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The Mechanisms Underlying Uterine Receptivity in Pigs and Women

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ABSTRACT

Successful implantation and pregnancy require synchronisation between a fully receptive endometrium and a well-developed embryo. In assisted reproductive technologies (ART), the embryo is created in artificial conditions while endometrial receptivity is affected by ovarian stimulation treatment. Therefore, one or both of these factors may be major contributors to embryo loss. Many supplementation regimes have been utilised to optimise this synchronisation and to provide luteal phase support in ART cycles.

In this study, the use of progestogen and oestradiol supplementations in women undergoing ART was assessed using a systematic review and meta-analysis approach. In addition, porcine models were used to evaluate the communication between embryo and endometrium during the peri-implantation period.

Meta-analysis studies were performed for women undergo ART cycles with fresh or frozen embryo transfer and in intrauterine insemination (IUI). All available trials that compared the effects of progestogen treatments alone or with oestradiol were included and all the studies did not meet the eligibility characteristics were excluded. In fresh embryo transfer, 211 studies with 29,357 women undergoing IVF/ICSI cycles were analysed. The use of progestogen supplementation improved the clinical pregnancy rate versus no supplementation ($p<0.001$ from 18% to 35% in treated cycles).

Starting progestogen supplementation 1-1.5 days after oocyte retrieval was more beneficial ($p<0.001$, 41%) than any other start time. Using the intramuscular route was significantly more beneficial ($p<0.001$, 43.8%) to clinical pregnancy rates than the vaginal route (35.8%). This meta-analysis study also showed that adding oestrogen to progestogen was beneficial ($p<0.01$) for clinical pregnancy rate. In frozen embryo transfer, 30 studies (5146 women)

were used to assess clinical pregnancy rate after progestogen supplementation. Regardless of the method used for endometrium preparation, progestogen supplementation as luteal phase support was beneficial ($p<0.01$) for clinical pregnancy outcome. In intrauterine insemination (IUI) cycles, there were 14 studies (1555 women) included in the analysis, which illustrated that using progestogens increased ($p<0.01$) clinical pregnancy rate after using gonadotrophins for ovarian simulation.

The first porcine study investigated the relationship between embryo development and number, luteal function and oestradiol concentration in uterine flushes on days 3-5, 11-12 and 13-14 of pregnancy (47 pigs in total). The number and weight of corpora lutea were similar in the animals that had a low (≤9) or high (>9) number of embryos. The total luteal progesterone content on pregnancy day 3-5 was significantly higher ($p<0.05$) in animals with a high number of embryos compared with those with a low number of embryos, while there were no differences on pregnancy days 11-12 and 13-14. Moreover, the result demonstrated that on pregnancy day 11-12, higher oestradiol levels in uterine flushes were significantly associated ($p<0.05$) with the presence of embryos at the filamentous stage. However, there was no association between the number of embryos and the uterine flush level of oestradiol on days 3-5 and 11-12 of pregnancy.

Immunohistochemistry demonstrated that the preimplantation disappearance of progesterone receptors (PGR) in luminal epithelial cells was associated with an increased expression of integrin αvβ1 (ITGA5/B1) in pregnant sows on days 11-12 and 13-14 of pregnancy. In addition, stanniocalcin 1 (STC1) protein was localised to the luminal epithelial and stroma cells of the endometrium.

On day 11-12 of pregnancy (n=6), there was no significant association between the endometrial progesterone content (high versus low) and STC1, osteopontin (OPN) or

mucin 1 (MUC1) expression in the endometrium. Explant culture was used to study the effects of progesterone and oestradiol treatment on STC1, OPN and MUC1 expression in endometrium from pregnant sows (n=8) on days 3-5, 11-12 and 13-14. To assess the regulation of STC1, OPN and MUC1 by steroid hormones, an endometrial explant tissue culture system was developed in which tissue structure, cell viability and PGR expression were maintained over several days without inducing high levels of apoptotic cell death.

Endometrial explant tissues were treated with 1) progesterone (0, 10 and 100 ng/ml) or 2) oestradiol (0, 0.1 and 1 ng/ml) in the absence or presence of 10 ng/ml progesterone in a 3 × 2 factorial design. Tissue was collected after 0, 48 and 96h in culture to determine the protein expression of STC1, OPN and MUC1 by Western blot. There was no evidence to support the hypothesis that STC1 expression was influenced by progesterone or oestradiol treatment. OPN protein was expressed at all experimental times, while MUC1 was only expressed at the start of culture.

A novel culture system was established to investigate angiogenesis in the porcine endometrium (non-pregnant sows in mid-luteal phase, n=4). This was utilised to examine the effect of STC1 (0, 5 and 50 ng/ml), prostaglandin F2α (PGF2α; 0, 10 and 100 ng/ml), prostaglandin E2 (PGE2: 0, 10 and 100 ng/ml) and uterine flushes from pregnant sows at day 11-12 and 13-14 (0, 20 and 200 µg/ml total protein) on endometrial endothelial cell (EC) network formation. These were investigated in basal conditions or with the addition of pro-angiogenic factors (VEGFA plus FGF2). STC1 increased the formation of EC networks significantly ($p<0.001$) and PGE2 had a positive effect on EC network formation in basal conditions ($p<0.05$). While PGF2α did not show any significant impact. In contrast to expectations, uterine flushes from both pregnancy days had a negative effect on EC

formation with 200µg/ml total protein blocking all EC formation in basal and angiogenic factors conditions.

In summary, progesterone supplementation has a fundamental positive impact on pregnancy outcome in women undergoing ART. More importantly, revised optimal time of starting supplementation (the day after oocyte retrieval) and route of progesterone (intramuscular) were identified. The communication between the endometrium and conceptus tissue is fundamental for successful pregnancy. The appropriate timing of uterine receptivity is dependent on the synchronised secretions from the ovary and embryo. These secretions are likely to regulate endometrial angiogenesis and thereby influence the likelihood of attachment and implantation occurring. Indeed, this study demonstrated that STC1 promoted angiogenesis to a greater degree than VEGFA/FGF2 and might be a therapeutic strategy by which endometrial receptivity could be improved.

DECLARATION

All the work in this thesis was completed by the author, except where acknowledged in the text. These studies were carried out under the supervision of Dr Bob Robinson and Dr Katie Woad, School of Veterinary Medicine and Science, and Dr George Mann, School of Biosciences, University of Nottingham.

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LIST OF CONFERENCE PRESENTATIONS

1) Amal Mohammed, Robert S. Robinson, Katie J. Woad & George E. Mann. Importance of progesterone supplementation timing on pregnancy outcome following assisted reproduction. Poster presentation, Fertility 2015. 7-9th January 2015 ICC Birmingham, UK.
2) Amal Mohammed, Kathryn Woad, George Mann and Robert Robinson. Efficacy of progestogen supplementation in women undergoing assisted reproductive technology treatment: a meta-analysis. Oral presentation, SRF Annual Conference 2016, 11-13th July 2016. University of Winchester, UK.
3) Amal Mohammed, Kathryn J Woad, George E Mann and Robert S Robinson. Establishment of a novel culture system to investigate angiogenesis and gland formation in the porcine endometrium. Poster presentation, Fertility 2017, 5-7th January 2017, EICC Edinburgh, UK.
4) Mohammed AA, Woad KJ, Mann GE and Robinson RS. The regulation and effect of stanniocalcin 1 in the porcine endometrium during the establishment of pregnancy. Poster presentation, Fourth World Congress of Reproductive Biology (WCRB2017), 27-29th September 2017 Okinawa Convention Centre Okinawa, Japan.

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LIST OF ABBREVIATION

Abbreviation	Full name
µg	Microgram
µl	Microlitre
3β-HSD	3β-hydroxysteroid dehydrogenase
ACTB	Actin Beta
AF-1	Activation function 1
AF-2	Activation function 2
ANOVA	Analysis of variance
ART	Assisted reproductive technology
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
CC	Clomiphene citrate
cDNA	Complementary DNA
CG	Chorionic gonadotrophin
CK	Cytokeratin
CL	Corpus luteum
cm	Centimetre
COX-2	Prostaglandin endoperoxide synthase
CVEC	Coronary venular endothelial cells
DAB	3, 3´-diaminobenzidine tetrahydrochloride
DBD	DNA-binding domain
DGE	Endometrial deep gland
DNA	Deoxyribonucleic acid
E2	Oestradiol
EC	Endothelial cell
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGM	Endothelial cell growth media
ESR	Oestrogen receptor
ESR1	Oestrogen receptor alpha
ESR2	Oestrogen receptor beta
ET	Embryo transfer

FBS	Fetal bovine serum
FET	Frozen-thawed embryo transfer
FGF	Fibroblast growth factor
FGF2	Fibroblast growth factor 2
FGF7	Fibroblast growth factor 7
FSH	Follicle stimulating hormone
g	Gram
GE	Glandular epithelium
GnRH	Gonadotrophin releasing hormone
h	Hour
H_2O_2	Hydrogen peroxide
hCG	Human chorionic gonadotrophin
HMG	Human menopausal gonadotrophins
HUVECs	Human umbilical vascular endothelial cells
ICM	Inner cell mass
ICSI	Intra-cytoplasmic sperm injection
IFN	Interferon
IFND	Interferon delta
IFNG	Interferon gamma
IGF1	Insulin-like growth factor-1
IGF2	Insulin-like growth factor 2
IgG	Immunoglobulin G
IL1B	Interleukin-1β
IM	Intramuscular
IRF2	Interferon regulatory factor 2
ITG	Integrin
ITGA5/B1	Integrin αvβ1
IUI	Intrauterine insemination
IVF	In vitro fertilisation
kD	kilo Dalton
LE	Luminal epithelium
LH	Luteinising hormone
LHCGR	LH/CG receptors
LIF	Leukaemia inhibitory factor
LPD	Luteal phase deficiency
ME	Middle East
min	Minute

ml	Millilitre
mm	Millimetre
MMP	matrix metalloproteinase
mPGES-1	Microsomal prostaglandin E synthase-1
mRNA	Messenger RNA
MUC1	Mucin 1
NFKB	Nuclear factor kappa B
ng	Nanogram
NGS	Normal goat serum
NHS	Normal horse serum
NSB	Non-specific binding
ºC	Celsius temperature
OHSS	Ovarian hyper-stimulation syndrome
OoR	Oocyte retrieval
OPN	Osteopontin
OR	Odds ratio
OXT	Oxytocin
P4	Progesterone
PAS	Plasminogen activator system
PBS	Phosphate buffer saline
PBS-T	Phosphate buffer saline with Tween
PCOS	Polycystic ovary syndrome
PCR	Polymerase chain reaction
PECAM	Platelet derived endothelial cell adhesion molecule
PGE2	Prostaglandin E2
PGF2α	Prostaglandin F2α
PGR	Progesterone receptors
PIGF	Placental growth factor
PLA2	Phospholipase A2, membrane associated
PTGS1	Prostaglandin-endoperoxide synthase-1
PTGS2	Prostaglandin-endoperoxide synthase 2
RCT	Randomised controlled trials
RNA	Ribonucleic acid
RT	Room temperature
SC	Subcutaneous
SEM	Standard error of the mean
SGE	Superficial glandular epithelium

SMA	Smooth muscle actin
SS	Superficial stroma
STC1	Stanniocalcin 1
TA	Total activity
TGFB1	Transforming growth factor β
TSP1	Thrombospondin
UK	United Kingdom
USA	United States of America
v	Volume
VEGFA	Vascular endothelial growth factor A
VEGFR	Vascular endothelial growth factor receptor
VWF	von Willebrand factor
w	Weight
WHO	World Health Organization
WOI	Window of implantation

CHAPTER 1 Literature Review

1.1 General Introduction

Progesterone (P4) is essential for implantation of the embryo and successful pregnancy and its role in early pregnancy maintenance in women was discovered by Allen and Doisy (1923). In addition, there is no doubt that progesterone deficiency leads to infertility (Young, 2013). However, diagnosis of insufficiency of progesterone in pregnant women is still controversial due to fluctuating levels in serum and the lack of an absolute endometrial receptivity marker that can assess the sufficiency of progesterone action. Combined with the profound ethical issues in collecting human embryos at implantation, studying the interactions between the endometrium and the human embryo *in vivo* has been unsurprisingly slow. Thus, information collected from animal models has assisted the understanding of the molecular basis of uterine receptivity and embryo implantation (Cha et al., 2012). Uterine receptivity is associated with molecular and structural changes, such as spatiotemporal expression patterns of the cell-extracellular matrix adhesion molecules called integrins (Lessey, 2011) and formation of micro protrusions (pinopodes or uterodomes) on the apical surface of the endometrial epithelial cells (Cha et al., 2012). Moreover, there are unique profile of genes that are up-regulated in the endometrium during implantation that might be considered as a uterine marker of receptivity (e.g. osteopontin, claudin 4, integrin alpha5,beta3) in humans (Horcajadas et al., 2004) and pigs (Kim et al., 2015).

Successful embryo implantation depends on the tightly controlled and synchronised action of oestradiol and progesterone on the endometrium (Cha et al., 2012). Implantation is a multi-factorial process mediated by a molecular interaction

between a receptive endometrium and an embryo. Receptivity of the endometrium occurs during the mid-luteal phase of the menstrual cycle and is also called the window of implantation (Rosario et al., 2003). The window of implantation normally lasts for only 4-5 days in women, but is likely to be even shorter in controlled stimulated ovarian cycles, which makes successful implantation a big challenge (Evans and Salamonsen, 2013).

The concerns over luteal phase deficiency (LPD) following assisted reproductive technology (ART) programmes has led to the frequent incorporation of progesterone supplementation in an attempt to improve pregnancy rates. However, the optimal regime for luteal phase support remains a debated issue in the clinical trials for infertility treatment (Ghanem and Al-Boghdady, 2012, Anita et al., 2017). Clinical studies have shown that poor endometrial receptivity might be the underlying cause (Heger et al., 2012).

Cyclic neovascularisation begins in the uterine endometrium of women after menstruation and continues until the end of the luteal phase. It is believed that endometrial neovascularisation generally occurs by angiogenesis, where the vasculature develops from the pre-existing blood vessels. During placentation, angiogenesis is initiated and is essential for the development of the extensive vasculature that is required to support the rapid embryonic and fetal growth (Torry et al., 2007). Both progesterone and oestradiol control angiogenesis in the endometrium through their regulation of angiogenic factor expression (Matsubara and Matsubara, 2012). There are many factors (e.g. vascular endothelial growth factor A (VEGFA), leukaemia inhibitory factor (LIF) and stanniocalcin 1 (STC1) (Allegra et al., 2009) that can affect and regulate angiogenesis in general; however

their effects have not yet been fully clarified in the endometrium. Hence, there is a need for controlled studies into this subject.

1.2 The anatomy of the reproductive tract

1.2.1 The ovary

The ovary is an endocrine gland that provides support for development of oocytes and the production of steroid hormones (progesterone and oestradiol). It is almond shaped in women and berry shaped in sows. The ovary consists of an epithelial surface layer with a connective tissue layer called tunica albuginea underlying the epithelium layer. The cortex is beneath the tunica albuginea and is where the follicles develop from the primordial to tertiary (Graafian) stages. Finally, the inner medulla is formed from loose connective tissue, and contains blood and lymph vessels along with nerves fibres (Fig.1.1) (Andersen, 1928, Hansel et al., 1973, Clement, 1987).

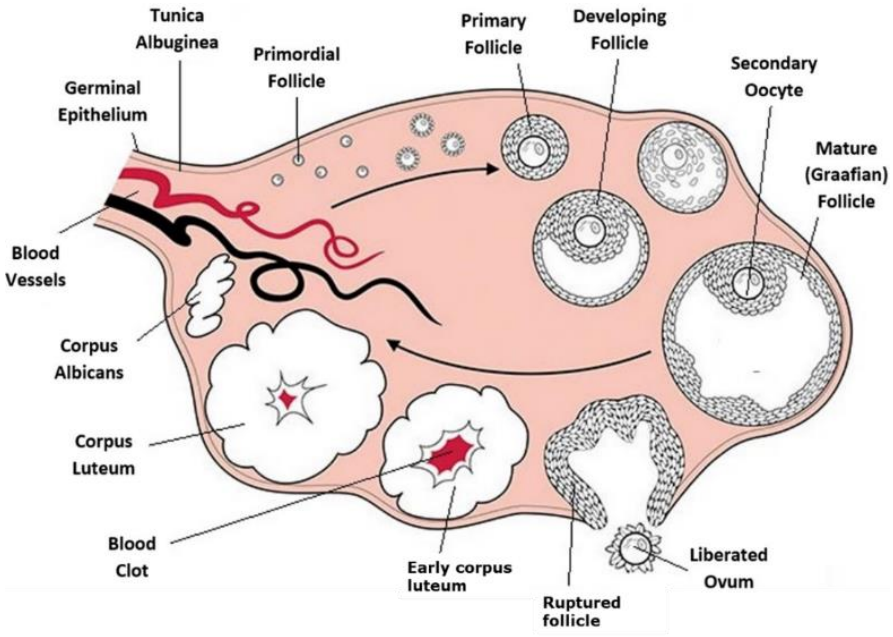


Figure 1.1: The structure of adult human ovary. The diagram illustrates the key stages of follicular and luteal development. The ovary consists of an epithelial surface layer with an underlying connective tissue layer. The ovarian cortex houses follicles and corpora lutea, whilst the medulla comprises of loose connective tissue, blood and lymph vessels with nerve fibres. Adapted and modified from Gaboka86-own work, CC BY-SA-4.0 , http://commons.wikimedia.org/w/index.php?curid=48731070

1.2.2 The uterus

The function of the uterus is profoundly influenced by sex steroid hormones and it is the organ where the fetus develops during gestation. The uterus is composed of three main layers: (1) the outer protective layer called the tunica serosa; (2) the middle layer or myometrium which is composed of two thin longitudinal layers of smooth muscle and a thick circular muscle layer; (3) inner endometrium layer which is characterised by the mucosa that is comprised of the luminal epithelium (LE), glandular epithelium (GE), stromal cells, immune cells and vascular elements

(Knobil, 1980). The uterine glandular secretions during the luteal phase support the early development of the embryo. While the luminal epithelium is where the embryo attaches during the establishment of pregnancy.

1.2.2.1 **Humans**

In humans, the uterus consists of mainly uterine body that has two small horns: the upper horns are connected to the fallopian tubes (uterine tube) and the lower body is connected with the cervix that then opens to the vagina (Fig. 1.2). In general, the endometrium displays four defined histological areas: a temporary functional layer that is represented by LE and a dense stromal layer (zone 1); the superficial glandular layer that characterises the upper third of uterine glands (zone 2); while zone 3 consists of the second third of the glandular tissues; the fourth zone is represented by the deep glandular layer, next to the myometrium (Fig. 1.3) (Rosario et al., 2003).

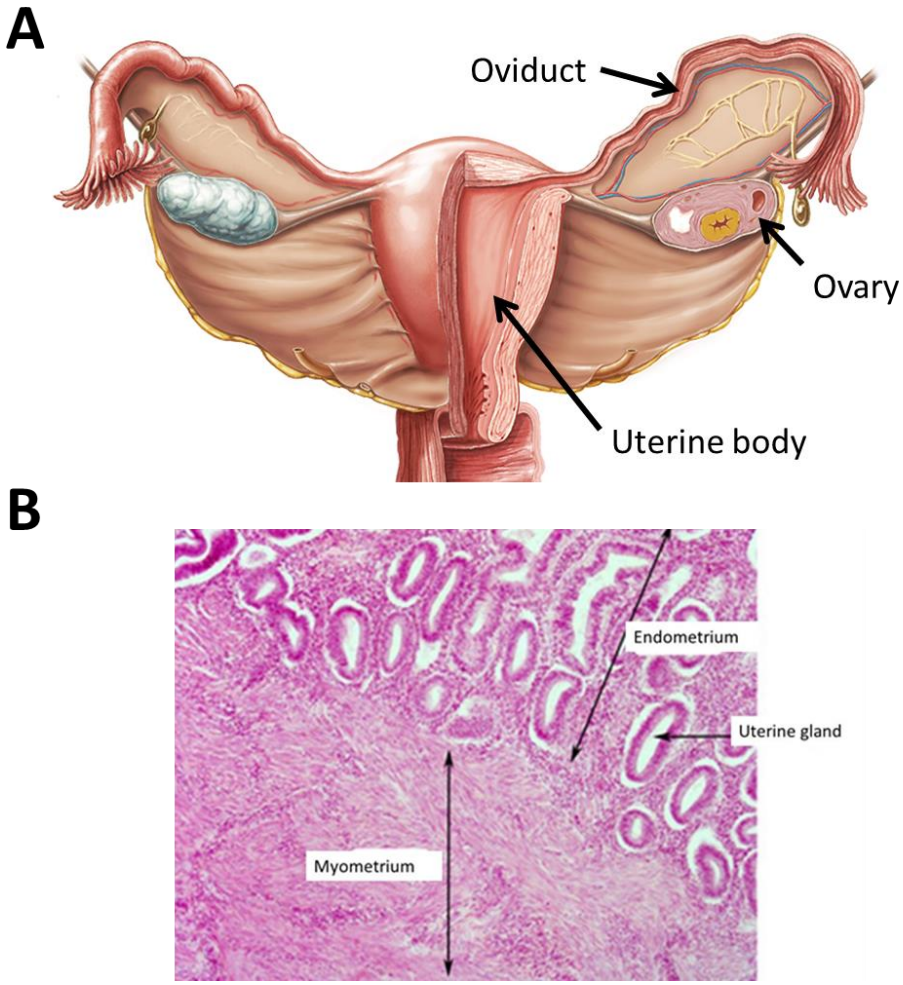


Figure 1.2: Female reproductive tract in women. A: shows an anatomical figure of the uterus and ovaries, Adapted from https://www.kenhub.com/en/library/anatomy/the-uterus, and B: shows a histological figure of the two key regions of the uterus, from https://mesa-anatomy.weebly.com/reproductive1.html.

1.2.2.2 **Pigs**

Sows and gilts have a bicornuate type of uterus, which is characterised by a very small uterine body and two long convoluted uterine horns. Uterine horn length may reach 60cm (up to 100cm when fully stretched out) which forms 90% of total tract length (Fig. 1.3) (Soloff and Swartz, 1974). The uterus needs to be this length to accommodate multiple embryos, which will develop in the folded areas. During implantation, critical morphological and biochemical changes occur in endometrial

cells (Kempisty et al., 2015). In pigs, progesterone and oestradiol are considered the main regulators of endometrial receptivity and the successful attachment of the embryo. These morphological changes include altered cell proliferation and tissue differentiation (Bazer et al., 2010). The endometrium is responsible for the attachment between conceptus and uterus with the placental attachment categorised as epitheliochorial. In this type of placenta, luminal epithelium tissue stays intact during pregnancy and the fetal tissue does not embed into the endometrium (Friess et al., 1980, Carter, 2007).

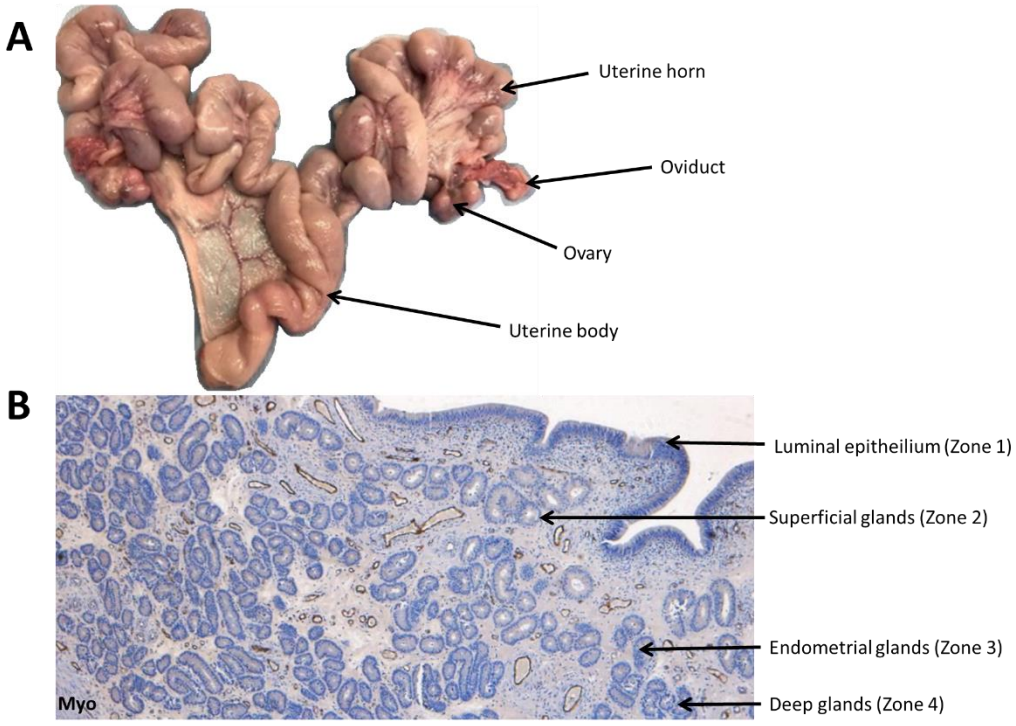


Figure 1.3: The female reproductive tract in pigs. (A): shows the uterus (body and uterine horns), fallopian tubes and ovaries. (B): histological image of the porcine uterus in cross section (myo=myometrium).

1.3 Hypothalamus-Pituitary and Ovarian Axis

The ovarian cycle is principally controlled by hormones of the hypothalamus and pituitary gland. Gonadotrophin releasing hormone (GnRH) is secreted from the hypothalamus and stimulates the anterior lobe of the pituitary to produce both follicle stimulating hormone (FSH) and luteinising hormone (LH). FSH and LH, in turn, stimulate folliculogenesis and corpus luteum development in the ovaries (Harris, 1951, Schally et al., 1973). There are both positive and negative feedback mechanisms that control the secretion of GnRH, FSH and LH by ovarian hormones (Fig.1.4).

GnRH is secreted in a pulsatile manner and with the interaction of steroid hormones, it controls secretion of both LH and FSH or just one of them according to the circumstances (Schally et al., 1971). Both FSH and LH are required to enhance the growth of follicles in the growing antral pool and enable their development and maturity. FSH is responsible for the recruitment of small antral follicles and secondly to stimulate the production of oestradiol as the follicle matures. After that, developed follicles secrete increasing amounts of oestradiol with LH essential for the later stages of follicular development, oocyte maturation and ovulation. LH will also ultimately stimulate progesterone secretion from the subsequent corpus luteum (Schwartz, 1974).

GnRH pulse frequency and amplitude is controlled by oestradiol and progesterone, as well as by some neuroendocrine factors such as kisspeptin and leptin (Barbieri, 2014). Generally, the lack of oestradiol increases GnRH pulse frequency (Kesner et al., 1987). While low levels of oestradiol and/or progesterone can reduce the frequency pulse of GnRH and thus FSH and LH (Soules et al., 1984, Barbieri, 2014).

However, oestradiol exerts both negative and positive feedback on GnRH and FSH secretion in both the hypothalamus and the pituitary gland. The effects of oestradiol induce an LH surge by acting positively on the hypothalamus (Barbieri, 2014). After ovulation, the residual follicular cells undergo luteinisation and secrete progesterone and some oestradiol that reduces both FSH and LH secretion. After luteolysis, progesterone concentrations decrease and the concentrations of both gonadotrophins increase and the cycle starts again.

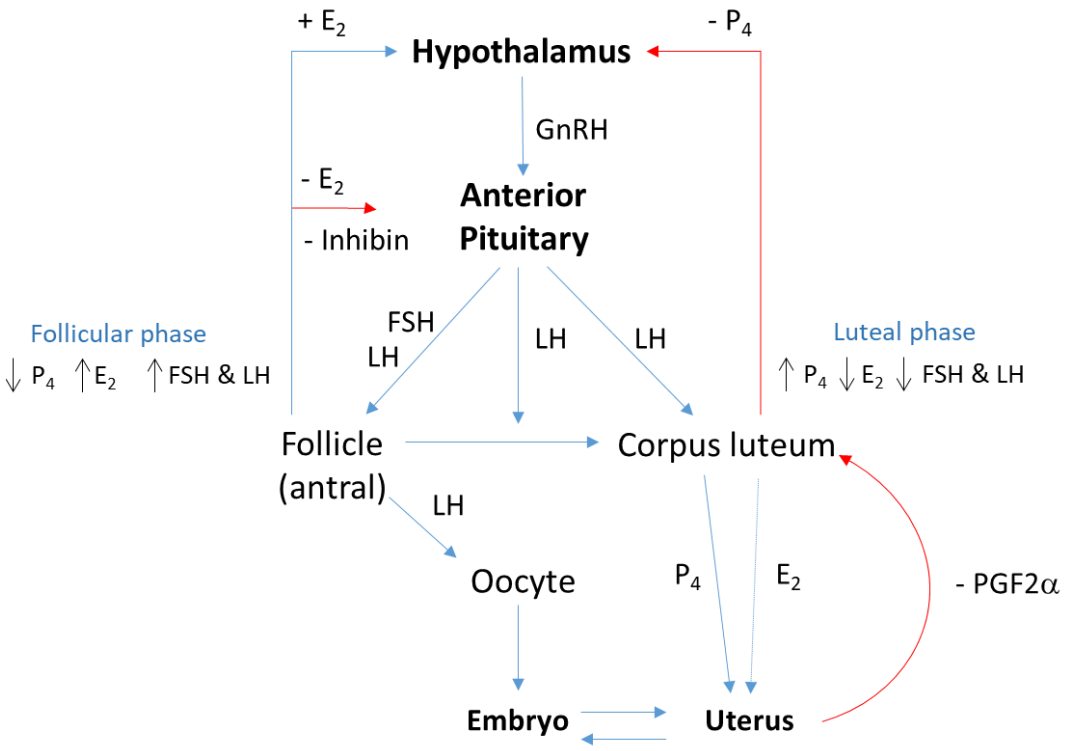


Figure 1.4: The outline of hypothalamus-pituitary-ovarian axis and its interactions with the embryo and uterus. The feedback mechanism between ovarian steroid hormones and hypothalamus and anterior pituitary gland is represented (negative, red; positive, blue). The levels of these hormones in the follicular and luteal phases are shown. The dashed line indicates the production of oestradiol (E2) that occurs in primates and pigs but not in other species. P4: progesterone, GnRH: gonadotrophin releasing hormone, LH: luteinising hormone, FSH: follicle stimulating hormone, PGF2α: prostaglandin F2α. Information gathered from (Hunter et al., 2004, Soede et al., 2011).

