The role and regulation of insulin-like peptide 3 (INSL3) in the female reproductive system

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Abstract

The peptide hormone Insulin-like peptide 3 (INSL3) shows important roles in the reproductive system. In the male, the testicular Leydig cell peptide INSL3 is involved in fetal testis descent and sperm maturation during the adulthood. In females of reproductive age INSL3 is mainly produced by the ovarian follicular theca interna cells. The current project aims to explore the detailed actions of INSL3 in the female reproductive system.

Firstly, the secretion pattern of the INSL3 peptide in the serum of young women and women attending an infertility clinic was assessed. The serum levels of INSL3 peptide appeared to increase during the follicular phase, decrease during the luteal phase, and drop to a nadir at menses. The secretion pattern corresponded to the growth of antral follicles within a follicular wave. Pathological conditions leading to an alteration of the antral follicle count, such as polycystic ovarian syndrome and low ovarian reserve, were accompanied by an increase or decrease in the serum INSL3 level, respectively.

The bovine system was adopted and validated as a model for the human in regard to the follicular production of INSL3, as the secretion pattern for INSL3 in bovine serum through estrous cycles was similar to that in women. Primary bovine theca interna cells were then used to study the regulation of the INSL3 gene and its secreted peptide products. At both peptide and mRNA levels

INSL3 production could be up-regulated by progesterone receptor signalling. Together with the analysis of the transcriptional activity of the bovine INSL3 gene promoter, it could be shown that estradiol (E2) – estrogen receptor alpha (ERα) signalling stimulated and E2-ERß signalling decreased INSL3 production. Dihydrotestosterone (DHT) acting through the androgen receptor (AR) and androstenedione (A4) probably acting through ERα and/or ERß both contribute to regulation of INSL3 production. Moreover, luteinising hormone (LH) from the anterior pituitary also influenced the INSL3 production, with the downstream pathway from low level LH stimulation probably involving synergy between cAMP-PKA and ER signalling.

The potential target of INSL3 was also identified in the female bovine reproductive system; full-length transcripts for the INSL3 receptor, RXFP2, were detectable in ovarian theca interna cells, in oocytes, as well as in the myometrium. Meanwhile, full-length transcripts for the closely related receptor, RXFP1, were detected in theca interna cells, endometrium and in myometrium, although the gene encoding the ligand for this receptor, relaxin, has been deleted during ruminant evolution.

When transfecting DNA expression plasmids encoding the individual RXFP2 and RXFP1 receptors into HEK293T cells, the expressed bovine RXFP2 could be activated by bovine and human INSL3 (EC50 = 0.7 & 0.6nM) and porcine and human relaxin (EC50 = 10.5 & 27.3nM). The expressed bovine RXFP1 could be activated by porcine and human relaxin (EC50 = 10.8 & 48.7nM) and

human relaxin 3 (EC50 = 97.9nM). Functional analysis showed that bovine myometrial cells were able to respond to both exogenous relaxin and INSL3, whereas theca interna cells could respond to INSL3 only. Together, however, these experiments support the view that endogenous RXFP2 and RXFP1 are both fully functional in the bovine even though the primary ligand for the latter is missing.

The results showed that for the female INSL3 is mainly produced by the theca interna cells of antral follicles, and is thus a potential biomarker of ovarian functionality. The expression of INSL3 can be regulated by sex steroid hormones in an auto/paracrine manner, as well as by LH in an endocrine manner. In conclusion, the INSL3-RXFP2 system acts in follicles and probably also the uterus to enable and orchestrate female reproductive physiology.

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Abbreviation

A4	Androstenedione
ABAM	Antibiotic-antimycotic
AFC	Antral follicle count
AR	Androgen receptor
bINSL3	Bovine Insulin-like peptide 3
BMP	Bone morphogenetic proteins
cAMP	Cyclic adenosine monophosphate
CL	Corpus luteum
DHT	Dihydrotestosterone
E2	Estradiol
ER	Estrogen receptor
ERE	Estrogen response element
FSH	Follicle-stimulating hormone
GVBD	Germinal vesicle breakdown
hINSL3	Human Insulin-like peptide 3
ICI	ICI 182,780
INSL3	Insulin-like peptide 3
LH	Luteinising hormone
LRR	Leucine-rich repeat
P4	Progesterone
P5	Pregnenolone
PCOS	Polycystic ovarian syndrome
PGF2α	Prostaglandin F2α
PKA	Protein kinase A
PR	Progesterone receptor
RT-PCR	Reverse transcription–polymerase chain reaction
RXFP1	Relaxin family peptide receptor 1
RXFP2	Relaxin family peptide receptor 2
SF-1	Steroidogenic factor 1
SFRE	Steroidogenic factor 1 response element
TICs	Theca interna cells
TRFIA	Time-resolved fluorescence immunoassay

Chapter 1:

Introduction/Literature review

1.1 Expression and effects of INSL3 in males

Insulin-like peptide 3 (INSL3) is a member of the relaxin family of peptides that belongs to the insulin superfamily. It acts mainly, though not exclusively, in the reproductive system, and has a marked sexually dimorphic physiology.

INSL3 was first identified from the boar as a gene transcript specifically expressed in the Leydig cells of the testes (Adham et al. 1993), and independently from the testes of mice (Pusch et al. 1996). It has long been considered as an almost exclusively male hormone. This is because it has much higher expression in males than in females, and because INSL3 has significant effects in facilitating testicular descent in the male fetus.

During male fetal development INSL3 is synthesised and secreted from the Leydig cells of the fetal testes, where it serves to stimulate the development of the gubernaculum via its cognate receptor relaxin/insulin-like family peptide receptor 2 (RXFP2, also called LGR8). Expansion of the gubernaculum leads to the anchoring of the fetal testes into the inguinal abdominal wall which results in the so-called transabdominal phase of testis descent as the rest of the abdomen grows in an antero-dorsal direction (Nef and Parada 1999; Kumagai et al. 2002). This stage is complete in humans and most mammals prior to birth. Genetic disruption of either INSL3 or RXFP2 causes cryptorchidism (failure of testis descent) (Zimmermann et al. 1999; Overbeek et al. 2001).

After birth in humans, there is a rise of INSL3 and testosterone production in early postnatal age (~3 months old, mini-puberty) which may contribute to some cases of spontaneous descent of testes that did not completely descend into the scrotum at birth (Bay et al. 2007; Ferlin et al. 2009). Circulating INSL3 levels then remain low and only after pubertal onset is there a significant increase in INSL3, reaching a maximum in early adulthood. In the adult, INSL3 appears to have a role in sperm maturation (Adham et al. 1997; Bullesbach et al. 1999) and bone metabolism (Ferlin et al. 2009). The circulating level of INSL3 declines progressively with age in men and rodents (Paust et al. 2002; Anand-Ivell et al. 2006; Anand-Ivell et al. 2009), reflecting a steady decline in Leydig cell functional capacity.

1.2 INSL3 expression through the life cycle in females

In female mammals, INSL3 is expressed mostly in the ovarian theca interna cells which like the testicular Leydig cells, are derived in the embryo from steroidogenic mesenchyme cells. Unlike in males, however, INSL3 peptide or its mRNA is not detectable at any stages of the female mouse embryo (Zimmermann et al. 1997), nor in the amniotic fluid from female human fetuses at least during mid-gestation (Anand-Ivell et al. 2008; Bay et al. 2008), nor in cord blood from baby girls at birth (Bay et al. 2007). Transgenic female mice with overexpression of INSL3 exhibit a descent of the ovaries to an inguinal position similar to that of the testes (Adham et al. 2002). Therefore, it is

important for INSL3 to remain silent during female embryo development to maintain a normal reproductive anatomy.

INSL3 remains undetectable in pre-pubertal girls (<8.7 years) (Bay et al. 2007). The specific transcript levels in the ovary begin to increase with the first wave of follicle development in immature rats (Zimmermann et al. 1997), and the circulating peptide levels increase as puberty progresses in girls (Hagen et al. 2015). Once the reproductive cycle is established, then INSL3 expression levels appear to be maximally expressed in the theca interna cells of small to medium-sized antral follicles, with a lower amount in large follicles and almost undetectable levels in very early corpora lutea, in the ruminant ovary (Bathgate et al. 1999). INSL3 becomes detectable again in large luteal cells of the mid- to late corpus luteum and then declines at luteolysis. If pregnancy occurs, INSL3 mRNA in the corpus luteum continues to rise until mid-gestation and remains elevated until shortly before parturition (Bathgate et al. 1999). In women, INSL3 expression persists until the menopause, when serum INSL3 levels become undetectable in post-menopausal women (Anand-Ivell et al. 2006).

Thus, it appears that INSL3 is synthesised and secreted starting from peri-puberty and ceasing at the menopause. Thereby, INSL3 should exert its potential roles during reproductive age. The aim of this project has been to explore in depth the physiological role of INSL3 in female reproduction.

1.3 The role of INSL3 in the female reproductive system

In regard to female reproductive function, INSL3 has been reported to be involved in folliculogenesis, oocyte maturation, as well as being linked to the corpus luteum and uterus.

1.3.1 Folliculogenesis and INSL3

Follicles develop through primordial, primary, secondary and antral stages (Fig 1.1). Growth of primordial follicles is initiated long before puberty. They can develop independently of gonadotropin action up to the early antral stage, though most of these will undergo atresia. Only after puberty onset, can antral follicles be rescued from atresia under the control of gonadotropins. The rescued antral follicles will further develop, and one (depend on species) will be selected as the dominant follicle and reach the pre-ovulatory stage. In response to a Luteinising Hormone (LH) surge, the dominant follicle eventually ovulates, leaving the empty follicle shell which will form a corpus luteum (McGee and Hsueh 2000). If the growth of the follicles are prematurely arrested, there will be a failure of the development of a dominant follicle and the consequent ovulation, resulting in polycystic ovarian syndrome (PCOS), causing anovulatory infertility (Gambineri et al. 2007). The process that includes follicle recruitment, development, ovulation, luteinisation and luteolysis cycles continuously throughout reproductive life until complete depletion of the primordial follicle reserve at menopause (Macklon and Fauser

1999). An early depletion of the primordial follicular pool, which could be caused by genetic, pathological, environmental and other factors, will lead to premature ovarian failure, resulting in premature menopause before the age of 40 (Vilodre et al. 2007).

INSL3，as the product of follicular theca interna cells, appears to be involved in ovarian physiology. The expression of INSL3 mRNA is first observed in large preantral follicles in the rat, or in early antral follicles in the bovine and in rhesus macaques (Irving-Rodgers et al. 2002; Hanna et al. 2010; Xue et al. 2012). It is suggested that INSL3 at this stage can promote follicle growth in vitro in the rat (Xue et al. 2014). Once the follicles reach the antral stage, INSL3 mRNA expression levels for the bovine are high, especially in small to medium-sized antral follicles, and low in atretic follicles (Bathgate et al. 1999). It is observed that there is accelerated follicle atresia in INSL3-deficient mice (Spanel-Borowski et al. 2001) and an inverse relationship between immunostained INSL3 and atresia in bovine antral follicles (Irving-Rodgers et al. 2002), together suggesting that INSL3 may protect follicular cells from apoptosis and atresia, and so increase the number of growing antral follicles; it might also be involved in follicle selection (Ivell and Bathgate 2002).

1.3.2 INSL3 facilitates oocyte maturation

The oocyte is surrounded by granulosa cells within the follicle, and together are enclosed by a semi-porous basement lamina. Outside the basement

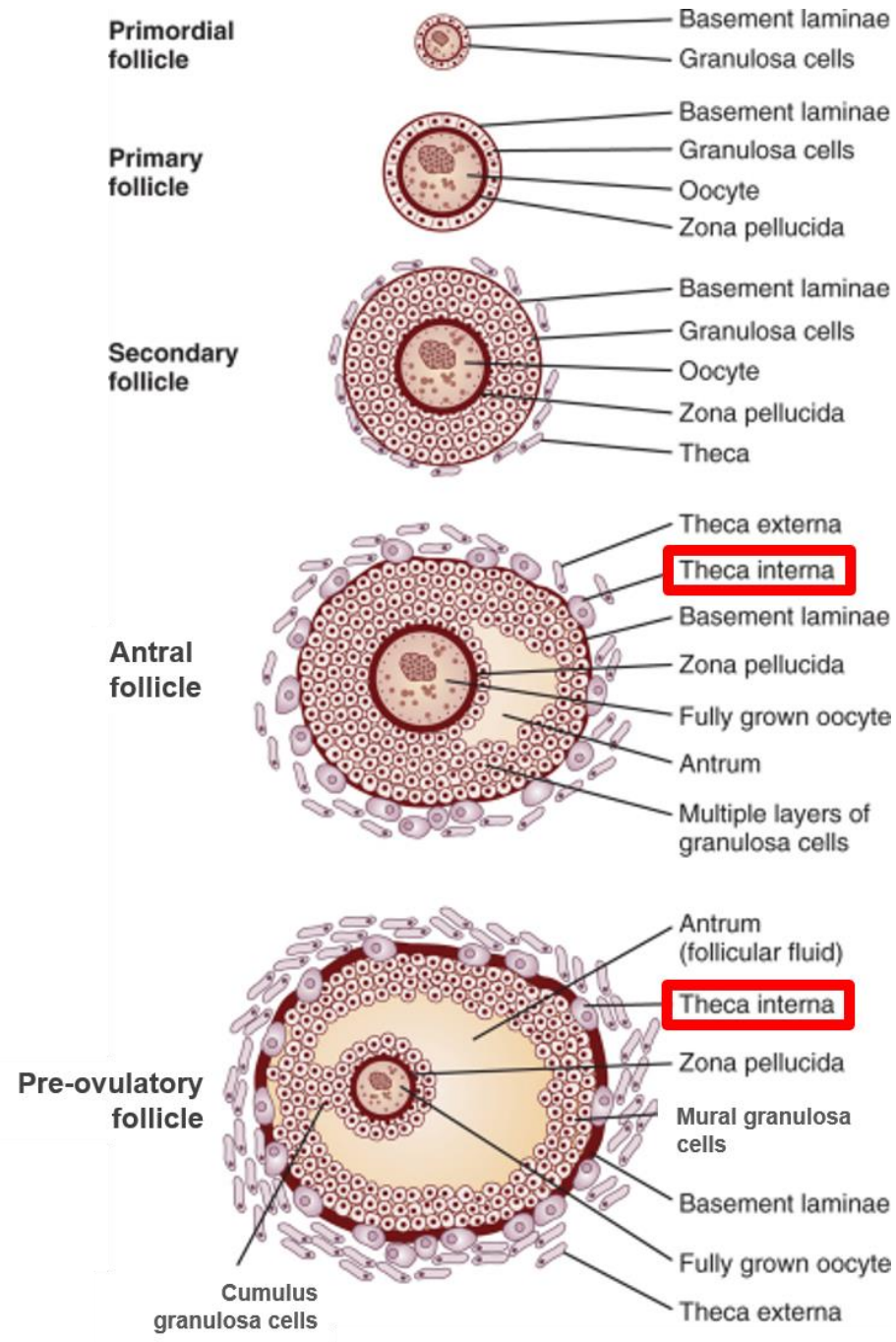


Figure 1.1. Folliculogenesis. When a primordial follicle forms, an oocyte is surrounded by a layer of follicular somatic cells, which then differentiate into granulosa cells. As the follicle develops, the oocyte grows with a zona pellucida presenting around the oocyte, and the granulosa cells proliferate (Knight and Glister 2006). Precursor theca cells from the ovarian stroma are recruited, forming an outer theca layer of the follicle (Young and McNeilly 2010). Theca cells further differentiate into theca interna and theca externa cells. The antrum starts to form and becomes filled with follicular fluid. As the follicle further enlarges, the antral space separates the cumulus granulosa cells from the mural granulosa cells (Sugiura et al. 2010).

lamina is a layer of theca cells (Fig 1.1). Theca formation accompanies the vascularisation in the theca layer (Fraser and Duncan 2009). The development of a vascular network in a follicle is essential for blood supply; delivering gonadotropins from the pituitary to the antral follicles thereby facilitating follicle recruitment, development, selection and ovulation; and transporting the hormones made locally in the follicle to distant target organs and tissues.

The theca cells then further differentiate into theca interna and theca externa cells at the tertiary antral stage. At the same time point the antrum starts to form and becomes filled with follicular fluid (Fig 1.1). The oocyte is therefore protected in a relatively isolated environment and is separated from the rest of the ovarian stroma. Under the support of granulosa cells and theca cells, the oocytes which have formed during prenatal life are arrested in meiotic prophase I. They are preserved like this until puberty onset, when they are gradually used for ovulation and fertilisation until the ovarian reserve is finally exhausted at menopause (McGee and Hsueh 2000).

Only the oocytes in the follicles which reach the pre-ovulatory stage can survive for ovulation. The others degrade as the follicles undergo atresia. In a pre-ovulatory follicle, following the LH surge, a decrease in intra-oocyte cAMP level is essential for resuming meiosis and so preparing a mature oocyte for ovulation (Tsafriri and Pomerantz 1986). This process accompanies germinal vesicle breakdown (GVBD) and the consequent first polar body extrusion.

Kawamura and colleagues (2004) showed that INSL3 might play a role in this process. In rat oocytes, RXFP2, the cognate receptor for INSL3, appears to couple with the inhibitory G protein (Gi). INSL3 binds and activates the receptor, thus causing a decrease in cAMP production, thereby supporting GVBD. This strongly suggests that INSL3 may be involved on promoting oocyte maturation. No effects of INSL3 were observed in relation to follicle rupture (Kawamura et al. 2004).

1.3.3 Other roles of INSL3

After ovulation, the empty follicle shell, comprising both theca and granulosa cells, luteinizes and forms a corpus luteum. Expression of mRNA for both INSL3 and RXFP2 has been detected within corpora lutea (Bathgate et al. 1999; Abe et al. 2013), and it has been reported that INSL3 shows a luteotropic effect, promoting progesterone production in bovine luteal cells (Abe et al. 2013). Moreover, there appears to be accelerated luteolysis in INSL3-deficient mice, further implying that INSL3 has an anti-apoptotic effect in this tissue (Spanel-Borowski et al. 2001).

In addition to the ovary, INSL3 mRNA and protein have been detected in human endometrium (Hombach-Klonisch et al. 2001), and full length RXFP2 mRNA has also been detected in the macaque uterus (Hanna et al. 2010). In mice deficient in RXFP2 on a Cav1-deficient background, there is increased incidence of endometrial cysts (Li et al. 2011). This suggests that

INSL3-RXFP2 signalling may be involved in preparing the endometrium for implantation of the blastocyst.

In summary, the available evidence supports a role for INSL3 in follicle development that prepares a mature oocyte for ovulation and later on fertilisation. In the event of pregnancy, INSL3 contributes to steroidogenesis in the corpus luteum and may also help to prepare the uterus for implantation of the blastocyst.

1.4 Regulation of INSL3 production

As introduced above, the actions of INSL3 are closely associated with female reproductive physiology, and it thus appears likely that appropriate INSL3 production is important for maintaining normal reproductive function.

1.4.1 Theca cells produce INSL3 and steroids

INSL3 mRNA and/or peptide have been detected in the hypothalamus, thyroid, mammary glands, corpora lutea, ovarian stroma, ovarian follicles, uterine, and placental tissues in various species (summarised in Ivell and Bathgate 2002). However, the theca interna cells of antral follicles appear to be the major source of INSL3 in the female mammal (summarised in Ivell and Bathgate 2002). INSL3 produced by theca interna cells appears to accumulate locally in follicular fluid, which in turn may act on follicular cells. In the macaque monkey, the INSL3 peptide level in follicular fluid is about 10 times higher than that in

peripheral serum (Hanna et al. 2010). INSL3 can also enter the circulation through blood capillaries in the theca layer, and hence impact on more distant organs (e.g. the uterus).

Theca interna cells are also the major source of sex steroid production in the female mammal. As shown in Fig 1.1, theca interna cells and granulosa cells are located next to each other within follicles, and these two types of cells form the steroid producing unit (Fig 1.2). Theca cells synthesize progesterone by using cholesterol. Progesterone can be converted into androstenedione which is then supplied to the neighbouring granulosa cells and is aromatised into estrogens (Liu and Hsueh 1986). The produced progesterone, androstenedione and estradiol not only enter the circulation and act on peripheral organs, but they also accumulate locally inside follicles. The detection of mRNA for steroid receptors within theca interna cells indicates their potential roles also in these cells (Vermeirsch et al. 2001; Berisha et al. 2002). Moreover, a previous study has shown that the rodent INSL3 promoter is active within the steroidogenic testicular Leydig cell-line MA-10, but not in the non-steroidogenic COS-7 cells, suggesting the requirement of steroids and/or transcription factors related to steroidogenesis for initiating transcriptional activity of the INSL3 promoter (Sadeghian et al. 2005).

1.4.2 Steroids regulate INSL3

Scattered evidence, mostly from male cells and tissues, suggests that some

steroid hormones may be able to regulate INSL3 production. For example, knock-out of the androgen receptor reduces INSL3 levels in mouse testis (Eacker et al. 2007). Moreover, testosterone appears to stimulate INSL3 mRNA expression in MA-10 mouse tumour Leydig cells, which are the main source of INSL3 in the males (Lague and Tremblay 2008), though this could not be shown by other researchers (Balvers et al. 1998; Sadeghian et al. 2005). The transcriptional activity of both human and rodent INSL3 promoters appeared to be enhanced by testosterone and inhibited by estradiol in these MA-10 cells (Lague and Tremblay 2009). Together such results suggest that in males androgen and estrogen signalling may be able to modulate INSL3 gene expression.

The only evidence for the female shows that in the context of intact large preantral rat follicles the androgen dihydrotestosterone (DHT) decreases INSL3 mRNA levels both in vivo and in vitro (Xue et al. 2014). Thereby, it seems there may be sexually dimorphic effects of steroids in regulating INSL3 production. In the current project, the detailed roles of different steroids have been examined in ovarian theca interna cells.

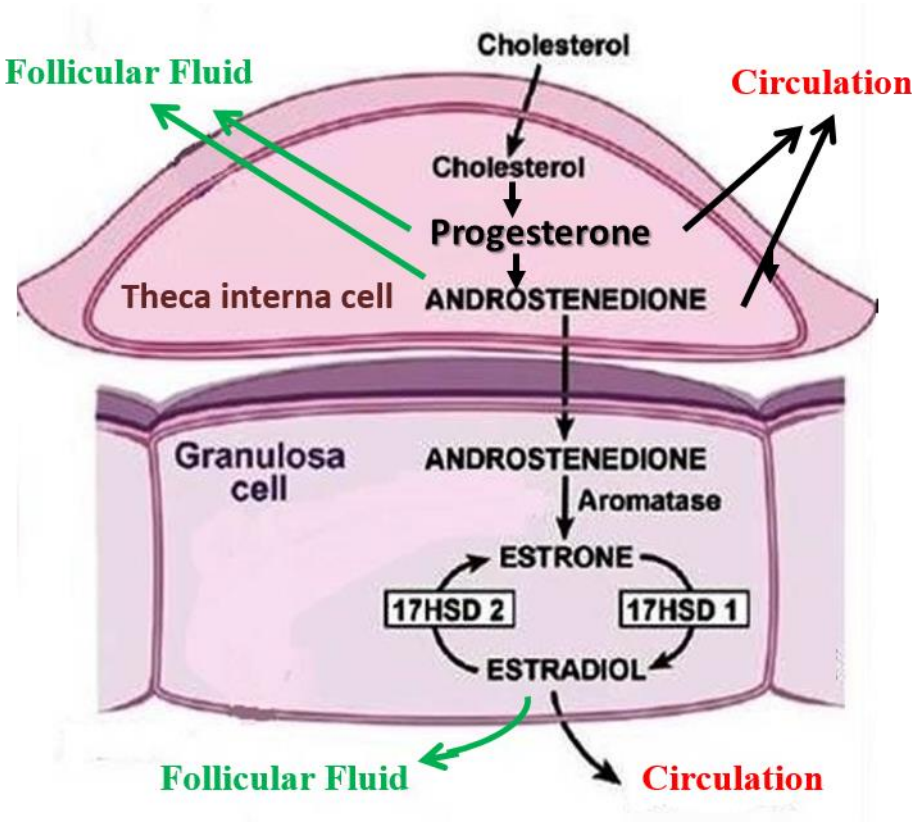


Figure 1.2. Steroidogenesis in theca and granulosa cells. Theca interna cells produce progesterone from cholesterol. Progesterone can be converted into androstenedione. Androstenedione diffuses to granulosa cells and is aromatised to estrone that is further converted into estradiol (Doshi and Agarwal 2013).

Chapter 2:
Objectives

This project has focused on the role of INSL3 in the physiology of the female reproductive system and the regulation in the female of INSL3 production. These specific objectives were as follows:

(1) To assess the physiological relevance of INSL3 in female reproduction, circulating INSL3 levels have been measured in human serum across the menstrual cycle in young healthy women of reproductive age. The secretion pattern was then compared with the bovine model. INSL3 was also assessed in women attending an infertility clinic, and thus subject to a range of reproductive-related pathology.

(2) Making use of cultured primary bovine theca interna cells, to assess the role of steroids in regulating INSL3 production. To confirm a role for steroids in regulating INSL3 production, the transcriptional activity of the bovine INSL3 gene promoter was examined in bovine theca interna cells.

(3) Finally, to characterize where INSL3 may be acting, the expression of its receptor RXFP2 and that of the structurally related receptor RXFP1 was examined in different tissues and cells of the bovine female reproductive system. Other potential ligands for these two receptors were also screened.

Chapter 3:

Material and Methods

3.1 Serum samples collections

3.1.1 Human samples

As part of a collaboration with Dr Georgina Hale from the University of Sydney, Australia, I was provided with samples of peripheral blood plasma that had been collected from 18 young women none of whom indicated any reproductive-related pathology. The selection criteria and detailed anthropometric data for these women are detailed in Hale et al. (2007) and Robertson et al. (2009). The 18 women were 28.9±4.4 years old with a history of smoking (though not at the time of sampling) for 29% of subjects. They had an average body mass index (BMI) of 24.5±3.7, and 0.2±0.08 pregnancies with first pregnancy at the age of 25.5±5 years old. Blood was collected for approximately 6 weeks at about 2 to 3-day intervals following menses. Sampling was defined as during menses (MEN), early follicular (EF), mid follicular (MF), late follicular (LF), ovulatory (OV), early luteal (EL), mid luteal (ML) or late luteal (LL) phases. Human INSL3 was measured in the plasma samples using sensitive in-house Time-Resolved Fluorescence Immunoassay (TRFIA), as described in section 3.3.

The second study comprised 909 samples of peripheral blood plasma from women who attended an infertility clinic in Adelaide, Australia. These samples were collected at first examination and were obtained as a part of a collaboration with Professor Kelton Tremellen (Repromed, Australia). Only one sample was collected per individual without consideration of time within a

menstrual cycle; about 80% corresponded to the follicular phase. The following infertility diagnoses were collated: male factor (n = 277), tubal block (n = 67), low ovarian reserve (n = 219), polycystic ovarian syndrome (PCOS; n = 134), endometriosis (n = 49), unexplained or other causes (e.g. recurrent miscarriage) (n = 173), or combinations of these. The antral follicle count (AFC), was defined as the total number of follicles with diameter of 2-10mm in both ovaries detectable by ultrasound (Tremellen and Kolo 2010).

The plasma samples were frozen and stored at -20°C until INSL3 analysis, using an in-house sensitive human INSL3 TRFIA (see 3.3). As a measure of quality control, the effect of repeated freeze-thaw cycles was checked using 10 randomly chosen samples. There was no reduction of INSL3 after at least three freeze-thaw cycles, and no effect of prolonged storage at -20°C.

Being retrospective analyses of anonymised samples, only local ethics advisory board approval was required. The study on the young healthy women was approved by the Institutional Review Boards at Family Planning, New South Wales, and the University of Sydney. All women had provided informed consent. The second study with samples obtained from women attending an infertility clinic was approved by the Scientific Advisory Committee of Repromed, Adelaide, Australia (SAC 4/11). All samples were previously checked as negative for HIV or Hep C.

3.1.2 Bovine samples

The bovine serum samples were collected by Professor Philip Knight's group at the University of Reading (Satchell et al. 2013). Estrous cycles of 5 healthy British Friesian heifers (~2 years old; ~400kg body weight) were synchronized by giving 3 injections of the PGF2α analog cloprostenol (0.5 mg), 14 days apart. The day of the third PG injection was designated as day 0 of the study. Two further PG injections were given on days 14 and 28 to induce luteolysis, resulting in 3 truncated cycles being studied per cow (n = 15 cycles in total). Blood samples (50 ml) were collected every day. The plasma samples were then stored at -20°C till INSL3 measurements, using the TRFIA specific for ruminant INSL3 (see 3.3). The animal experiment were carried out in full compliance with United Kingdom Home Office guidelines and the Animals (Scientific Procedures) Act 1986.

3.2 Primary bovine cell isolation and culture

3.2.1 Primary bovine theca interna cell culture

3.2.1.1 Theca interna cell isolation

Tissue collection in this project was in accordance with German, British and European laws and provisions for ethical regulation. The isolation protocol essentially followed Glister et al. (2013). Bovine ovaries were collected from the local slaughterhouse. They were transported to the laboratory in warm

DPBS (Gibco, Thermo Fisher, Paisley, UK) supplied with 2% antibiotic-antimycotic (ABAM, Sigma, St. Louis, USA). Follicles sized from 4mm to 6mm were dissected from the ovaries without an obvious corpus luteum to ensure they had not been luteinised due to exposure to the LH surge. Follicles were excised and opened, and granulosa cells which might still be attached to the inside of the follicle shell were scraped off. The theca interna layer was peeled away, and pooled in Medium 199 (Sigma) with additional collagenase IV (1mg/ml) (Sigma) and trypsin inhibitor (100µg/ml) (Sigma). Digestion of the theca interna layer was carried out in a shaking water bath at 38°C at 60rpm. After 45min shaking incubation cells were dissociated by repeated pipetting up and down. The digestion mixture was returned to the shaking water bath and incubated for another 15min. At the end of digestion cells were once more pipetted up and down. The tube was allowed to stand for 2min to allow large undigested fragments to settle down, and the liquid phase only was transferred to Medium 199 additionally containing 1% of ABAM. After brief centrifugation at 1500 *xg* for 5min the cell pellet was re-suspended in DPBS, 2 volumes of autoclaved deionised water was quickly added on top to break any red blood cells, and immediately add 2 volumes of 2x DPBS (Ambion, Thermo Fisher, Grand Island, USA) to restore the isotonicity. The resulting cells were re-suspended in culture-medium, comprising McCoy's 5A medium (Sigma), with 1% ABAM, 2mM L-glutamine (Gibco), 10mM HEPEs (Sigma), 0.1% bovine serum albumin (BSA, Biomol, Hamburg, DE), 10ng/ml

bovine insulin (Sigma), 5μg/ml bovine apo-transferrin (Sigma), and 5ng/ml sodium selenite (Sigma). Cells were maintained at 38°C with 5% CO_2.

3.2.1.2 Stimulation experiments in theca interna cells

After theca interna cells had been isolated, they were seeded at ~180,000 cells / well (48-well plate) (Thermo Scientific, Naerum, DK). The cells were then incubated for about 30 hours and then medium was completely changed to fresh culture-medium containing stimulants, followed by incubation for a further 48 hours at 38°C with 5% CO_2. When treating the cells, the inhibitors (i.e. onapristone and RU 486 (kind gift from Professor Walter Elgar, Berlin, DE), bicalutamide (Sigma), ICI 182,780 (ICI, Tocris, Minneapolis, USA), and H89 (Sigma)) were added first, and immediately followed by estradiol (E2) (Sigma), androstenedione (A4) (Fluka), 5 alpha-dihydrotestosterone (DHT) (Sigma), 8Br-cAMP (Sigma), or LH from sheep pituitary (Sigma). After 48 hours' incubation, the culture-medium from individual wells was collected and stored at -20°C until INSL3 measurement (see 3.3). The measured INSL3 level was normalised using the INSL3 level in the control or vehicle condition, and represented as Relative INSL3 Level. Each treatment was performed in triplicate. The cell in triplicate wells were washed with DPBS, and then pooled in TriZol (Ambion) for later RNA extraction (see 3.4.1). Each experiment was repeated three times.

The methodology for culturing theca interna cells in the current study was

modified from that of Glister et al. (2013). The culture time was reduced from 6 days to 4 days. For INSL3 secretion experiments, cells were plated in 48-well plate at a density of 180,000cells / well rather than in 96-well plate at a density of 75,000 cells / well. Also, the culture time was reduced from 6 days to 4 days. In general, this revised protocol led to similar or improved INSL3 production (not shown).

3.2.1.3 MTT assay for toxicity in theca interna cells

The isolated theca interna cells were seeded at ~80,000cells / well (96-well plate) (Thermo Scientific) and were cultured for 30 hours at 38°C with 5% CO_2. Each chemical was used to treat the cells for 48 hours, and then cell viability was measured with the MTT assay (Invitrogen, Thermo Fisher, USA), following the manufacturer's protocol. Briefly, culture-medium in individual wells was removed and changed to 100µl of fresh culture-medium, 10µl of MTT solution (5mg/ml) was added, and the cells were incubated at 37°C for 4 hours. Then 100µl of SDS solution in 0.01M HCl was added, and incubation continued at 37°C for 18 hours. The cell viability was then determined by measuring absorbance at 570nm using a Victor 3TM 1420 multilabel counter (PerkinElmer, Waltham, USA). Cell viability is positively correlated with the absorbance.

3.2.1.4 Transfection experiments in theca interna cells

The plasmid DNA used in this set of transfection experiments were human estrogen receptor alpha (hERα) and beta (hERß), bovine ERß (bERß), bovine SF-1 and LacZ expression plasmids that were in vector pRcCMV, as previously described (Stedronsky et al. 2002), and were a generous gift from Dr Norbert Walther, Hamburg, Germany. The human androgen receptor (AR) in the vector pCMV4 expression plasmid was a kind gift from Dr Yvonne Giwercman, Lund, Sweden, as previously described (Nenonen et al. 2010). The sequence of the bovine INSL3 gene and upstream promoter was selected from the latest version of the bovine genome project for chromosome 7 (AC_000164.1). In analogy to the identified promoter sequence for human and rat (Koskimies et al. 1997; Robert et al. 2006b), the proximal promoter of the bovine INSL3 gene was considered to be the upstream region of approximately 1800bp of the bovine INSL3 exon 1 immediately following the last exon 23 of the JAK3 gene. This sequence was independently verified by PCR and sequencing from bovine genomic DNA (W. Suehrbier, unpublished Dr Vet Med thesis; University of Hannover, Germany). This sequence terminating 3' end at the putative transcription start site was inserted into the multiple cloning site of the reporter construct pGL3-B (Promega) to create the full-length promoter-reporter construct bI3_1L. The construct bI3_FL contains a further 30 nucleotides of the 5' UTR of the INSL3 mRNA sequence. Subsequently, a series of 5' deletion promoter fragments of the bI3_1L were

prepared (bI3_2L – bI3_8L). All constructs were custom-made and verified by GeneArt (Thermo Fisher, Regensburg, DE).

