is specific for the target antigen (cAMP). The tracer is Eu^{3+}-labelled antigen that competes with antigens from a sample to bind with the 1Ab. The Eu^{3+} from the bound tracer will then be dissociated and emit fluorescence that can be detected. The intensity of the fluorescence inversely reflects the number of antigen molecules bound to the 1. Ab.

2.9 RNA Seq experiments.

2.9.1 In vivo mouse uterus subject to relaxin- or vehicle treatment

Total RNA from mouse uterus had been archived at -80^{o}C and was available for further analysis. This RNA resulted from experimentation to explore the role of relaxin in vivo to elicit a pro-inflammatory response. The details of this experimentation and its results were published in Glynn et al. (2017). Four samples of total RNA from mouse uterus treated with intra-uterine injection of human relaxin were available, as were four comparable samples prepared in parallel from vehicle-treated uterus. All 8 samples were first checked for amount and quality by nanodrop spectrophotometry and gel electrophoresis to assess the integrity of the ribosomal RNA bands, respectively.

3 μg of each RNA sample were then sent to Genewiz GmbH (Leipzig, Germany) for RNA-Seq analysis using the Illumina NovaSeq, 2x150bp configuration, and with an estimated data output of approximately 20M raw paired-end reads per sample.

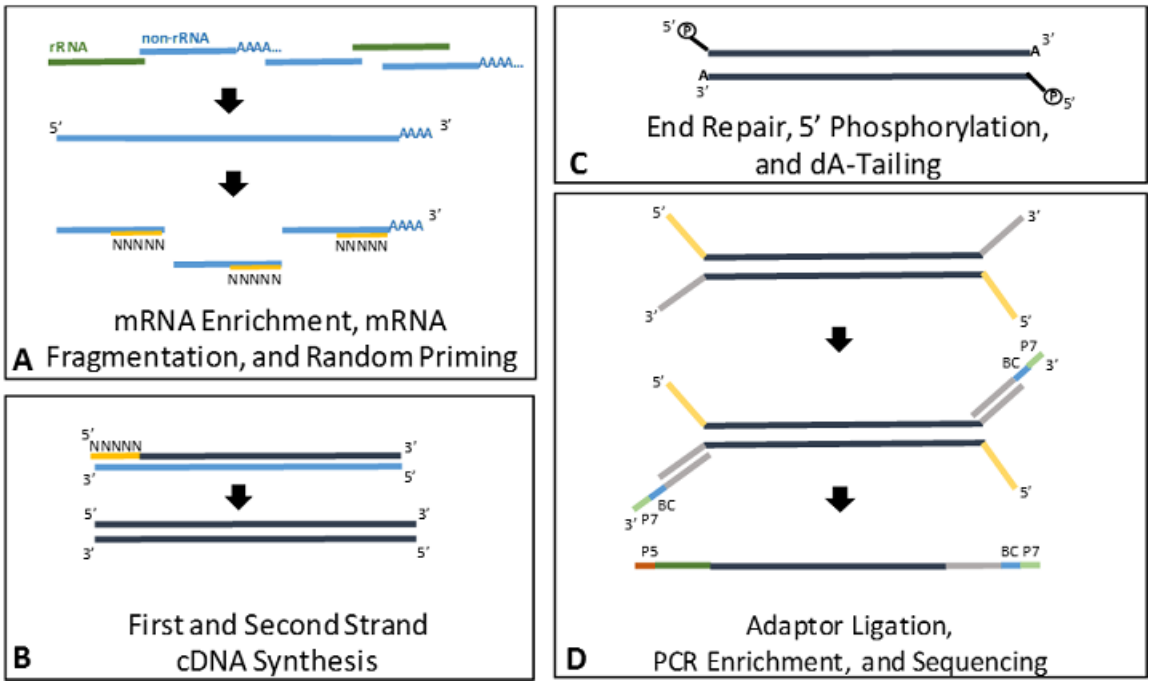


Figure 2.3 RNA-seq library preparation workflow (*from Genewiz*). mRNA sequencing via polyA selection

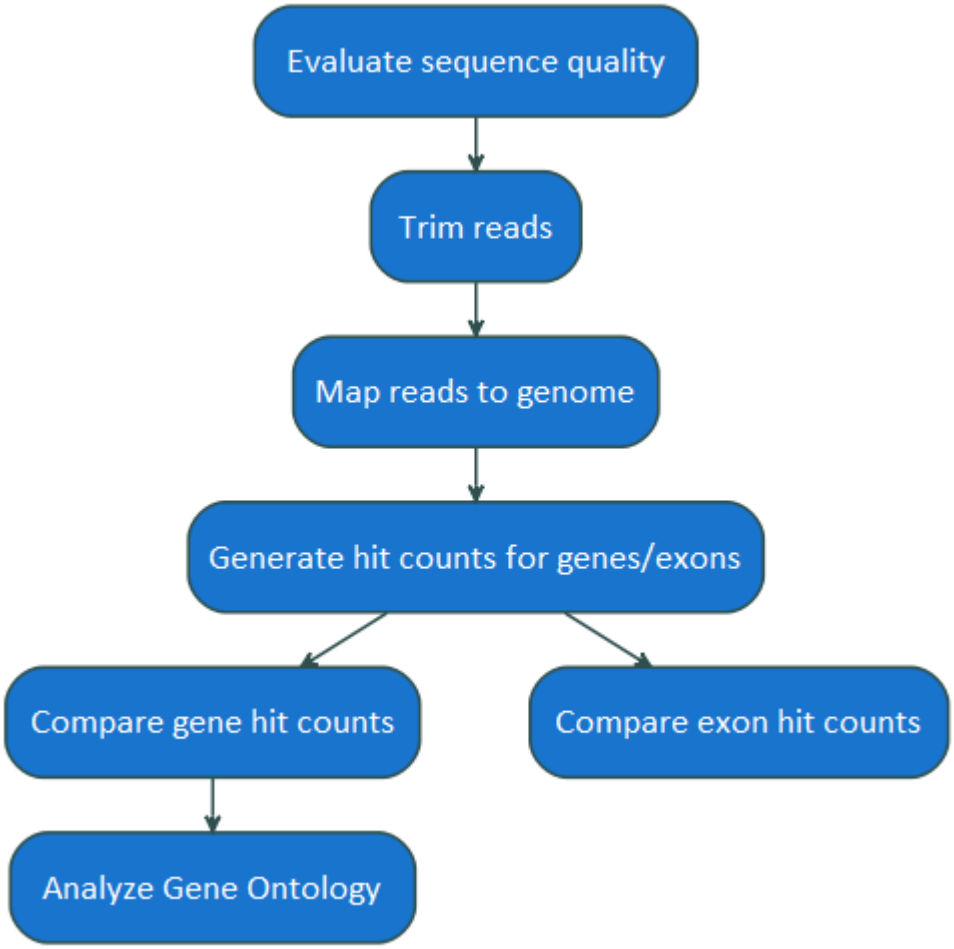


Figure 2.4 Bioinformatics analysis workflow (from Genewiz)

The company provided full sequence information of 20-30 million reads per sample, as well as quantified transcript expression for all samples, tabulating all RNA transcripts significantly different in expression between relaxin-treated and vehicle-treated samples. This information was then subjected to a modified Integrated Pathway Analysis (NIPA) also linked to the KEGG pathway database (cooperation with Professor Richard Emes, University of Nottingham) to give rise to the results summarized in chapter 7.

2.9.2 NIPA analysis of unpublished microarray results available for human primary endometrial stromal cells treated or not with relaxin.

In order to gain further information on possible novel endpoints of relaxin action on uterine cells and tissues, a database of human Affymetrix microarray data obtained previously by the Ivell/Anand-Ivell group examining the effects of relaxin-treatment or not of human endometrial stromal cells was similarly analysed by NIPA (cooperation with Professor Richard Emes, University of Nottingham). Altogether 4 samples of relaxin-treated cells and 4 samples of vehicle-treated cells were compared, differentially expressed RNA transcripts compared and subjected to NIPA and KEGG analyses, as above.

2.10 Statistical analysis

All data were analysed using the Graphpad Prism versions 7 and 8. Descriptive column statistics were used to define the mean, and the standard error (SEM). pTrend was additionally calculated using Graphpad version 8 and takes account of multiple dose-responsiveness where individual doses may not be significantly different from the basal. Data were compared using ANOVA followed by Neumann-Keuls comparison or an unpaired T-test.

Chapter 3: Expression of the ruminant RXFP1 and RXFP2 receptors in uterine cells.

Chapter.3

Expression of the ruminant RXFP1 and RXFP2 receptors in uterine cells.

3.1 Introduction

RXFP1 and RXFP2 receptors are similar to the glycoprotein hormone receptor subfamily, in that they contain similar large ectodomains comprising multiple leucine-rich regions (Scott et al., 2007). It is possible that RXFP1 and RXFP2 derived from related glycoprotein hormone receptors prior to modern vertebrates. Functional studies have indicated that RXFP1 receptors have similar signal transduction pathways to the glycoprotein hormone receptors (Hsu et al., 2002). RXFP1, which is the cognate receptor for H2-relaxin, shares approximately 60% of its amino acid sequence with RXFP2, which is the cognate receptor for INSL3 (Hsu et al., 2002). RXFP1 and RXFP2 belong to the subfamily of leucine-rich repeat-containing G protein-coupled receptors (LGR), which are part of the larger rhodopsin-like group of receptors. RXFP1 and RXFP2 each consist of three domains (Fig 3.1): the large ectodomain, which makes up the majority of the receptor,

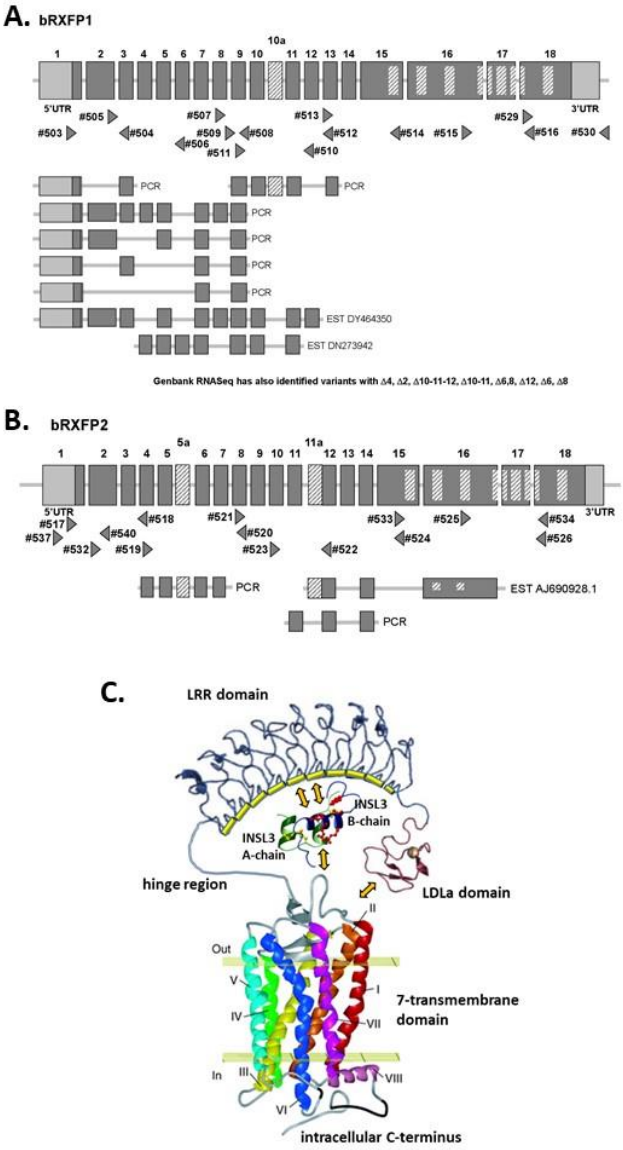


Figure 3.1: Structure and organization of the ruminant RXFP1 and RXFP2 receptors. **A**. Exon-intron structure of bRXFP1 indicating the positions of PCR primers used for RT-PCR (arrowheads), as well as structures of splice variants (below) identified by RT-PCR or from EST sequences. From Dai et al. (2017a). **B**. Exon-intron structure of bRXFP2 indicating the positions of PCR primers used for RT-PCR (arrowheads), as well as structures of splice variants (below) identified by RT-PCR or from EST sequences. From Dai et al. (2017a). **C**. Structure of RXFP2 and its relationship to INSL3 binding (LRR, leucine-rich repeat domain; LDLa, lipoprotein D-like; roman numerals identify individual transmembrane regions; double arrows indicate interaction sites between the receptor and INSL3). From Ivell & Anand-Ivell (2018).

the seven transmembrane domain region, and the cytoplasmic domain of the C-terminal tail (Fig 3.1C). The largest domain (the ectodomain) comprises two separate sections: the first comprises the ten leucine-rich repeats (LRRs) and their capping structures, and then there is a cysteine-rich low-density lipoprotein class A or LDL-A module at the extreme amino terminus of RXFP1 and RXFP2 (Hsu et al., 2003; Hsu et al., 2002; Bathgate et al., 2013).

RXFP1 and RXFP2 each contain two complex ligand binding sites that are required to activate the receptors (Fig 3.1C). These comprise certain residues in the LRRs of the ectodomain which contact individual B-chain residues in relaxin or INSL3, and which lead to a conformational change in the receptor. This then allows the second extracellular loop of the transmembrane region to form a second low affinity ligand-binding site, which then contacts part of the relaxin A-chain (Sudo et al., 2003a; Halls et al., 2005b).

As is often the case with GPCRs, there can be alternative splicing of the gene transcripts for RXFP1 and RXFP2, which can lead to an alteration or loss of the encoded receptor function. The 18 exons each for RXFP1 and RXFP2 mean that alternative splicing can produce a large number

of variations for RXFP1 and RXFP2 (Fig 3.1A and 3.1B) (Muda et al., 2005; Kern and Bryant-Greenwood, 2009), as also for the alternative members of the LGR family, including the luteinising hormone receptor (LHR), the thyroid-stimulating hormone receptor (TSHR) (Ando et al., 2001) and the follicle-stimulating hormone receptor (FSHR) (Gerasimova et al., 2010; Song et al., 2002). Some of the products of alternative splicing consist of transmembrane domains but others may comprise purely ectodomain fragments. Upon closer inspection of these variations it becomes apparent that some are expressed on the surface of the cell whereas some are held inside the endoplasmic reticulum (ER) and others may be incompletely secreted, even acting as secreted antagonists by their ability to sequester hormone (Kern et al., 2008; Muda et al., 2005). However, only a minority of the splice variants identified appear capable of binding to the ligand (Kern and Bryant-Greenwood, 2009; Muda et al., 2005).

If a splice variant does not have an LDL-A module and is expressed at the surface of the cell, they will be capable of binding to the ligand but unable to transfer a signal (Scott et al., 2005). Furthermore, some splice variants operate as dominant-negative complements to wild type RXFP1. Such variants can inhibit receptor homo-dimerization, maturation and trafficking to the surface of the cell, thereby depriving

the receptor of its ability to function (Kern et al., 2008; Scott et al., 2005; 2006)

By using different combinations of specific PCR primers from all regions of the RXFP1 and RXFP2 transcripts, this study has aimed to show the presence of full-length RXFP1 and RXFP2 in different cells of the bovine and sheep uterus. The sequences of all primers were checked that they would recognize equally both ovine and bovine receptor sequences.

3.2 Results

While relaxin has been shown to be produced by the female reproductive system of most mammals, there is a large deletion in the genomes of cattle and sheep which have evidently lost a functional relaxin gene in these species (Dai et al., 2017a). Yet preliminary analysis by the Anand-Ivell group at the University of Nottingham has shown that the specific receptor for relaxin, RXFP1, and the closely related receptor RXFP2 are both expressed and appear to be functional in cows. RXFP1, but not RXFP2, is reported as having an increased EC50 (Dai et al., 2017) for its specific ligand. Here, I have first assessed the expression of RXFP1 and RXFP2 gene transcripts in individual uterine cell types derived from sheep or cows, to determine any cell specificity of expression. Combinations of different PCR primers were used to determine whether or not there may be any splice variants present.

3.2.1 Expression of RXFP1, RXFP2 in Secondary cell-lines

Semi-quantitative RT-PCR was carried out to determine the expression of RXFP1 in 3 different cell lines derived from sheep uterus: ovine glandular epithelial cells (OGE), ovine luminal epithelial cells (OLE) and ovine stromal cells (OST) (Fig 3.2).

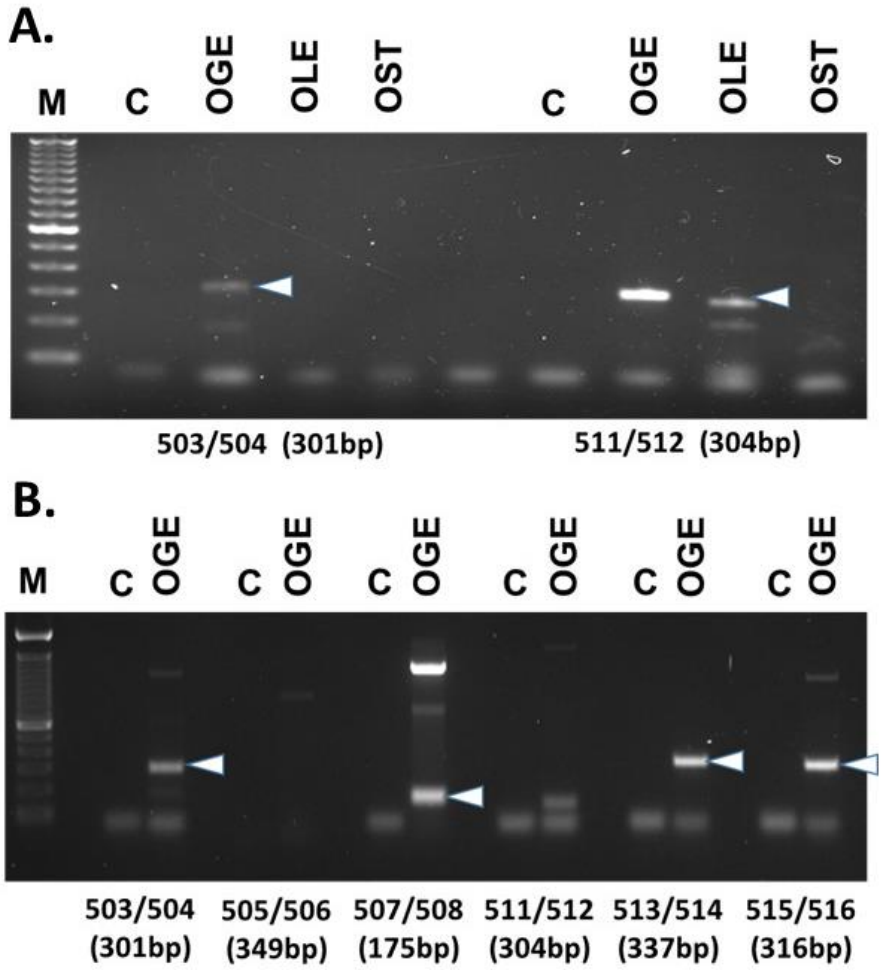


Figure 3.2 A.Semi-quantitative RT-PCR analysis for different regions of the RXFP1 gene in the ovine endometrial cell-lines OGE, OLE, and OST using primer pairs 503/504 and 511/512. **B**. The ovine endometrial cell-line OGE was used for more detailed analysis of different regions of RXFP1, using the primer pairs 503/504, 505/506, 507/508, 511/512, 513/514 and 515/516. (M, DNA markers; C, negative control; arrowheads indicate correctly sized bands; expected size of the PCR product is given in parentheses beside or below the primer numbers). The relative positions of the primers used are given in Fig 3.1A. The DNA marker ladder (M) indicates increments of 100bp with the central prominent band being 600bp.

Using two sets of RXFP1-specific primers, of the different sheep cell-lines tested, only OGE cells indicated consistent expression (Fig 3.2A), with possibly also OLE cells also expressing RXFP1-specific transcripts.

OST cells were negative for RXFP1. Expression was then confirmed for OGE cells only using further primer combinations (Fig 3.2B). Most, though not all primer combinations supported the expression of RXFP1 in the OGE cell-line.

Similarly, semi-quantitative RT-PCR was performed to detect RXFP2 gene transcripts in the same cells (Fig 3.3A). Only OST cells indicated any expression of RXFP2. RXFP2 has been shown to be expressed in numerous splice variant forms (Heng et al., 2008), some of which may be expressed and inhibitory. As for RXFP1, also for RXFP2, combinations of different primers were applied to confirm the expression of potentially functional receptors in OST cells (Fig 3.3B). As in Figure 3.3A, cDNA from bovine ovarian theca cells served here as a positive control (Dai et al., 2017a). The results showed that only two out of five primer pairs supported the expression of apparently normal RXFP2 transcripts.

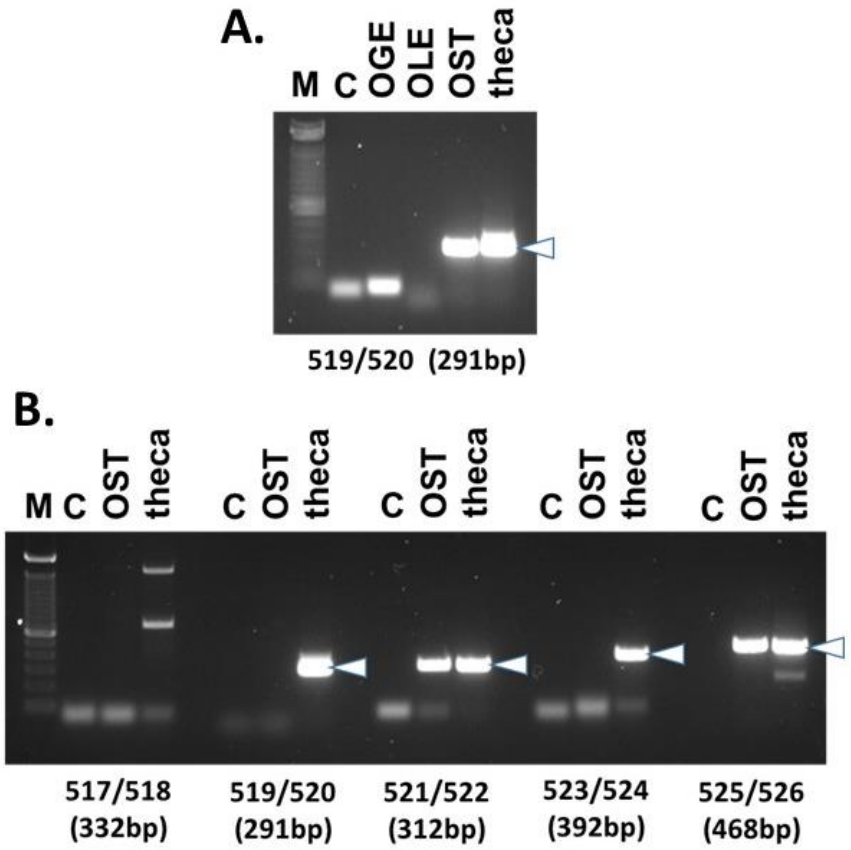


Figure 3.3 A. Semi-quantitative RT-PCR analysis for different regions of the RXFP2 gene in ovine endometrial cell-lines OGE, OLE, and OST using the primer pair 519/520. **B.** Ovine stromal cells (OST) were used for more detailed analysis of different regions of RXFP2 using the primer pairs 517/518, 519/520, 521/522, 523/524 and 525/526 (M, DNA markers; C, negative control; theca, positive control; arrowheads indicate correctly sized bands; expected size given in parentheses beside or below the primer numbers). The relative positions of the primers used are given in Fig 3.1B.

3.2.2 Expression of RXFP1, RXFP2 in bovine primary cell cultures

Stromal cells and epithelial cells were isolated from bovine endometrium (two independent animals), and a similar combination of PCR primers to that used above was used to determine the expression of RXFP1 and RXFP2 gene transcripts. cDNA from bovine ovarian theca cells served as a positive control, although the expression of RXFP1 here is substantially weaker than for RXFP2 (Dai et al., 2017b). For all PCR primers, RXFP1 appears to be consistently expressed in at least one preparation of bovine primary stromal cells (ESC), whereas primary bovine endometrial epithelial cells (EEC) are mostly negative (Figure 3.4A-C).

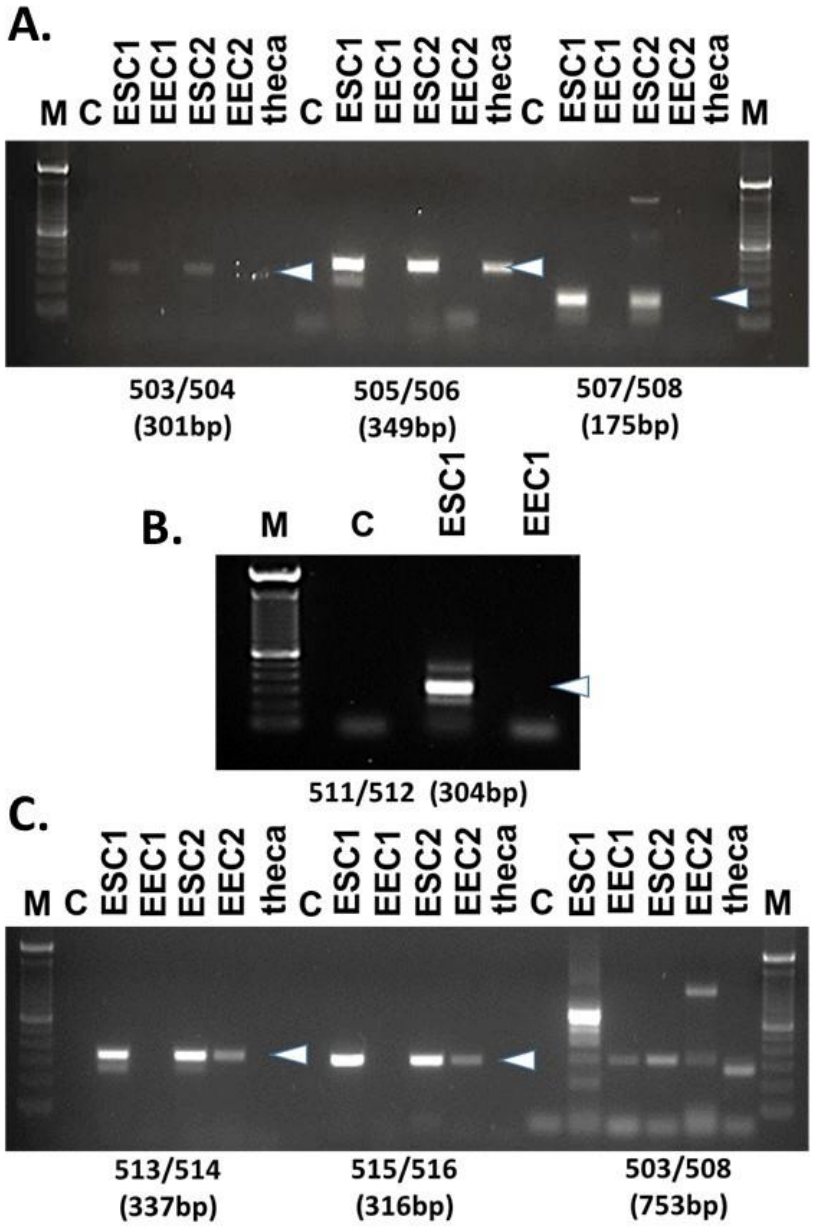


Figure 3.4. Semi-quantitative RT-PCR analysis for different regions of the RXFP1 gene in bovine primary cells. **A**. using primer pairs 503/504, 505/506 and 507/508. **B**. using primer pair 511/512. **C**. using primer pairs 513/514, 515/516 and 503/508. M, DNA markers; C, negative control; theca, positive control; ESC1(Animal1), ESC2,(Animal2), endometrial stromal cells; EEC1(Animal1), EEC2,(Animal2) endometrial epithelial cells; arrowheads indicate correctly sized bands (expected size given in parentheses beside or below the primer numbers). The relative positions of the primers used are given in Fig 3.1A.