1.4 **Menstrual Cycle in Women**

The menstrual cycle describes the systematic changes that happen in the female reproductive system (Fig. 1.5). The menstrual cycle includes three phases: 1) Proliferative (follicular) phase: that starts from the first sign of menstruation. In this phase, follicles grow in response to FSH and LH. Serum concentrations of oestradiol and progesterone are very low throughout the early follicular phase (Baird and Fraser, 1974). Serum oestradiol concentrations increase in parallel with growth of the pre-ovulatory follicle (Knobil, 1980). 2) Ovulation, when ovarian oestradiol from development follicles dramatically increases which in turn increases LH and induces the maturation of oocyte and ovulation. 3) Luteal phase: the corpus luteum is derived from the residual part of mature ovulated follicle. Theca cells differentiate into small luteal cell and granulosa cells into the large luteal cells in developing corpus luteum. Both steroidogenic cell types secrete progesterone while large luteal cells can also convert androgens to oestradiol (Kohen et al., 2003, Barbieri, 2014).

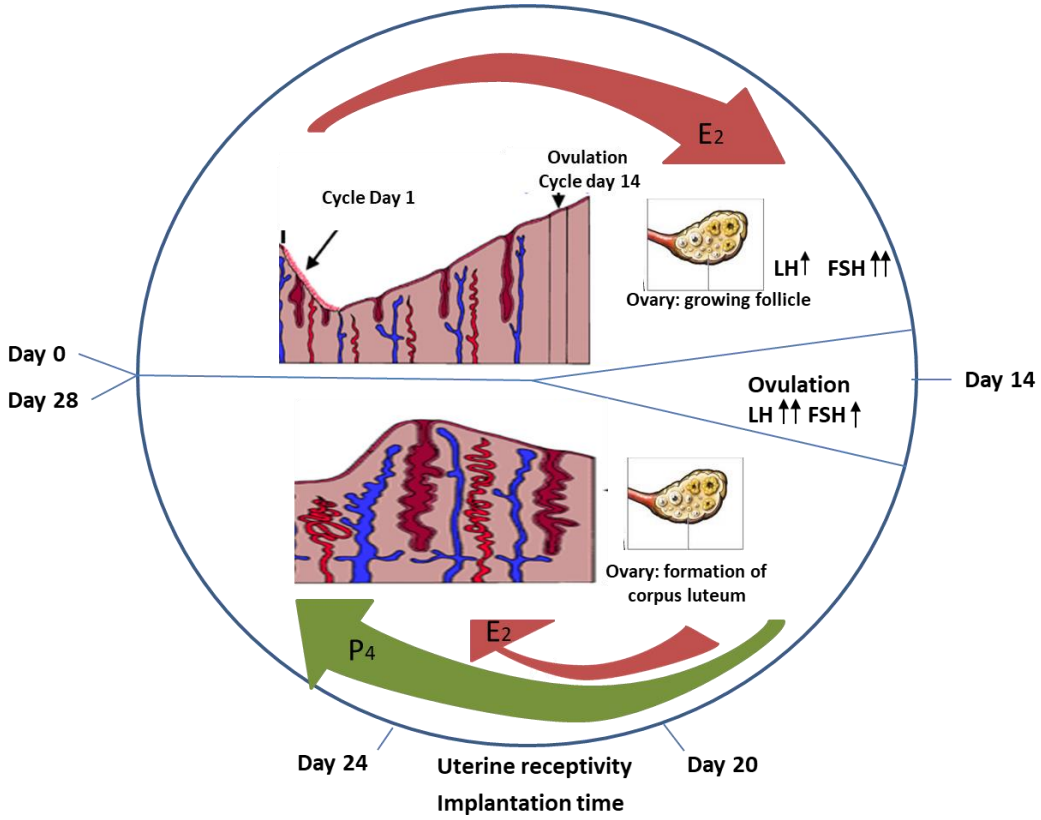


Figure 1.5: Menstrual cycle in women. The figure demonstrates the major patterns of hormone secretion, and the associated changes in uterine and ovarian structures observed. E2: oestradiol; P4: progesterone; LH: luteinising hormone; FSH: follicle stimulating hormone. Information gathered from https://www.early-pregnancy-tests.com/ovulatoryphase, and (Cha et al., 2012).

1.5 The Oestrous Cycle of Pigs

Gilts reach puberty within 150-220 days after birth. The oestrous cycle (lasts around 21 days) in the pig has two main phases, the follicular and luteal phases (Fig 1.6) (Soede et al., 2011). As a polytoccous species, pigs produce a large number of offspring (>10) with around 15-30 oocytes ovulated in each cycle. These oocytes are ovulated as a group over several hours to enable synchronous embryonic development (within 12 hours) in the oviduct and then the uterus (Helmond et al., 1986). Usually, gilts are inseminated 2 or 3 cycles after puberty has been attained which is followed by 114-116 days of pregnancy. After farrowing, pigs will enter a period of lactational anoestrus until weaning. In the UK, weaning usually takes place

28-40 days after farrowing. Thereafter, the sows will rapidly enter the follicular phase and will be in oestrus 4-6 days after weaning. During pregnancy and lactational anoestrus, the growth of follicles is suppressed by progesterone or prolactin leading to a lack of gonadotrophin stimulation and any follicular growth that is gonadotrophin-independent. A wave of FSH stimulates the recruitment of antral follicles that will continue to develop or degenerate by atresia (Knox, 2005).

1.5.1 Follicular phase

The follicular phase in the pig lasts for 4-6 days. During the luteal phase, the number of recruited antral follicles increases while their diameter remain limited to around 3 mm (Hazeleger et al., 2005). That is why ovaries usually have small follicles and are dominated by corpora lutea at this time.

In the follicular phase, a large number of follicles (30-50) in a pool of follicles (2-4 mm) start to develop and many of them undergo atresia (Guthrie and Cooper, 1996). Both FSH and LH regulate follicular development and ovulation. FSH controls and maintains the pool of medium-sized antral follicles and in those, whose granulosa cells become LH-responsive will be selected to develop into ovulatory follicles (Knox, 2005, Soede et al., 2011). The co-dominant antral follicles (often >20) secrete oestradiol, which positively feeds back to the hypothalamus to induce the LH surge as well as stimulating oestrus behaviour (classified as day 0 of the cycle) then ovulation. Ovulation occurs at 33 ± 3 hours after the peak of the LH surge and 44 ± 3 hours from the onset of oestrus (Soede et al., 1994).

In pigs, plasma oestradiol levels are low (<20pg/ml) during the luteal phase until day 17. Thereafter, oestradiol concentrations increase during the follicular phase and will reach >50pg/ml at oestrus (Henricks et al., 1972).

1.5.2 Luteal phase

The luteal phase begins immediately after ovulation and lasts approximately 16 days. It is characterised by the formation of the corpus luteum (CL) from the residual cells of the ovulated follicles. The CL reaches its maximal mass of between 0.4-0.5g (total luteal mass 6-10g) (Soede et al., 2011) (Fig 1.6).

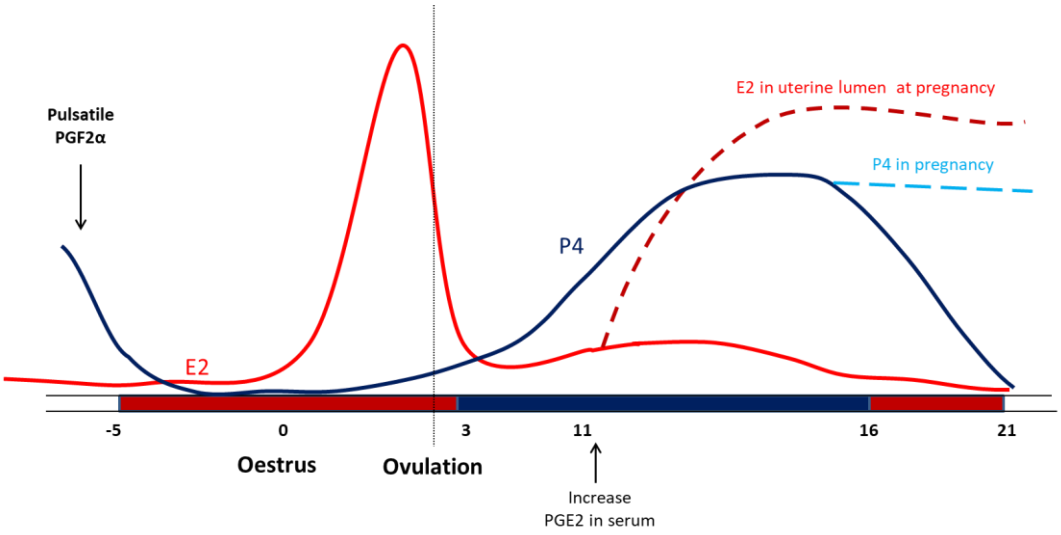


Figure 1.6: Hormonal profiles during the oestrous cycle and early pregnancy in pigs. It shows progesterone (P4, blue) and oestradiol (E2, red) levels at different times of the oestrous cycle (solid line) and during early pregnancy (dashed line). Prostaglandin F2α (PGF2α) induces luteolysis at day 16 of cycle and prostaglandin E2 (PGE2) increases on day 11 of pregnancy. Red: follicular phase, blue: luteal phase. Information gathered from (Knox, 2015) and results of Chapter 3.

After ovulation, there is considerable reorganisation and remodelling of residual tissues, and cellular differentiation that ultimately result in the development of a fully functional corpus luteum. In pigs, plasma progesterone concentration is < 2ng/ml on day 0 (day of oestrus) and then gradually increases to ~20-25ng/ml at day 6 and then

continues to peak at day 8 or 9 with >30ng/ml (Henricks et al., 1972). In the absence of successful fertilisation, the corpora lutea undergo luteolysis that is initiated by uterine-derived prostaglandin F2α. This results in the structural and functional degeneration of the corpora lutea, and is accompanied by a rapid reduction in circulating progesterone concentrations (De Rensis et al., 2012).

1.6 Endometrial Receptivity

Endometrial receptivity is a limited period of time when the uterus is conducive to accept a competent blastocyst and implantation can occur. There are many signals that confer receptivity and the time of maximal endometrial receptivity is called the window of implantation (Bourgain and Devroey, 2003, Makker and Singh, 2006).

In pigs, the attachment of the conceptus starts on day 14 of pregnancy and lasts until day 19. Successful attachment requires the participation of both uterine epithelial cells and embryonic trophoblast in the adhesion process (Waclawik, 2011). In most species, progesterone receptors (PGR) are down-regulated in the uterine luminal and glandular epithelium in the window of implantation due to the prolonged progesterone action in the early luteal phase (Mathew et al., 2011). In pigs, the down-regulation of PGR in epithelial cells has been detected on day 10 (Mathew et al., 2011, Waclawik, 2011). However, PGR expression in the stromal cells is maintained at this time. Loss of PGRs is synchronised with the activation of nuclear factor kappa B (NFKB) in the luminal and glandular epithelial cells (Ross et al., 2010). This initiation of NFKB expression is a key component during the attachment window. It is also associated with increased prostaglandin-endoperoxide synthase 2 (PTGS2, also known as cyclo-oxygenase 2 [COX2]) expression and prostaglandin production on day 12 (Geisert et al., 2006, Waclawik, 2011).

Down-regulation of endometrial PGR expression by progesterone is believed to suppress mucin 1 expression in the luminal pinopodes (micro protrusions) and stimulate the expression of integrins to enable embryo adhesion (Bowen et al., 1996). In contrast, mucin 4 expression is maintained in the luminal and glandular epithelial which plays a key role in keeping the luminal epithelial intact from invasion (Ostrup et al., 2010). Mucin 4 expression in non-pregnant pigs increases in the luminal and glandular epithelium on day 12 and peaks on day 15 before declining if the sow re-enters the follicular phase (Ferrell et al., 2003). However, mucin 4 expression in the pregnant endometrium is greater than in equivalent cyclic endometrium. This timeframe corresponds to the period of conceptus attachment to the luminal epithelium therefore supporting the role of mucin 4 in regulating this.

Facilitation of conceptus–endometrium interactions at implantation and restriction of trophoblast cells are regulated by extracellular matrix (ECM) molecules, integrins, proteases and protease inhibitors. Transforming growth factor β (TGFB1) is a key regulator of the expression of these factors (Jaeger et al., 2005). It is thus not surprising that TGFB1 is particularly evident during the pre-implantation period and is involved in the maternal-embryo interactions (Massuto et al., 2010).

Integrins and ECM interactions assist with the adhesion of trophoblast to the endometrial epithelium. Progesterone alone or in conjunction with oestradiol regulate the expression of integrin subunits (A4, A5 and B1) (Kim et al., 2010). The endometrial expression of these integrin subunits increased between days 11-15 of the pregnant or non-pregnant sow (Bowen et al., 1996). During apposition, oestradiol induced the expression of osteopontin (OPN; also known as secreted phosphoprotein 1) in luminal epithelium while progesterone regulates expression of OPN in the

glandular epithelium (White et al., 2005). OPN can bind to integrin A5/B3 or integrin A5/B1 in trophoblast cells and thus affect cell adhesion and migration. This interaction can alter focal adhesions as well as MTOR, P38 and ERK1/2 signalling pathways (Kim et al., 2010). Growth factors are critical in the endometrium-embryo interaction and conceptus growth. There is evidence that in the pre-implantation endometrium, both oestradiol and progesterone increase the expression of fibroblast growth factor 7 (FGF7) and insulin-like growth factor-1 (IGF1) in pig (Waclawik et al., 2009). FGF7 expression peaked at oestrus and during the attachment period between day 12 and 20 of pregnancy (Ka et al., 2000). Furthermore, progesterone induced FGF7 expression in stromal cells (PGR remains present in stromal cells at this time) while oestradiol, from conceptus, stimulated the maximal expression of FGF7 in luminal epithelium (Ka et al., 2007).

1.6.1 Preparation for attachment and implantation

1.6.1.1 Progesterone receptors (PGR)

The progesterone receptor is a member of the nuclear receptor superfamily of transcription factors that includes the androgen, oestrogen, mineralocorticoid and glucocorticoid receptors (Conneely et al., 1987, Evans, 1988). The human progesterone receptor exists in target tissues in two isoforms: PGRA and PGRB, with molecular weights of 94 and 114kD, respectively. They are similar except that PGRB contains an extra 164 amino acids at the N-terminus. Geisert et al (1994) used ligand-binding assays to demonstrate that the concentration of PGR changed during the oestrous cycle of the pig. PGR concentrations were highest between days 0 and 5, followed by a decrease in its level until days 10 to 12 in both pregnant and cyclic animals. On days 0-12 of the oestrous cycle, Western blot analysis detected two PGR

isoforms: at 117kD (B isoform) and 90kD (A isoform). Conversely, no visible bands were present on days 15-18 in cyclic or pregnant animals (Geisert et al., 1994).

Similarly, immunostaining for PGR (A and B isoforms) showed that it was present on days 0-12 of the oestrous cycle, but was absent from epithelial cells on day 15 in both cyclic and pregnant gilts. Specifically, there was intense localisation of PGR in luminal and glandular epithelium from day 0 until day 5. However, PGR localisation decreased on day 7-10 of the oestrous cycle and disappeared by day 12 in the luminal epithelium and day 15 in the glandular epithelium in both cyclic and pregnant gilts. PGR, though were present in the deep and superficial stroma and myometrium throughout the oestrous cycle and early pregnancy (Geisert et al., 1994).

1.6.1.2 **Oestrogen receptors (ESR)**

The action of oestradiol is mediated by oestrogen receptors (ESR): oestrogen receptor alpha (ESR1 or as previously known: ERα) and oestrogen receptor beta (ESR2 or as previously known: ERβ) (Matthews and Gustafsson, 2003, Lee et al., 2012). ESR1 and ESR2 are encoded by different genes that are found on different chromosomes (Kong et al., 2003). Human ESR1 has a molecular weight of 66kD and is comprised of 595 amino acids, while the molecular weight of ESR2 is 54kD with the full-length protein comprised of 530 amino acids.

Oestrogen receptors have five structural domains each with different roles in ligand binding and signal transduction (Skafar and Zhao, 2008). The N-terminal of the ESR subunit A and B includes an activation function 1 (AF-1) sequence, which is responsible for the activity of ESR. It is also an essential domain for interaction with co-regulators. Namely the p160 family was identified as the co-regulator that can

bind with AF-2, while the co-activators p300 and AD2 can bind AF-1(Fig 1.7). There is 30% identity in the AF-1 domain between ESR1 and 2. In many physiological studies, AF-1 activity is higher in ESR1 than ESR2. Both domains contain specific amino acids that can stimulate AF-1 activity through post transcriptional modification (Kong et al., 2003). The C domain includes a DNA binding domain that is required for sequence-specific binding of the receptor with the target DNA sequence and subsequently regulate gene expression (Geserick et al., 2005). The D domain, which is a pivotal region, contains specific amino acid sequences that regulate nuclear expression and modify the signalling of ESR. The C-terminal region of ESR contains the E/F domains that hold a ligand-binding domain and AF-2 activity. AF-1 and AF-2 switch the transcriptional regulatory action of ESR according to the cells' response to the environment around the cells such as presence the co-regulators or hormones (Edwards, 2000).

There is up-regulation of ESR1 expression after oestrus between days 0 and 12, which is likely to assist in the preparation of the endometrium for establishment of pregnancy. While there was no difference in endometrial *ESR1* mRNA expression between days 10 to 15 in pregnant and cyclic sows (Geisert et al., 1993), there was a different spatial distribution of ESRs in the endometrium (Geisert et al., 1993, Joyce et al., 2007). In addition, ESR1 showed intense staining in the stromal and luminal and glandular epithelial cells during the follicular phase. While in luteal phase (days 5 to 15), there was strong staining in glandular epithelial cells but more moderate staining in luminal epithelium and stromal cells. The intensity of staining started to decline after day 15 in luminal epithelium and was absent in stromal cells. During pregnancy, ESR1 showed positive immunoreactivity in epithelial cells on days 10 to 12, and was detected in stromal cells. The positive staining started to

decrease in epithelial cells by day 15 of pregnancy. On Day 18 of pregnancy, ESR1 staining remained detectable in the epithelium and was absent in the stroma or glandular epithelium (Geisert et al., 1994).

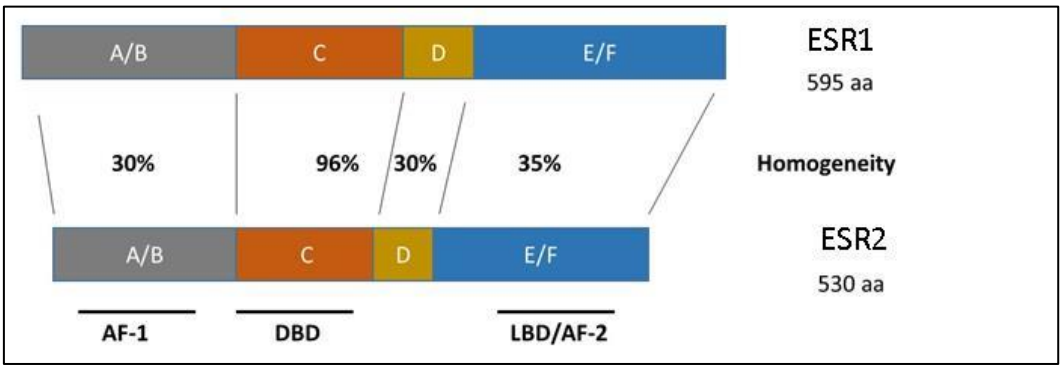


Figure 1.7: Structure of human oestrogen receptor ESR1 and ESR2. It shows the four functional domains: a DNA-binding domain (DBD), and two transcriptional activation functions (AF-1 and AF-2) and a ligand-binding domain (LBD). The percentage of homology in these domains between ESR1 and ESR2 is indicated. Modified from (Roman-Blas et al., 2009).

1.6.1.3 **Mucin 1**

Mucin 1 is a glycoprotein located on the apical surface of epithelial cells in several organs such as the uterus (Ren et al., 2010) and ovary (Deng et al., 2013). It is encoded by the MUC1 gene (Meseguer et al., 1998). As MUC1 is an apical surface glycoprotein, it provides a barrier to infection and acts as a molecule providing lubrication (Lagow et al., 1999). In the uterus, MUC1 is an active inhibitor of the interaction between different cell types, as well as between cells and the ECM (Ren et al., 2010). This indicates that MUC1 expression in the luminal epithelium prevents embryo attachment during endometrial receptivity. The expression of MUC1 must therefore be down-regulated to enable endometrial receptivity at implantation (Braga and Gendler, 1993).

In pigs, intense MUC1 immunostaining in endometrial tissue was detected on day 0 of the oestrous cycle but it was absent by day 10 in pregnant and cyclic animals (Bowen et al., 1996). The same study reported that, in ovariectomised sows, MUC1 was not affected by oestradiol treatment, while progesterone and a combination of progesterone and oestradiol decreased MUC1 expression. Another study indicated that the expression of *MUC1* mRNA in the area between attachment sites was greater than at attachment sites (Ren et al., 2010). Therefore, the presence of some MUC1 expression in luminal epithelium during pregnancy might assist to keep the luminal epithelium intact and enable the placenta to be non-invasive in sows.

In animal models, MUC1 as an anti-adhesion molecule forms a barrier on the endometrial surface, that resists implantation (Aplin, 2000). It was suggested that, in rabbit, the local down regulation of MUC1 is mediated by the effects of embryo before implantation (Hoffman et al., 1998). However, MUC1 appears to be up regulated during endometrial receptivity in women. Moreover, MUC1 expression in both the luminal and glandular epithelium is lower in women with recurrent implantation failure than in fertile women and those with recurrent miscarriage (Wu et al., 2018). This might explain the rejection of poor embryo from implantation (Aplin, 1996).

1.6.1.4 **Integrins**

The integrin (ITG) family are transmembrane receptors that assist in the adhesion of cell to the ECM and this interaction is showed in the next section (Fig. 1.8). An integrin molecule is composed of two sub-units, α and β, and many cell type–specific factors can react with integrins according to different ligand-binding specificities (Giancotti and Ruoslahti, 1999). There are 22 integrin heterodimers with at least 16

α subunits and 8 β subunits (Gullberg et al., 1995). The ability of adhesive properties is influenced by divalent ions with magnesium promoting whereas calcium decreases cell adhesion.

Integrin expression is often indirectly regulated by both oestradiol and progesterone. For example, in human endometrial culture, integrin heterodimers A5/B3 and A5/B5 were up-regulated by LIF (Chung et al., 2016) which is in turn regulated by oestradiol and progesterone (Yang et al., 1996). In an immunohistochemical study of the different integrin subunits (ITGA1, ITGA3, ITGA4, ITGA5 as well as ITGB1 and ITGB3), their expression was at its lowest level on day 0 and the highest was between days 10 and 15 of the oestrous cycle in pig endometrium (Rashev et al., 2005). There were no differences in their expression between pregnant and cycling animals on days 10–15. At this time, the expression of subunits ITGA1 and ITGA3 was low; but ITGA5 and ITGB3 expression was high, while ITGA4, ITGA5 and ITGB1 were moderately expressed. The same study showed that ITGA3 and ITGB3 expression was not affected by sex steroids. However, the expression of ITGA4, ITGA5 and ITGB1 was low after oestradiol treatment but increased after progesterone treatment or a combination of progesterone and oestradiol (Bowen et al., 1996). In addition, mRNA expression of *ITGA4* and *ITGB3* was high in the endometrium on day 18 of pregnancy and did not correlate significantly with the level of steroid hormones in serum (Lin et al., 2007). Collectively, this suggested some integrin subunits are regulated by progesterone and integrin is one of the molecules that support true-epitheliochorial placenta in pigs.

1.6.1.5 **Osteopontin**

Osteopontin (OPN) is an acidic phosphoprotein that is secreted by most cell types. This extracellular protein contains a cell-adhesion sequence and intermediates in the interaction between ECM components and cells (White et al., 2005). OPN is a monomer consisting of 264 to 301 amino acids according to the species. The binding region of OPN contains the amino acid sequence Arg-Gly-Asp (RGD) that binds with ITG A5/B5, ITG A5/B1, ITG A4/B1 and ITG A5/B3 (Hu et al., 1995). In order for attachment between the conceptus and endometrium, OPN interacts with integrins which are expressed in the cell membranes of the luminal epithelium and trophectoderm cells of the conceptus (Fig. 1.8) (Johnson et al., 2014).

In pigs, OPN is expressed in both endometrial epithelial cells and immune cells. Its expression increases after day 25 to 30 of pregnancy and remains elevated until day 85 of pregnancy. OPN protein can be detected in the apical luminal epithelium and in uterine flushes (Garlow et al., 2002). Furthermore using *in situ* hybridisation, *OPN* mRNA was present in the luminal epithelium from day 15 of pregnancy while *OPN* mRNA was first localised in the glandular epithelium on day 35 of pregnancy. Its expression was influenced by both steroid hormones and the presence of the conceptus. For example, in sheep pregnant with singletons, both the gravid and non-gravid horns expressed *OPN* mRNA, but the presence of the conceptus further stimulated *OPN* expression (Dunlap et al., 2008). It was also found that progesterone alone in the absence of an embryo was not sufficient to induce OPN in the porcine endometrium (Bailey et al., 2010). Moreover, in pigs, OPN was regulated by conceptus-derived oestradiol in luminal epithelial cells and by progesterone in glandular epithelial cells (White et al., 2005).

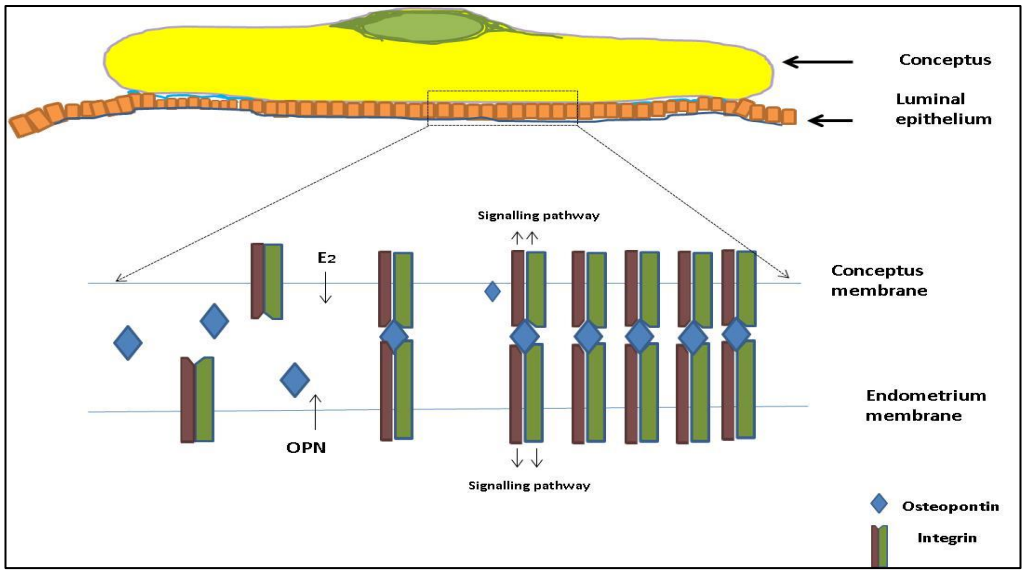


Figure 1.8: Regulation and proposed function of osteopontin (OPN) and integrin in the endometrium and porcine conceptus: elongated porcine conceptuses secrete oestradiol (E2) that induce the secretion of OPN from endometrium epithelium. Integrin subunits are expressed on both conceptus and luminal epithelium cells. OPN binds between integrin subunits in both sides (conceptus and endometrium). Modified from (Johnson et al., 2014).

1.6.1.6 **Stanniocalcin 1**

Stanniocalcin 1 (STC1) is a glycoprotein hormone which was first discovered in the corpuscles of the stannius gland in bony fish (Yeung et al., 2012), where it regulates calcium and phosphate homeostasis. STC1 has also been detected in a wide range of mammalian tissues, but it is undetectable in serum, suggesting that STC1 acts primarily as an autocrine / paracrine factor. The regulation of calcium and phosphate by STC1 is thought to be conserved in mammals, with additional functions suggested in ion transport, steroidogenesis and cell metabolism (Chang et al., 1995).

Stanniocalcin is upregulated in a number of cancers and it has been suggested that it acts as an angiogenic factor in tumour tissue, by upregulating VEGFA expression (He et al., 2011). In addition, STC1 stimulates angiopoietin and VEGFA signalling pathways (Law and Wong, 2013).

STC1 gene showed a high expression level in endometrial tissue at the time of implantation in cycling women (Allegra et al., 2009), sheep (Song et al., 2006), rats (Xiao et al., 2006) and pigs (Song et al., 2009). In women, STC1 was further up-regulated in the endometrium during the implantation window of pregnant women (Allegra et al., 2009). Song et al (2006) and (2009) stated that STC1 has a functional role in implantation and embryo development as a result of its mRNA being expressed in the endometrium of ewes and sows. In pigs, S*TC1* expression was not affected by the day of the oestrous cycle; however, its mRNA expression was affected by the day of pregnancy. *STC1* expression was detected at day 9 of pregnancy and increased steadily until day 14 of pregnancy. Thereafter, its expression decreased to non-detectable by day 25 of pregnancy (Song et al., 2009). The same study suggested that STC1 expression might be regulated by progesterone and oestradiol *in vivo*. This was demonstrated in pseudopregnant and ovariectomised sows, whereby progesterone was required for STC1 expression in luminal epithelium. In addition, in this study STC1 was down-regulated in luminal epithelium by oestrogen after a long-term progesterone treatment.