For transfection experiments, the isolated theca interna cells were cultured at a density of 1.6 million in a T75 flask (Sarstedt, Nümbrecht, DE) for 18 hours. The cells were trypsinised and washed twice with DPBS (without Ca^{2+} or Mg^{2+}) (Gibco). Following the manufacturer's protocol of the Neon transfection system (Invitrogen, Thermo Fisher, Seoul, KR), the cells were transfected with varied combinations of DNA in the ratio of 6µg DNA / 500,000 cells. The cells in an amount ranging from 500,000 up to 2,000,000 were re-suspended in 100µl of Buffer R for transfection with a 100µl Neon Tip, using a single pulse of 1500 volts for 20 milliseconds. After each transfection, the resultant cell suspension in Buffer R was diluted in antibiotic-free medium at 250,000 cells in 500µl volume per well (48-well plate). The ratio of promoter-reporter (luciferase) plasmid : receptor expression plasmid : LacZ expression vector was 2 : 3 : 1 unless otherwise reported in the results section. As a negative control for the promoter-reporter plasmids, the pGL3-Basic (pGL3-B) plasmid was used which contains neither promoter nor transcriptional enhancer sequences. The empty vector p0GH was used to make up any DNA deficit so as to have equal DNA to cells ratio. The transfected cells were then treated with various stimulants and cultured for 24 hours in antibiotic-free culture-medium. The Neon transfection programs used in the current project had been previously optimised using GFP-expression plasmids followed by

microscopic analysis to determine percentage GFP-expression cells (not shown). The program that gave minimal cell death and maximal transfection efficiency was selected. This was used throughout for the same cell type.

After 24 hours' incubation, the supernatant was removed and the cells were lysed using 70µl of 1× passive lysis buffer (Promega, Madison, USA). The transcriptional activity of promoters was determined by measuring the expression level of the reporter gene luciferase. Briefly, 25µl of cell lysate was mixed with 50µl of Luciferase Assay Substrate (Luciferase assay system, Promega). The generated luminescence was measured immediately using a SIRIUS Luminometer (Berthold Detection System, Pforzheim, Germany). The transfection efficiency was determined by normalising from the β-galactosidase expression levels of the co-transfected LacZ-expressing plasmids. Using the β-galactosidase Enzyme Assay System (Promega), 25µl of cell lysate was mixed and incubated with 25µl of Assay 2X buffer at 37ºC for 30min. Then 75µl of sodium carbonate (1M) was added to stop the reaction, and the absorbance measured at 450nm in a Victor 3TM 1420 multilabel counter. Values are expressed as the luciferase level normalised against the β-galactosidase level, represented as Relative Light Units (RLU). For each experiment, at least three replicates were used per treatment, and each set of treatments was repeated 2-4 times.

3.2.2 Isolation of primary bovine granulosa cells, oocytes and cumulus cells

The bovine ovaries which were used for theca interna cell isolation, were also used for obtaining granulosa cells. Follicular fluid containing granulosa cells and the cumulus-oocyte-complex (COC) was aspirated and collected from follicles ranging from 6mm to 10mm. The COCs were manually removed and the residual cell suspension subjected twice to unit sedimentation. The granulosa cells were then harvested by centrifugation at 150 *xg* for 10min. Cells were washed once and cultured in granulosa cell culture-medium, containing DMEM/F-12 (1:1) (without phenol red) (Gibco), 1% fetal bovine serum (charcoal stripped) (CCS) (PAA, Cölbe, DE), 1% ABAM, and 2mM L-Glutamine, at 38°C with 5% CO_2.

Eight isolated COCs were pooled and cumulus cells mechanically separated from oocytes using a mouth pipe and glass pipette followed by a 2min incubation in 1× trypsin-EDTA solution (Gibco). Oocytes were transferred to another petri dish containing 3mg/ml Polyvinyl alcohol (Sigma) in DPBS. The isolated granulosa cells, oocytes, and cumulus cells were centrifuged at 150 *xg* for 10min. The individual cell pellets were frozen immediately on dry ice. RNA was extracted using the RNAqueous-micro total RNA isolation kit (Ambion; see 3.4.1).

3.2.3 Primary bovine myometrial cells

The following bovine tissues were also collected from the local slaughterhouse. Uterus was obtained from healthy post-pubertal cows. The uterus was transported to the laboratory on ice. Before dissection, it was quickly washed in 70% EtOH. The ovary was then cut open and rinsed in DPBS (with 1% Pen/Strep and 1% Amphotericin B (Sigma)). The bovine endometrial tissue that includes both epithelial and stromal cells was stripped away from the myometrium, and collected in liquid nitrogen, pulverized and lysed in TriZol for RNA extraction (see 3.4.1). Myometrial tissue was then isolated from the uterus and finely chopped into 3-5mm^3 pieces in DPBS containing 1% ABAM. The tissue was then transferred to 5ml of digestion solution containing 0.4mg/ml Collagenase (type 2) (Worthington, Lakewood, USA), 10U/ml deoxyribonuclease (type I) (Sigma) and 1% ABAM in DMEM/F12 (1:1) (without phenol red) and incubated in a petri dish at 37°C and 5% CO_2 for 13 hours. The digestion solution was diluted with myometrial culture-medium (1% ABAM, 2mM L-glutamine and 10% fetal bovine serum (FBS) (PAA) in DMEM/F-12 (1:1) without phenol red). The resultant cell suspension was subjected to unit sedimentation twice to remove large pieces, followed by centrifugation at 150 *xg* for 5min. The myometrial cells were cultured in a T75 flask using myometrial culture-medium. After two days' culturing, the cells were trypsinised, washed and plated at 6,000cells / well in a 48-well plate and were cultured at 37°C with 5% CO_2 for overnight before stimulation. The Trizol

method was used to extract RNA (see 3.4.1).

3.2.4 Other bovine tissues and cells

Two cell embryos, 4-8 cell embryos, and blastocysts obtained after in vitro fertilisation were a generous gift from Dr Dana Janowski (Leibniz Institute for Farm Animal Biology) (Janowski et al. 2012), and the RNA was extracted using RNAqueous-micro total RNA isolation kit (see 3.4.1). RNA from primary bovine mammary epithelial cells (MEC) was a kind gift from Prof Hans-Martin Seyfert, Leibniz Institute for Farm Animal Biology, Germany.

3.2.5 HEK-293T cell culture

The HEK-293T cells permanently transfected with human RXFP1 or human RXFP2 were as previously described (Sudo et al. 2003) and maintained in HEK-293T 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.

The basic HEK 293T cells that were used for expressing the transiently transfected bovine RXFP1 (bRXFP1) or bovine RXFP2 (bRXFP2) were cultured in parallel using the same HEK-293T culture-medium. The full-length sequences for bRXFP1 and bRXFP2 were determined based on PCR sequencing results within our laboratory (Chapter 7) as well as from available

sequences in the NCBI database. To improve expression, the natural leader sequences of the receptors were replaced by that of the bovine prolactin gene with an intermediate short FLAG sequence, as previously described for the human equivalent receptors (Hsu et al. 2000), the reconstructed sequences were chemically synthesized and inserted into the expression vector pcDNA3.1/Zeo (+) (custom gene synthesis by GeneArt).

To transfect bRXFP1 or bRXFP2 expression plasmids into basic HEK-293T cells, the plasmids were mixed with cells in a ratio of 2μg DNA per 500,000 cells. The cells of an amount ranging from 500,000 up to 2,000,000 were re-suspended in 100μl Buffer R and transfected with a 100μl Neon Tip, using a single pulse of 1300 volts for 30 milliseconds. After each transfection, the resultant cell suspension in Buffer R was diluted in antibiotic-free HEK-293T culture-medium at 50,000cells in 500μl volume per well (48-well plate). After 24 hours' incubation, the cells were stimulated in fresh antibiotic-free culture-medium as follows.

3.2.6 Stimulation for cAMP production

The sources of various relaxin family peptides were: porcine relaxin (pRLN) from Professor David Sherwood (University of Illinois, Urbana-Champain, USA); recombinant human H2 relaxin (rhRLN), human INSL3 (hINSL3), and bovine INSL3 (bINSL3) from Professor John Wade (Florey Institute of Neuroscience, University of Melbourne, Australia); human relaxin 3 (hRLN3)

from Phoenix Pharmaceuticals (Burlingame, USA).

Primary bovine ovarian theca interna cells, primary bovine uterine myometrial cells and different HEK 293T cells were seeded at a concentration of 20,000cells, 6000cells and 50,000cells per well (48-well plate), respectively, 20-30 hours prior to stimulation. The culture medium in individual wells was aspirated and the cells were treated with stimulants in fresh medium. Wherever the phosphodiesterase inhibitor IBMX (isobutylmethylxanthine, 1mM, Sigma) was used, the cells were pre-treated with the inhibitor for 10-20min. The stimulation with various relaxin family peptides was then made for 20min. The reaction was stopped by adding cold absolute ethanol (EtOH) (Fisher Scientific, Loughborough, UK) on top in each well. The total cAMP from the conditioned culturing medium and the cells was extracted using 85-87% EtOH (Heng et al. 2008). After centrifugation at 2000 *xg* for 45min, the liquid phase containing cAMP was then separated and dried at 60°C using 180mbar vacuum for 90min with a 30% speed in a RapidVap Labconco evaporator (Labconco Corporation, Kansas, USA). The content was then re-suspended in 500µl assay matrix comprising 50% of MEM buffer in deionised water (Gibco), and was measured using a highly sensitive cAMP TRFIA (see 3.3).

3.3 Time-resolved fluorescence immunoassay (TRFIA)

As shown in figure 3.1, in a TRFIA reaction, the secondary antibodies (Rockland, Limerick, USA) are pre-coated in the well of a microtitre plate (Thermo Scientific). The primary antibodies are then added and can bind to the secondary antibodies. Tracers in the form of Eu^{3+} labelled antigens compete with the antigens contained within a sample and bind to the primary antibodies. After washing, the Eu^{3+} from the bound tracer is then dissociated and can be excited by pulsed light at 340nm with emission of intense fluorescent light at 614nm which can be detected using the Victor3TM 1420 multilabel counter in delayed-response mode. The intensity of fluorescence generated from the bound tracer inversely reflects the amount of antigen present within a sample.

In the current project, I have used three in-house designed and previously validated Time-Resolved Fluorescence Immunoassays (TRFIAs) to detect very low levels of human INSL3 (hINSL3) (Anand-Ivell et al. 2006), bovine INSL3 (bINSL3) (Anand-Ivell et al. 2011) and cyclic adenosine monophosphate (cAMP) (Anand-Ivell et al. 2007). After re-establishing these assays in our laboratory in Nottingham, I validated them by running standards and spiking tests, and calculating the intra- and inter-plate coefficient variation. The procedures for these three assays are summarised in Table 3.1. The standard curves for each assay are displayed in Figure 3.2.

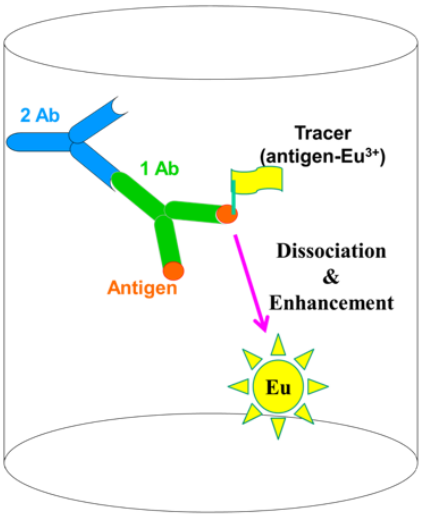


Figure 3.1. Principle of Time-resolved fluorescence immunoassay (TRFIA). A pre-coated second antibodies (2 Ab) can bind to first antibody (1 Ab) that are specific for the target antigens (i.e. hINSL3, bINSL3, and cAMP). Tracer is Eu^{3+} labelled antigen that compete with antigens from a sample to bind with 1Ab. The Eu^{3+} from the bound tracer will then be dissociated and emit fluorescent that can be detected. The intensity of the fluorescent reversely reflects the number of antigens bound with the 1 Ab.

Table 3.1. Summary of the procedures and controls for the Time-resolved fluorescence immunoassays (TRFIAs) that detects human INSL3 (Anand-Ivell et al. 2013), bovine INSL3 (Glister et al. 2013), and cAMP (a. sensitive assay; b. ultra-sensitive assay).

TRFIA	Human INSL3	Bovine INSL3	cAMP
Secondary Antibody	Goat-anti-Rat	Goat-anti-Rabbit	Goat-anti-Rabbit
Primary Antibody	rat-anti-human INSL3 (Ivell Lab)	rabbit-anti-bovine INSL3 (Ivell Lab)	rabbit-anti-cAMP (Ivell Lab)
Detection Range	(ng/ml) 0.005 – 2	(ng/ml) 0.02 – 20	(pmol/ml) a. 1 – 243 b. 0.04 – 9.72
Procedure	+ 100µl Sample + 100µl Antiserum ↓ Overnight at 4ºC Wash once + 200µl Tracer ↓ 3h at 4ºC Wash 4 times + 200µl Enhancement ↓ 60min Record time resolved fluorescence	+ 50µl Sample + 100µl Antiserum ↓ Overnight at 4ºC Wash once + 150µl Tracer ↓ 2h at 4ºC Wash 4 times 200µl Enhancement ↓ 60min Record time resolved fluorescence	+ 50µl Sample + 50µl Tracer + 100µl Antiserum ↓ Overnight at 4ºC Wash 4 times + 200µl Enhancement ↓ 45min Record time resolved fluorescence
Intra-plate CV%	<3%	<2%	a. <4% b. <3%
Inter-plate CV%	<10%	<10%	a. <8% b. <4%

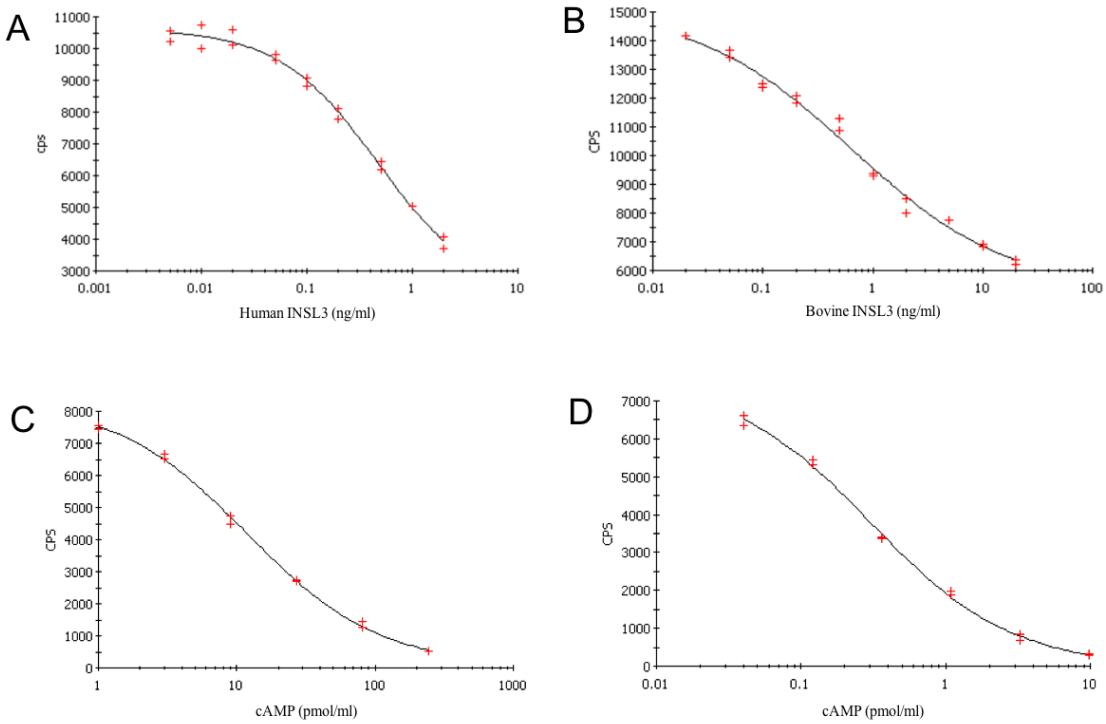


Figure 3.2. Standard curves for Time-resolved fluorescence immunoassays (TRFIAs). Here show the standard curves for human INSL3 TRFIA (A), bovine INSL3 TRFIA (B), cAMP TRFIA (C), and ultra-sensitive cAMP TRFIA (D).

3.4 Gene expression

3.4.1 RNA extraction

When the RNAqueous-micro total RNA isolation kit (Ambion) was used for RNA extraction, the company protocol was strictly followed. Briefly, the cell sample was lysed with 100µl of Lysis Solution, and mixed well with 50µl of 100% EtOH (Sigma). The lysate/EtOH was then passed through a Micro-Filter Cartridge by centrifugation at 12,000 *xg* for 1min, with the RNA binding to the filter. The bound RNA was washed with 180µl of Wash Solution 1 (30% in EtOH) and then with 180µl of Wash Solution 2/3 (20% in EtOH) twice, and all wash steps were performed by centrifugation at 12,000 *xg* for 1min. The Micro-Filter Cartridge was then transferred from the Collection tube to a Micro Elution Tube. The bound RNA was then eluted with 8µl of preheated (75ºC) Elution Solution by centrifugation at 12,000 *xg* for 30sec. A second elution gave a better RNA yield. The RNA was stored at -80ºC untill use. Wherever TriZol was used for RNA extraction, 0.2 volumes of chloroform (Sigma) was used to separate RNA in an aqueous phase from the organic phase by centrifugation at 2000 *xg* for 10min. This aqueous phase that contains RNA was then transferred into a fresh 1.5ml tube. The RNA could then be precipitated with 0.5 volumes of isopropanol (Sigma), and be pelleted by centrifugation at 12,000 *xg* and 4ºC for 10min. The RNA pellet was then washed by 1 volume of 80% EtOH twice with centrifugation at 12,000 *xg* and 4ºC for 10min. After removing all the EtOH, the RNA pellet was air-dried and

then re-suspended in RNAse free water (Ambion), which was then stored in -80ºC till use.

3.4.2 Deoxyribonuclease (DNAse) treatment

To avoid any DNA contamination that may lead to erroneous results in the subsequent PCR, the extracted RNA was then subjected to DNAse treatment using the TURBO DNAse-free kit (Ambion). Following the manufacturer's protocol, the RNA sample was incubated with 0.1 volume of 10x TURBO DNAse Buffer and 4 units of TURBO DNAse at 37ºC for 20min. The reaction was inactivated by adding 0.2 volumes of DNAse Inactivation Reagent and incubating at room temperature for 2min. The reaction mixture was centrifuged at 10,000 *xg* at 4ºC for 5min, and so the supernatant could then be transferred to a fresh 1.5ml tube and stored at -20ºC.

3.4.3 RNA quantitation

The RNA sample was diluted 1:50 in 10mM sodium phosphate buffer (pH 7.0) in a final volume of 100µl. The concentration of the diluted RNA sample was measured using an Eppendorf Biophotometer (Eppendorf, North Ryde, AU). A ratio of OD_{260}/OD_{280} (optical density at 260nm to that at 280nm) between 1.8 and 2.0 indicates good quality of RNA.

3.4.4 RNA qualification

The quality of an RNA sample was further checked by gel electrophoresis

using 1% agarose gels (Fisher Scientific) containing 0.25% guanidine thiocyanate (Sigma) and 1% ethidium bromide (EtBr) in 1x TAE buffer (4.84g/L Tris Base, 0.12% Acetic acid, 0.2% EDTA (0.5M) (all from Sigma)). A 2:1 ratio of sharp and clear EtBr stained 28S:18S ribosomal RNA bands in a sample indicates high quality of RNA.

3.4.5 Synthesis of complementary DNA (cDNA)

The cDNA was synthesised from either 1µg or 3µg RNA (within any experiment, the same amount of RNA was used for cDNA synthesis) using the Superscript III reverse transcriptase kit (Invitrogen). An RNA sample was primed with oligo (dT) 12-18 primer (Invitrogen) at 70ºC for 5min, and then mixed with 0.5 volumes of 5x First Strand Buffer, 0.1 volumes of 0.1M DTT (all are supplied by the manufacturer), 0.5mM of Deoxynucleotide (dNTP) Mix (Sigma) and 200units of Superscript III Reverse Transcriptase, making up to a total volume of 25µl with RNAse free water. The reaction was carried out at 42ºC for 90min in a Shake 'n' Stack hybridisation oven (Thermo Scientific, Darmstadt, DE). The synthesised cDNA was then diluted 1:5 with RNAse free water, and stored at -20ºC until use.

3.4.6 Polymerase chain reaction (PCR)

RT-PCR was carried out using the primers listed in Table 3.2, designed using the Primer3 software applied to sequences XM_002694415 (bovine RXFP1),

NM_001304277 (bovine RXFP2), NM_174778 (bovine S27a), NM_001001443 (bovine ERα), NM_174051 (bovine ERβ), NM_001244127 (bovine AR), NM_001205356 (bovine PR), NM_174403 (bovine SF-1), NM_174365 (bovine INSL3). For each reaction, DNA was denatured at 95ºC for 45 seconds, annealing was carried out at the temperature indicated in the figure legends, with elongation at 72ºC for 1 minutes, for a total of 35 cycles. Ribosomal protein S27a, which has been shown to be expressed consistently in mammalian tissues (CV≤ 0.61%) (Lee et al. 2002), was used as the housekeeping gene in the real-time RT-PCR analysis. The positions of different RXFP1/2 primers are shown in Fig 7.1 and Fig 7.2. In each reaction, 0.25μM of the primer pair (Eurofins, Jena, DE) were used to amplify 1μl of cDNA, using EmeraldAMP MAX HS PCR master mix (Takara-Clontech, Saint-Germain-en-Laye, France) for semi-quantitative RT-PCR in a MJ Research PTC-200 DNA Engine Thermal Cycler (Bio-Rad, Hercules, USA), or using Takara Sybergreen Premix II (Takara, Shiga, JP) for real-time qRT-PCR in a Rotor-Gene 3000 machine (Corbett Research, Sydney, AU).

3.4.7 Agarose gel electrophoresis for PCR products

A PCR product (8μl) was mixed with 2μl of 1x TrackIT cyan/orange Loading Buffer (Invitrogen) and analysed by gel electrophoresis on a 2% agarose gel (Fisher Scientific, Pittsburgh, USA) with 1% Ethidium bromide (Sigma) in 1x TAE buffer, followed by florescence digitisation using a Bio-Rad GelDoc XR

system (Bio-Rad, Gladesville, AU). The TrackIT 100bp DNA Ladder (2µl, Invitrogen) was loaded in each gel to determine the size of every DNA band. For RXFP1 and RXFP2 expression experiments, the DNA was extracted from each gel band, using the MinElute Gel Extraction Kit (Qiagen, Hilden, DE). Following the company protocol, 3 volumes of Buffer QG was used to dissolve 1 volume of gel at 50ºC for 10min. The mixture was mixed with 1 volume of isopropanol, which was then allowed to pass through a MinElute Column by centrifugation at 10,000 *xg* for 1min. The Column with bound DNA was washed with 500µl of Buffer QG followed by 750µl of Buffer PE, both by centrifugation at 10,000 *xg* for 1min. The bound DNA was then eluted with 10µl of Buffer EB by centrifugation as above, and then custom sequenced by Eurofins (DE). The results were then compared with the bovine RXFP1 variant X1 (XM_002694415.4) and bovine RXFP2 (NM_001304277.1).

3.4.8. Analysis of real-time RT-PCR results

The real-time PCR results were calculated using the following equation:

$$NE = \frac{(E_{Reference})^{CT_{Reference}}}{(E_{Target})^{CT_{Target}}}$$

where $E_{Reference}$ and E_{Target} are the PCR amplification efficiencies of the target and housekeeping gene (bovine S27a), respectively; $CT_{Reference}$ and CT_{Target} are the threshold cycles of the PCR amplification of the target gene and

Table 3.2. List of primers used for RT-PCR.

Transcript	No.	Orientation	Sequence (5' - 3')
Bovine RXFP1	503	Forward	tgacatctcgtcctgtcttcc
	504	Reverse	ggagaaccgaccaagcattc
	505	Forward	cgaggacaactgtggagacatc
	506	Reverse	cagtcctcggaaagcatagaca
	507	Forward	gaagccgggtgtttttgaag
	508	Reverse	gggcatgtgctgacaaagag
	509	Forward	taatgtccttacccgcttgc
	510	Reverse	ctagtttctggagaggtgcaaatg
	511	Forward	ccgcttgcctgacaaatctc
	512	Reverse	ggcttgaattgtctggattgg
	513	Forward	cccaatccagacaattcaagc
	514	Reverse	ggcatgcagcttgttctcag
	515	Forward	ggagcccagatttattcagtgg
	516	Reverse	gaatcgggttcaaagcactgt
	529	Forward	acagtgctttgaacccgattc
	530	Reverse	gtttcaagacaggcaaagctg
Bovine RXFP2	517	Forward	cttcagccgcagattgattg
	518	Reverse	cagttcctttgcacacacagc
	519	Forward	gtgccagtggtttctagcaatg
	520	Reverse	caaccgctgtgaaattctgc
	521	Forward	gcagaatttcacagcggttg
	522	Reverse	tgaaaatgtggggtggtagc
	523	Forward	tccacctttctgtcgtgcag
	524	Reverse	ccgtcagtcaagggcataca
	525	Forward	gttcctggccgttgtctttc
	526	Reverse	acgacccaggaggtgattgt
	532	Forward	tgtgggaatctctcccagtg
	533	Forward	tgtatgcccttgactgacgg
	534	Reverse	acgacccaggaggtgattgt
	537	Forward	tcagttgctgtgaacccattg
	540	Reverse	gccgtcacagtgaaaagcac
Bovine S27a	501	Forward	tcctcctgaccagcaaagactg
	502	Reverse	tgcttgttcttcttgggagtgg
Bovine ERα	566	Forward	agggaagctcctatttgctcc
	567	Reverse	cggtggatgtggtccttctct
Bovine ERβ	568	Forward	gcttcgtggagctcagcctg
	569	Reverse	aggatcatggccttgacacaga
Bovine AR	570	Forward	cagatggcagtcattcag
	571	Reverse	cttggtgagctggtagaag
Bovine PR	593	Forward	gggatgaagcatcaggctgt
	594	Reverse	ttctctggcttagggcttgg

Bovine SF-1	599	Forward	agctgctggtttttgaccac
	600	Reverse	cgaggctgaagagaatgagg
Bovine INSL3	574	Forward	gcgcctgagaaactgtgtgg
	575	Reverse	gggttgatggcagttgctcg
Bovine Nur77	597	Forward	ctgtgtgtggggacaatgct
	598	Reverse	gcatcctctttcccgaagtg

housekeeping gene, respectively; and NE is normalised expression of target gene.

3.5 Plasmid preparation

All the DNA plasmids used in this project were prepared as follows.

3.5.1 Preparation of competent cells

XL-1 blue E. Coli cells were a kind gift from Professor Hans-Martin Seyfert. A single colony of cells was cultured in 5ml of LB broth (Gibco) at 37°C with shaking at 130rpm for about 16 hours. This starting culture (1.6ml) was diluted 1:100 in LB broth (160ml) (Gibco), and incubated at 37°C with shaking at 145rpm for about 90-120min. When the OD_{600} value of the culture reached 0.4-0.5, as measured in an Eppendorf BioPhotometer, the bacteria were placed on ice for 30min. The bacterial cells were then harvested by centrifugation at 4000 ×*g* and 4°C for 10min. The cell pellet was re-suspended in 40ml of cold TB buffer, comprising 10mM Hepes, 15mM $CaCl_2$, 250mM KCl and 55mM $MnCl_2$ (all from Sigma) with pH of 6.7. The cells were placed on ice for 10min and harvested again as above. The cell pellet was re-suspended in 10ml of cold TB buffer containing 7% DMSO (Sigma) and then allowed to cool down on ice for 10min. The resulting competent cells were then immediately frozen in liquid nitrogen and stored at -80ºC.

3.5.2 Transformation of plasmid DNA into competent E. Coli

Competent cells (50µl) were thawed on ice for 30min and then mixed with 1µl plasmid DNA (~100ng), and kept on ice water for a further 30min. Tubes were then heat-shocked at 42ºC in a water bath for 50sec and then immediately cooled on ice water. These were then mixed with 9 volumes of S.O.C medium (Invitrogen) and incubated at 37ºC with a shaking speed of 90rpm for 1h. After this incubation, about 5µl of the bacterial suspension was spread on to a 90mm LB agar plate containing the desired antibiotic and incubate at 37ºC for 16 hours. The DNA plasmids that were ampicillin (50µg/ml, Gibco)-resistant included ERα, hERß, SF-1, p0GH, pGL3-B, LacZ, bRXFP1 and bRXFP2. The plasmids that were kanamycin (30µg/ml, Gibco)-resistant included bI3_FL, bI3_1L, bI3_2L, bI3_3L, bI3_4L, bI3_5L, bI3_6L, bI3_7L and bI3_8L.

After 16 hours' incubation, a single colony (~2mm in size) was picked and cultured in 5ml LB broth with the desired antibiotic at 37ºC and a shaking speed of 160rpm for 8 hours. This starting culture was then diluted 1:1000 in LB broth with antibiotic and cultured at 37ºC with a shaking speed of 160rpm for a further 16 hours.

3.5.3 Extraction of plasmid DNA

Bacteria were harvested by centrifugation at 4000rpm and 4ºC for 30min. Plasmid DNA was then extracted using the Qiagen Midi Kit (Qiagen) following the manufacturer's protocol. Briefly, bacteria harvested from 45ml culture were

re-suspended in 4ml of cold Buffer P1 (containing RNase A and LyseBlue), then lysed in 4ml Buffer P2, and the reaction was stopped by adding 4ml of cold Buffer P3. The mixture was centrifuged at 4000rpm and 4ºC for 30min, the supernatant that contained the DNA plasmids was allowed to pass through a QIAGEN-tip 100 which had been pre-equilibrated with 4ml of Buffer QBT. The QIAGEN-tip with bound plasmids DNA was then washed with10ml of Buffer QC twice by letting the buffer pass through the QIAGEN-tip by gravity. Buffer QF (5ml) was added to the QIAGEN-tip to elute the bound plasmids. Isopropanol (3.5ml) was then used to precipitate the plasmid DNA by centrifugation at 4000rpm and 4ºC for 30min. The DNA pellet was then washed with 70% EtOH (Sigma, USA) twice by centrifugation at 13,000 *xg* for 10min. After completely removing the EtOH, the DNA pellet was air-dried and re-suspended in 200µl TE buffer (pH 8.0, Ambion). The concentration was measured with an Eppendorf BioPhotometer.

The extracted plasmids were digested with the appropriate restriction enzymes. The resulting fragments of plasmid DNA were separated by agarose gel electrophoresis and compared with the restricted fragments from the initial plasmid preparations (Eurofins).

3.6 Statistical analysis

All data were analysed using the Graphpad Prism version 6 package. For the human serum INSL3 data, descriptive column statistics were used to define

mean, and standard error (SEM). Data were compared using the unpaired t-test (two-tailed) following ANOVA. Regressions used the Spearman r value. Statistical analysis for bovine plasma INSL3 concentrations was performed by repeated measures ANOVA. For primary theca interna cell experiments, results were analysed using one-way ANOVA followed by Dunnett's multiple comparisons test. For relaxin-family peptides activating RXFP1 and RXFP2 experiments, the EC50 values were calculated from the non-linear regression (curve fit) log (agonist) vs response (three parameter).

For results examining INSL3 serum levels in women and cows, figures are representing almost exclusively biological variance and are expressed as means±SEM. For all cell culture experiments, results from representative experiments are shown with means±SEM expressing almost exclusively technical variance. For these experiments, the biological variance from repeated experiments using different and independent batches of cells, is calculated and described in the corresponding text.

Chapter 4:
Circulating INSL3 levels in women and cows

The results from the current chapter have been published in:

*Anand-Ivell R, Tremellen K, **Dai Y**, Heng K, Yoshida M, Knight P, Hale G, Ivell R. 2013. Circulating insulin-like factor 3 (INSL3) in healthy and infertile women. Human Reproduction 28:3093-3102.*

*Satchell L, Glister C, Bleach E, Glencross R, Bicknell A, **Dai Y**, Anand-Ivell R, Ivell R, Knight P. 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.*

4.1 Introduction

As described in chapter one, INSL3 is synthesised and secreted during reproductive age in female mammals. In order to assess the physiological relevance of INSL3 in female reproduction, circulating INSL3 concentrations were measured in sera from a group of healthy young women collected across their menstrual cycles as well as in a large cohort of women attending an infertility clinic. Changes of the INSL3 levels were then compared with those in a bovine model.

4.1.1 Reproductive cycles in human and bovine

The cow has been used as a model for studying ovarian follicular and luteal development with relevance for the human for several decades (Adams et al. 2012). There are many similarities between human and bovine physiology in regard to ovarian cyclicity. As shown in Fig 4.1 A, in both species, a cohort or a wave of small antral follicles are recruited by the increased level of Follicular Stimulating Hormone (FSH), under the control of the hypothalamus-pituitary-gonadal axis (McGee and Hsueh 2000). In these small antral follicles, INSL3 transcripts have been observed in the follicular theca intern cells (TIC), at least in bovine and rhesus macaques (Irving-Rodgers et al. 2002; Hanna et al. 2010). As the cycle progresses, some of the follicles enlarge, while others become atretic with the follicular cells gradually undergoing apoptosis. Eventually, one follicle grows faster than the others and

survives as the dominant Graafian follicle, which can ovulate when the pituitary Luteinising Hormone (LH) level increases at the LH surge. The LH surge also initiates luteinisation in granulosa cells and theca cells, and so leads to the formation of a corpus luteum (CL) in the luteal phase (Young and McNeilly 2010). A complete cycle terminates with luteolysis (CL regression) (Fig 4.1 A). In women, a new cycle starts with menses, accompanied by endometrial shedding, whereas in the cow the endometrium is rather reabsorbed without shedding (Adams et al. 2012).