No consistent expression of RXFP2 mRNA could be detected in either endometrial stromal cells or endometrial epithelial cells (Figure 3.5). However, since also the theca cell RNA used here as positive control was also negative for some primer combinations, it suggests that the PCR conditions used were not optimal. These experiments need repeating with altered PCR conditions.

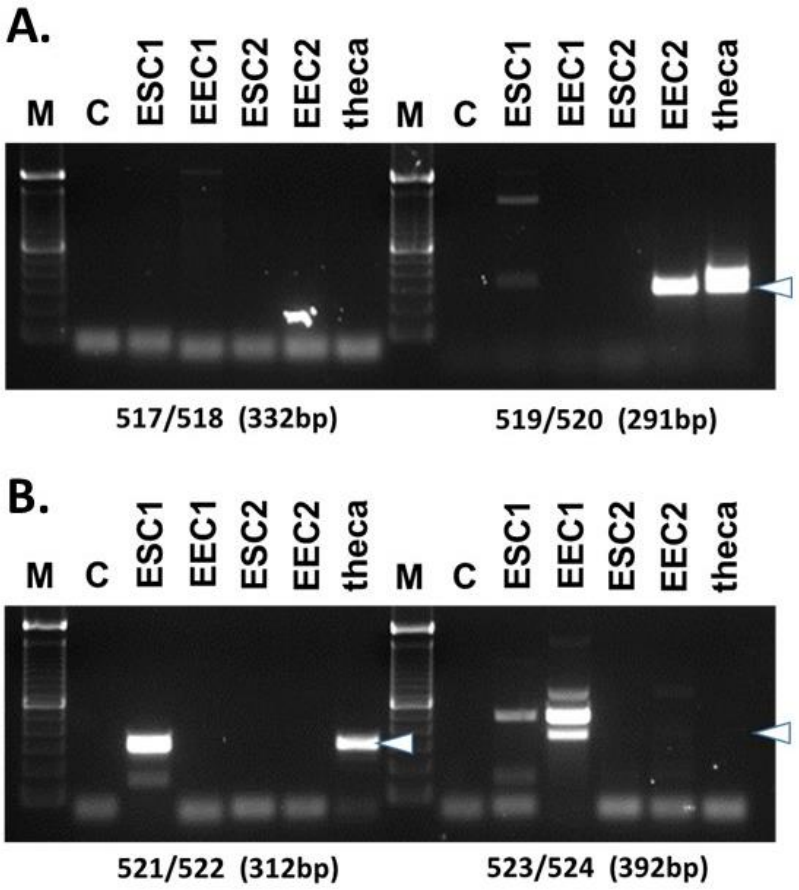


Figure 3.5. Semi-quantitative RT-PCR analysis for different regions of the RXFP2 gene in bovine primary cells. **A**. using primer pairs 517/518 and 519/520. **B**. using primer pairs 521/522 and 523/524. M, DNA markers; C, negative control; theca, positive control; ESC1 (Animal1), ESC2 (Animal2), endometrial stromal cells; EEC1 (Animal1), EEC2 (Animal2), endometrial epithelial cells. arrowheads indicate correctly sized bands (expected size given in parentheses beside the primer numbers). The relative positions of the primers used are given in Fig 3.1B.

3.2.3 Expression of RXFP1, RXFP2 in bovine primary myometrial cell cultures.

Using similar sets of RXFP1-specific primers for primary bovine myometrial cells (Myo) indicated fairly consistent expression for all primer combinations (Fig 3.6A), thus supporting the expression of RXFP1 in these cells.

In contrast to RXFP1, using two pairs of specific PCR primers there appeared to be no consistent expression of RXFP2 mRNA in these primary myometrial cells (Fig 3.6B). PCR products were seen using the RXFP2-specific primers, but these were not of the correct size. These products will need sequencing to determine whether these represent the results of alternative splicing of the RXFP2 gene, or whether these are unspecific.

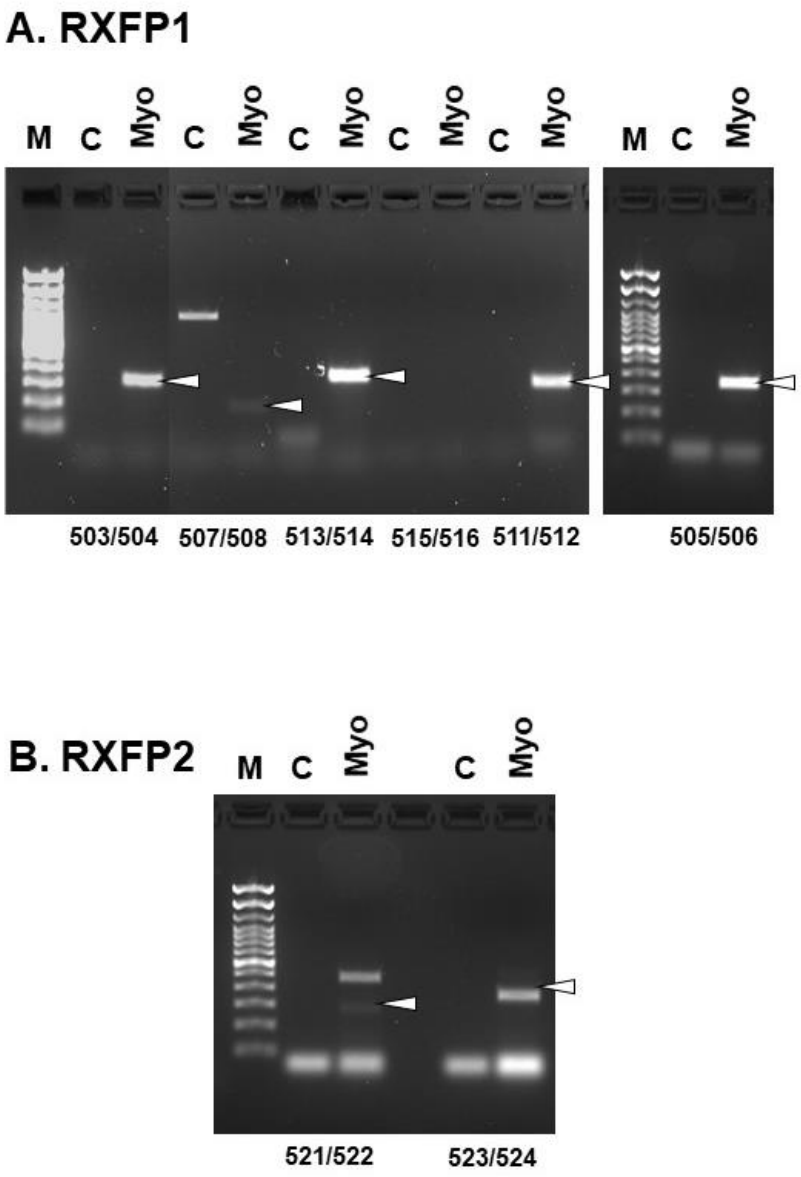


Figure 3.6. Semi-quantitative RT-PCR analysis for different regions of the RXFP1 (upper panel **A**) and RXFP2 (lower panel **B**) genes in primary cultures of bovine myometrial cells (Myo). M, DNA markers; C, negative control; arrowheads indicate correctly sized bands (expected size given for the respective primer pairs in the legends to Figs 3.4 and 3.5). The relative positions of the primers used are given in Fig 3.1A. Note that only a weak positive PCR product is seen for primer pair 507/508; the band evident in the control is unspecific. None of the products observed for the RXFP2 reactions have the correct size.

RXFP1	OGE	OLE	OST	ESC	EEC	Myo
#503 / #504	+	-	-	+	-	+
#505 / #506	+	nd	nd	+	-	+
#507 / #508	+	nd	nd	+	-	+
#511 / #512	+	+	-	+	+	+
#513 / #514	+	nd	nd	+	+	+
#515 / #516	+	nd	nd	+	-	-
#503 / #508	nd	nd	nd	+	+	nd

Table 3.1. Summary of semi-quantitative RT-PCR results for different regions of the RXFP1 gene in glandular epithelial cells (OGE), ovine luminal epithelial cells (OLE), ovine stromal cells (OST), primary cultures of endometrial stromal cells (ESC); primary cultures of endometrial epithelial cells (EEC) and primary cultures of bovine myometrial cells (Myo). (+, positive expression of correctly sized product; -, no correct expression; nd; not determined.

RXFP2	OGE	OLE	OST	ESC	EEC	Myo
#517 / #518	nd	nd	+	-	-	-
#519 / #520	-	-	+	+	+	-
#521 / #522	nd	nd	-	+	-	-
#523 / #524	nd	nd	-	+	+	-
#525 / #526	nd	nd	+	nd	nd	nd

Table 3.2. Summary of semi-quantitative RT-PCR results for different regions of the RXFP2 gene in glandular epithelial cells (OGE), ovine luminal epithelial cells (OLE), ovine stromal cells (OST), primary cultures of endometrial stromal cells (ESC); primary cultures of endometrial epithelial cells (EEC) and primary cultures of bovine myometrial cells (Myo). (+, positive expression of correctly sized product; -, no correct expression; nd, not determined.

3.3 Discussion

The RT-PCR results imply that the gene for RXFP1 is indeed expressed in the sheep OGE cell-line, representing glandular epithelial cells, but not in either the stromal or luminal epithelial cell-lines. In contrast, using primary cultures of bovine epithelial, stromal, and myometrial cells it was shown that RXFP1 is expressed consistently on the stromal cells and myometrial cells, similar to what has been observed in the human (Heng et al., 2008), though not reliably on epithelial cells. The finding of RXFP1 expression in these cell types is in accord with the results of Dai et al (2017a) who showed transcript expression in bovine endometrial fragments.

Regarding the closely related receptor RXFP2, transcripts for this gene were found to be possibly expressed in bovine endometrial epithelial cells and in the ovine stromal cell-line (OST) though apparently no other cell type. Also, Dai et al (2017a) showed only very low levels of RXFP2 expression in bovine myometrial cells compared to the higher level of expression of RXFP1 in the same cells. Although not shown in the earlier study, this is plausible, since often RXFP1 and RXFP2 are shown to be expressed in different cell types (Bathgate et al., 2013). These studies

need further confirmation, also by sequencing of the RT-PCR products to confirm product identity.

RXFP1 has been shown to be expressed consistently in myometrial, epithelial and stromal endometrial cells of other mammalian species such as human, mouse, bovine, and cat (Dai et al., 2017a; Braun et al., 2012; Ivell et al., 2003; Li et al., 2011; Vodstrcil et al., 2010), though in differing amounts. Less information is available for the expression of RXFP2 in uterine cells of other species. In human, it was stated that RXFP2 was found in myometrium and endometrial epithelium (Li et al., 2011), and in rat, it was found that RXFP2 was expressed in myometrium and endometrium (Li et al., 2011).

Most earlier published studies have not assessed the possibility of alternative splice products for the RXFP1 and RXFP2 genes in tissues and cells of any species, relying often only on a single primer combination from the downstream, transmembrane-encoding region of the genes, which does not exhibit the splicing variation of the ectodomain-encoding region. Only Dai et al. (2017a) has assessed alternative splicing for RXFP1 or RXFP2 in any ruminant tissue. In that study a shorter PCR product could be faintly observed for RXFP1 with the primer combinations 511/512 and 513/514 in primary myometrial

cells, similar to that observed here for the OGE cell-line, though no such secondary product was seen in myometrial cells in the present study, possibly because the PCR conditions may not have been optimal in the current investigation. Based on the size difference, and location of these primers, this could suggest that there is weak expression of a transcript which is missing one of the short LRR-encoding exons. As mentioned earlier, such exons are important for correct ligand-receptor interaction and subsequent conformational change of the receptor ectodomain, though the relatively low expression of these alternative transcripts suggest that their protein products may not be physiologically relevant. For RXFP2, Dai et al. (2017a) found no evidence for alternative splicing products in any of the cells and tissues they looked at, except for oocytes, where a larger secondary product was observed. In the present study most RXFP2 PCR products, where observed, were correctly sized except where weakly expressed alternatives were seen sporadically and not reproducibly, as in primary endometrial epithelial and stromal cells (Fig 3.5). However, in the current study, it was not possible to sequence these alternative products, so that a definitive conclusion as to possible relevance cannot be drawn. Since the PCR products of the RXFP2 gene in myometrial cells appeared to be quite aberrant, though also not yet sequenced, the expression of this gene here requires further investigation. Dai et al. (2017a) indicated only very weak or no

expression of RXFP2 in myometrial cells, so that it seems likely that improving PCR conditions further may not yield a reproducible positive outcome.

Taken together, the results presented here indicate consistent expression of RXFP1 gene transcripts in ruminant primary endometrial stromal and myometrial cells, as well as in the sheep OGE cell-line. RXFP2 is expressed more sporadically, though, besides in ovarian theca cells, most consistently in the sheep OST cell-line. Unfortunately, the latter cells are extremely slow-growing and consequently less suited to experimentation.

Chapter 4: cAMP production in diverse uterine cells treated with Relaxin or INSL3

Chapter 4

cAMP production in diverse uterine cells treated with Relaxin or INSL3

4.1 Introduction

4.1.1 Signaling pathways of RXFP1

The cell-type specific activation of various pathways is generated by the binding of relaxin to its cognate GPCR, RXFP1 (Kong et al., 2010). These pathways include the production of cyclic 3'-5' adenosine monophosphate (cAMP) and/or nitric oxide (NO), phosphorylation of mitogen activated protein kinases (MAPKs), engagement with the glucocorticoid receptor (GR), and activation of peroxisome proliferator-activated receptor gamma (PPARgamma) (Bathgate et al., 2013). The NO pathway appears to be mostly restricted to vascular endothelial cells or cardiomyocytes(McGuane et al., 2011) .

The increase of extracellular and intracellular cAMP has been greatly analysed, with extensive research also on the expression of RXFP1 in cells activated by the main signalling pathways (Bartsch et al., 2001; Fei et al., 1990; Dai et al., 2017b). It is being accepted that heightened degrees of cAMP may be biphasic. The receptor appears to be involved with as much as three mutually exclusive kinds of G-protein coupling, namely Gαs, GαoB and Gi3 (Halls et al., 2006).

Halls et al. (2006) and Heng et al. (2008) mentioned that INSL3, similar to relaxin, is fully responsible for catalyzing the manufacturing of cAMP by activating adenylyl

cyclase in human embryonic kidney cells transfected with an RXFP2 expression construct (Halls et al., 2006; Heng et al., 2008). Moreover, cAMP increases also after the stimulation of RXFP2-expressing rat gubernacular cells by INSL3, and in primary human osteoblasts (Ferlin et al., 2009). However, RXFP2 may also couple with Gi/Go proteins in oocytes or in testicular germ cells, leading to decreased cAMP. Consequently, the type of such cell and the scheme of expression of the appropriate G protein can influence the response of cAMP (Kong et al., 2009).

As a result, the treatment of different uterine cells with relaxin and/or INSL3 and the measurement of resulting cAMP in the cell cultures should provide important information concerning the functionality of the RXFP1 or RXFP2 receptor systems.

4.2 Results

From the PCR analysis, it would appear that the OGE cell-line as well as primary bovine endometrial stromal cells and primary myometrial cells all likely express the RXFP1 relaxin receptor, though confirmation is required at the protein level. It is known for the human that both stromal and epithelial endometrial cells, as well as myometrial cells also express the relaxin receptor (Anand-Ivell et al., 2013). Equally it is known that immortalization of cells, as was used to produce the OGE, OLE and OST cell-lines, can lead to loss of some specific gene expression. Consequently, it was decided first to test the functionality of both the primary bovine endometrial stromal cells (ESC) as well as the OGE cell-line, by stimulation with porcine relaxin. Porcine relaxin was chosen since, as ungulates, pigs are evolutionarily closer to ruminants than either humans or rodents, and thus might be expected to have a closer affinity for the ruminant receptor. It is to be noted that since porcine relaxin was obtained by extraction from pig corpora lutea and thus cannot be 100% pure, concentration is given in ng/ml. Actual purity assessed by electrophoresis suggested the preparations to be approximately 85% pure. Both human recombinant relaxin as well as chemically synthesized INSL3, though of much higher purity are given either in ng/ml for comparative purposes, or in molar units. For human

endometrial stromal cells, relaxin stimulation leads to a large increase in the production of cAMP (Bartsch et al., 2004). Thus, in these experiments cAMP production was measured as a functional primary end-point in the presence or not of either the phosphodiesterase inhibitor IBMX or forskolin (Fig 4.1). The latter is an allosteric activator of adenylyl cyclase (Kocan et al., 2017).

4.2.1 cAMP production in bovine endometrial stromal cells (ESC)

There appears to be a biphasic response in all three independent experiments. A relatively high concentration of porcine relaxin was used given that the measured EC50 for the bovine RXFP1 is almost 50-fold greater than for the equivalent human receptor (Dai et al., 2017b). There appears to be initially a consistent, though small and within experiments not statistically significant stimulation at the relatively low concentration of 100ng/ml porcine relaxin. This is followed by a subsequent inhibition or lack of effect at higher concentrations, irrespective of whether or not forskolin or the phosphodiesterase inhibitor IBMX was also applied (Fig 4.1). Using the pTrend statistic, which takes account of the variance at all three relaxin concentrations, this reduction in effect of relaxin is statistically significant within individual experiments, though not for the pooled data.

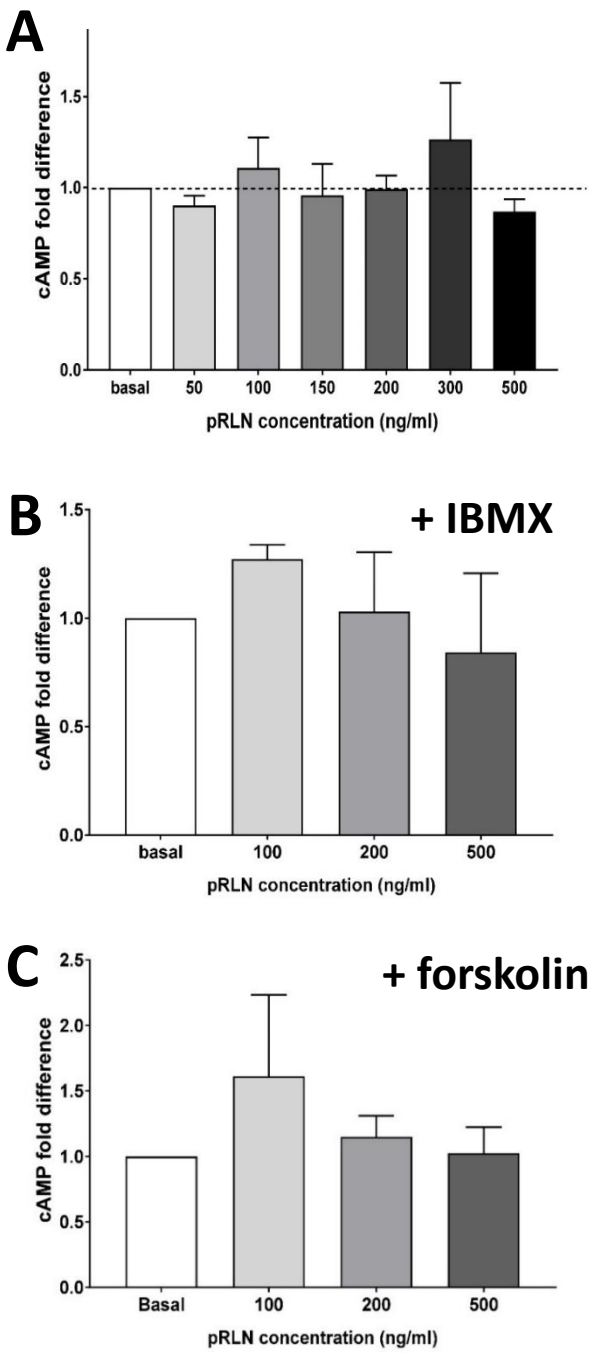


Figure 4.1. Effect of porcine relaxin (pRLN) on cAMP production by primary bovine endometrial stromal cells. **A**. In the absence of further effectors, pRLN has no significant effect (data combined from 3 independent experiments). **B**. In the presence of IBMX, there appears to be a stimulation at 100 ng/ml, but no significant effect at higher concentrations (data combined from 3 independent experiments). **C**. In the presence of forskolin (as in B, data combined from 3 independent experiments). Results are presented as means ± SEM.

4.2.2 cAMP production in the ovine glandular epithelial (OGE) cell-line

Similar experiments were carried out using the OGE cell-line (Figure 4.2), this time using a larger concentration range of porcine relaxin. The results showed that OGE exhibited an inhibition of the basal cAMP with relaxin, which was significant using the pTrend statistic on this pooled data, as well as within individual experiments (not shown). This might indicate that the RXFP1 receptor is using Gi or G0 as the specific receptor-linked G-protein.

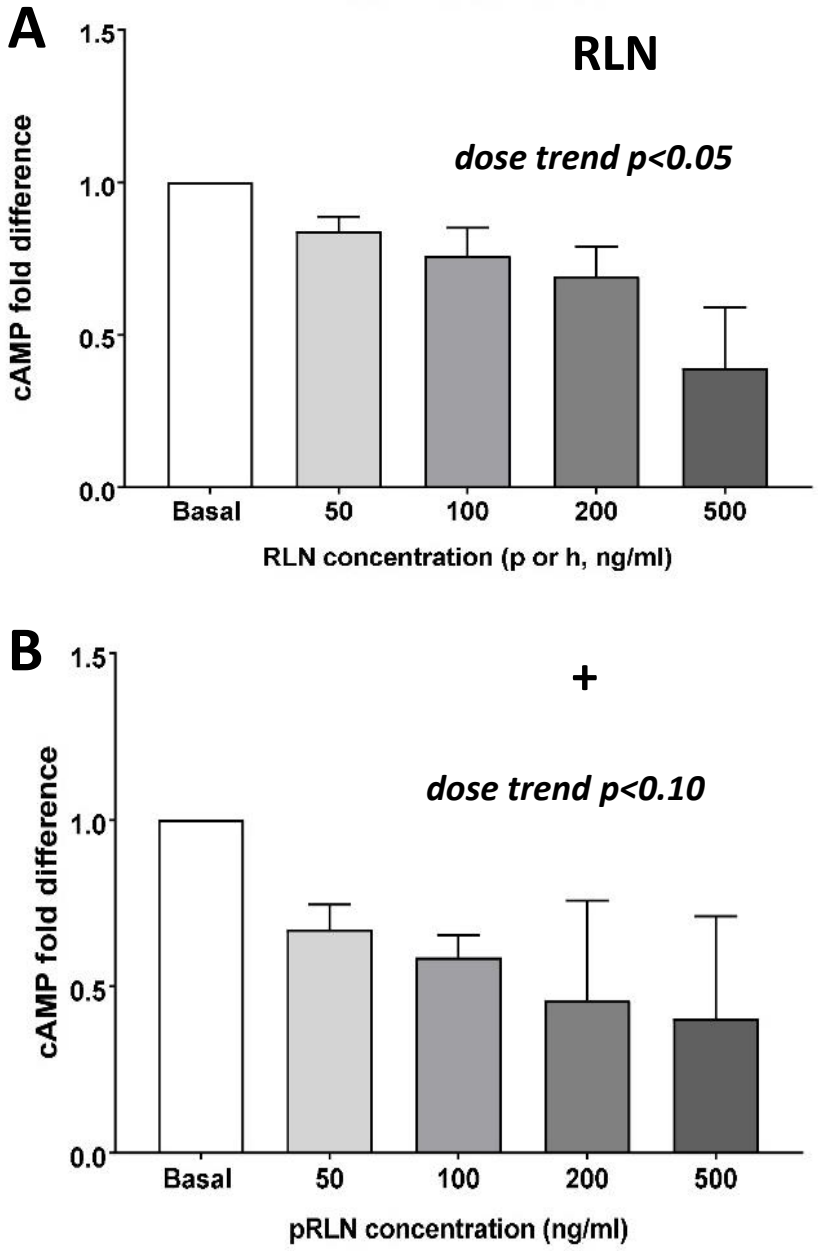


Figure 4.2. Effect of relaxin on cAMP production by the OGE cell-line. A. In the absence of additional effectors (triplicate experiments), RLN shows a significant inhibitory trend. Human relaxin (hRLN) was used instead of porcine relaxin (pRLN) in some experiments. They had identical EC50 values for the cloned and transfected bovine RXFP1 receptor (Dai et al., 2017a). B. In the presence of IBMX, the inhibitory trend just failed to achieve significance. Results are presented as means $\pm$ SEM.

4.2.3 Effect of INSL3 on cAMP production by the ovine stromal (OST) cell-line.

Also similar experiments were carried out using the OST cell-line, which from the RT-PCR experiments was shown to express only the RXFP2 receptor (see chapter 3). Unfortunately, this cell-line (Fig 4.3) grew only very slowly, with a cell doubling-time of several weeks, and prevented these experiments (assessment of cAMP production) from being repeated. The OST cells exhibited a clear inhibition of basal cAMP with INSL3, suggesting that the RXFP2 receptor in these cells is using Gi or G0 as the specific receptor-linked G- protein.