1.7 Establishment of Pregnancy

Establishment of pregnancy requires a reciprocal communication between blastocyst, endometrium and corpus luteum. These communications start before the window of attachment and are more likely to begin during the embryonic period (Waclawik, 2011).

In the pig, an intra-uterine mixture of adhesion factors, nutrient molecules, growth factors, enzymes, cytokines and other proteins known as histotroph supports embryo development and implantation. Histotroph is secreted by endometrial epithelial cells

(luminal and glandular) and results in the promotion of adhesion and trophectoderm cell migration (Gray et al., 2002, Dunlap et al., 2008, Erikson et al., 2009).

1.7.1 Early embryogenesis (humans)

Fertilisation occurs within 24 hours after ovulation. Once fertilisation happens, the oocyte finishes meiosis II and the second polar body appears. Then the zygote will undergo many mitotic divisions (cleavage) with the first division completed (two-cell embryo) 30 hours after fertilisation. Within three days, the embryo reaches 16 cells in size and becomes a morula (64 cells) by day four. At the morula stage, the inner cells establish the inner cell mass that will form the three embryo germ layers, while the outer cells of the morula will form the trophoblast that will be a part of placenta after implantation. Implantation occurs on day six after ovulation, when the morula reaches the uterus, and at the same time fluid will accumulate within the zona pellucida between the inter-cellular space and the inner cell mass. In so doing, it forms the blastocele cavity (Whelan, 2009). At that time, the inner cell mass gathers in one pole to form the embryoblast and the outer cell mass forms the trophoblast and this embryonic stage is termed blastocyst. When the blastocyst hatches from the zona pellucida, it becomes ready for implantation (Sadler, 2011). Then, the embryo attaches to the endometrium by the trophoblast cells apposing and adhering to the endometrial luminal epithelium.

The endometrium, under the influence of ovarian steroids, prepares a receptive environment for the incoming embryo while in the oviduct the embryo undergoes cleavage. For example, oestradiol increases the thickness of the endometrium and progesterone stimulates histotroph secretion and regulates the molecular signature required for implantation (Young, 2013).

The first step of implantation is the apposition of trophectoderm cells such that they are in close contact with the luminal epithelial cells and in so doing stimulate the breakdown of the basement membrane. Next, thin folds of trophectoderm cells will invade in between the endometrial epithelial cells and in the process, they will fuse to form syncytia. Consequently, the syncytia will proliferate and degrade the endometrial ECM. Finally, the interior part of trophectoderm cells form placental villi (Bischof and Campana, 1996). Successful implantation needs cross talk between embryo and endometrium. The invading blastocyst has many paracrine signals such as human chorionic gonadotrophin (hCG), insulin-like growth factor 2 (IGF2) and interleukin 1, that can control the physiological function of endometrial cells (Simon et al., 1994, Simon et al., 1998).

1.7.2 **Early embryogenesis (pigs)**

Ovulation takes place, on average, 44h after the start of oestrus, which lasts 24 to 72h. Fertilisation occurs in the oviduct at the ampullary-isthmic zone, before the embryos enter the uterus at the 4-cell stage on day 4 (Bazer and First, 1983). Then, they develop into the morula stage, before reaching the blastocyst stage by day 6. Early blastocysts cannot develop in the oviduct due to a lack of essential elements and the presence of embryostatic factors (Bazer and Johnson, 2014). On day 6 and 7 of pregnancy, blastocyst hatches out from the zona pellucida and extends in length from 0.5-1 mm to 2-6 mm (Bazer and Johnson, 2014). In this elongation stage, a compact band of cells, called the elongation zone, form from the trophectoderm and endoderm cells of the inner cell mass (ICM) and these migrate/proliferate towards the ends of the spherical embryo.

Porcine blastocysts undergo huge morphological development that enables the attachment of the trophoblast to the luminal epithelium. These transitions include the change from spherical to tubular and then elongation to filamentous stages (Fig 1.9). These changes occur between days 10-12 of pregnancy. These morphological changes include increasing the length of the blastocyst from around 4-9mm to 15-50mm at the tubular stage, then 100-200mm in the filamentous stage within a few hours between day 10-12 of pregnancy, and ultimately 800-1000mm on day 15 (Geisert et al., 1982, Bazer, 2013, Geisert et al., 2014). It should be noted that at any time, all three embryonic stages (spherical, tubular and filamentous) can be observed within the same uterine horn (Geisert et al., 1982, Bazer, 2013). This reflects the rapid morphological changes that are occurring during this period. It is estimated that by day 25 of pregnancy, 30-40% of embryos fail to develop and are lost (Perry and Rowlands, 1962). It is believed that the majority of the loss occur due to failure of the elongation process and thus fail to achieve sufficient contact area with the luminal epithelium (Perry and Rowlands, 1962).

Intriguingly, the total DNA and RNA content in the conceptus increases between days 10 and 16, but on a given day, there is no difference in total DNA between the spherical, tubular and early filamentous stages (Geisert et al., 1982). The mitotic index for spherical blastocysts decreases 40% prior to the elongation period. This might indicate that elongation is responsible for increasing the attachment area between conceptus and endometrium.

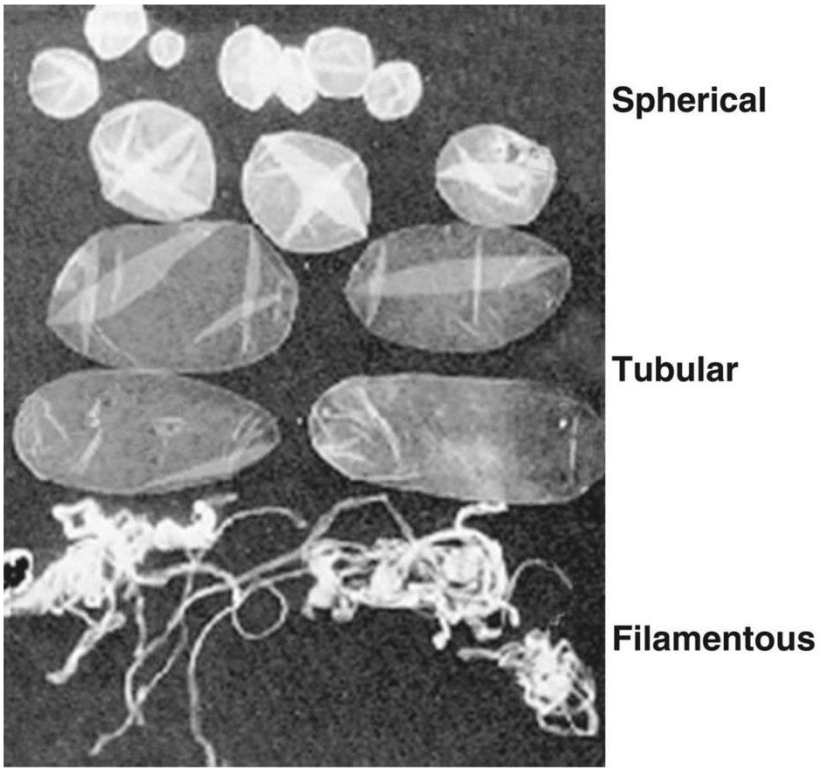


Figure 1.9: The development of the porcine embryo during the elongation stage (days 10-12 of pregnancy). From (Geisert et al., 2014).

1.7.3 Maternal recognition of pregnancy

Human

In women, human chorionic gonadotrophin hormone (hCG) is the maternal recognition hormone and the first significant increase in maternal circulation of hCG occurs 9 to 13 days after ovulation; just after the beginning of implantation of the blastocyst (Catt et al., 1975). hCG is secreted from syncytiotrophoblast cells in placental villi (Midgley and Pierce, 1962) and its secretion is controlled by placental GnRH (Kelly et al., 1991, Lin et al., 1995). More recent studies indicated that hCG can be detected in 8-cell embryos in culture and can be detected in serum one day before implantation (Kumar and Magon, 2012).

hCG plays a critical role in the communication between the mother and embryo, due to its action to rescue the corpus luteum from luteolysis and thereby maintain progesterone secretion (Stouffer et al., 1977). Moreover, hCG increases angiogenesis in the decidua of the uterus during pregnancy by acting through its receptors on the spiral arteries (Zygmunt et al., 2002, Theofanakis et al., 2017).

In infertility treatment, hCG is used for the final maturation of oocytes and triggering ovulation in stimulated cycles, because hCG mimics LH and its action is mediated through the same receptor (Cole, 2010). Moreover, the half-life of hCG is longer than LH thus making the use of hCG more preferable (Theofanakis et al., 2017).

Pig

Oestradiol is the maternal recognition of pregnancy signal in pigs, and its secretion is responsible for maintenance of functional CL and prevention of luteolysis. Conceptus-derived oestradiol acts by re-directing the endometrial release of PGF2α away from the uterine vasculature and towards the uterine lumen instead (Keys and King, 1990). Therefore, PGF2α is prevented from reaching the ovary and causing luteolysis (Bazer and Thatcher, 1977, Bazer, 2013).

There are two phases of oestradiol secretion by the porcine conceptus. The initial production of oestradiol is detected during the spherical stage and thereafter increases gradually until day 12 of pregnancy. After that secretion of oestradiol declines between days 13-14, but increases again between days 15 and 25 (Bazer et al., 1986). In the uterus, PGF2α causes local contractions, which helps to distribute embryos throughout the whole uterine horn whilst this is not thought sufficient to reach the ovary and induce luteolysis (Raheem, 2017). Polge et al. (1966) suggested

that at least four embryos in the sow are required to maintain pregnancy, while there are other reports that at least one embryo in each uterine horn is required (Dumesnil, 1961, Polge et al., 1966, Miller and Dziuk, 1968). Indeed, in the case of an unilateral pregnancy, the non-pregnant horn is required to be removed before attachment (day 14) for pregnancy to be maintained (Dumesnil, 1961). This implies that PGF2α production from a non-pregnant horn is sufficient to act in an endocrine manner to induce luteolysis.

Oestradiol from the conceptus is also likely to stimulate the release of nutrients from the uterus to nourish the developing embryos with proteins, ions, and carbohydrates. Moreover, it stimulates the production of carbohydrate-rich glycocalyx, in the uterine luminal cells, which is required for attachment of the conceptus membrane with the endometrium (Keys and King, 1990, Geisert et al., 1993).

In pregnant sows, oestradiol concentrations increase dramatically in the uterine lumen at the time of rapid embryonic elongation. However, there is only a slight change in the plasma oestradiol concentrations during early pregnancy. Oestradiol exerts its effects on the uterus during both the oestrous cycle and early pregnancy via the nuclear steroid receptor ESR1 (Henricks et al., 1972, Geisert et al., 2012). Therefore, successful implantation is achieved, when an adequate embryonic signal occurs when the uterus is receptive.

1.7.4 **The role of the conceptus**

Adhesion between the embryo and endometrium are likely to be mediated through macromolecular interactions that regulate growth, movement and gene expression. These interactions include heterodimeric transmembrane integrin receptors such as

ITGA5, ITGB3 and ITGB1, which transfer the various signals between the ECM and the actin cytoskeleton (Burghardt et al., 2009, Bazer and Johnson, 2014).

The ECM contains secreted OPN and FGF7 that stimulate cell proliferation, differentiation, movement and angiogenesis. Both OPN and FGF7 are up-regulated by oestradiol in the luminal epithelium and influence the adhesion of the trophectoderm (Waclawik, 2011, Bazer, 2013).

In the pig, the initial attachment between embryo and endometrium starts by day 13 and is completed by day 24 (Massuto et al., 2010). Gene expression in endometrial epithelial cells is likely to be modulated by oestradiol and other conceptus-derived proteins such as interferons (IFN) during the establishment of pregnancy. The production of interferons by the conceptus is common in mammals. However, the pig is the only known mammalian species, which produces both IFND and IFNG in the pre-implantation period. The concentration of IFN in the uterine flushing increases after day 10 of pregnancy and peaks on day 16 (La Bonnardiere et al., 1991). IFNG represents 75% of the total IFN between days 13-20 of pregnancy with IFND representing the remaining 25% (Lefèvre et al., 1998).

In the pig, there is a complex interplay between endometrium and conceptus during pregnancy recognition and implantation. STAT1 activity in the pregnant endometrium tissue is regulated in a cell-type specific manner by the embryonic-derived IFN and oestrogen. Joyce et al. (2007) stated *in utero* treatment with dialysed media from cultured embryos that contained interferons (IFND and IFNG) induced STAT1 in stroma cells. Whilst oestradiol induced STAT1 expression in luminal epithelial cells. This, in turn enabled the remodelling of the luminal epithelium required for attachment. The same study also indicated that the premature treatment

of pregnant sows with oestrogen on day 10 led to the loss of all embryos. In addition, the *in utero* administration of interferons D and G, isolated from conceptus culture spent medium, increased PGE2 secretion and expression of STAT1 in stromal cells (Harney and Bazer, 1989, Bazer and Johnson, 2014). Thus, oestrogen and interferons regulate STAT1 in different manner according to cell type indicating the complex communication between the endometrium and conceptuses at time of implantation time.

Interleukin-1β (IL1B) is also produced by the conceptus during the dramatic morphological transformation period (10-12 days), but its levels are decreased by day 15 (Ross et al., 2003). The endometrial response to IL1B is to modulate the effect of oestradiol (Bazer, 2013a). The chronological expression pattern of ILIB indicates that it might play a role in both elongation and establishment of pregnancy.

The establishment of pregnancy is also dependent on the ability of the conceptus to prepare the endometrial epithelium and stromal cells for successful attachment. Oestradiol is likely to play a role in this by inducing the expression of an array of genes including cluster of differentiation 24, neuromedin beta, aldo-keto reducing family member 1 and interferon regulatory factor 2 (IRF2) which are known to remodel the uterus for implantation and placentation.

1.7.5 **The influence of progesterone**

Successful attachment is required for the extension of the luteal phase. Progesterone stimulates the endometrium to secrete factors such as cytokines and growth factors (Rosario et al., 2003) that are required for embryo implantation. The maximal concentrations of ESR1 and PGR in the endometrium occur during the follicular

phase and at oestrus in particular. Thereafter, ESR1 levels decrease throughout the luteal phase. This implies that oestradiol enhances PGR and ESR1 expression, while progesterone down-regulates these receptors (Fujishita et al., 1997). In addition, PGR is temporally and spatially-regulated during the luteal phase. In the luminal epithelium and, partially in the superficial epithelial gland cells, PGR will disappear during the luteal phase but is up-regulated in the deep glands and stromal cells (Geisert et al., 2014).

1.7.6 **The influence of prostaglandins**

Prostaglandins (PGs) are vital factors regulating the oestrous cycle and pregnancy, including luteolysis, implantation and embryonic growth (Morawska et al., 2012). In pigs, prostaglandins are produced and secreted from the endometrium as well as the conceptus.

In the normal oestrous cycle of sows, luteolysis starts as a result of increasing prostaglandin F2α (PGF2α) concentrations on day 15-16. However, exogenous administration of PGF2α cannot induce luteolysis before day 10 (Zorrilla et al., 2009). In contrast, during pregnancy, the action of endometrial-derived PGF2α is redirected from endocrine to exocrine therefore; PGF2α does not reach the corpus luteum. Indeed, PGF2α concentrations in the utero-ovarian vein are lower in pregnant pigs compared to cyclic counterparts. While, PGF2α concentrations in the uterine lumen are elevated in pregnant pigs. Theoretically, there is retrograde transfer for PGF2α from the uterine vein and lymph vessels back to the lumen of the uterus (Krzymowski and Stefańczyk-Krzymowska, 2004).

It has been suggested that PGF2α might regulate uterine angiogenesis because it enhances the expression of pro-angiogenic factors (e.g. VEGFA) in the endometrium. In turn, VEGFA has been shown to increase the proliferation of endometrial endothelial cells (Sales et al., 2005, Kaczynski et al., 2016).

In addition, PGE2 acts as a luteotrophic hormone that assists to maintain corpus luteum function during early pregnancy (Christenson et al., 1994). Consequently, several studies have suggested that PGE2 is a second maternal recognition of pregnancy factor (Cao et al., 2005). Blitek and colleagues demonstrated that, culturing luminal epithelial and stromal cells with early embryo conditioned media increased PGE2 production (Blitek et al., 2011). In the three days before implantation, the PGE2/PGF2α ratio was elevated compared with a non-conceptive cycle (Weems et al., 2006, Waclawik and Ziecik, 2007). In addition, it has been suggested that the PGE2/PGF2α ratio affects the formation and differentiation of luminal epithelial cells, myometrial motility as well as uterine blood flow and vascular permeability (Cao et al., 2005, Waclawik and Ziecik, 2007).

Oxytocin (OXT) plays a role in controlling PGF2α synthesis. Its role is well recognised in the ruminants. In contrast, in the porcine, OXT might play a role in the pulsatile production of PGF2α, but there is no evidence that OXT has a role in luteolysis (Przygrodzka et al., 2014). In pregnant gilts, OXT concentrations increase in the uterine lumen during the preimplantation period compared to cyclic sows. Moreover, OXT induced production of PGE2 in the pregnant uterus (Waclawik et al., 2010).

1.8 Angiogenesis

Angiogenesis is the process of new growth of blood vessels from endothelial cells. Angiogenesis occurs physiologically in the embryo but rarely in adults except in the corpus luteum, endometrium and in response to wound healing. Angiogenesis is also important pathologically, in conditions such as tumour growth, endometriosis and chronic inflammatory disorders (Gargett and Rogers, 2001).

In adults, angiogenesis is under strict control. Thus, angiogenesis is only triggered under defined circumstances. These restrictions are essential for normal development, because both unnecessary creation of new blood vessels or their inadequate formation might lead to serious conditions (Karamysheva, 2008). Therefore, normally, there is a balance between stimulation and inhibition of angiogenesis and it is likely that the formation of new vessels is suppressed in the normal state.

1.8.1 The angiogenic process

Angiogenesis takes place by one of four alternative mechanisms: sprouting of endothelial cells, intussusception or division of a vessel lumen, elongation of an existing vessel and incorporation of endothelial progenitor cells (Gargett and Rogers, 2001).

1) In sprouting angiogenesis, endothelial cells release proteases such as the plasminogen activator system (PAS) and matrix metalloproteinases (MMPs) to breakdown the basement membrane and destroy the ECM (Rundhaug, 2005, Bogaczewicz et al., 2006). Endothelial cells then migrate and proliferate to form visible tubules. After that, a new basement membrane is laid down and pericytes are

employed to stabilise the new microvessels. Angiogenesis in the corpus luteum and during tumour growth is thought to occur primarily by sprouting (Risau, 1997). 2) Intussusception occurs when a contact zone is formed between opposing capillary walls that leads to reorganisation of inter-endothelial cell junctions enabling central perforation of the bilayer and the formation of a lumen. In so doing, a network of vessels is produced as observed in lung development (Risau, 1997). 3) Existing vessels elongate and grow wider in many growing tissues. 4) Finally, circulating bone marrow-derived stem cells or endothelial progenitor cells also participate in blood vessel formation at the sites of vessel injury, in postnatal neovasculogenesis and tumour tissue (Asahara et al., 1999, Qiu et al., 2006).

1.8.2 Regulation of angiogenesis

Angiogenesis is a complex process which is controlled by numerous pro-angiogenic and anti-angiogenic factors which act together to keep vessel growth within tight bounds.

1.8.2.1 Vascular endothelial growth factor A

The vascular endothelial growth factor (VEGF) family plays a key role in regulating angiogenesis in many body systems. It is comprised of six members, namely VEGFA, VEGFB, VEGFC, VEGFD, VEGFE and placental growth factor (PlGF) (Smith, 2001). Amongst this family, VEGFA is considered the most important pro-angiogenic factor and has received most attention.

VEGFA is a homodimer glycoprotein with a molecular weight of 45kD in humans (Carmeliet, 2005). Its biological effects are mediated by two receptors, VEGFR-1 (formerly FLT1) and the receptor tyrosine kinase VEGFR-2 (formerly KDR) that

differ according to their signalling features (Ferrara et al., 2003). VEGFR2 is considered the major mediator of VEGFA-stimulated effects on mitogenesis, angiogenesis and vascular permeability. VEGFA and its receptors are the key inducing system that control the proliferation and movement of endothelial cells to initiate micro-vessel formation (Karamysheva, 2008). VEGFA stimulates EC proliferation and migration directly from existing blood or lymph vessels in numerous systems *in vitro* (Robinson et al., 2009, Woad and Robinson, 2016) and *in vivo* (Carmeliet, 2005). Moreover, VEGFA prevents endothelial cell apoptosis and acts as a survival factor (Karamysheva, 2008). VEGFA also enhances vascular permeability, and it is this activity which may further support the pathological responses to VEGFA such as inflammation and tumour metastasis (Senger et al., 1993). After angiogenesis, the immature blood vessels need to be stabilised by the recruitment of mural cells (pericytes) (Benjamin et al., 1999, Darland and D'Amore, 1999).

1.8.2.2 **Fibroblast growth factor 2**

The fibroblast growth factor (FGF) family is one of the most multi-purpose growth factor signalling systems, playing important roles in regulating many cell types including endothelial cells (Maciag et al., 1979). It has been recognised that there are 22 different FGF ligands and four tyrosine kinase receptors in humans and mice (Cross and Claesson-Welsh, 2001).

In vitro, FGF2 acts an effective and potent mitogen for many types of cells, such as endothelial cells and fibroblasts. In cultured endothelial cells, FGF2 increased the angiogenic process by inducing proteinase molecules, proliferation and migration (Seghezzi et al., 1998). It has been shown that FGF2 also induces VEGFA

expression in endothelial cells and then stimulates angiogenesis (Seghezzi et al., 1998). FGF2 induces angiogenesis, in part by upregulation of VEGFR-2, while VEGFA can release FGF2 from the ECM in the endometrium (Smith, 2001). Moreover, *in vitro*, VEGFA has a synergetic potent effect with FGF2 at stimulating angiogenesis (Pepper et al., 1992). In pigs, FGF2 is secreted by conceptuses and endometrium before and during the implantation period of pregnancy as well as in the oestrous cycle (Lim et al., 2016).

1.8.2.3 **Other pro-angiogenic factors**

Angiogenesis is primarily induced through the actions of VEGFA and FGF2 on endothelial cells and pericytes. There are, though, several other factors that stimulate angiogenesis either via FGF2 and VEGFA or independently and these will be highlighted in this section.

The contribution of steroid hormones (progesterone and oestrogen) to the stimulation of angiogenic processes have been stated by several studies (Shifren et al., 1996, Fraser, 1999, Jaffe, 2000). Both progesterone and oestrogen can induce the angiogenic process through the up-regulation of VEGFA expression in breast cancer tissue (Botelho et al., 2015), as well as in the ovary and endometrium (Shifren et al., 1996). In humans, Shifren et al (1996) reported that *VEGFA* mRNA levels were 3.6 fold higher in the proliferative phase compared to mid-luteal phase. In the same study, oestradiol, medroxyprogesterone acetate and both hormones combined increased *VEGFA* mRNA expression by 3.1, 2.8, and 4.7 fold, respectively in endometrial cells.

Hypoxia, the inadequate supply of tissue oxygen, is a potent stimulator of angiogenesis by the up regulating the expression of VEGFA, VEGFR2 as well as numerous other genes. Hypoxia leads to the stabilisation and thus activation of the transcription factor, hypoxia-inducible factor 1A (HIF1A) which binds to the hypoxia response element of numerous genes such as VEGFA (Krock et al., 2011). Recently, it was shown that during menstruation endometrial oxygen concentrations were reduced, which was associated with increased HIF1A activity and stimulation of endometrial repair. Presumably, the latter involved the activation of angiogenesis (Maybin et al., 2018).

Macrophages regulate angiogenesis by participation in cell-to-cell interactions with endothelial cells, pericytes, and vascular smooth muscle cells. These interactions have been shown to regulate the development of the vasculature in both embryos and adults (Corliss et al., 2016). Moreover, activated tissue macrophages produce VEGFA in the endometrium which is controlled by steroid hormones (McLaren et al., 1996). It is feasible that transforming growth factor alpha and beta (TGFA and TGFB) production from macrophages that mediates the upregulation of VEGFA in the endometrium (Woidacki et al., 2015). Moreover, during normal pregnancy TGFB expression at the embryo-maternal interface is important for the induction of angiogenesis that occurs at implantation and early pregnancy (Larsson et al., 2001). Similarly, epidermal growth factor (EGF) is expressed during implantation and is known to stimulate the development of new blood vessels in the endometrium as well as being important for successful implantation (Torry et al., 1996). Therefore, the paracrine and endocrine factors that regulate angiogenesis have the ability to increase angiogenesis through inducing VEGFA production or its cellular pathway.

Intriguingly, there are several studies concerning the role of STC1 in promoting angiogenesis in tumours and other tissues (He et al., 2011). It is feasible that these effects are mediated by increasing VEGFA and/or VEGFR2 signalling as observed in human umbilical vascular endothelial cells (HUVECs) (Law and Wong, 2013). Equally, it has been observed that STC1 and 2 promoted angiogenesis in HUVECs directly. It appeared that STC2 induced angiogenic sprouting while STC1 mediated its effects through VEGFA/VEGFR2 pathways (Law and Wong, 2013).

1.8.2.4 **Inhibitors of angiogenesis**

An angiogenesis inhibitor is a substance that obstructs the formation of new blood vessels. Generally, angiogenesis inhibitors either are exogenously administered (drugs), or are endogenously produced as a part of the body's control of the angiogenesis process. This section will highlight endogenous inhibitors of angiogenesis.

Angiogenesis is regulated by a delicate balance between the various pro- and anti-angiogenic factors (Fig. 1.10). It is thought that, in normal adults, formation of new vessels is generally suppressed (except in the female reproductive tract) but if this balance is disturbed then there is increased blood vessel formation as seen in some pathologic conditions (such as tumour, optical disorders). Nyberg et al. (2005) proposed that the endogenous inhibitors of angiogenesis are essential regulators to switch off pathological angiogenesis.

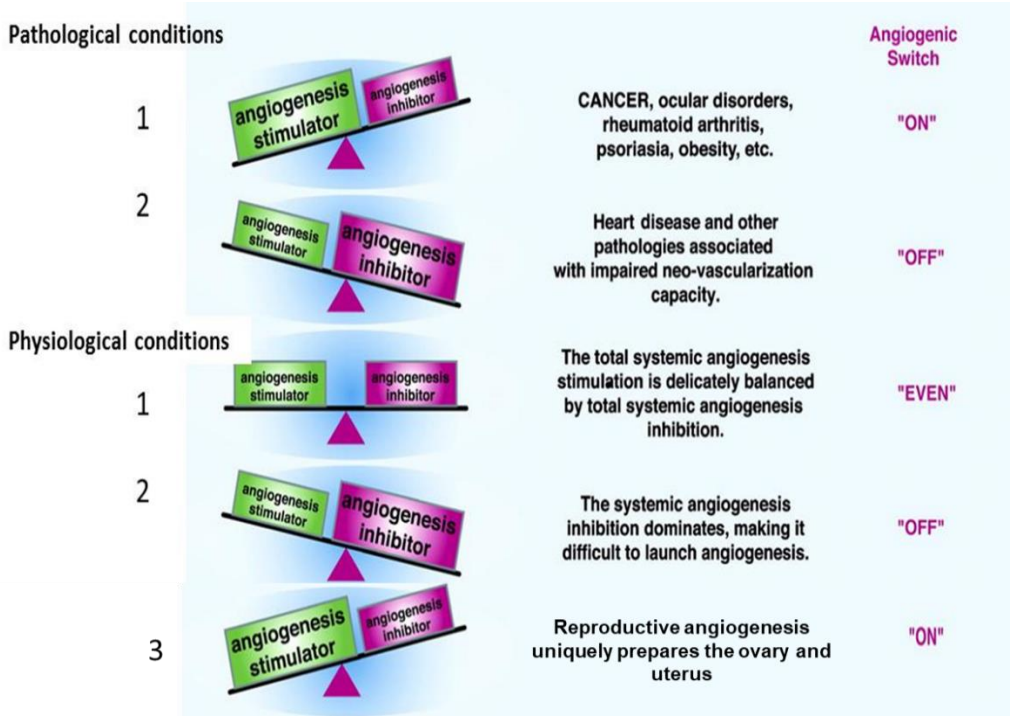


Figure 1.10: The balancing of angiogenesis with angiogenic stimulators and inhibitors in pathological and physiological states. Adapted from (Nyberg et al., 2005).

To recognise how angiogenesis is balanced under physiological conditions, a clarification of the existing angiogenesis inhibitors molecules is required.

It is recognised that components of the vascular basement membrane can regulate endothelial cell proliferation and migration (Darland and D'Amore, 1999). Based on this, Nyberg et al (2005) classified the endogenous inhibitors into two types: the matrix-derived and non-matrix derived inhibitors of angiogenesis. The majority of studies have concentrated on the effects of these inhibitors in cancer and their potential as a treatment. However, this next section will explain those that are related to the female reproductive system.

Matrix-derived inhibitors of angiogenesis

1) Thrombospondin 1 (TSP1) is a major anti-angiogenesis factor, produced by fibroblast cells (Good et al., 1990) that downregulates FGF2 and platelet derived endothelial cell adhesion molecule 1 (PECAM 1) expression. TSP1 is located in the stroma cells of the endometrium and is regulated by progesterone (Smith, 2001).