In women a wave of follicles appears to emerge in the early follicular phase, then develops giving rise to a dominant Graafian follicle that can be selected for ovulation (Adams et al. 2012) (Fig 4.1 B). In the bovine, when the dominant follicle from a wave emerges at the onset of luteolysis, this follicle wave is an ovulatory one (Adams et al. 2012) (Fig 4.1 B). In addition to the ovulatory wave there are one or two anovulatory waves in women and in cows that are initiated by transient rises in FSH levels during ovulation and the luteal phase (Baerwald et al. 2003; Aerts and Bols 2010). These follicles can develop in the presence of a dominant follicle in the respective wave. However, none of the dominant follicles can proceed to ovulation, probably due to insufficient FSH supply in the human or to a low level of LH that is supressed by CL-produced progesterone in the bovine (Adams et al. 2012). Eventually, these anovulatory waves of follicles undergo atresia.

Due to the presence of multiple waves of growing antral follicles in both human

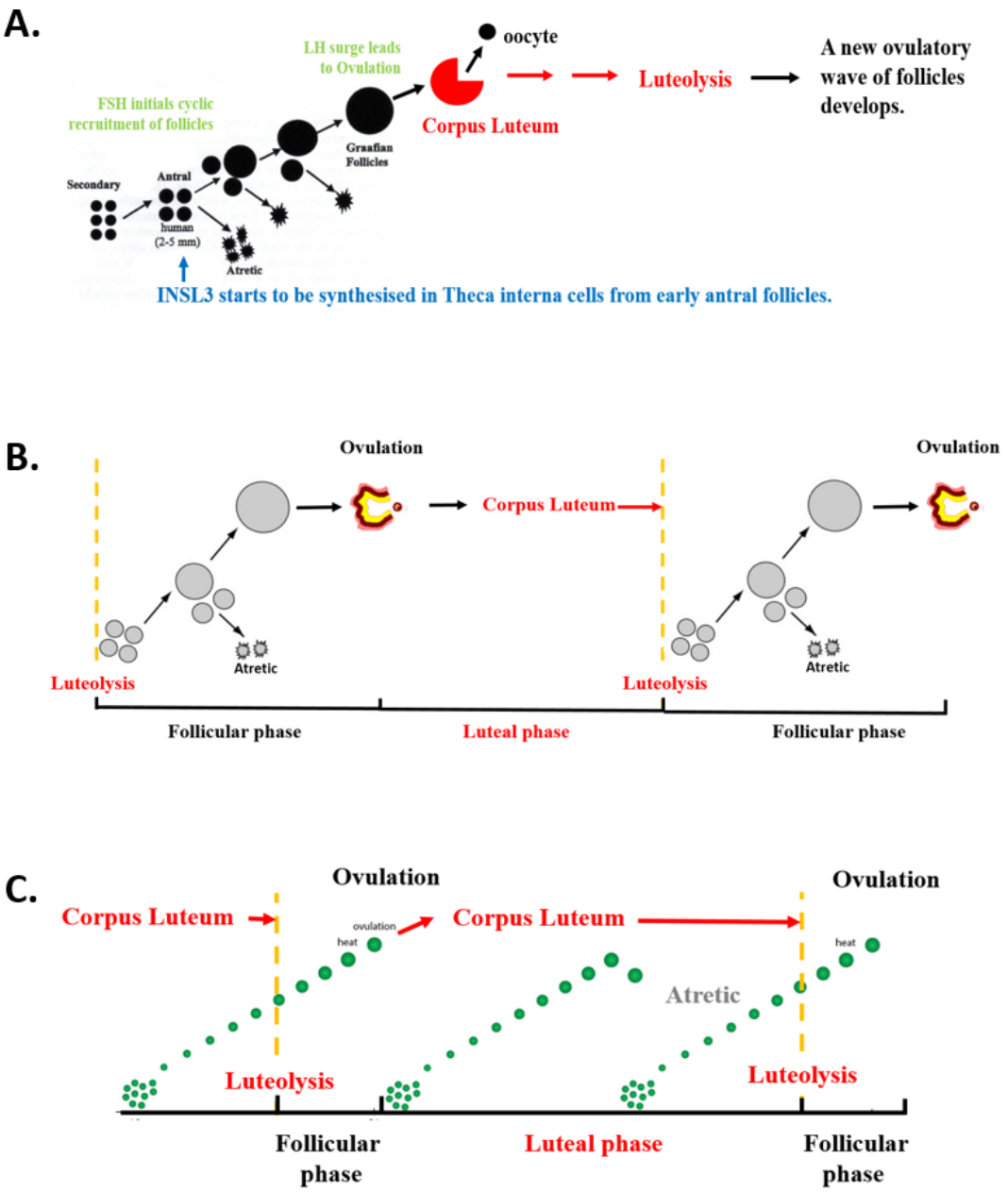


Figure 4.1. Reproductive cycles. (A) An ovulatory wave of follicles develops in both human and bovine. In a reproductive cycle, a cohort or a wave of growing antral follicles are recruited by the elevated FSH concentration. As they develop, most of the antral follicles undergo atresia, and only one of the surviving follicles is selected as the dominant follicle with the fastest growth rate. Induced by an LH surge, the dominant Graafian follicle ovulates and the follicle shell transforms into a corpus luteum (CL), entering the luteal phase (McGee and Hsueh 2000). INSL3 is believed to be expressed in follicles from the early antral stage in bovine and rhesus macaques (Hanna et al. 2010, Irving-Rodgers et al. 2002). (B) Human menstrual cycle: a cohort of small antral follicles are recruited in the early follicular phase. CL forms after ovulation, and so it enters the luteal phase. Luteal phase terminates with luteolysis, and a new cycle then starts with the recruitment of another cohort of small antral follicles. There are equal length of follicular phase and luteal phase of 14 days on

average. (C) Bovine estrous cycle: here shows a bovine estrous cycle with an ovulatory and an anovulatory wave. Only in the absence of a CL, is the selected dominant follicle from the growing wave able to develop to ovulate and form a CL. The follicular wave emerging during ovulation is normally anovulatory and undergoes atresia (Aerts and Bols 2010). Unlike the symmetric follicular and luteal phases in women, the cow has a relatively short follicular phase of 3-4 days, compared to a luteal phase of 16-18 days (Adams et al. 2012).

and bovine, the number and the size of the follicles change dynamically during a reproductive cycle. Follicles from the early antral stage start to express INSL3, at least in the bovine and in rhesus macaques (Irving-Rodgers et al. 2002; Hanna et al. 2010). Consequently, the total available theca interna cell mass in the ovaries, which is the source of INSL3, is also dynamically changed through the reproductive cycle. Although the expression levels of INSL3 transcripts have been examined in individual follicles of varying status (i.e. different sizes, healthy, atretic, and during luteinisation) (Bathgate et al. 1999), the total production of INSL3 from a combination of follicles present in ovaries at the same time point through a reproductive cycle has not been previously assessed. As follicular theca interna cells are thought to be the major source of INSL3 in the female (Ivell and Bathgate 2002), the levels of INSL3 circulating in peripheral blood should reflect the INSL3 output from the ovaries with all the available antral follicles.

In this section, serum samples collected from healthy young women of reproductive age through the menstrual cycle were used to assess the natural dynamics of ovarian INSL3 secretion. Serum levels of INSL3 were also measured in the bovine through synchronized and truncated estrous cycles and compared with the human in regard to follicular production of INSL3.

4.1.2 Infertility

In the current chapter, the serum levels of INSL3 were also measured from

women attending an infertility clinic. They were diagnosed as having infertility due to a dysfunction of the ovary (i.e. polycystic ovarian syndrome (PCOS) and low ovarian reserve), dysfunction of the uterus (i.e. endometriosis), tubal block, male factor, or other unknown reasons.

So far, only PCOS has ever been shown to be associated with altered levels of circulating INSL3 (Havelock et al. 2005; Gambineri et al. 2007; Gambineri et al. 2011; Szydlarska et al. 2012). PCOS is an ovarian dysfunction that affects 6-10% women of reproductive age (Tsilchorozidou et al. 2004). It is observed that an increased number of follicles ranging from 2 to 9mm exists in each of the ovaries (Jonard et al. 2003). These follicles are arrested in an early antral stage that results in the failure of dominant follicle development, and therefore can lead to anovulatory infertility in women (Jonard et al. 2003). The increased number of such antral follicles in the ovary is believed to be the cause of the hyperandrogenism and elevated circulating INSL3 observed in these women. A positive correlation between INSL3 and androgens has also been observed (Havelock et al. 2005; Gambineri et al. 2007; Gambineri et al. 2011; Szydlarska et al. 2012).

The other pathological conditions were examined here for the first time for their relevance in regard to serum levels of INSL3.

4.2 Results

4.2.1 Peripheral INSL3 concentration across the menstrual cycle in healthy young women of reproductive age

By using serum samples supplied by our collaborator from the University of Sydney, Australia, Dr Georgina Hale, I was able to measure the INSL3 concentration in serum through one menstrual cycle and a half from 18 healthy young women of reproductive age, with regular cycles. The INSL3 levels were consistently low at menses but showed a significant increase (2-3 folds) during each follicular phase (Fig 4.2). Mean INSL3 concentrations dropped slightly during the luteal phase but the difference was not statistically significant (Fig 4.2) (Anand-Ivell et al. 2013).

4.2.2 Peripheral INSL3 concentration across synchronised estrous cycles in the bovine

As mentioned in the introduction, only when the CL undergoes luteolysis, can the dominant follicle from the wave of growing antral follicles further develop to ovulate. Prostaglandin F2α (PGF2α) from the uterine endometrium is an important factor that involved in inducing luteolysis (Forde et al. 2011), and injections of the PGF2α analogue cloprostenol have been shown to cause luteolysis (Flint and Sheldrick 1982). Cloprostenol is therefore used in this study for synchronising estrous cycles in 5 heifers with 3 truncated (14 days) cycles. The serum samples were collected by our collaborator at the University

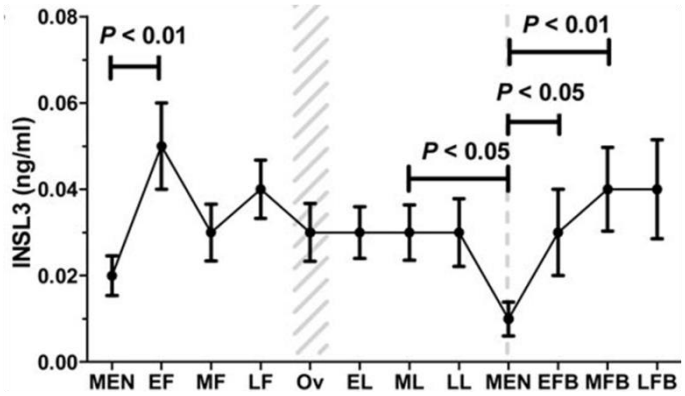


Figure 4.2 Peripheral INSL3 concentration (Mean ± SEM) in 18 healthy young women of reproductive age. The serum samples were collected every 2-3 days. MEN, menses; EF, early follicular phase; MF, mid follicular phase; LF, late follicular phase; Ov, ovulation; EL, early luteal phase; ML, mid luteal phase; LL, late luteal phase; EFB, MFB and LFB represent early, mid and late follicular phases of the subsequent cycle. The cross-hatched vertical bar indicates ovulation, whereas the dashed line indicates the second menses. The combined menses samples are significantly different from all luteal phase samples (not shown) (Anand-Ivell et al. 2013).

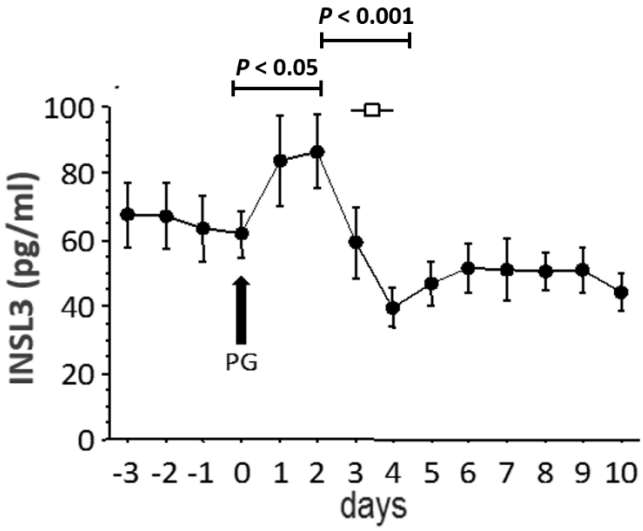


Figure 4.3 Plasma INSL3 profile during synchronised estrous cycles in heifers. Circulating INSL3 concentration was measured in 5 heifers with 3 truncated estrous cycles (total 15 cycles) which were induced by the PGF2α analogue cloprostenol (PG). The Mean ± SEM values of INSL3 through 15 truncated cycles were plotted. The PG administration (Day 0) is indicated by an arrow. The LH surge (Day 3.5 ± 0.6) is indicated by the open square symbol (Satchell et al. 2013).

of Reading, Professor Phillip Knight.

As shown in Fig 4.3, after cloprostenol was injected to induce luteolysis on day 0, the mean INSL3 level was 62.9pg/ml. It increased ($P < 0.05$) up to 86.6pg/ml on day 2, during the follicular phase, just prior to the LH surge. Around ovulation, as indicated by the presence of the LH surge, the INSL3 level dropped significantly ($P < 0.001$) to about 39.8pg/ml and then remained at this level from day 4 onwards, i.e. during the luteal phase (Fig 4.3) (Satchell et al. 2013).

4.2.3 Peripheral INSL3 concentration in a cohort of women attending an infertility clinic

Unlike the cohort of healthy young women whose blood samples could be collected through one and a half cycles, the women seeking medical help for infertility were only sampled once on entry by our collaborator, Professor Kelton Tremellen, from the Repromed infertility clinic (Adelaide, Australia), thus offering a cross-sectional view of a much larger but more heterogeneous population. Based on estimated time after last menstruation, these women were sampled at different times relative to menses, though ~80% of samples were collected during the follicular phase.

Women whose partners were diagnosed with infertility due to a male factor had circulating INSL3 concentrations indistinguishable from those with tubal block (0.079 ± 0.005ng/ml, n = 277 versus 0.073 ± 0.008ng/ml, n = 67);

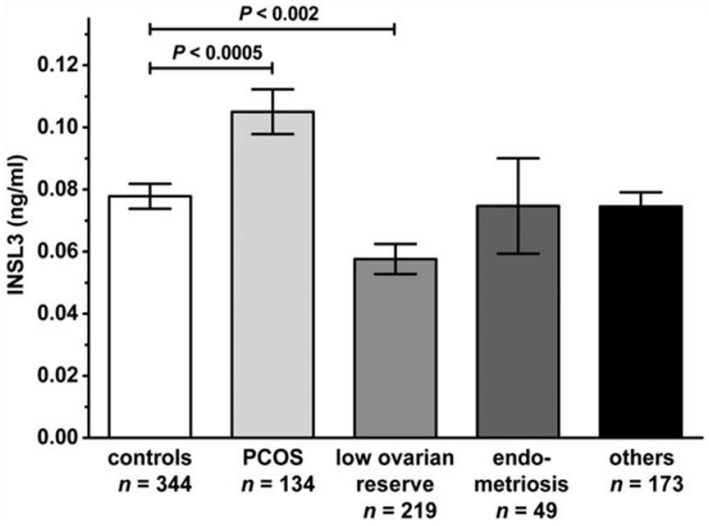


Figure 4.4 INSL3 levels in infertile women. Aggregate INSL3 levels (Mean ± SEM) in 909 women attending an infertility clinic. Subjects are grouped into controls (comprising both male factor and women with tubal obstruction), diagnosed Polycystic Ovarian Syndrome (PCOS), women with low ovarian reserve, diagnosed endometriosis, and women with infertility due to unknown reasons or of other origin (Anand-Ivell et al. 2013).

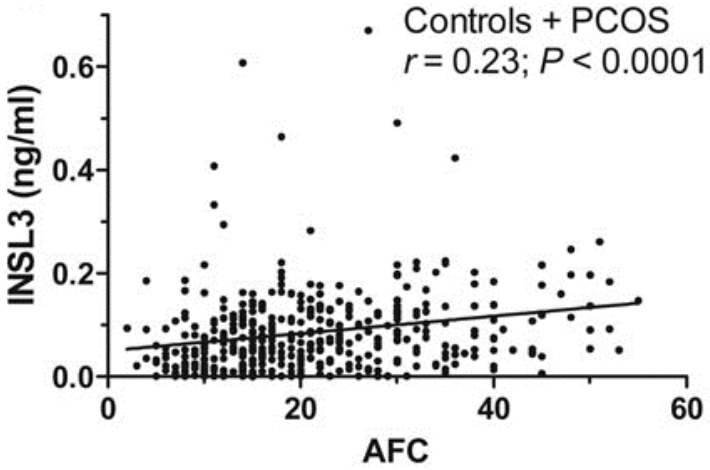


Figure 4.5 Scatter plot correlation of INSL3 against antral follicle count (AFC) for individual infertile women. INSL3 levels were positively correlated with AFC for the control (male factor + tubal block) and PCOS groups combined (n=478) (Anand-Ivell et al. 2013).

therefore, both groups could be assumed to have healthy ovarian function and INSL3 values were combined to form a normal control group with which to compare the other diagnostic categories (Fig 4.4). The results showed that women diagnosed with PCOS had higher peripheral INSL3 ($P < 0.0005$), and women with depleted ovarian reserve had lower INSL3 ($P < 0.005$). In contrast, women with endometriosis or with infertile of unknown etiology did not differ from the control group (Fig 4.4) (Anand-Ivell et al. 2013).

The antral follicle counts (AFCs) (2-5mm) from this cohort of women had been measured previously by ultrasound (Tremellen and Kolo 2010). Correlation analysis amongst the control group (male factor + tubal block) showed that INSL3 levels were positively correlated with AFC ($r = 0.21$, $P < 0.0003$) (not shown). When the control and PCOS groups were combined the significance of the correlation between INSL3 and AFC was increased ($P < 0.0001$), though the correlation coefficient stay the same ($r = 0.23$) (Fig 4.5) (Anand-Ivell et al. 2013).

4.3 Discussion

4.3.1 Circulating INSL3 levels showed a cyclic pattern in both human and bovine.

This is the first time that the circulating INSL3 concentrations has been measured across reproductive cycles. The results were able to show a cyclic

pattern of circulating INSL3 levels in both human and bovine.

Together with other hormone profiles across menstrual cycles in the human (Appendix Fig 9.1), it showed that INSL3 levels started to increase when a cohort of growing antral follicles was recruited, as indicated by the rise of FSH levels. The increase in INSL3 also accompanied the elevation of estradiol (E2), which was more obvious in the second follicular phase (Appendix Fig 9.1). The elevated E2 production reflected the development of the recruited follicles having enhanced aromatase activity in granulosa cells that can convert the androstenedione produced by the theca interna cells to E2 (Liu and Hsueh 1986). INSL3 levels then drop when the LH surge occurred, and the level remained low when P4 levels were high (Appendix Fig 9.1). The LH surge induces ovulation and causes luteinisation of the follicular cells, to form a corpus luteum (CL). The luteinised follicular cells therefore shifted from producing E2 to producing progesterone (P4) in the luteal phase (Baerwald et al. 2012). Therefore, INSL3 levels drop just before ovulation, remained low during the luteal phase, and further fall to reach a nadir at menses.

A similar scenario was observed in the heifers (Appendix Fig 9.2), although there was only an incomplete luteal phase due to synchronisation, compared to a natural cycle of approximately 21 days (Aerts and Bols 2010). The CL is the main source of P4 during the luteal phase. After cloprostenol was injected to induce luteolysis on day 0, P4 levels drop dramatically (Appendix Fig 9.2). A wave of antral follicles was then free to develop and increasingly produced E2

(Appendix Fig 9.2). This accompanied elevated levels of INSL3. Both E2 and INSL3 started to drop just before the LH surge that induced ovulation. INSL3 levels remained low when P4 increased again, indicating the formation of a new CL (Appendix Fig 9.2).

According to the observations illustrated above, INSL3 levels increased during the follicular phase and were low in the luteal phase in both species. The rise in circulating INSL3 after menses in women and after luteolysis in the bovine likely reflect the total production from healthy antral follicles which were recruited by the inter-cycle rise in FSH. Although INSL3 mRNA but not protein has been detected in CL from different species, including human and bovine (Bathgate et al. 1996; Bamberger et al. 1999; Satchell et al. 2013), the levels of circulating INSL3 during the luteal phase were low. This is consistent with the observation by Satchell et al. (2013) that the mRNA levels for INSL3 and its receptor RXFP2 in bovine CL were only about 1/10 and 1/100 of that in follicles respectively, indicating the much reduced requirements for functional INSL3-RXFP2 signalling in the CL. This further confirms that antral follicles are indeed the major, if not exclusive, source of circulating INSL3 within non-pregnant female mammals.

As the bovine showed systemic INSL3 levels essentially comparable to those in the human, the bovine model, which has already been validated for understanding ovarian function in the human (Adams et al. 2012), also appears to be suitable for researching the role of INSL3 in female

reproduction.

4.3.2 INSL3 and infertility

As shown in Fig 4.4, circulating INSL3 levels in women only appeared to be affected by infertility when there was a dysfunction within the ovary, such as PCOS or low ovarian reserve, but not when there was endometriosis or tubal blockage. AFC is relatively high in PCOS patients (Jonard et al. 2003), and low in women with low ovarian reserve (Tremellen and Kolo 2010). The circulating INSL3 was significantly elevated in the former, and reduced in the latter (Fig 4.4). Correlation analysis (Fig 4.5) further confirms at the population level that there is a biological relationship between serum INSL3 level and antral follicle count, though it is unlikely that INSL3 is sufficiently sensitive to be of use in diagnosing the number of follicles within individuals.

The increased INSL3 seen in the PCOS group agrees with previously published studies (Havelock et al. 2005; Gambineri et al. 2007), and probably reflects the greater amount of theca interna tissue which gives rise to both androgens and INSL3. Nevertheless, it is also possible that the theca interna cells of women with PCOS may have increased individual INSL3 expression, as also increased activity of the steroidogenic pathway which gives rise to androgens (Nelson et al. 1999).

Although INSL3 mRNA and protein have been detected in human endometrium (Hombach-Klonisch et al. 2001), endometriosis had negligible

effect on circulating INSL3 levels (Fig 4.4). This would suggest that the uterus is not contributing significantly to circulating INSL3 compared to what is produced by ovarian antral follicles.

In conclusion, INSL3 is produced largely by growing antral follicles, and circulating levels are influenced by the number of such follicles in the ovaries. Thus, circulating INSL3 measurements could be used as a new clinical parameter to assess antral follicle growth and development in women of reproductive age.

Chapter 5:

Steroids regulate INSL3 expression in bovine ovarian theca interna cells

5.1 Introduction

Based on the observations in the previous chapter, circulating INSL3 appears to be mainly produced by the cyclically recruited antral follicles before exposure to the LH surge. The theca interna cells (TIC) of the antral follicles appear to be the major source of INSL3 (Ivell and Bathgate 2002). INSL3 mRNA and protein are detectable in TICs from both human and bovine antral follicles (Fig 5.1 & 5.2) (Anand-Ivell et al. 2013; Glister et al. 2013).

Moreover, TICs are steroidogenic cells that synthesises progesterone (P4) and androstenedione (A4) (Fig 5.3). The neighbouring granulosa cells can use A4 supplied by the TICs to make estrogens (Liu and Hsueh 1986) (Fig 5.3). This biosynthesis of steroids is under the control of gonadotropins that reach the follicles through capillaries in the theca interna layer (Young and McNeilly 2010), as well as local intra-ovarian factors, such as members from TGF-β superfamily and WNT family (Knight and Glister 2006; Hernandez Gifford 2015). TICs therefore reside in a micro-environment that contains abundant sex steroid hormones and gonadotropins. As has been described in chapter 1.4.2, some steroids appear to be able to modulate INSL3 expression in the testis or in testicular Leydig cells; much less is known for the female side. Therefore, bovine TICs isolated from healthy medium sized antral follicles that had not been exposed to the LH surge were used for examining the regulation of INSL3 production.

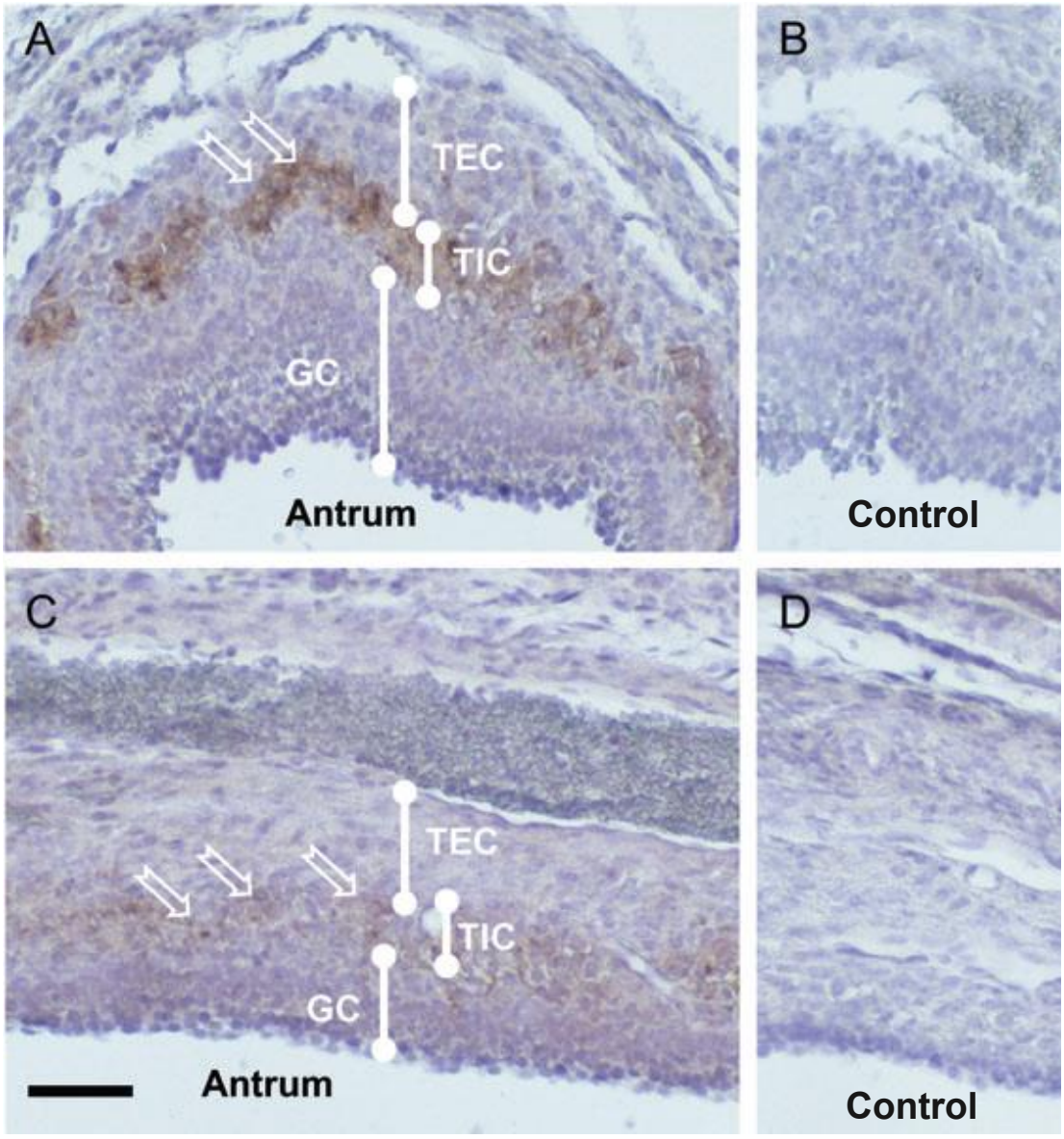


Figure 5.1. Immunohistochemical staining for INSL3 in theca interna cells in human ovarian follicles. INSL3 proteins were stained in brown colour (indicated by arrows) in TICs from both a small antral follicle (~2mm) (A) and a large follicle (~10mm) (C), with more intense staining in the former (A). B and D are negative controls where the corresponding pre-immune serum at the same concentration was used instead of the primary antibody. GC, granulosa cells; TIC, theca interna cells; TEC, theca externa cells. Scale bar = 100μm (Anand-Ivell et al. 2013).

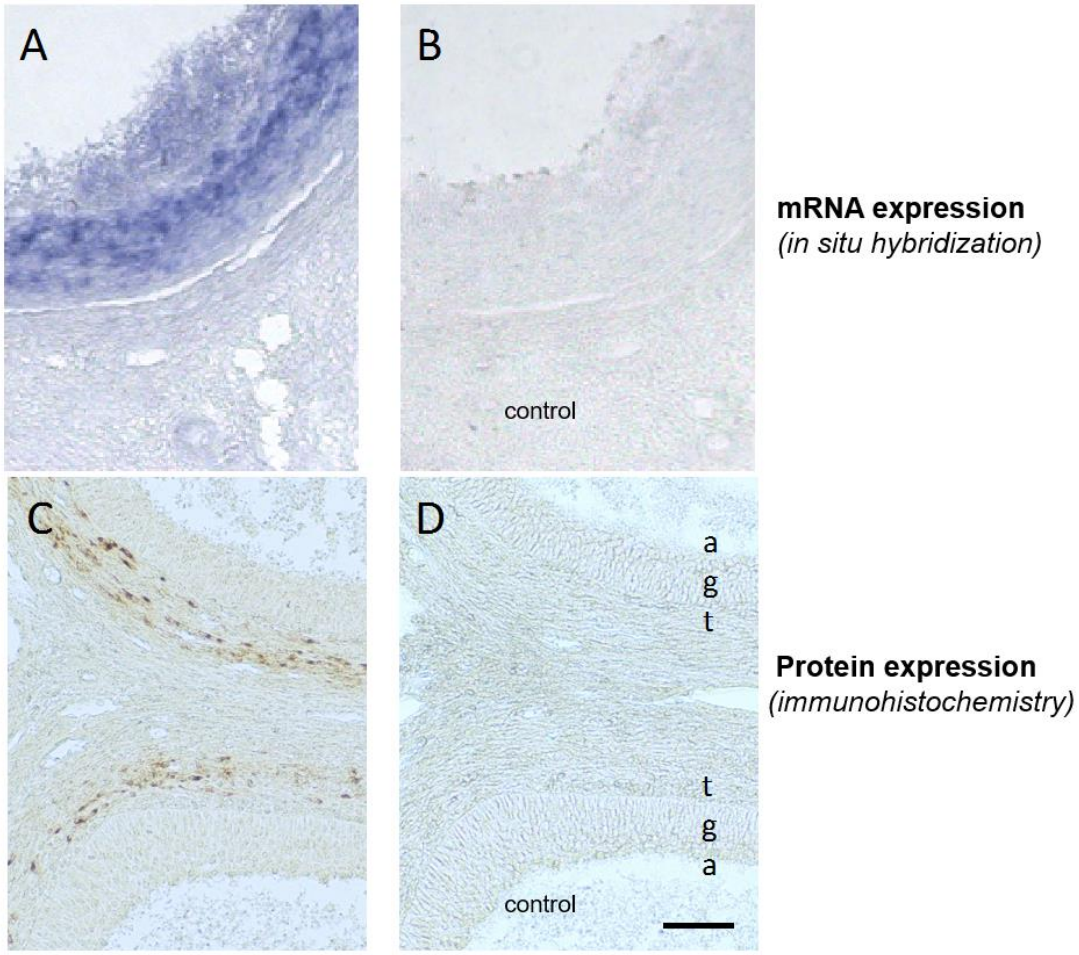


Figure 5.2. Localisation of INSL3 in bovine antral follicles. INSL3 mRNA (in situ hybridization; blue staining) (A) and INSL3 protein (immunohistochemistry; brown staining) (C) were observed in theca cells in bovine antral follicles. The corresponding controls were incubated with a sense riboprobe (B) or nonimmune serum (D). a, antrum; g, granulosa layer; t, theca layer. Scale bar =100µm (Satchell et al. 2013).

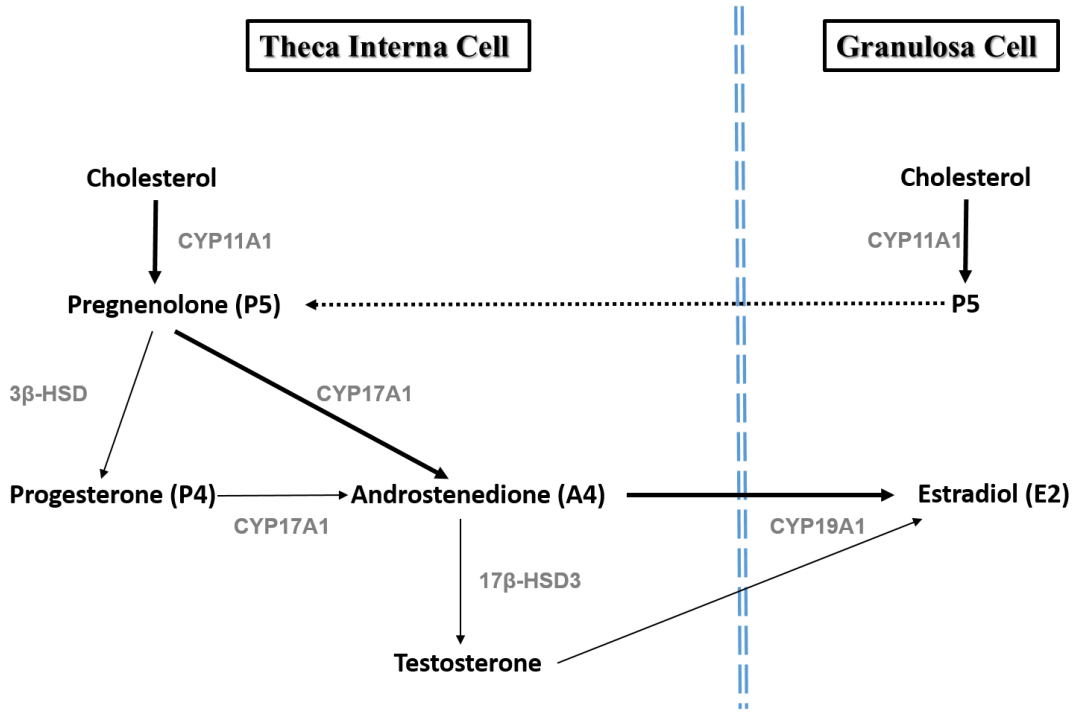


Figure 5.3. Steroidogenesis in bovine follicular theca interna cells (TIC) and granulosa cell (GC). During the follicular phase, prior to the LH surge, cholesterol can be used by both TIC and GC to synthesis pregnenolone (P5). However, most P5 is supplied from the GC (Fortune 1986). Part of the P5 can be converted into progesterone (P4) by the enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD) within the TIC. P5 and P4 are then converted into androstenedione (A4) by the enzyme CYP17A1. Some of the A4 can also be used to make testosterone by 17β-Hydroxysteroid dehydrogenases (17β-HSD), and most of the A4 as well as testosterone diffuse to GC where they can be converted into estrogens by CYP19A1 (aromatase).