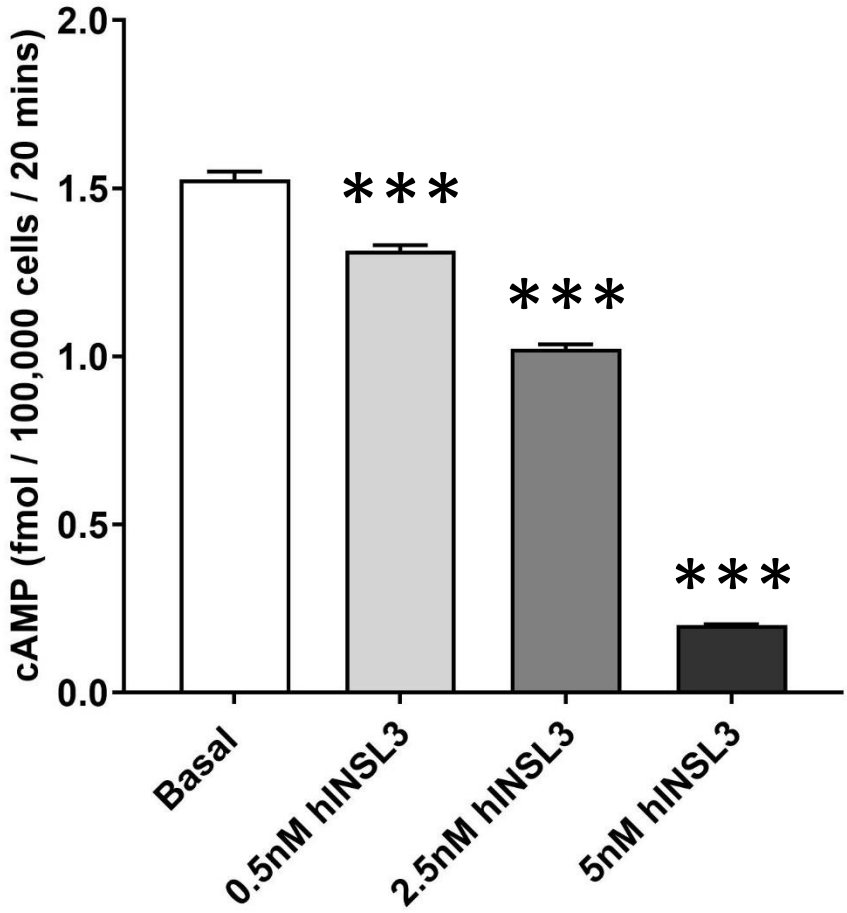


Figure 4.3. Effect of bINSL3 on cAMP production by the ovine endometrial stromal (OST) cell-line. Data from one experiment using triplicate independent wells. Results are presented as means + SEM. Bovine INSL3 evidently acts on the OST cell-line to inhibit cAMP production in the absence of either IBMX or forskolin (*** indicates result significantly different from basal; $p<0.0001$).

4.2.4 cAMP production in bovine primary myometrial cells.

Finally, as further confirmation of the findings, experiments made use of primary bovine myometrial cells. Initially cells were prepared from the uteri of slaughtered dairy cows. However, this tissue proved very inconsistent in the preparation of myometrial cells (yield and responsiveness). Later experiments exclusively used tissue from Angus beef cows, which are slaughtered as heifers at a younger age and yielded much more consistent myometrial cell preparations. The bovine myometrial cells indicated a general inhibitory effect of relaxin on basal cAMP production, similar to what was seen with bovine endometrial stromal cells at higher relaxin concentrations, suggesting a predominantly Gi or G0 linked RXFP1 receptor (Fig 4.4A-C). The inhibition was significant using the pTrend statistic on this pooled data, as well as within individual experiments (not shown). INSL3 also was applied to this culture system, and similarly suggested a Gi- linked receptor (Fig 4.4D). It is to be noted that as predicted from the more artificial, transiently transfected receptor expression constructs, bRXFP1 required a higher concentration of ligand than bRXFP2.

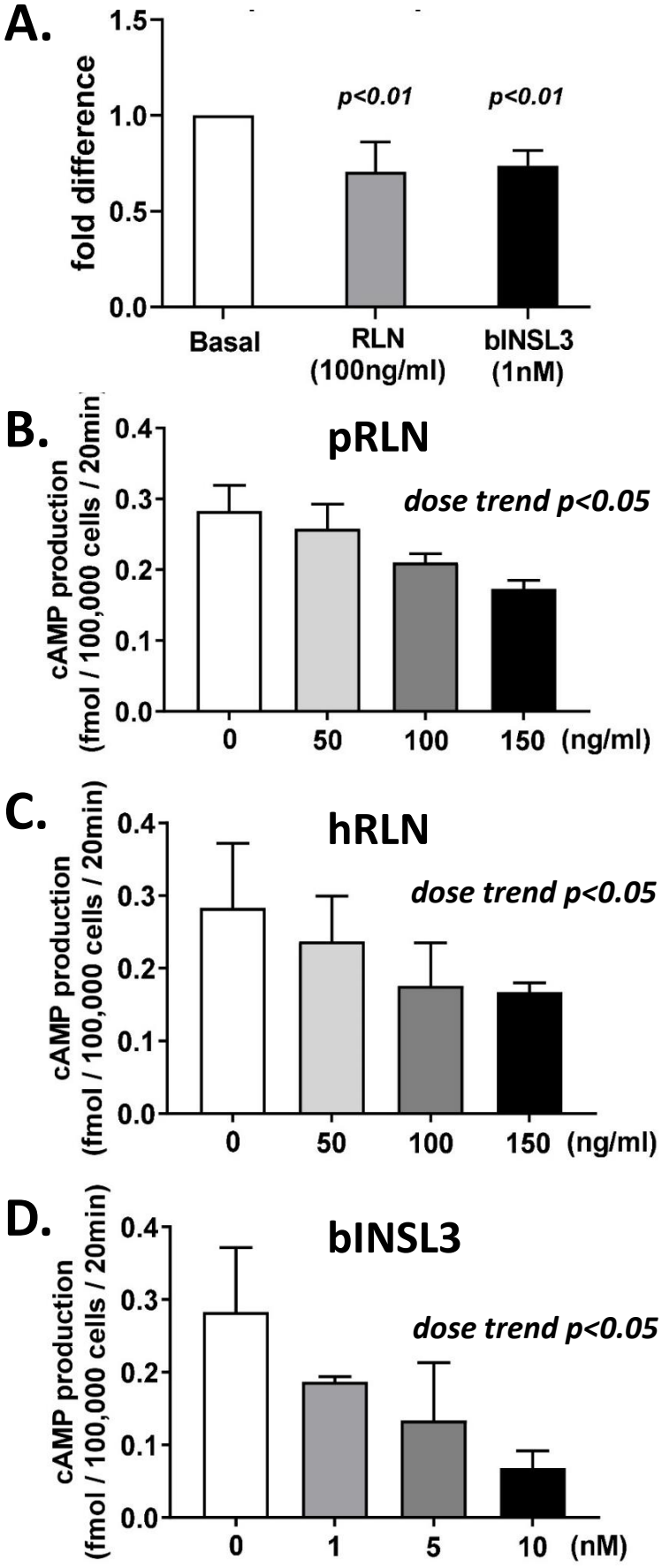

A.
fold difference
1.5
1.0
0.5
0.0
p<0.01
p<0.01
Basal
RLN
(100ng/ml)
bINSL3
(1nM)
B.
pRLN
dose trend p<0.05
cAMP production
(fmol / 100,000 cells / 20min)
0.4
0.3
0.2
0.1
0.0
0
50
100
150
(ng/ml)
C.
hRLN
dose trend p<0.05
cAMP production
(fmol / 100,000 cells / 20min)
0.4
0.3
0.2
0.1
0.0
0
50
100
150
(ng/ml)
D.
bINSL3
dose trend p<0.05
cAMP production
(fmol / 100,000 cells / 20min)
0.4
0.3
0.2
0.1
0.0
0
1
5
10
(nM)

Figure 4.4. Effect of relaxin and INSL3 on cAMP production by primary bovine myometrial cells. **A**. Summary of multiple independent experiments, using either human or porcine relaxin and bovine INSL3, at a concentration close to the EC50 for the cloned bovine RXFP1 and RXFP2 receptors. **B**. Dose-response inhibition by pRLN of cAMP production. **C**. Dose-response inhibition by hRLN of cAMP production. **D**. Dose-response inhibition by bINSL3 of cAMP production. All experiments were carried out at least in triplicate in the absence of either IBMX or forskolin. Results are presented as means + SEM.

4.3 Discussion

Following on from the RT-PCR analysis (chapter 3) which strongly supported the expression of RXFP1 and RXFP2 in a range of ruminant primary cells and cell-lines, this preliminary functional analysis appears to show that cultured bovine endometrial stromal cells (ESC) cells are able to respond to exogenous relaxin in the presence or absence of the phosphodiesterase inhibitor IBMX or forskolin with a bimodal response. At least in the primary bovine endometrial stromal cells, there is an increase in cAMP at 100ng/ml, but not at higher concentrations. In the other cell types, and at higher concentrations also in the bovine endometrial stromal cells, we see a generally inhibitory effect of relaxin and/or INSL3 upon the basal production of cAMP. This initially supports a role for relaxin, possibly in first acting at low concentrations via Gs, and then switching over to Gi or G0 as the specific receptor-linked G-protein at higher concentrations. Alternatively, the higher concentrations may be causing a desensitization/internalization of the RXFP1 receptor protein. In the sheep glandular epithelial (OGE) cell-line (OGE), as well as in primary myometrial cells there appears to be only

an inhibition of the basal cAMP production with relaxin. This might indicate that in ruminants, and unlike in other species, the RXFP1 receptor is here exclusively using Gi or G0 as the specific receptor-linked G-protein.

INSL3 was also used in the primary myometrial culture system (Fig 4.4), and similarly suggested a Gi- linked RXFP2 receptor, as also when applied to the slow-growing ovine stromal cell-line (OST), in both of which cell systems there was a significant inhibition of cAMP production.

There is little data in the literature with which to compare these results. In human endometrial stromal cells and human myometrial cells, relaxin has been shown only to stimulate the production of cAMP, with or without the addition of IBMX or forskolin (Bartsch et al., 2004; Heng et al., 2008). Whether there are shifts in G-protein coupling upon relaxin stimulation, as occurs in a time-dependent manner in the human monocytic THP1 cell-line (Nguyen & Dessauer, 2005), cannot be ascertained from these experiments, but seems plausible. INSL3 appeared to be without effect in human myometrial primary cell cultures (Heng et al., 2008).

Taken together, these results, using primary and secondary ruminant cell systems, indicate that RXFP1 and/or RXFP2 receptors are not only being expressed at the transcript level, but appear to be expressed as proteins and functionally linked with G-proteins in an appropriate fashion. That the mode of G-protein interaction is not consistently stimulatory via Gs, as mostly in other species, is to be expected, since the evolutionary loss of the natural receptor ligand in ruminants is unlikely to be without some consequences. Moreover, immortalization of cells, as in the production of the OGE cell-line, may also influence the mode of GPCR signaling. Further experimentation, preferably using primary ruminant cell culture systems, and using more temporally and pharmacologically defined signaling models, for example using pharmacological kinase inhibitors, siRNA, or mutated G-protein expression constructs, together with western blotting of diverse signaling components, will be required to examine this relaxin signaling in much more detail. Importantly, however, the present preliminary experiments underscore the fundamental functionality of RXFP1 and RXFP2 in the ruminant.

Chapter 5: Effect of the small molecule RXFP1 agonists, ML290 and analogues, on transfected human and bovine receptors

Chapter 5

Effect of the small molecule RXFP1 agonists, ML290 and analogues, on transfected human and bovine receptors

5.1 Introduction

ML290 is the first selective small molecule agonist for the human RXFP1 receptor to be identified (Hu et al., 2016; Xiao et al., 2013). It elevates cAMP levels and increases the expression of VEGF in cells that endogenously express human RXFP1 (Xiao et al., 2013). This effect is however not observed in cells that express human RXFP2 or RXFP3.The half-life of the natural relaxin hormone is less then 10 minutes (Yoshida et al., 2012). However, ML290 has a half-life of 8.56 hours in mice with no apparent toxicity (Xiao et al., 2013). ML290 is an agonist of RXFP1 in human, monkeys and pigs but has no action at the mouse orthologue (Hu et al., 2016). It does not appear to directly compete for the orthosteric ^{125}I-H2 binding to RXFP1 in humans, indicating an allosteric site of action (Xiao et al., 2013). Current research has shown that the site of binding of ML290 is in a binding pocket within the TM (transmembrane) domains with a strong hydrophobic interaction at the extracellular terminus of TM7 to form a crucial hydrogen bond interaction with G659/T660 residues of ECL (extracellular loop) 3 (Hu et al., 2016). Currently, there is no detailed information on the mechanisms of signal transduction used by ML290 in recombinant cell lines or in cells that express RXFP1.

Increased binding of ML290 to RXFP1 does not compete directly with the orthosteric binding of the natural hormone relaxin, whose binding kinetics do not appear to be affected (Fig 6.1) (Kocan et al., 2017). In HEK293 cells that express the human RXFP1 receptors, it is reported that ML290 leads to a rise in cAMP and phosphorylation of p38MAPK. However, there is no rise in cGMP or phosphorylation of ERK1/2 (Kocan et al., 2017). If ML290 is added before, the response of pERK1/2 to relaxin is increased. In smooth muscle cells and primary human vascular endothelial cells that express RXFP1 endogenously, ML290 increases the levels of both cGMP and cAMP as opposed to p-ERK1/2 (Kocan et al., 2017). ML290 increases cGMP levels in human cardiac fibroblasts but had no effect on p-ERK1/2 in these cells. When ML290 was given chronically, MMP-2 expression was activated and TGF-β1 induced Smad2 and Smad3 phosphorylation. In vascular endothelial cells, ML290 was 10 times more effective in increasing cGMP levels and p-p38MAPK than in the accumulation of cAMP (Kocan et al., 2017). ML290 results in strong coupling of RXFP1 to $G\alpha_s$ as well as to $G\alpha_{oB}$, but weaker coupling to $G\alpha_{i3}$. This signaling preference of ML290 at the human RXFP1 receptor suggests that it possesses both vasodilator and anti-fibrotic properties.

HEK293 cells transfected with human RXFP1	
Pathway affected	**Type of Impact**
cAMP	Activation
p38MAPK	Activation
cGMP	No activation
p-ERK1/2	No activation
smooth muscle cells and primary human vascular endothelial cells	
Pathway affected	**Type of Impact**
cAMP	Activation
cGMP	Activation
p-ERK1/2	No activation
human cardiac fibroblasts	
Pathway affected	**Type of Impact**
p-ERK1/2	No activation(given chronically)
MMP-2	Activation(given chronically)
TGF-β1	Inhibition(given chronically)
p- Smad2	Activation(given chronically)
p- Smad3	Activationgiven chronically)

Table 5.1. summary of ML 290 effects in different cell types

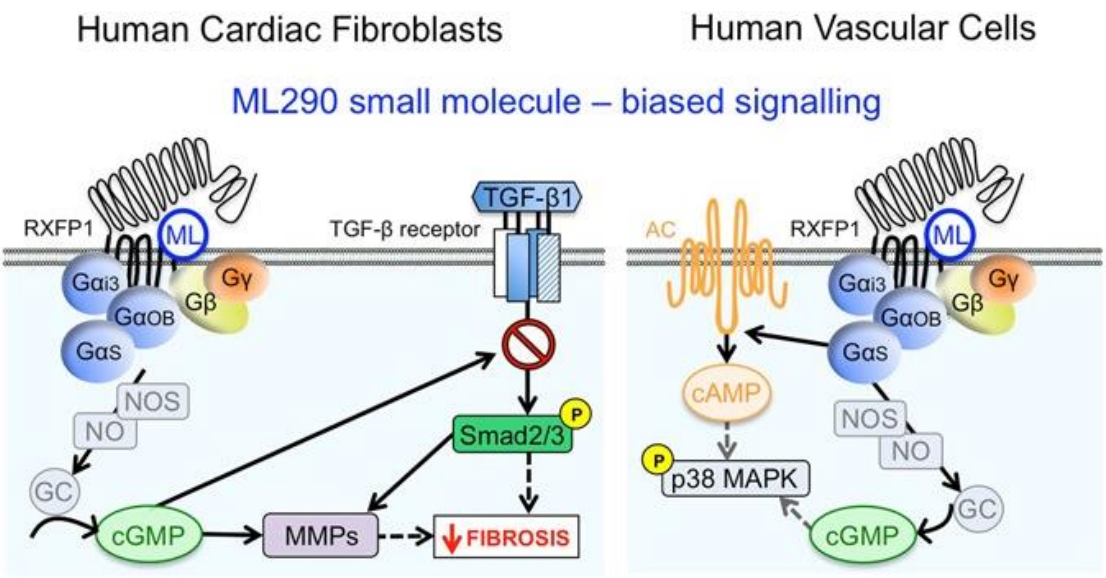


Figure 5.1. RXFP1 signalling pathways activated by ML290 in human primary vascular cells and myofibroblasts. ML290 causes anti-fibrotic activity in human primary myofibroblasts, with ML290 activating cGMP and cAMP, but not pERK (Kocan et al., 2017).

The majority of the experiments described above were carried out using the human RXFP1 receptor. It was therefore of great interest to determine whether similar effects were evident also for the bovine RXFP1 receptor. As positive control, experiments were also carried out using the human RXFP1 receptors, permanently transfected into HEK293T cells, to verify or not the previously published results. In addition to ML290 itself, three similar compounds, designed as modifications of the original lead compound (McBride et al., 2017), were also used (Table 5.1). All four compounds had been found to be active at the human RXFP1 receptor (McBride et al., 2017).

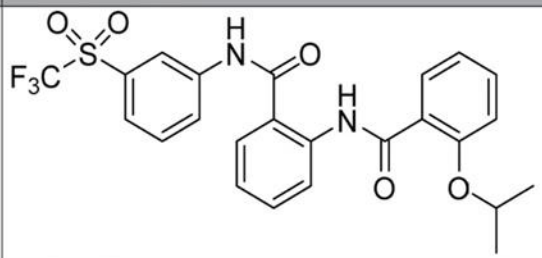
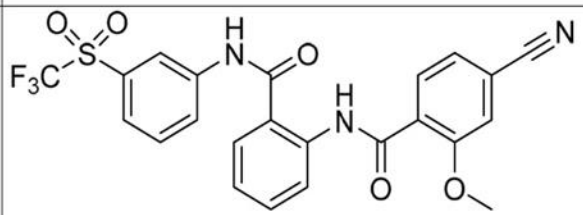
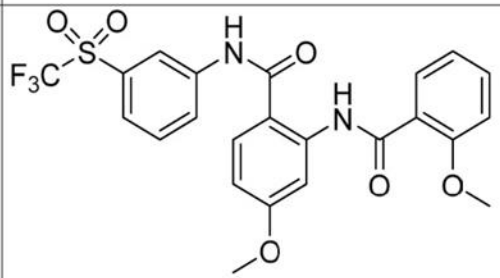
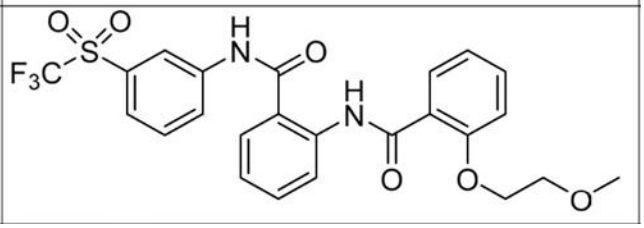
Compound Name	Chemical Structure
ML290	
Compound 1	
Compound 2	
Compound 3	

Table 5.2. Structures of ML290 and structurally derived compounds 1 to 3, according to McBride et al., 2017.

5.2 Results

5.2.1 Dose-response experiments for human recombinant relaxin in HEK293T cells permanently transfected with a human RXFP1 expression construct or transiently transfected with a bovine RXFP1 expression construct.

Figure 5.2 shows the dose-response relationships for human recombinant H2 relaxin at the human RXFP1 (hRXFP1) receptor (panel A) and the bovine RXFP1 (bRXFP1) receptor (panel B). As shown previously, relaxin reacts with hRXFP1 with high affinity to yield an EC50 in the low nanomolar range with regard to cAMP production (Dai et al., 2017b). In contrast, for bRXFP1 (panel B), human relaxin, and also porcine relaxin (not shown), indicate an EC50 for cAMP production of approximately 50nM, reflecting the absence of the hormone in the cow and consequent mutation of the receptor (Dai et al., 2017a).

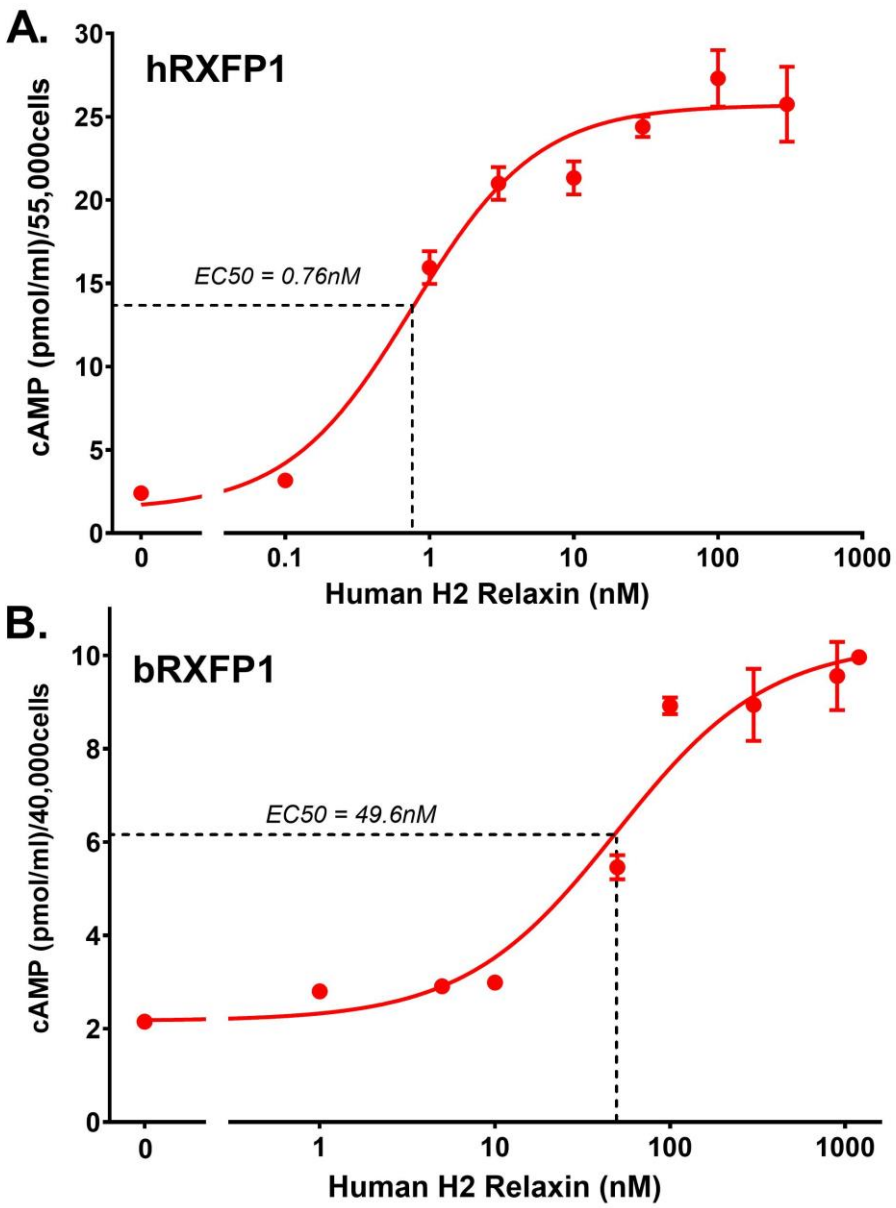


Figure 5.2. Dose-response curve for cAMP production by HEK293T cells permanently transfected with human RXFP1(hRXFP1) (panel A) or transiently transfected with bovine RXFP1 (bRXFP1) (panel B) and stimulated by increasing concentrations of recombinant human H2 relaxin. The EC50 doses are shown by the dashed lines.

5.2.2. Dose-response experiments for ML 290 in HEK293T cells permanently transfected with a human RXFP1 expression construct or transiently transfected with a bovine RXFP1 expression construct.

When these systems are tested with the four ML290 related small molecule compounds (Fig 5.3) at concentrations varying between 10 and 1000nM, there appears to be no effect whatsoever on cAMP production, with all values not significantly different from the basal responses. This was evident for both the human as well as the bovine RXFP1 receptor.