2) Arresten, an anti-angiogenic collagen fragment, is derived from the basement membrane of blood vessels (Timpl, 1996). Its production is increased significantly in the second and third trimester (from 16 weeks gestation), and this is particularly noticeable in pre-eclampsia (Yong et al., 2015). Arresten might block angiogenesis by interacting with integrin A1/B1 and blocking the binding site for type I collagen (Colorado et al., 2000).

3) Matrix metalloproteinases (MMPs) have been indicated as both pro- and anti-angiogenic factors. For example, MMPs can inhibit angiogenesis by releasing inhibitors of angiogenesis that are associated with the extracellular matrix. For example, MMP can proteolytically cleave certain collagen chains and plasminogen as well as modifying cell receptor signalling by cleaving their ligand-binding domains (Rundhaug, 2005).

Non-matrix-derived inhibitors of angiogenesis

1) Angiostatin inhibits the proliferation and migration of endothelial cells (O'Reilly et al., 1994). Angiostatin is cleaved from plasminogen different MMPs. The inhibition of MMP activity on plasminogen, reduced the release of angiostatin in the human endometrium. This, in turn, created a pro-angiogenic environment (Perchick and Jabbour 2003). While, Laschke and Menger (2012) suggested using angiostatin with a combination of other drugs in the treatment of endometriosis.

2) Chondromodulin-1 is one of the most potent anti-angiogenic factors known (Kusafuka et al., 2002). It was originally thought to be cartilage specific but was subsequently detected in the early pregnant mice uterus. Here, chondromodulin-1 had an inhibitory action on the embryo implantation by regulating tissue remodelling and angiogenesis at the fetal-endometrium interface. In so doing, it inhibited trophoblast migration and invasion (Miura et al., 2011).

3) Interferons can inhibit angiogenesis in tumour cells (Mitsuyasu, 1991), with its effects being mediated by inhibiting *VEGFA* gene transcription (von Marschall et al., 2003). Thus, it was suggested that interferons might have a role in pre-eclampsia with elevated interferon α impairing the interactions between the endothelial cells and trophoblast as well as suppressing the remodelling of spiral arteries (Andrade et al., 2015). The interaction between the angiogenic factors and angiogenic inhibitors can open new promises in treatment of pathological conditions of the reproductive system and might improve pregnancy rates in fertility clinics.

1.8.3 **Angiogenesis and vascularisation changes in the uterus**

The mechanism of new blood vessel growth in the endometrium takes place by elongation during the proliferative phase, while the development of the capillary plexus occurs though intussusception in the proliferative and secretory phases. Immunohistochemistry studies have not demonstrated any sprouting angiogenesis in the human endometrium and a sprouting biomarker (integrin A5/B3) has only been detected in blood vessels that already exist (Asahara et al., 1999). This would suggest that sprouting is not involved in the endometrial angiogenesis process.

1.9 Early Pregnancy Loss

Even when there is connection between the embryo (blastocyst) and the endometrium, unsuccessful implantation often occurs. There are many possible reasons for this failure: absence of embryo implantation when the endometrium sheds (Simon and Laufer, 2012). In natural cycles, there is a 30% chance that a woman achieves a successful pregnancy (Zinaman et al., 1996) and around 60% in women with a positive pregnancy test (hCG) at week 20 of gestation (Norwitz et al., 2001). Among the pregnancy losses, implantation failures represent approximately 75% of these failures (Wilcox et al., 1988).

The loss of embryos has been well stablished in pigs, but the specific pathway for these losses is still not yet recognised. It is estimated that between 20-40% of embryos and fetuses are lost before gestational day 40 (Bidarimath and Tayade, 2017). However, 89% of the eggs released at ovulation can be recovered from the oviduct/uterus and approximately 95% of these recovered eggs are fertilised. Henceforth, around 22% of these fertilised eggs degenerate between pregnancy days 6 and 9, with greater losses of around 28% of embryos occurring between days 13 and 18 of pregnancy (Perry and Rowlands, 1962). The causes for these high losses are believed to limited uterine space, defective embryos (e.g. chromosomal defects, absence of mitotic figures; Mcfeely (1967)) or abnormal uterine environments (Webel and Dziuk (1974). Moreover, alterations or imbalance of intrauterine factors, including, angiogenic and antiangiogenic growth factors (Keys and King, 1990), cytokines (Geisert et al., 2014) and chemokines (Bidarimath et al., 2016) are highly likely to play an important role in conceptus health and hence the prevention of its loss.

1.10 Manipulation of Reproduction to Improve Pregnancy Success

1.10.1 Assisted reproductive technologies

Assisted reproductive technology (ART) is a term that describes all of the technologies that are used to achieve pregnancy. ART is defined according to the World Health Organization (WHO) as:

"*All treatments or procedures that include the in vitro handling of both human oocytes and sperm, or embryos, for the purpose of establishing a pregnancy. This includes, but is not limited to, in vitro fertilization and embryo transfer, gamete intra-fallopian transfer, zygote intra-fallopian transfer, tubal embryo transfer, gamete and embryo cryopreservation, oocyte and embryo donation, and gestational surrogacy. ART does not include assisted insemination (artificial insemination) using sperm from either a woman's partner or a sperm donor*" (Zegers-Hochschild et al., 2009).

This thesis will consider both *in vitro* fertilisation (IVF) and intra-cytoplasmic sperm injection (ICSI) (Fig. 1.11). IVF is the procedure of enabling fertilisation of the sperm and ovum to take place in artificial oviduct environments (i.e. in the laboratory). ICSI is a treatment for male factor infertility, that is defined according to the WHO as: "*a procedure in which a single spermatozoon is injected into the oocyte cytoplasm*" (Zegers-Hochschild et al., 2009).

Both IVF and ICSI involve the same procedures in terms of the preparation of women for oocyte retrieval (OoR) and embryo transfer (ET). Women usually undergo controlled ovarian stimulation that uses exogenous gonadotrophins (FSH

alone or with LH) to stimulate the ovaries after the endogenous production of gonadotrophins has been inhibited. This inhibition is induced through the continued use of gonadotrophin releasing hormone agonist or antagonist. Consequently, this pharmacological intervention induces the development of multiple ovarian follicles that can be aspirated to obtain multiple oocytes (Zegers-Hochschild et al., 2009).

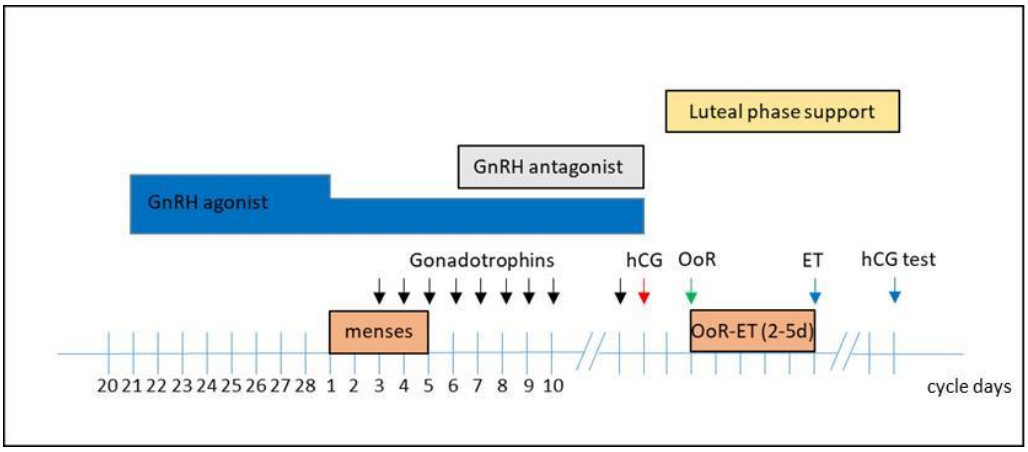


Figure 1.11: Protocol for ART procedures. Represents the alternative regimens of a long induction protocol (GnRH agonist) or short induction protocol (GnRH antagonist). Human chorionic gonadotrophin (hCG), oocyte retrieval (OoR) and embryo transfer (ET). The x-axis is day relative to first day of menstruation.

1.10.2 **Therapeutic use of progesterone supplementation**

Luteal phase deficiency (LPD) is a condition where there is either insufficient progesterone secretion to allow normal endometrial preparation for embryo implantation, or the uterine cells do not respond to progesterone (Mesen and Young, 2015). It is very important to understand the heterogeneity within LPD and its overlap with other reproductive disorders.

The mechanism of LPD is not fully understood, but there are many physiological and pathological conditions that are associated with this disorder. LPD is usually associated with stimulated ovarian cycles and a consequence of using exogenous

gonadotrophin hormones (Yding Andersen and Vilbour Andersen, 2014), polycystic ovary syndrome (Joseph-Horne et al., 2002), endometriosis (Cunha-Filho et al., 2003), or thyroid disorders (Practice Committee of the American Society for Reproductive Medicine, 2015). Moreover, progesterone deficiency might be a consequence of aging (Prior, 2005) or stress (Kajantie and Phillips, 2006). This study will focus on luteal phase deficiency in stimulated cycle following ART procedures.

A stimulation cycle is the primary reason for LPD due to the high level of steroid synthesis that follows controlled ovarian hyper-stimulation (Fatemi et al., 2007, Polyzos et al., 2010). There are several mechanisms that have been proposed to explain LPD in ART. Firstly, the premature regression of the corpus luteum as a result of reduced steroidogenic capacity (Hoff et al., 1983, Fatemi et al., 2007). Secondly, premature endometrial development that occurs due to elevated progesterone immediately after oocyte collection that leads to asynchrony between embryo and endometrium receptivity. Third, reduced LH secretion after the continued use of GnRH agonist or antagonist therapies thus providing less luteotrophic support (Devoto et al., 2009).

1.10.2.1 **Progesterone Supplementation in ART**

In IVF/ICSI cycles, luteal phase deficiency occurs after ovarian induction in both GnRH agonist and antagonist regimes (Beckers et al., 2003). Therefore, providing luteal phase support via progesterone or hCG will rescue normal corpus luteum function and increase pregnancy and birth rates in ART cycles (Leth-Moller et al., 2014). There have been reports that luteal support with hCG might cause ovarian hyper stimulation syndrome in some conditions, hence the use of hCG to rescue LPD has been discouraged, (Fatemi et al., 2007). Therefore, progestogen supplementation

is by far the most commonly used luteal phase support to improve implantation rates. Consequently, there has been a wide range of luteal phase support regimes developed to assist with early pregnancy and it is now routine to provide exogenous progesterone to women undergoing fresh or frozen embryo transfers (Kaser et al., 2012).

Progestogen treatment can be given intramuscularly (IM), vaginally (Oler et al., 2017), subcutaneously (SC) (Klement et al., 2018), orally (Lee et al., 2017) or rectally (Harb and Coomarasamy, 2017). Oral progesterone is easy to take, but it has many complaints about its uncomfortable side effects (e.g. dizziness and feeling unwell) and so its uptake has been low (Licciardi et al., 1999).

Several studies have shown that the IM route may achieve higher pregnancy rates, but it has lower tolerability by the women undergoing the treatment. This is likely to be due to the need for daily injection for a long period of time (Propst et al., 2001). More recently, the most widely accepted form of progestogen supplementation is the vaginal route. It has fewer reported side effects and limited meta-analyses have indicated that pregnancy outcome after vaginal supplementation was similar to that for IM treatment (Zarutskie and Phillips, 2009).

1.11 Overarching hypothesis

The receptivity of the endometrium to the embryo is crucial for the establishment of pregnancy in most mammals including women and pig. The overarching hypothesis was that the timing of endometrial receptivity is regulated by the existence of the embryo (and its secretion) as well as the steroid hormones produced by the ovary (or administered exogenously). The inadequate or inappropriate timing of these secretions will lead to a lack of synchrony between the ovary, endometrium and embryo resulting in implantation failure. The hypothesis was investigated using clinical data, *ex vivo* tissue and novel *in vitro* culture systems.

1.11.1 Aims of the PhD Thesis

1) To conduct an extensive and comprehensive meta-analysis of the specific benefits of progestogen supplementation in different ART regimens to clinical pregnancy rates in women. *There is a wide range of different progestogen supplementation regimen used in clinical practice with limited evidence-based data as to which is optimal. Thus, this analysis initially aims to establish the relative benefit of progestogen supplementation relative to untreated women. The next step was then to compare 1) the different routes of administration, 2) the start time of progestogen supplementation and whether it is influenced by route, 3) the duration of supplementation and 4) the relative benefit of adding oestrogen co-treatment to the progestogen supplementation.*
2) To elucidate the association between endometrial progesterone levels and embryo development (number, stage and oestradiol secretion) during the establishment of pregnancy in the pig. *This will establish the inter-*

relationship between these crucial factors during early pregnancy providing the framework for subsequent experiments.

3) To define the temporal-spatial expression of key endometrial receptivity markers (namely ITGA5/B1, PGR, ESR1 and STC1) during the porcine peri-implantation period and determine their direct regulation by steroid hormones *in vitro*.

4) To determine the effects of stanniocalcin 1, prostaglandin E2, prostaglandin F2α and pregnant uterine flushes on the formation of endometrial endothelial cell networks *in vitro*. *These molecules are produced by the endometrium and/or embryo during early pregnancy and are known to regulate angiogenesis. Angiogenesis is an important component in establishment of pregnancy and placentation and thus these investigations will provide critical information as to how these are regulated.*

CHAPTER 2 Efficacy of progestogen supplementation in women undergoing assisted reproductive technology treatment: A meta-analysis

2.1 **Introduction**

Every year, around one million couples undergo IVF/ICSI procedures worldwide (Zegers-Hochschild et al., 2009). In these treatments, luteal phase deficiency (LPD) is a common occurrence after the ovarian stimulation and ovulation induction regimes. This inadequate and inappropriate production of progesterone (P4) during the subsequent luteal phase results in the inappropriate functioning of the endometrium (i.e. altered receptivity marker expression) and ultimately the failure of synchronisation between the embryo transfer and the window of implantation. This has been suggested to be the single most crucial factor limiting a successful pregnancy outcome in ART (Daya, 2009, Ebrahimi et al., 2010).

The most plausible reasons for the luteal phase deficiency are: 1) premature regression of the corpus luteum as a consequence of increased oestradiol concentrations. This will lead to long-term LH suppression and inhibition 3β-hydroxysteroid dehydrogenase (3β-HSD) expression (Hoff et al., 1983, Beckers et al., 2003, Fatemi, 2009, Yding Andersen and Vilbour Andersen, 2014). 2) Markedly reduced secretion of LH secretion for 10 days after the use of GnRH agonist or antagonist ovarian induction protocols (Tavaniotou et al., 2001, Devoto et al., 2009).

In ART cycles, hCG treatment is usually used to mimic the LH surge for final oocyte maturation and to trigger ovulation. However, hCG has a longer half-life than LH (>24h vs <60 minutes) (Practice Committee of the American Society for Reproductive Medicine, 2008). This means hCG will increase LH/CG receptors

(LHCGR) stimulation for ovulation but ultimately will result in the earlier down-regulation of LHCGR in the luteal phase (Evans and Salamonsen, 2013). However, in natural cycles, there are increases in both LH and FSH at ovulation. Consequently, the use of GnRH agonists to induce ovulation has been suggested since it stimulates the release of both LH and FSH as well as avoiding ovarian hyper-stimulation. This is, in part, due to GnRH agonists also having a shorter half-life than hCG. Despite this, pregnancy rates after GnRH treatment were not improved versus hCG treatment (Beckers et al., 2003, Fatemi and Popovic-Todorovic, 2013).

In recognition of LPD, the therapeutic use of hCG supplementation during the luteal phase was developed to maintain the corpus luteum for longer and to further stimulate progesterone and oestradiol secretion. This would allow the blastocyst more time to develop and implant (Pritts and Atwood, 2002). However, unsurprisingly luteal support with hCG was associated with ovarian hyper-stimulation syndrome in some women (Hoff et al., 1983) and therefore its use in LPD was discouraged (Fatemi et al., 2007). This ultimately led to the development of therapeutic progestogen supplementation to replace hCG treatment. Today, progestogen supplementation is by far the most widely used form of luteal phase support for women undergoing ART with fresh or frozen embryo transfer (van der Linden et al., 2011, Kaser et al., 2012, Palomba et al., 2015).

Many different routes of administration for progestogen supplementation have been suggested and developed. The most commonly used routes are intramuscular (IM) or vaginal, but oral, subcutaneous (SC) and rectal routes have been used (Zarutskie and Phillips, 2009, Tomic et al., 2015). The oral route is the easiest to administer but it has many complaints for its uncomfortable side effects (e.g. tiredness and motion

sickness) (Gibbons et al., 1998, Tavaniotou et al., 2000). Furthermore, there are reports that pregnancy rates after oral micronized progesterone were significantly lower than via IM or vaginal routes (Licciardi et al., 1999). On the contrary, the administration of a different progestogen (dydrogesterone) orally yielded similar pregnancy rates as vaginal micronized P4 with improved patient satisfaction and tolerability (Saharkhiz et al., 2016). Tomic et al. (2015) went further to recommend this as the routine regime for luteal support.

There are several studies stating that the highest pregnancy rates were achieved with IM progestogen supplementation. However, there are reports of lower tolerability because it needs prolonged and daily injections (Propst et al., 2001). Conversely, a small meta-analysis study indicated that pregnancy rates were similar with intramuscular or vaginal administration of progestogen (Zarutskie and Phillips, 2009). Given, the uncomfortable side effects from both IM and oral routes and perceived comparable pregnancy rates between IM and vaginal routes, the most widely accepted route currently is vaginal (Polyzos et al., 2010). However, a comprehensive analysis of the full benefit of using progestogen as luteal phase support is required and there is a lack of information about the optimum doses, start times and duration of progestogen supplementation for luteal phase support (Dodd et al., 2005, Russell et al., 2015).

For example, Tay and Lenton (2002) stated that the dose and time of progestogen supplementation after ovulation induction is often not efficient to prevent suboptimal levels of progestogen during the window of implantation. They recommended not starting treatment until day 4 or 5 after oocyte retrieval. Indeed, a recent meta-

analysis suggested that more control trials are required to elucidate the optimal starting time (Connell et al., 2015).

In terms of duration of supplementation, luteal phase support for the first 12 weeks delayed miscarriage but did not in fact improve live birth rate compared with 2 weeks of luteal phase support (Proctor et al., 2006). A study of UK fertility clinics demonstrated that there is no agreement on the duration of treatment, with 24% of clinics stopping luteal support at the time of hCG test, while 40% sustained treatments up to 12 weeks and the remaining clinics prolonged treatments for > 12 weeks (Russell et al., 2015).

It is clear that progestogen treatment provides a significant improvement to pregnancy outcomes (Palomba et al., 2015). However, continued research is required to critically evaluate the optimal routes, dose and timing of onset of supplementation and the potential combination with other drugs. Therefore, the focus of this meta-analysis study was to determine which routine timings and routes of progesterone are most effective in terms of luteal support.

2.2 Methods

2.2.1 Search strategy

An extensive literature search was performed using the following websites: Google Scholar, PubMed, Medline and Web of Science (of publications 1980 to July 2017). For this purpose, the principal search term was “progestogen supplementation” in conjunction with “vaginal, intramuscular, oral or rectal”. A separate search was conducted with the following terms “assisted reproductive techniques or ART”, “*in vitro* fertilisation or IVF”, “intra-cytoplasmic sperm injection or ICSI”, “intrauterine

insemination or IUI" or "fresh embryo transfer or frozen embryo transfer" in the title, abstract or keywords section. The next stage was to reduce these to those that were randomised controlled trials (RCT) or retrospective studies. In this regard, the majority of the RCT studies did not include a "control" group and more commonly two or more progestogen treatments were compared in the RCT. Additionally, manual searching was performed from all the relevant review articles, Cochrane reviews and previous meta-analysis studies.

The PRISMA guidelines of preferred reporting items for systematic reviews and meta-analyses were used (Moher et al., 2009) to confirm the eligibility of the articles. Initially, the title, abstract and keywords were screened and this excluded 327 articles. Then, the full text of each article was retrieved and was carefully reviewed.

Inclusion criteria: Articles were included where the following standards were met: 1) Included women undergoing assisted reproductive technology (ART) with fresh or frozen embryo transfer or intra-uterine insemination; 2) Used ovarian stimulation to promote follicular development and ovulation; 3) Stated an assessment of clinical pregnancy (e.g. presence of a gestational sac) including the number of women that were pregnant in each control or treatment group.

Exclusion criteria: 1) assignment of luteal phase support progestogen treatment group after a positive hCG pregnancy test; 2) the hCG treatment was the only luteal support.

These articles were then categorised in either fresh embryo transfer (ET), frozen ET or intra-uterine inseminations (IUI) for the separate meta-analysis. There were 94 articles utilising fresh ET, 13 articles with frozen ET and 6 articles with IUI (Fig

2.1). Within the articles, the different reported treatments were assigned a unique number or study group. This meant that in each article there were between two and four study groups resulting in 211 study groups.

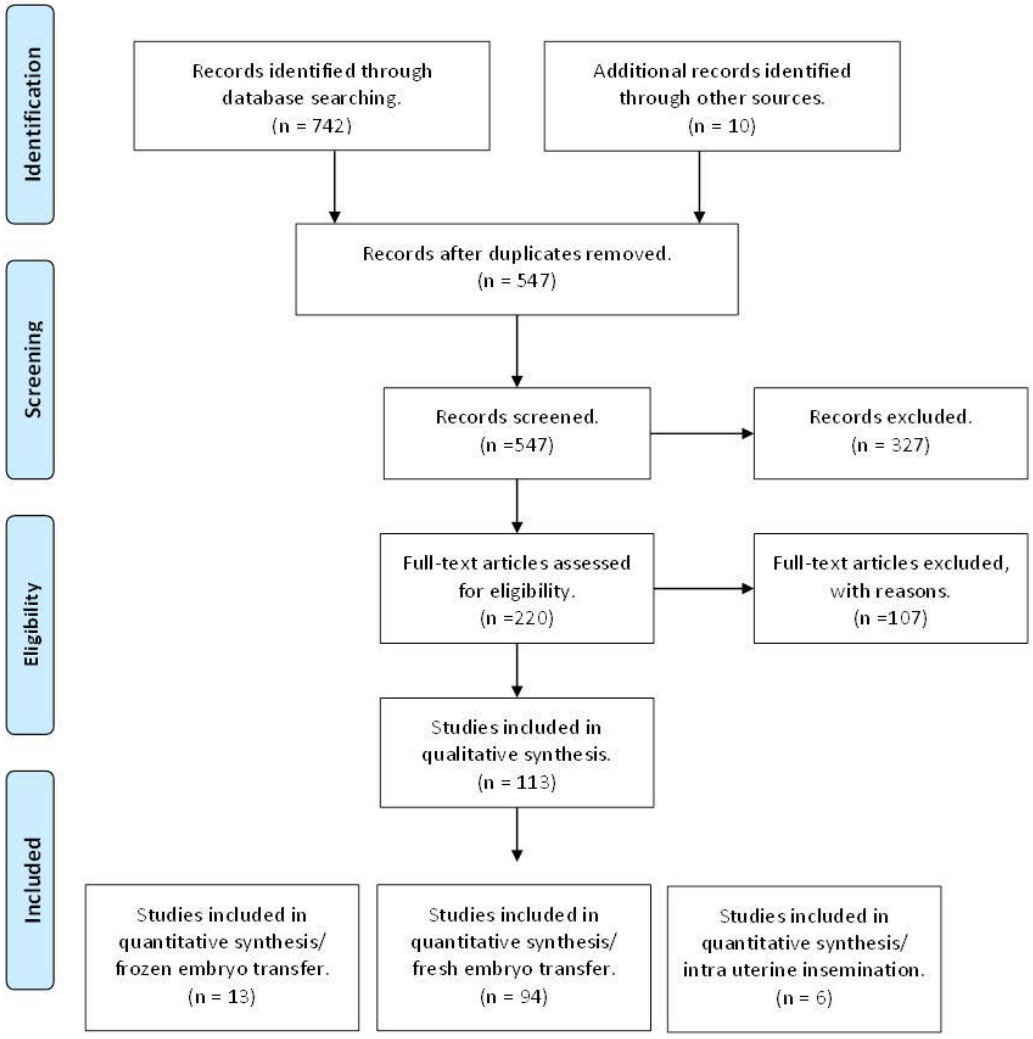


Figure 2.1: A PRISMA flow chart for the identification of articles to include in the meta-analysis.

Table 2.1 shows the distribution of the study groups with fresh ET weighted according to the number of women that were involved in each one. There were 7 study groups including more than 500 women, 17 study groups with between 301 and 500 women, 16 study groups were between 201-300 women. However, the

majority of the study groups had between 20 and 200 women (101-200 women, n=53; 51-100, n=68 women and 20-50 women, n=43). Only 7 study groups had less than 20 women.

Overall, there were 115 study groups where supplementation was via the vaginal route, 58 study groups via IM route and, 15 study groups via oral route. Two study groups for each rectal and SC route and eight study groups where no treatment (control) was administered. The remaining 11 study groups, multiple routes (mixed) were used for supplementation of progestogen (see Appendix 1).

Table 2.1: The distribution of study groups according to the number of women and progestogen route in ART where fresh embryo transfer was employed.

NUMBER OF WOMEN IN EACH	ROUTE OF PROGESTOGEN SUPPLEMENTATION	NUMBER OF STUDY GROUPS
>500	IM	1
	VAGINAL	6
301-500	IM	3
	MIXED	1
	ORAL	3
	SC	2
	VAGINAL	8
201-300	IM	6
	VAGINAL	10
101-200	CONTROL	1
	IM	15
	MIXED	3
	ORAL	2
	VAGINAL	32
51-100	CONTROL	3
	IM	19
	MIXED	3
	ORAL	4
	RECTAL	2
	VAGINAL	37
20-50	CONTROL	4
	IM	13
	MIXED	4
	ORAL	5
	VAGINAL	17
<20	IM	1
	ORAL	1
	VAGINAL	5

2.2.2 **Processing of data**

During this process, the following information was extracted from the accepted articles: route of progestogen supplementation/formulation, dose of progestogen administered, the starting time of progestogen supplementation and duration of treatment (this information was used to calculate total dose of progestogen

administered). Additional information was used to create the database: (a) demographic information (e.g. place, and date of research, number of cycles or women, time of randomisation and the age of participants; (b) type of ART procedure and aetiology of infertility and (c) other treatment information including, gonadotrophin used, the ovulation induction regime (oocyte trigger) type of GnRH agonist protocol. It was not possible to obtain all information for each study group from the article since key information was frequently omitted in the original manuscript.

Progestogen supplementation in IVF/ICSI cycles with fresh embryo transfer

Classification of groups:

1. Route of administration

The relative benefits of using different routes to administer progestogen on clinical pregnancy were compared with the routes classified as follows:

- Intramuscular (IM)
- Vaginal
- Oral
- Rectal
- Subcutaneous (SC)
- Control (no progesterone supplementation)

The control group was used as the reference group in the initial analyses. In the subsequent analysis, the vaginal route was used as the comparator since this was the route in which most women were treated. When comparing the different routes of

administration, study groups in which multiple routes were employed (mixed) were excluded from that analysis only. Further details on the articles are in Appendix 1.

2. Time to commence progestogen supplementation

The commencement of progestogens supplementation was the key component of this meta-analysis. Five subgroups were created based on the time of progestogen supplementation was commenced in each study group and are as follows:

- Before oocyte retrieval
- The day of oocyte retrieval: (at oocyte retrieval or the evening of oocyte retrieval).
- Between oocyte retrieval and embryo transfer.
- On the day of embryo transfer.
- After embryo transfer.

The control group was used as the reference group in the initial analyses. In the subsequent analysis, the "at oocyte retrieval" group was used as the comparator since this was the time in which treatment was commenced in most women. Further details on the articles are in Appendix 1.

3. Exact day when progestogens supplementation commenced:

The study groups were categorised according to the day on which progestogen supplementation commenced relative to the day of oocyte retrieval as follows: (1) at oocyte retrieval (the day and evening of), or on (2) the first, (3) second and (4) third day after oocyte retrieval. This data was mostly generated from that stated directly in the article. However, in some articles, it was necessary to utilise other information

as the oocyte retrieval to embryo transfer time. The study group was excluded if there was insufficient information to state the exact day.

4. The co-treatment with oestrogen:

For this analysis, only those articles that directly compared the potential benefit of oestrogen co-treatment with progestogen supplementation were incorporated. This analysis did not take into account the oestrogen route of administration and/or starting time of treatment. The study groups with oestrogen treatment were used as the comparator "control group".

5. Duration of progestogen supplementation:

The duration of progestogen supplementation was classified in to two groups (≤3 and >3-10 weeks) for both clinical pregnancy and live birth rates.

Progestogen supplementation in IVF/ICSI cycles with frozen embryo transfer

In this analysis, the effects of progestogen supplementation on the clinical pregnancy rate was investigated with respect to the route of supplementation, which were as follows:

- Vaginal route.
- Other route.
- Control (no progestogen supplementation)

Further details on the articles are in Appendix 2.

Progestogen supplementation in intrauterine insemination IUI

The relative benefit of progestogen supplementation in the luteal phase was compared with a classic control (no treatment) group. The study groups were further sub-divided based on the treatment that was used for ovarian stimulation as follows:

- Clomiphene citrate
- FSH

Further details on the articles are found in Appendix 3.

2.2.3 **Statistical analysis**

The statistical analysis was performed after consultation with Dr Jim Craigon (School of Biosciences, University of Nottingham). There was considerable heterogeneity in the experimental design between the different articles, thus a classical meta-analysis statistical approach with Forest plots was not feasible. Binominal logistic regression was performed in GenStat 17th Edition (VSNi, Hemel Hempstead, UK) to determine odds ratio, its significance and confidence interval. In each analysis, the "number of subjects" was the number of women treated, "number of successes" was the number of women with a clinical pregnancy for that study group and the "model to be fitted" included the appropriate factor as described below. The initial meta-analysis determined the odds ratio of the benefit of progestogen supplementation vs "no progestogen supplementation" control group on clinical pregnancy. The control group contained those women treated with placebo or patients who received no treatment under a randomisation protocol.