5.2 Results

5.2.1 Steroid receptor expression in TIC

In order to examine whether steroids are able to regulate INSL3 expression directly in TIC, the presence of certain steroid receptors was first checked using real-time qRT-PCR. Bovine progesterone receptor (PR), androgen receptor (AR), estrogen receptor alpha (ERα), and estrogen receptor beta (ERβ) were all expressed in bovine TICs, as shown in Fig 5.4. This figure also includes the expression level of transcription factor steroidogenic factor 1 (SF-1, also named as NR5A1) and Nur77 (also named as NR4A1) which had been shown to regulate INSL3 expression previously (Lague and Tremblay 2009). These two will be described in Chapter 6 and Chapter 8, respectively.

5.2.2 Toxicity test

In the following experiments, various steroid agonists, antagonists and inhibitors were used to test their ability to regulate INSL3 expression. To exclude any effects caused by cell death, the toxicity of these compounds was first examined using the MTT assay. The doses tested here were the highest concentrations to be used in the subsequent experiments. All the compounds and the vehicles showed no effects on cell viability, except for 50μM of the PKA inhibitor H89 which significantly reduced TIC number ($P < 0.0001$) (Fig 5.5). De-attachment of cells was also observed when TICs were cultured with 50μM H89 (not shown).

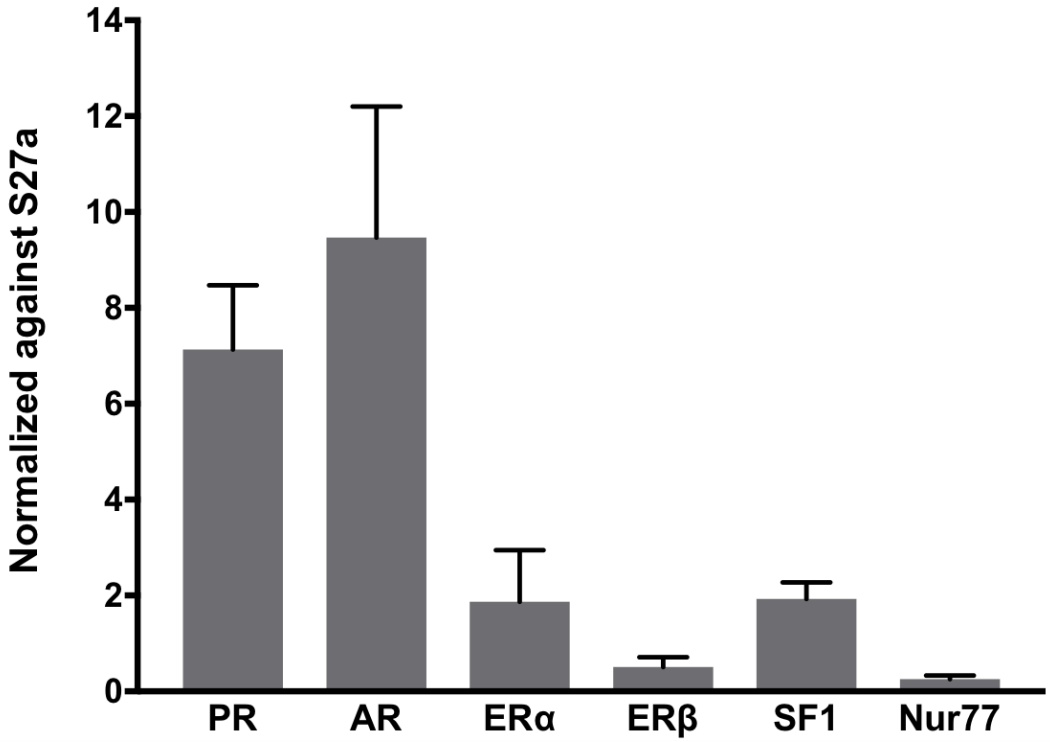


Figure 5.4. Expression of transcription factors in bovine TIC. By using real-time qRT-PCR, transcripts for bovine progesterone receptor (PR), androgen receptor (AR), estrogen receptor alpha (ERα), estrogen beta (ERβ), SF-1 and Nur77 were detectable in bovine TICs. The expression levels were normalised against bovine S27a mRNA expression levels. Values are Mean ± SEM, for n=3 biological replicates. The annealing temperature for PR and Nur77 are 61ºC; for ERα, ERβ and S27a are 62ºC, for AR is 60-54 touchdown, and SF-1 are 60ºC,

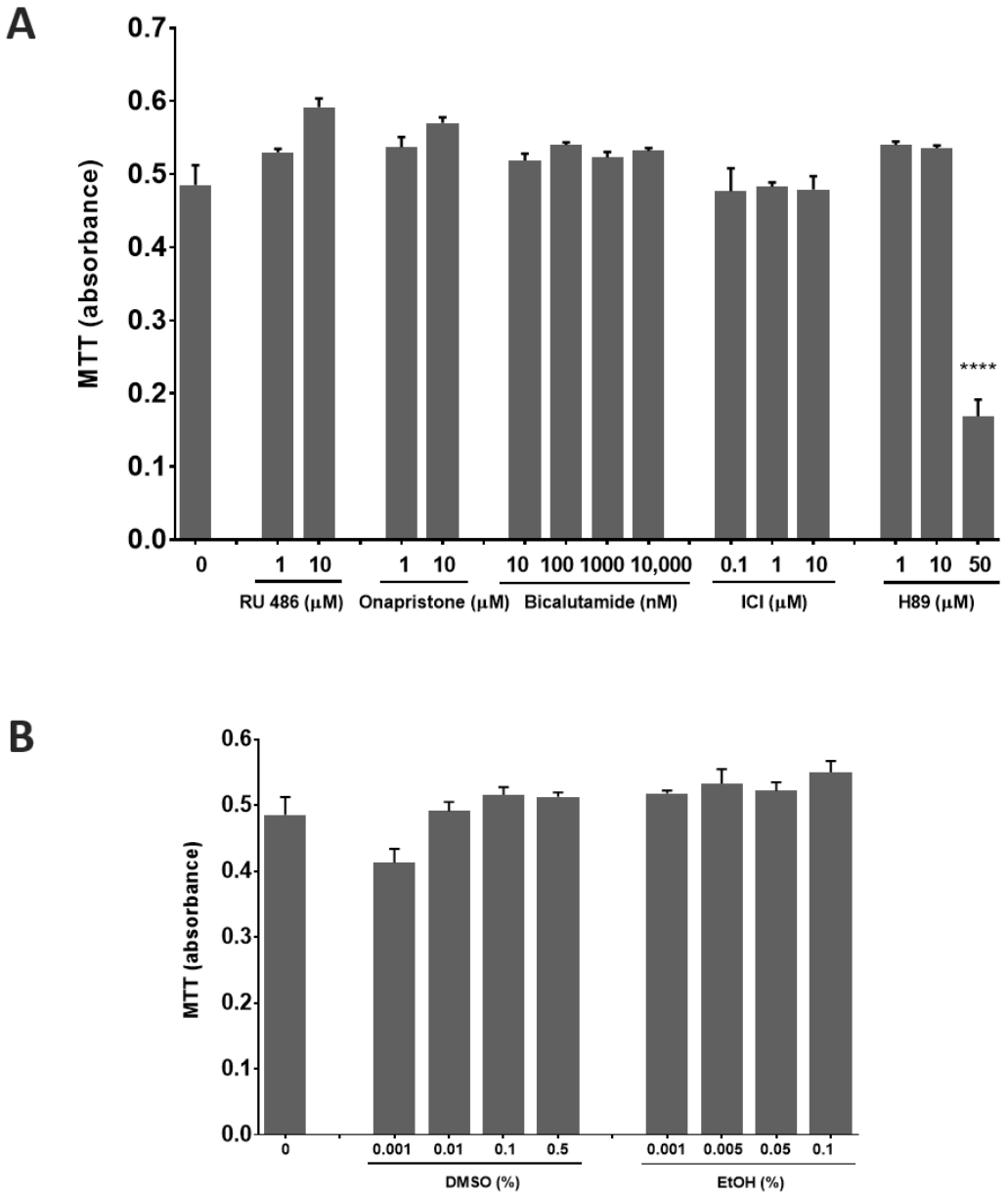


Figure 5.5. Toxicity of chemicals and vehicles used in these experiments. Bovine TICs were treated with increasing concentrations of (A) RU 486, Onapristone, Bicalutamide, ICI 182,780 (ICI), H89, (B) DMSO and EtOH. Cell viability is positively correlated with the absorbance detected in the MTT assay. Values are Mean ± SEM (n=4 technical replicates); ****: $P < 0.0001$ vs 0 treatment.

5.2.3 LH regulated INSL3 peptide level.

The method for culturing bovine TICs was modified from that used by Glister et al. (2013). Preliminary results from these experiments were essentially similar to those generated by our collaborators Glister et al. (2013) and Satchell et al. (2013). Results from both groups showed INSL3 levels were significantly increased by low dose LH (150pg/ml in the Reading lab; 100-1000pg/ml in the Nottingham lab) (Fig 5.6 A & C), and were reduced by high dose LH (1500pg/ml in the Reading lab; 10,000pg/ml LH in the Nottingham lab) (Fig 5.6 B & C). Therefore, the Nottingham primary bovine TIC culture system behaved similarly to that from the University of Reading, though there were differences in the absolute INSL3 values, which might be due to our protocol modifications (see chapter 3.2.1.2). Moreover, the INSL3 peptide levels in culture media were significantly correlated with the endogenously expressed INSL3 mRNA levels ($P < 0.05$), implying that the measured INSL3 peptide secreted into the culture media represented a good surrogate for the endogenously expressed gene (Fig 5.7).

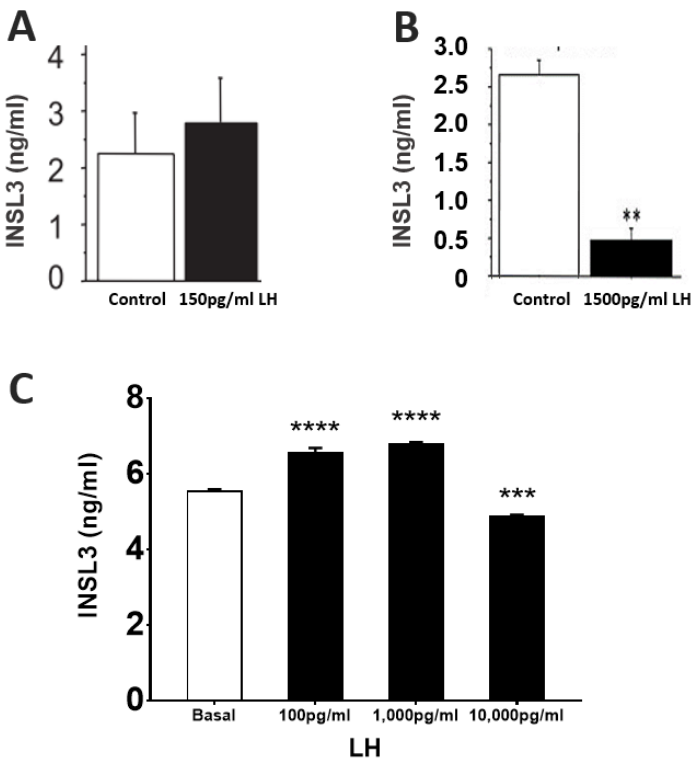


Figure 5.6. LH regulates INSL3 peptide levels. Results from our collaborators showed (A) 150pg/ml of LH tended to increase INSL3 level (Mean ± SEM) (Glister et al. 2013), and (B) 1500pg/ml of LH significantly reduced INSL3 level (Mean ± SEM; ** P<0.01; n=3 biological replicates) (Satchell et al. 2013). (C) In our lab, INSL3 levels (Mean ± SEM, *** P<0.001, **** P<0.0001 vs basal; n=3 technical replicates) were significantly increased by 100pg/ml and 1000pg/ml of LH, and were reduced by 10,000pg/ml of LH.

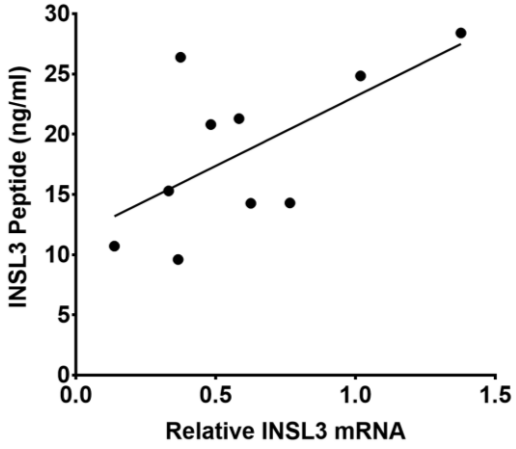


Figure 5.7. Secreted INSL3 peptide levels are correlated with expressed INSL3 mRNA levels in bovine TICs. Both INSL3 peptide in culture medium and INSL3 mRNA (normalised by the house-keeping gene bovine S27a expression level) in cell lysate from the same experiment were measured and correlated well with each other ($P < 0.05$). The annealing temperature for INSL3 is 62ºC.

5.2.4 Progesterone signalling

Since TICs naturally produce progesterone as a product of the side chain cleavage enzyme, CYP11A1, and 3-beta-hydroxysteroid dehydrogenase (Fig 5.3), it can be assumed that endogenously expressed PR is already activated by locally synthesised P4. Therefore, the PR antagonists RU 486 and onapristone were used to block any PR signalling. Both antagonists inhibited INSL3 production in a dose-responsive manner (Fig 5.8). Repeated experiments (n=2) confirmed levels of inhibition for RU486 at 10μM of 0.495 fold on average. Since RU 486, but not onapristone, can also block glucocorticoid receptors, additional glucocorticoid agonist dexamethasone (0.1–10μM) was added to check for any effects caused by glucocorticoid signalling. However, all doses of dexamethasone could not reverse the inhibitory effects of RU 486 (Fig 5.8 B). This shows that progesterone signalling, but not glucocorticoid signalling, indeed promotes INSL3 production in cultured TIC.

5.2.5 Androgen signalling

In TICs androgens are made from P4 and P5 by the action of the enzyme CYP17A1 (Fig 5.3). Using the bovine INSL3 TRFIA, I was able to measure INSL3 peptides in the media supplied from our collaborators at the University of Reading. The result showed that inhibition of CYP17A1 in TIC by the inhibitor abiraterone (100nM) reduced the production of INSL3, although this

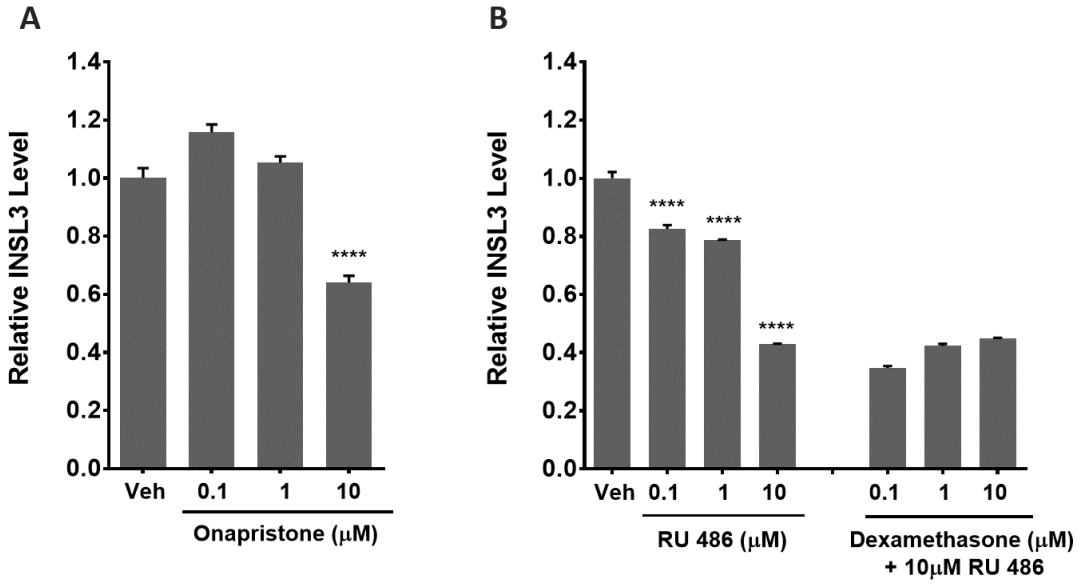


Figure 5.8. Suppression of progesterone signalling reduced INSL3 production. Treatment with the PR antagonists onapristone (0.1-10μM) (A) and RU 486 (0.1-10μM) (B) significantly reduced INSL3 levels (Mean ± SEM; n=3 technical replicates) in TIC. Additional dexamethasone (0.1-10μM) (B) was unable to reverse the inhibitory effect of 10μM of RU 486. The vehicle value was considered as 1–fold in each group. ****: $P < 0.0001$ vs vehicle.

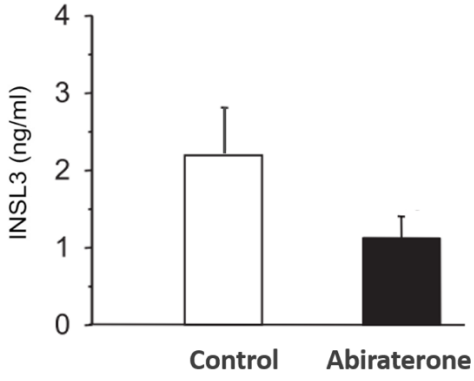


Figure 5.9. Abiraterone tended to suppress INSL3 production. The CYP17A1 inhibitor abiraterone (100nM) reduced the INSL3 peptide level (Mean ± SEM) in the culture medium (Glister et al. 2013).

was not statistically significant (Fig 5.9). This suggested that androgens might stimulate INSL3 production. To further explore the role of androgen signalling, the following experiments were carried out.

5.2.5.1 AR signalling inhibited INSL3 production

Just as for progesterone signalling, androgen signalling should also be already activated in TIC since both androgens and AR are synthesised locally. The anti-androgen bicalutamide was used to prevent androgen from binding to AR, thereby to attenuate androgen signalling. However, this led to a significant increase in INSL3 levels (Fig 5.10 A). Biological replicate experiments (n=4) confirmed this effect at 10nM (1.46±0.14 fold, $P<0.05$) and 100nM (1.51±0.15 fold, $P<0.05$). This stimulatory effect caused by 100nM bicalutamide could be reversed by 1nM DHT , the most potent natural androgen (Nacusi and Tindall 2011), although 1nM DHT alone did not elicit any obvious effect (Fig 5.10 B).

5.2.5.2 AR signalling may integrate with ER signalling to regulate INSL3 production.

When increasing doses (0.1 – 10nM) of DHT were applied, the level of secreted INSL3 peptide tended to increase; and in the presence of the ER antagonist ICI (1μM), at all concentrations of DHT the INSL3 levels were dramatically reduced by an average of 49% (Fig 5.11 A). This was repeated for 4 biological replicates ($P< 0.001$, n=4).

A dose response relationship appears to be evident for INSL3 secretion in

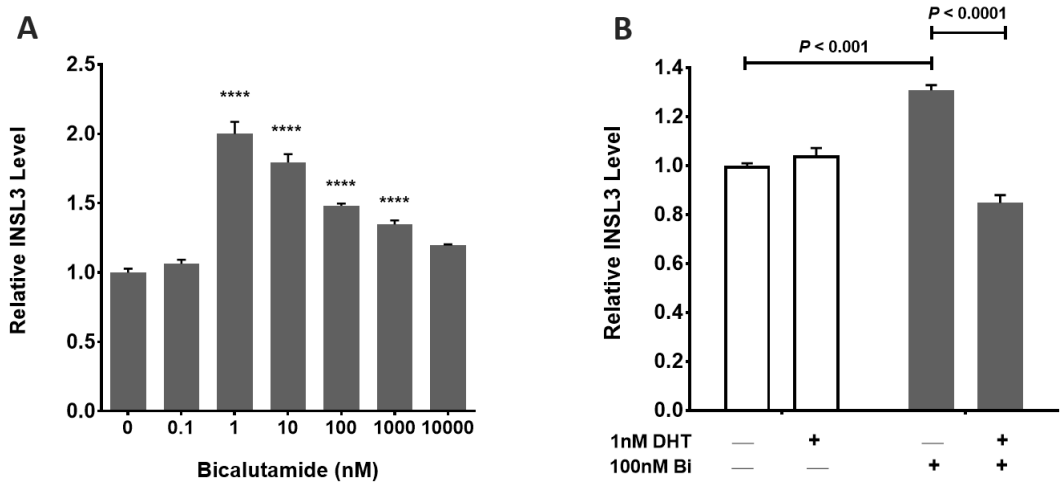


Figure 5.10. Androgen receptor signalling decreased INSL3 production (Mean ± SEM; n=3 technical replicates). (A) TICs were treated with increasing concentrations of the anti-androgen bicalutamide. ****: $P < 0.0001$ vs 0nM bicalutamide. (B) Cells were treated with 1nM DHT in the presence or absence of 100nM bicalutamide (Bi).

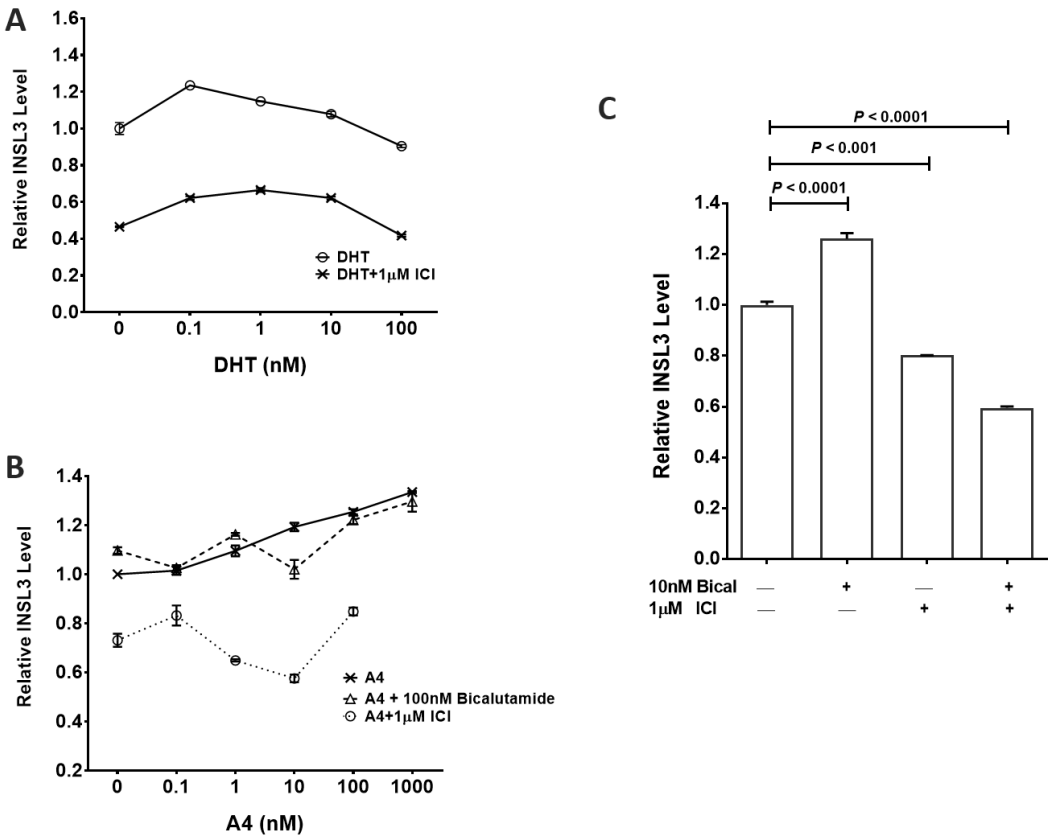


Figure 5.11. Androgen signalling was affected by the estrogen antagonist ICI 182,780 (ICI) and anti-androgen bicalutamide (Bical). TICs were treated with increasing doses of DHT (A) or androstenedione (A4) (B) in the presence or absence of 1μM of ICI (A & B) or 100nM of bicalutamide (B). (C) TICs were treated with 10nM of bicalutamide, 1μM of ICI, or the combination of the two. Secreted INSL3 levels are represented as Mean ± SEM (n=3 technical replicates).

response to A4 (Fig 5.11B), with a statistically significant elevation, observed also in repeated experiments at 1000nM (1.38±0.14 fold, P<0.05, n=3 biological replicates). Bicalutamide (100nM), which showed stimulatory effects under basal conditions in Fig 5.10 A, had no obvious effects on INSL3 production when cells were co-treated with A4 (0.1nM, 1nM, 100nM, and 1000nM) (Fig 5.11 B). ICI (1μM) again decreased the INSL3 levels induced by all concentrations of A4 (0.1 – 1000nM). Calculated from 4 biological replicates, the reduction was by 29% on average (P < 0.01).

In the presence of both AR antagonist bicalutamide and ER antagonist ICI, INSL3 levels were further inhibited (~0.6 fold, n=2 biological replicates) compared to when ICI was used alone (~0.73 fold, n=2 biological replicates); the stimulatory effect caused by bicalutamide alone was not observed when ICI was also included (Fig. 5.11 C). Therefore, both AR and ER appear to be involved convergently in regulating INSL3 production by DHT and A4.

5.2.6 Estrogen signalling

5.2.6.1 Estrogen signalling increased INSL3 production.

Estrogen is produced in granulosa cells rather than by TIC, and is thus normally absent in TIC culture (Fig 5.3). Therefore, additional estradiol (E2) should be needed to activate endogenous ERs in TIC. The exogenous E2 indeed increased INSL3 production in a biphasic manner (Fig 5.12). Biological replicate experiments confirmed this pattern and with the highest stimulatory

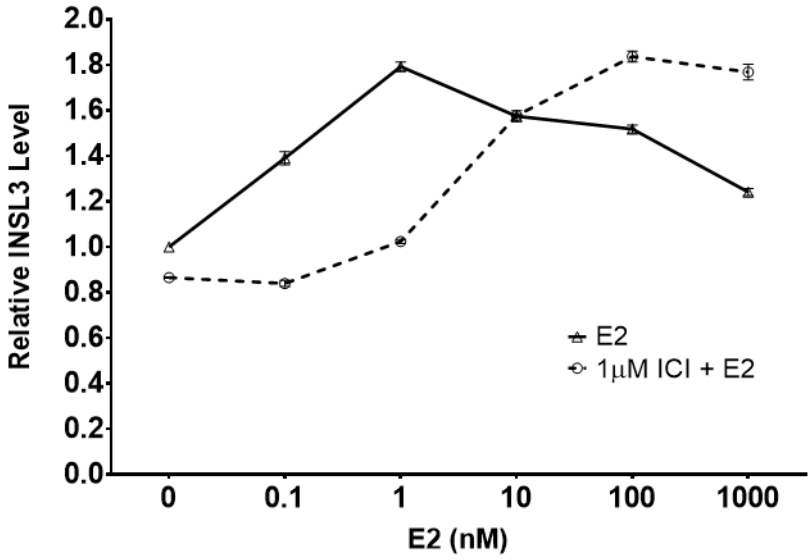


Figure 5.12. Estrogen signalling stimulated INSL3 production. TICs were stimulated with increasing doses of E2 (1 – 1000nM) with or without the presence of 1μM of ICI. INSL3 level is presented as Mean ± SEM (n= 3 technical replicates).

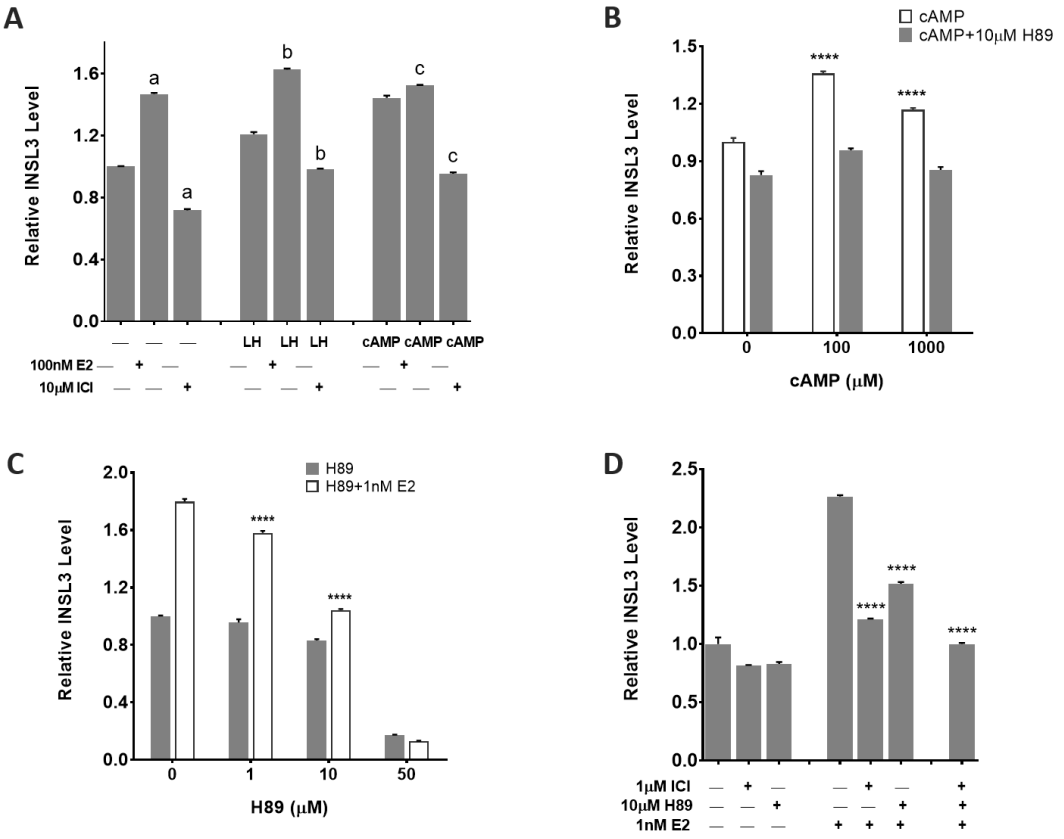


Figure 5.13. LH-cAMP-PKA signalling integrates with estrogen signalling. (A) TICs were treated with or without 10μM of ICI, and were then stimulated with or without 100nM E2, together with or without 150pg/ml of LH or 100μM of 8Br-cAMP (cAMP). a, b: $P < 0.0001$ vs control, LH, respectively; c: $P < 0.001$ vs cAMP. (B) Cells were firstly treated with 10μM of H89, and then treated with cAMP (100μM and 1000μM). ****: $P < 0.0001$ vs 0. (C) Cells were firstly treated with increasing concentrations of H89, and then treated with 1nM E2. ****: $P < 0.0001$ vs 0μM H89 + 1nM E2. (D) Cells were firstly treated with 1μM of ICI and/or 10μM of H89, and then stimulated with 1nM E2. ****: $P < 0.0001$ vs 1nM E2. All INSL3 levels are expressed as Mean ± SEM for n=3 technical replicates.

effect at 1nM E2 (1.9±0.11 fold, $P < 0.0001$, n=3 biological replicates). In the presence of the ER antagonist ICI, which competes with E2 for binding to ER, about 100 times more E2 was required to achieve similar effects (Fig 5.12). This experiment was repeated with virtually identical results.

5.2.6.2 LH-cAMP-PKA signalling integrates with estrogen signalling

As already shown above (Fig 5.12 & Fig 5.6), 100nM E2 and low dose of LH (150pg/ml) stimulated INSL3 secretions (Fig 5.13 A), observed in biological repeated experiments as 1.48±0.03 fold ($P<0.001$, n=3) and ~1.17 fold (n=2), respectively. The main downstream effector of LH signalling is cAMP. The cell-soluble analogue of cAMP, 8Br-cAMP (cAMP) at 100μM also increased INSL3 levels (Fig 5.13 A), observed in biological replicated experiments as 1.48±0.14 fold ($P<0.001$, n=4). E2 (100nM) could further increase the stimulatory effects of LH (150pg/ml) and cAMP (100μM) (Fig 5.13 A). Both the stimulation of INSL3 production by LH (150pg/ml) and by cAMP (100μM) was blocked by the ER antagonist ICI (10μM) (Fig 5.13 A). The main downstream effector of cAMP signalling is protein kinase A (PKA). Firstly, as a positive control, the PKA inhibitor H89 (10μM) could block the INSL3 production induced by 100μM cAMP (Fig 5.13 B). The high dose of cAMP (1000μM) showed a weaker stimulatory effect than that caused by the low dose of cAMP (100μM) (Fig 5.13 B), while the biological replicate experiments showed an even inhibitory effect (0.88±0.06 fold, n=7), which is similar to the effect of a high dose of LH (10,000pg/ml) (Fig 5.6 C). H89 (10μM) then further enhanced

the inhibitory effect of 1000 μM cAMP (down to 0.85-fold). This supports the view that LH-cAMP-PKA signalling is regulating INSL3 expression (Fig 5.13 B).

However, not only did H89 block the effects of cAMP, it also significantly reduced the INSL3 level induced by 1nM E2 (Fig 5.13 C), equivalent to 1.90±0.11 fold at 1nM E2 for n=3 biological replicates and an average of 1.40 fold at 10μM H89 for n=2 biological replicates. The dramatic reduction observed upon using 50μM of H89 in the presence and absence of 1nM E2 (Fig 5.13 C) was most probably due to the significant cell death caused at this concentration of inhibitor, as shown in Fig 5.5 A

Furthermore, whereas 1μM ICI completely blocked the effect of 1nM E2 (Fig 5.13 D) (reduced from 1.9±0.1 fold to about 1.17 fold for 3 and 2 biological replicates, respectively), 10μM H89 was only partially able to inhibit the effect of estrogen (Fig 5.13 D), equivalent to an average of 1.4 fold over basal for 2 biological replicates. When both inhibitors were used together, INSL3 concentration in the medium was reduced to that level seen with 1μM ICI alone (Fig 5.13 D). Together, these results suggest that E2-ER signalling integrates with LH-cAMP-PKA signalling to regulate INSL3 production in bovine TICs, and that probably LH-cAMP-PKA signalling is modulating the action of the ERs.

5.3 Discussion

In the previous chapter, it was shown that the peptide hormone INSL3 had a cyclic expression pattern through the reproductive cycle. In this chapter, hormonal regulators that may contribute to this cyclicity were identified. The results showed, for the TICs where INSL3 is synthesised, that its expression is under the control of the hypothalamus-pituitary-ovary axis via LH, as well as intra-ovarian factors such as sex steroids.