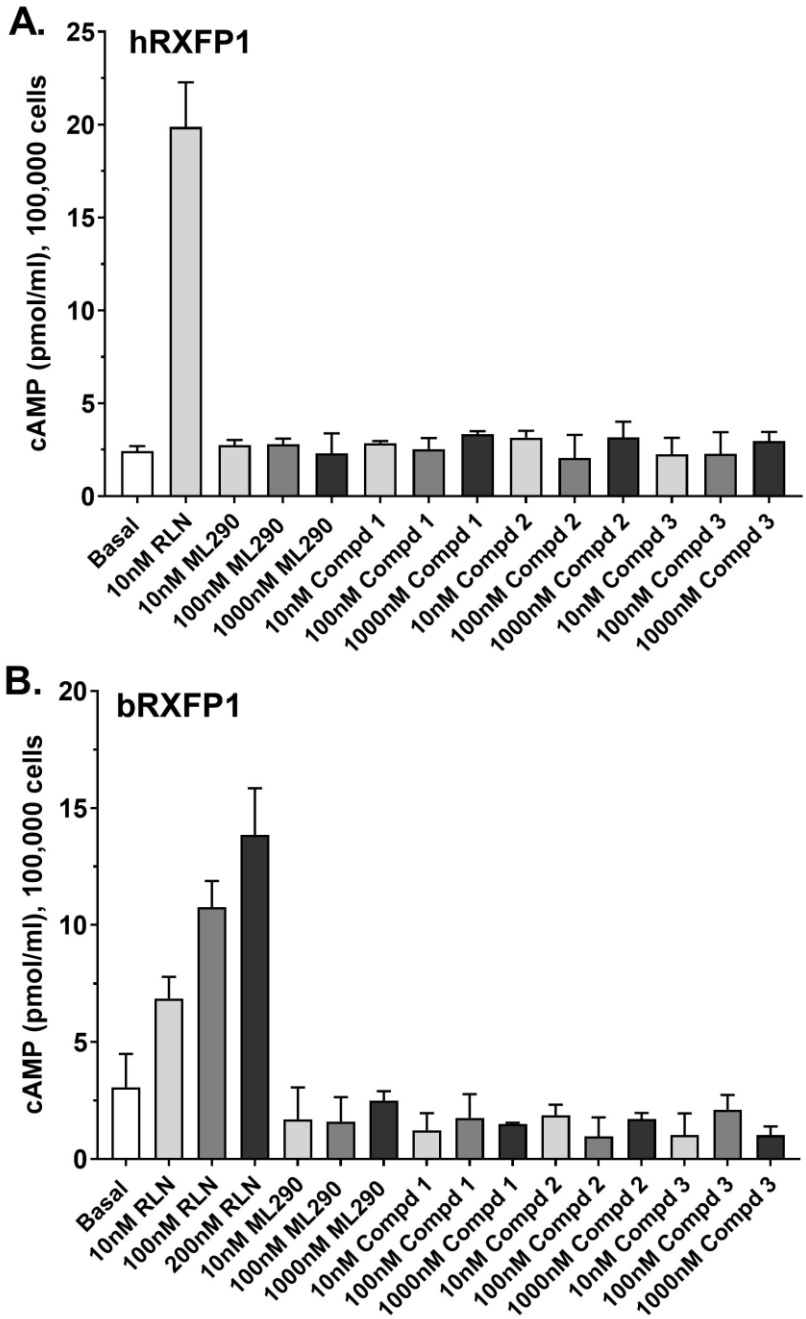


Figure 5.3. cAMP production by transfected cells by hRXFP1 in panel A and bRXFP1 in Panel B after 20min stimulation. One concentration of Relaxin was used and three different concentrations of ML290 and Compounds 1-3.The x-axis shows the concentration of cAMP in pmol/ml after 20min stimulation and the y-axis RLN, ML290 and compounds (analogues; see Table 5.1) 1-3 in different concentrations and one basal.

5.3 Discussion

The positive control experiments (Fig 5.2) clearly showed that both human and bovine RXFP1 cell systems functioned as indicated previously in response to the natural hormone relaxin on various occasions in the literature (Bartsch et al., 2001; Fei et al., 1990; Dai et al., 2017b). However, there was no effect at all of either the original small molecule compound ML290, or its derivatives, at least on cAMP production, by these systems, neither on the human receptor nor on the bovine homologue. In regard to the human receptor, this is in marked contrast to the published studies (Kocan et al., 2017; McBride et al., 2017). It is possible that other cell signaling end-points may have been positively affected, but it was not possible to test that here. Further studies are needed, including repeating these experiments using differently sourced small molecule compounds, as well as the assessment of alternative end-points.

Chapter 6: Effect of relaxin and relaxin-like peptides on gene expression by the Ovine Glandular Epithelial (OGE) cell-line and primary bovine myometrial cells.

Chapter 6

6.1 Introduction

6.1.1 The intracellular signal pathways in ruminant endometrial epithelial cells (and their surrogate OGE cells) involved in the maternal recognition of pregnancy

The OGE cell-line was created by immortalisation of primary endometrial preparations of sheep glandular epithelial cells (Johnson et al., 1999). Importantly, these cells possess many of the phenotypic characteristics of endometrial epithelial cells during the estrous cycle, which are able to respond to the production by the new embryo of the cytokine interferon-tau (IFN tau). This is part of the ruminant mechanism for the maternal recognition of pregnancy (Fig 6-1).

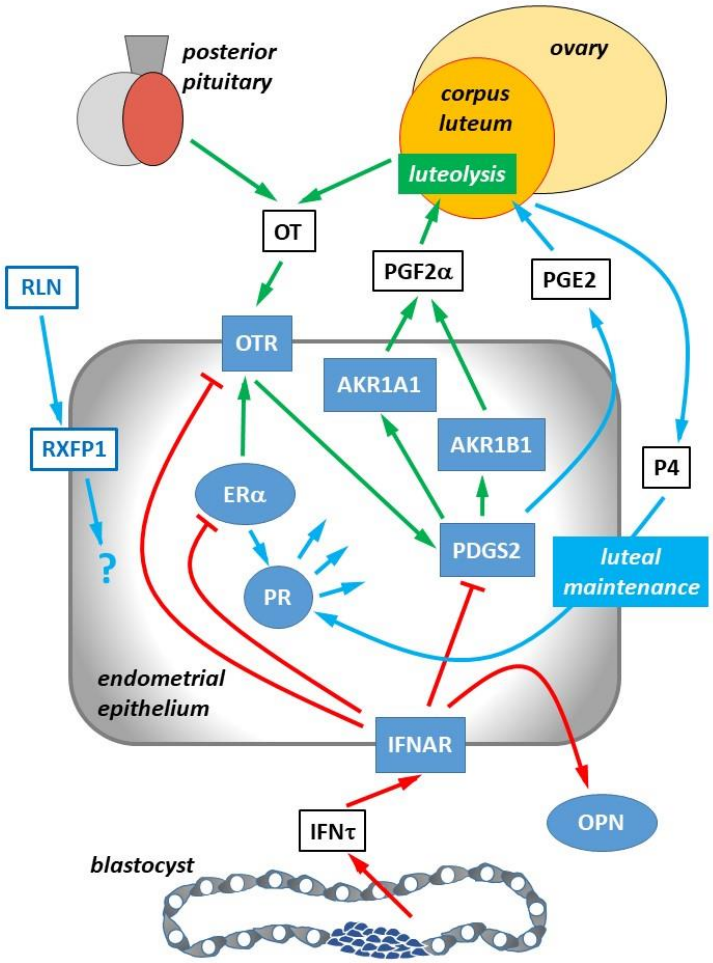


Figure 6.1. Scheme to show the intracellular pathways in ruminant endometrial epithelial cells (and their surrogate OGE cells) involved in the maternal recognition of pregnancy. In the non-conceptive cycle, oxytocin (OT) from the ovarian corpus luteum causes the formation of prostaglandin F2a (PGF2a) via the enzymes prostaglandin synthase 2 (PDGS2), and the aldoketo-reductases 1A1 and 1B1 (AKR1A1, AKR1B1). In the event of conception, interferon-tau (IFN tau) from the blastocyst activates the interferon receptor (IFNAR) which then reduces the expression of the oxytocin receptor (OTR) and estrogen receptor, ERα, shifts prostaglandin production to the luteotrophic prostaglandin E2 (PGE2), and induces the secretion of osteopontin (OPN). Not known is whether and how relaxin (RLN), acting through its specific receptor RXFP1, might influence these signalling pathways. (P4, progesterone; PR, progesterone receptor).

At the end of the normal, non-conceptive estrous cycle in ruminants the corpus luteum produces large amounts of the peptide hormone oxytocin (OT) in a pulsatile fashion (Fuchs, 1987). This OT interacts with specific receptors (OTR) on the endometrial epithelium, causing these cells to increase production of the prostaglandin PGF2a, presumably by up-regulation of the enzyme PDGS2 (prostaglandin synthase 2; also known as cyclooxygenase 2, COX2) as well as the aldoketo-reductase enzymes AKR1B1 and AKR1A1 (Bresson et al., 2011; Pépin et al., 2013). This PGF2a then acts back on the corpus luteum to induce luteolysis (the destruction of the corpus luteum) and hence a decline in the progesterone production needed to maintain uterine function. In the event of pregnancy and the formation of a blastocyst, which enters the oviduct and uterus (Fig 6.1), the blastocyst secretes very large quantities of the cytokine IFN-tau, maximally at around day 20 after fertilization. This interferon interacts with a class A interferon receptor (IFNAR) on the surface of the endometrial epithelial cells, represented by the OGE cell-line, and initiates a cascade of events leading to the down-regulation of OTR on these cells, possibly via a down-regulation of the estrogen receptor ERα (Spencer and Bazer, 2004), though possibly also directly on OTR gene transcription via IFN-responsive transcription factors (Telgmann et al., 2003). Concomitantly, there is

also a switch in prostaglandin production from PGF2a to PGE2, presumably by reducing the activity or expression of AKR1B1 and AKR1A1 (Arosh et al., 2016). Whereas PGF2a acts luteolytically on luteal cells by activating PI3-kinase signaling pathways, PGE2 acts luteotrophically by activating instead adenylyl cyclase-dependent cell systems within luteal cells. Consequently, IFN-tau leads to a persistence of the corpus luteum and the maintenance of progesterone (P4) production essential to support uterine function and early pregnancy.

Additionally, IFN-tau is also able to induce the production by the endometrial epithelium and also by the OGE cell-line of the major uterine secretory product osteopontin (OPN; also known as secretory phosphoprotein 1, SPP1). This protein plays a key role in the implantation of the blastocyst in ruminant species by promoting appropriate cell-cell interactions (Johnson et al., 2014).

Therefore, in order to test the activity and functionality of relaxin (RLN) and its specific receptor (RXFP1) in the ruminant, the OGE cell-line was used, and the transcription of some of the key genes involved in the maternal recognition of pregnancy employed as functionally relevant end-points (Fig 6.1). Synthetic human relaxin was used for these experiments, instead of porcine relaxin. Though evolutionarily more

distant from ungulate relaxin, human relaxin was found to have a similar EC50 to that for porcine relaxin when applied to transfected bovine RXFP2 receptors (Fig 5.2) (Dai et al., 2017a).

6.1.2 Intracellular signaling pathways in ruminant myometrial cells

The smooth muscle cells of the myometrium, even within the non-pregnant cycle, are subject to two contradictory physiological constraints: contraction and quiescence (Fig 6.2).

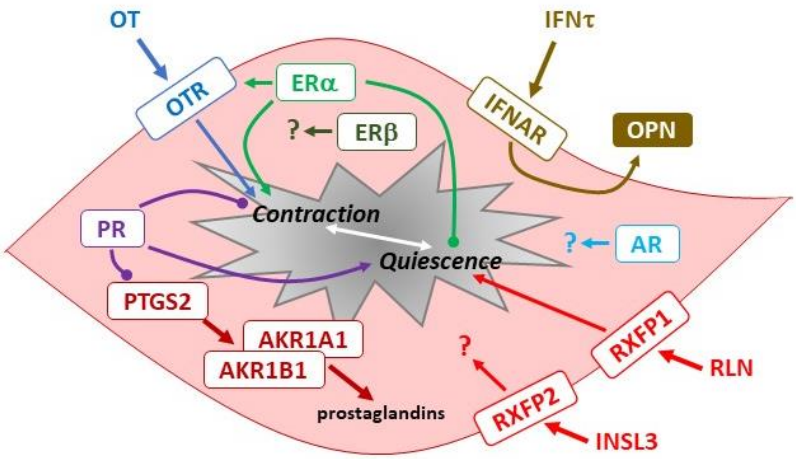


Figure 6.2. Schematised image of a typical myometrial smooth muscle cell indicating the relationships between specific gene expression and the contractile-quiescent cycle. Abbreviations are as in the legend to Fig 6.1. ? indicates a lack of knowledge of the specific roles of the receptors AR (androgen receptor), ERβ and RXFP2. Conventional arrowheads indicate stimulation, whereas the round line-heads indicate inhibition.

Regulated contraction is necessary not only for labour during pregnancy, but peristaltic contraction is implicated in sperm transport, menstrual flow in women, as well as embryo spacing in multiparous species (Hama et al., 2007; Kunz and Leyendecker, 2002; Smith and Kaunitz, 2017) . The regulation of such contraction involves essentially also regulated quiescence, inhibiting contractile signaling cascades where these are inappropriate. Simplistically, contraction of myometrial smooth muscle cells is associated with the increase and release of intracellular Ca^{2+} from the sarcoplasmic reticulum. This is particularly induced, for example during labour, by the action of oxytocin (OT) on membrane oxytocin receptors (OTR). These are GPCRs which specifically activate PI3-kinase and Ca^{2+}-linked signalling pathways. Prostaglandin PGF2a acts in a similar way. In contrast, factors which elevate intracellular cAMP, such as the hormones relaxin (RLN) and/or PGE2 appear to induce quiescence in the myometrium, particularly in rat and pig (Bani et al., 1999; MacLennan and Grant; 1991, Arosh et al., 2004), and physiologically suppress OT-induced and spontaneous contractions (Sherwood, 2004). These signaling mechanisms are also under steroidal control, with progesterone in general promoting quiescence and inhibiting contraction, though this also depends very much on the balance in protein expression between the longer progesterone receptor

(PR) variant, PRB (stimulatory), and the shorter variant PRA (inhibitory) (Kidder and Winterhager, 2015), both derived from the same gene. Estrogens, acting through either estrogen receptor ERα or ERβ, appear to support the opposite physiological state, promoting contractility and suppressing quiescence (Welsh et al., 2012). Finally, it has been recently reported that receptors for the trophoblast cytokine interferon tau (IFN-tau) are also present on myometrial cells and, just as for endometrial epithelial cells, their specific activation in early ruminant pregnancy may also lead to increased secretion of osteopontin (OPN) (Johnson et al., 2014). Consequently, for the analysis of bovine myometrial primary cell cultures, it has been possible to make use of a similar set of indicator genes and effectors to those used earlier for the analysis of endometrial epithelial cells (see above).

I have shown earlier that primary cultures of bovine myometrial smooth muscle cells express what appears to be normal, full-length RXFP1 gene transcripts, encoding the RLN receptor, and more weakly also RXFP2, the receptor for the closely related peptide hormone INSL3 (Chapter 4; Dai et al., 2017a). Therefore, in the present study, both RLN and INSL3 were used as effectors, together with OT and IFN-tau (Fig 6.2).

In preliminary studies, it was found that preparations of primary myometrial cell cultures derived from Holstein dairy cows, and acquired at the local slaughterhouse, indicated considerable inconsistency in yields, cell survival and cell physiology. This may have been due to the fact that dairy cows are usually being slaughtered for a range of reasons linked to poor uterine health, low milk yield, infertility, and age. Consequently, for all subsequent studies on bovine myometrium, uterine tissues were used which derived exclusively from young beef (Angus) heifers, which were generally healthy and slaughtered at a much younger age. The primary myometrial cells from these animals were altogether more consistent in all pragmatic culture parameters.

6.2 Results

6.2.1 Effect of relaxin on gene expression by the Ovine Glandular Epithelial (OGE) cell-line.

The transcripts investigated (Fig 6.1) are expressed in the OGE cell-line at quite different levels (Fig 6.3), with the enzymes PDGS2, AKR1A1, and AKR1B1 at relatively high abundance relative to the transcript S27a (small ribosomal protein 27a), being used for transcript normalisation (Lee et al., 2002). Although not shown here, the secreted protein OPN is similarly expressed at a high level. In contrast, the receptors OTR, ERα and PR are expressed at much lower levels, with particularly ERβ close to the limits of detection.

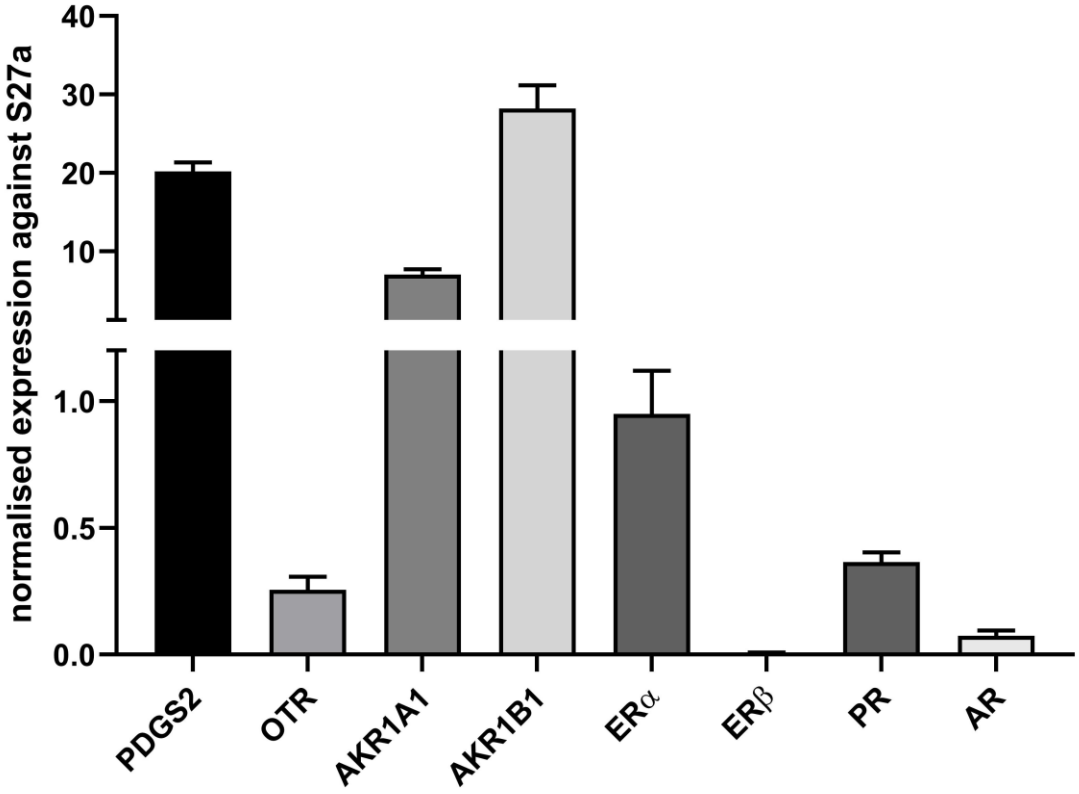


Figure 6.3. Relative transcript expression in the OGE cell-line determined by real-time quantitative RT-PCR (qRT-PCR) for key genes involved in ruminant maternal recognition of pregnancy (see Fig 6.1). PDGS2, prostaglandin synthase 2; OTR, oxytocin receptor; AKR1A1 and AKR1B1, aldoketo-reductases 1A1 and 1B1, respectively; ERα and ERβ, estrogen receptors a and b, respectively; PR, progesterone receptor; AR, androgen receptor. Results are presented as means ± SEM.

6.2.2 Time course study of specific gene expression in OGE cells under the influence of relaxin and other effectors.

In the first set of experiments, individual replicate wells of OGE cells (200,000 cells per well) were cultivated for 8 hours, 24 hours, and 48 hours before complete RNA extraction to measure specific gene expression. This time course study was essential to determine the most appropriate incubation time for subsequent experiments. As effectors, only RLN (100nM), OT (10nM) or the second messenger analogue 8Br-cAMP (1mM) were applied here. At 8 hours incubation, only OTR mRNA was found to be reduced relative to S27a (Fig 6.4), with all three effectors shown to have a similar statistically significant inhibition. At 24 hours, AKR1A1, AKR1B1 and PR were all found to be inhibited by 8Br-cAMP compared to the basal without any treatments, with RLN showing a significant inhibitory effect on PR only at this time-point. Although triplicate wells were used for each experimental paradigm, the variance between wells was still quite considerable. As a result, for several other end-points and for other effectors at 24 hours, similar reductions were also seen (e.g. for OTR, AKR1A1, AKR1B1, ERα, PR, and AR) though these did not achieve statistical significance. By 48 hours, it appears that none of the effectors showed any difference from the basal condition for any of the measured gene transcripts. It should be noted

that where RLN was shown to exert a statistically significant effect either at 8 hours (OTR) or 24 hours (PR), this was similar to the effect induced by 8Br-cAMP. Therefore, for all subsequent experiments, a time-point of 24 hours was chosen as the most likely to indicate any influence of the chosen effectors on the cultured OGE cell-line.

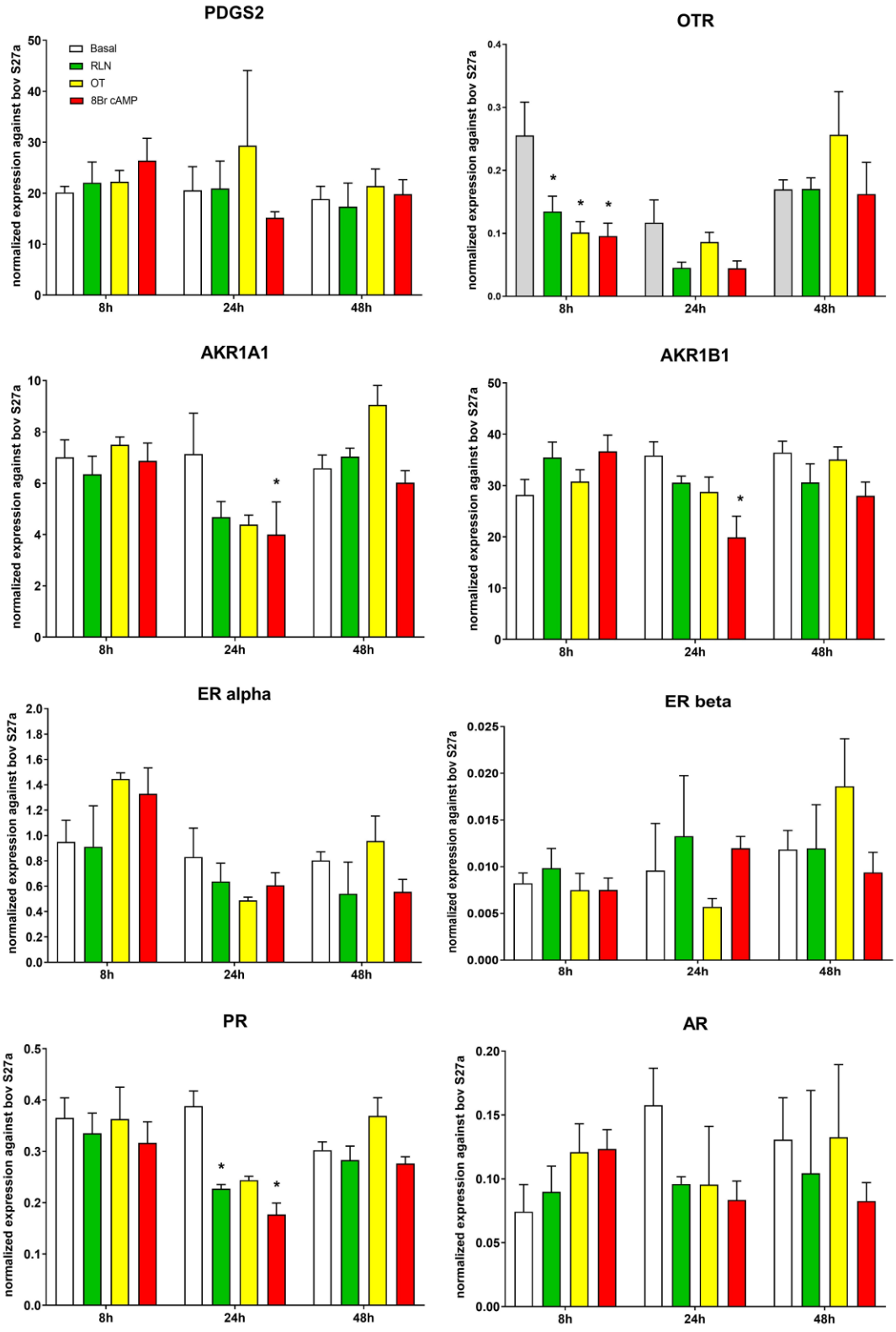

PDGS2
Basal
RLN
OT
8Br cAMP
normalized expression against bov S27a
8h
24h
48h
OTR
AKR1A1
AKR1B1
ER alpha
ER beta
PR
AR

Figure 6.4. Time course for specific transcript expression in OGE cells harvested at 8, 24 and 48 hours of cultivation with the effectors: relaxin (RLN, 100ng/ml), oxytocin (OT, 10nM), and 8Br-cAMP (1mM). Results are presented as means $\pm$ SEM. * indicates statistically significant difference from Basal for that time-point ($p<0.05$). PDGS2, prostaglandin synthase 2; OTR, oxytocin receptor; AKR1A1 and AKR1B1, aldoketo-reductases 1A1 and 1B1, respectively; ERα and ERβ, estrogen receptors a and b, respectively; PR, progesterone receptor; AR, androgen receptor.

6.2.3 Assessment in cultures of OGE cells of the functionality of Relaxin at its specific receptor RXFP1.

The second set of experiments (Fig 6.5) applied RLN, OT or IFN-tau either alone or in combination with other effectors at single optimal concentrations based on the literature (Wang and Goff, 2002; Gullam et al., 2009; Heng et al., 2008). For these experiments, after 24 hours of treatment, triplicate wells were pooled for RNA extraction, and all experiments repeated on separate occasions over a period of several months. The data in Fig 6.5 are combined from 4 fully independent experiments. As above, in spite of replication, there was still considerable variance between wells, with the result that only certain effectors and their combinations proved to have statistically significant impact. For PDGS2 only the combinations of IFN-tau and RLN, or OT and RLN indicated significantly increased expression above basal. Interestingly, the addition of RLN to IFN-tau itself caused the significant increase in PDGS2 expression, suggesting that RLN indeed is effective in these cells but only in synergy with IFN-tau. Similar results were also obtained for AKR1B1, ERα, and PR (Fig 6.5). For PR, however, it appears

that IFN-tau on its own is also effective with the additional RLN having little augmenting impact. As for the preceding experiment, between-well variance was still too high for some treatments to indicate statistical significance. So, for example, for OTR, it is interesting to note that just as previously at 8 hours both RLN and OT reduce specific gene expression relative to the basal, though just failing to achieve significance. Also, for OPN, it can be seen that RLN and IFN-tau achieve a synergy which just fails to achieve a statistically significant increase over basal (Fig 6.5).