1) Route of administration: initially, the effect of different routes of progestogen supplementation (IM, oral, rectal, SC and vaginal) were compared with

the no treatment (control) group. There were a further 11 study groups that used multiple routes therefore these were excluded from this analysis but were used for different analysis such as time of supplementation. This analysis was used as an initial analysis for the whole dataset and to establish whether progestogen supplementation was beneficial.

2) Time to commerce supplementation: the initial comparison compared different time of supplementation (before OoR, at OoR, between OoR and ET, at ET and after ET) with no treatment group acting as the comparator. The follow-up analysis used the most common commencement time (at OoR) as the comparator against the other starting times (before OoR, between OoR-ET, at ET and after ET).

3) Intramuscular vs vaginal comparison: the mostly commonly used routes for luteal phase progestogen supplementation were IM and vaginal routes. The effect of different start times in each route (IM and vaginal independently) were compared with at oocyte retrieval (OoR) group as the comparator. This was followed up by directly comparing IM and vaginal routes according to the different start time of supplementation (before OoR, at OoR, between OoR-ET, at ET and overall). In each case, the vaginal route was the comparator. Following that, an additional comparison between IM and vaginal routes was performed with the articles stratified by publication date (1990-1999, 2000-2009 and 2010-2017). The search did not show any study with vaginal route in the 1980s.

4) The optimal start day: The database was further examined to compare the different exact treatment start day with commencing treatment on the day of oocyte retrieval. The times categorised were day of oocyte retrieval, +1 to 1.5 days, +2 to 2.5 days and +3 to 4 days.

5) The effect of co-treatment with oestrogen: In this analysis, only those articles that compared no oestrogen with oestrogen treatment were included. The data was analysed in two separate ways: (1) with the data categorised by progestogen supplementation route: vaginal, IM or all routes combined and (2) with data categorised into "at oocyte retrieval" or "between oocyte retrieval and embryo transfer". In all cases, the comparator was the no oestrogen treatment group.

6) The effect of duration of progestogen supplementation: In this analysis, progestogen supplementation for 3 weeks and less (the comparator group) was compared with supplementation lasting for between 3-10 weeks. Initially, all routes of administration were included, but this was then split down according to either intramuscular or vaginal route of administration. The effect of duration of progestogen supplementation on clinical pregnancy and live birth rates were analysed.

7) The effect of luteal phase support on clinical pregnancy rate after frozen ET: In this analysis, the relative benefit of progestogen supplementation was compared with no treatment control group. The data was further split down to only include the most common route of administration (vaginal, N=17 study groups).

8) The effect of progestogen supplement after intrauterine insemination (IUI): In this analysis, the benefit of using progestogen supplementation was compared with no treatment (control) group. The data was further split down according to the type of ovulation induction used (clomiphene citrate or FSH).

The data are presented as odds ratio (OR) with 95% confidence intervals (CI) alongside the number of study groups (N) and women (n). In each case, the odds

ratio (with 95% CI) and statistical significance are relative to the comparator group rather than between groups.

2.3 Pregnancy outcomes of progestogen supplementation in IVF/ICSI cycles with fresh embryo transfer

2.3.1 Objectives

1) Compare the benefits of progestogen supplementation vs no treatment (control) on clinical pregnancy rate, according to route and start time of treatment.

2) Elucidate the optimal start day after oocyte retrieval for progestogen supplementation to be commenced.

3) Determine the differences between IM and vaginal routes for progestogen supplementation on clinical pregnancy rate at optimal start times

4) Determine the efficacy of incorporating oestrogen treatment within the progestogen luteal phase support regime on clinical pregnancy rates.

2.3.2 Results

2.3.2.1 The characteristics and demographics of the included study groups

In total, 94 full text articles were assessed for eligibility (211 study groups with 29,357 women) and fitted the selection criteria. In the search methodology, the term "randomised control trial" was used, however the majority of these articles were not classical "control" studies as they did not have a non-treated or control group. In fact, there were only 8 control study groups across the meta-analysis. The studies were often randomised clinical trials in which comparisons were made between two or more different treatments or treatment regimes. Articles in which retrospective

analysis of two or more progestogen treatments were performed were also included in the meta-analysis.

The studies were conducted across the World with the greatest proportion originating from continental Europe (37%). A relatively low percentage of these were performed in Africa and UK (1% and 4% respectively). The others were from USA (21%), Asia Pacific (12%) and the Middle East (24%; Fig 2.2).

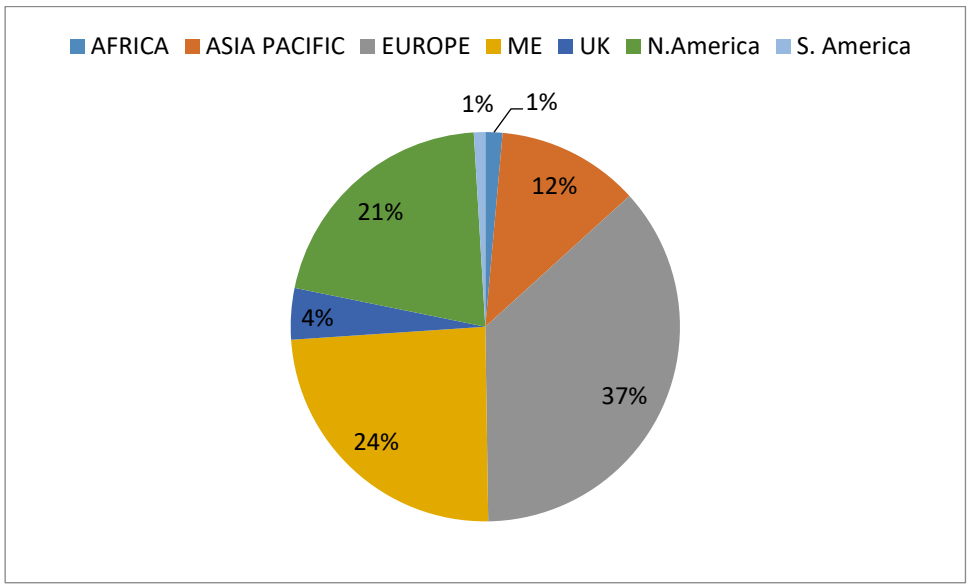


Figure 2.2: Global distribution of the study groups that investigated the effects of progestogen supplementation on clinical pregnancy rates in women undergoing assisted reproduction. In total, 211 study groups were identified. ME is Middle East and UK is United Kingdom

The age range across the whole dataset was as expected, with the youngest individual being 18 whilst the oldest woman was 42. In the vast majority of studies, there was matching of age groups across the different treatments. The mean age was 32.5 years across all the studies. The fertilisation rates and number of embryos transferred were generally stated but not in all study groups. In most cases, the ovarian stimulation protocol was described with the majority using long induction protocols (N=124), a short antagonist protocol being conducted in 18 study groups. There were a further

16 study groups with short flare-up protocols. In the remaining study groups, either both long and short protocols were used or it was not described. A variety of ovarian induction hormones were used within these protocols including FSH (N=88), human menopausal gonadotrophins (HMG) (N=23), both FSH and HMG (N=47) while 53 study groups did not mention which gonadotrophin type was used. The most common treatment for triggering final oocyte maturation was hCG (N=190 study groups), however GnRH was used in 7 study groups and the other 14 study groups did not state the method of oocyte triggering.

Aetiology of the specific infertility was mentioned in only 17 study groups. Two study groups included women with endometriosis and two groups included polycystic ovary syndrome (PCOS) women. The remaining 13 study groups were women at risk of ovarian hyper-stimulation syndrome (OHSS). Moreover, there was insufficient information to dissect the benefits of progestogen supplementation with different underlying aetiologies to warrant further investigation.

2.3.2.2 **The benefit of progestogen supplementation via different routes of administration on clinical pregnancy rate in women undergoing ART in comparison to untreated women**

In current clinical practise, progestogen supplementation is routinely performed however; there is remarkably limited to support the many different regimens employed. This initial analysis addressed this and quantified the benefit. There was a significant benefit of progestogen supplementation compared to the untreated control women. All routes of progestogen supplementation improved clinical pregnancy outcome compared to control women: IM (OR=3.57; $p<0.001$), SC (OR=2.62; $p<0.001$), vaginal (OR=2.52; $p<0.001$), oral (OR=1.86; $p<0.001$) or rectal routes (OR=1.75; $p<0.05$). In this analysis, the relative benefit of the different routes was not compared. It should be noted that there were only two study groups in each of the SC and rectal routes. In terms of clinical pregnancy rates, this was equivalent to an increase from 18% in untreated women (N=8 study groups) to 43% in IM group (N=58), 29% in oral group (N=15), 27% in rectal group (N=2), 36% in SC group (N=2) and 35% in vaginal group (N=115; Fig. 2.3). The 11 study groups, where progestogen was administered by multiple different routes were excluded from this particular analysis as these could not be assigned to a particular category. They were though included in subsequent analysis. This initial analysis revealed that all routes of administration increased the pregnancy outcome. The subsequent analysis then directly compared the relative benefit of intramuscular and vaginal routes of administration.

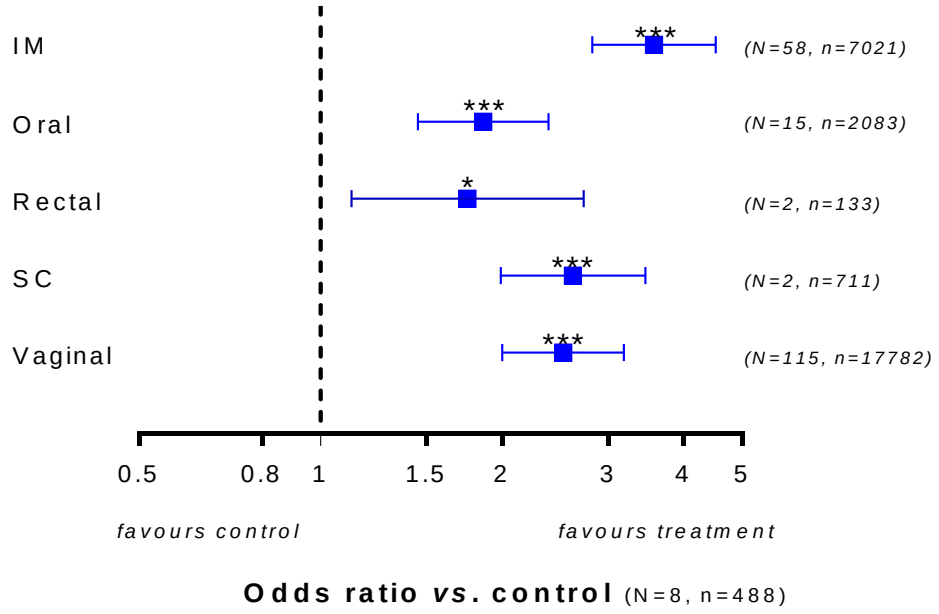


Figure 2.3: The odds ratio (± 95% confidence intervals) for the comparative increase in clinical pregnancy rate across different routes of progestogen supplementation versus no progestogen treatment in women undergoing ART with fresh embryo transfer. The different routes were intramuscular (IM), oral, rectal, subcutaneous (SC) and vaginal. Significant differences are indicated as *, p<0.05, ***, p<0.001 following binomial logistic regression analysis. The comparator was the no progestogen supplementation group (dashed line). The relative differences between the routes were not compared. For each route, N=number of study groups and n=total number of women treated.

2.3.2.3 The effect of different times at which progestogen supplementation commenced affected clinical pregnancy rate in comparison to untreated women

This study next compared whether different start times of progestogen supplementation improved clinical pregnancy outcome compared with the untreated control women (Fig. 2.4). There was a clear benefit to progestogen supplementation at all start times (before OoR [OR=1.57, p<0.01], at OoR (OR=2.76, p<0.001), OoR-ET (OR=3.37, p<0.001), at ET [OR=2.17, p<0.001] and after ET [OR=2.45, p<0.001]).

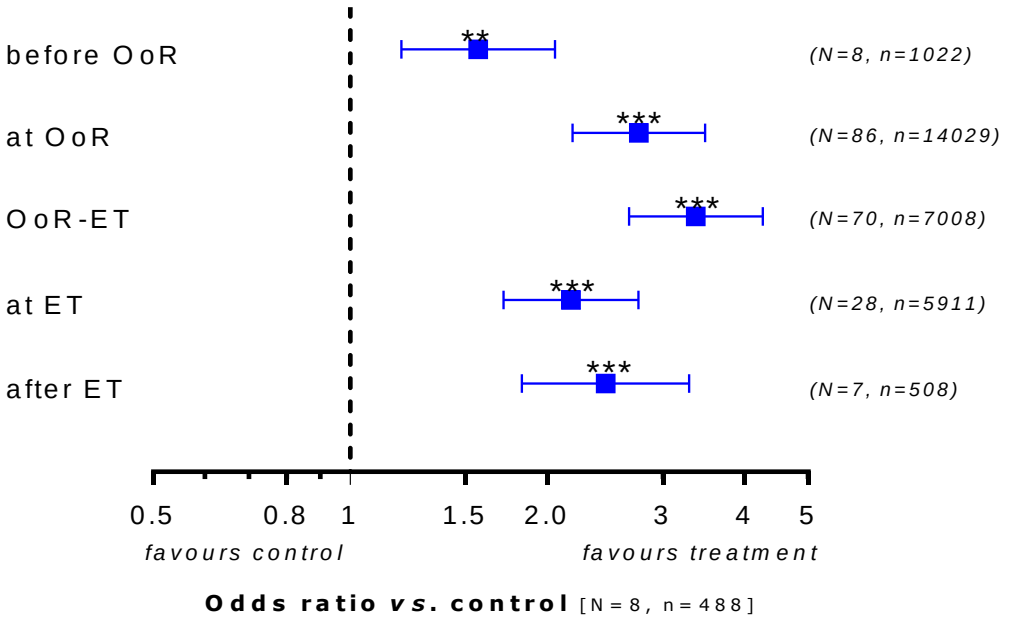


Figure 2.4: The odds ratio (± 95% confidence interval) for the relative benefit to clinical pregnancy rate for the different start time of progestogen supplementation in comparison with no progestogen treatment in women undergoing ART with fresh embryo transfer. The administration times were before oocyte retrieval (before OoR), at oocyte retrieval day (at OoR), between oocyte retrieval and embryo transfer (OoR-ET), at embryo transfer (at ET) and after embryo transfer (after ET) N=number of study groups, n=number of women. The dotted line represents the comparative odds ratio for the control group. There were significant treatment effects: **, p<0.01; ***, p<0.001 versus untreated control women.

A primary focus of this meta-analysis was the start time of progestogen supplementation, which was classified as; before oocyte retrieval (N=8 study groups), the day of oocyte retrieval (N=86), between oocyte retrieval and the embryo transfer (N=70), on day of embryo transfer (N= 28) and after embryo transfer (N=7 study groups). Having established, that there was a clear benefit to clinical pregnancy rates of progestogen supplementation at all start times, the next step was to compare the different start times to the most commonly used start time, namely at oocyte retrieval. Starting progestogen supplementation at OoR-ET increased clinical pregnancy outcome (OR=1.22; p<0.001) while clinical pregnancy outcome was significantly lower when progestogen supplementation was started before OoR

(OR=0.57; p<0.001) or at ET (OR=0.79; p<0.001). There was no statistical difference between at ET and at OoR groups (OR=0.89; p>0.05) (Fig 2.5).

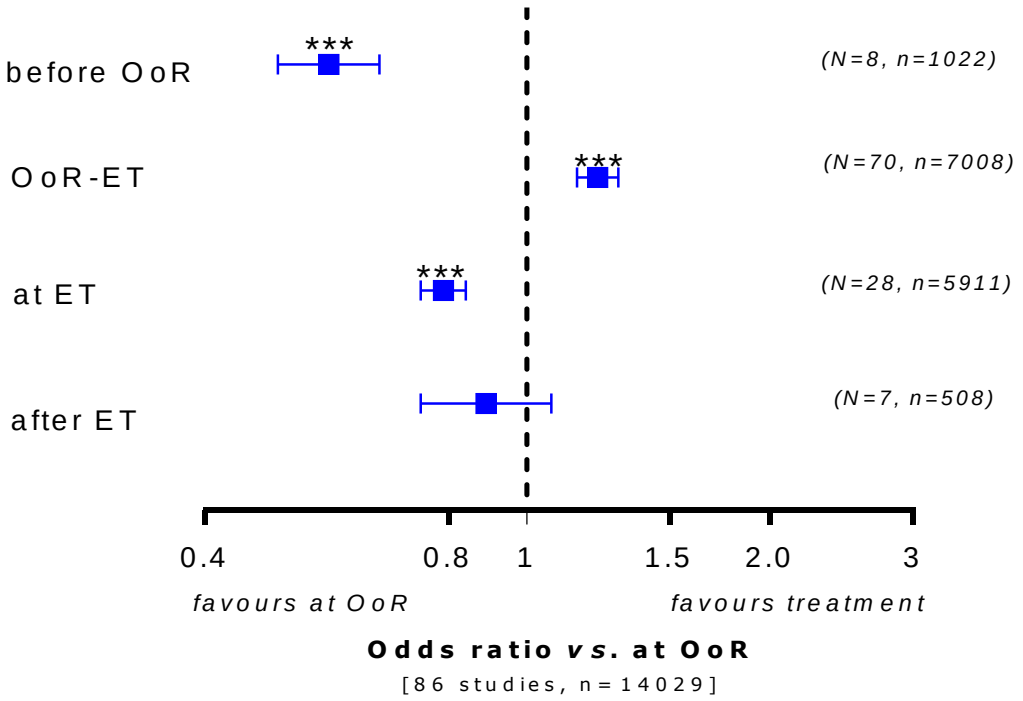


Figure 2.5: The odds ratio (± 95% confidence interval) for the relative benefit to clinical pregnancy rate for the different start time of progestogen administration in comparison to at oocyte retrieval (at OoR). The times were before oocyte retrieval (before OoR), between oocyte retrieval and embryo transfer (OoR-ET), at embryo transfer (at ET) and after embryo transfer (after ET) N=number of study groups, n=number of women. The dotted line represents the comparative odds ratio for the at OoR group. Significant differences are indicated by ***, p<0.001.

The result of this section highlighted the effects of starting progestogen supplementation at different times with between oocyte retrieval and embryo transfer being the optimal. The next analysis, concentrated on the effect of start time with the study groups split into the two most common routes of administration (IM and vaginal). This analysis also directly compared the relative benefit of intramuscular versus vaginal at the different start times.

2.3.2.4 **The direct comparison between IM and vaginal routes of administration of progestogen supplementation on clinical pregnancy rates when supplemented at different optimal times**

Comparisons were performed to determine the optimal time of supplementation for two principal routes. Figure 2.6 represents the odds ratio of clinical pregnancy outcome after progestogen supplementation via the IM route only. The comparator was starting supplementation at oocyte retrieval. The results showed a significant increase in clinical pregnancy outcome in the OoR-ET group (OR=1.23; p<0.001) versus OoR. Whereas, there were significant decreases when supplementation was started before OoR (OR=0.31; p<0.001), at ET (OR=0.75; p<0.01) and after ET (OR=0.36; p<0.001).

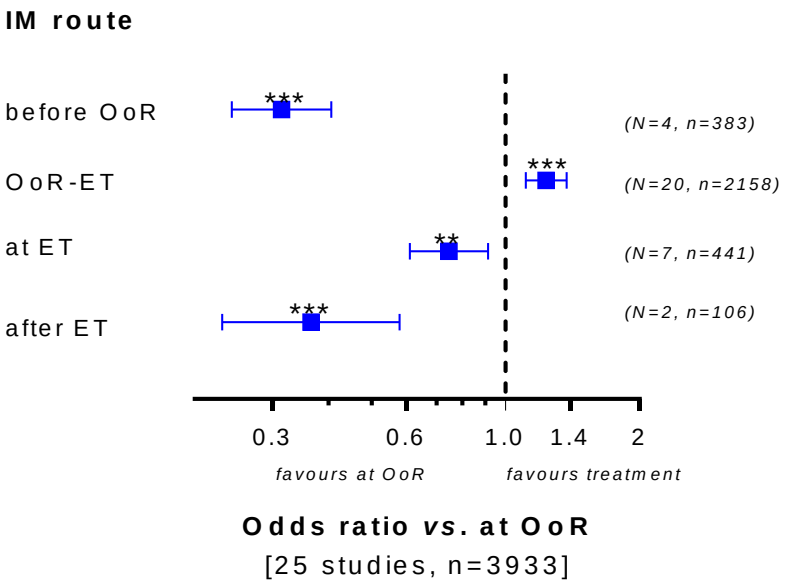


Figure 2.6: The odds ratio (± 95% confidence interval) for the relative benefit to clinical pregnancy rate for the different start time of progestogen administration (via IM route). The comparator was progestogen supplementation at oocyte retrieval (OoR). ET is embryo transfer, while N=number of study groups, n=number of women. The dotted line represents the comparative odds ratio for at OoR group. Significant differences are shown as ***, p<0.001 and **, p<0.01.

A separate analysis was performed for when progestogen was supplemented via the vaginal route. The clinical pregnancy outcome when supplementation started at OoR-ET (OR= 1.29; p<0.001) was greater than when started at OoR. While, at embryo transfer and before oocyte retrieval, the odds ratio for clinical pregnancy rate was decreased in comparison to starting supplementation at oocyte retrieval (Fig 2.7).

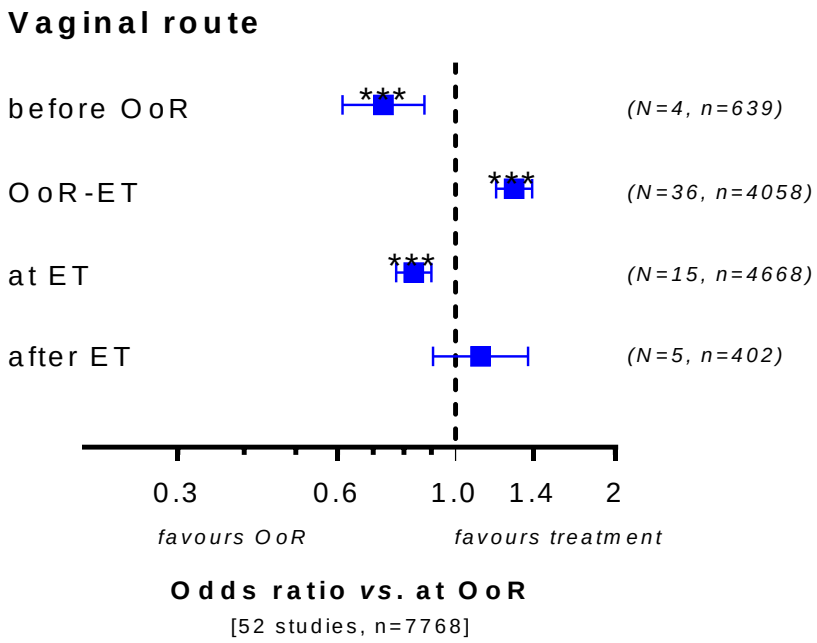


Figure 2.7: The odds ratio (± 95% confidence interval) for the relative benefit to clinical pregnancy rate of the different start time of progestogen administration via vaginal route. The comparator was progestogen supplementation at oocyte retrieval (OoR). ET is embryo transfer, while N=number of study groups, n=number of women. The dotted line represents the comparative odds ratio for the OoR group. Significant differences are shown as ***, p<0.001.

Next, vaginal and IM routes were compared at the different start time. Overall, the odds ratio for a successful clinical pregnancy was significantly higher if progestogens were supplemented via the IM route compared with supplementation via the vaginal route (OR= 1.42; p<0.001; Fig. 2.8). The overall group included all time-points (i.e. before OoR, at OoR). Also, according to each time of

supplementation IM was more beneficial than the vaginal route, i.e. at OoR (OR=1.44; p<0.001), OoR-ET (OR=1.38; p<0.001) and at ET (OR=1.29; p<0.001) (Fig 2.8). The lowest benefit was observed when progestogen supplementation was started at ET.

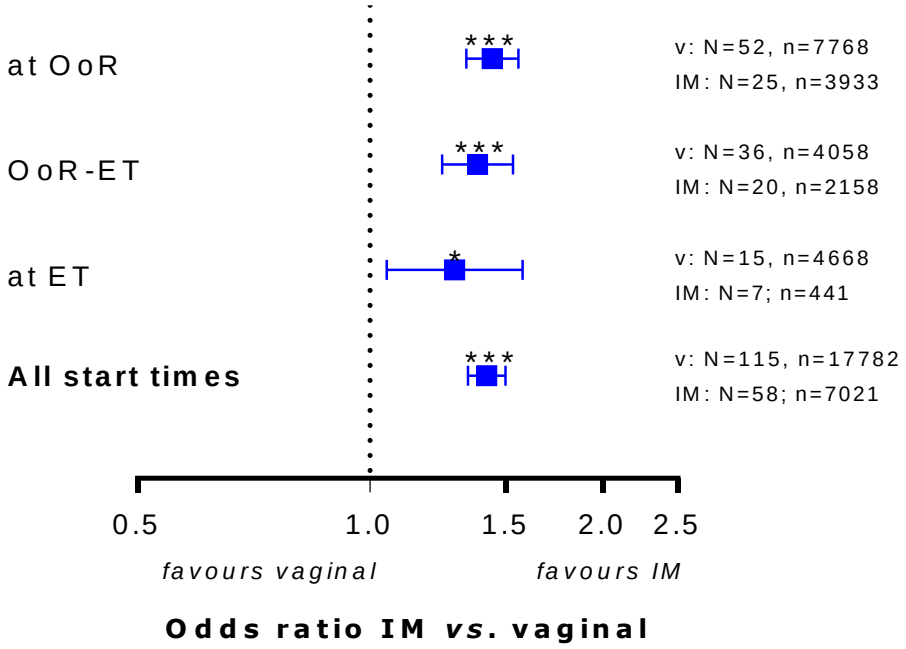


Figure 2.8: The odds ratio (± 95% confidence interval) for the relative benefit to clinical pregnancy rate for intramuscular (IM) route of progestogen supplementation over vaginal (v) route at the different start times. At oocyte retrieval (at OoR), between oocyte retrieval and embryo transfer (OoR-ET) and at embryo transfer (at ET). N=number of study groups, n=number of women. The dotted line represents the comparative odds ratio for vaginal route. Significant differences are shown as *, p<0.05; ***, p<0.001.

Table 2.2 shows the comparison in clinical pregnancy rate between IM and vaginal routes of administration across three different decades 1990-2017. The number of women reported in the published literature using the vaginal route has increased over this time. However, the relative efficacy of IM compared with vaginal route showed that in each decade IM had a greater benefit to clinical pregnancy rate (p<0.001). This indicated the benefit of intramuscular progestogen supplementation over

vaginal route was consistent over the decades despite the advances in the delivery progestogen supplementation vaginally.

Table 2.2: The relative benefit of intramuscular (IM) compared to vaginal routes of progestogen administration for luteal phase support on clinical pregnancy rates across different decades.

Decade	Number of study groups, number of patients	Odds ratio[1] (*95% confidence interval*)	Sig.
1990-1999	N=27, n=3026 (vaginal) N=25, n=2343 (IM)	**1.37** (*1.22-1.54*)	p<0.001
2000-2009	N=63, n=11497 (vaginal) N=21, n=3458 (IM)	**1.41** (*1.31-1.53*)	p<0.001
2010-2017	N=19, n=2905 (vaginal) N=10, n=1114 (IM)	**2.20** (*1.91-2.53*)	p<0.001

[1]reference route of administration is vaginal

2.3.2.5 What is the optimum day after oocyte retrieval to start progestogen supplementation?

The meta-analysis in the previous section revealed that across all routes the optimum start time was between oocyte retrieval and embryo transfer. Starting luteal phase support after oocyte retrieval was evaluated in 78 articles. The interval between oocyte retrieval and embryo transfer can be 2-5 days, this time window was further dissected to the exact day after oocyte retrieval. The data were categorised into 3 groups with the reference level used for this analysis being the "at OoR" group (Fig 2.9). There was a significant difference between all groups ($p<0.001$), with OoR +1 to 1.5 day start time offering the greatest benefit (OR=1.18; $p<0.001$). Starting progestogen supplementation at OoR +2 to 2.5 days also yield higher clinical pregnancy rates (OR=1.10; $p<0.05$) compared to initiating treatments at oocyte

retrieval. Conversely, starting progestogen supplement on days OoR +3 to 4 days was associated with decreased pregnancy rates (OR=0.63, p<0.001).

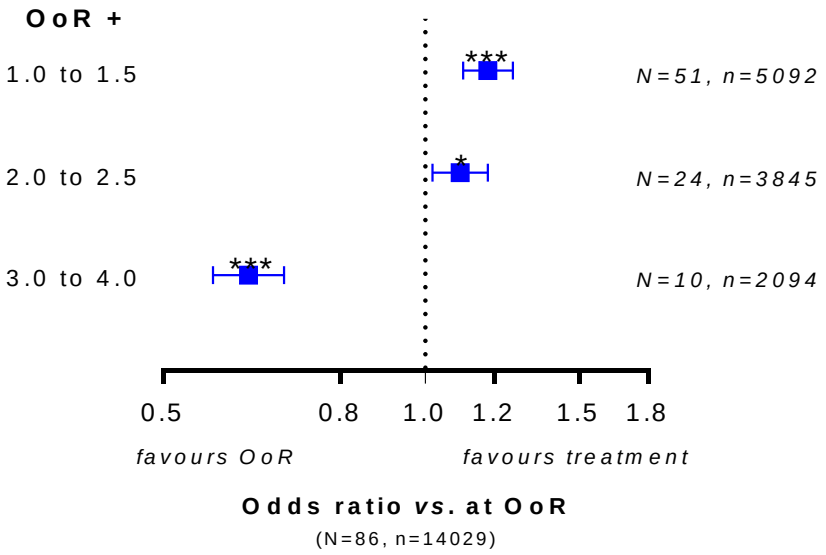


Figure 2.9: The odds ratio (± 95% confidence interval) for the relative benefit to clinical pregnancy rate of the different start days of progestogen supplementation after oocyte retrieval (OoR) in comparison to "at OoR" group. N=number of study group, n=number of women. The dotted line represents the comparative odd ratio for at OoR start time. Significant differences are shown as *, p<0.05; ***, p<0.001.