5.3.1 INSL3 production is controlled by LH

Theca cells acquire the LH receptor (LHR) at about the stage of the earliest antral follicles (McNatty et al. 1984), which coincides with the first observation of INSL3 in bovine and macaque follicles (Irving-Rodgers et al. 2002; Hanna et al. 2010). In vivo, LH circulates at a moderately low level across the non-conceptive reproductive cycle, increasing at the LH surge just before ovulation (Appendix Fig. 9.1). In bovine theca cells, it is observed that LH increases A4 production in a biphasic manner with a maximal effect at 100pg/ml LH, and from ~1500pg/ml upwards, LH is no longer able to stimulate A4 production; this is in contrast to its effect on progesterone that increases consistently in a dose-dependent manner (<3200pg/ml of LH) (Glister et al. 2005). This shift from A4 to P4 production in TIC mirrors the luteinisation of theca cells in response to a high dose (≥1500pg/ml) of LH that mimics the pre-ovulatory LH surge in vivo. Moreover, the highest LH concentration

observed during the LH surge in the bovine is 10.83 ± 0.76 ng/ml (Mondal et al. 2006). Therefore, the high doses of LH (1500pg/ml and 10000pg/ml) used in the current study are most likely mimicking the conditions when TICs are exposed to the LH surge.

In order to examine how the dynamically changing LH may regulate INSL3 levels, different doses of LH were used to treat TICs. Low doses of LH (<1000pg/ml) or its downstream effector analogue 8Br-cAMP (100μM), equivalent to the in vivo situation early in the estrous cycle, led to increased production of INSL3. In contrast, a high dose of LH (1000 – 10000pg/ml) or 8Br-cAMP (1000μM), equivalent to the in vivo LH surge, appears to have an inhibitory effect. The inhibitory effect of high dose LH (10000pg/ml) has also been observed at the mRNA level in TIC, with the decline in INSL3 mRNA accompanying increased CYP11A1 expression; this is indicative of theca cell luteinisation that starts to produce large amounts of P4 (Bathgate et al. 1999; Walther et al. 2006).

In conclusion, during follicular development, the low level of circulating LH appears to promote INSL3 production by TICs; and towards ovulation, the higher level of LH, especially the LH surge, supresses INSL3 production. This change in INSL3 production during a reproductive cycle may be also modulated by the dynamically changed production of sex steroids (to be discussed in the following sections).

5.3.2 INSL3 production is regulated by sex steroids

As shown in Fig 5.3, in the follicular phase and prior to ovulation, TICs produce both progesterone and androgens, such as testosterone and A4, whereas in vivo estradiol is produced via cooperation with the neighbouring granulosa cells. Here I have shown the expression of the steroid receptors PR, AR, ERα and ERβ as transcripts within bovine TICs (Fig 5.4), suggesting that the sex steroids may exert their effects in an autocrine and/or paracrine manner.

Within the relatively short-term culture (48 hours) adopted in the present study, we can assume that PR is already activated by endogenous P4, and that the PR antagonists onapristone and RU 486 should impair progesterone signalling. These PR antagonists significantly inhibited INSL3 production, implying that endogenous progesterone is part of a stimulatory theca cell signalling pathway. Since RU 486 can also inhibit glucocorticoid receptors (GR), additional glucocorticoid agonist dexamethasone was applied, with no effect, showing that indeed PR and not GR was implicated here.

5.3.2.1 Androgen signalling involves both AR and ER

During the follicular phase of the reproductive cycle, both P4 and P5 can act as precursors for androgen synthesis via the enzyme CYP17A1. Inhibiting CYP17A1 by using abiraterone could reduce total androgen production and thus lead to impaired production of INSL3.

However, androgens seem to be acting in a more complicated manner. When

DHT was used to treat TICs, it did not have any significant effect on INSL3 production. When the AR antagonist bicalutamide was applied, INSL3 production was increased; and the increase could be reversed by addition of DHT. This is probably because the AR might have already been saturated with endogenous androgens in the cultured TICs, and thus exogenous DHT cannot show any further effect. Only when AR-dependent signalling was inhibited by bicalutamide, the inhibitory effect of DHT through AR could be observed. This is consistent with the observation in the rat that DHT reduces total INSL3 mRNA production in preantral follicles both in vivo and in vitro (Xue et al. 2014).

The ER antagonist ICI dramatically reduced INSL3 levels in the presence of DHT, suggesting that ERα or ERβ may be involved in DHT signalling (Fig 5.11 A). Similarly, ERα or ERβ might also be involved in A4 signalling in TICs, as ICI also reduced INSL3 levels in the presence of A4 (Fig 5.11 B). Unlike DHT, A4 showed a dose-dependent stimulatory effect on INSL3 production, and this effect could not be influenced by the AR antagonist bicalutamide (Fig 5.11 B). This suggests that the stimulatory effect of A4 may not act through AR.

It thus appears that, DHT may inhibit INSL3 production via AR, and A4 increases INSL3 production probably via ERα and/or ERβ. It is also possible that DHT may also act in some way via ERα and/or ERβ to increase INSL3 level. More experiments are needed to explore these mechanisms further.

5.3.2.1.1 Other ligands may activate ER

From the results shown here, ICI inhibits INSL3 production by ~30-50% under basal conditions (Fig 5.11). Either there must be other ligands that can activate ER, or this receptor is acting in a completely autonomous and unliganded fashion. In previous studies, the DHT metabolite 5α-androstane-3β,17β-diol (3β-Adiol) is able to induce ERβ-mediated transcriptional activity in the neuronal cell line HT-22 as well as in the prostate cell lines DU145 and PC3 (Guerini et al. 2005; Pak et al. 2005). In the prostate cell lines, 3β-Adiol - ERβ signalling effectively inhibits prostate cancer cell migration (Guerini et al. 2005). Moreover, the A4 precursor Dehydroepiandrosterone (DHEA) can directly bind to ERβ to elicit transcriptional activity in the prostate cancer cell line LNCaP (Arnold and Blackman 2005). Thus, one possibility is that these androgens precursors and/or metabolites may be acting endogenously through ERβ, and in this way contributing to INSL3 production. This needs to be tested further in TICs.

5.3.2.1.2 AR can both inhibit and stimulate INSL3 production

The results generally showed an inhibitory effect of activated AR, reflected by an elevated INSL3 production upon the treatment with the AR antagonist bicalutamide (Fig 5.10 A). However, in the presence of the ER antagonist ICI, bicalutamide showed further suppression of INSL3, suggesting a stimulatory influence of AR (Fig 5.11 C). Therefore, there may be at least two ways for AR

to act to regulate INSL3 synthesis.

One possible mechanism has been reported previously that may explain the stimulatory effects through AR. AR can directly interact with ERα or ERβ in a cytoplasmic ternary complex with Src in breast cancer cells (i.e. Src/AR/ERα complex) and prostate cancer cells (i.e. Src/AR/ERβ complex) under the stimulation of EGF (Migliaccio et al. 2000; 2005). The ternary complex dependent DNA synthesis can be increased by either AR or ER agonists, and be inhibited by either AR (bicalutamide) or ER antagonist (ICI) (Migliaccio et al. 2005). Also, the heterodimer of AR with ERα has been shown to occur in MCF-7 cells (Migliaccio et al. 2005). Therefore, it is possible that a similar AR/ER complex might also occur in TICs, and that the activation of the complex could be controlled by both estrogens and androgens.

5.3.2.2 Estrogen signalling integrates with LH-cAMP-PKA signalling

I have shown that exogenous E2 activating through ER in the TIC can lead to increased INSL3 production in a biphasic manner (Fig 5.12). The addition of the ER antagonist ICI competes with E2 to bind with ER, and this competitive effect can be compensated by giving 100 times more E2 (Fig 5.12).

Meanwhile, as discussed previously in 5.3.1, a low dose of LH (150pg/ml) or the cAMP analogue 8Br-cAMP can also increase INSL3 production. Such a stimulatory effect is also observed when the cells are co-treated with E2. More

interestingly, the elevated INSL3 levels caused by LH or 8Br-cAMP can be inhibited by the ER antagonist ICI, suggesting a cross-talk between LH signalling and E2 signalling.

LH-LHR signalling increases cAMP production, which in turn activates Protein kinase A (PKA) (Chin and Abayasekara 2004). The activation of PKA can be inhibited by the compound H89 (Hidaka et al. 1991). When INSL3 was used as the endpoint, H89 (10μM) completely abolished the stimulatory effects caused by cAMP, confirming that LH-cAMP-PKA signalling indeed controls INSL3 production.

Moreover, H89 (1μM and 10μM) can also reduce the INSL3 production caused by E2, without affecting cell viability. However, there is no further inhibition beyond that imposed by the addition of 1μM ICI. These observations suggest that PKA could modulate ER status in the cytoplasm, which in turn might alter the transcriptional activity of the INSL3 gene in the nucleus. This is possibly caused by the phosphorylation of ER at serine residue 305 by PKA (Chen et al. 1999; de Leeuw et al. 2013). This, however, needs to be confirmed by further experiments.

5.4 Conclusion:

Theca interna cells are an important component of an ovarian follicle and are supplied by vigorous capillary vascularisation. They are not only exposed to the regulators from TICs themselves, from granulosa cells, oocytes, and from

neighbouring follicles, but are also exposed to the systemic regulators from the pituitary and other endocrine organs (i.e. adrenal gland). Together, these hormonal signals influence TICs so that they are able to support the selection and growth of a dominant antral follicle, and the maturation of the oocyte, leading to ovulation and the synchronous preparation of the oviduct and uterus for pregnancy. The results from the current chapter show that as a major product of TIC, and as a biomarker of their functionality, the expression of INSL3 is also influenced by a range of important extrinsic regulators.

From mid-follicular phase, prior to the LH surge, P4 is synthesised in TICs by converting its precursor pregnenolone (P5), which is supplied mainly by granulosa cells as well as TICs themselves (Fortune 1986) (Fig 5.3). P5 is also used for making A4, and the enzyme CYP17A1 is involved in this process (Fig 5.3). As shown in Fig. 5.14, in terms of autocrine-paracrine regulation, P4 acts on endogenous PR in the TIC that in turn stimulates INSL3 production; moreover, DHT/A4 may act on ER to enhance, and DHT, and probably the other AR agonist testosterone, can act on AR to inhibit INSL3 production.

Furthermore, by measuring bINSL3 peptide in TIC culture medium supplied from our collaborators at Reading University, I was able to assess the impact of BMPs on bINSL3 production. The result shows BMP-4 and -7, produced locally by TIC, BMP-2 and -6, produced by granulosa cell and/or the oocyte can dramatically inhibit bINSL3 production (Appendix Fig. 9.3) (Glister et al. 2013). This suggests that intra-ovarian BMPs suppress INSL3 production in

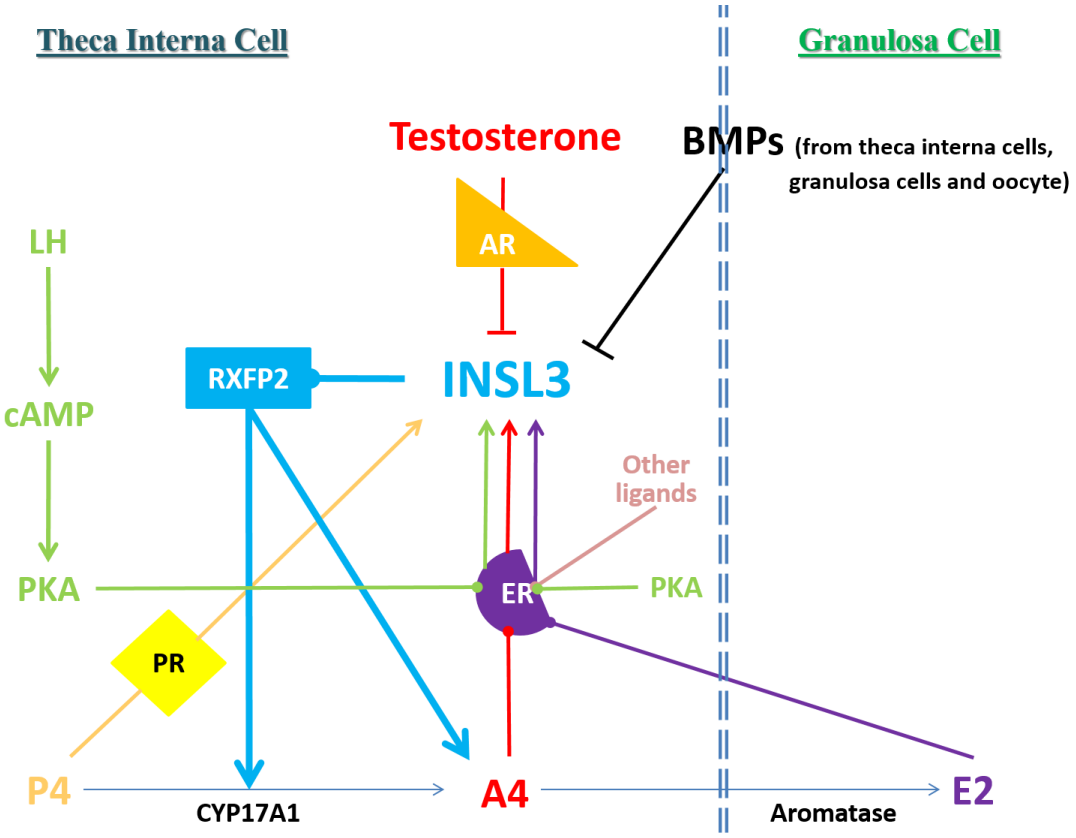


Figure 5.14. Regulation of INSL3 in theca interna cells. A4 can be converted from P4 by enzyme CYP17A1 in TICs. A4 can diffuse to granulosa cells to be aromatised into E2. P4 acts on PR; and A4 and E2 act on ER in TICs to upregulate INSL3 production. Androgen, like testosterone, may act on AR to reduce INSL3 levels. INSL3 in turn activate its cognate receptor RXFP2 to enhance the production of A4, which may be partly through increasing the production of CYP17A1, the enzyme involved in producing A4 (Glister et al. 2013). ER can also be activated by other unknown ligands and via phosphorylation by PKA that can be produced from LH-cAMP-PKA signalling (Chen et al. 1999, de Leeuw et al. 2013). BMPs produced by theca cells, granulosa cells and/or oocyte had inhibitory effects on INSL3 production (Glister et al. 2013).

an autocrine and/or paracrine manner (Fig 5.14). In another experiment, knock-down of either INSL3 or its receptor RXFP2 in TICs dramatically reduced A4 production, probably in part by inhibiting the expression of CYP17A1 (Appendix Fig. 9.4) (Glister et al. 2013). This demonstrates the existence of a regulatory loop in TICs between A4 and INSL3 (Fig. 5.14).

In terms of paracrine regulation, E2, which is converted by aromatase in granulosa cells from A4 supplied from TICs, acts on ERα and/or ERβ in TICs to positively regulate INSL3 production. It is possible that there are also other ligands that can activate ER to promote INSL3 production in TICs. In return, BMPs that are synthesised by the TICs themselves, the neighbouring granulosa cells and/or the oocyte can feed back to TICs to reduce INSL3 production.

LH, the key hormone, besides FSH, from the hypothalamus-pituitary-ovary axis, stimulates INSL3 synthesis at a low dose comparable to that in the circulation during follicle development, and may inhibit INSL3 production at the high dose of the LH surge just before ovulation. Importantly, there is cross-talk in TICs between PKA signalling, activated by LH-cAMP or other signalling pathways, and E2 signalling (Fig. 5.14).

Taken together, it appears that INSL3 expression is regulated by steroids in a complex manner. The production of ovarian steroids across the reproductive cycle changes dynamically and can also be affected by pathological conditions.

For example, steroid production is shifted from E2 to P4 when the ovary moves from the follicular phase to the luteal phase; the E2 to P4 ratio changes dramatically in atretic follicles when granulosa cells are forced into apoptosis; and women with polycystic ovarian syndrome (PCOS) have much higher serum levels of A4 and testosterone. All such changes in steroid levels, including also of their precursor and metabolites can potentially influence the net production of INSL3. This might help to explain some observations, for example, that there is almost undetectable INSL3 in the newly formed corpus luteum, or that INSL3 level is low in atretic follicles, but elevated in the serum of women with PCOS (Fig 4.4) (Bathgate et al. 1999; Irving-Rodgers et al. 2002; Anand-Ivell et al. 2013). Thus, not only does INSL3 have a probable role in maintaining follicular function and development, but it also acts as a powerful biomarker reflecting ovarian function.

Chapter 6:

Transcriptional regulation of the bovine INSL3 gene in theca interna cells (TICs)

6.1 Introduction

The INSL3 gene is located within the genome of most mammalian species in close proximity or even within the 3' end of the Janus Kinase 3 (JAK3) gene (Koskimies et al. 1997; Safford et al. 1997; Spiess et al. 1999). These two genes presumably have discrete promoter regions based on their independent expression pattern and methylation conditions. For this reason, the upstream region of approximately 1800bp of the bovine INSL3 exon 1 immediately following the last exon of the JAK3 gene is considered as the probable promoter for the INSL3 gene. To date, INSL3 upstream promoter regions from the human, rodent and dog have been looked at, and the transactivation of INSL3 promoter-reporter plasmids have been examined mostly in the testicular Leydig cells of the male. The results from previous studies suggest that the INSL3 promoter activity is mostly dependent on the orphan transcription factor steroidogenic factor 1 (SF-1) via one or more responsive elements for SF-1 (SFRE) which are located in the promoter regions of INSL3 from human, rodent and dog (Zimmermann et al. 1998; Truong et al. 2003; Sadeghian et al. 2005; Robert et al. 2006a). This activity appears to be modulated also by sex steroid hormones, where at least for Leydig cells it has been suggested that testosterone (T4) may increase and estradiol (E2) inhibit transcriptional activity (Lague and Tremblay 2008; Lague and Tremblay 2009; Tremblay et al. 2009), though other authors have not been able to confirm this (Sadeghian et al. 2005).

The current project has, for the first time, analysed the bovine INSL3 (bINSL3) promoter, and its transcriptional activity has been examined in follicular theca interna cells, which are the principal source of this hormone in the female. The results suggest a marked difference in the regulation between these female cells and the male Leydig cells, as described previously. Whereas in the previous chapter, I have shown how the sex steroids may regulate bINSL3 peptide production, in the current chapter this is assessed from the point of view of the available sex steroid receptors and the bovine INSL3 gene promoter.

6.2 Results

6.2.1 Analysis of the bINSL3 promoter

The INSL3 gene (Gene ID: 281870) is located on bovine chromosome 7 and is approximately 1800bp downstream of the JAK3 gene. This ~1800bp sequence, which is immediately upstream of the first exon of the INSL3 gene and the transcriptional start site, had previously been shown specifically to bind nuclear proteins extracted from bovine TIC (W. Suehrbier, unpublished Dr Vet Med thesis; University of Hannover, Germany) (Fig 6.1). Consequently, this sequence was considered to constitute the bovine INSL3 promoter region.

Bioinformatic analysis of the bINSL3 promoter, using the Transfac database (Matys et al. 2006), revealed three potential sites for SF-1 binding (SFRE) as well as putative steroid receptor half-sites (Fig 6.1). This promoter mapping is

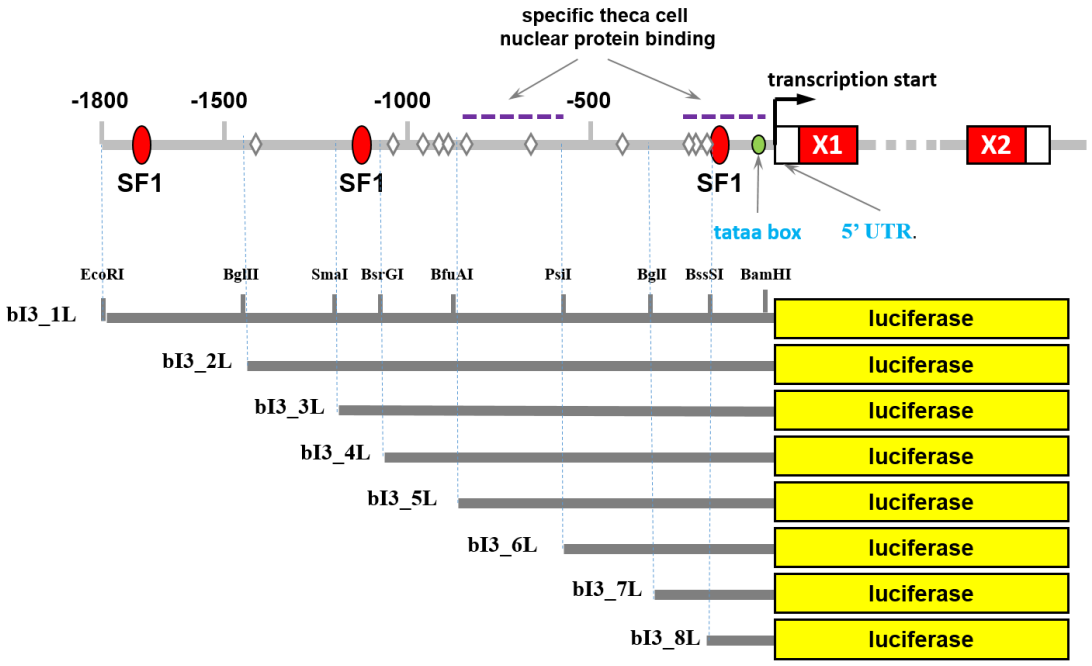


Figure 6.1. Analysis of bovine INSL3 gene promoter. The bovine INSL3 (bINSL3) gene contains two exons (X1 and X2). Approximately 1762bp sequence upstream of X1, which includes the transcription start and the 5' untranslated sequence from the 5'UTR, was considered as the full-length promoter and was inserted into the pGL-3B vector which uses luciferase as reporter gene. This plasmid is named bI3_FL. Plasmid bI3_1L contains the same full length promoter except that the 5' untranslated sequence has been excised. Bioinformatic analysis revealed 3 potential sites for SF-1 binding (red oval) as well as a number of so-called steroid receptor half-sites (rhombus). A previous study in our laboratory had identified two regions that specifically bound theca cell nuclear proteins (purple dotted line) (W. Suehrbier,unpublished). Based on the putative locations of transcription factor binding sites, a series of promoter-deletion constructs were also made as indicated (bI3_2L to bI3_8L) in the reporter vector pGL3-B.

consistent with previous observations on the INSL3 promoters from other species, though the binding sites may be located differently within the promoter (Zimmermann et al. 1997; Sadeghian et al. 2005; Tremblay and Robert 2005).

In order to determine the possible importance of the various trans-acting responsive elements for the action of steroid receptors and the other signalling molecules, a series of 5' deletion promoter-reporter constructs were made in pGL3-B plasmids, using luciferase as reporter gene (Fig 6.1). Two full-length constructs had been made which differed only in their 3' sequences: bI3_1L, like all deletion constructs ended at the transcription start site, whereas bI3_FL additionally included 10bp of the 5' UTR of the first exon. In all subsequent experiments bI3-1L and bI3-FL behaved identically.

6.2.2 The role of steroids in activating the bINSL3 promoter

In the following experiments, various bINSL3 promoter-reporter constructs were co-transfected together with different transcription factors in order to evaluate their ability with diverse steroids to regulate the bINSL3 promoter.

As shown in figure 6.2 (p0GH group), the full-length bINSL3 promoter (bI3_FL) could be activated (~2 folds) in the absence of any additional transcription factors and/or steroids, and this activation was enhanced by 10nM E2 (further ~0.5 fold), although such enhancement was not statistically significant. This indicated that there was endogenous signalling within the TICs that was able

to activate the introduced bINSL3 promoter, and that such endogenous signalling might respond to additional E2.

The transcription factor SF-1 was endogenously expressed in theca interna cells (Fig 5.4). By adding exogenous SF-1, without any ligands, the transcriptional activity of bI3_FL was increased in a dose-dependent manner, with a maximal effect of about 5-fold at 3× SF-1. This maximal effect seemed to be inhibited by 10nM of E2 by about 40% (Fig 6.2 SF-1 group).

Additional ERα, in the presence of 10nM E2, dramatically stimulated the transcriptional activity of the INSL3 promoter, with a maximal effect of about 10-fold achieved with 2× of ERα (Fig 6.2 ERα group).

Unlike ERα, both human and bovine ERβ (hERβ and bERβ) appeared to have inhibitory effects. While hERβ appeared to inhibit promoter activation even in the absence of E2, the bERβ required the steroid for this effect (Fig 6.2 hERβ and bERβ groups).

6.2.3 Activation of the bINSL3 promoter by estrogens.

As shown in Fig 6.3 A, E2 activated ERα-mediated transactivation of the bINSL3 promoter occurs in a dose-dependent manner, with an EC50 value of 1.03nM for E2. As previously, hERβ did not respond to E2 at low concentrations (0.1 – 10nM), but E2 at concentrations of 100nM–1000nM reduced the transcriptional activity (Fig 6.3 B). Bovine ERβ appeared to have inhibitory effects at all doses E2 tested (0.01 – 1000nM) (Fig 6.3 C).

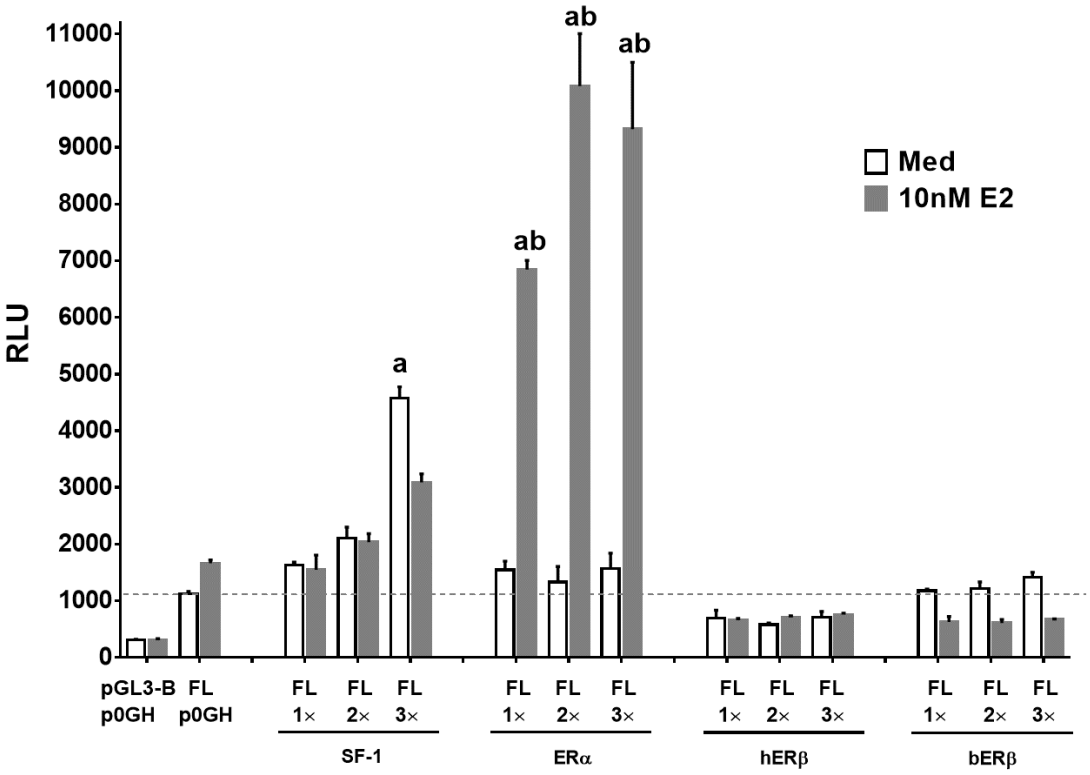


Figure 6.2. Activation of the bovine INSL3 gene promoter by various transcription factors. Primary bovine TICs were transfected with the full-length bovine INSL3 promoter plasmids bI3_FL (FL) with increasing amounts (1×, 2×, 3×) of various transcription factors, SF-1, ERα, human ERβ (hERβ), and bovine ERβ (bERβ); LacZ expression plasmids were always co-transfected as a control for transfection efficiency. Vector p0GH was used to complement any DNA amount difference. As negative control, FL plasmids were replaced by pGL-3B that contains neither promoter nor transcriptional enhancer sequences and so could not give any reporter gene expression, and the transcription factor expression plasmid was replaced by p0GH. The transfected cells were treated with or without (Med) 10nM E2 for 24 hours. Values are Mean ± SEM for n=3 technical replicates: a: $P < 0.0001$ vs FL + p0GH in medium; b: $P < 0.0001$ vs med in each treatment.

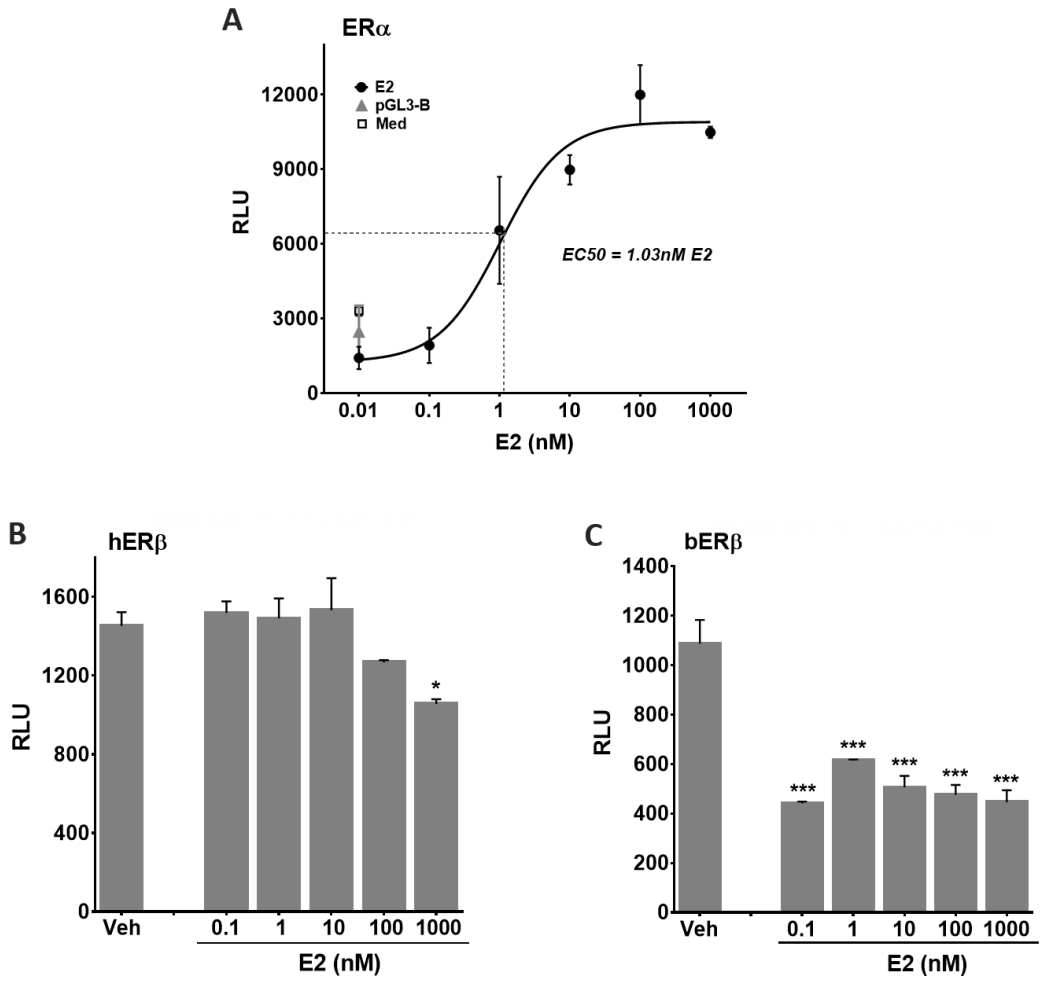


Figure 6.3. Effect of estradiol on the bINSL3 promoter transcriptional activity. TICs were transfected with bI3_FL together with ERα (A), hERβ (B), or bERβ (C), and then were stimulated with increasing doses of E2 (0.1 – 1000nM). EtOH (0.001%) was used as vehicle control. Values are Mean ± SEM for n=3 technical replicates (n=3): *: $P < 0.05$ vs Vehicle. ***: $P < 0.001$ vs Vehicle.

6.2.4 Androgens appear to act through both ER and AR to activate the bINSL3 promoter

Results from the previous chapter suggested that A4 and DHT might utilise both ER and AR to regulate INSL3 peptide production. To examine this hypothesis, the bINSL3 promoter was tested for its responsiveness to various steroid receptors that were activated by either DHT or A4.

As shown in Fig 6.4, the bINSL3 promoter could be activated in all cases, except when hERβ transfected cells were treated with A4 which did not elicit any activation (Fig 6.4 B). In all cases, A4 seemed to activate the receptors ERα, bERβ and AR only at very high concentration (≥1000nM), and DHT appeared to activate the ERα, hERβ, bERβ, and AR at lower concentrations (≥1nM). Similar results were also obtained when the alternative full-length INSL3 promoter-reporter constructs (bI3_FL or bI3_1L) were applied (not shown). In the control experiment, where no exogenous steroid receptor was applied, A4 showed a small elevation at 100nM (Fig 6.4 E). This indicated that in the transfected TICs there was endogenous signalling responding to A4 that contributed to the net effects of the increase in the transcriptional activity of the exogenous bINSL3 promoters.

6.2.5 Steroid receptors use different binding sites in the bINSL3 promoter to modulate transcriptional activity

In this set of experiments, various combinations of ligand and receptor were

used to test their ability to activate different regions of the bINSL3 promoter. In response to SF-1, only the full-length promoter (bI3_1L and bI3_FL) was able to elicit full transcriptional activity, indicating that the distal SFRE might be the only one mediating the action of SF-1 (Fig 6.5 A & 6.6).

In the case of E2-activated ERα, responsiveness was indicated even from the shortest promoter construct, bI3_8L, indicating that the proximal SFRE might be positively contributing to promoter transactivation (Fig 6.5 B). The increase in promoter activity persisted from bI3_8L to bI3_6L, and a second enhancement was observed from bI3_5L to bI3_4L (Fig 6.5 B). These results suggested that the promoter region between 600bp and 1000bp upstream, a region with several steroid receptor half-sites (Fig 6.6), might be mediating the promoter transactivation induced by E2-activated ERα. A decrease in promoter activity was observed from bI3_4L to bI3_2L, suggesting that the middle SFRE and the half-site at about 1400bp upstream might be mediating the suppression of promoter activity caused by E2-activated ERα (Fig 6.5 B).