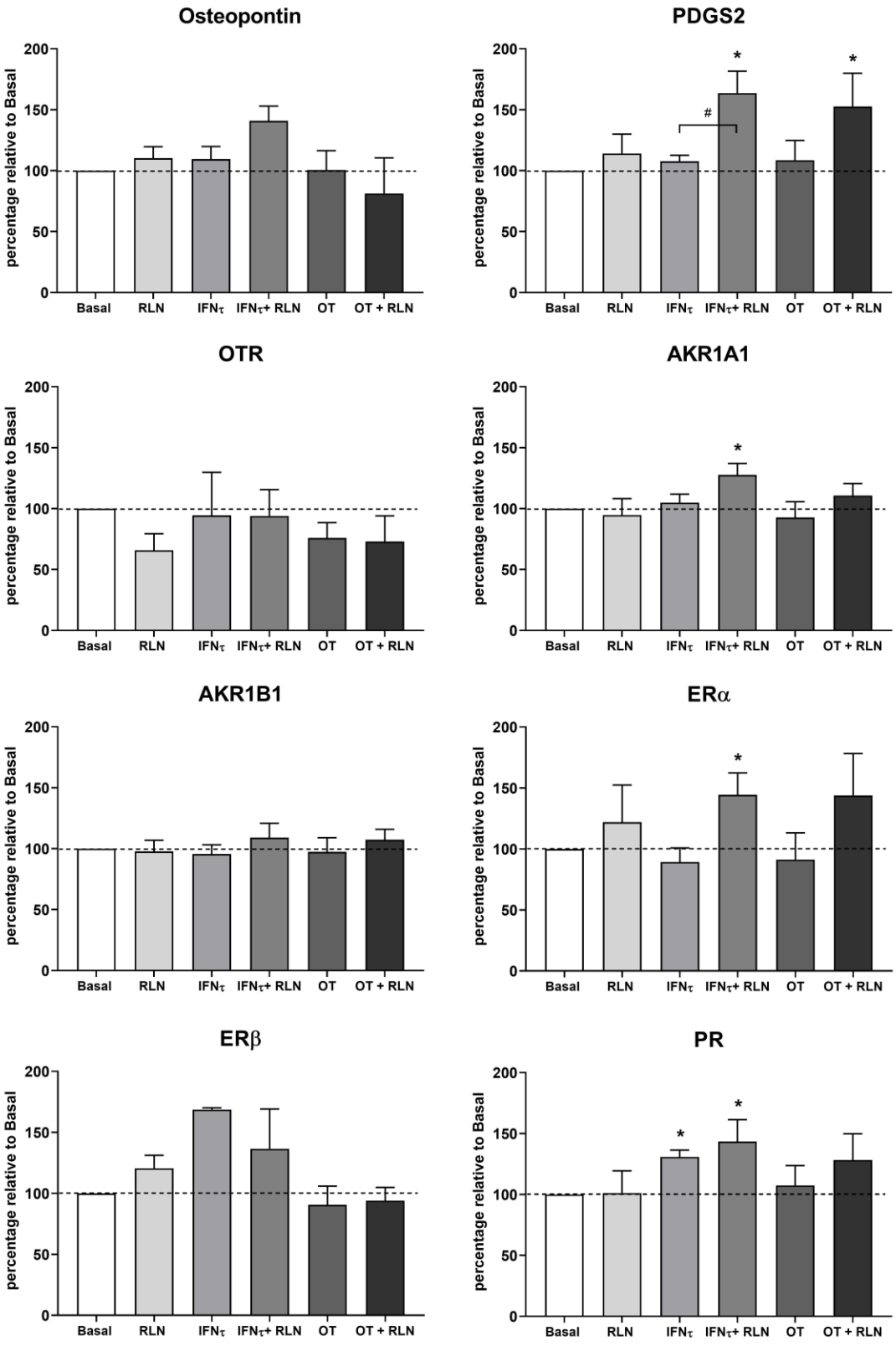

Osteopontin
PDGS2
OTR
AKR1A1
AKR1B1
ERα
ERβ
PR
percentage relative to Basal
0
50
100
150
200
Basal
RLN
IFNτ
IFNτ+ RLN
OT
OT + RLN
*
#

Figure 6.5. Specific gene transcripts measured by qRT-PCR after 24 hours of OGE cell culture, normalised to the mRNA for ribosomal protein S27a, and expressed relative to Basal conditions (100 percent). Results collated from 4 completely independent experiments and presented as means $\pm$ SEM. * indicates statistically significant difference from Basal ($p<0.05$). # indicates statistically significant difference from IFN-tau treatment alone. PDGS2, prostaglandin synthase 2; OTR, oxytocin receptor; AKR1A1 and AKR1B1, aldoketo-reductases 1A1 and 1B1, respectively; ERα and ERβ, estrogen receptors a and b, respectively; PR, progesterone receptor; AR, androgen receptor.

6.2.4 Effect of relaxin-like peptides and other effectors on myometrial cell gene expression

Quantitative RT-PCR was used to assess the levels of expression of the indicator genes identified above and which had been used in previous experiments (see 6.2.2) when assessing endometrial epithelial cells.

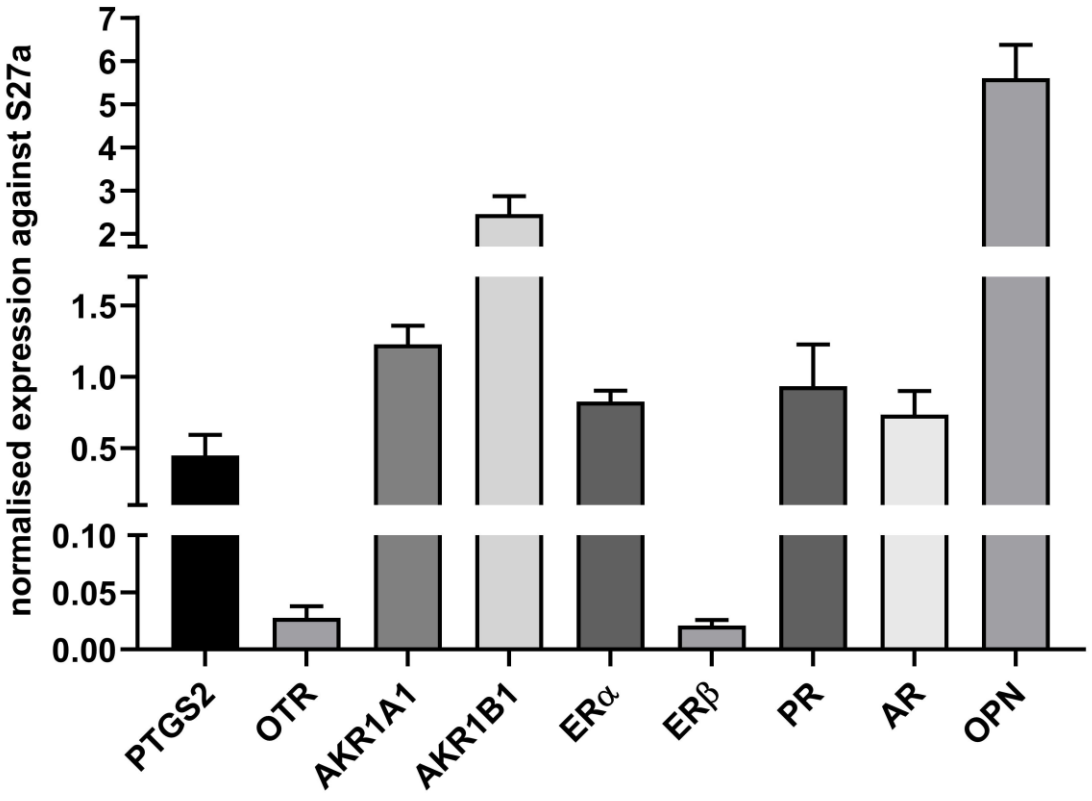
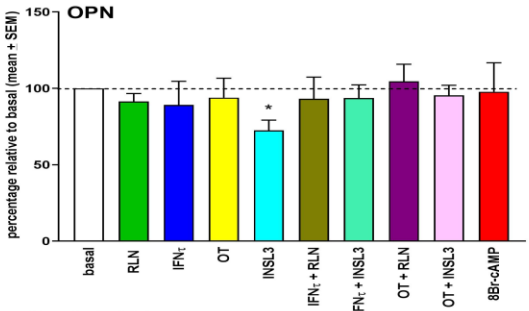


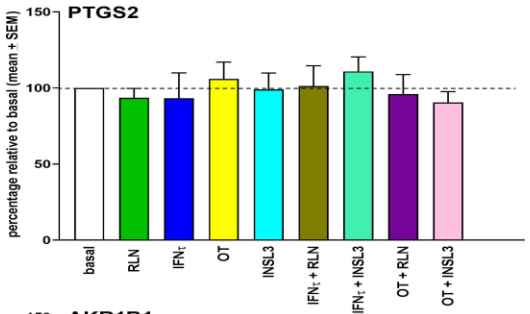
Figure 6.6 Relative expression by qRT-PCR of selected indicator genes in primary cultures of bovine myometrial smooth muscle cells harvested at 24 hours under basal conditions. Transcript quantity (mean ± SEM for 3-7 independent experiments) is expressed relative to the level of transcripts for the small ribosomal protein S27a (x10). PDGS2, prostaglandin synthase 2; OTR, oxytocin receptor; AKR1A1 and AKR1B1, aldoketo-reductases 1A1 and 1B1, respectively; ERα and ERβ, estrogen receptors a and b, respectively; PR, progesterone receptor; AR, androgen receptor; OPN, osteopontin.

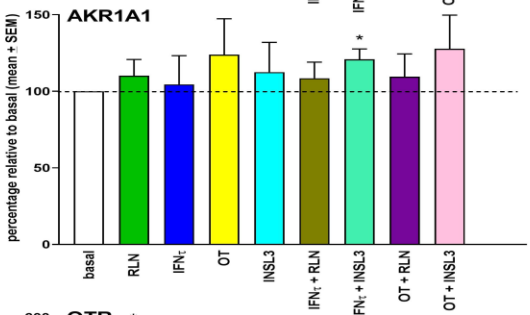
The data illustrated in Fig 6.6 are normalized relative to the expression of transcripts for the bovine small ribosomal protein S27a (Dai et al., 2017a) and represent the expression levels in the basal controls after 24 hours of culture. As expected, and as seen also for the OGE cells, the OPN and AKR1B1 genes are both highly expressed, whereas the

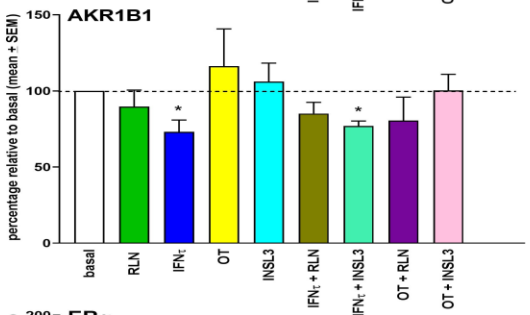
AKR1A1, PTGS2, ERα and PR genes are all moderately expressed. Also, as in the OGE cells, both OTR and ERβ genes are expressed only at relatively low levels. The androgen receptor (AR) appears to be more highly expressed in the myometrial cell cultures, compared to the OGE cell-line.

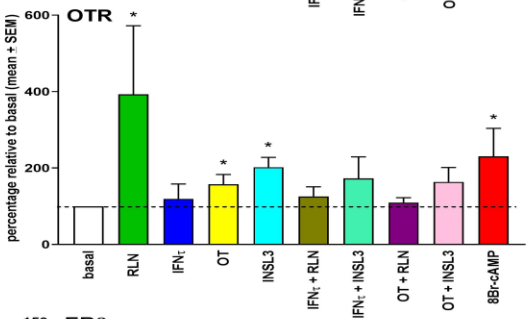
For the specific analysis of the different indicator genes, results were collated from 4 completely independent experiments, derived from a similar number of independent animals (Fig 6.7). Gene expression, normalized to S27a, is here converted to percentage of basal (100%) to allow for appropriate collation, comparison, and statistics.

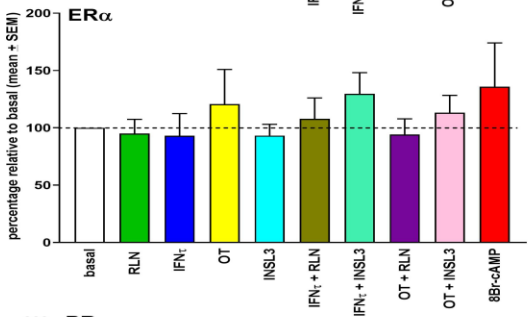

OPN
percentage relative to basal (mean + SEM)
150
100
50
0
basal
RLN
IFNτ
OT
INSL3
IFNτ + RLN
IFNτ + INSL3
OT + RLN
OT + INSL3
8Br-cAMP

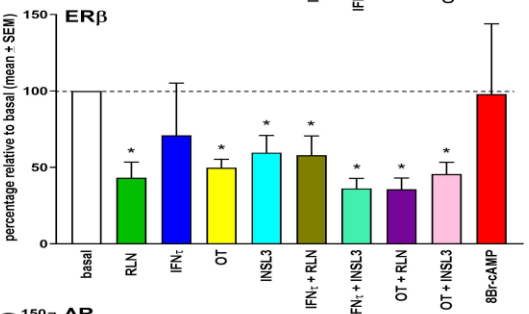

PTGS2
percentage relative to basal (mean + SEM)
150
100
50
0
basal
RLN
IFNτ
OT
INSL3
IFNτ + RLN
IFNτ + INSL3
OT + RLN
OT + INSL3

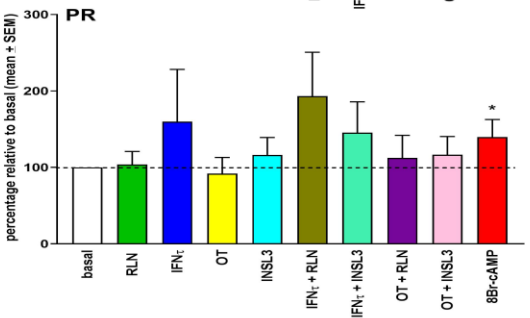

AKR1A1
percentage relative to basal (mean + SEM)
150
100
50
0
basal
RLN
IFNτ
OT
INSL3
IFNτ + RLN
IFNτ + INSL3
OT + RLN
OT + INSL3


AKR1B1
percentage relative to basal (mean + SEM)
150
100
50
0
basal
RLN
IFNτ
OT
INSL3
IFNτ + RLN
IFNτ + INSL3
OT + RLN
OT + INSL3


OTR
percentage relative to basal (mean + SEM)
600
400
200
0
basal
RLN
IFNτ
OT
INSL3
IFNτ + RLN
IFNτ + INSL3
OT + RLN
OT + INSL3
8Br-cAMP


ERα
percentage relative to basal (mean + SEM)
200
150
100
50
0
basal
RLN
IFNτ
OT
INSL3
IFNτ + RLN
IFNτ + INSL3
OT + RLN
OT + INSL3
8Br-cAMP


ERβ
percentage relative to basal (mean + SEM)
150
100
50
0
basal
RLN
IFNτ
OT
INSL3
IFNτ + RLN
IFNτ + INSL3
OT + RLN
OT + INSL3
8Br-cAMP


PR
percentage relative to basal (mean + SEM)
300
200
100
0
basal
RLN
IFNτ
OT
INSL3
IFNτ + RLN
IFNτ + INSL3
OT + RLN
OT + INSL3
8Br-cAMP

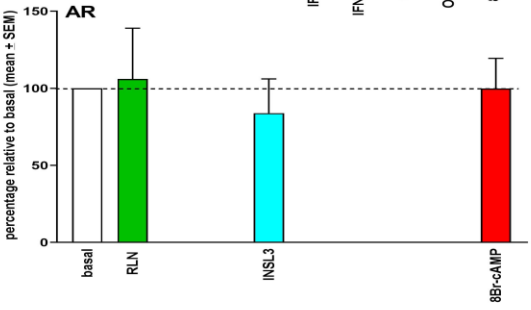

AR
percentage relative to basal (mean + SEM)
150
100
50
0
basal
RLN
INSL3
8Br-cAMP

Figure 6.7. Relative expression by qRT-PCR of selected indicator genes in primary cultures of bovine myometrial smooth muscle cells grown for 24 hours under basal conditions, or stimulated with the effectors RLN (100nM), IFN-tau (100nM), OT (1 nM), INSL3 (10 nM) or their combinations, as indicated. Results are expressed as mean + SEM from 4 independent experiments. Data are normalised against S27a gene expression and converted to percentage of the basal value. *indicates statistically significant difference ($p<0.05$) from basal. PDGS2, prostaglandin synthase 2; OTR, oxytocin receptor; AKR1A1 and AKR1B1, aldoketo-reductases 1A1 and 1B1, respectively; ERα and ERβ, estrogen receptors a and b, respectively; PR, progesterone receptor; AR, androgen receptor. For AR, not all effectors or their combinations were applied.

The principal aim of this study was to determine whether the RLN receptor, RXFP1, is still appropriately functional despite the genetic loss of its ligand. The EC50 for an exogenous ligand (e.g. porcine or human RLN) is indeed almost 2 orders of magnitude greater than in other species, presumably reflecting genetic drift towards loss of function in the absence of specific ligand (Fig 5.2; and Dai et al., 2017a). Application of synthetic human RLN at 100ng/ml to primary cultures of myometrial smooth muscle cells alone (Fig 6.7) only indicated effects on the expression of OTR (stimulatory) and ERβ(inhibitory), with no effects on any of the other measured gene transcripts. For OTR, also OT, INSL3 and 8Br-cAMP were similarly stimulatory. Since both PI3-kinase (OT) as well as cAMP (INSL3, 8Br-cAMP) appear to be equally stimulatory, this suggests a convergence of pathways, probably at the level of downstream kinases and transcription factors. This would be in agreement with an earlier study of the bovine OTR gene promoter

(Telgmann et al., 2003) in which no conventional cAMP-responsive element was found and estrogenic stimulation also appeared to be via unconventional non-canonical mechanisms.

Just as for the action of these effectors on the OTR gene, also for ERβ we see that RLN is acting in the same way as OT and INSL3, and their combinations, in this case to inhibit gene expression. Here 8Br-cAMP appears to be without effect. Besides acting on these two genes, INSL3 at 10 nM concentration also appeared to be the only factor to influence the expression of OPN (inhibition), though combined with IFNt, it interestingly resulted in a stimulation of AKR1A1 and an inhibition of AKR1B1. Also, IFN-tau alone had no effect on OPN, nor on any other of the tested genes, except for AKR1B1 (inhibition). These results are opposite to some previous studies (Johnson et al., 2000; Brooks et al., 2015), but in agreement with another previous study (Johnson et al., 2003).

Of interest, there was no significant influence of any of the tested effectors on the expression of ERα, AR, PTGS2, and PR (except for 8Br-cAMP).

6.3 Discussion

The aim of the experiments using OGE cells comprised two principal components: firstly, control experiments were carried out using effectors known to be able to influence endometrial epithelial cells in the context of the maternal recognition of pregnancy in ruminants. Thus, we can confirm that 8Br-cAMP and OT are able to reduce the expression of OTR, AKR1B1 and/or PR, or that IFN-tau is alone able to increase the expression of PR. However, we were not able to reproduce the previously documented inhibition by IFN-tau of OTR (Mann et al., 1999), though it is important to note from the literature that very high concentrations of IFN-tau are needed to see such effects, and it is possible that the concentration used in the present study was not sufficient.

The second and main purpose of these studies was to examine the functionality of RLN at its specific receptor RXFP1, which I showed earlier was expressed in the OGE cell-line probably as the full-length mRNA transcript (see chapter 3). In this context, it can be seen from the first experiment that 100nM RLN indeed significantly reduces the expression of OTR at 8 hours, as also PR at 24 hours. From the second set of experiments, whilst RLN appeared to have no statistically significant

effect on OGE cells alone at 24 hours, it indeed showed a significant effect in synergy with IFN-tau and/or OT on the expression of PDGS2, AKR1A1, and ERα. Taken together, therefore, these results do support the *in vitro* results obtained from the transiently transfected bovine RXFP1 (see chapter 5), that RLN can induce intracellular pathways within cells expressing the ruminant receptor.

The relatively high between-well experimental variance observed with these experiments using the OGE cell-line necessitates the further repetition of these studies, thereby to improve and verify the statistical validity of the findings. However, because of the Covid-19 pandemic and the resultant closure of the laboratory, this was not possible at the present time.

In the myometrium of other species, such as pig or rat, relaxin is considered to be a factor promoting quiescence (Fig 6.2). It is thus somewhat surprising to observe a stimulation of OTR gene transcription and a reduction in ERβ mRNA. If ERβ acts like ERα to stimulate OTR expression (Telgmann et al., 2003), then this would suggest a contradictory mechanism. However, there is no information at all on the targets of ERβ in the myometrium, or whether these differ from those for ERα. Further experimentation would be necessary to elucidate such

information. However, it is consistent that the factors which all stimulate OTR all have the opposite effect on ERβ, suggesting a coherence to the mechanisms involved. Interestingly, IFN-tau does not behave at all like these factors, being notably without effect on these genes. The opposite is true for AKR1B1; here IFN-tau is significantly inhibitory alone or with INSL3, whereas OT, RLN and INSL3 alone are all without effect. This supports strongly the notion that the two categories of effectors are operating through quite discrete and non-overlapping signaling pathways in these cells.

In many ways, it is a comforting result that for most genes and treatments there is no significant change in expression. This emphasizes the technical accuracy of experimentation, and thereby highlights the few results which are indeed significantly different from basal. There is nevertheless some degree of variance that might be reduced with further experiments, which might show up additional significant effects.

Comparing the primary cultures of myometrial cells with the previous OGE cell-line, both subjected to a similar set of effectors, it is very clear that there are some marked similarities and differences. For example, in the OGE cells at 8 hours of incubation, OTR is significantly inhibited by RLN, OT and 8Br-cAMP, whereas for all the same effectors OTR is

stimulated in the myometrial cells at 24 hours. Thus, while the impact is opposite, the pathways being used are evidently similar and shared in common between RLN, OT and 8Br-cAMP. In a similar vein, PR is inhibited at 24h by 8Br-AMP in OGE cells, but significantly stimulated at 24 hours in the myometrial cells. Interestingly, IFN-tau stimulates PR gene expression in OGE cells, and similarly increases PR expression in myometrial cells (though not quite reaching significance). This reinforces the notion that IFN-tau is using a quite discrete signaling system from the other effectors.

What is evident from the preliminary results so far obtained, is that OTR, PR, and possibly ERβ, are the three genes most likely to be influenced by RLN, and which need further exploration. These new experiments would need to assess both dose- and time-responsiveness, as well as possible interactions with steroid hormones as additional effectors. Moreover, it would be interesting to see for PR, whether the ratio of PRA to PRB (by western blot) is also influenced by RLN stimulation.

Chapter 7: Application of global transcript analysis to determine novel relaxin targets in the uterus.

Chapter 7

Application of global transcript analysis to determine novel relaxin targets in the uterus.

7.1 Introduction

The preceding chapters have indicated that, although certain gene transcripts were found to be up- or down-regulated in either the OGE cell-line or in bovine primary myometrial cells upon relaxin stimulation, the number of significant end-points was relatively small. Much of what we know about the influence of relaxin on physiology and gene expression stems from earlier work on the role of relaxin in fibrosis (Bennett, 2009) or in regulating the cardiovascular system (Sarwar et al., 2017). This is summarized in the KEGG pathway for RXFP1 signalling (Fig 7.1) registered with the international KEGG database. However, uterine physiology may involve many other specialist pathways, not previously identified in other contexts. In order to identify further relaxin-dependent endpoints which would indeed be relevant for uterine physiology, also in the cow, archived RNA samples from previous studies of uterine cells and tissues in the Anand-Ivell laboratory, have been made use of, retrospectively re-analyzing these using either modern RNA-Seq technology or older microarray analysis in order to obtain a global profile of transcriptome changes consequent upon relaxin stimulation.

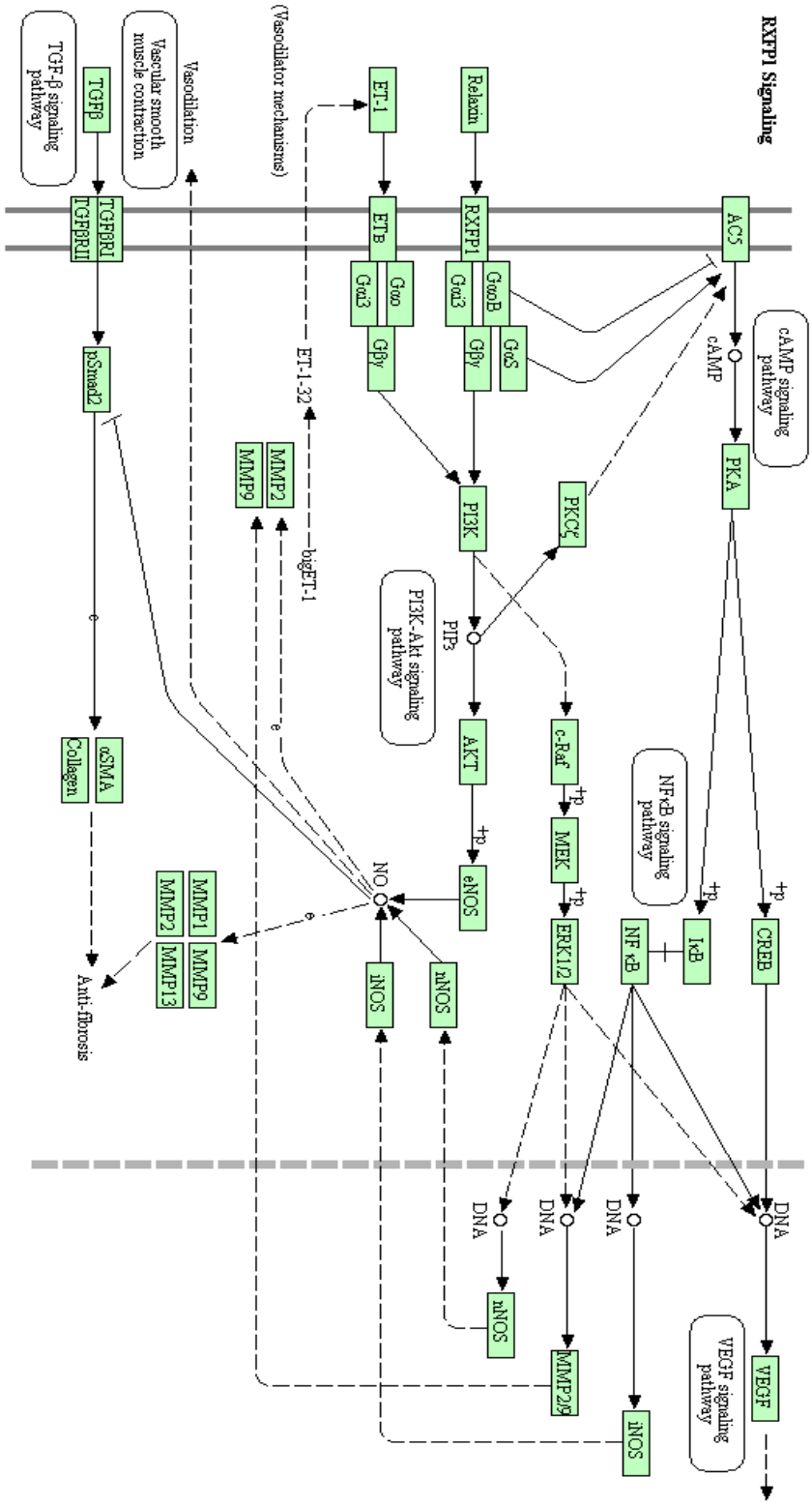


Figure 7.1 The KEGG pathway for RXFP1 signalling registered with the international KEGG database

In the first study, wild type mouse uterus from the time of estrus was subjected *in vivo* to intrauterine gavage with a physiological concentration of human relaxin (Glynn et al., 2017) . 10 hours later the uterus was removed and subjected to RT-PCR and protein analysis for a selected cytokine, CXCL1 (alternatively known as KC). In other, related studies using relaxin knockout mice it had been shown that relaxin was able to influence a number of proinflammatory cytokines linked to the uterine immune response to mating and pregnancy (Glynn et al., 2017). Importantly, the relaxin applied in the uterine gavage was able to influence not only uterine luminal epithelial cells but also underlying endometrial glandular and stromal cells, and probably also cardiovascular and myometrial tissues. Furthermore, immunohistochemistry using two different immune cell markers, as well as RT-PCR for CD45, showed that within the time-period of the experiment there had been no influx of extra-uterine immune cells (Glynn et al., 2017), so that all transcript responses identified can be assumed to originate from the uterine cells themselves.