2.3.2.6 **The effect of co-treatment with oestrogen during progestogen supplemented luteal phase support on clinical pregnancy rates**

For this analysis, only those study groups that specifically compared the benefit of adding oestrogen treatment were included. This meta-analysis study showed that overall the addition of oestrogen to the progestogen supplementation during luteal phase support was beneficial to clinical pregnancy rates (OR=1.13; p<0.001). When comparing the benefit of oestrogen supplementation in combination with progestogen supplementation via vaginal or IM route, it was discovered that there was a difference between the two routes. Namely, adding oestrogen was beneficial

to pregnancy outcome when progestogen was supplemented via the vaginal (OR=1.54, p<0.001) but not the intramuscular (OR=0.92, p>0.05) route (Fig. 2.10).

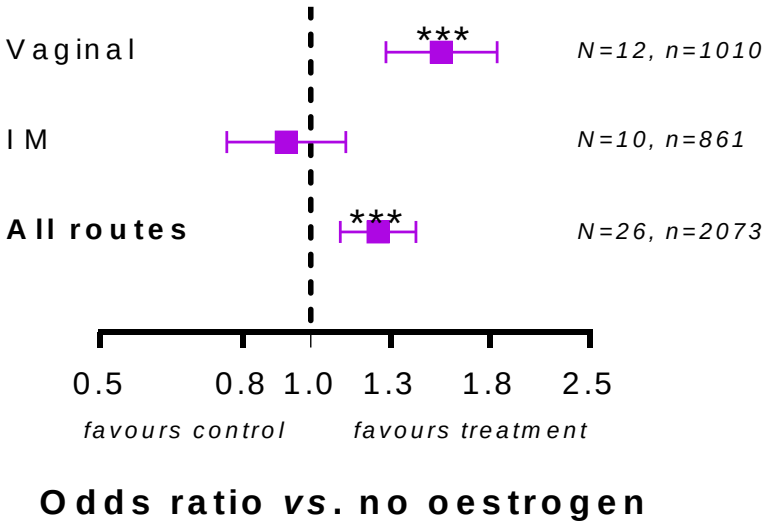


Figure 2.10: The odds ratio (± 95% confidence interval) for the relative benefit to clinical pregnancy rate of adding oestrogen treatment to progestogen supplementation. The data were categorised on whether progestogen supplementation was via intramuscular (IM) or vaginal routes. The overall group includes all routes of progestogen supplementation. The comparator for the analysis was no oestrogen treatment and this is represented by the dashed line. Significant difference are indicated as ***, p<0.001.

Figure 2.11 shows the benefit on clinical pregnancy rate of adding oestrogen to the progestogen supplementation according to the start time of progestogen supplementation. The results indicated that adding oestrogen increased the clinical pregnancy outcome but only when progestogen supplementation was started at oocyte retrieval (OR=1.53, p<0.001).

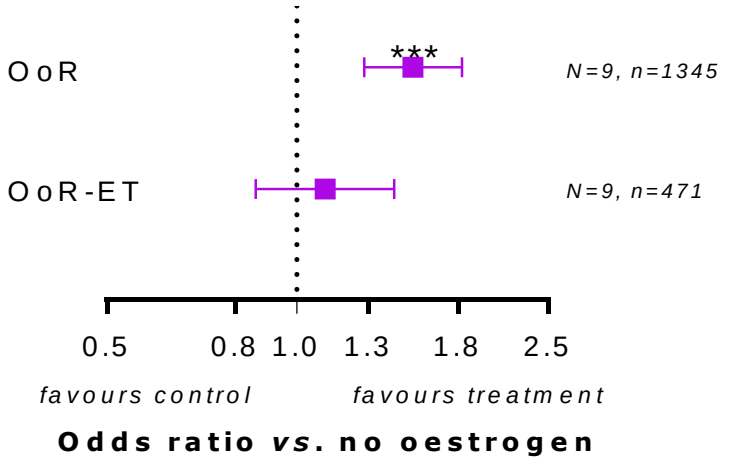


Figure 2.11: The odds ratio (± 95% confidence interval) for the relative benefit to clinical pregnancy rate of adding oestrogen treatment to progestogen supplementation. The data was categorised based on whether progestogen supplementation was started at oocyte retrieval (OoR) or between oocyte retrieval and embryo transfer (OoR-ET). The comparator was the no oestrogen treatment group, which is represented by the dotted line. Significant difference are represented by ***, p<0.001.

2.3.2.7 **The effect of progestogen supplementation duration on clinical pregnancy rates**

This meta-analysis investigated the duration of progestogen supplementation on clinical pregnancy rates. For this, the data were divided into two groups; <3 weeks and 3 to 10 weeks. The result indicated that continuing progestogen supplementation for more than 3 weeks overall did not increase clinical pregnancy rates versus shorter supplementation (OR=1.03; p>0.05). When the data were sub-divided based on whether progestogen supplementation was via intramuscular or vaginal routes then extending progestogen supplementation was beneficial when it was administered intramuscular (OR= 1.18, p<0.01) but not vaginally (OR= 0.98, p<0.05; Fig 2.12).

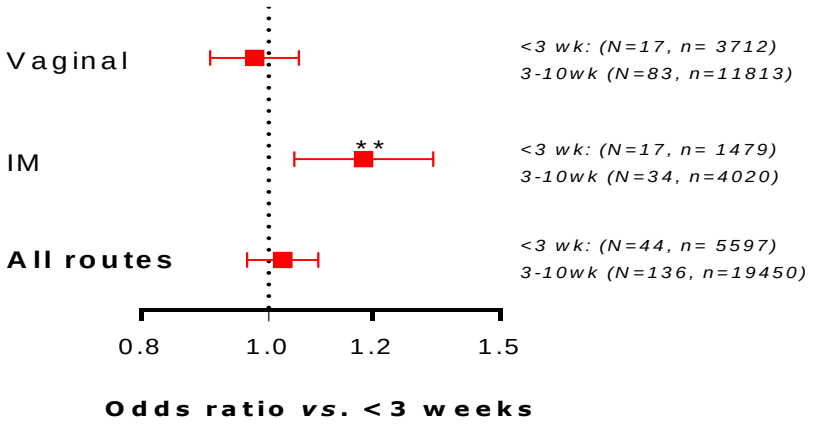


Figure 2.12: The odds ratio (± 95% confidence interval) for the relative benefit of different duration of progestogen supplementation to clinical pregnancy rates. The benefit of extending supplementation beyond 3 weeks (up to 10 weeks) in comparison to <3 weeks was conducted. The data was further categorised whether progestogen was supplemented via intramuscular (IM) or vaginal routes alongside all routes of supplementation combined (overall). The dotted line represents the comparative odds ratio for <3 weeks group. Significance difference are indicated as *, p<0.05; **, p<0.01.

2.3.2.8 **The effect of progestogen supplementation duration on live birth rates**

This dataset was more limited as there were fewer study groups that reported live births. It did show that overall continuing progestogen supplementation for >3 weeks increased live birth rates slightly (OR=1.10, p<0.05). When the data were sub-divided based on whether progestogen supplementation was via intramuscular or vaginal routes then extending progestogen supplementation was beneficial when administered vaginally (OR=1.16, p<0.01). However, when administered intramuscularly for >3 weeks live birth rates were adversely affected (OR= 0.78, p<0.01; Fig 2.13).

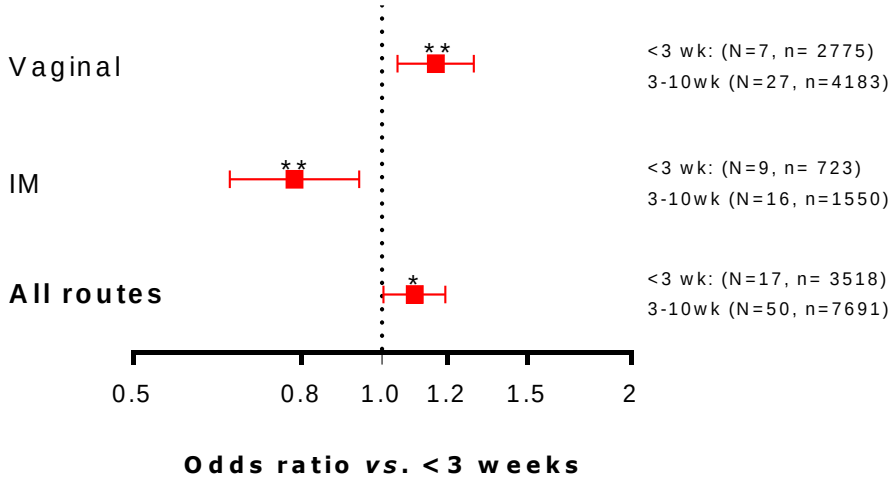


Figure 2.13: The odds ratio (± 95% confidence interval) for the relative benefit of different durations of progestogen supplementation to live birth rates. The benefit of extending progestogen supplementation beyond 3 weeks (up to 10 weeks) was compared to supplementation for <3 weeks. The data was further categorised whether progestogen was supplemented via intramuscular (IM) or vaginal routes alongside all routes of supplementation combined (overall). The dotted line represents the comparative odds ratio for <3 weeks group. Significance difference are indicated as *, $p<0.05$; **, $p<0.01$.

2.4 Pregnancy outcomes of progestogen supplementation in IVF/ICSI cycles with frozen-thawed embryo transfer (FET)

2.4.1 Objective

To determine the benefit to clinical pregnancy rate of luteal phase support compared with no treatment in women undergoing ART with biological or donor frozen embryos.

There was insufficient data to compare the different routes and start time of progestogen supplementation. There was though sufficient data to determine the effect of progestogen supplementation vaginally on clinical pregnancy rates.

2.4.2 Results

2.4.2.1 The characteristics of the study groups

There were 27 study groups (4946 women) that described the use of frozen embryos, which were identified from 14 eligible articles. These were conducted in the Middle East (22%), Europe (30%), Asia Pacific (26%) and USA (22%) (Fig 2.15). In these, 6 study groups involved donor oocyte/embryo while 19 study groups used frozen embryos. There were in total, 4 study groups where luteal phase support was not performed and these were used as the comparator control group. The majority of the study groups used progestogen supplementation vaginally (N=17), but intramuscular (N=4), oral (N=1) and mixed routes (N=1) were used. In terms of the endometrial receptivity preparation before embryo transfer, 2 study groups' utilised natural cycles, 12 were with oestrogen and three used gonadotrophins. The remainder did not state which treatment if any was performed (for further information, see Appendix 2).

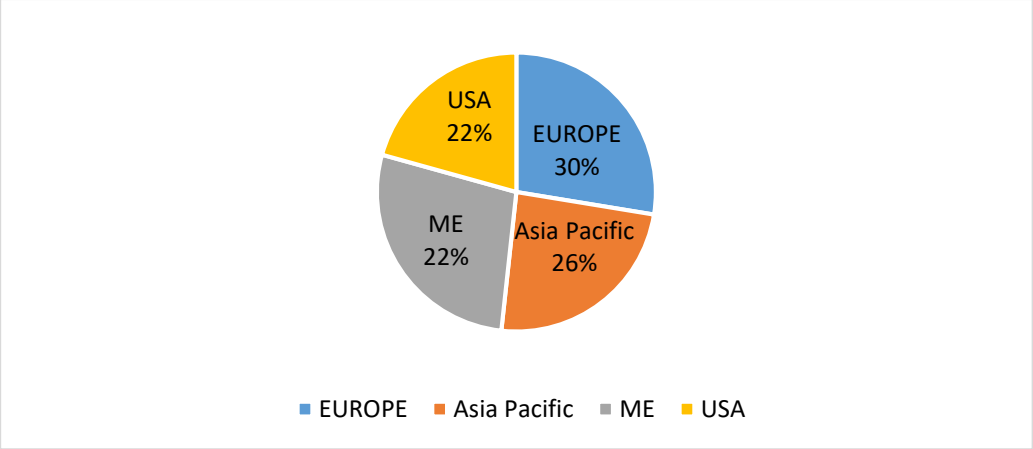


Figure 2.14: Global distribution of randomised clinical trials that have reported the effects of progestogen supplementation on clinical pregnancy rates in women undergoing frozen embryo transfer. In total, 27 study groups were identified which used frozen embryo transfer. ME (Middle East).

2.4.2.2 **The effect of luteal phase support on clinical pregnancy rates in ART where frozen embryo transfer was employed.**

There was a significant benefit (OR=1.96; p<0.001) in terms of clinical pregnancy outcome to including progestogen supplementation versus no treatment when transferring frozen embryos (Fig. 2.15). That is equivalent to increasing the pregnancy rate from 23.6% for untreated women to 37.7% in luteal phase support, regardless of start time or route of progesterone supplementation. A similar benefit to overall was observed when progestogen was administered vaginally compared to overall (OR= 1.68, p<0.001). There were too few women in the intramuscular study group to make an accurate comparison.

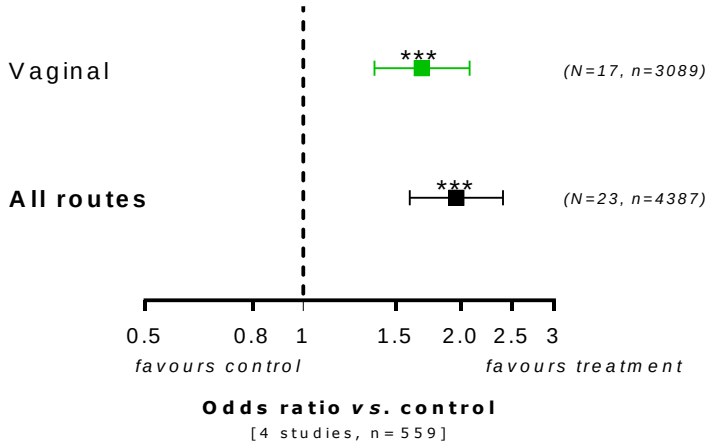


Figure 2.15: The odds ratio (± 95% confidence intervals) for the benefit to clinical pregnancy rate of progestogen supplementation on clinical pregnancy rates in women undergoing ART with frozen embryos. N=number of study groups, n=number of women. The comparator was no progestogen treatment (dashed line). Significant difference are indicated with ***, $p<0.001$.

2.5 Intra-uterine insemination (IUI)

2.5.1 Objectives

This study investigated progestogen supplementation in IUI cycles and utilised binomial logistic regression analysis in the following objectives:

1) Determine the benefit of luteal phase support over no treatment in IUI cycles.

2) To compare the effect of progestogen supplementation after different ovulation induction protocols on clinical pregnancy rate in IUI cycles

2.5.2 Results

2.5.2.1 The characteristics of the study groups

A total of 14 study groups were identified (n = 1555 women) as using progestogen supplementation in IUI cycles. Eight study groups were performed in Middle East, four in USA and two in Asia Pacific. Half of the study groups had no luteal phase support, while progestogen supplementation was used via the vaginal (N=5) or intramuscular route (N=1) or route was not described (N=1).With respect to ovulation induction, the following hormones were used: FSH (N=6), clomiphene citrate (CC, N=4), or aromatase inhibitors (N=2). The final two study groups did not mention the type of ovulation induction (for further information, see Appendix 3)

2.5.2.2 The effect of luteal phase support with progestogens on clinical pregnancy outcomes after IUI

Progestogen treatment as luteal phase support was significantly beneficial (OR=1.83; $p<0.001$) for clinical pregnancy rate after IUI regardless of the route of administration. Numerically, this was equivalent to increasing mean pregnancy rates from 15% for control group (untreated) to 24% for the progestogen supplemented

cycles (Fig .2.16). Moreover, progestogen supplementation increased pregnancy rates significantly in studies that used FSH for ovulation induction (OR= 2.49; p<0.001), while luteal phase support did not show significant benefit in IUI cycle after using CC as the ovulation induction treatment (OR=1.12 p>0.05).

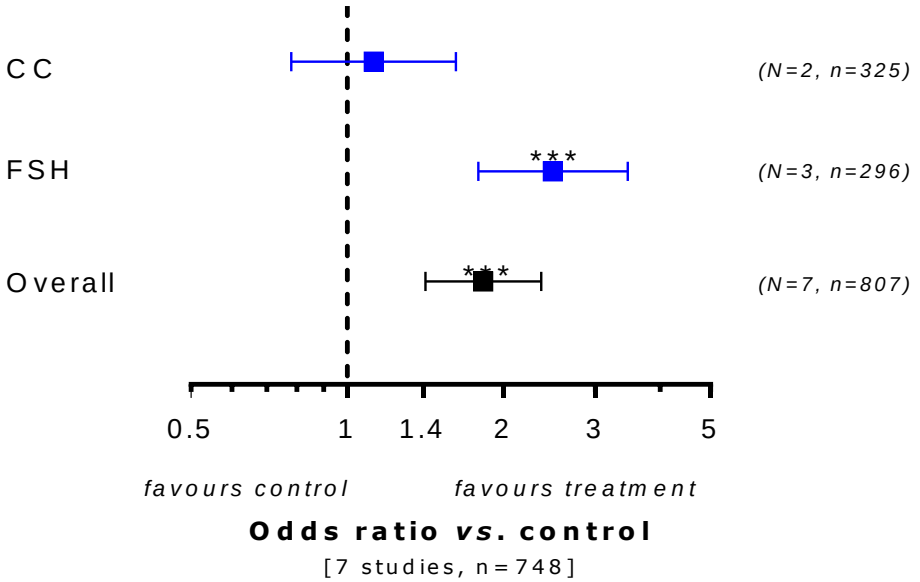


Figure 2.16: The odds ratio (± 95% confidence intervals) for the comparative increase in clinical pregnancy of progestogen supplementation following ovulation induction with clomiphene citrate (CC) or follicle stimulating hormone (FSH) or overall versus no progestogen treatment in women undergo intra-uterine insemination (IUI) cycles. Dotted line represents no treatment group (control). Significant difference are indicated with ***, p<0.001. N=number of study groups, n=number of women.

2.6 Discussion

This meta-analysis clearly demonstrated that progestogen supplementation is essential to maximise clinical pregnancy rates after IVF or ICSI/ET. This finding was true for all routes of administration and the different start times. The time of starting progestogen supplementation was a particular focus of this study; starting treatment in a window after oocyte retrieval but before embryo transfer (OoR-ET) had the greatest benefit for clinical pregnancy rate. Additional interrogation of the data revealed that within this interval (OoR-ET), the optimal timing was 1 to 1.5 days after oocyte retrieval for progestogen supplementation to be initiated.

When the analysis focussed on the two principally used progestogen routes (IM and vaginal), the results showed that the intramuscular route was more beneficial than the vaginal route at increasing clinical pregnancy rates. Additionally, there was variation between these routes when the start time of supplementation was compared. Namely, the intramuscular route of progestogen supplementation resulted in higher pregnancy rates compared with the vaginal route at all start times. Conversely, extending progestogen supplementation beyond 3 weeks increased the live birth rate if administered via the vaginal route while the birth rate was decreased significantly when given intramuscularly. Another observation was that the addition of oestrogen to the progestogen supplementation regime significantly increased clinical pregnancy rates compared with no oestrogen treatment. Further analysis showed that oestrogen supplementation was beneficial only if progestogen supplementation was administered vaginally but not when via the intramuscular route.

There are several systematic and meta-analysis studies that have also investigated this subject. A study by Soliman et al. (1994) investigated the benefit of progestogen

for luteal support after oocyte retrieval in eighteen trials and stated that using hCG with progestogen was superior to progestogen alone. A subsequent meta-analysis focusing on hCG and the intramuscular route found increased pregnancy outcome in both groups compared with no treatment (Pritts and Atwood, 2002). In contrast, previous meta-analysis did not find sufficient evidence to recommend progestogen supplementation for luteal phase support after oocyte retrieval (Daya, 1988).

There are several previous meta-analyses assessing the association between different routes of progestogen for luteal support in ART. There is no agreement regarding the optimal routes of progestogen, with some studies showing that intramuscular offers the greatest benefit to clinical pregnancy rate compared with vaginal and oral routes (Pritts & Atwood, 2002). This study was limited and only included five trials with 891 women. Other meta-analyses have indicated that the IM route improved the outcomes of ongoing pregnancy and live birth rates over the vaginal route (Daya, 1988, Daya, 2009).

In contrast, another meta-analysis study indicated that using vaginal progestogen was equivalent to using 50 mg intramuscular progestogen as a luteal support on clinical pregnancy rates (Zarutskie and Phillips, 2009). It consisted of nine studies (1,620 subjects) and yielded an odds ratio of 0.91 (CI 0.74-1.13) when vaginal was compared to intramuscular route. However, it did not consider the start time of progestogen supplementation.

Progesterone in an oil-based solution is rapidly absorbed after intramuscular administration with its plasma concentration increasing within 2 hours, and peaking after 8 hours (29ng/ml). On the other hand, vaginal progesterone peaked within 3-9 hours (4.8ng/ml) after administration of vaginal micronized progesterone. Its half-

life is around 13 hours (Levy et al., 2000). Progestogen has the ability to promote the luteinisation process and the expression of LHCGR, steroidogenic enzymes, and matrix metalloproteinase (MMP) and thus the control of luteolysis (Devoto et al., 2009). Therefore, progestogen administered by the IM route could play a systemic role in the corpus luteum rescue process and regulate the expression of endogenous ovarian factors.

The current meta-analysis also considered the optimal start time of progestogen treatment. A clear strength of this study was the large number of study groups for each start time. Moreover, the results analysed the relationship between the time of supplementation and the progestogen route. A limitation of the present study was determining the exact start time according to the day of oocyte retrieval and embryo transfer as there was limited information in several of the original articles (as explained in the methods).

Generally, starting progestogen luteal phase support during the OoR-ET interval achieved the greatest benefit ($p<0.001$) in terms of clinical pregnancy outcome. By splitting this window of supplementation (OoR-ET) further, the meta-analysis indicated that the day after oocyte retrieval (OoR+1 to1.5) was the ideal time to start progestogen treatment, with benefit still found on OoR day+2 to 2.5.

One previous meta-analysis report studied the effect of the start time for progestogen treatment on the pregnancy outcome in five trials with 1,010 women (Connell et al., 2015) Their result was in partial agreement with the current study: suggesting the potential advantage of starting progestogen treatment 2 days after oocyte retrieval to maximise the synchronisation between embryo development and endometrium receptivity.

The optimal time of supplementation may be explained by the relationship between the window of implantation (WOI) and timing of ET. This window is considered as the most crucial factor for achieving a successful pregnancy in IVF/ICSI cycles (Bourgain and Devroey, 2003). For a successful pregnancy, embryo transfer needs to be performed at the WOI (Wilcox et al., 1999). In unstimulated cycles, the window of implantation starts on day LH+6 and ends by LH+10 (hCG injection for triggering oocyte maturation in IVF/ICSI cycles) (Miller et al., 2012). The formation of pinopodes and integrin A5/B3 expression are the most cited factors associated with this window. These factors are related to the level of progesterone and the down-regulation of progesterone receptor in the endometrial epithelial cells (Stavreus-Evers et al., 2001, Kiliçdag et al., 2010). In stimulated cycles, the formation of epithelial pinopodes occurs 1-2 days in advance of that in the natural cycle (Creus et al., 2003). Furthermore, integrin A5/B3 expression was decreased in luminal epithelium of women in stimulated cycles (Thomas et al., 2002). This evidence indicates that endometrial receptivity is earlier in stimulated cycles than normal cycle. This premature endometrial receptivity is even more obvious in women with elevated progesterone on the day that pregnancy hCG is first detected (Bourgain and Devroey, 2003).

It is well established that oestrogen levels in the mid-luteal phase are different between the pregnant and non-pregnant women in IVF/ICSI cycles (Edgar, 1995). Thus, the progesterone:oestradiol ratio on the day of embryo transfer is considered an indicator as to whether there will be successful pregnancy (Gruber et al., 2007). Several systematic reviews have considered the benefit of adding oestrogen to progestogen supplementation on the establishment and maintenance of pregnancy. In 2002, Pritts and Atwood showed that the addition of oestrogen was beneficial to

pregnancy outcome in three trials (Pritts and Atwood, 2002). However, there are still some disagreements around the role of the oestrogen treatment after oocyte retrieval. A later meta-analysis with 10 randomised controlled trials (RCT) stated that there was no beneficial outcome on the ongoing pregnancy and implantation rates when oestrogen was added to the luteal phase support after oocyte retrieval (Gelbaya et al., 2008). Similarly, another meta-analysis (4 RCT with 587 patients) suggested that the addition of oestrogen to progestogen supplementation did not increase pregnancy outcome (Kolibianakis et al., 2008). A similar report by Jee et al. (2010) also stated a lack of efficacy of additional oestrogen on pregnancy rates. However, the present large study indicated a benefit of including oestrogen particularly if vaginal progestogen supplementation was used.

The current meta-analysis found that extending luteal phase support longer than 3 weeks did further improve clinical pregnancy rates when the vaginal route and all routes combined were considered. In contrast, when progestogens were supplemented intramuscularly for 3 weeks or less, then this was more beneficial than extending beyond 3 weeks. Extending progestogen supplementation beyond 3 weeks increased live birth rates, when the vaginal route and all routes combined were considered. While, the live birth rate was decreased when progestogen treatment via the IM route was extended beyond 3 weeks. Although there is no strong evidence to support the continuation of luteal phase support until week 12 of gestation, this practice is used worldwide in IVF/ICSI cycles (Liu et al., 2012).

Overall, using progestogen as luteal support is essential to maximise the pregnancy outcome in infertility clinics, but the type and timing of progestogen supplementation are considered the main challenges. Clinical pregnancy outcomes

were clearly greatest when progestogen was supplemented intramuscularly compared to other routes, including vaginal, irrespective of the start time. Combined with the better tolerability by women, vaginal progestogens are clearly still an option. Therefore, an alternative regimen would be to supplement with progestogen intramuscularly during the first two weeks and then sustain progestogen supplementation via the vaginal route. This certainly warrants randomised controlled trials to investigate the viability and benefit of this strategy. The addition of oestrogen offers some benefit to pregnancy rates (e.g. with vaginal progestogen) but not in all cases. A consideration that has not been investigated is the progesterone/oestrogen ratio before the addition of oestrogen for luteal phase support.

Frozen embryo transfer

It has been found that pregnancy outcome of sub-fertile couples undertaking frozen thawed embryo transfer is lower than fresh embryo transfer (Ghobara et al., 2017). However, frozen embryo transfer raises the cumulative pregnancy outcome per couple and thus can reduce total cost compared to the IVF/ICSI cycles with fresh embryo transfer. Frozen embryo transfer is achieved by using different regimes for endometrium preparation: natural cycle (no treatment), endometrium prepared by progesterone and oestrogen; as hormone therapy for FET cycles; and ovulation induction for FET cycles (Ghobara and Vandekerckhove, 2008).

In this current meta-analysis, progestogen supplementation was beneficial for clinical pregnancy outcome following frozen embryo transfer regardless of its formulation and route. This is similar to the findings for fresh embryo transfer, where progestogen was essential for IVF/ICSI cycles due to luteal phase deficiency as a consequence of the controlled ovarian induction. However, a meta-analysis study

had shown that there is no consistent superiority of any endometrial preparation for frozen embryo transfer (Yarali et al., 2016).

Intra-uterine insemination

This meta-analysis pooled original data in published literature regarding the use of progestogen as luteal phase support in IUI cycles and compared it to the non-treated control group. The result showed a significant increase in clinical pregnancy rates in women receiving progestogen as luteal phase support, when the type of ovulation induction treatment was not accounted for. However, these differences were only observed in the subgroup of women who received FSH to induce ovulation and not in those women treated with CC; this is in agreement with a previous meta-analysis study (Miralpeix et al., 2014).

Luteal phase deficiency will typically follow ovulation induced by gonadotrophins (Beckers et al., 2003, Fatemi, 2009, Yding Andersen and Vilbour Andersen, 2014) and therefore, progestogen supplementation is considered essential (Zelinski-Wooten et al., 1992). However, using CC for ovulation induction works by increasing endogenous FSH and LH secretion, and consequently the luteal phase is often more similar to that observed in the natural cycle. Therefore, the need for luteal phase support is markedly reduced. In summary, the results indicated that luteal phase support is essential for successful implantation and therefore increased clinical pregnancy outcome, in fresh and frozen embryo transfer as well as IUI.

This Chapter has also highlighted the critical importance of the effects of progestogen on the endometrium and in particular endometrial receptivity to the embryo. This progesterone-influenced interaction between the embryo and

endometrium will be critical in the establishment of pregnancy. However, the mechanism underlying how progesterone is exerting its action is unclear. It is not logistically feasible or ethically appropriate to collect endometrium from women undergoing IVF/ICSI and embryo transfer. Thus, it was decided to utilise the pig as a model species to investigate the embryonic-endometrial interactions that occur during attachment and the establishment of pregnancy. While it is recognised that there are clear differences between humans and pigs (i.e. ovulation numbers, placentation and maternal recognition of pregnancy signals), there are definite similarities between pigs and women undergoing ART. The key features are that the endometrium is influenced by oestrogens (from the CL and embryo), the local process of attachment and progesterone profile. These factors are investigated in the following experimental Chapters.

CHAPTER 3 Conceptus-endometrial communication in the pre-implantation period in pigs

3.1 Introduction

There are several aspects of reproductive physiology and their pathological responses in pigs and humans that are similar (Walters et al., 2012, Bazer and Johnson, 2014). Indeed, pigs have been classed as the optimal model for human research in several body systems including urinary (Mock et al., 1997), skin (Summerfield et al., 2015), as well as reproduction (Constantinescu, 2017).