Like the E2-activated ERα, E2-activated bERβ could stimulate the shortest promoter bI3_8L, and elicited a maximal response in the bI3_4L construct. Moreover, the full-length promoters bI3_1L and bI3_FL also showed full responsiveness to E2-activated bERβ (Fig 6.5 C). In this case, the increase in

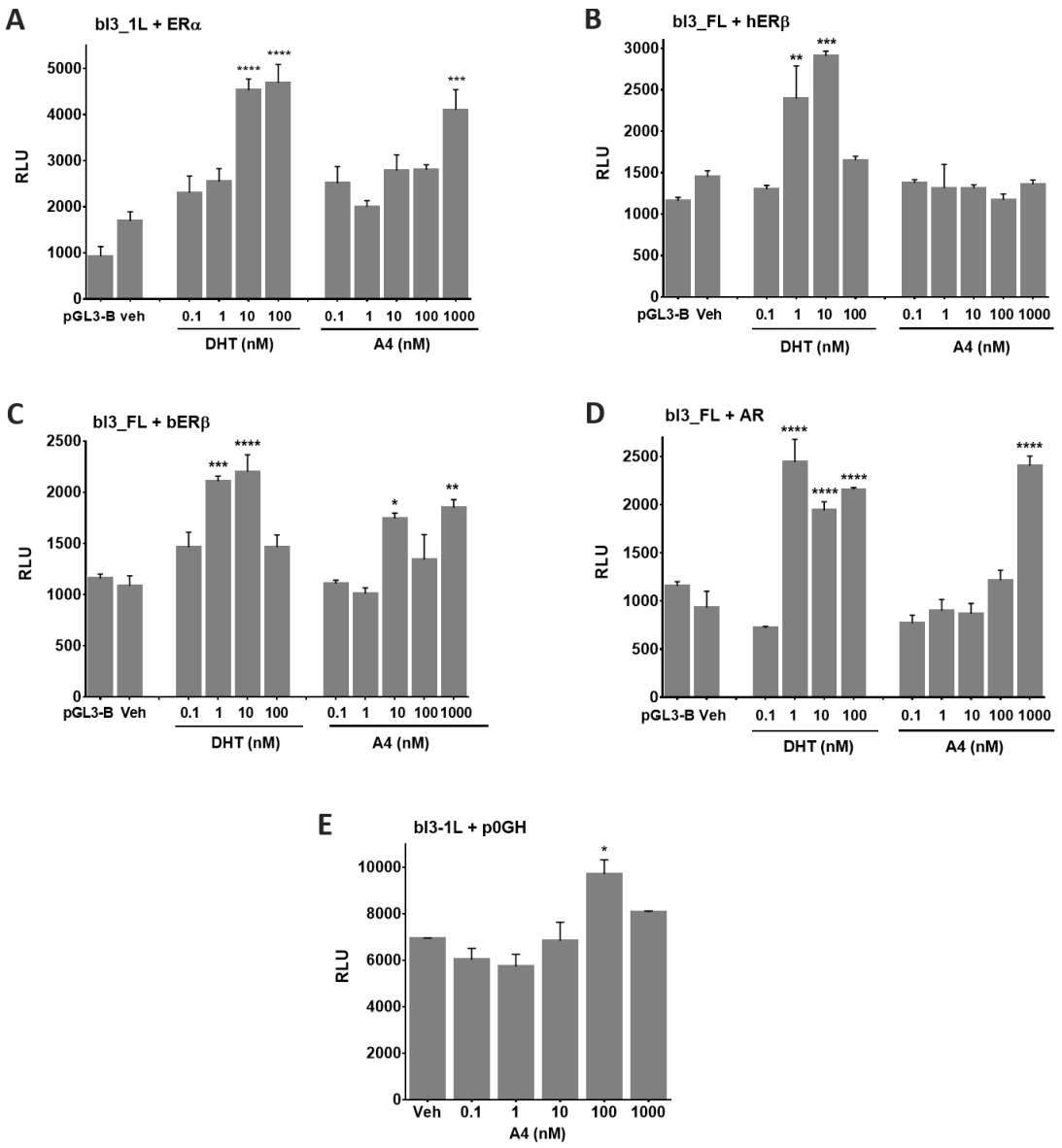


Figure 6.4. Androgens activate bINSL3 promoter via different receptors. TICs were transfected with either bI3_1L (A & E) or bI3_FL (B, C & D). Steroids receptor expression vectors for ERα (A), hERβ (B), bERβ (C), AR (D), or empty vector p0GH (E) were also co-transfected into the TICs. The transfected cells were stimulated with DHT (1 – 100nM) or A4 (0.1 – 1000nM). Values are Mean ± SEM for n=3 technical replicates. *: $P < 0.05$ vs Vehicle. **: $P < 0.01$ vs Vehicle. ***: $P < 0.001$ vs Vehicle. ****: $P < 0.0001$ vs Vehicle.

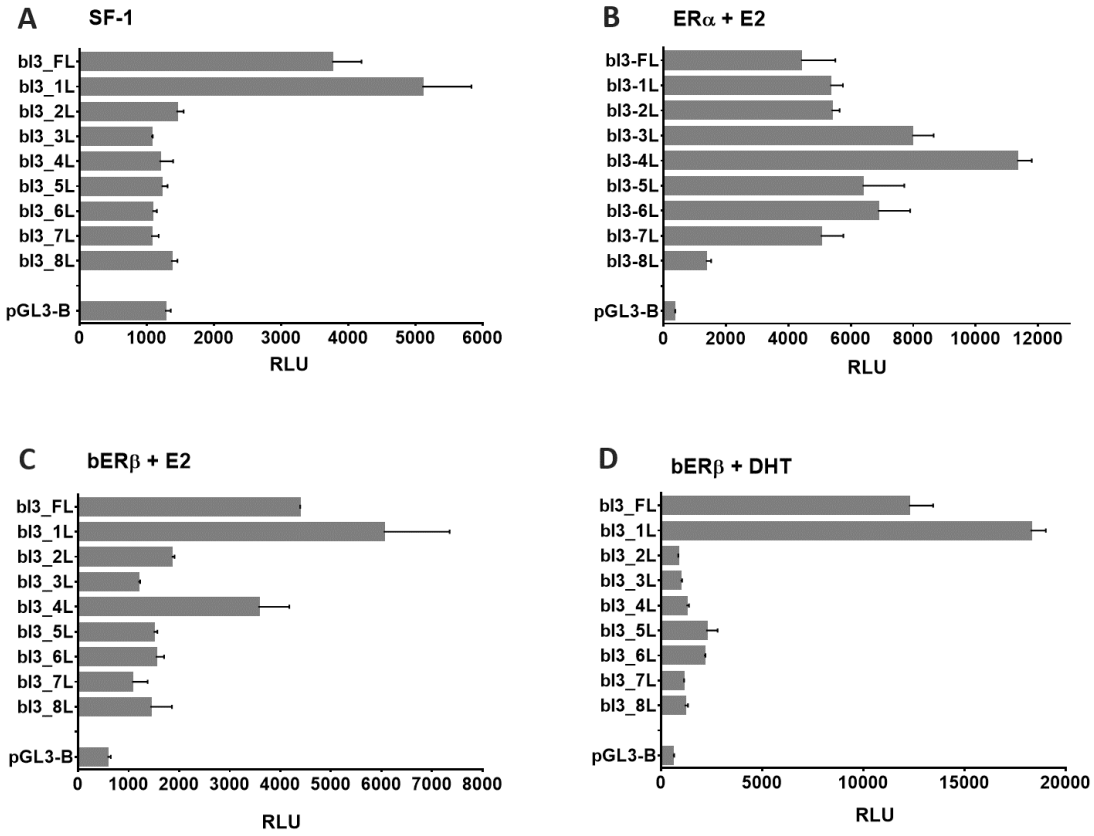


Figure 6.5. Activation of a series of deletion constructs for the bINSL3 promoters. A series of bINSL3 promoter deletion constructs (from bI3_8L to bI3_FL) (see Fig 6.1) were transfected into TICs with SF-1 (A), ERα (B) or bERβ (C & D) expression plasmids, and then stimulated without (A) or with 50nM E2 (B), 0.1nM E2 (C), 1nM of DHT (D), as indicated. The pGL3-B empty vector with various transcription factors was used as negative control. Values are Mean ± SEM, (n=3 technical replicates).

transactivation was observed from bI3_5L to bI3_4L and bI3_3L to bI3_1L, and a decrease was observed from bI3_4L to bI3_3L (Fig 6.5 C). This suggested that the promoter region around 1000bp upstream, containing half-sites, and the promoter region from about 1200bp to 1800bp upstream, containing a half-site at about 1400bp upstream, as well as the distal SFRE, might be positively mediating the effect of E2-activated bERβ; and the promoter region around the middle SFRE might be involved in an inhibitory effect (Fig 6.6).

For DHT-activated bERβ, similar to the responses to the other steroid receptors, even the shortest promoter bI3_8L indicated some activation; however, full transactivation could be achieved only with the full-length promoters (Fig 6.5 D). These results imply that both the proximal SFRE as well as the distal SFRE are likely involved in promoter activation, with the latter having the most prominent effect.

6.3 Discussion

The results presented in this chapter show how steroid receptors in response to various steroids are able to regulate transcriptional activity of bINSL3 promoter constructs transfected into follicular theca interna cells. Briefly, the transcriptional activity of the bINSL3 promoter could be stimulated by E2-activated ERα and be inhibited by E2-activated ERβ; DHT and A4 could activate not only AR but also ERα and ERβ, thereby increasing bINSL3

promoter activity. SF-1 also induced bINSL3 promoter activity. All these steroid-receptor combinations appeared to act on the bINSL3 promoter in different ways to elicit transactivation, whereby the identified SFREs and maybe the steroid receptor half-sites are playing important roles.

In figure 6.2 and 6.4 E, the results showed that there is endogenous signalling which is also contributing to the final activation of the bINSL3 promoter. However, the magnitude of the induction caused by endogenous factors appears relatively smaller than that caused by exogenous signalling (Fig. 6.2 and 6.4). Therefore, the net results from transfection experiments involving co-expressed transcription factors is likely caused by these and the effects of their ligand-induced activation.

6.3.1 E2-ER activated bINSL3 promoter activity

In a previous chapter, a biphasic effect of E2 in stimulating INSL3 peptide production was observed, supposedly through endogenously expressed ERα and/or ERβ (Fig 5.12). In the present chapter I have shown that exogenous ERα and ERβ may have opposite effects in response to E2 by increasing or inhibiting INSL3 promoter activity, respectively (Fig 6.3). This antagonistic action between ERα and ERβ has once been observed also in the promoter of another follicular gene Anti-müllerian hormone (AMH), whose transcriptional activities were examined in the mouse luteinised granulosa cells line KK1 (Grynberg et al. 2012). Like the INSL3 promoter, the AMH promoter contains

only an estrogen response element (ERE) half-site as well as SF-1 binding sites, though no classic palindromic ERE (Guerrier et al. 1990; Watanabe et al. 2000). Similar results were obtained that E2 could enhance ERα-mediated AMH promoter activity with an EC50 value of about 1nM, and E2-activated ERβ showed inhibitory effects at least from 0.1nM to 1000nM (Grynberg et al. 2012) (Fig 6.3). Therefore, both INSL3 and AMH may have adopted a similar regulatory system in regard to estrogen signalling.

Moreover, Grynberg et al. (2012) showed an increase in AMH promoter activity when ERα was dominantly expressed in the host cells, and a decrease in AMH promoter activity when ERβ was dominantly expressed. Thus, the expression ratio of ERα to ERβ may determine the E2-induced net transcriptional activity of a promoter. Furthermore, previous study showed the ratio of bovine ERα to ERβ transcripts in TIC decreases when follicles develop towards the pre-ovulatory stage (Schams and Berisha 2002). During follicular development, E2 levels keep increasing due to the enhanced aromatase activity in granulosa cells. Furthermore, E2 at 100nM appear to reduce the ratio of ERα to ERβ transcripts at least in human luteinised granulosa cells (Grynberg et al. 2012). Therefore, as follicles develop, E2 levels increase which may lead to a reduction in the ratio of ERα to ERβ, and so the regulation of INSL3 production caused by E2 might shift from E2-activated ERα to E2-activated ERβ, and thus from a positive to a negative control. This would be consistent with the results of previous chapters, that a low concentration of

E2 stimulated INSL3 peptide production and that this stimulatory effect became weaker as E2 concentrations increase further (Fig 5.12). This might contribute to the fall in circulating INSL3 peptide levels in the late follicular phase (Fig 4.2 & 4.3).

6.3.2 Androgen stimulated bINSL3 promoter activity

Results from the previous chapter showed that the androgen DHT might act through AR to inhibit INSL3 production, and that both DHT and A4 may act through ER to stimulate INSL3 production (Fig 5.10 & 5.11). In the promoter transactivation experiments described here, the results confirmed that both DHT and A4, at various concentrations, can increase INSL3 promoter activity via ERα (Fig. 6.4 A). Not only ERα, but also b/hERβ and AR could mediate the DHT/A4 effects (except for A4-activated hERβ) to enhance INSL3 promoter activity (Fig. 6.4 B, C & D). These results are similar to the observations in mouse testicular Leydig cells, where the transfected human and rodent INSL3 promoter-reporter constructs could be activated by testosterone (Lague and Tremblay 2008; Tremblay et al. 2009). Though they did not define which receptors were involved, the use of the AR antagonist hydroxyflutamide to supress the stimulatory effects caused by testosterone, suggested that in these cells the action of testosterone might indeed be via AR (Lague and Tremblay 2008).

Although there was no observed inhibitory effect caused by DHT or A4 via AR

that reduced the transcriptional activity of the INSL3 promoter to below the basal level, one study using mouse preantral follicles showed that INSL3 transcripts could be reduced by 1μM of DHT in vitro (Xue et al. 2012). Therefore, the inhibitory effects of DHT do exist in the ovarian follicle, though the mechanism to explain this is not yet clear.

6.3.3 Mapping the upstream bINSL3 promoter region for the effects of various transcription factors.

Based on the results shown in Fig. 6.5, the region of the bovine INSL3 promoter that appears to respond to variously liganded steroid receptors and transcription factors can be mapped as in Fig 6.6. Just as in other species, the transcription factor SF-1 strongly promotes transcriptional activity of bovine INSL3 promoter, most probably through the distal SFRE, though probably not via the two more proximal sites. E2 and DHT could activate the bINSL3 promoter in the absence of any classically palindromic steroid response element. Rather, the proximal SFRE would appear to mediate the basic transactivation induced by both E2-activated ERα and E2 or DHT-activated ERβ. The region of the middle SFRE probably mediates the suppressive effects caused by E2-activated ERα/β. Meanwhile the region of the distal SFRE responded positively to ERβ that was activated by E2 or DHT. Most of the identified half-sites corresponded to positive signals for E2-activated ERα/β, except for the one located at around -1400bp that was associated with

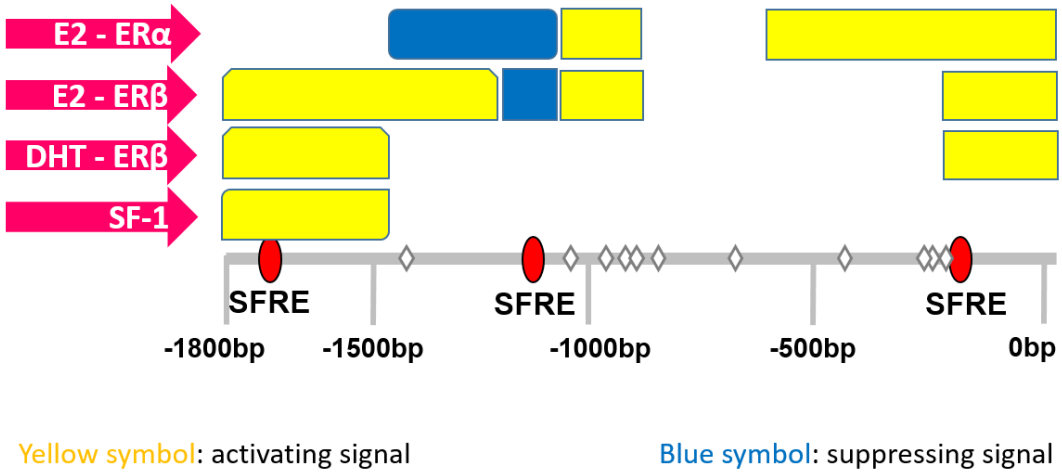


Figure 6.6. Mapping the DNA regions responding to various transcription factors. The diagram shows the bovine INSL3 upstream promoter region of about 1800bp, containing 3 potential sites for SF-1 binding (SFRE) (red oval) as well as steroid receptor half-sites for steroid receptors binding (rhombus). The host theca interna cells were treated with additional SF-1 expression plasmids (SF-1), ERβ expression plasmids in the presence of DHT (DHT-ERβ), ERβ expression plasmids in the presence of E2 (E2-ERβ), or ERα expression plasmids in the presence of E2 (E2-ERα). Different combinations of promoter regions respond to different stimulations that either increase (yellow rectangle) or decrease (blue rectangle) the transcriptional activity of the bovine INSL3 promoter.

a decreased transcriptional activity induced by E2-activated ERα (Fig 6.6).

It is evident that both ER isoforms, ERα and ERβ could be activated by E2, though they involve different regions of the promoter. When ERβ was activated by DHT, the region around -1000bp containing several half-sites, which was responsible for a maximal activation induced by E2-activated ERα/β, was no longer essential. Rather, the distal 300bp containing the SFRE retained its importance to elicit the maximal transactivation as for E2-activated ERβ. Therefore, ERβ might possess some intrinsic features of ERα in response to E2, but these features are masked when it is activated by DHT (Fig 6.6). These results suggest that ERβ might have a different conformation when activated by E2 or by DHT, and so they can interact with different regions on the bINSL3 promoter directly or indirectly by recruiting other transcription factor/s (see more in chapter 8.2).

The region responding to DHT-activated ERβ partially overlaps the region responding to SF-1 (Fig 6.6). Although there is no previous evidence showing that ERβ can cooperate with SF-1 in activating promoter activity, results in Fig. 6.2 showed that E2 tended to reduce the transcriptional activity of the INSL3 promoter induced by a high amount (3×) of SF-1; this suggests that excessive SF-1 might cooperate with the E2-activated endogenous ERβ to negatively regulate transactivation, probably in a form of a multiunit complex. Clearly, the present experiments have only provided indirect evidence for such DNA

interactions. More detailed experiments, involving site-specific mutagenesis of the various promoter regions as well as ChIP analyses of the putatively involved transcription factors will be required.

The results from the current chapter, by further dissecting how sex steroids might be regulating bINSL3 production at the transcriptional level of the gene promoter support the observations from the previous chapter describing steroid regulation of INSL3 peptide production.

Chapter 7:

The role of bovine RXFP1 and RXFP2 signalling in the bovine female reproductive system

The results from current chapter had been published in:

***Dai Y**, Ivell R, Liu X, Janowski D, Anand-Ivell R. 2017. Relaxin-family peptide receptors 1 and 2 are fully functional in the bovine. Frontiers in Physiology **8**: 359.*

7.1 Introduction:

INSL3 belongs to the relaxin peptide family. Other members from this family include relaxin, relaxin 3, INSL5 and INSL6 (Bathgate et al. 2001). In higher primates and human, the relaxin family also includes relaxin 1 and INSL4 (Bathgate et al. 2001). These peptides share a similar structure that consists of an A-chain and B-chain linked by two inter-chain disulphide bonds and one intra-chain disulphide bond present within the A-chain (summarised in Chan et al. 2011). The relaxin family peptides bind to and activate G-protein coupled receptors classified as relaxin family peptide (RXFP) receptors. RXFP1, RXFP2, RXFP3, and RXFP4 are the cognate receptors for relaxin, INSL3, relaxin 3 and INSL5, respectively (Bogatcheva et al. 2003; Liu et al. 2003; Sudo et al. 2003; Scott et al. 2004; Liu et al. 2005; Kong et al. 2010). The receptor for INSL6 has not yet been identified.

The actions of the relaxin 3 - RXFP3 and INSL5 - RXFP4 systems appear to be restricted to the brain and gut, respectively (summarised in Halls et al. 2015). Although the receptor for INSL6 has not yet been identified, INSL6 has been shown to be involved in male germ cell development (Burnicka-Turek et al. 2009), skeletal muscle regeneration (Zeng et al. 2010; Zeng et al. 2014), and its expression is also detectable in human and macaque placenta (Eidem et al. 2016). The INSL3-RXFP2 system has been studied in reproductive tissues, bone, and kidney; while relaxin-RXFP1 signalling has effects in reproductive and cardiovascular tissues, brain, bone, breast, kidney, lung, liver,

and blood cells; most of which have been examined in human and rodent systems (summarised in Bathgate et al. 2013). Extensive searching using a range of molecular techniques has failed to identify a relaxin homologue in ruminants, including cattle, sheep and goats (Hartung et al. 1995), except for a partially deleted sequence representing part of the A-chain in sheep (Roche et al. 1993). A chromosomal synteny analysis comparing the corresponding regions of the bovine, human, ovine, equine and porcine genomes indicated a significant deletion in the bovine genome on chromosome 8, where the relaxin gene should have been located (Dai et al. 2017). Nevertheless, a relaxin-dependent physiology appears to persist at least in the bovine. Administration of porcine relaxin to beef cows before term induces a classic pelvic canal expansion and cervical relaxation, and also contributes to luteolysis, leading to premature parturition (Musah et al. 1986; Musah et al. 1989). This leads to the question whether, in the absence of relaxin, its cognate receptor RXFP1 is still functionally expressed in the bovine.

Moreover, besides their cognate receptors, some relaxin family peptides have been shown to bind to and activate other related receptors, though with lower affinity and efficiency. For example, human relaxin could activate RXFP2 (the cognate receptor for INSL3) in mouse calvarial osteoblasts to elicit osteogenic and collagenolytic effects in vitro (Duarte et al. 2014). This may be due to the high level of homology between RXFP1 and RXFP2, as both receptors have a large extracellular N-terminus containing leucine-rich-repeats for ligand

binding, and the homology between human RXFP1 and RXFP2 is 80% (Bathgate et al. 2013). This leads to the possibility that other receptors, such as RXFP2, may be responsible for relaxin-dependent physiology in the bovine. In another case, synthetic relaxin 3 can bind to and activate human RXFP1 (cognate receptor for relaxin) in vitro, and relaxin 3 - RXFP1 signalling may be responsible for water drinking in the rat in vivo (Bathgate et al. 2006). Human INSL3 binds to mouse RXFP1 in vitro, and human INSL3 increases the activity of human relaxin in widening the symphysis pubis in a mouse model (Bullesbach and Schwabe 1995). Together this suggests that another relaxin-like peptide might be substituting for the missing relaxin in the bovine to activate RXFP1.

The current study aimed to examine the presence of full-length RXFP1 and RXFP2, and their expression patterns in various cells of the bovine reproductive system. In order to check for receptor functionality, and since activation of human RXFP1 and RXFP2 leads to the production of cAMP (Hsu et al. 2002; Heng et al. 2008), cAMP was used as endpoint to identify possible ligands for individual bovine receptors.

7.2 Results:

7.2.1 Expression of RXFP1 and RXFP2 in the bovine female reproductive system.

In order to assess the expression of the full-length transcripts for bovine

RXFP1 (bRXFP1) and bRXFP2, various combinations of oligonucleotide primer pairs were used in RT-PCR reactions to ensure that the entire 18 exons could theoretically be amplified (Fig 7.2, Fig 7.3, Fig 7.5 & Fig 7.6). The positions of different primers for bRXFP1 and bRXFP2 are shown in Fig 7.1 and Fig 7.4, respectively. A range of tissues and cells obtained from the bovine female reproductive system were used to screen for the expression of bRXFP1 and bRXFP2 transcripts. Not all successful PCR primer combinations are illustrated below. The semi-quantitative RT-PCR results showed that bRXFP1 is consistently shown to be expressed in endometrium, myometrial cells and theca interna cells, though not in granulosa cells, oocytes, mammary epithelial cells, or early embryos (not shown) (Fig 7.2). A detailed analysis of the expression of bRXFP1 transcripts in myometrial cells is illustrated in Fig 7.3. In contrast, RXFP2 is expressed only in theca interna cells, oocytes, and weakly in myometrial cells (Fig 7.5). Detailed RT-PCR analysis of the expression of bRXFP2 transcripts in bovine oocytes is shown in Fig 7.6. Arrowheads indicate the correctly sized and sequenced PCR products; alternatively sized products, evident in most samples, and mostly as quantitatively minor bands, proved on sequencing to represent splice variants.

Indeed, in the bovine both RXFP1 and RXFP2 genes exhibited multiple splice variants, in which one or more exons are missing, as well as alternative novel exons (Fig 7.1 & Fig 7.4). Some of the missing and novel exons have been observed previously in archived Expressed Sequence Tags (EST DY464350;

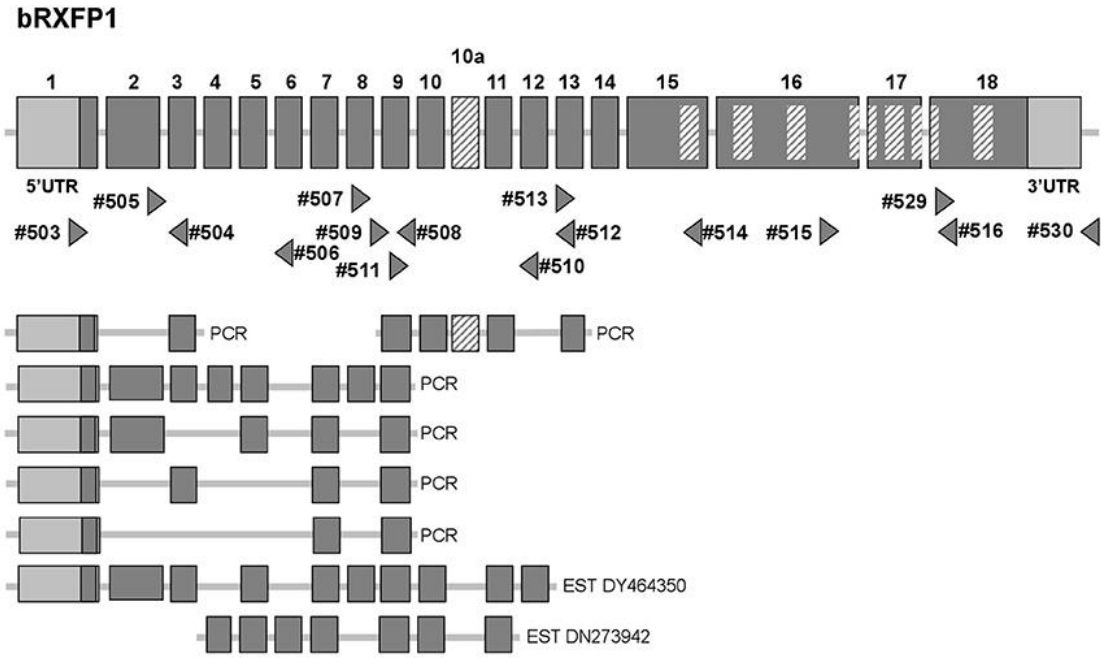


Figure 7.1. Schematic exon organisation of the bovine RXFP1 (bRXFP1) gene. Bovine RXFP1 contains 18 exons (rectangles in the first row). 10a (cross-hatched rectangle): the novel exon detected in the current project. The internal cross-hatched rectangles represent the encoded transmembrane regions. A number with arrowhead indicates the position of a primer. Below the primers are the splice variants detected during semi-quantitative RT-PCR analysis or previously detected splice variants (EST DY464350; DN273942). The grey line that links two exons represents respective exon/s that was/were not detectable in a splice variant (Dai et al. 2017).

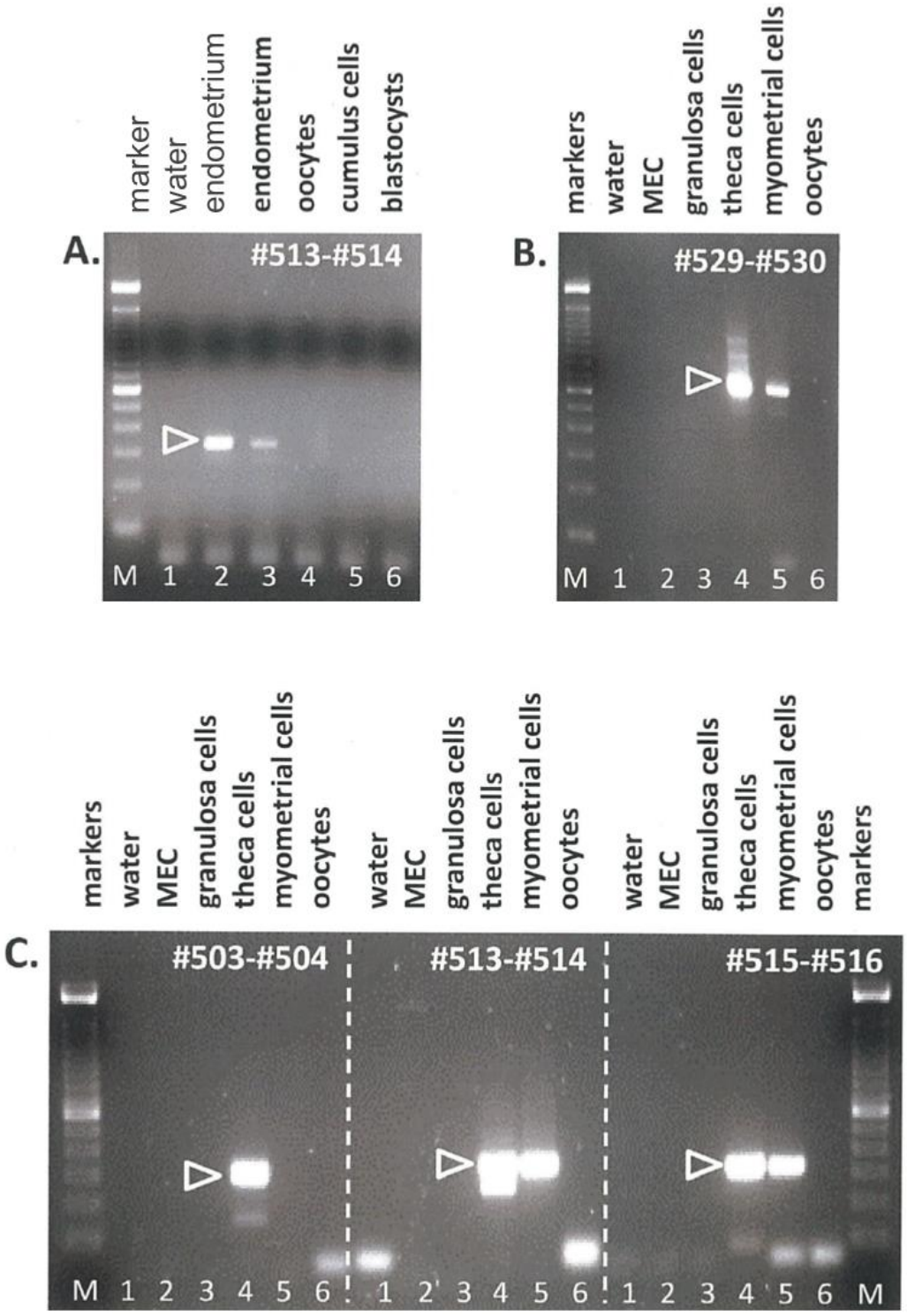


Figure 7.2. Semi-quantitative RT-PCR analysis of bovine RXFP1. Various combinations of primers were used to amplify bRXFP1 in different bovine reproductive tissues and cells. The primer number refers to Fig 7.1. Arrowheads indicate the correctly sized PCR product. MEC: primary bovine mammary epithelial cell. M: 100bp DNA ladder marker. PCR conditions (primers, annealing temperature, expected size): #503-#504, 62ºC, 301bp (C); #513-#514, 62ºC, 337bp (A & C); #515-#516, 62ºC, 316bp (C); #529-#530, 62ºC, 692bp (B) (Dai et al. 2017).

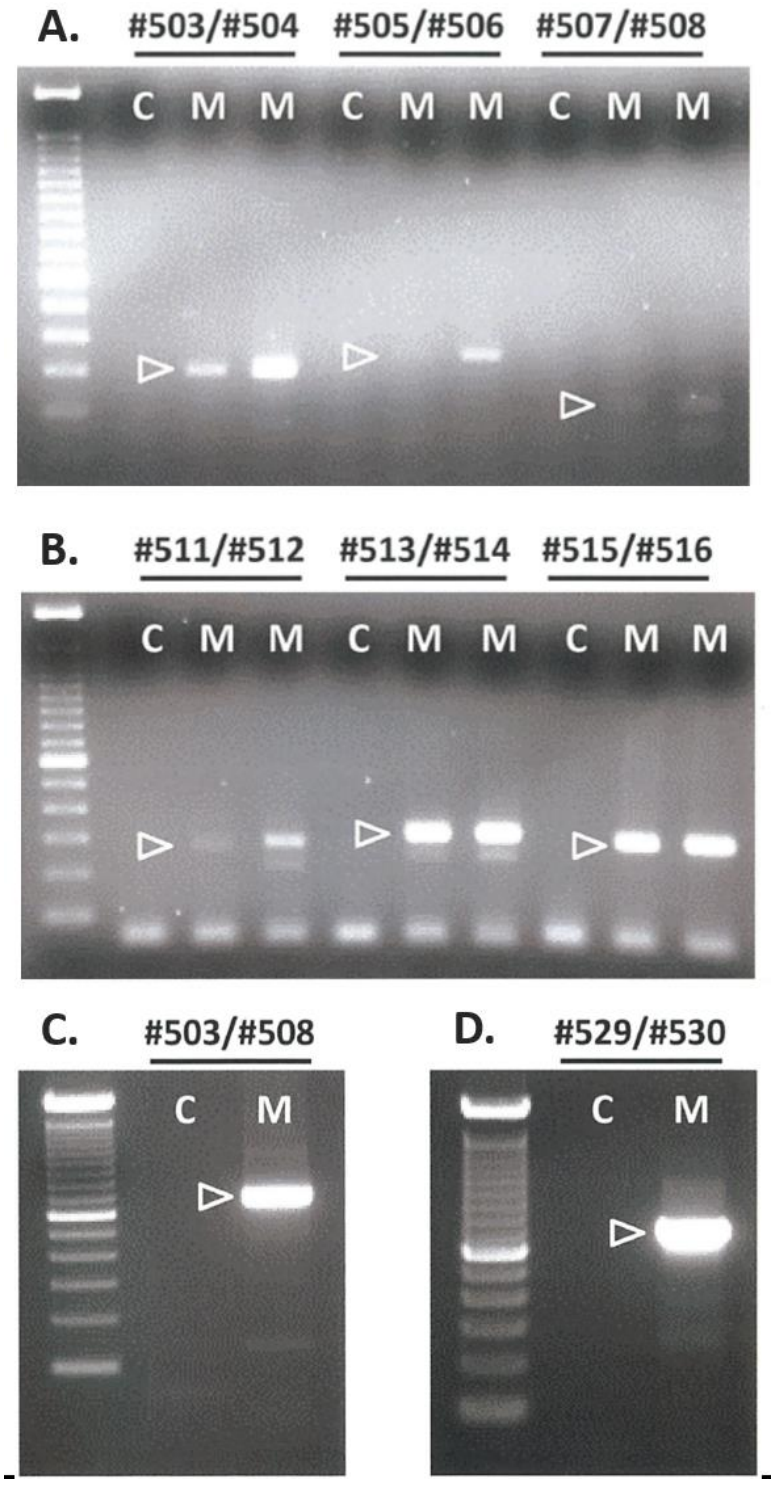


Figure 7.3. Expression of bovine RXFP1 transcripts in bovine myometrial cells. Various combinations of primers were used to amplify bRXFP1 cDNA from bovine myometrial cells. The primer number refers to Fig 7.1. Arrowheads indicate the correctly sized PCR product. C: water control. M: myometrial cells from different batches. PCR conditions (primer, annealing temperature, expected size). #503-#504, 62ºC, 301bp (A), #505-#506, 62ºC, 349bp (A); #507-#508, 62ºC, 175bp (A); #503-#508, 62ºC, 753bp (C); #511-#512, 62ºC, 304bp (B); #513-#514, 62ºC, 316bp (B); #515-#516, 62ºC, 316bp (B); #529-#530, 62ºC, 692bp (D) (Dai et al. 2017).