The second, older study involved the culture of primary endometrial stromal cells derived at hysterectomy from women being operated to resolve uterine pathology (fibroids, endometriosis). These relatively pure primary cultures respond to relaxin by up-regulating cAMP and

undergoing decidualization *in vitro*, very similar to that which occurs *in vivo* during the luteal phase under the influence of progesterone (Bartsch et al., 2004; Anand-Ivell et al., 2007). Primary stromal cell cultures were stimulated overnight with relaxin, then RNA prepared and initially used for RT-PCR for the key endometrial products prolactin and IGFBP1. In culture, it can be observed that the cells stop dividing, lose their fibroblast-like morphology and acquire the typical polygonal appearance of decidualized cells.

Although properly stored at -80C for several years, all RNA was checked for integrity before subjecting to RNA-Seq (mouse uterus) or GeneChip microarray (human endometrial stromal cells). The resulting transcriptome profiles were analyzed using bioinformatic procedures precisely to identify novel statistically significantly altered physiological pathways, rather than to focus on individual gene products for which at this time there would be little or no physiological context, especially in regard to translating results to the ruminant uterus. Moreover, the identification of a pathway, comprising at least two or more altered gene transcripts offers greater statistical robustness in regard to its interpretation, than might a single altered gene out of context.

7.2 Results

7.2.1 Significantly altered transcript pathways in mouse uterus subjected *in vivo* to relaxin.

Four RNA replicates derived from four individual mice whose uteri were treated with relaxin were compared with a similar number of vehicle-treated uteri. The RNA samples were then subjected to RNA-Seq analysis (Genewiz GmbH, Leipzig, Germany) with more than 20-fold genome coverage. The resulting reads were then quantitatively analyzed to yield a file of significantly up- or down-regulated gene transcripts. Since individual gene identities were difficult to allocate to specific physiological pathways, it was decided to focus only on those significantly regulated genes which were recognizably part of previously described pathways (comprising a minimum of two significantly altered transcripts) as listed in the KEGG database. This analysis made use of the NIPA software for Integrated Pathway Analysis (courtesy Professor Richard Emes, Nottingham University). The identified and significantly regulated KEGG pathways are listed in Table 7.1. Pathways are sorted based on statistical probability (p value; pval). It should be noted that the titles given to each pathway are of less interest than the fact that these transcripts have been shown to be co-regulated in other

previously published contexts. It is also to be noted that many of the pathways overlap with one another, including several genes in common.

Of the up-regulated genes, the most prominent pathways relate to cytokines, chemokines and their receptors, reinforcing the notion of relaxin as part of the uterine cytokine response in pregnancy. Also significant, and as identified already in Fig 7.1, we see the up-regulation of the NF-kappa B and Nos2-linked signalling pathways, as well as pathways involving Ptgs2 and prostaglandin production. Of particular interest are also the activation of the osteoclast differentiation and the Toll-like receptor signaling pathways, both markers for cell differentiation in response to the changing uterine cycle and early pregnancy. Finally, the increase in certain cell adhesion molecules should be noted: a further indication of altered uterine differentiation.

Of the significantly down-regulated gene pathways, the majority are associated with the cell cycle, indicating a cessation of cell proliferation and a shift to cell differentiation. Of interest we also see a link to progesterone- and p53-mediated pathways which lead to cell arrest (Engeland, 2018), both of which are known to be involved in uterine differentiation in the human, as also the FoxO pathway (Lynch et al., 2009; Gellersen and Brosens, 2014).

Table 7.1 Different KEGG pathways in mouse uterus exposed to relaxin in vivo

KEGG Pathway UP

Pathway	pval	External.ids
mmu04657 IL-17 signaling pathway	8.93E-15	S100a8, S100a9, Csf2, Ptgs2, Ccl7, Cxcl5, Cxcl3, Cxcl1, Mapk11, Il1b
mmu04668 TNF signaling pathway	1.04E-13	Vcam1, Csf2, Ptgs2, Cxcl5, Cxcl3, Cxcl1, Mapk11, Ccl5, Fas, Il1b
mmu04060 Cytokine-cytokine receptor interaction	8.39E-13	Csf2, Il27, Ccl7, Cd40, Cxcl5, Cxcl3, Cxcl1, Tnfrsf11b, Il1rn, Ccl5, Ccl4, Fas, Il1b
mmu04064 NF-kappa B signaling pathway	1.15E-10	Vcam1, Ptgs2, Cd40, Cxcl3, Cxcl1, Bcl2a1a, Ccl4, Il1b
mmu05140 Leishmaniasis	2.82E-09	Fcgr4, Ptgs2, Ncf4, Mapk11, Nos2, Il1b
mmu05323 Rheumatoid arthritis	1.34E-08	Csf2, Cxcl5, Cxcl3, Cxcl1, Ccl5, Il1b
mmu05167 Kaposi's sarcoma-associated herpesvirus infection	3.79E-08	Csf2, H2-T22, Ptgs2, Cxcl3, Cxcl1, Mapk11, Fas
mmu04380 Osteoclast differentiation	2.12E-07	Fcgr4, Ncf4, Tnfrsf11b, Nox1, Mapk11, Il1b
mmu04620 Toll-like receptor signaling pathway	8.91E-07	Cd40, Mapk11, Ccl5, Ccl4, Il1b
mmu05142 Chagas disease (American trypanosomiasis)	1.06E-06	Mapk11, Ccl5, Nos2, Fas, Il1b
mmu04514 Cell adhesion molecules (CAMs)	1.5E-06	Vcam1, Cldn1, H2-T22, Cd40, Cd274

KEGG Pathway DOWN

Pathway	pval	External.ids
mmu04110 Cell cycle	8.58E-12	Ccnb2, Ttk, Plk1, Ccnb1, Ccna2, Cdc25c, Cdkn2a, Bub1, Bub1b, Cdk1
mmu04914 Progesterone-mediated oocyte maturation	2.63E-10	Ccnb2, Aurka, Plk1, Ccnb1, Ccna2, Cdc25c, Bub1, Cdk1
mmu04114 Oocyte meiosis	3.23E-09	Ccnb2, Sgo1, Aurka, Plk1, Ccnb1, Cdc25c, Bub1, Cdk1
mmu04115 p53 signaling pathway	1.65E-05	Ccnb2, Ccnb1, Cdkn2a, Cdk1
mmu04218 Cellular senescence	1.97E-05	Ccnb2, Foxm1, Ccnb1, Ccna2, Cdkn2a, Cdk1
mmu00512 Mucin type O-glycan biosynthesis	0.000259	Galnt5, Galnt13
mmu04068 FoxO signaling pathway	0.000305	Ccnb2, Plk1, Ccnb1, Prkaa2
mmu01524 Platinum drug resistance	0.000412	Top2a, Birc5, Cdkn2a
mmu03460 Fanconi anemia pathway	0.00153	Fanci, Fancd2
mmu05166 HTLV-I infection	0.004934	Ccnb2, Ccna2, Cdkn2a, Bub1b
mmu04512 ECM-receptor interaction	0.007158	Lama1, Hmmr
mmu05410 Hypertrophic cardiomyopathy (HCM)	0.007849	Lama1, Prkaa2

Table 7.1. KEGG pathways are indicated by their code and name as first identified for the compilation of the KEGG database. pval indicates the probability of the pathway being selected by chance and is a better measure of the relevance of the involved gene transcripts in uterine physiology than transcript amount or fold-change. Only pathways with a pval $\leq$ 0.01 are shown. The External.ids indicate the individual transcript identities within the pathways which are significantly altered comparing relaxin-treated with vehicle-treated controls.

7.2.2 Significantly altered transcript pathways in human endometrial stromal cells in response to relaxin.

Far fewer gene transcripts can be identified using the older GeneChip microarrays, which made use of only approximately 20,000 oligonucleotide sequences to identify expressed gene transcripts. Nevertheless, also for these relatively pure cultures of endometrial stromal cells (4 replicates each for treatment and controls), we see similar patterns of significant gene expression (Table 7.2) when analyzed using the NIPA software, looking for significantly altered KEGG pathways. In particular, we should note the up-regulation of the prolactin-, estrogen-, and chemokine-signaling pathways, as well as those involved in cAMP- and prostaglandin-signaling, all of which are linked to protein secretion and cell differentiation. Also, and just as for the mouse uterus, relaxin induces a considerable reduction in expression of those genes involved in the cell cycle and proliferation (Table 7-2), as well as p53-mediated and FoxO signaling and cAMP pathways (Gellersen and Brosens, 2014). And we see the up-regulation of the leucine and isoleucine degradation pathway which plays a role in the receptive goat endometrium (Zhang et al., 2017). The analysis also revealed the up-regulation of protein processing in the endoplasmic reticulum pathway. It has been suggested that endoplasmic reticulum stress plays a role in the endometrial changes during the menstrual cycle (Guzel et al., 2017)

Table 7.2 Different transcript pathways in human endometrial stromal cells exposed to relaxin.

KEGG Pathway UP

Pathway	pval	External.ids
hsa00280 Valine, leucine and isoleucine degradation	0.000117	HMGCS1, ALDH2
hsa04141 Protein processing in endoplasmic reticulum	0.000324	CRYAB, HSPA2, DNAJB11
hsa04360 Axon guidance	0.000449	CXCL12, WNT5A, TRPC6
hsa04612 Antigen processing and presentation	0.000494	LGMN, HSPA2
hsa04622 RIG-I-like receptor signaling pathway	0.00036	ISG15, IFIH1
hsa04917 Prolactin signaling pathway	0.00036	CGA, SHC2
hsa04915 Estrogen signaling pathway	0.002563	SHC2, HSPA2
hsa05162 Measles	0.002563	IFIH1, HSPA2
hsa05224 Breast cancer	0.003063	SHC2, WNT5A
hsa05034 Alcoholism	0.005731	SHC2, HDAC4
hsa04062 Chemokine signaling pathway	0.006171	SHC2, CXCL12
hsa05205 Proteoglycans in cancer	0.00761	TIMP3, WNT5A
hsa01100 Metabolic pathways	0.008396	DPM3, HMGCS1, TPK1, DHRS3, PDE4B, PTGDS, ALDH2
hsa04024 cAMP signaling pathway	0.008892	CGA, PDE4B

KEGG Pathway DOWN

Pathway	pval	External.ids
hsa04110 Cell cycle	2.82E-10	CDC45, MCM5, MCM3, CDC20, PTTG1, GADD45B, ORC6
hsa01230 Biosynthesis of amino acids	6.3E-07	PSAT1, TKT, SHMT2, CS
hsa01200 Carbon metabolism	5.7E-06	PSAT1, TKT, SHMT2, CS
hsa04115 p53 signaling pathway	1.86E-05	RRM2, GADD45B, SERPINE1
hsa00630 Glyoxylate and dicarboxylate metabolism	3.79E-05	SHMT2, CS
hsa03030 DNA replication	6.61E-05	MCM5, MCM3
hsa00260 Glycine, serine and threonine metabolism	9.09E-05	PSAT1, SHMT2
hsa00240 Pyrimidine metabolism	0.000262	RRM2, TK1
hsa03018 RNA degradation	0.000685	EXOSC9, HSPD1
hsa05212 Pancreatic cancer	0.000612	RAD51, GADD45B
hsa05220 Chronic myeloid leukemia	0.000612	GADD45B, SHC1
hsa04068 FoxO signaling pathway	0.002928	KLF2, GADD45B
hsa04024 cAMP signaling pathway	0.011629	OXTR, SSTR1

Table 7.2. KEGG pathways are indicated by their code and name as first identified for the compilation of the KEGG database. pval indicates the probability of the pathway being selected by chance and is a better measure of the relevance of the involved gene transcripts in uterine physiology than transcript amount or fold-change. Only pathways with a pval $\leq$ 0.01 are shown. The External.ids indicate the individual transcript identities within the pathways which are significantly altered comparing relaxin-treated with vehicle-treated controls.

7.3 Discussion

Although the tissues, cells and species are quite different in the two sets of global transcript changes that are seen upon relaxin stimulation, there are indeed considerable areas of overlap which give confidence to the extrapolation of the identified genes and pathways to assess the cells of the ruminant uterus. Moreover, it is useful to note that there are few overlaps between the pathways identified and those explored on an ad hoc basis in the preceding analyses of OGE and myometrial cells. This provides a degree of optimism to future analyses for novel target gene transcripts of the latter ruminant cells. It is to be noted that the many pathways have immune roles which may help in the context of implantation sites (Mor et al., 2011).

Of the potential relaxin-responsive target genes highlighted by the above global transcriptome studies that could be used for ruminant cells and tissues, in addition to those already identified, such as OTR, one could suggest using a selection of uterine chemokines or cytokines, such as CXCL1, CXCL3 or CXCL12 as well as a differentiation marker such as WNT5a. Additionally, since a shift from proliferation to differentiation appears to be a hallmark of relaxin action in uterine tissues, then a selection of cell cycle markers, such as CDC20 or CDK1 might be

appropriate. Furthermore, since the KEGG pathway analysis reinforces the role of NF-kappa B and NOS pathways (Fig 7.1) in the actions of relaxin, known targets of these expressed specifically within myometrial or epithelial cells should also be explored. The completion of this project is being pursued by other members within the Anand-Ivell laboratory.

Chapter 8: Discussion.

Chapter 8

8. Discussion

The incidence of dystocia is observed to be relatively high in cattle (Mee, 2008) as well as in sheep (Jacobson et al., 2020) compared to pigs or other species. Dystocia is defined as a difficulty in giving birth often due to an insufficiency of pelvic and/or cervix distension at the time of labour and can lead to substantial perinatal and maternal mortality. Because the condition of dystocia in other species such as the pig can be improved by administration of the reproductive hormone relaxin (RLX), it was logical to investigate the expression and role of relaxin also in ruminants (reviewed in (Hartung et al., 1995)). In addition to parturition-related incidents, high performance dairy cows in particular have been shown to be increasingly subject to poor fertility largely due to endometrial incompetence and accompanying implantation failure or miscarriage (Walsh et al., 2011). These are all symptoms which in other species, such as the human, have been linked to the action of relaxin produced by the ovary, endometrium and/or placenta (Anand-Ivell & Ivell, 2014). The notion that the poor reproductive performance in ruminants might be linked to the physiology of relaxin in those species was confirmed recently when it was shown by synteny analysis that the gene encoding relaxin was partially or completely deleted in sheep and cows (Dai et al., 2017). However, this lack of relaxin expression does

not preclude a consideration of using exogenous relaxin as a therapeutic option in the treatment of these conditions in ruminants. The aim of the present study was to investigate firstly the expression and functionality of the potential relaxin receptors bRXFP1 and bRXFP2 in cells and tissues of the female ovine and/or bovine reproductive systems, and to determine how these might be regulated by application of exogenous hormones.

8.1 Expression of RXFP1 and RXFP2 in uterine cells and tissues

As a first estimate for the expression of functional RXFP1 and RXFP2 receptors, we used RT-PCR to identify RNA transcripts. Real-time PCR results indicated that the RXFP1 gene is abundantly expressed in the sheep glandular epithelial cell-line (OGE), though not in either an ovine endometrial stromal cell-line (OS) or the corresponding luminal epithelial cell-line, OLE. However, RXFP1 RNA was identified in primary bovine endometrial stromal cells (see chapter 3). Mazella et al (2004) also noted the expression of RXFP1 in glandular epithelial cells in the human and observed lower expression in stromal cells than glandular cells. This conflicts somewhat with studies using specific RXFP1 and RXFP2 antibodies in human uterine tissues where most RXFP1 expression was observed in stromal cells, with less in the glandular epithelium (Ivell et al., 2003).

RXFP1 RNA was also identified in primary bovine myometrial cells, consistent with its physiological role in other species to maintain uterine quiescence (Griss et al., 1967; MacLennan et al., 1986) This is similar to the findings from human myometrial cells (Heng et al., 2008). Taken together, these results largely reflect what is known about the physiology of relaxin from other species and are in agreement with the observations of RXFP1 transcript expression in bovine endometrial tissue fragments by Dai et al (2017a). Future studies should additionally use immunohistochemistry and/or western blotting to confirm such receptor expression at the protein level.

Regarding RXFP2, transcript expression for this receptor gene was observed in primary bovine endometrial epithelial cells. This is similar to the findings from mouse endometrial epithelial cells (Li et al., 2011) in primary stromal cells (one sample), and possibly in myometrial cells. This would be in agreement with a previous study (Dai et al., 2017). In addition, RXFP2 transcripts were identified in the sheep OST cell-line. Because of the inconsistencies in these results, these experiments need repeating for different RNA preparations and using different PCR primer combinations. The fact that RXFP1 transcripts proved easier to detect

than those for RXFP2 probably reflects also the scarcity of the latter receptors in the cells and tissues concerned.

It is clear that transcript expression assessed by RT-PCR is not complete evidence for the functionality of the expressed receptors. Here I have firstly attempted to use a range of PCR primers in order to cover much of the transcript sequence which elsewhere in the literature has been shown to be prone to alternative splicing (Muda et al., 2005; Anand-Ivell et al., 2006; Heng et al., 2008; Dai et al., 2017a). Such alternatively spliced products are often missing one or more of the small exons encoding the leucine-rich repeats essential for hormone-binding, or they may introduce new sequence including a stop codon. Either way, such products, if expressed as proteins, are likely to be without positive function such as signaling capacity in their own right. Nevertheless, there is evidence from experimental studies that such abbreviated receptors may by dimerization interfere with the proper functioning of correctly spliced receptors, thus acting as inhibitory molecules in the relaxin/RXFP1 or INSL3/RXFP2 systems (Svendsen et al., 2008; Kern and Bryant-Greenwood, 2009; Rivero-Müller et al., 2010).

8.2 cAMP production in diverse uterine cells

As discussed in earlier sections, the primary mode of action for the transfected RXFP1 and RXFP2 receptors is to activate Gs and hence adenylyl cyclase, resulting in an increase of intracellular cAMP. Reality in vivo may not be as simple as this. For naturally RXFP1-expressing cells, such as the monocytic THP1 cell-line or primary human endometrial stromal cells, RXFP1 signaling has been suggested to be more complex. On the one hand there may be temporal switching for coupling between different G-proteins, such that depending on the timing of the response one might see activation of both PI3-kinase as well as cAMP (Halls et al., 2005a). For the primary human endometrial stromal cells, it was definitively shown that cAMP expression also required the concomitant activation of a tyrosine kinase activity (Bartsch et al., 2001), normally not represented for classical Gs-dependent activation of adenylyl cyclase. Furthermore, in vascular cell types, relaxin has been shown to activate other signalling pathways such as that for NOS and endothelin (Leo et al., 2017) (see Fig 7.1).

In the present investigation, it was shown that in regard to cAMP production, relaxin acting on naturally RXFP1-expressing bovine or ovine cells, did indeed elicit statistically significant effects, though different from what we might have expected (see above). Irrespective

of the effects, what this first shows is that the RXFP1 receptor protein, though having a higher EC50 for the ligand (Dai et al., 2017b), nevertheless is functional. For primary cultured bovine endometrial stromal cells (ESC), these appear to respond to exogenous relaxin in a bimodal fashion. At concentrations close to the EC50 there seems to be an increase in cAMP (100ng/ml) in the presence or absence of the phosphodiesterase (PDE) inhibitor IBMX or forskolin, which however is not significant. At higher concentrations, relaxin fails to show any stimulation but rather supports an inhibitory trend for these cells, as well as for all the other cell systems used. A relaxin-dependent increase in cAMP was also seen with human endometrial stromal cells (Bartsch et al., 2001). However, the prevailing inhibition of basal cAMP production seen in the majority of RXFP1-expressing cell systems used here is different and may be a feature unique to the ruminant. This suggests that in ruminants, and unlike in other species, the RXFP1 receptor in vivo is predominantly acting in an inhibitory manner, using Gi and G0 as the specific G-proteins coupled to the receptor. These results therefore differ somewhat from those reported previously for human and bovine myometrial and endometrial glandular epithelial cells (Heng et al., 2008; Dai et al., 2017b; Chen et al., 1988).

In both the primary bovine myometrial cells as well as in the sheep OST cell-line INSL3 was applied and like the effects of relaxin on the RXFP1 receptor appeared to be Gi-linked, thereby significantly inhibiting cAMP production. This differs from the reported action of INSL3 on human myometrial cells, where INSL3 had no effect on cAMP production (Heng et al., 2008) .

8.3 The effects of ML290 and analogues on transfected human and bovine receptors

On the assumption that the application of exogenous relaxin might have therapeutic potential in the treatment of cows and sheep, it was important to explore the possibility of alternative RXFP1 ligands. Currently, relaxin itself can only be produced in sufficient quantities for therapeutic applications in humans using recombinant techniques (Cimini et al., 2017) or high-throughput synthetic chemistry (Canova-Davis et al., 1990). Even then, its application is limited to acute rather than chronic conditions, largely due to the expense of its production and the limited half-life in vivo of the biological molecule. For human applications, even though extensive small molecule resources had been searched both physically and in silico, only one compound and its derivatives had shown any activity at the human RXFP1 receptor. This compound, ML290, like the natural relaxin, caused an increase in cAMP

production, presumably by activating adenylyl cyclase in a Gs-linked manner (Kocan et al., 2017). It has been shown, however, that this molecule does not bind to the conventional hormone binding pocket of RXFP1, but rather acts allosterically to trigger cell signaling (Agoulnik et al., 2017). We were fortunate to obtain samples of ML290 and three chemically modified derivatives from Professor Fallowfield at Edinburgh University (McBride et al., 2017). It was therefore disappointing that, in spite of taking precautions to account for solubility and other properties of the new ligands, we could see no effect of these compounds on cAMP production in HEK293T cells transfected not only with bovine RXFP1 expression constructs, but also in those cells transfected with human RXFP1 constructs as positive controls. From the literature it is evident that whilst cAMP appears to be the main signaling end-point for these molecules, other end-points such as NOS activation may also be apparent (Kocan et al., 2017). Having said that, the signaling pathways activated by ML290 do appear to differ from those activated by natural relaxin (Kocan et al., 2017), probably due to its allosteric mode of action at the RXFP1 receptor. At present, we cannot explain our failure to detect any effect at all of ML290. For future experiments it would be necessary to obtain alternative sources of the compound, and to try alternative experimental paradigms, conditions, and end-points.

8.4 The search for alternative relaxin end-points, applying specific gene expression analysis.

While providing evidence for specific RXFP1 activation by porcine relaxin, the preceding studies were less convincing in terms of the conventional relaxin signaling end-point: an elevation of intracellular cAMP. In order to explore possible alternative signaling pathways and end-points, it was decided to take advantage of the known physiology of the two cell systems from which we had obtained the most interesting results, the sheep OGE cell-line and the primary cultures of bovine myometrial cells.

As indicated in chapter 5, the OGE cell-line has been used successfully to study the actions of oxytocin and interferon-tau in regulating the maternal recognition of pregnancy (MRP) in ruminants (Bowen and Burghardt, 2000; Bazer et al., 1997; Gimpl and Fahrenholz, 2001). Since I have shown that these cells also express RXFP1 and in other species RXFP1 is similarly expressed on endometrial epithelial cells (Ivell et al., 2003) , I logically looked at key components of the Maternal Recognition of Pregnancy (MRP) system to see whether these could be influenced by relaxin at the level of gene expression. In the OGE system it appears from the preliminary studies that, of the end-point gene transcripts tested, only OTR, PR, ERα and PDGS2 seem to be suitable

candidates. However, more experiments would be needed to elaborate in greater detail just how RXFP1 activation is influencing these genes.

For the myometrial cells, a similar network of endocrine effectors is acting. Pulsatile pituitary oxytocin is the essential trigger at the end of pregnancy to induce myometrial contraction and initiate labour (Gimpl and Fahrenholz, 2001). In tissues and primary cell culture, oxytocin acting via OTR triggers myometrial cell contraction by inducing the release of Ca^{2+} from intracellular stores in the sarcoplasmic reticulum (Molnár and Hertelendy, 1990). In the rat and pig, this induced contraction (as well as spontaneous contractions) can be inhibited by the application of relaxin (Bani et al., 1999; MacLennan and Grant, 1991). Prostaglandins can similarly influence myometrial contractility: in general, an elevation of PI3-kinase and intracellular Ca^{2+} favours contractility, whereas an increase in cAMP favours quiescence. Moreover in rats at least the increase in OTR that is observed in the term myometrium seems to involve the actions of estrogen receptors ERα and possibly ERβ (Zingg et al., 1998). Treatment of pregnant rats with the estrogen receptor antagonist Tamoxifen slows the increase of uterine OTR expression and in vitro estradiol has been shown to increase OTR expression in the OGE cell-line (Telgmann et al., 2003). Similarly, studies in knockout mice show that ERα is necessary to induce the

expression of OTR in the brain and elsewhere. Relaxin is also able to induce ERα in the rat uterus (Pillai et al., 1999), suggesting that one effect of relaxin may be to regulate OTR through ER activation. Indeed, in mice with deletion of the relaxin gene, it was shown that both ERα and OTR were both expressed at significantly lower levels in the term myometrium (Siebel et al., 2003). In contrast to these findings the microarray study of human endometrial stromal cells, reported in Table 7.2, showed that OTR was significantly down-regulated by relaxin in these cells. Together, all these results reinforce the connection between activated relaxin receptors and both ERα and OTR expression and presumably function, though not necessarily in the same way in all cells, tissues, times, and species.