In the pig, embryos reach the uterus shortly after the 4-cell stage and will then undergo development to the blastocyst/hatched blastocyst stage while being free-living in the uterine lumen (Bazer and Johnson, 2014). The initial attachment between embryo and endometrium begins on day 13 of pregnancy and implantation is completed by day 24 (Massuto et al., 2010). The conceptus plays a critical role in the establishment of pregnancy through its secretions. Firstly, the maternal recognition of pregnancy signal in pigs is oestrogen and in particular 17β oestradiol (Bazer and Thatcher, 1977, Bazer, 2013, Bazer and Johnson, 2014). The conceptus needs to secrete oestradiol until at least day 25 in order to block luteolysis and preserve the corpora lutea (Geisert et al., 1990). Conceptus-derived oestradiol prevents luteolysis via shifting the pattern of prostaglandin E2 and prostaglandin F2α (PGE2/PGF2α) secretion within the endometrium. More specifically, in the non-pregnant cycle, PGF2α secretion is directed away from the uterine lumen and towards the circulation where it can exert an endocrine effect. Conversely, in pregnancy, PGF2α secretion is directed in an exocrine manner towards the uterine lumen.

The transcriptome profile of the endometrial epithelial cells is modulated by oestrogen and other conceptus-derived proteins such as interferons (IFN; particularly IFND and IFNG) that are involved in the intermediate recognition and establishment of pregnancy. Indeed, IFN levels increase in uterine flushing after day 10 of pregnancy and peak on day 16 (La Bonnardiere et al., 1991, Lefèvre et al., 1998). Importantly, interferons are not the maternal recognition signal in pigs as they are in ruminants.

The establishment of pregnancy depends on the ability of the conceptus through its secretions, to prepare the epithelial and stromal cells for successful attachment. In a fully receptive uterus, the endometrium has a particular temporal-spatial distribution of key proteins that might be considered as markers of the receptive uterus. These include integrins (Germeyer et al., 2014) and progesterone receptors (Geisert et al., 2006). This Chapter explores this further by testing the hypothesis that the uterine luminal fluid oestradiol content is influenced by endometrial progesterone content, and embryo development and that, in turn, endometrium receptivity markers are regulated by oestradiol and progesterone.

3.2 Aims

The aim of this study was to investigate the communication between conceptus, endometrium and corpus luteum throughout the pre-implantation period and time of uterine receptivity. This study addressed the following questions:

1) What is the relationship between luteal weight, luteal and endometrial progesterone content, number of embryos and uterine fluid concentration of oestradiol during early pregnancy?
2) Is there a specific expression pattern of uterine receptivity markers (progesterone and oestradiol receptor, stanniocalcin 1 and integrin A5/B1) during preimplantation period of pregnancy?

3.3 Objectives

Based on this, the following objectives were developed:

1) Collect and record physical characteristics of embryos, uterine flushes, endometrium and corpora lutea samples from pregnant sows during the pre-implantation period (days 3-5, 11-12 and 13-14).
2) Assess the degree of vascularisation in the corpora lutea by von Willebrand factor (VWF).
3) Analyse the progesterone content of the luteal and endometrial tissue in relationship to the concentration of oestradiol in uterine flushing.
4) Assess the relationship between these different parameters
5) Elucidate the temporal-spatial distribution of the progesterone receptor (PGR), oestrogen receptor alpha (ESR1), integrin$\alpha v\beta 1$ (ITGA5/B1) and stanniocalcin 1 (STC1) by immunohistochemistry during the pre-implantation endometrium.

3.4 Materials and methods

3.4.1 Animals

This project was ethically approved by the School of Biosciences Ethics Review Committee, University of Nottingham. The management of the sows was co-ordinated by Dr Ramiro Alberio, University of Nottingham, who kindly gifted the samples in this project. Crossbred Large White and Landrace sows (2-3 years old; n=47 in total) were observed daily and sows were artificially inseminated at 12 and 24h after the first sign of oestrus. The sows were housed under normal and husbandry management and no interventions were performed. The sows were then sacrificed at specific time points with insemination day being defined as day 0. Reproductive tracts were collected on day 3-5 (n=7), day 11 (n=24), day 12 (n=9), day 13 (n=4) and day 14 (n=3) of pregnancy. Subsequently, two animals were discovered to be non-pregnant. These were on day 11 (n=1) and 13 (n=1) of pregnancy and these animals were not included in the analysis.

At the slaughterhouse, both ovaries and uterus were collected from each animal. Typically, there were 3-5 animals per slaughter time point and the study was conducted from November 2014 until December 2016. The conceptuses were collected by flushing each uterine horn twice with 30 ml of phosphate buffer solution (PBS) containing 1% (v/v) fetal bovine serum (FBS). The uterine flushings were clarified by centrifugation (3000g for 10min). The supernatant was collected, then aliquoted, and frozen at -80ºC until analysis. On return to the laboratory, corpora lutea were individually dissected from the ovary, then counted and weighed. From the pool of corpora lutea per animal, two were randomly selected and together with a cross-section of uterine horn, they were fixed overnight (16h) in Bouin's solution

before being transferred to 70% (v/v) ethanol prior to paraffin embedding. A part of the uterine horn (10cm) was opened and two strips of endometrium tissue (2cm in length) were isolated from the underlying myometrium. The remaining corpora lutea and strips of endometrium were snap frozen, and then stored at -80ºC for molecular analysis.

3.4.2 Determination of steroid hormone content in the corpus luteum, endometrium and uterine flush.

3.4.2.1 Steroid hormone extraction from luteal and endometrial tissue

The extraction of steroids hormones (principally progesterone) from luteal and endometrial tissue was performed as previously described (Tsang et al., 1990). Briefly, ice–cold PBS (1 ml) was added to 200 mg tissue, and then homogenised using a tissue grinder (Polytron homogeniser) for approximately 30 seconds and then immediately returned to ice. This was repeated 3 times for luteal and 5 times for endometrial tissue. Once fully homogenised, 200 µl of homogenised liquid transferred to a glass extraction tube and 2 ml of petroleum ether (Acros, New Jersey, USA) was added. The tubes were vortexed vigorously for 5min and then snap-frozen in methanol on dry ice. The ether liquid was then transferred to a borosilicate tube (12x75 mm). The ether was air-dried in a fume hood for 36 hours. The extraction was then reconstituted in 500 µl of PBS containing 0.1% (w/v) bovine serum albumin (BSA). This was vortexed for 1min and then stored at -20ºC until analysis.

3.4.2.2 Quantification of progesterone concentration by ELISA.

Progesterone analysis was performed by ELISA according to the manufacturer's procedure (Ridgeway Science Ltd, Alvingdon, UK). The progesterone ELISA has been previously validated for use in the pig (Picton et al., 1999). The 96-well ELISA

plates were supplied pre-coated with progesterone antibody and stored at 4˚C. Plates were prepared by removing the liquid and blot drying onto tissue paper. Standards of progesterone (0, 0.25, 0.5, 1, 2, 5, 10 and 20 ng/ml) in buffer were provided with the kit. The samples were diluted with PBS (1 in 750-2000 for luteal tissue; 1 in 3-5 for endometrial tissue). Samples and standards were brought to room temperature (RT) prior to adding 10 µl of standards or samples to the appropriate well in duplicate. Next, 200 µl of progesterone enzyme label was added to each well and then incubated at RT for 90min. After the incubation, the wells were emptied and washed with tap water 3 times, then blotted dry. Then, 200 µl of substrate solution (supplied with kit) was added to each well and incubated for 30min in the dark at RT. After colour development, the absorbance was read at 570 nm using an automated plate reader (Labsystems Multiskan Ascent 354). Specific software (Ascent Software Version 2.6, Thermo Labsystems) was used to determine progesterone concentration using a 4-parameter logistic regression. The mean intra-assay coefficient of variation was 6.7%. All the samples were performed on a single plate for each tissue source.

3.4.2.3 **Quantification of oestradiol concentration by ELISA**

Oestradiol concentration was evaluated using a commercial competitive ELISA kit for 17β-oestradiol (Cayman Chemicals) as previously used by Luiking et al. (2015). The antibody has 100% cross-reactivity with oestradiol, 14.5% with oestradiol-3-sulphate, 14% with oestradiol-3-glucuronide, 12% with oestrone, 10% with oestradiol-17-glucuronide and all other steroids <0.3%. The uterine flushing fluid was diluted 1 in 5 with PBS prior to analysis. ELISA buffer (100 µl; supplied with kit) was added to the non-specific binding (NSB) wells and 50 µl to the maximum binding (B0) wells. Standards (6.6–4000 pg/ml oestradiol) or samples (50 µl) were

added to the appropriate wells in triplicate. Oestradiol-conjugated AChE tracer (supplied with kit) was added to all wells except the total activity (TA) well and blank wells. Then, oestradiol antiserum was added to each well except the TA, NSB and blank wells. After 1h incubation in the dark, wells were rinsed in wash buffer. Then, the substrate (Ellman's reagent) was added to the each well and oestradiol AChE tracer (50 µl) to the TA well. The absorbance was read at 405 nm after 60-90min of development. The standard curve was generated by subtracting the NSB from each standard and sample (Fig 3.1). The absorbance was then converted to %B/B0 and a 4-parameter logistic curve fitted using Graph Pad Prism to determine the unknowns.

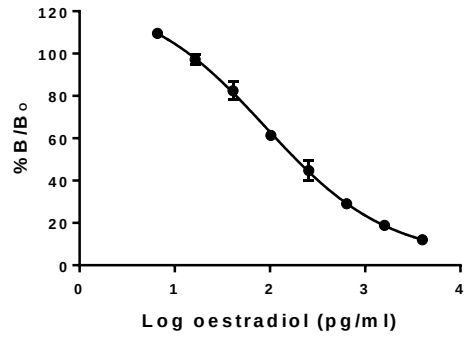


Figure 3.1: A composite standard curve for the oestradiol quantification by ELISA.

3.4.3 **Immunohistochemistry analysis for PGR, ITGA5/B1, ESR1, VWF and STC1**

Tissue preparation

Endometrial in cross section and two corpora lutea from each animal were fixed in Bouin's solution for 16h then washed and stored in 70% (v/v) ethanol. The fixed tissue was transferred to a cassette and then dehydrated in a series of increasing ethanol concentrations in an automatic tissue processor (TP1020, Leica, Milton

Keynes, UK) as follows: 70% (v/v) for 2h; 80% (v/v) for 2h; 95% (v/v) for 2h and 100% (v/v) for 2 × 2h. Next was 50% ethanol, 50% histoclear for 2h, followed by histoclear for 2h and finally molten paraffin wax for 2 × 2h. The samples were blocked and kept in a dry, cold place until analysis. Embedded tissue was sectioned using a microtome (HM355, Micron, Walldrof, Germany) and then adhered to electrostatic slides. Slides were left over a hot plate (50°C) for 30min and then overnight at RT to dry. On the day of immunohistochemistry, slides were placed in histoclear to remove paraffin wax for 3 × 5min and then rehydrated in a series of decreasing ethanol: 100% (v/v) for 5min, 95% (v/v) for 2min and 70% (v/v) for 5min. Finally, the slides were washed in PBS 2 ×5min.

Antigen retrieval

There were two main methods employed to restore immunoreactivity of the target epitopes: 1) Protease-induced epitope retrieval was used for VWF immunohistochemistry, in which slides were incubated in 0.04% (w/v) trypsin in 0.025% (w/v) $CaCl_2$ in PBS for 15min at 37ºC, and then washed in running water for 30 sec and PBS for 5min. 2) Heat-induced epitope retrieval was used for all other immunohistochemistry protocols: Slides were immersed in pre-warmed 10 mM citrate buffer pH 6.0 and then heated in 850W microwave for 2 x 5min followed by cooling for 30min. The slides then were washed in PBS for 2 × 5min.

Blocking

Any endogenous peroxidase was blocked by immersing slides in 3% (v/v) H_2O_2 in methanol for 10min at RT. Then slides were washed in PBS for 2 × 5min. After that, slides were incubated with blocking serum [20% of normal goat serum (NGS) or 4%

of normal horse serum (NHS) as appropriate based on the secondary antibody] for 30min at RT (see Table 3.1 for details).

Primary antibody

Slides were incubated with primary antibody (Table 3.1) for 2h at RT in a humidified chamber before being washed with PBS for 2 × 5min to remove any unbound antibody. For VWF immunohistochemistry, the first wash was 0.1% Triton × 100 in PBS for 5min followed by a PBS wash for 5min. Control slides were prepared with mouse or rabbit IgG at the same concentration as the primary antibody that was used.

Table 3.1: Primary antibodies utilised in immunohistochemistry for Chapter 3.

Primary antibody	Supplier	Blocking serum	Conc/dilution	Species
Von Willebrand Factor	Dako	20% NGS	20 µg/ml	Rabbit
Progesterone receptor	Leica	4% NHS	5 µg/ml	Mouse
Integrin A5/B1	Dako	20% NGS	1:20	Rabbit
Stanniocalcin 1	Thermos Fisher Scientific	20% NGS	5 µg/ml	Rabbit
Oestrogen receptor 1	Dako	4% NHS	2 µg/ml	Mouse

Secondary antibody

The Vector ABC Elite method was used to detect the primary antibody as per manufacturer's instructions. Briefly, biotinylated goat anti-rabbit IgG or biotinylated horse anti-mouse IgG (both Vector Laboratories Ltd, Peterborough, UK) were added as appropriate. The secondary antibodies were diluted 1 in 200 in 2% (v/v) normal goat serum (rabbit primary antibody) or normal horse serum (mouse primary antibody) in PBS. The sections were incubated in a humidified chamber for 30min at RT. Slides were washed in PBS for 2 ×5min.

Avidin-Biotin complex

Avidin-biotin complex (Vector) was prepared (as per instructions) by adding 1 drop of A solution then 1 drop of B solution to 5 ml of PBS. Then, 250 µl was added to the sections for 30min in a humidified chamber at RT, and afterwards washed with PBS for 2 x 5min.

Detection of antigen

Immunoreactions were detected with peroxidase substrate 3, 3´-diaminobenzidine tetrahydrochloride (DAB, Vector) by adding DAB (250 µl) to each section for 1-5min dependent on colour development. The reaction was stopped by immersing the sections in running tap water for 10min.

Counterstaining and mounting

The nuclei of cells were counterstained by immersing slides in Harris's haematoxylin for 30 sec with excess stain washed away in running tap water for 5min. The haematoxylin stain was blued by dipping slides in 1% (v/v) ammoniated water 8 times. Sections were dehydrated with a series of ethanol (70%, 95%, and 100% v/v) for 2min each, then placed in Histoclear or xylene 2 × 5min with histological mounting medium (Omnimount; National Diagnostic) or DPX (Raymond A Lamb, East Sussex, UK) mounting media.

3.4.4 Quantification of the immunohistochemical staining

For quantification of degree of luteal VWF staining, ten images (field of views) from each section were captured randomly across the whole section under 20x objective lens using a Leica microscope. Quantification of the area of VWF staining was

performed as previously described (Robinson et al., 2009). The specific brown colour was selected using Image Pro-Plus 6.2 (Media Cybernetics, Wokingham, UK). The software than recorded the total area of brown staining and the percentage of staining. This was recorded for each image. For each section, the mean from the 10 images was generated and two sections per animal were analysed.

For PGR, ITGA5/B1. ESR1 and STC1 immunohistochemistry, each section was extensively reviewed under the microscope to assess the spatial localisation of each protein and to semi-quantify the intensity of the stain on a scale of absent (-) to intense (+++). No quantification using image analysis was performed since similar analysis have been conducted previously.

3.5 The association between embryo development and endometrial/luteal steroid content during the establishment of pregnancy.

3.5.1 Aim

The aim was to investigate the associations between embryonic development (number of embryos, their developmental stage and oestradiol concentration in uterine flushes) with luteal and endometrial progesterone and oestradiol content and the degree of vascularisation in the corpus luteum

3.5.2 Experimental design

Animals (as described in section 3.4.1) were grouped according to two factors, (1) number of embryos and (2) day of pregnancy. Firstly, the number of embryos were classified as to whether high (>9 embryos) or low (≤9 embryos). All animals with embryos in the filamentous stage were excluded (from low and high number of embryo analysis) as it was difficult to determine the exact number of embryos

present. Secondly, animals were categorised based on the day of pregnancy, thus for the different analyses, the following groups were created:

1) Corpus luteum characteristics and progesterone content:

- According to different days of pregnancy, day 3-5 (n=7 in total), day 11-12 (n=26 in total) and day 13-14 (n=7).
- According to number of embryos: day 3-5 (n=7 in total) and day 11-12 (n=11 in total). *Day 14 was excluded, as there was a large number of filamentous embryos.*
- Degree of vascularisation in or corpora lutea tissue on days 11-12 and 13-14.

Day 3-5 was excluded, as their histological appearance did not allow quantification.

2) Uterine flush oestradiol content and its relationship to other parameters:

- Oestradiol concentration in uterine flushes was compared across different days of pregnancy. These were divided into three groups: day 3-5; day 11-12 and day 13-14.
- Oestradiol concentration in the uterine flushes from pregnancy with low ($n \leq 9$) or high ($n > 9$) number of embryos. There were a total of 7 animals on day 3-5 of pregnancy and 11 on day 11-12 of pregnancy.
- Association between stage of embryonic development on day 11-12 of pregnancy and uterine flush oestradiol content. The animals were divided into two groups as follows: animals with embryos at filamentous stage (n=7) and animals did not have embryo at filamentous stage (n=19).

The experimental design is summarised in Figure 3.2.

3.5.2.1 **Statistical analysis**

The data were checked for homogeneity of variance (by Levene's test) and normality (by residual plots) and were log10 transformed where necessary. GenStat 17th Edition (VSNi, Hemel Hempstead, UK) was used to perform the statistical analysis while Graph Pad Prism 6.3 (GraphPad, California, USA) was used to plot the data. All values are presented as mean + SEM. One-way ANOVA with no blocking was performed where there were 3 groups, and multiple t-tests were performed to determine where the differences lay. Where there were only 2 groups to compare, an unpaired t-test was used. The effect of embryo number or developmental stage was determined at each time-point separately.

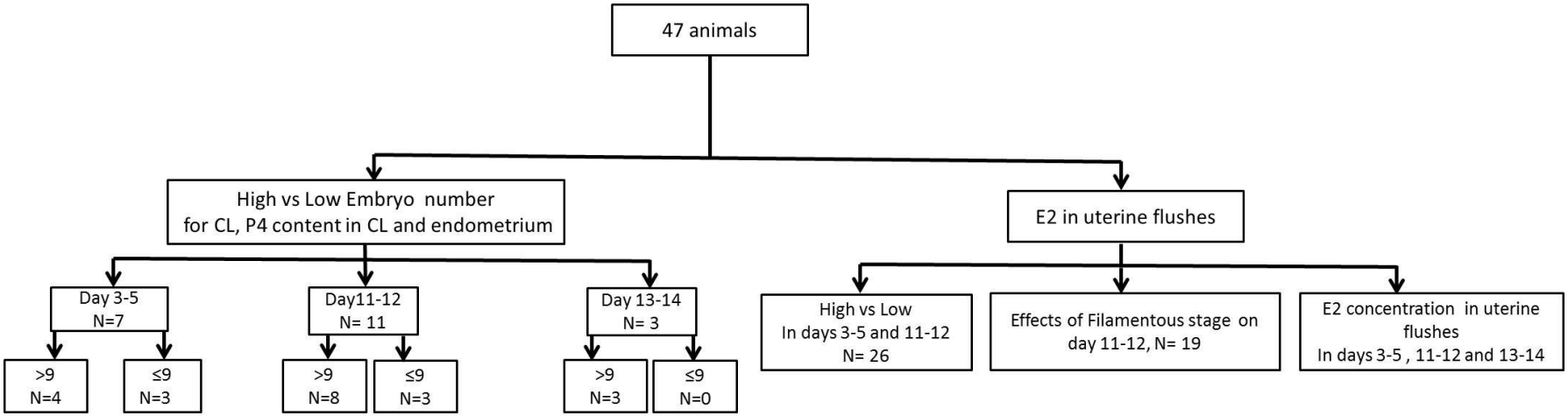


Figure 3.2: Diagram showing the experimental design for measurement of progesterone (P4) in both endometrium and corpus luteum, oestradiol (E2) in uterine flushes and corpus luteum (CL) parameters (number and weight). Filamentous stage was excluded for statistics in high number of embryos vs low number of embryos.

3.5.3 Results

3.5.3.1 Corpus luteum and embryo characteristics

The mean number of CL per animal across the study was 28.1 with a similar number of CL observed at each time point ($p>0.05$). The number and total luteal weight were not different between the low and high number of embryos groups on days 3-5 and 11-12 of pregnancy ($p>0.05$; Fig. 3.3). As expected, the total luteal weight was lower on days 3-5 compared with days 11-12 and 13-14 ($p<0.001$). The total luteal weights on days 11-12 and 13-14 were similar at approximately 11.5g.

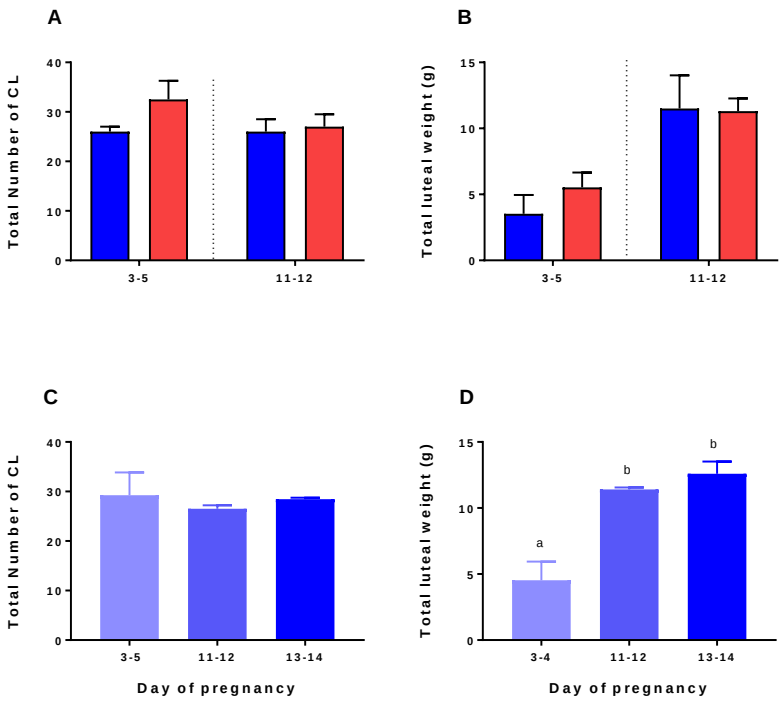


Figure 3.3: The association between number of embryo (low [blue] and high [red]) with corpus luteum (CL) characteristics. (A) Shows the total number of CL and (B) the total weight of CL on days 3-5, 11-12 and 13-14 of pregnancy. (C) Shows the total number of CL and (D) shows the total luteal weight on days 3-5, 11-12 and 13-14 of pregnancy. Data values = mean + SEM, a<b, $p<0.05$, n=3-7 per group.

3.5.3.2 **Luteal and endometrial progesterone content during the peri-implantation period**

The mean luteal and endometrial progesterone content on the different days of pregnancy and number of embryos are shown in Figure 3.4. The luteal progesterone content was similar on the different days of pregnancy, whereas the total luteal progesterone content increased as pregnancy progressed ($p<0.05$). The endometrial progesterone content was similar on the different days of pregnancy. The level of endometrial and luteal progesterone content were similar with different number of embryos ($p>0.05$). Intriguingly, on day 3-5 of pregnancy, the total progesterone content in animals with high number of embryos was greater than those with low number of embryos ($p<0.05$). While, there were no differences on days 11-12 and 13-14 of pregnancy.

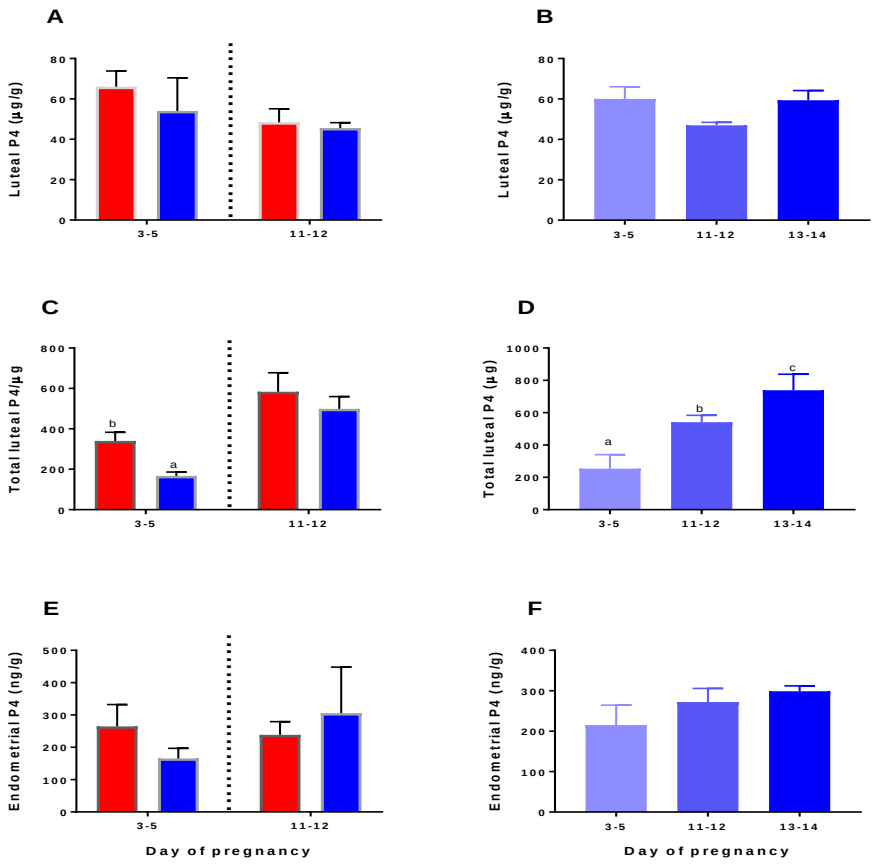


Figure 3.4: Association between number of embryos (low [blue] and high [red]) and progesterone (P4) content in (A): Corpus luteum per gram of tissue, (C): Total content in corpus luteum (µg) and (E): Endometrial tissue per gram of tissue, on day 3-5 and 11-12 of pregnancy. Progesterone content in different days of pregnancy 3-5, 11-12 and 13-14 in (B): Corpus luteum per gram of tissue, (D): Total content in corpus luteum (µg) and (F): Endometrial tissue per gram of tissue. Data values = mean + SEM, n=3-7 per group, a<b<c; $p<0.05$.

3.5.3.3 **The association between oestradiol uterine flushes level and number of embryos during the peri-implantation period**

The oestradiol content in the uterine flushes (regardless of the embryonic stage or number of embryos) was significantly greater on days 11-12 and 13-14 of pregnancy compared with day 3-5 of pregnancy ($p<0.001$). The level of oestradiol was numerically lower on day 13-14 than on day 11-12 of pregnancy but this failed to reach significance (Fig 3.5A).

The number of embryos did not affect the oestradiol concentration in the uterine flush on day 3-5 and 11-12 of pregnancy (Fig 3.5B). There was no analysis for day 13-14 because all animals with a low number of embryos had filamentous stage of embryos and thus were excluded.

On day 11-12 of pregnancy, animals with embryos that were at the filamentous stage had a greater concentration of oestradiol in the uterine flushing ($p<0.05$) when compared to those animals where the embryos were at the ovoid stage (Fig. 3.5C).

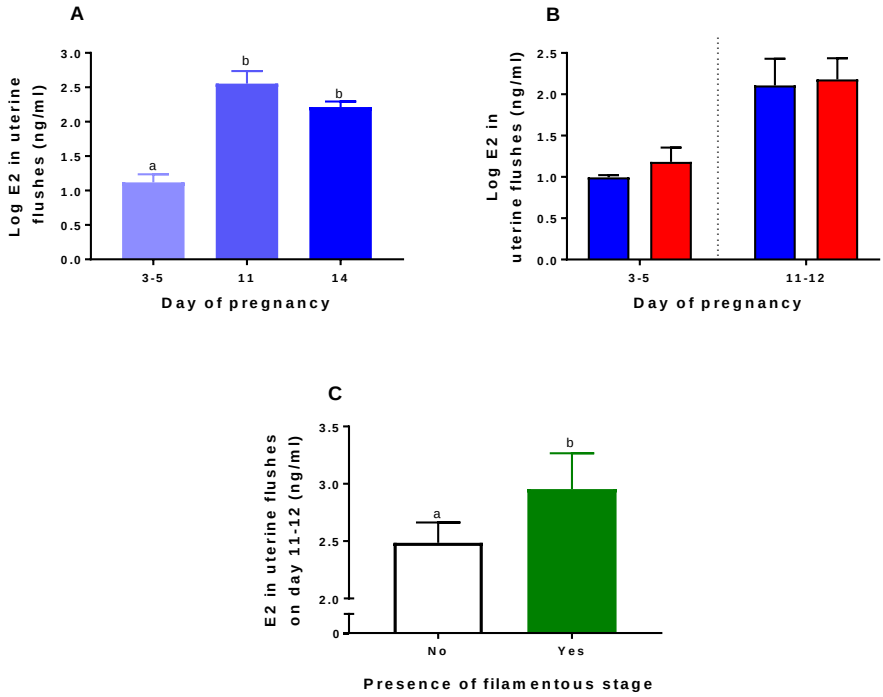


Figure 3.5: The association between uterine flush oestradiol concentration and embryonic number and stage. (A) Shows the 17β oestradiol (E2) concentration in uterine flush on days 3-5, 11-12 and 13-14 of pregnancy. (B) Shows the association between number of embryo (low and high) with E2 content in uterine flush on days 3-5 and 11-12 of pregnancy. (C) Shows the association between E2 and embryo developmental stage (yes=has a filamentous embryos) on day 11-12 of pregnancy. Data values = mean + SEM with n=3-7 per group; a<b: $p<0.05$.