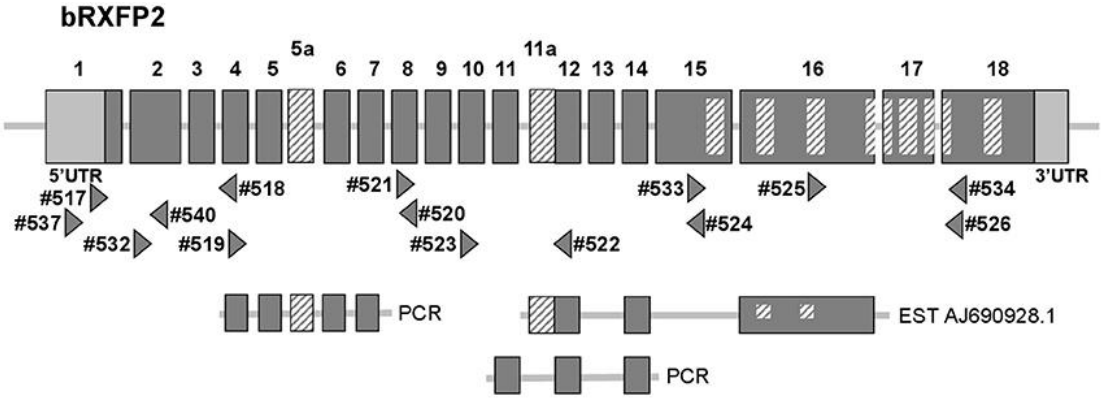


Figure 7.4. Schematic exon organisation of the bovine RXFP2 (bRXFP2) gene. Bovine RXFP2 contains 18 exons (rectangles in the first row). 5a & 11a (cross-hatched rectangles): the novel exons detected in the current experiments. The internal cross-hatched rectangles represent the encoded transmembrane regions. A number with arrowhead indicates the position of an oligonucleotide primer. Below the primers are the splice variants detected during semi-quantitative RT-PCR (PCR) analysis or previously detected splice variant (EST AJ690928.1). The grey line that links two exons represents respective exon/s that was/were not detectable in a splice variant (Dai et al. 2017).

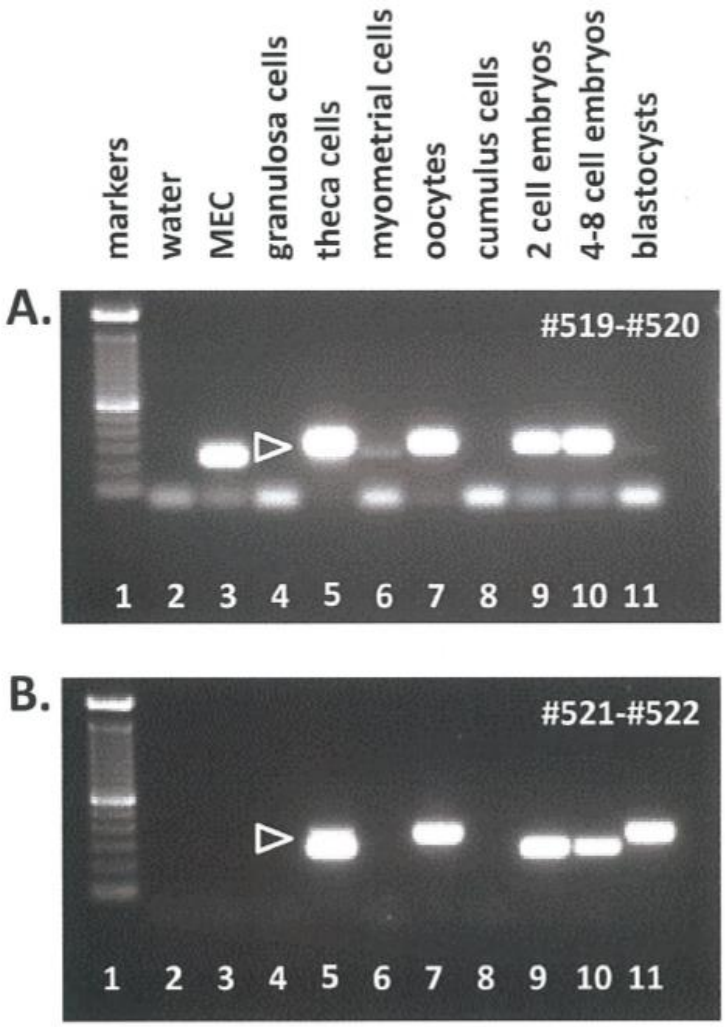


Figure 7.5. Semi-quantitative RT-PCR analysis of bovine RXFP2. Various combinations of primers were used to amplify bRXFP2 transcripts in different bovine reproductive tissues and cells. Primer number refers to Fig 7.4. Arrowheads indicate the correctly sized PCR product. MEC: primary bovine mammary epithelial cells. PCR conditions (primers, annealing temperature, expected size): #519-#520, 62–58ºC touchdown, 291bp (A); #521-#522, 62–60ºC touchdown, 312bp (B) (Dai et al. 2017).

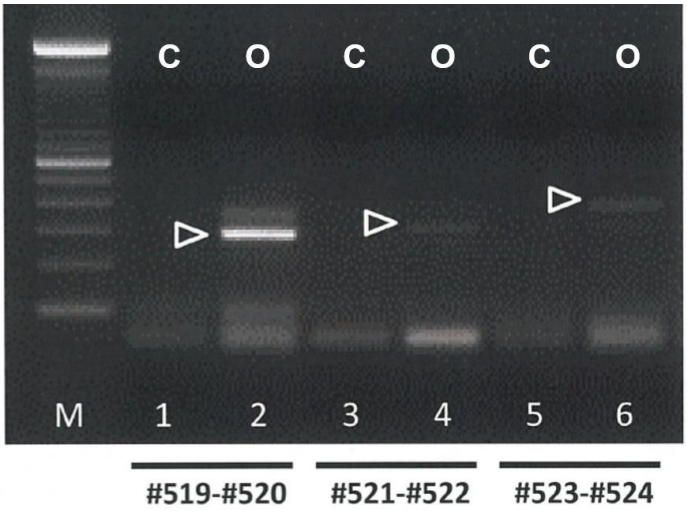


Figure 7.6. Expression of bovine RXFP2 transcripts in bovine oocytes. Three sets of primer pairs were used to amplify bRXFP2 cDNA from bovine oocytes. The primer number refers to Fig 7.4. Arrowheads indicate the correctly sized PCR product. C: water control. O: oocytes. PCR conditions (primers, annealing temperature, expected size): #519-#520, 62–58ºC touchdown, 291bp; #521-#522, 62–60ºC touchdown, 312bp; #523-#524, 62ºC, 392bp (Dai et al. 2017).

DN273942; AJ690928.1). It is to be noted that none of the deleted exons lead to a frame-shift or in-frame stop codons. In contrast, where there appears to be the introduction of a novel exon (bRXFP1 exon 10a; bRXFP2 exons 5a and 11a) this indeed leads to an in-frame stop codon in each case.

7.2.2 Stimulation of primary cultures of bovine myometrial cells and theca interna cells.

Based on the semi-quantitative RT-PCR analysis, both bRXFP1 and bRXFP2, as full-length transcripts, appear to be expressed in both myometrial and theca interna cells. To confirm if these transcripts can be successfully translated into functional protein receptors, both types of cells were assessed for their abilities to respond to the cognate ligands for these receptors, relaxin and INSL3. The phosphodiesterase inhibitor IBMX that prevents cAMP break-down was used to pre-treat both types of cells, and so increases the basal level of cAMP. The acute cAMP production caused by a receptor ligand was used to determine the receptor activation.

For myometrial cells, both peptides increased cAMP production in a dose-responsive manner (Fig 7.7 A). For theca interna cells, though both porcine relaxin (pRLN) and recombinant human relaxin (rhRLN) appeared to have no effects, bovine INSL3 (bINSL3) increased cAMP production in a dose-dependent manner (Fig 7.7 B).

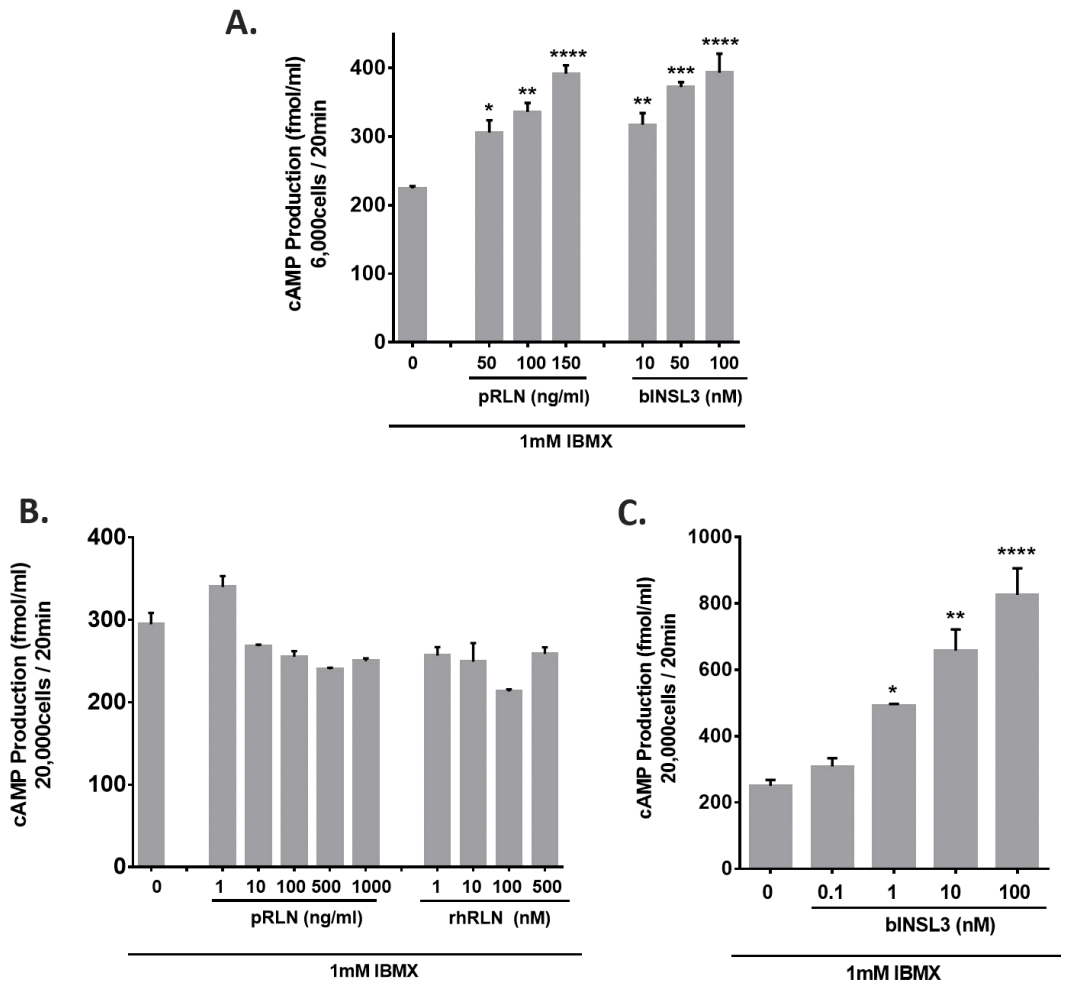


Figure 7.7. Stimulation of primary bovine myometrial cells and theca interna cells with relaxin and INSL3. Primary bovine myometrial cells (A) and bovine theca interna cells (B & C) were pre-treated with 1mM IBMX (for 30min for myometrial cells, and 10min for theca interna cells), and then were treated with porcine relaxin (pRLN), recombinant human relaxin (rhRLN), or bovine INSL3 (bINSL3) for 20min. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, ****: $P < 0.0001$ vs 0 + 1mM IBMX. Values are Mean ± SEM for n=3 technical replicates (Dai et al. 2017).

7.2.3 Identification of the potential ligands for bRXFP1 and bRXFP2

To further characterise the action of the individual receptors, eukaryotic expression vectors encoding the full-length bRXFP1 and bRXFP2 transcripts were respectively transfected and expressed in HEK-293T cells, and various putative ligands used to treat these transfected cells. Results showed that bRXFP1 was activated by both porcine as well as recombinant human relaxin (pRLN & rhRLN) and by human relaxin 3 (hRLN3), but not by INSL3 up to 1000nM; in contrast bRXFP2 was activated by human and bovine INSL3 (hINSL3 & bINSL3), and by high doses of pRLN and rhRLN, but not by hRLN3 up to 1500nM (Fig 7.8). All such stimulations occurred in a dose-dependent manner. In parallel, similar stimulations were carried out using permanently transfected cells expressing human RXFP1 (hRXFP1) and hRXFP2, and the results summarised in Table 7.1.

The EC50 values for pRLN, rhRLN and hRLN3 in activating bRXFP1 were about one order of magnitude higher than when activating hRXFP1; with the lowest values generated by pRLN (10.8nM for bRXFP1, and 0.4nM for hRXFP1), followed by rhRLN (48.7nM for bRXFP1, and 0.9nM for hRXFP1), and with the highest values generated by hRLN3 (97.9nM for bRXFP1, and 14.6nM for hRXFP1) (Table 7.1). In contrast, EC50 values were largely comparable between bRXFP2 and hRXFP2 (Table 7.1). For the cognate ligand INSL3, whether human or bovine, the EC50s were consistently 0.6-0.7nM, and for RLN of different sources EC50 values ranged from

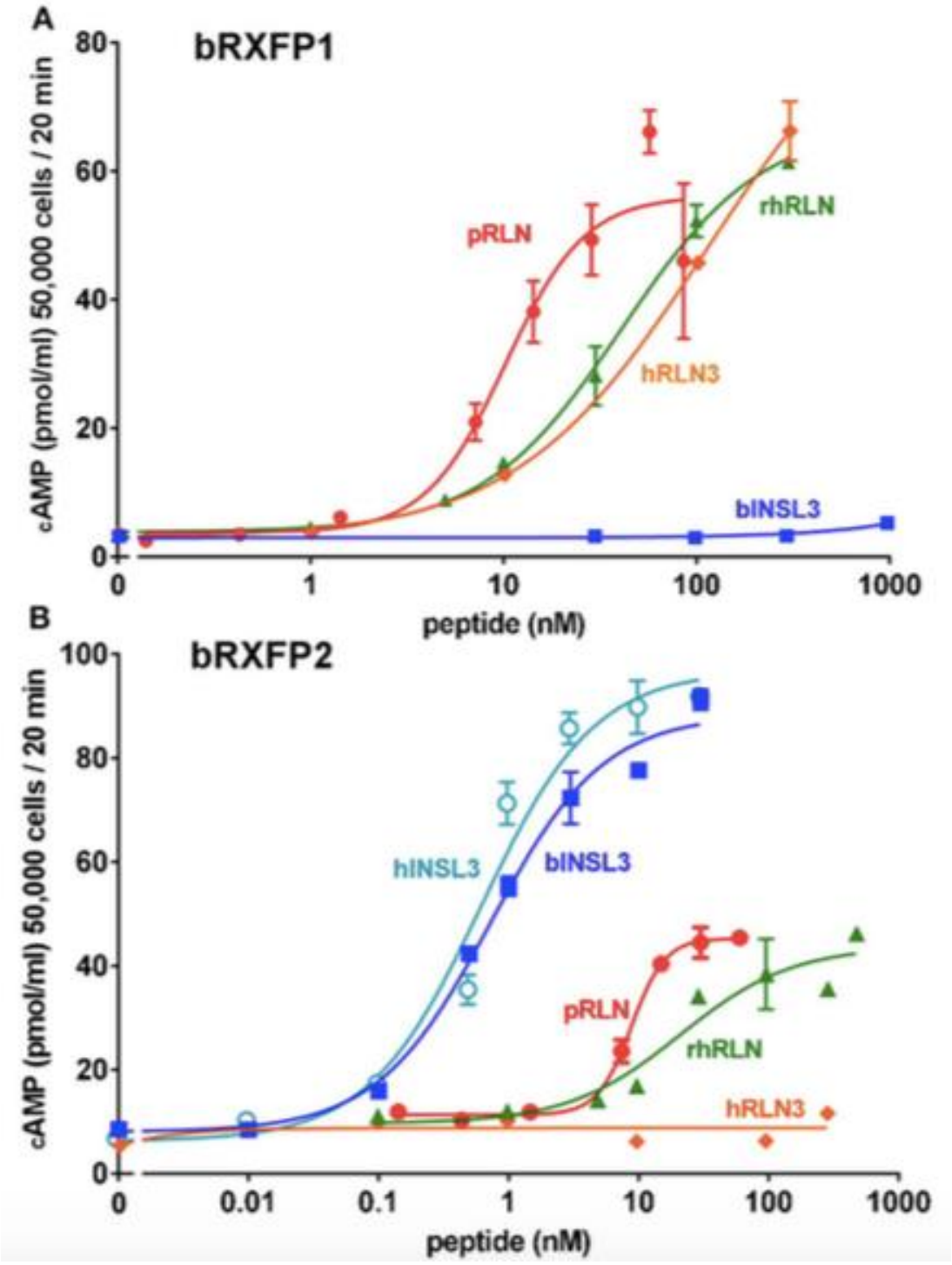


Figure 7.8. Activation of transfected bRXFP1 and bRXFP2 with various relaxin-family peptides. Full-length bRXFP1 (A) and bRXFP2 (B) expressing plasmids were transfected and expressed in HEK-293T cells, and then were stimulated with porcine relaxin (pRLN), recombinant human relaxin (rhRLN), human relaxin 3 (hRLN3), human INSL3 (hINSL3), or bovine INSL3 (bINSL3) for 20min. The acute production of cAMP was measured in order to assess the activation of each receptor by the different ligands. Values are Mean ± SEM, (n=3 technical replicates) (Dai et al. 2017).

Table 7.1. EC50 values for human and bovine RXFP1 and RXFP2. [1] Concentrations for pRLN have been converted to nM assuming a MW of 6kD and a purity of 85%. [2] Dose-response curve had not reached an asymptote at the highest concentration tested. [3] n.d.: not determined; no significant stimulation could be detected at the highest concentration applied (Dai et al. 2017).

Peptide	bRXFP1	hRXFP1	bRXFP2	hRXFP2
pRLN	10.8nM[1]	0.4nM[1]	10.5nM[1]	>12nM[1,2]
rhRLN	48.7nM	0.9nM	27.3nM	53.9nM
hRLN3	97.9nM	14.6nM	n.d. [3]	n.d. [3]
bINSL3	>1000nM[2]	n.d. [3]	0.7nM	0.6nM
hINSL3	n.d. [3]	n.d. [3]	0.6nM	0.7nM
Insulin	n.d. [3]		n.d. [3]	
IGF1	n.d. [3]		n.d. [3]	
IGF2	n.d. [3]		n.d. [3]	

10-50nM (Table 7.1). In addition, insulin, IGF1 and IGF2, which are also similarly structured members of the relaxin-insulin family, could not activate bRXFP1 or bRXFP2 at any of the doses applied (1-100nM) (Table 7.1).

7.3 Discussion:

The results in the current chapter show that both bRXFP1 and bRXFP2 receptors are expressed in cells of the bovine female reproductive system, and that they appear to be functional in primary myometrial and/or theca cells with appropriately increased cAMP production. By using a transiently transfected HEK-293T cell system, their principal preferred ligands were confirmed to be porcine relaxin and bINSL3 for bRXFP1 and bRXFP2, respectively.

7.3.1 Potential role of INSL3-RXFP2 signalling

By examining the expression of bRXFP2, it was possible to identify putative targets for INSL3 action within the bovine female reproductive system. Bovine RXFP2 is expressed at a high level in ovarian theca interna cells, and INSL3 induces a dose-dependent increase in cAMP production in these cells, consistent with the role for INSL3-RXFP2 to promote androstenedione synthesis (Glister et al. 2013). RXFP2 is also expressed in the oocyte. As shown in a rat model, INSL3 may act via RXFP2 in the oocyte to cause a reduction in the intracellular cAMP level that is essential for meiotic resumption

(Kawamura et al. 2004). There is also low level expression of RXFP2 in bovine myometrial cells, as shown previously also for the equivalent cells in the human (Heng et al. 2008). Unlike in human myometrial cells, however, where INSL3 is unable to activate cAMP production (Heng et al. 2008), bovine INSL3 (10nM, 50nM, and 100nM) enhances cAMP production in bovine myometrial cells to the level similar to that achieved by porcine relaxin (1.42 to 1.75-fold) (Fig 7.7 A).

Moreover, besides the cognate ligand INSL3, it has been observed that RLN can also activate bRXFP2 receptors that are transfected and so expressed in HEK-293T cells. This is consistent with previous in vitro studies where a high dose of RLN can activate human RXFP2 expressed in HEK-293T cells, though with lower binding affinity and efficacy (Hsu et al. 2002; Sudo et al. 2003; Bathgate et al. 2006). However, these high concentrations are unlikely to be found in vivo (Anand-Ivell and Ivell 2014b), so that any physiological relevance of RLN-RXFP2 coupling in vivo should be considered cautiously.

In bovine, the expression pattern and cellular localisation of INSL3 transcripts in corpora lutea are very similar to that of relaxin transcripts in the pig (Denning-Kendall et al. 1989; Bathgate et al. 1999). Moreover, human relaxin induces widening of the symphysis pubis in mouse, and this effect can be enhanced by additional human INSL3, which may act through a direct interaction between human INSL3 and mouse RXFP1 (Bullesbach and Schwabe 1995). It has been speculated that INSL3 could be a substitute for

relaxin in the bovine to trigger classical relaxin-dependent physiology. However, this speculation is not consistent with the observation in HEK-293T cells, where bINSL3 of up to 1000nM was unable to activate the expressed bRXFP1 (Fig 7.8 A). Similarly, hINSL3 seems not to activate the expressed hRXFP1 in HEK-293T cells (Table 7.1). Therefore, INSL3 is unlikely to be the substitute for relaxin that activates RXFP1 in the bovine.

7.3.2 Role of bRXFP1 signalling

Though the relaxin gene is absent in the bovine, its cognate receptor bRXFP1 appears to be expressed and to function normally as in other species. In bovine myometrial cells, where endogenous bRXFP1 is expressed, pRLN (50ng/ml – 150ng/ml) induced a 1.5 to 1.7-fold increase in cAMP production. This magnitude of increment is similar to that observed in human myometrial cells (Heng et al. 2008), although the EC50 value for relaxin in activating bRXFP1 (10nM – 50nM) is higher than that in activating hRXFP1 (<1nM) in transfected HEK-293T cells. (Table 7.1). This may explain why administration of a high dose of pRLN in the bovine could elicit a relaxin-dependent physiology, including pelvic canal expansion and cervical relaxation (Musah et al. 1986; Musah et al. 1989). Similarly, the EC50 value for hRLN3 (97.9nM) in activating bRXFP1 is also higher than that in activating hRXFP1 (14.6nM). Although there is no evidence yet to show that RLN3 is expressed in the reproductive system, RXFP1 may be a natural receptor for RLN3 at least in

the brain (Bathgate et al. 2006). Besides these relaxin family peptides, also an unrelated peptide derived from C1q-tumor necrosis factor-related protein 8 (CTRP8) has been found to function as a ligand for RXFP1, stimulating cAMP production in human glioblastoma brain tumour cells (Glogowska et al. 2013; Klonisch et al. 2017). This suggests that there may be other potential ligands in vivo that might activate bRXFP1 to initiate a physiological response.

In previous studies, heterodimerisation between hRXFP1 and hRXFP2 has been observed in vitro (Svendsen et al. 2008). This opens up the possibility that, in myometrial cells and theca cells where both bRXFP1 and bRXFP2 are co-expressed, bRXFP1-bRXFP2 form a heterodimer that can be activated by INSL3 through RXFP2, even though the cognate ligand for RXFP1 is absent in the bovine. Further experiments will be needed to examine this in greater detail.

7.3.3 Splice variants of receptors

The splice variants of bRXFP1 detected in various bovine reproductive cells show evidence of the loss of one or more 5' exons. These are the exons transcribing the extracellular domain, in particular the individual leucine-rich repeats (LRR) that are essential for ligand binding (Kern et al. 2008). Since the missing exons did not lead to any frame-shift or in-frame stop codon, such variants could result in translated proteins with defective ligand-binding and signalling properties, but whose transmembrane and intracellular domains are

perfectly intact. Such transcript variants might act as inhibitors in a receptor dimerization situation (Scott et al. 2005). Again, further research will be necessary to elucidate these details.

7.3.4 Downstream signalling of RXFP1 and RXFP2

The downstream signalling of RXFP1 and RXFP2 is complex. Not only can these receptors activate more than just adenylyl cyclase and the cAMP pathway in various cells, but also the resulting cAMP production seems to be the net effect of both stimulatory and inhibitory G protein-coupled RXFPs. In HEK-293T cells where hRXFP1 or hRXFP2 are expressed, both receptors are found to be coupled with stimulatory G protein ($G\alpha_s$) as well as inhibitory G protein/s ($G\alpha_{oB}$, $G\alpha_{i3}$) (Halls et al. 2006; Halls et al. 2007). If there is different involvement of stimulatory and inhibitory G proteins in various cell types, this could lead to the observation that cAMP production levels can be very different in different cells. In the experiments described here using transfected HEK-293T cells, activation of the expressed bRXFP1 and bRXFP2 lead to a marked increase in cAMP production; in contrast, the observed elevation of cAMP in primary myometrial and theca interna cells is very modest at maximally 2-fold. Moreover, even a decrease of cAMP production has previously been observed in the rat oocyte via RXFP2 (Kawamura et al. 2004). Thus, the detailed mechanisms of RXFP1 and RXFP2 signalling in specific cell types needs to be explored further.

In conclusion, this study has found that bRXFP1 is indeed functionally expressed in the bovine, even though its cognate ligand relaxin appears to have been deleted during ruminant evolution. However, RLN3, as a possible alternative ligand, was identified as able to activate this receptor in vitro, though any physiological role in vivo still needs to be confirmed. Targets for INSL3-RXFP2 signalling were identified in the bovine female reproductive system and imply that INSL3 is mainly acting in an autocrine/paracrine manner within the ovarian follicle involving both theca cells and oocytes, and additionally with possible effects on uterine myometrial cells.

Chapter 8

Discussion

8.1 Potential roles of INSL3

In females, the synthesis of INSL3 appears to be restricted to the reproductive age (see chapter 1.2). In non-pregnant females, both in human and bovine, the circulating INSL3 level increases during the follicular phase and decreases in the luteal phase (Fig 4.2 & 4.3). In the human, the serum INSL3 level is positively correlated with antral follicle count (Fig 4.5), supporting the view that ovarian follicles are the major producer of INSL3 in females. INSL3 is specifically produced by the follicular theca interna layer in both human and bovine (Fig 5.1 & 5.2), and isolated bovine theca interna cells are able to produce and secrete INSL3 peptide (see chapter 5). As the theca interna layer is highly vascularised, the produced INSL3 can not only accumulate in follicular fluid but also be delivered to neighbouring follicles and distant organs in the body via the circulation. The expression of the full-length transcript for the INSL3 receptor RXFP2 in bovine theca interna cells and oocytes (Fig 7.5 & 7.6) and the production of cAMP induced by activation of INSL3-RXFP2 signalling (Fig 7.7 & 7.8) indicate that INSL3 could act as an intra-ovarian factor to regulate follicle development.

First of all, INSL3 can increase the growth of preantral follicles in vitro, which has been demonstrated in a rat model (Xue et al. 2012; Xue et al. 2014). A possible mechanism behind this is that the activation of INSL3 signalling increases cAMP production in the preantral follicle which in turn promotes the synthesis of GDF9 by the oocyte (Xue et al. 2014). GDF9 can further act on

granulosa cells to enhance cell proliferation and so follicle enlargement. Androgen receptor signalling appears to be positively involved in the down-stream process of INSL3-GDF9-mediated preantral follicle growth in the rat (Vitt et al. 2000; Xue et al. 2014). In women with polycystic ovarian syndrome (PCOS), where increasing numbers of follicles are arrested at the antral stage (2-9mm), a low expression level of the GDF9 transcript is observed in oocytes (Wei et al. 2012). This lack of GDF9 may, at least, partly result in the failure of follicle growth. Although I have shown that the serum level of INSL3 increases in women with PCOS (Fig 4.4), no study has ever shown INSL3 production from an individual follicle obtained from PCOS patients. Therefore, at present we cannot determined whether the high level of INSL3 in PCOS serum is simply a result of the increased number of antral follicles or due to an enhanced ability of individual follicles to produce INSL3. Furthermore, experiments should be carried out to examine if the reduced GDF9 production is due to the reduced INSL3-RXFP2 signalling or impaired sensitivity to INSL3 in antral follicles from women with PCOS.

Secondly, INSL3 could protect follicles from atresia and so support the development of the dominant follicle. There is a decrease in INSL3 level in atretic follicles in the bovine, and an increase in follicular atresia is observed in INSL3 knock-out mice (Spanel-Borowski et al. 2001; Irving-Rodgers et al. 2002). Moreover, BMP-2, which is produced by granulosa cells, and BMP-6, which is generated by granulosa cells and oocytes, have been shown to

suppress the production of INSL3 by bovine theca interna cells (Glister et al. 2004; Glister et al. 2010; Glister et al. 2013). Both BMP-2 and BMP-6 have a higher expression level in atretic follicles than in dominant follicles in the rat (Erickson and Shimasaki 2003). This possibly contributes to the low level of INSL3 in atretic follicles and the higher level in the dominant follicle. The detailed mechanisms by which INSL3 might protect the dominant follicle from atresia need to be further explored. Furthermore, in bovine theca interna cells, INSL3-RXFP2 signalling causes an increase in the production of androstenedione, which is the precursor for estradiol (Glister et al. 2013). Thus, a high level of INSL3 in the dominant follicle should contribute to the increased production of estradiol in this follicle.

Thirdly, INSL3 is involved in steroidogenesis in ovarian follicles. The first detection of pro-INSL3 is coincident with the expression of steroidogenic enzymes CYP11A1 and 3ß-HSD in the theca interna layer of very small antral follicles in the bovine (Irving-Rodgers et al. 2002). This is the stage at which theca cells acquire the LH receptor (LHR) in bovine follicles (McNatty et al. 1984). Thus when LH signalling from the hypothalamus-pituitary-ovary axis starts to act on theca interna cells to promote steroid production via the LHR, INSL3 signalling also commences since RXFP2 transcripts are detectable in these cells at small antral stage (Satchell et al. 2013). The effect of INSL3-RXFP2 signalling to regulate androstenedione production, which has previously been observed in medium-sized antral follicular theca interna cells

(Glister et al. 2013), most probably persists throughout the antral stage. In return, androstenedione could increase the production of INSL3, forming a positive feedback regulating mechanism in theca interna cells (Fig 5.11B, 5.14 & 6.4). Since androstenedione is the precursor for making estradiol by follicular granulosa cells, INSL3-RXFP2 also indirectly controls estradiol production. A high level of estradiol feeds back positively to the anterior pituitary, triggering the LH surge (Beshay and Carr 2013), which in turn inhibits INSL3 production (Fig 5.6). Moreover, a high level of estradiol has a weaker stimulatory effect in producing INSL3 (Fig 5.12). Together, this regulation could result in a reduced level of INSL3 around ovulation, which in turn impairs the production of estradiol (together with reduced aromatase expression) and so LH levels return to baseline. Therefore, INSL3 signalling facilitates the hypothalamus-pituitary-ovary axis to regulate steroid production in both theca and granulosa cells, at least, during the follicular phase and up to ovulation. These pre-ovulatory events need to be further explored in Graafian follicles before the LH surge.

Fourthly, INSL3 may influence oocyte physiology in dominant follicles. In the rat, INSL3-RXFP2 signalling appears to reduce cAMP production in the oocyte in vitro, and additional INSL3 appears to increase germinal vesicle breakdown (GVB) in both isolated cumulus-enclosed oocytes and preovulatory follicles, with the maximal effects achieved at 10nM - 100nM of INSL3 (Kawamura et al. 2004). The follicular fluid concentration of INSL3 in medium- and large-sized

bovine antral follicles is about 60μM and 20μM, respectively, though the concentration in preovulatory follicles has not yet been determined (Dai et al. 2017). This concentration range substantially exceeds the effective doses of INSL3 for the oocyte, and would imply that the oocyte RXFP2 is either permanently occupied by INSL3, or permanently desensitised, or both, during the antral stage. This tallies with the observation that INSL3 induction of oocyte maturation is restricted to rat preovulatory follicles but not antral follicles isolated before priming with pregnant mare serum gonadotropin (which has LH like activity) (Kawamura et al. 2004). The concentration of INSL3 in follicular fluid from preovulatory follicles needs to be measured to determine whether it might decrease to within an effective range to activate the oocyte RXFP2, thereby to reduce cAMP production and encourage oocyte maturation. At the same time, INSL3-RXFP2 signalling in theca interna cells stimulates cAMP production (Fig 7.7), some of which may be secreted and could reach the oocyte. As described previously, the concentration of INSL3 in follicular fluid appears to decrease as the follicle develops (Dai et al. 2017). Moreover, towards ovulation, the LH surge suppresses INSL3 production (Fig 5.6), and decreases gap junction permeability (Norris et al. 2008). Together, such factors could contribute to the low level of intra-oocyte cAMP, which is essential for initiating resumption of meiosis and so GVB (Tsafriri and Pomerantz 1986).