Interestingly, for the myometrial cell cultures, relaxin had a clearly significant effect on the expression of RNA transcripts for ERβ (down-regulated) as well as OTR (up-regulated). Together with the preceding results from the OGE cell-line, it would appear that relaxin in the ruminant uterus is able to influence (albeit differently in different cell types) the expression of the key steroid receptors ERα, ERβ, and PR, as well as the receptors (OTR) for one of the key peptide modulators of uterine function. Considering that cows and sheep have been lacking a natural ligand for the relaxin receptor for several million years at least,

these findings are strongly suggestive that the remaining RXFP1 relaxin receptors are nevertheless still functional and linked to key uterine signaling pathways.

Since INSL3 appears able to activate the bovine RXFP1 receptor at modest concentrations (Dai et al., 2017a), it was important to compare the effects of relaxin on these cell systems with those of INSL3. In the available time, this was only possible for the primary myometrial cells. Importantly, it was observed that INSL3, just like relaxin, increased the expression of OTR and down-regulated ERβ. Therefore, whilst the expression of RXFP2 in these cells is still unsure, it is quite possible that INSL3 may be acting in a similar way to relaxin, and maybe via the RXFP1 receptor, in addition to RXFP2. Furthermore, INSL3 also appears to be the only effector able to influence (down-regulate) the expression of osteopontin (OPN).

8.5 Application of global transcript analysis to determine novel relaxin target genes in the uterus.

The preceding sections have succeeded in confirming the potential pharmacological relevance of relaxin-dependent stimulation of RXFP1 pathways in ruminant uterine cells. However, the effects seen do not appear to be substantial or sufficiently essential to justify in themselves an application of relaxin in a therapeutic context. Part of this scepsis,

however, resides not in the ineffectiveness of relaxin, but simply in our lack of understanding of relaxin action even in those species where we have a well characterized and dramatic physiological effect. So, for example, we do not fully understand the molecular mode of action of relaxin acting on human endometrial stromal cells to induce decidualization (Bartsch et al., 2004), nor precisely how the hormone is able to induce quiescence in the rat uterus (Porter, 1979). Therefore, in order to begin to address this lack of molecular understanding, two classical relaxin-dependent systems from other species have been used in combination with modern NGS-sequencing or microarray analysis to gain a global appreciation of the transcriptome changes that result from relaxin stimulation, presumably via uterine RXFP1 receptors. The systems that were used were quite different from one another. In the mouse uterus model, whole uterine tissues were examined in vivo at the time of oestrus (Glynn et al., 2018). Therefore, significant changes in gene expression could reflect impacts on luminal epithelial cells, on glandular cells, on stromal or myometrial cells, on resident immune cells, or even on pervading blood vessels. In the second model, relatively pure endometrial stromal cells from human hysterectomy tissue were subject to relaxin stimulation in vitro, under circumstances where we know that cAMP- and NF-kappa B- dependent pathways are being activated (Dschietzig et al., 2003; Heng et al., 2008).

Perhaps surprisingly, and most importantly, there were considerable overlaps in the pathways and patterns of gene expression changes observed in both systems, at least in terms of KEGG pathway analysis. At this stage of the analysis, changes in individual genes were felt to be less important, since possibly representing more tissue- or species-specific effects. First and foremost, relaxin represents a key differentiation switch: it causes a shut-down of genes involved in cell division and the cell cycle; concomitantly it causes an induction of a more differentiated state and, in particular, for both systems - and in two very divergent species – this involves the expression of pro-inflammatory cytokines, chemokines, and their receptors. This transition would lead in vivo to an accumulation of new immune cells into the uterus, which are essential to allow implantation to occur in a healthy fashion.

This could also offer an explanation for relaxin action on the cervix and uterus at term of pregnancy, where pro-inflammatory cytokines are believed to be involved in the induction of labour (Shynlova et al., 2020) and cervical softening (Kelly, 1996), and where historical experiments with porcine relaxin in cows did indeed suggest a positive effect on cervical relaxation (Musah et al., 1986). Together, such findings lend

support to a future pharmaceutical application of relaxin or related agonists to improve cervical relaxation and aid delivery.

Thus, for future studies of the bovine uterine cells and tissues which were the object of the current investigation, it would be very worthwhile to consider as new endpoints of relaxin action, the measurement of the relative expression of cell cycle markers, such as CDK1 or CDC20, as well as the transcript levels of pro-inflammatory markers like CXCL1, CXCL3, or CXCL12, and other differentiation indicators such as WNT5a. These would be in addition to the steroid hormone receptors and OTR, identified earlier.

8.6 An evolutionary view of gene loss.

Comparative genomic analysis suggests that the relaxin gene may have been rendered inactive and eventually lost some 30 million years ago during Artiodactyl evolution and expansion. Whilst a relaxin homologue can be identified in Camellids, as well as in horses, pigs, and whales, there is no evidence for the presence of a functional relaxin gene in any modern ruminant. There is a clear deletion in the syntenic genome sequence corresponding to relaxin in all other mammals (Dai et al., 2017a). Interestingly, this is the same time when it is assumed that the interferon-tau gene also evolved in ruminants and adopted its role in the maternal recognition of pregnancy (Ealy and Wooldridge, 2017).

Given that relaxin appears to have evolved many of its reproductive roles in mammals in response to the uniquely mammalian aspects of internal fertilization, placentation, and pregnancy recognition (Anand-Ivell et al., 2013), it may be that the two events were physiologically linked. Darwinian evolution and natural selection can only act where a protein is expressed and functional (negatively or positively). One of the first events that occurs upon loss of gene function is mutational inactivation of control regions in a gene, so that a transcript is not produced, or if produced is unable to encode a protein. This is followed by further mutation and loss of sequence. For a hormone, this is then followed by corruption of the receptor and its specific signaling, unless there is an alternative ligand, which can maintain downstream physiological relevance. We still have too little information about normal relaxin signaling pathways in uterine cells to be able to state whether or not what we see in uterine cells of cows and sheep is consistent with a physiological function or not. What is apparent is that the bovine RXFP1 receptor does appear to be connected within similar intracellular networks to those reported for other non-ruminant species, including those implicated in interferon-tau function. Further research is needed to provide more detail about these connections, and whether or not an alternative ligand for the RXFP1 receptor, potentially such as INSL3,

may be involved. Until we understand these relationships more fully, any pharmacological approach in vivo should be met with caution.

8.7 Future studies

Unfortunately, the completion of my thesis work was interrupted by the Covid-19 outbreak and the necessity of me returning home prematurely to Saudi Arabia. Many of the final experiments that would be needed to affirm my results and to provide additional reassurance of the conclusions drawn must now await a later time and opportunity. These proposed experiments include the following:

1. Repeat RT-PCR using alternative PCR primers to verify the presence and/or absence of potentially functional full-length transcripts for RXFP1 and RXFP2 in all cells used, with sequencing of all PCR products.
2. To apply western blotting using pERK and/or alternative signalling moieties on cells stimulated or not with relaxin.
3. To follow up on the possible involvement of INSL3 in the stimulation of OGE and myometrial cells, especially to determine whether this relaxin-like peptide might be using RXFP1-linked pathways in these cells. This could be analysed using a co-transfection approach with siRNA specific for RXFP1 and RXFP2 and downstream effectors. This

might thus offer an explanation why ruminant RXFP1 has retained a degree of functionality in spite of having lost its specific ligand for many generations.

4. To repeat experiments using the small molecule RXFP1 agonist, ML290, trying alternative stimulation protocols, and compound provenence.
5. As cited above, and based on the global transcriptomics approach, to re-analyse RNA derived from the cell culture experiments by qRT-PCR using PCR primers encoding selected pro-inflammatory cytokines, cell cycle markers, etc.

8.8 conclusions

The present study was undertaken to determine whether in ruminants, which have lost the gene encoding the hormone relaxin, its receptor RXFP1 is still functional at a cellular level in vivo, and consequently whether exogenously applied relaxin analogues might offer a novel therapeutic option to address reproductive problems in cows and sheep.

I have succeeded in showing that the bovine and/or sheep RXFP1 receptor is indeed functional in a selection of primary and secondary cell systems derived from ruminant uterine tissues. However, the apparent mode of signal transduction from this receptor, predominantly via a Gi-linked pathway, seems to differ from what would have been expected based on earlier studies using, for example, human endometrial stromal cells. Part of the problem, however, lies in our incomplete understanding of relaxin action in uterine tissues from any species. For that reason, it was necessary to embark on a more global approach, initially looking for gene expression profiles in ruminant and other species, which could highlight novel pathways and molecular mechanisms of action shared amongst all mammals. Further research will need to elaborate on these pathways, particularly in responsive uterine cell systems, to show what physiological effect application of exogenous relaxin in ruminants might have.

Of particular interest was the preliminary observation for myometrial cells that the structurally related peptide hormone INSL3 appears to induce a similar profile of gene expression to relaxin, even though initial RT-PCR studies suggested only limited or no expression of the specific INSL3 receptor, RXFP2, in myometrial cells. The possibility that INSL3 might be acting through RXFP1-linked pathways has considerable relevance not only for the therapeutic considerations discussed here, but also of course for the natural physiology of ruminants. Could endogenous INSL3 be effectively replacing relaxin in other important relaxin-dependent physiological systems in ruminants such as the heart and cardiovascular system? This also must be pursued in future studies.

References

ADHAM, I. M., BURKHARDT, E., BENAHMED, M. & ENGEL, W. 1993. Cloning of a cDNA for a novel insulin-like peptide of the testicular Leydig cells. *Journal of Biological Chemistry,* 268**,** 26668-26672.

AGOULNIK, A. I., AGOULNIK, I. U., HU, X. & MARUGAN, J. 2017. Synthetic non-peptide low molecular weight agonists of the relaxin receptor 1. *British journal of pharmacology,* 174**,** 977-989.

ANAND-IVELL, R., DAI, Y. & IVELL, R. 2013. Neohormones as biomarkers of reproductive health. *Fertility and Sterility,* 99**,** 1153-1160.

ANAND-IVELL, R., HENG, K., BARTSCH, O. & IVELL, R. 2007. Relaxin signalling in THP-1 cells uses a novel phosphotyrosine-dependent pathway. *Molecular and cellular endocrinology,* 272**,** 1-13.

ANAND-IVELL, R., HIENDLEDER, S., VINOLES, C., MARTIN, G. B., FITZSIMMONS, C., EURICH, A., HAFEN, B. & IVELL, R. 2011. INSL3 in the ruminant: a powerful indicator of gender- and genetic-specific feto-maternal dialogue. *PLoS One,* 6**,** e19821.

ANAND-IVELL, R. & IVELL, R. 2014. Regulation of the reproductive cycle and early pregnancy by relaxin family peptides. *Molecular and cellular endocrinology,* 382**,** 472-479.

ANAND-IVELL, R. J., RELAN, V., BALVERS, M., COIFFEC-DORVAL, I., FRITSCH, M., BATHGATE, R. A. & IVELL, R. 2006. Expression of the insulin-like peptide 3 (INSL3) hormone-receptor (LGR8) system in the testis. *Biology of reproduction,* 74**,** 945-953.

ANDO, S., SARLIS, N. J., KRISHNAN, J., FENG, X., REFETOFF, S., ZHANG, M. Q., OLDFIELD, E. H. & YEN, P. M. 2001. Aberrant alternative splicing of thyroid hormone receptor in a TSH-secreting pituitary tumor is a mechanism for hormone resistance. *Molecular Endocrinology,* 15**,** 1529-1538.

ANUMBA, D. O., EL GELANY, S., ELLIOTT, S. L. & LI, T. C. 2009. Serum relaxin levels are reduced in pregnant women with a history of recurrent miscarriage, and correlate with maternal uterine artery Doppler indices in first trimester. *European Journal of Obstetrics & Gynecology and Reproductive Biology,* 147**,** 41-45.

AROSH, J., BANU, S., KIMMINS, S., CHAPDELAINE, P., MACLAREN, L. & FORTIER, M. A. 2004. Effect of interferon-τ on prostaglandin biosynthesis, transport, and signaling at the time of maternal recognition of pregnancy in cattle: evidence of polycrine actions of prostaglandin E2. *Endocrinology,* 145**,** 5280-5293.

AROSH, J. A., BANU, S. K. & MCCRACKEN, J. A. 2016. Novel concepts on the role of prostaglandins on luteal maintenance and maternal recognition and establishment of pregnancy in ruminants. *Journal of dairy science,* 99**,** 5926-5940.

BALVERS, M., SPIESS, A. N., DOMAGALSKI, R., HUNT, N., KILIC, E., MUKHOPADHYAY, A. K., HANKS, E., CHARLTON, H. M. & IVELL, R. 1998. Relaxin-Like Factor Expression

as a Marker of Differentiation in the Mouse Testis and Ovary*. *Endocrinology,* 139**,** 2960-2970.

BAMBERGER, A.-M., IVELL, R., BALVERS, M., KELP, B., BAMBERGER, C. M., RIETHDORF, L. & LöNING, T. 1999. Relaxin-Like Factor (RLF): A New Specific Marker for Leydig Cells in the Ovary. *International Journal of Gynecological Pathology,* 18**,** 163-168.

BANI, D., BACCARI, M. C., NISTRI, S., CALAMAI, F., BIGAZZI, M. & SACCHI, T. B. 1999. Relaxin up-regulates the nitric oxide biosynthetic pathway in the mouse uterus: involvement in the inhibition of myometrial contractility. *Endocrinology,* 140**,** 4434-4441.

BARTSCH, O., BARTLICK, B. & IVELL, R. 2001. Relaxin signalling links tyrosine phosphorylation to phosphodiesterase and adenylyl cyclase activity. *MHR: Basic science of reproductive medicine,* 7**,** 799-809.

BARTSCH, O., BARTLICK, B. & IVELL, R. 2004. Phosphodiesterase 4 inhibition synergizes with relaxin signaling to promote decidualization of human endometrial stromal cells. *The Journal of Clinical Endocrinology & Metabolism,* 89**,** 324-334.

BATHGATE, R., BALVERS, M., HUNT, N. & IVELL, R. 1996. Relaxin-like factor gene is highly expressed in the bovine ovary of the cycle and pregnancy: sequence and messenger ribonucleic acid analysis. *Biol Reprod,* 55.

BATHGATE, R., HALLS, M., VAN DER WESTHUIZEN, E., CALLANDER, G., KOCAN, M. & SUMMERS, R. 2013. Relaxin family peptides and their receptors. *Physiological reviews,* 93**,** 405-480.

BATHGATE, R., MONIAC, N., BARTLICK, B., SCHUMACHER, M., FIELDS, M. & IVELL, R. 1999. Expression and Regulation of Relaxin-Like Factor Gene Transcripts in the Bovine Ovary: Differentiation-Dependent Expression in Theca Cell Cultures1. *Biology of Reproduction,* 61**,** 1090-1098.

BATHGATE, R. A., IVELL, R., SANBORN, B. M., SHERWOOD, O. D. & SUMMERS, R. J. 2006. International Union of Pharmacology LVII: recommendations for the nomenclature of receptors for relaxin family peptides. *Pharmacological reviews,* 58**,** 7-31.

BATHGATE, R. A., SAMUEL, C. S., BURAZIN, T. C., LAYFIELD, S., CLAASZ, A. A., REYTOMAS, I. G. T., DAWSON, N. F., ZHAO, C., BOND, C. & SUMMERS, R. J. 2002. Human relaxin gene 3 (H3) and the equivalent mouse relaxin (M3) gene Novel members of the relaxin peptide family. *Journal of Biological Chemistry,* 277**,** 1148-1157.

BAZER, F. W., SPENCER, T. E. & OTT, T. L. 1997. Interferon tau: a novel pregnancy recognition signal. *American Journal of Reproductive Immunology,* 37**,** 412-420.

BENNETT, R. G. 2009. Relaxin and its role in the development and treatment of fibrosis. *Translational Research,* 154**,** 1-6.

BOWEN, J. A. & BURGHARDT, R. C. 2000. Cellular mechanisms of implantation in domestic farm animals. *Seminars in Cell & Developmental Biology,* 11**,** 93-104.

BRAUN, B. C., VARGAS, A. & JEWGENOW, K. 2012. The molecular detection of relaxin and its receptor RXFP1 in reproductive tissue of Felis catus and Lynx pardinus during pregnancy. *Reproduction,* 143**,** 399-410.

BRAVO, P., STEWART, D., LASLEY, B. & FOWLER, M. 1996. Hormonal indicators of pregnancy in llamas and alpacas. *Journal of the American Veterinary Medical Association,* 208**,** 2027-2030.

BRESSON, E., BOUCHER-KOVALIK, S., CHAPDELAINE, P., MADORE, E., HARVEY, N., LABERGE, P. Y., LEBOEUF, M. & FORTIER, M. A. 2011. The human aldose reductase AKR1B1 qualifies as the primary prostaglandin F synthase in the endometrium. *The Journal of Clinical Endocrinology & Metabolism,* 96**,** 210-219.

BROOKS, K., BURNS, G. & SPENCER, T. E. 2015. Conceptus elongation in ruminants: roles of progesterone, prostaglandin, interferon tau and cortisol. *Journal of Animal Science and Biotechnology***,** 5.

BULLESBACH, E. E., RHODES, R., REMBIESA, B. & SCHWABE, C. 1999. The relaxin-like factor is a hormone. *Endocrine,* 10**,** 167-169.

CANOVA-DAVIS, E., BALDONADO, I. P. & TESHIMA, G. M. 1990. Characterization of chemically synthesized human relaxin by high-performance liquid chromatography. *Journal of Chromatography A,* 508**,** 81-96.

CHASSIN, D., LAURENT, A., JANNEAU, J.-L., BERGER, R. & BELLET, D. 1995. Cloning of a new member of the insulin gene superfamily (INSL4) expressed in human placenta. *Genomics,* 29**,** 465-470.

CHEN, G., HUANG, J. R. & TSENG, L. 1988. The effect of relaxin on cyclic adenosine 3′, 5′-monophosphate concentrations in human endometrial glandular epithelial cells. *Biology of reproduction,* 39**,** 519-525.

CIMINI, D., DELLA CORTE, K., FINAMORE, R., ANDREOZZI, L., STELLAVATO, A., PIROZZI, A., FERRARA, F., FORMISANO, R., DE ROSA, M. & CHINO, M. 2017. Production of human pro-relaxin H2 in the yeast Pichia pastoris. *BMC biotechnology,* 17**,** 1-11.

CONKLIN, D., LOFTON-DAY, C. E., HALDEMAN, B. A., CHING, A., WHITMORE, T. E., LOK, S. & JASPERS, S. 1999. Identification of INSL5, a new member of the insulin superfamily. *Genomics,* 60**,** 50-56.

CONRAD, K. P. 2011. Maternal vasodilation in pregnancy: the emerging role of relaxin. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology,* 301**,** R267-R275.

DAI, Y., IVELL, R., LIU, X., JANOWSKI, D. & ANAND-IVELL, R. 2017a. Relaxin-family peptide receptors 1 and 2 are fully functional in the bovine. *Frontiers in physiology,* 8**,** 359.

DAI, Y., IVELL, R., LIU, X., JANOWSKI, D. & ANAND-IVELL, R. 2017b. Relaxin-Family Peptide Receptors 1 and 2 Are Fully Functional in the Bovine. *Frontiers in Physiology,* 8.

DSCHIETZIG, T., BARTSCH, C., RICHTER, C., LAULE, M., BAUMANN, G. & STANGL, K. 2003. Relaxin, a Pregnancy Hormone, Is a Functional Endothelin-1 Antagonist: Attenuation of Endothelin-1–Mediated Vasoconstriction by Stimulation of Endothelin Type-B Receptor Expression via ERK-1/2 and Nuclear Factor-κB. *Circulation research,* 92**,** 32-40.

EALY, A. D. & WOOLDRIDGE, L. K. 2017. The evolution of interferon-tau. *Reproduction,* 154**,** F1-F10.

EINSPANIER, A., LIEDER, K., HUSEN, B., EBERT, K., LIER, S., EINSPANIER, R., UNEMORI, E. & KEMPER, M. 2009. Relaxin supports implantation and early pregnancy in the marmoset monkey. *Annals of the New York Academy of Sciences,* 1160**,** 140-146.

ENGELAND, K. 2018. Cell cycle arrest through indirect transcriptional repression by p53: I have a DREAM. *Cell Death & Differentiation,* 25**,** 114-132.

FEI, D. T., GROSS, M. C., LOFGREN, J. L., MORA-WORMS, M. & CHEN, A. B. 1990. Cyclic AMP response to recombinant human relaxin by cultured human endometrial cells—a specific and high throughput in vitro bioassay. *Biochemical and biophysical research communications,* 170**,** 214-222.

FERLIN, A., MENEGAZZO, M., GIANESELLO, L., SELICE, R. & FORESTA, C. 2012. Effect of relaxin on human sperm functions. *Journal of andrology,* 33**,** 474-482.

FERLIN, A., PEPE, A., GIANESELLO, L., GAROLLA, A., FENG, S., FACCIOLLI, A., MORELLO, R., AGOULNIK, A. I. & FORESTA, C. 2009. New roles for INSL3 in adults. *Annals of the New York Academy of Sciences,* 1160**,** 215-218.

FUCHS, A.-R. 1987. Prostaglandin F2alpha and oxytocin interactions in ovarian and uterine function. *Journal of steroid biochemistry,* 27**,** 1073-1080.

GELLERSEN, B., BROSENS, I. A. & BROSENS, J. J. Decidualization of the human endometrium: mechanisms, functions, and clinical perspectives. Seminars in reproductive medicine, 2007. © Thieme Medical Publishers, 445-453.

GELLERSEN, B. & BROSENS, J. J. 2014. Cyclic decidualization of the human endometrium in reproductive health and failure. *Endocrine reviews,* 35**,** 851-905.

GERASIMOVA, T., THANASOULA, M. N., ZATTAS, D., SELI, E., SAKKAS, D. & LALIOTI, M. D. 2010. Identification and in vitro characterization of follicle stimulating hormone (FSH) receptor variants associated with abnormal ovarian response to FSH. *The Journal of Clinical Endocrinology & Metabolism,* 95**,** 529-536.

GIMPL, G. & FAHRENHOLZ, F. 2001. The oxytocin receptor system: structure, function, and regulation. *Physiological reviews,* 81**,** 629-683.

GLYNN, D. J., HENG, K., RUSSELL, D. L., SHARKEY, D. J., ROBERTSON, S. A., ANAND-IVELL, R. & IVELL, R. 2017. Male seminal relaxin contributes to induction of the post-mating cytokine response in the female mouse uterus. *Frontiers in physiology,* 8**,** 422.

GOLDSMITH, L. T., WEISS, G., PALEJWALA, S., PLANT, T. M., WOJTCZUK, A., LAMBERT, W. C., AMMUR, N., HELLER, D., SKURNICK, J. H. & EDWARDS, D. 2004a. Relaxin regulation of endometrial structure and function in the rhesus monkey. *Proceedings of the National Academy of Sciences of the United States of America,* 101**,** 4685-4689.

GOLDSMITH, L. T., WEISS, G., PALEJWALA, S., PLANT, T. M., WOJTCZUK, A., LAMBERT, W. C., AMMUR, N., HELLER, D., SKURNICK, J. H. & EDWARDS, D. 2004b. Relaxin regulation of endometrial structure and function in the rhesus monkey. *Proceedings of the National Academy of Sciences,* 101**,** 4685-4689.

GRANOT, I., GNAINSKY, Y. & DEKEL, N. 2012. Endometrial inflammation and effect on implantation improvement and pregnancy outcome. *Reproduction,* 144**,** 661-668.

GRISS, G., KECK, J., ENGELHORN, R. & TUPPY, H. 1967. The isolation and purification of an ovarian polypeptide with uterin-relaxing activity. *Biochimica et Biophysica Acta (BBA)-Protein Structure,* 140**,** 45-54.

GUICO-LAMM, M. L. & SHERWOOD, O. 1988. Monoclonal Antibodies Specific for Rat Relaxin. II. Passive Immunization with Monoclonal Antibodies throughout the Second Half of Pregnancy Disrupts Birth in Intact Rats*. *Endocrinology,* 123**,** 2479-2485.

GULLAM, J. E., BLANKS, A. M., THORNTON, S. & SHMYGOL, A. 2009. Phase-plot analysis of the oxytocin effect on human myometrial contractility. *European Journal of Obstetrics & Gynecology and Reproductive Biology,* 144**,** S20-S24.

GUZEL, E., ARLIER, S., GUZELOGLU-KAYISLI, O., TABAK, M. S., EKIZ, T., SEMERCI, N., LARSEN, K., SCHATZ, F., LOCKWOOD, C. J. & KAYISLI, U. A. 2017. Endoplasmic reticulum stress and homeostasis in reproductive physiology and pathology. *International journal of molecular sciences,* 18**,** 792.

HALLS, M. L., BATHGATE, R. A. & SUMMERS, R. J. 2005a. Signal switching after stimulation of LGR7 receptors by human relaxin 2. *Annals of the New York Academy of Sciences,* 1041**,** 288-291.

HALLS, M. L., BATHGATE, R. A. & SUMMERS, R. J. 2006. Relaxin family peptide receptors RXFP1 and RXFP2 modulate cAMP signaling by distinct mechanisms. *Molecular pharmacology,* 70**,** 214-226.

HALLS, M. L., BOND, C. P., SUDO, S., KUMAGAI, J., FERRARO, T., LAYFIELD, S., BATHGATE, R. A. & SUMMERS, R. J. 2005b. Multiple binding sites revealed by interaction of relaxin family peptides with native and chimeric relaxin family peptide receptors 1 and 2 (LGR7 and LGR8). *Journal of Pharmacology and Experimental Therapeutics,* 313**,** 677-687.

HALLS, M. L. & COOPER, D. M. 2010. Sub-picomolar relaxin signalling by a pre-assembled RXFP1, AKAP79, AC2, β-arrestin 2, PDE4D3 complex. *The EMBO Journal,* 29**,** 2772-2787.

HALLS, M. L., HEWITSON, T. D., MOORE, X. L., DU, X. J., BATHGATE, R. A. & SUMMERS, R. J. 2009. Relaxin activates multiple cAMP signaling pathway profiles in different target cells. *Annals of the New York Academy of Sciences,* 1160**,** 108-111.

HALLS, M. L., VAN DER WESTHUIZEN, E. T., BATHGATE, R. & SUMMERS, R. J. 2007. Relaxin family peptide receptors–former orphans reunite with their parent ligands to activate multiple signalling pathways. *British journal of pharmacology,* 150**,** 677-691.

HAMA, K., AOKI, J., INOUE, A., ENDO, T., AMANO, T., MOTOKI, R., KANAI, M., YE, X., CHUN, J. & MATSUKI, N. 2007. Embryo spacing and implantation timing are differentially regulated by LPA3-mediated lysophosphatidic acid signaling in mice. *Biology of reproduction,* 77**,** 954-959.

HANNA, C. B., YAO, S., PATTA, M. C., JENSEN, J. T. & WU, X. 2010. Expression of insulin-like 3 (INSL3) and differential splicing of its receptor in the ovary of rhesus macaques. *Reproductive Biology and Endocrinology,* 8**,** 150.

HARTUNG, S., KONDO, S., ABEND, N., HUNT, N., RUST, W., BALVERS, M., BRYANT-GREENWOOD, G. & IVELL, R. 1995. The search for ruminant relaxin. *Progress in relaxin research*, 439-456.

HAYES, E., CURNOW, E., TROUNSON, A., DANIELSON, L. & UNEMORI, E. 2004. Implantation and pregnancy following in vitro fertilization and the effect of recombinant human relaxin administration in Macaca fascicularis. *Biology of reproduction,* 71, 1591-1597.

HENG, K., IVELL, R., WAGAARACHCHI, P. & ANAND-IVELL, R. 2008. Relaxin signalling in primary cultures of human myometrial cells. *Molecular human reproduction,* 14, 603-611.

HOMBACH-KLONISCH, S., ABD-ELNAEIM, M., SKIDMORE, J. A., LEISER, R., FISCHER, B. & KLONISCH, T. 2000. Ruminant relaxin in the pregnant one-humped camel (Camelus dromedarius). *Biology of reproduction,* 62, 839-846.

HSU, S. Y., NAKABAYASHI, K., NISHI, S., KUMAGAI, J., KUDO, M., SHERWOOD, O. D. & HSUEH, A. J. 2002. Activation of orphan receptors by the hormone relaxin. *Science,* 295, 671-674.

HSU, S. Y. T., NAKABAYASHI, K., NISHI, S., KUMAGAI, J., KUDO, M., BATHGATE, R. A., SHERWOOD, O. D. & HSUEH, A. J. 2003. Relaxin signaling in reproductive tissues. *Molecular and cellular endocrinology,* 202, 165-170.

HU, X., MYHR, C., HUANG, Z., XIAO, J., BARNAEVA, E., HO, B. A., AGOULNIK, I. U., FERRER, M., MARUGAN, J. J. & SOUTHALL, N. 2016. Structural insights into the activation of human relaxin family peptide receptor 1 by small-molecule agonists. *Biochemistry,* 55, 1772-1783.

HUDSON, P., HALEY, J., JOHN, M., CRONK, M., CRAWFORD, R., HARALAMBIDIS, J., TREGEAR, G., SHINE, J. & NIALL, H. 1983. Structure of a genomic clone encoding biologically active human relaxin. *Nature,* 301, 628-631.

HUDSON, P., JOHN, M., CRAWFORD, R., HARALAMBIDIS, J., SCANLON, D., GORMAN, J., TREGEAR, G., SHINE, J. & NIALL, H. 1984. Relaxin gene expression in human ovaries and the predicted structure of a human preprorelaxin by analysis of cDNA clones. *The EMBO journal,* 3, 2333.

IVELL, R. 1997. Biology of the relaxin-like factor (RLF). *Reviews of Reproduction,* 2, 133-138.

IVELL, R., BALVERS, M., POHNKE, Y., TELGMANN, R., BARTSCH, O., MILDE-LANGOSCH, K., BAMBERGER, A.-M. & EINSPANIER, A. 2003. Immunoexpression of the relaxin receptor LGR7 in breast and uterine tissues of humans and primates. *Reproductive biology and endocrinology,* 1, 114.

IVELL, R., HENG, K. & ANAND-IVELL, R. 2007. Diverse signalling mechanisms used by relaxin in natural cells and tissues: the evolution of a “neohormone”. *Relaxin and Related Peptides*, 26-33.

IVELL, R., HENG, K. & ANAND-IVELL, R. 2014. Insulin-like factor 3 and the HPG axis in the male. *Frontiers in endocrinology,* 5, 6.

IVELL, R., KOTULA-BALAK, M., GLYNN, D., HENG, K. & ANAND-IVELL, R. 2010. Relaxin family peptides in the male reproductive system—a critical appraisal. *MHR: Basic science of reproductive medicine,* 17, 71-84.

JACOBSON, C., BRUCE, M., KENYON, P. R., LOCKWOOD, A., MILLER, D., REFSHAUGE, G. & MASTERS, D. G. 2020. A review of dystocia in sheep. *Small Ruminant Research*, 106209.

JAMES, R., NIALL, H., KWOK, S. & BRYANT-GREENWOOD, G. 1977. Primary structure of porcine relaxin: homology with insulin and related growth factors. *Nature,* 267, 544-546.

JAUNIAUX, E., JOHNSON, M. R., JURKOVIC, D., RAMSAY, B., CAMPBELL, S. & MEURIS, S. 1994. The role of relaxin in the development of the uteroplacental circulation in early pregnancy. *Obstetrics & Gynecology,* 84, 338-342.

JOHNSON, G. A., BURGHARDT, R. C. & BAZER, F. W. 2014. Osteopontin: a leading candidate adhesion molecule for implantation in pigs and sheep. *Journal of animal science and biotechnology,* 5, 1-14.

JOHNSON, G. A., BURGHARDT, R. C., BAZER, F. W. & SPENCER, T. E. 2003. Osteopontin: Roles in Implantation and Placentation1. *Biology of Reproduction,* 69, 1458-1471.

JOHNSON, G. A., BURGHARDT, R. C., NEWTON, G. R., BAZER, F. W. & SPENCER, T. E. 1999. Development and characterization of immortalized ovine endometrial cell lines. *Biology of reproduction,* 61, 1324-1330.

JOHNSON, G. A., SPENCER, T. E., BURGHARDT, R. C., TAYLOR, K. M., GRAY, C. A. & BAZER, F. W. 2000. Progesterone modulation of osteopontin gene expression in the ovine uterus. *Biology of Reproduction,* 62, 1315-1321.

KELLY, R. W. 1996. Inflammatory mediators and parturition. *Reviews of reproduction,* 1, 89-96.

KERN, A. & BRYANT-GREENWOOD, G. D. 2009. Mechanisms of relaxin receptor (LGR7/RXFP1) expression and function. *Annals of the New York Academy of Sciences,* 1160, 60-66.

KERN, A. S., HUBBARD, D., AMANO, A. & BRYANT-GREENWOOD, G. D. 2008. Cloning, expression, and functional characterization of relaxin receptor (leucine-rich repeat-containing g protein-coupled receptor 7) splice variants from human fetal membranes. *Endocrinology,* 149, 1277-1294.

KIDDER, G. M. & WINTERHAGER, E. 2015. Physiological roles of connexins in labour and lactation. *Reproduction,* 150, R129-R136.

KLONISCH, T., BIALEK, J., RADESTOCK, Y., HOANG-VU, C. & HOMBACH-KLONISCH, S. 2007. Relaxin-like ligand-receptor systems are autocrine/paracrine effectors in tumor cells and modulate cancer progression and tissue invasiveness. *Relaxin and Related Peptides.* Springer.

KOCAN, M., SARWAR, M., ANG, S. Y., XIAO, J., MARUGAN, J. J., HOSSAIN, M. A., WANG, C., HUTCHINSON, D. S., SAMUEL, C. S. & AGOULNIK, A. I. 2017. ML290 is a biased allosteric agonist at the relaxin receptor RXFP1. *Scientific reports,* 7, 1-14.

KONG, R. C., SHILLING, P. J., LOBB, D. K., GOOLEY, P. R. & BATHGATE, R. A. 2010. Membrane receptors: structure and function of the relaxin family peptide receptors. *Molecular and cellular endocrinology,* 320, 1-15.

KUNZ, G. & LEYENDECKER, G. 2002. Uterine peristaltic activity during the menstrual cycle: characterization, regulation, function and dysfunction. *Reproductive biomedicine online,* 4, 5-9.

LEE, P. D., SLADEK, R., GREENWOOD, C. M. & HUDSON, T. J. 2002. Control genes and variability: absence of ubiquitous reference transcripts in diverse mammalian expression studies. *Genome research,* 12**,** 292-297.

LEO, C. H., JELINIC, M., NG, H., MARSHALL, S., NOVAK, J., TARE, M., CONRAD, K. & PARRY, L. 2017. Vascular actions of relaxin: nitric oxide and beyond. *British journal of pharmacology,* 174**,** 1002-1014.

LI, Z., FENG, S., LOPEZ, V., ELHAMMADY, G., ANDERSON, M. L., KAFTANOVSKAYA, E. M. & AGOULNIK, A. I. 2011. Uterine cysts in female mice deficient for caveolin-1 and insulin-like 3 receptor RXFP2. *Endocrinology,* 152**,** 2474-2482.

LOK, S., JOHNSTON, D. S., CONKLIN, D., LOFTON-DAY, C. E., ADAMS, R. L., JELMBERG, A. C., WHITMORE, T. E., SCHRADER, S., GRISWOLD, M. D. & JASPERS, S. R. 2000. Identification of INSL6, a new member of the insulin family that is expressed in the testis of the human and rat. *Biology of reproduction,* 62**,** 1593-1599.

LUU, V. V., HANATATE, K., TANIHARA, F., SATO, Y., DO, L. T. K., TANIGUCHI, M. & OTOI, T. 2013. The effect of relaxin supplementation of in vitro maturation medium on the development of cat oocytes obtained from ovaries stored at 4 C. *Reproductive biology,* 13**,** 122-126.

LYNCH, V. J., BRAYER, K., GELLERSEN, B. & WAGNER, G. P. 2009. HoxA-11 and FOXO1A cooperate to regulate decidual prolactin expression: towards inferring the core transcriptional regulators of decidual genes. *PloS one,* 4**,** e6845.

MACLENNAN, A. & GRANT, P. 1991. Human relaxin. In vitro response of human and pig myometrium. *The Journal of Reproductive Medicine,* 36**,** 630-634.

MACLENNAN, A., GRANT, P., NESS, D. & DOWN, A. 1986. Effect of porcine relaxin and progesterone on rat, pig and human myometrial activity in vitro. *The Journal of reproductive medicine,* 31**,** 43-49.

MANN, G., LAMMING, G., ROBINSON, R. & WATHES, D. 1999. The regulation of interferon-tau production and uterine hormone receptors during early pregnancy. *Journal of reproduction and fertility. Supplement,* 54**,** 317.

MCBRIDE, A., HOY, A. M., BAMFORD, M. J., MOSSAKOWSKA, D. E., RUEDIGER, M. P., GRIGGS, J., DESAI, S., SIMPSON, K., CABALLERO-HERNANDEZ, I. & IREDALE, J. P. 2017. In search of a small molecule agonist of the relaxin receptor RXFP1 for the treatment of liver fibrosis. *Scientific Reports,* 7**,** 1-11.

MCGUANE, J. T., DEBRAH, J. E., SAUTINA, L., JARAJAPU, Y. P., NOVAK, J., RUBIN, J. P., GRANT, M. B., SEGAL, M. & CONRAD, K. P. 2011. Relaxin induces rapid dilation of rodent small renal and human subcutaneous arteries via PI3 kinase and nitric oxide. *Endocrinology,* 152**,** 2786-2796.

MEE, J. F. 2008. Prevalence and risk factors for dystocia in dairy cattle: A review. *The Veterinary Journal,* 176**,** 93-101.

MOLNáR, M. & HERTELENDY, F. 1990. Regulation of intracellular free calcium in human myometrial cells by prostaglandin F2 α: comparison with oxytocin. *The Journal of Clinical Endocrinology & Metabolism,* 71**,** 1243-1250.

MONK, J., LEONARD, S., MCBEY, B. & CROY, B. 2005. Induction of murine spiral artery modification by recombinant human interferon-gamma. *Placenta,* 26**,** 835-838.

MOR, G., CARDENAS, I., ABRAHAMS, V. & GULLER, S. 2011. Inflammation and pregnancy: the role of the immune system at the implantation site. *Annals of the New York Academy of Sciences,* 1221**,** 80.

MUDA, M., HE, C., MARTINI, P. G., FERRARO, T., LAYFIELD, S., TAYLOR, D., CHEVRIER, C., SCHWEICKHARDT, R., KELTON, C. & RYAN, P. L. 2005. Splice variants of the relaxin and INSL3 receptors reveal unanticipated molecular complexity. *Molecular human reproduction,* 11**,** 591-600.

MUSAH, A., SCHWABE, C., WILLHAM, R. & ANDERSON, L. 1986. Relaxin on induction of parturition in beef heifers. *Endocrinology,* 118**,** 1476-1482.

NGUYEN, B. T. & DESSAUER, C. W. 2005. Relaxin stimulates protein kinase C ζ translocation: requirement for cyclic adenosine 3′, 5′-monophosphate production. *Molecular Endocrinology,* 19**,** 1012-1023.

NISTRI, S. & BANI, D. 2003. Relaxin receptors and nitric oxide synthases: search for the missing link. *Reproductive Biology and Endocrinology,* 1**,** 1-2.

OHLETH, K. M. & BAGNELL, C. A. 1999. Relaxin Secretion and Gene Expression in Porcine Granulosa and Theca Cells Are Stimulated during In Vitro Luteinization1. *Biology of Reproduction,* 60**,** 499-507.

PARRY, L. J. & VODSTRCIL, L. A. 2007. Relaxin physiology in the female reproductive tract during pregnancy. *Relaxin and Related Peptides***,** 34-48.

PEPIN, N. L., CHAPDELAINE, P. & FORTIER, M. A. 2013. Evaluation of the prostaglandin F synthase activity of human and bovine aldo-keto reductases: AKR1A1s complement AKR1B1s as potent PGF synthases. *Prostaglandins & other lipid mediators,* 106**,** 124-132.

PICCINNI, M.-P. 2006. T cells in normal pregnancy and recurrent pregnancy loss. *Reproductive biomedicine online,* 13**,** 840-844.

PICCINNI, M. P., BANI, D., BELONI, L., MANUELLI, C., MAVILIA, C., VOCIONI, F., BIGAZZI, M., BANI SACCHI, T., ROMAGNANI, S. & MAGGI, E. 1999. Relaxin favors the development of activated human T cells into Th1-like effectors. *European journal of immunology,* 29**,** 2241-2247.

PILLAI, S. B., ROCKWELL, L. C., SHERWOOD, O. & KOOS, R. D. 1999. Relaxin Stimulates Uterine Edema via Activation of Estrogen Receptors: Blockade of its Effects Using ICI 182,789, a Specific Estrogen Receptor Antagonist. *Endocrinology,* 140**,** 2426-2429.

PORTER, D. 1979. The myometrium and the relaxin enigma. *Animal Reproduction Science,* 2**,** 77-96.

PUSCH, W., BALVERS, M. & IVELL, R. 1996. Molecular cloning and expression of the relaxin-like factor from the mouse testis. *Endocrinology,* 137**,** 3009-3013.

RAHEEM, K. A. 2017. An insight into maternal recognition of pregnancy in mammalian species. *Journal of the Saudi Society of Agricultural Sciences,* 16**,** 1-6.

RIVERO-MULLER, A., CHOU, Y.-Y., JI, I., LAJIC, S., HANYALOGLU, A. C., JONAS, K., RAHMAN, N., JI, T. H. & HUHTANIEMI, I. 2010. Rescue of defective G protein–coupled receptor function in vivo by intermolecular cooperation. *Proceedings of the National Academy of Sciences,* 107**,** 2319-2324.

ROCHE, P. J., CRAWFORD, R. J. & TREGEAR, G. W. 1993. A single-copy relaxin-like gene sequence is present in sheep. *Molecular and cellular endocrinology,* 91**,** 21-28.

ROGERS, P., MURPHY, C., SQUIRES, K. & MACLENNAN, A. 1983. Effects of relaxin on the intrauterine distribution and antimesometrial positioning and orientation of rat blastocysts before implantation. *Journal of reproduction and fertility,* 68**,** 431-435.

SARWAR, M., DU, X. J., DSCHIETZIG, T. B. & SUMMERS, R. J. 2017. The actions of relaxin on the human cardiovascular system. *British journal of pharmacology,* 174**,** 933-949.

SATCHELL, L., GLISTER, C., BLEACH, E. C., GLENCROSS, R. G., BICKNELL, A. B., DAI, Y., ANAND-IVELL, R., IVELL, R. & KNIGHT, P. G. 2013. Ovarian Expression of Insulin-Like Peptide 3 (INSL3) and Its Receptor (RXFP2) During Development of Bovine Antral Follicles and Corpora Lutea and Measurement of Circulating INSL3 Levels During Synchronized Estrous Cycles. *Endocrinology,* 154**,** 1897-1906.

SCHUMACHER, M., SCHWARZ, M. & BRäNDIE, W. 1984. Desensitization of the cAMP system in mouse Leydig cells by hCG, cholera toxin, dibutyryl cAMP and cAMP: localization of the 'lesion'to the guanine nucleotide regulatory protein-adenylate cyclase complex. *Molecular and cellular endocrinology,* 34**,** 67-80.

SCOTT, D., TREGEAR, G. & BATHGATE, R. 2005. LGR7-Truncate Is a Splice Variant of the Relaxin Receptor LGR7 and Is a Relaxin Antagonist in Vitro. *Annals of the New York Academy of Sciences,* 1041**,** 22-26.

SCOTT, D. J., LAYFIELD, S., YAN, Y., SUDO, S., HSUEH, A. J., TREGEAR, G. W. & BATHGATE, R. A. 2006. Characterization of novel splice variants of LGR7 and LGR8 reveals that receptor signaling is mediated by their unique low density lipoprotein class A modules. *Journal of Biological Chemistry,* 281**,** 34942-34954.

SCOTT, D. J., WILKINSON, T. N., ZHANG, S., FERRARO, T., WADE, J. D., TREGEAR, G. W. & BATHGATE, R. A. 2007. Defining the LGR8 residues involved in binding insulin-like peptide 3. *Molecular Endocrinology,* 21**,** 1699-1712.

SEGAL, M. S., SAUTINA, L., LI, S., DIAO, Y., AGOULNIK, A. I., KIELCZEWSKI, J., MCGUANE, J. T., GRANT, M. B. & CONRAD, K. P. 2012. Relaxin increases human endothelial progenitor cell NO and migration and vasculogenesis in mice. *Blood, The Journal of the American Society of Hematology,* 119**,** 629-636.

SHERWOOD, O. D. 2004. Relaxin's physiological roles and other diverse actions. *Endocrine reviews,* 25**,** 205-234.

SHYNLOVA, O., NADEEM, L., ZHANG, J., DUNK, C. & LYE, S. 2020. Myometrial activation: Novel concepts underlying labor. *Placenta,* 92**,** 28-36.

SIEBEL, A. L., GEHRING, H. M., REYTOMAS, I. G. T. & PARRY, L. J. 2003. Inhibition of oxytocin receptor and estrogen receptor-α expression, but not relaxin receptors (LGR7), in the myometrium of late pregnant relaxin gene knockout mice. *Endocrinology,* 144**,** 4272-4275.

SMITH, R. P. & KAUNITZ, A. M. 2017. Patient education: Painful menstrual periods (dysmenorrhea)(Beyond the Basics).

SONG, G. J., PARK, Y.-S., LEE, Y. S., LEE, C. C. & KANG, I. S. 2002. Alternatively spliced variants of the follicle-stimulating hormone receptor gene in the testis of infertile men. *Fertility and sterility,* 77**,** 499-504.

SPANEL-BOROWSKI, K., SCHAFER, I., ZIMMERMANN, S., ENGEL, W. & ADHAM, I. M. 2001. Increase in final stages of follicular atresia and premature decay of corpora lutea in Insl3-deficient mice. *Mol Reprod Dev,* 58.

SPENCER, T. E. & BAZER, F. W. 2004. Conceptus signals for establishment and maintenance of pregnancy. *Reproductive Biology and Endocrinology,* 2**,** 49.

STEWART, D. R., NEVINS, B., HADAS, E. & VANDLEN, R. 1991. Affinity purification and sequence determination of equine relaxin. *Endocrinology,* 129**,** 375-383.

STEWART, D. R. & VANDEVOORT, C. A. 1997. Simulation of Human Luteal Endocrine Function with Granulosa Lutein Cell Culture*. *The Journal of Clinical Endocrinology & Metabolism,* 82**,** 3078-3083.

SUDO, S., KUMAGAI, J., NISHI, S., LAYFIELD, S., FERRARO, T., BATHGATE, R. A. & HSUEH, A. J. 2003a. H3 relaxin is a specific ligand for LGR7 and activates the receptor by interacting with both the ectodomain and the exoloop 2. *Journal of Biological Chemistry,* 278**,** 7855-7862.

SUDO, S., KUMAGAI, J., NISHI, S., LAYFIELD, S., FERRARO, T., BATHGATE, R. A. D. & HSUEH, A. J. W. 2003b. H3 relaxin is a specific ligand for LGR7 and activates the receptor by interacting with both the ectodomain and the exoloop 2. *Journal of Biological Chemistry,* 278**,** 7855-7862.

SVENDSEN, A. M., VRECL, M., ELLIS, T. M., HEDING, A., KRISTENSEN, J. B., WADE, J. D., BATHGATE, R. A., DE MEYTS, P. & NøHR, J. 2008. Cooperative binding of insulin-like Peptide 3 to a dimeric relaxin family peptide receptor 2. *Endocrinology,* 149**,** 1113-1120.

TANG, X.-M. & CHEGINI, N. 1995. Human fallopian tube as an extraovarian source of relaxin: messenger ribonucleic acid expression and cellular localization of immunoreactive protein and 125I-relaxin binding sites. *Biology of reproduction,* 52**,** 1343-1349.

TELGMANN, R., BATHGATE, R. A., JAEGER, S., TILLMANN, G. & IVELL, R. 2003. Transcriptional regulation of the bovine oxytocin receptor gene. *Biology of reproduction,* 68**,** 1015-1026.

THATCHER, W., STAPLES, C., DANET-DESNOYERS, G., OLDICK, B. & SCHMITT, E. 1994. Embryo health and mortality in sheep and cattle. *Journal of Animal Science,* 72**,** 16-30.

UNEMORI, E. N., ERIKSON, M. E., ROCCO, S. E., SUTHERLAND, K. M., PARSELL, D. A., MAK, J. & GROVE, B. H. 1999. Relaxin stimulates expression of vascular endothelial growth factor in normal human endometrial cells in vitro and is associated with menometrorrhagia in women. *Human Reproduction,* 14**,** 800-806.

UNEMORI, E. N., LEWIS, M., CONSTANT, J., ARNOLD, G., GROVE, B. H., NORMAND, J., DESHPANDE, U., SALLES, A., PICKFORD, L. B. & ERIKSON, M. E. 2000. Relaxin induces vascular endothelial growth factor expression and angiogenesis selectively at wound sites. *Wound Repair and Regeneration,* 8**,** 361-370.

VANDEVOORT, C. A., MTANGO, N. R., LATHAM, K. E. & STEWART, D. R. 2011. Primate preimplantation embryo is a target for relaxin during early pregnancy. *Fertility and sterility,* 96**,** 203-207.

VODSTRCIL, L. A., SHYNLOVA, O., VERLANDER, J. W., WLODEK, M. E. & PARRY, L. J. 2010. Decreased expression of the rat myometrial relaxin receptor (RXFP1) in late pregnancy is partially mediated by the presence of the conceptus. *Biology of reproduction,* 83**,** 818-824.

WALSH, S., WILLIAMS, E. & EVANS, A. 2011. A review of the causes of poor fertility in high milk producing dairy cows. *Animal reproduction science,* 123**,** 127-138.

WANG, B. & GOFF, A. Interferon-tau stimulates secretion of macrophage migration inhibitory factor from bovine endometrial epithelial cells. Biology of Reproduction, 2002. SOC STUDY REPRODUCTION 1603 MONROE ST, MADISON, WI 53711-2021 USA, 118-119.

WELSH, T., JOHNSON, M., YI, L., TAN, H., RAHMAN, R., MERLINO, A., ZAKAR, T. & MESIANO, S. 2012. Estrogen receptor (ER) expression and function in the pregnant human myometrium: estradiol via ERa activates ERK1/2 signaling in term myometrium. *J Endocrinol,* 212**,** 227-238.

WILKINSON, T. N., SPEED, T. P., TREGEAR, G. W. & BATHGATE, R. A. 2005. Evolution of the relaxin-like peptide family. *BMC evolutionary biology,* 5**,** 14.

XIAO, C. W., MURPHY, B. D., SIROIS, J. & GOFF, A. K. 1999. Down-Regulation of Oxytocin-Induced Cyclooxygenase-2 and Prostaglandin F Synthase Expression by Interferon-τ in Bovine Endometrial Cells1. *Biology of Reproduction,* 60**,** 656-663.

XIAO, J., HUANG, Z., CHEN, C. Z., AGOULNIK, I. U., SOUTHALL, N., HU, X., JONES, R. E., FERRER, M., ZHENG, W. & AGOULNIK, A. I. 2013. Identification and optimization of small-molecule agonists of the human relaxin hormone receptor RXFP1. *Nature communications,* 4**,** 1-7.

YOSHIDA, T., KUMAGAI, H., SUZUKI, A., KOBAYASHI, N., OHKAWA, S., ODAMAKI, M., KOHSAKA, T., YAMAMOTO, T. & IKEGAYA, N. 2012. Relaxin ameliorates salt-sensitive hypertension and renal fibrosis. *Nephrology Dialysis Transplantation,* 27**,** 2190-2197.

ZHANG, L., LIU, X., LIU, J., MA, L., ZHOU, Z., SONG, Y. & CAO, B. 2017. The developmental transcriptome landscape of receptive endometrium during embryo implantation in dairy goats. *Gene,* 633**,** 82-95.

ZINGG, H., GRAZZINI, E., BRETON, C., LARCHER, A., ROZEN, F., RUSSO, C., GUILLON, G. & MOUILLAC, B. 1998. Genomic and non-genomic mechanisms of oxytocin receptor regulation. *Vasopressin and Oxytocin.* Springer.