Besides the ovary, INSL3 seems also to target the uterus. Apparently,

full-length RXFP2 transcripts are detectable in myometrial cells in the bovine, macaque, and human (Fig 7.5) (Heng et al. 2008; Hanna et al. 2010). Previous studies have shown there to be possible local production of INSL3 in the marmoset uterus as well as in the human endometrium, with the expression level increased in the proliferative phase as compare to the secretory phase (Hombach-Klonisch et al. 2001; Ivell and Bathgate 2002). This cyclical change of expression of INSL3 in the uterus is consistent with the change of circulating INSL3 level in serum through the reproductive cycle (Fig 4.2 & 4.3). Irrespective of whether INSL3 is produced locally within the uterus or by the ovaries, it could activate RXFP2 in the uterus to produce cAMP, at least in the myometrium (Fig 7.7). Together with relaxin-RXFP1 signalling, where RXFP1 is also detectable in both endometrium and myometrial cells (Fig 7.2), both systems may contribute to uterine quiescence, as both can increase cAMP production which is essential for maintaining uterine relaxation (Fig 7.7) (Price and Bernal 2001; Heng et al. 2008). This may facilitate a successful implantation during the secretory phase. More experiments are required to understand the exact roles of the INSL3-RXFP2 system in the uterus in the presence and absence of pregnancy. Besides this potentially direct role, INSL3-RXFP2 signalling may also be indirectly responsible for endometrial development by regulating the production of steroids by the ovary, as discussed above.

The direct effects of INSL3 in the female reproductive system are summarised

Table 8.1. This table summarises the observed direct effects of INSL3 in the female reproductive system from different species. These results are collected from the published studies and the results from current project (Spanel-Borowski et al. 2001; Kawamura et al. 2004; Heng et al. 2008; Glister et al. 2013; Xue et al. 2014). A4: androstenedione; HPO: hypothalamus-pituitary-ovary; LH: luteinising hormone; P4: progesterone; E2: estradiol; BMP: bone morphogenetic proteins; +: positive effect; - : negative effect.

	RXFP2	Direct Effects of INSL3		
Theca interna cells	Yes	Steroidogenesis	+ A4 +CYP17A1	+ cAMP
Granulosa cells	No			
Oocytes	Yes	Germinal vesicle breakdown		- cAMP
Follicles	Yes	Growth Anti-atresia	+ Testosterone	+ GDF9
Myometrial cells	Yes			+ cAMP

in Table 8.1. In addition to the reproductive system, INSL3 appears to have effects in other organs as well, including bone, brain and kidney (summarised by Bathgate et al. 2013). In the human, INSL3-RXFP2 signalling appears to regulate bone metabolism. As shown by Ferlin et al. (2009), dysfunction of RXFP2 in both mice and human is associated with osteopaenia, and additional INSL3 increases the proliferation of osteoblasts and stimulates mineralisation. A detailed PCR study further showed INSL3-RXFP2 signalling most probably acts through a MAPK cascade to induces the expression of key osteoblast genes involved in osteoblast differentiation, matrix deposition and osteoclastogenesis (Ferlin et al. 2011). Consequently, the absence of INSL3 may theoretically contribute to the increased prevalence of osteoporosis in postmenopausal women (Anand-Ivell and Ivell 2014a).

In the rat brain, the INSL3-RXFP2 system appears to alter the activity of the parafascicular nucleus that may be involved in sensorimotor, limbic and cognitive functions (Shen et al. 2005; Sedaghat et al. 2008). In the rat kidney, this signalling regulates mesangial cell proliferation that may be involved in maintaining the structure and function of the glomerular capillary ultrafiltration apparatus (Fu et al. 2005).

During pregnancy, at least in ruminants, INSL3 of male fetal origin is able to cross the placenta and can be measured in maternal blood (Anand-Ivell et al. 2011). Whilst this has not yet been shown for the human, the high levels of male fetal INSL3 detected in amniotic fluid does suggest that INSL3 may

influence maternal and/or placental physiology and might be linked to the onset of pre-eclampsia (Anand-Ivell et al. 2008).

In conclusion, there is strong evidence to suggest that INSL3-RXFP2 signalling may have a number of systemic effects in the body. In regard to the roles within the female reproductive system, INSL3-RXFP2 signalling supports follicle development, possibly protects follicles from atresia, and helps to prepare the oocyte for maturation and ovulation. At the same time, the intra-ovarian INSL3-RXFP2 system facilitates LH signalling from the hypothalamus-pituitary-ovary axis to regulate steroidogenesis in ovarian follicles. These ovary-produced steroids can further impact on the uterus to promote endometrial development and differentiation. Therefore, INSL3-RXFP2 signalling directly and/or indirectly prepares the uterus for the potential implantation of an embryo. Additionally, when pregnancy occurs, INSL3 produced by the mid-gestational male fetus may cross the placenta and potentially contribute to the maternal circulating INSL3, and thus possibly influence the physiology of other target organs. Taken together, INSL3-RXFP2 signalling appears to play an important role in female physiology. Understanding the mechanisms and effects of INSL3-RXFP2 signalling could contribute to maintaining the health of women, particularly following the menopause, when all sources of INSL3 are absent. More research is required to assess this aspect in greater detail.

8.2 Possible mechanisms of sex steroid action

In chapters 5 and 6, I have shown that the expression of INSL3 in theca interna cells is controlled by intra-ovarian sex steroid hormones (Fig 5.8 – Fig 5.12, Fig 6.3 & Fig 6.4). This does not only depend on the concentration of steroid (Fig 5.12) but also the expression pattern of different types and subtypes of steroid receptors (Fig 6.3 & Fig 6.4). Moreover, some steroids rather than acting in the nucleus to regulate transcriptional activities, also appear to interact with cytoplasmic second messenger pathways (Fig 5.13). Together, such observations suggest that steroid hormones in ovarian follicles may be acting in a non-classical manner to regulate the expression of INSL3.

In the classical pathway, the steroid receptors are activated by their cognate ligands in the cytoplasm then move to the nucleus to interact with their respective steroid response elements in the promoter region of a target gene, thereby eliciting transcriptional activation (Fig 8.1 A). However, such steroid response elements are absent from the INSL3 gene promoter region (Chapter 6), suggesting that non-classical mechanisms may mediate the action of steroids in theca interna cells to regulate INSL3 gene expression. Based on previous studies, possible non-classical pathways are summarised in Fig 8.1 B & C, where a direct interaction between the activated receptor and the gene promoter is absent. Rather, it seems likely that certain transcription factor/s form a complex with the steroid-activated receptor monomer, and that these other transcription factors can then physically interact with the DNA and so

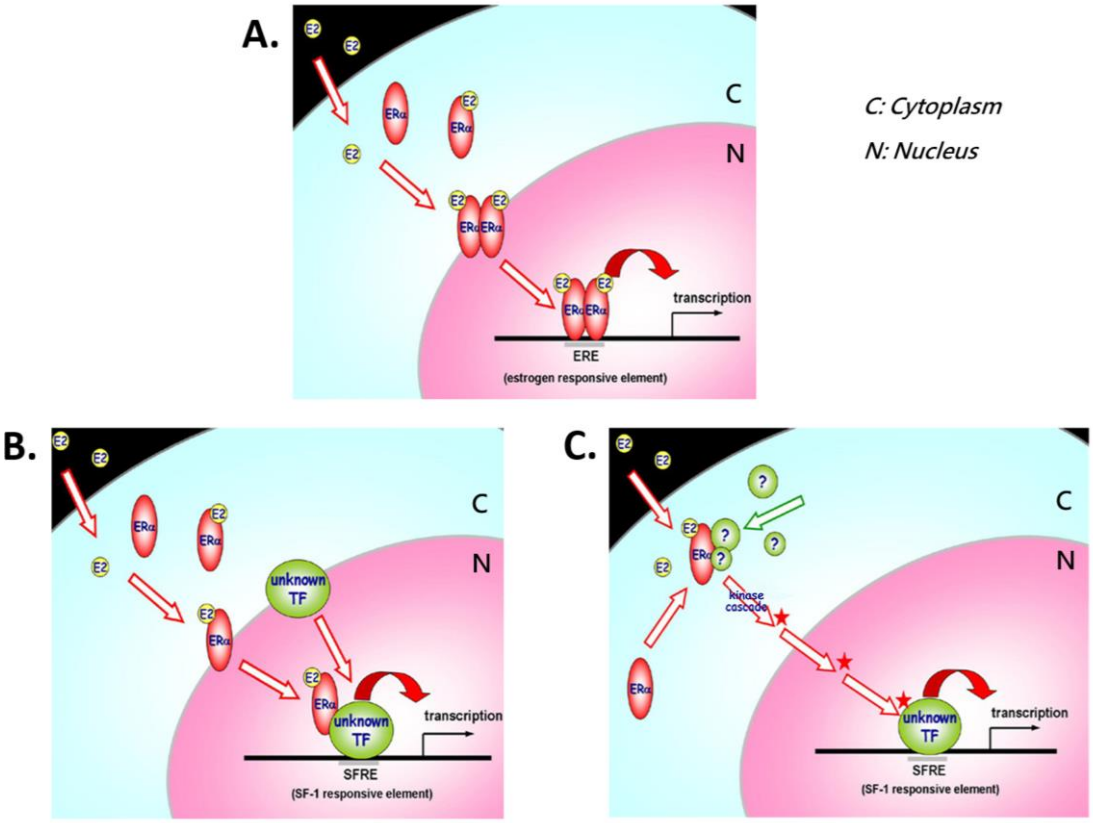


Fig 8.1. Possible ways of action for ligand-activated estrogen receptors (ER). (A) The classical model in which there is direct binding of estradiol (E2)-activated ERα homodimer to a canonical palindromic estrogen response element (ERE). (B) The tethered receptor model: a non-classical model in which a ligand-activated ERα does not bind itself to the DNA, but interacts indirectly with a promoter via an unknown intermediate transcription factor. (C) An indirect non-classical model involving a ligand-activated ERα forming part of a larger protein complex, possibly in the cytoplasm. This protein complex leads to activation of a specific kinase cascade which in turn targets other transcription factors, triggering a specific transcriptional response. In both (B) and (C), an SF-1 response element (SFRE) is used as an example to illustrate a possible targeting site in a gene promoter that mediates the action of non-classical steroid signalling.

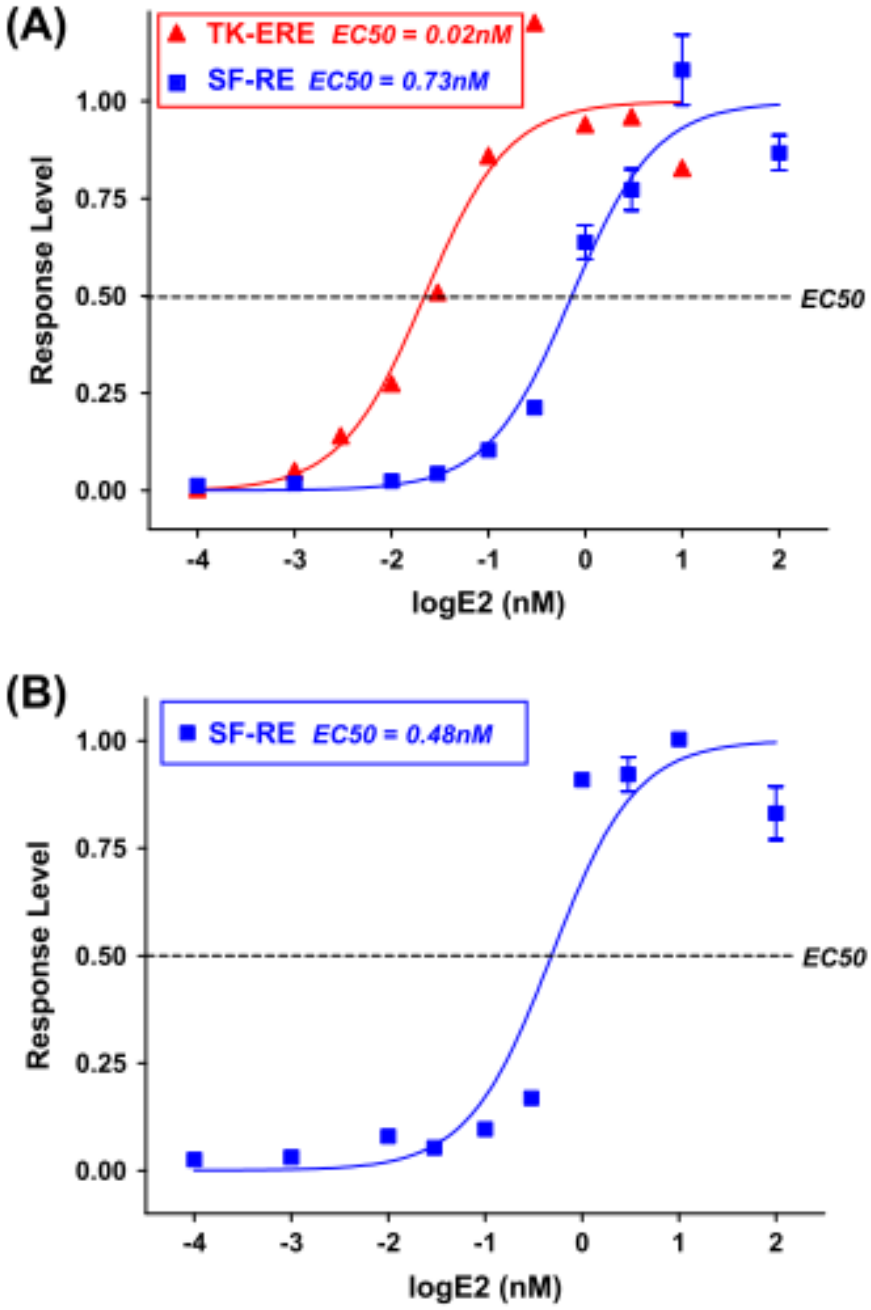


Fig 8.2. Dose-response relationship for E2-activated estrogen receptor alpha (ERα) via classical (TK-ERE) and non-classical (SF-RE) pathways. In HT1080 cells, where ERα is not expressed endogenously, the expression plasmids of wild type human ERα (A) and mutated human ERα (B), in which the DNA-binding domain is disrupted, were transiently transfected and expressed. The receptors were activated by increasing doses of E2 which in turn stimulated the promoter-luciferase reporter plasmids. The palindromic estrogen response element (ERE)-dependent thymidine kinase promoter (TK-ERE) represents the classical system (A), and an SF-1 response element (SFRE)-dependent promoter (SF-RE) here for the oxytocin gene, represents the non-classical steroid signalling system (A) & (B) (Ivell et al. 2014).

initiate transcription (Fig 8.1 B). This mechanism is also referred to as the 'tethered receptor' model. Another 'indirect' model shows activation of a steroid receptor occurring within the cytoplasm, and this in turn interacts with and activates other cytoplasmic components, leading to the stimulation of a kinase cascade whose end-point includes specific gene activation in the nucleus (Fig. 8.1 C). In the INSL3 gene promoter, a classic estrogen response element (ERE) is absent, and a mutagenesis study indicated that the common binding sites for both Nur77 and SF-1 (SFRE) are essential for mediating estradiol (E2)-regulated transactivation in mouse MA-10 Leydig cells (Lague and Tremblay 2009). This response element SFRE is also found in the promoters of other E2-ERα dependent genes, which similarly implicate an SFRE in mediating E2-ERα stimulated gene transactivation. Such genes include the oxytocin, AMH, osteopontin, lactoferrin, and LHβ genes (Vanacker et al. 1999; Teng et al. 2002; Koohi et al. 2005; Luo et al. 2005; Grynberg et al. 2012). In preliminary studies, I have examined the regulation of the promoter of the oxytocin gene by E2 (Ivell et al. 2014). The results showed that both normal ERα, as well as a mutated ERα, whose DNA binding domain had been disrupted, are able to mediate the activation of the oxytocin gene promoter with similar EC50s for E2 (Fig 8.2). This indicates that a physical interaction between the DNA of the gene promoter and ERα is not necessary for E2-dependent activation of the oxytocin gene promoter via an SFRE. Thus, an SFRE could be a possible platform to direct non-classical steroid signalling in

the regulation of transcriptional activity for a target gene.

In regard to the ‘tethered receptor’ model, several transcription factors appear to be able to interact with an SFRE and form a complex with steroid receptors. For example, the transcription factor SF-1 can bind testosterone-activated androgen receptor (AR) in regulating the transcriptional activity of the LHβ gene promoter (Jorgensen and Nilson 2001), COUP-TFI can form a complex with E2-activated ERα to enhance that receptor’s activity (Metivier et al. 2002), and DAX-1 can physically interact with ERα or ERβ to suppress ER signalling (Zhang et al. 2000). In my results (Fig 6.2), it seems that SF-1 (3×) is indeed able to integrate with an E2-activated endogenous receptor to regulate the transactivation of the INSL3 gene promoter, although a direct physical interaction has not yet been examined. There are also other response elements mediating non-classical signalling. For example, DHT-activated AR can compete with a specific coactivator to physically interact with Nur77 and so inhibit the transcriptional activities of steroidogenic enzymes induced by Nur77 in mouse Leydig cells (Song et al. 2012).

The third so-called ‘indirect’ model involves the interaction between an activated steroid receptor and certain kinase-dependent cascades. Here I showed in chapter 5.2.6.2 that E2-dependent signalling integrates with cAMP-PKA signalling to regulate INSL3 peptide production in theca interna cells (Fig 5.13), though the down-stream effector that acts on the gene promoter still needs to be identified. Another example shows that the

transcriptional activity of the oxytocin gene promoter activated by E2-ERα signalling can be inhibited by the MAP kinase kinase inhibitor PD98059 in MDA-MB 231 cells (Koohi et al. 2005). As summarised by Maruyama and Yoshimura (2008), both progesterone receptors (PR) PR-A and PR-B can interact with a variety of cytoplasmic kinases and molecules that are involved in cAMP-dependent and other signalling during endometrial decidualization.

The classical and non-classical models of steroid action appear to require different concentrations of steroids to regulate their endpoints. As shown in Fig 8.2 A, the thymidine kinase gene promoter, containing a single estrogen response element (TK-ERE), represents the classical model. It can be activated by ERα-mediated estradiol in concentrations ranging from 1pM to about 1nM (Fig 8.2 A) with an EC50 of approximately 0.02nM. In contrast the oxytocin gene promoter, containing a single SFRE (SF-RE) and representing the non-classical model requires almost two orders of magnitude more estradiol to stimulate transcriptional activity via ERα (Fig 8.2 A; EC50 0.5-0.7nM). A similar difference in the functional range of steroid concentration can be observed also when comparing effects in steroid-synthesising tissues, such as the ovary, with effects in distant, peripheral tissues and cells. For example, the range for estradiol to increase INSL3 gene promoter activity in follicular theca interna cells or to increase AMH gene promoter activity in mouse luteinised granulosa cells, both via ERα, is from 10pM – 1μM (Grynberg et al. 2012) (Fig 6.3 A). In contrast, in endometrial cells, estradiol

increases PR synthesis with a maximal effect achieved at only about 1nM (Chen and Jow 1989). Clearly, there are different mechanisms mediating estradiol action in different cell systems, with the non-classical mechanisms evident in steroid source tissues having higher EC50s.

Moreover, in the ovary, not only estradiol, but also progesterone, with a role in regulating primordial follicle assembly, or testosterone, promoting the transition from primary to secondary follicle, appear to utilise up to micromolar concentrations of the steroid (Kezele and Skinner 2003; Yang and Fortune 2006). The high levels of steroids required for these physiological activities in the ovary match the concentrations of steroids available in follicular fluid. The highest level for steroids in follicular fluid can reach about 4μM for estradiol, about 1μM for androstenedione, and about 700nM for testosterone (Dieleman et al. 1983; Fortune and Hansel 1985). In comparison, the circulating levels of steroids in serum are generally only in the picomolar to low nanomolar range (Appendix Fig 9.1 and Fig 9.2). Therefore, it seems likely that the non-classical models of action will be commonly used by steroids in their source organs (i.e. ovary, testis and adrenal gland), where steroids can be synthesised and accumulated at locally high concentrations.

As a corollary to this argument, these classical and non-classical modes of steroid action also suggest possible mechanisms to explain the tissue-specific effects of selective estrogen receptor modulators (SERM), selective androgen receptor modulators (SARM) and selective progesterone receptor modulators

(SPRM). It has been shown for the non-classical estrogen-activated regulation of the oxytocin gene that SERMs such as tamoxifen which are full antagonists in the classical system, act as agonists when mediating their effect by the non-classical pathway (Koohi et al. 2005).

Moreover, previous studies assessing global gene activation by specific steroids suggest that not all the steroid-dependent genes have a canonical steroid response element within their upstream regulatory region. A recent study using a global ChIP-Seq approach showed that only about 40% of ERα-dependent genes in the uterus have a conventional ERE within a reasonable distance of their promoter regions (Hewitt et al. 2012), implying that non-classical mechanisms, which utilise response elements for other transcription factors in the gene promoters, could be a common mode of regulation for many steroid-controlled genes.

Besides these nuclear steroid receptor mediated actions, previous studies have shown there to be also membrane-linked steroid receptors, such as membrane-bound ERα and ERß, and G protein-coupled receptor GPR30, that can also transduce steroid signalling (summarised by Vinas et al. 2012). Rather than regulating the transcriptional activity of a target gene, these membrane receptors are most probably responsible for the rapid events that occur within seconds to minutes, such as ion fluxes, prolactin release and nitric oxide release, via kinase-dependent cascades, including MAPK and PI3K (summarised by Vinas et al. 2012). It remains to be assessed whether

such membrane steroid receptors are also expressed and activated in theca interna cells, and if their signalling is directly and/or indirectly involved in regulating INSL3 production and/or other endpoints.

In further studies, we not only need to understand which mechanism is utilised by individual steroids, but also need to examine if different mechanisms co-exist and how they might cooperate with each other to achieve the final effect. For example, for the observed non-monotonic dose response of estradiol in regulating INSL3 production in theca interna cells (Fig 5.12), could this be due to different expression levels of the stimulatory ERα and inhibitory ERß at various stages of folliculogenesis (Fig 6.3)? E2-ER signalling integrates with cAMP-PKA signalling (Fig 5.13); are these systems mutually obligatory? As the concentration of steroids in the ovary is much higher than that in the periphery, will the regulatory mechanisms for INSL3 in theca interna cells be different from that in, for example, endometrial cells? Again, further experiments will be required to elucidate these questions.

Appendix

The supporting results shown in this section are from the following publications, and these results were contributed by the other co-authors. They were used for supporting and discussing the results presented in different chapters from the current thesis.

Anand-Ivell R, Tremellen K, **Dai Y**, Heng K, Yoshida M, Knight P, Hale G, Ivell R. 2013. Circulating insulin-like factor 3 (INSL3) in healthy and infertile women. *Human Reproduction* 28:3093-3102.

Glister C, Satchell L, Bathgate R, Wade JD, **Dai Y**, Ivell R, Anand-Ivell R, Rodgers R, Knight P. 2013. A functional link between bone morphogenetic protein and insulin-like peptide 3 signalling in modulating ovarian androgen production. *Proceedings of National Academy of Science USA* 110 (15): E1426–E1435.

Satchell L, Glister C, Bleach E, Glencross R, Bicknell A, **Dai Y**, Anand-Ivell R, Ivell R, Knight P. 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.

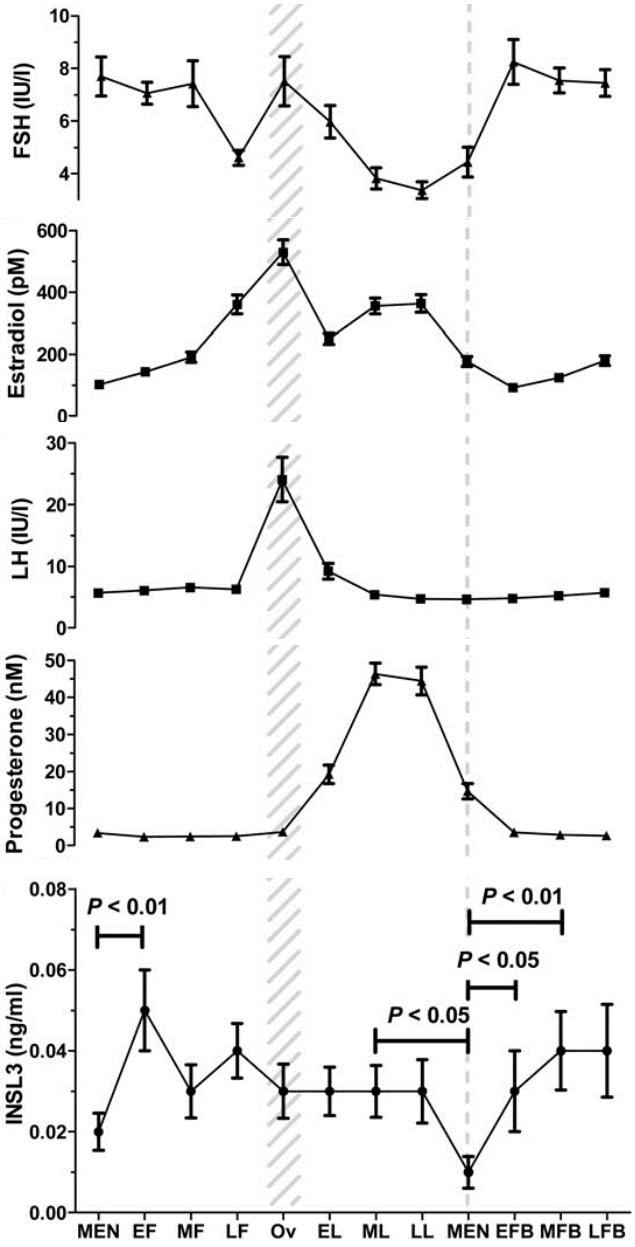


Figure 9.1. Averaged hormone profiles. Serum levels (Means+SEM) of estradiol, LH, progesterone and INSL3 from 18 healthy young women of reproductive age were measured throughout the menstrual cycle. MEN, menses; EF, early follicular phase; MF, mid follicular phase; LF, late follicular phase; Ov, ovulation; EL, early luteal phase; ML, mid luteal phase; LL, late luteal phase; EFB, MFB and LFB represent the corresponding early, mid and late follicular phases of the following cycle. The cross-hatched vertical bar indicates ovulation, and the dashed line indicates the second menses. In regards to the INSL3 concentrations, when samples from both menses are pooled, all luteal phase samples are significantly higher than those at menses (not shown) (Anand-Ivell et al. 2013).

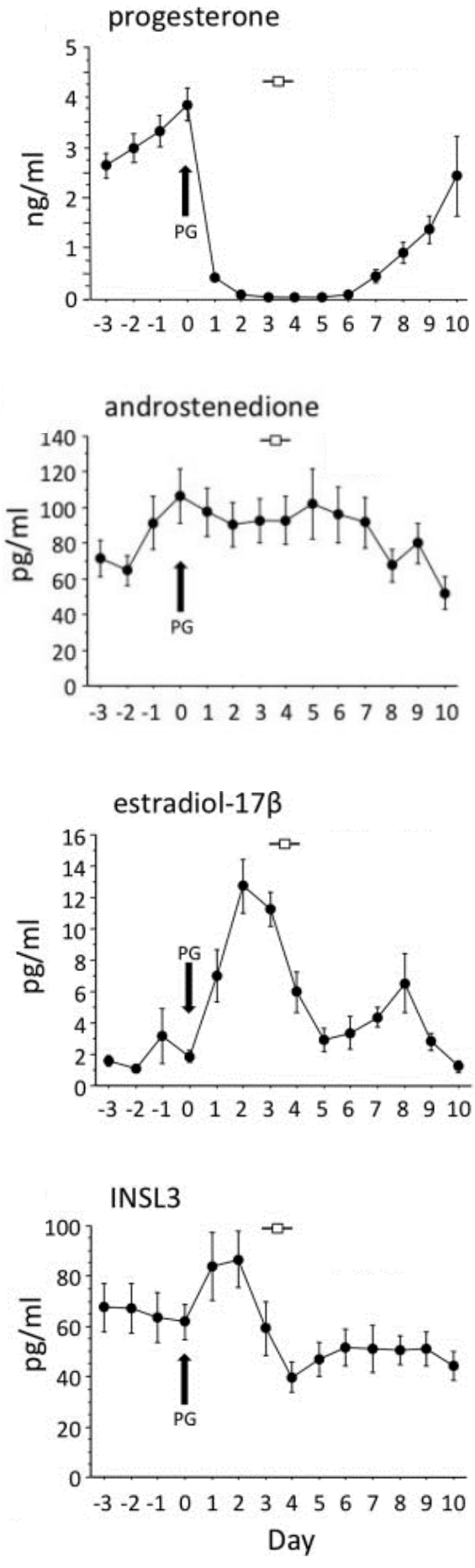

progesterone
ng/ml
PG
androstenedione
pg/ml
PG
estradiol-17β
pg/ml
PG
INSL3
pg/ml
PG
-3 -2 -1 0 1 2 3 4 5 6 7 8 9 10
Day

Figure 9.2 Hormone profiles across truncated estrous cycles in bovine. Mean (± SEM) plasma concentrations of progesterone, androstenedione, estradiol and INSL3 across 15 synchronised cycles (3 truncated cycles in 5 heifers). PGF2α analogue cloprostenol (PG) was injected to induce luteolysis on day 0, indicated as the arrow. The mean (± SEM) time of the LH surge (3.5 ± 0.6) is indicated by open square symbol (Satchell et al. 2013).

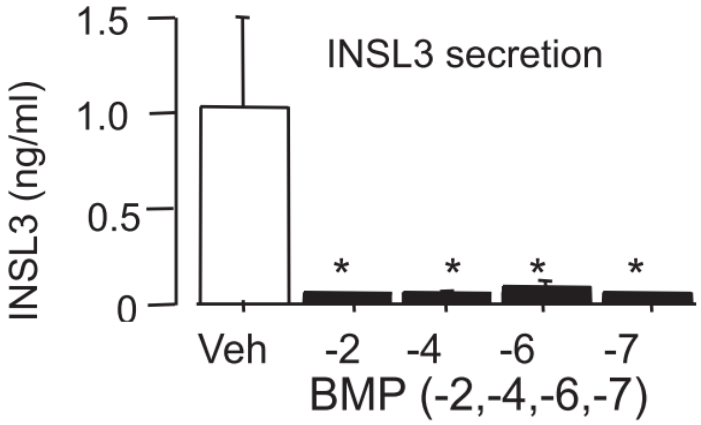


Figure 9.3. BMPs inhibit INSL3 at both peptide and mRNA levels. The primary bovine TICs were treated with 10ng/ml of BMP2, -4, -6, and -7. The secreted INSL3 peptide (Mean ± SEM) were inhibited in all cases. *: $P < 0.05$ vs vehicle control (Glister et al. 2013).

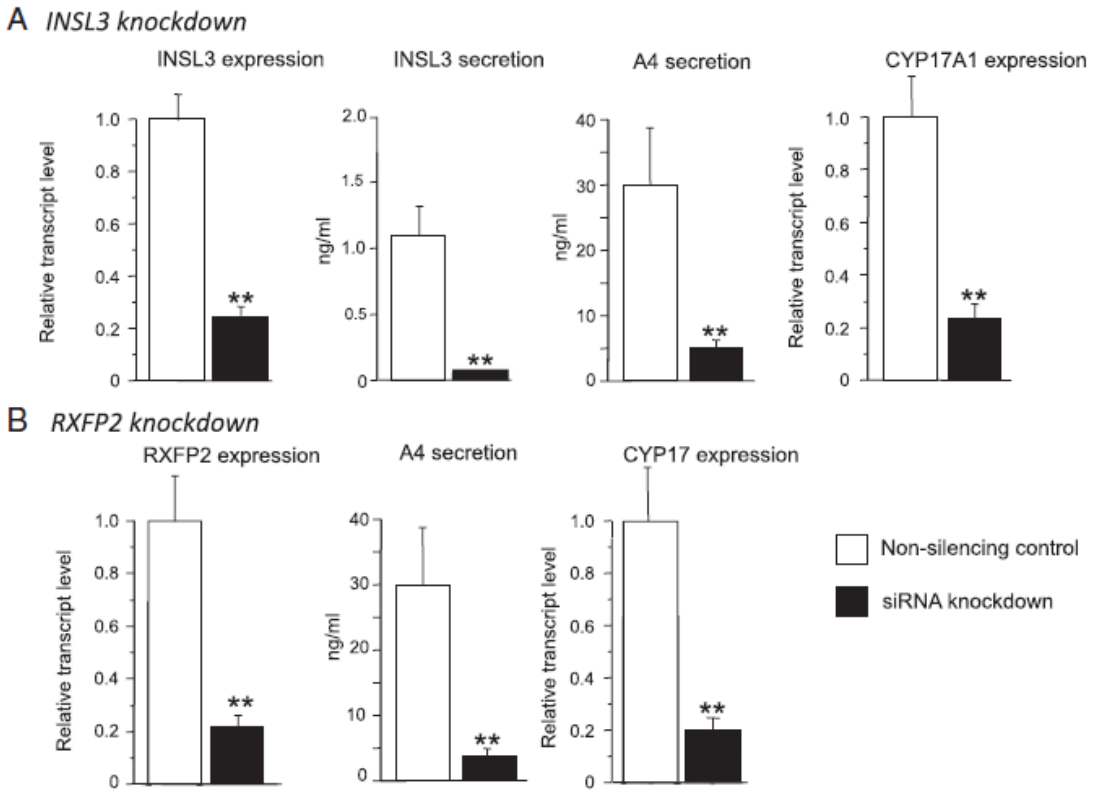


Figure 9.4. INSL3-RXFP2 signalling controls androstenedione production. INSL3 (A) or RXFP2 (B) were knocked-down in primary theca interna cells with siRNA. INSL3 mRNA and protein levels (A) and RXFP2 mRNA levels (B) were measured to ensure successful knock-downs. In both cases, A4 levels were dramatically reduced by about 80%, and the expression of the enzyme that converts progesterone to androgens were also reduced (A & B). Values are Mean ± SEM. **: $P < 0.01$ vs control (Glister et al. 2013).

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