3.5.3.4 **Luteal vascularisation**

Extensive microvasculature was observed across the whole corpus luteum section, with endothelial cells in close proximity to luteal steroidogenic cells (Fig 3.6A and B). This pattern was evident on both days 11-12 and 13-14 of pregnancy. There was no specific staining in the immunohistochemistry control (Fig 3.6C). The staining was similar between days 11-12 and 13-14 of pregnancy and between those with high and low numbers of embryos (Fig. 3.6D and F). On day 3-5, the histological appearance of CL did not allow quantification.

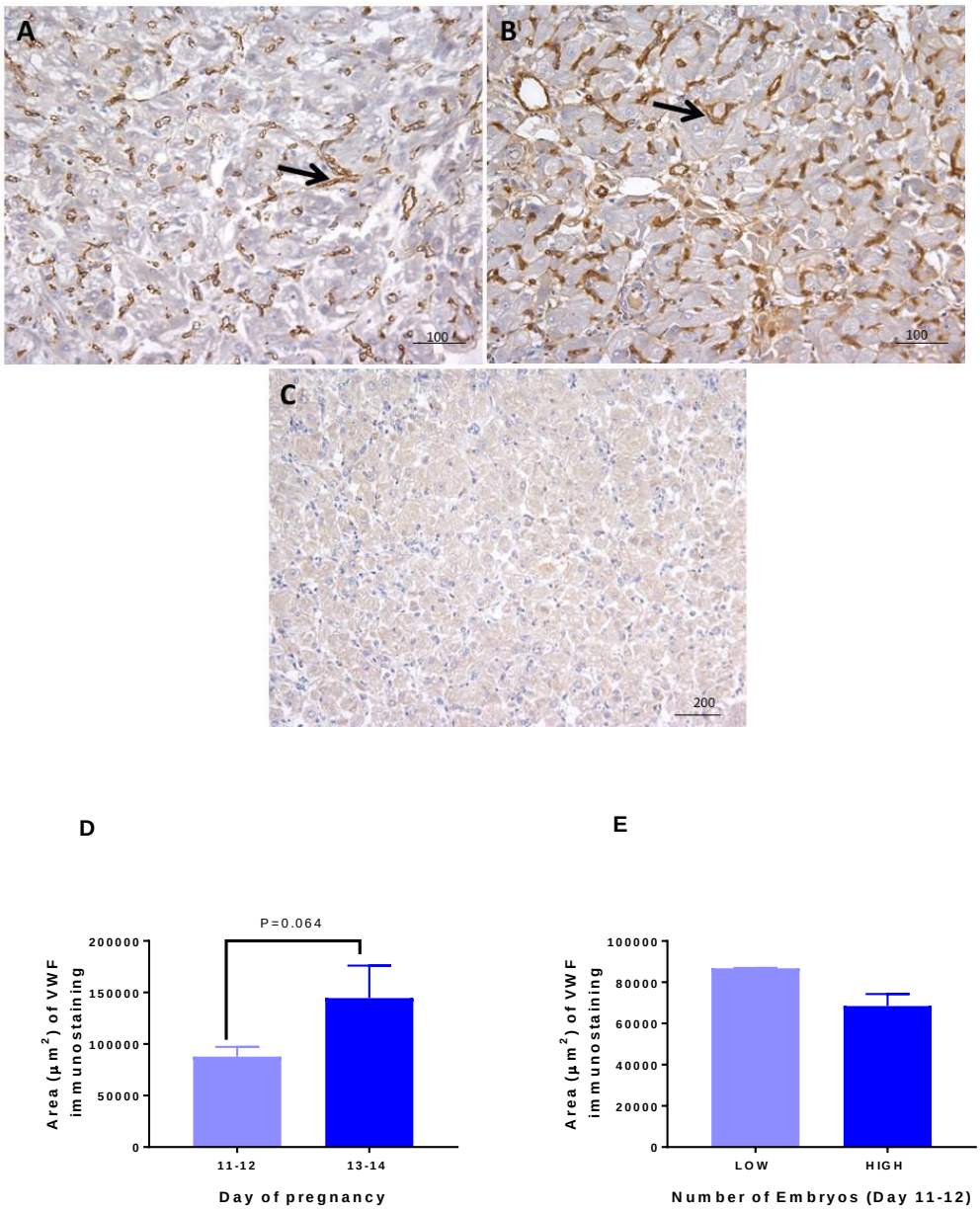


Figure 3.6: Immunohistochemical localisation of von Willebrand factor (VWF) in the corpus luteum from (A) day 11-12 and (B, C) day 13-14 of pregnancy. (C) Shows luteal tissue staining with the control rabbit IgG (primary antibody absent). Arrows indicate area with positive VWF staining. (D) Shows quantification of area of VWF staining on day 11-12 and 13-14 of pregnancy. (E) Shows the comparison between animals with low (n≤9) and high (n>9) embryos on day 11-12 of pregnancy. Data are mean + SEM.

3.6 Temporal-spatial assessment of endometrium markers during the establishment of pregnancy.

3.6.1 Experimental design

From the animals described in 3.4.1, animals were grouped according to day of pregnancy as follows; day 3-5 (n=3), day 11-12 (n=10) and day 13-14 (n=3).

3.6.2 Results

3.6.2.1 Temporal changes in progesterone receptor expression

The temporal-spatial assessment of progesterone receptors (PGR) in luminal epithelium is shown in Figure 3.7. Nuclear PGR was located in the luminal epithelium of the endometrium (Fig 3.7A). Specifically, on day 3-5 of pregnancy the expression of PGR was very intense in this region but was completely absent on days 11-12 and 13-14 of pregnancy (Fig 3.7B and C; Table 3.2). Superficial stroma cells lying just below the luminal epithelium were positive for PGR across all the pregnancy days studied. Visual quantification showed that its intensity was higher on day 3-5 comparing with days 11-12 and 13-14 of pregnancy (Table 3.2).

The degree of PGR immunostaining varied in the epithelial cells of superficial glands across the time points (Fig. 3.8 and Table 3.2). Namely, on day 3-5 (Fig 3.8A) PGR expression was very high compared with reduced expression on day 11-12 (Fig 3.8B) and low in some samples on day 3-5 (Fig 3.8C) of pregnancy.

Progesterone receptor was immunolocalised to the epithelial cells of endometrial deep glands on all days of pregnancy (Fig. 3.8). The intensity of the staining on day 3-5 was lower than that on days 11-12 and 13-14 of pregnancy (Table 3.2).

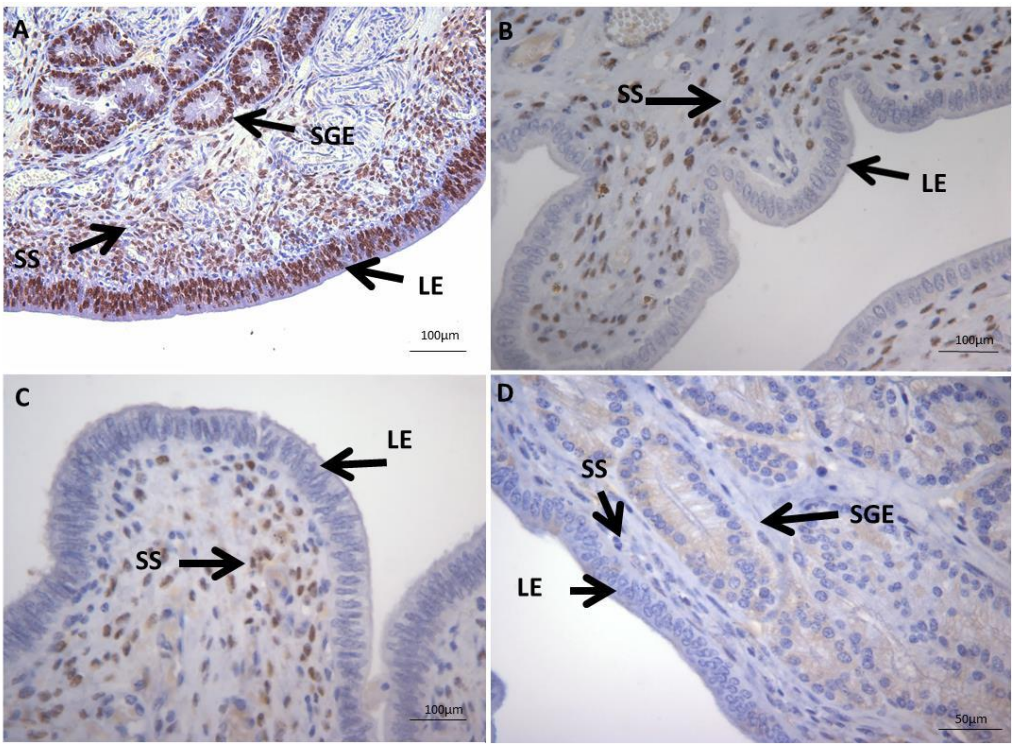


Figure 3.7: Immunohistochemical localisation of progesterone receptor in the luminal region of the endometrium of pregnant sows. Representative images are shown for (A): pregnancy day 3-5; (B): pregnancy day 11-12; (C) pregnancy day 13-14, while (D): shows negative mouse IgG control where no staining was evident (control). The appearance of progesterone receptor immunostaining was dense and extensive in all cells of the luminal epithelium (LE) and superficial stroma layers (SS) on day 3-5, while it was absent in the luminal epithelium on days 11-2 and 13-14. Superficial glandular epithelium (SGE). Scale bars = 100 and 50µm.

Table 3.2: Staining intensity for progesterone receptor protein in porcine endometrium during early pregnancy. It was based on a score of '-'no staining, '+' weak staining,'++' moderate staining and '+++' strong staining.

Day of pregnancy	Luminal epithelium	Superficial glandular epithelium	Deep glandular epithelium	Superficial stroma	Deep stroma
3-5	+++	+++	+	++/+++	+
11-12	-	+	+++	+/++	+/++
13-14	-	-/+	+++	+/++	+/-

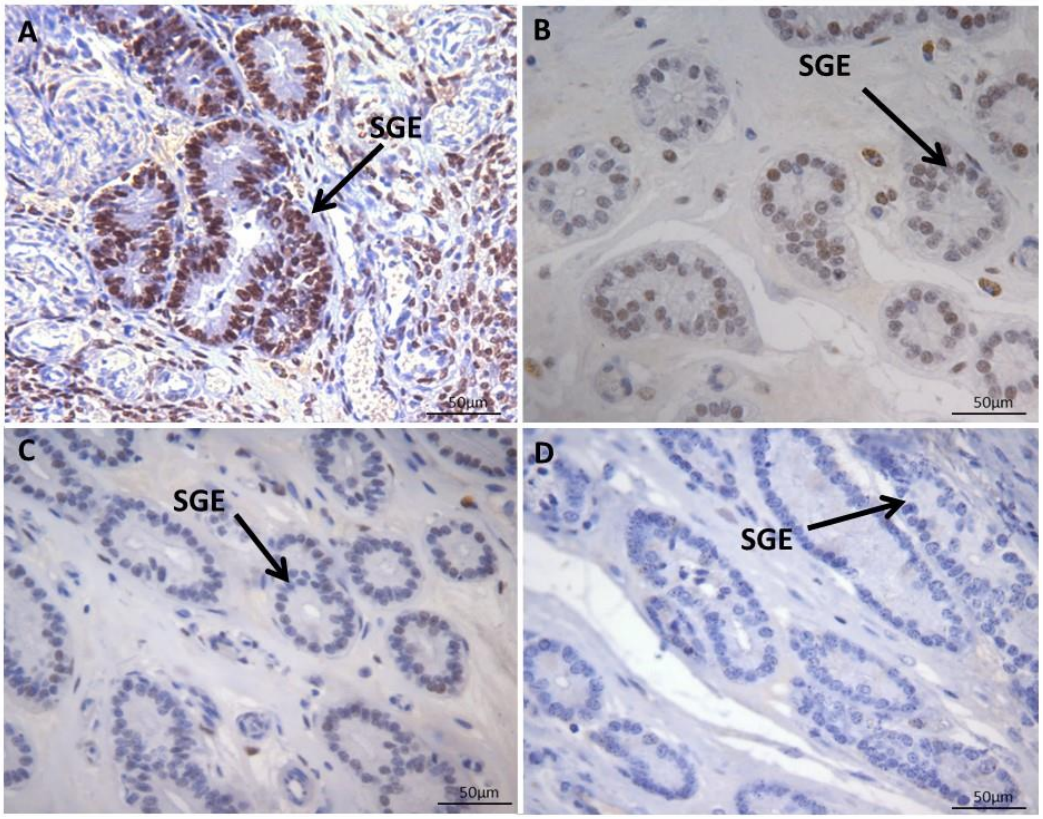


Figure 3.8: Immunohistochemical localisation of progesterone receptor in the endometrial superficial glands of pregnant sows. Representative images are shown in (A): pregnancy day 3-5; (B): pregnancy day 11-12; (C) pregnancy day 13-14 and (D): shows negative mouse IgG control where no staining was evident. The appearance of progesterone receptor staining was dense and extensive in all epithelial cells of superficial (SGE) on day 3-5, while it was much lower on day 11-12 and hardly detected on day 13-14. Scale bar= 50µm.

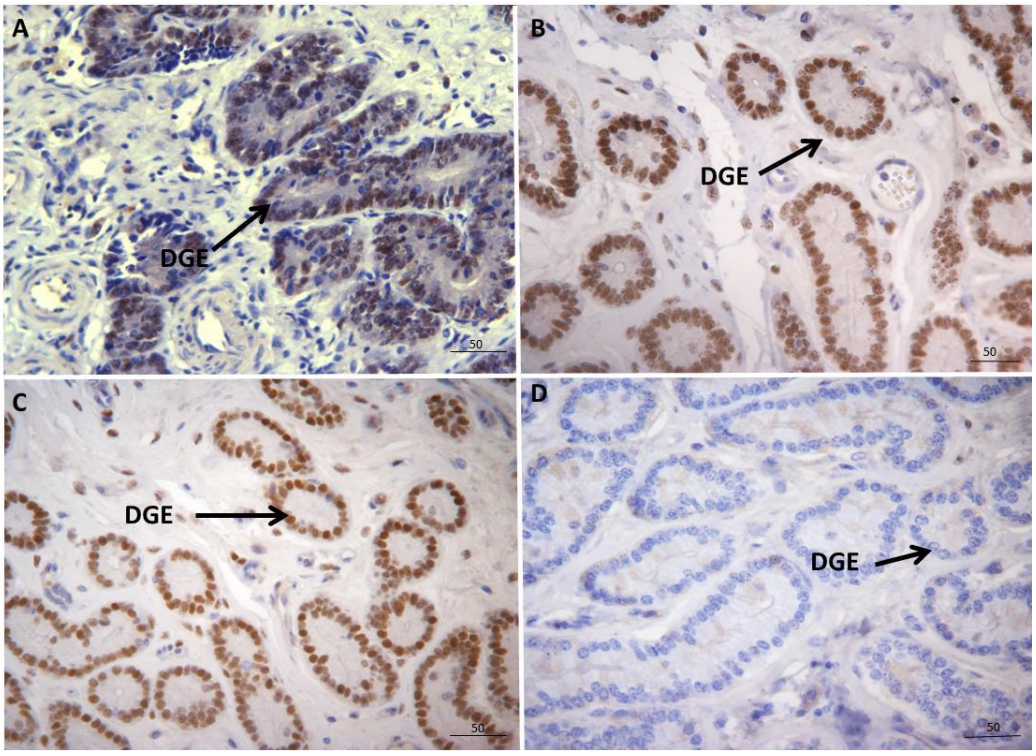


Figure 3.9: Immunohistochemical localisation of progesterone receptor in endometrial deep glands (DGE) of pregnant sows. Representative images are shown for (A): pregnancy day 3-5; (B): pregnancy day 11-12; (C) pregnancy day 13-14 and (D): shows negative mouse IgG control where no staining was evident. The appearance of progesterone receptor antibody staining was moderate to intense on all days studied. Scale bar = 50µm.

3.6.2.2 **Immunolocalisation of oestrogen receptor**

Immunostaining for oestrogen receptor 1 (ESR1) was detected in the cell nucleus in endometrial luminal epithelial tissue of pregnant sows during the peri-implantation period (Fig 3.10). On days 3-5 of pregnancy (Fig 3.10A), neither luminal epithelium nor stroma cells showed any positive staining. However, in sows on day 11-12 of pregnancy, some luminal epithelial cells and stroma cells were positive (Fig 3.10B). While on day 3-5 of pregnancy, ESR1 expression was observed in most of the stroma cells (Fig 3.10C). There was also some cytoplasmic staining for ESR1 in glandular epithelial cells on day 13-14 of pregnancy. The immunohistochemical controls were blank.

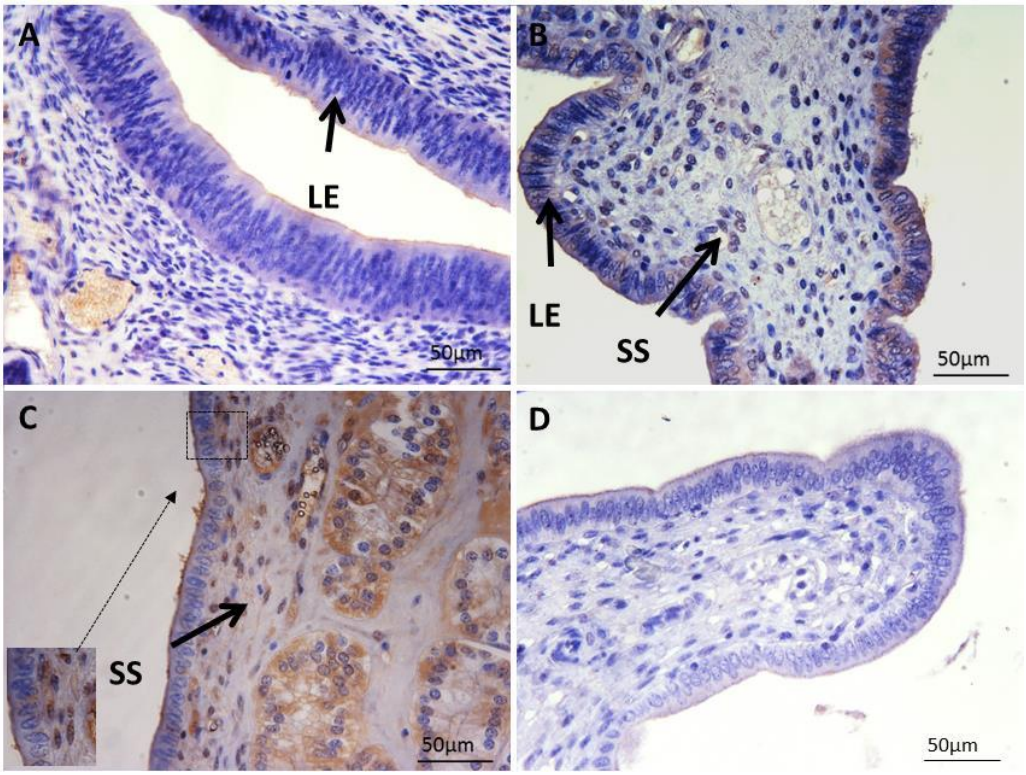


Figure 3.10: Immunohistochemical localisation of oestrogen receptor 1 (ESR1) in luminal epithelium (LE) and superficial stroma (SS) layers of the endometrium in pregnant sows. Representative images are shown of (A): pregnancy day 3-5; (B): pregnancy day 11-12; (C) pregnancy day 13-14 and (D): shows negative mouse IgG control where no staining was evident. ESR1 was detected in the nucleus of some of the luminal epithelium and superficial stroma cells on days 11-12 and 13-14 of pregnancy. There was some cytoplasmic staining of ESR1 present (C). Scale bar = 50µm

In contrast, the number of cells that were positive in deep glandular epithelium was very limited. Again, there was no positive staining on day 3-5 of pregnancy (Fig. 3.11A). In glandular epithelium, ESR1 was detected on days 11-12 and 13-14 of pregnancy (Fig. 3.11B and C respectively) and it was more intensive on day 11-12 than day 13-14.

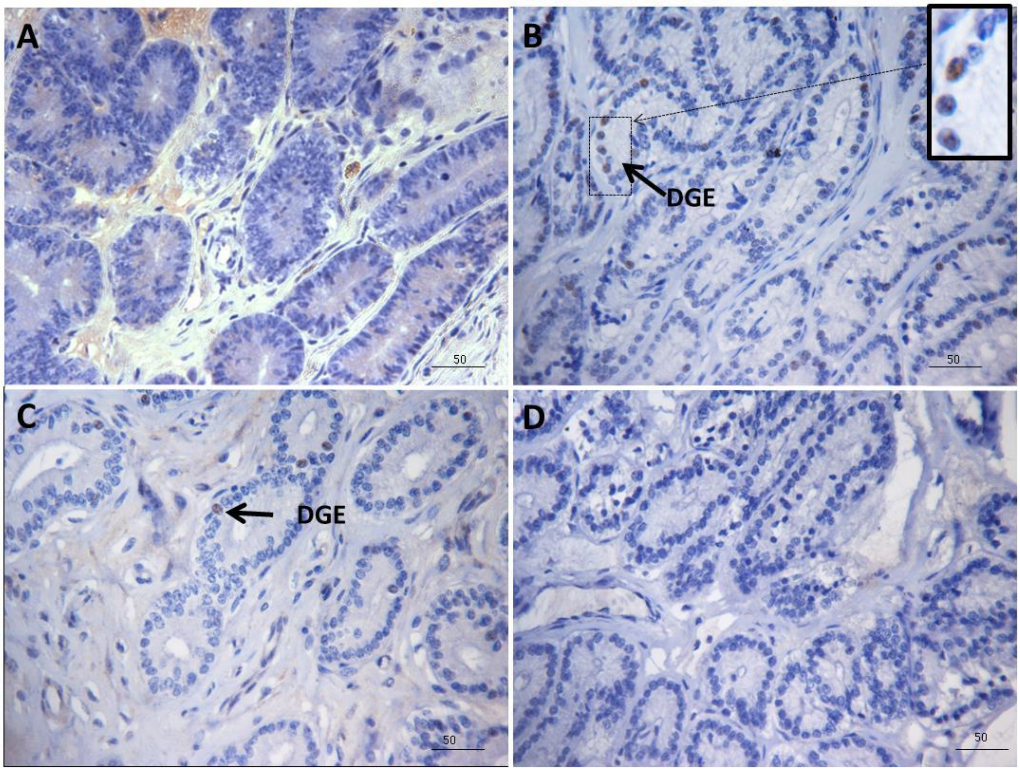


Figure 3.11: Immunohistochemical localisation of oestrogen receptor 1 (ESR1) in the endometrial deep glands (DGE). Representative images from pregnant sows on (A) day 3-5; (B): day 11-12 and (C) day 13-14 of pregnancy are shown. (D) shows the negative mouse IgG control where no staining was evident. The appearance of ESR1 nuclear staining was detected in a few glandular epithelial cells on days 11-12 and 13-14 of pregnancy. Scale bar = 50µm.

3.6.2.3 **Stanniocalcin 1 in the porcine endometrium**

There was expression of stanniocalcin 1 (STC1) in the luminal epithelial layer of endometrium (Fig 3.12). STC1 expression was detected on all days of pregnancy investigated. It appeared to increase gradually as the pregnancy progressed. Moreover, STC1 expression was more noticeable in stroma cells on days 11-12 and 13-14 than day 3-5 of pregnancy. Intriguingly, the luminal epithelial cells from day 11-12 pregnant sows showed moderate staining across the cytoplasm while the staining was much more localised in the apical surface of the luminal epithelial cells on day 13-14 of pregnancy.

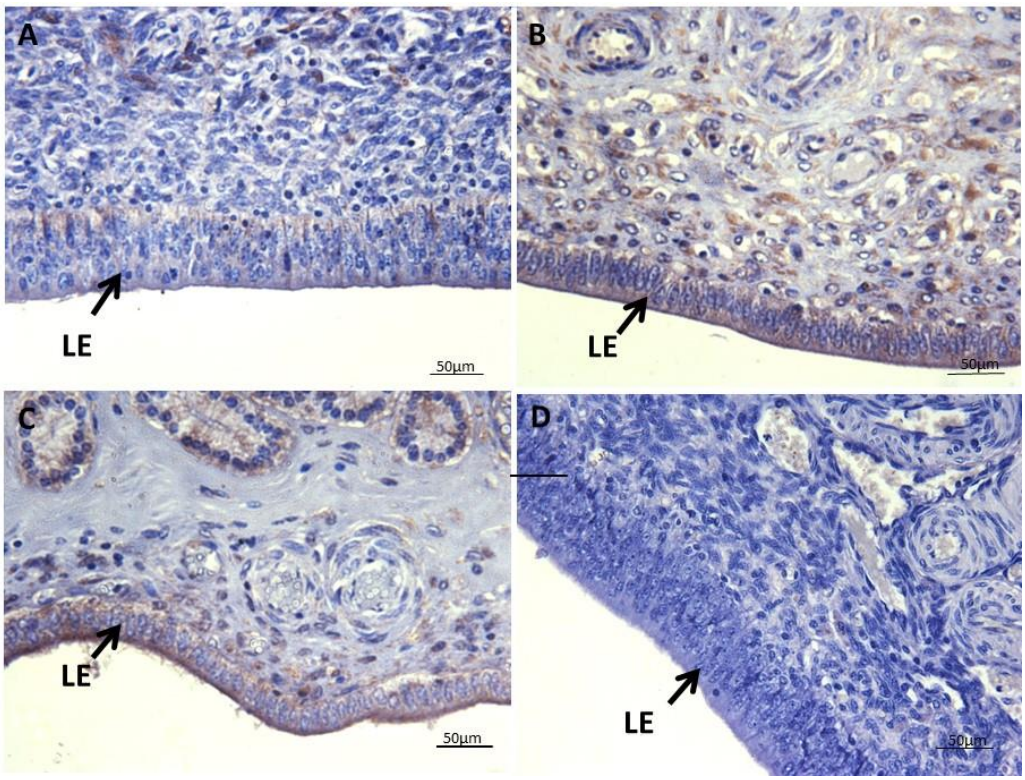


Figure 3.12: Immunohistochemical localisation for stanniocalcin 1 (STC1) to the luminal epithelium (LE) layer of the endometrium in pregnant sows. Representative images from (A) day 3-5; (B): day 11-12; and (C) day 13-14 of pregnancy are shown. (D) Shows the negative rabbit IgG control where no staining was evident. STC1 was present on all days with its expression in the luminal epithelium increasing with day of pregnancy. Scale bar = 50µm.

3.6.2.4 **Immunolocalisation of integrin A5/B1 in endometrial luminal epithelium**

Immunostaining of integrin A5/B1 (ITGA5/B1) in endometrial tissue was present on day 11-12 and 13-14 of pregnancy (Figure 3.13A and B, respectively). Samples from day 3-5 of pregnancy were not analysed. On day 11-12 of pregnancy, ITGA5/B1 expression was observed in the luminal epithelium in both the cytoplasm and along the apical surface. While on day 13-14 of pregnancy, the positive staining in luminal epithelium appeared more on apical surface than the cytoplasm.

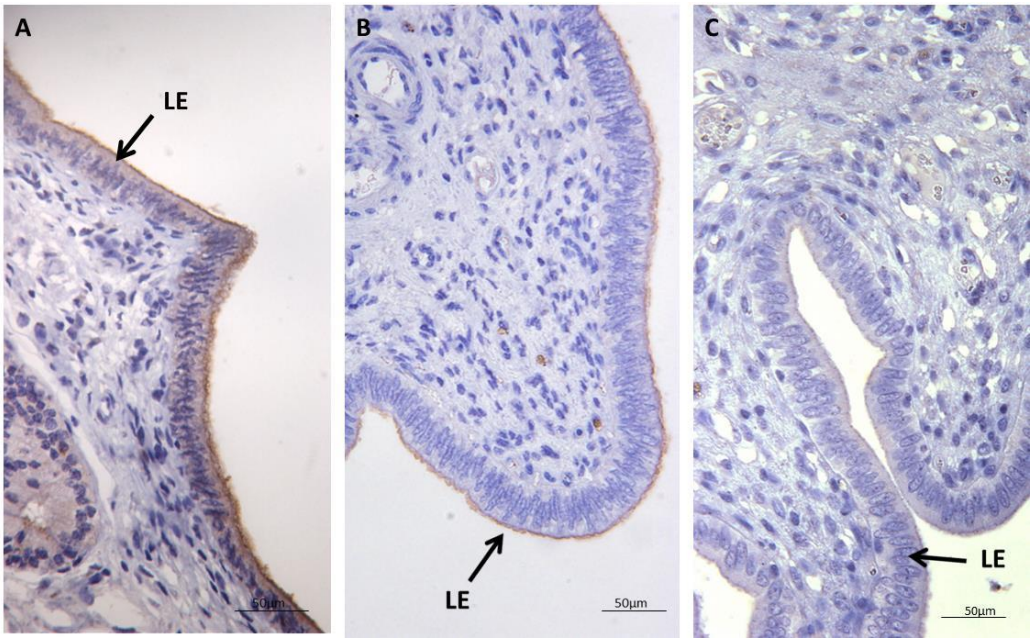


Figure 3.13: Immunohistochemical staining for ITGA5/B1 in the luminal epithelium (LE) of the pregnant sow endometrium. Representative images from (A) day 11-12; (B) day 13-14 of pregnancy with the immunohistochemical control shown in (C). ITGA5/B1 protein was expressed on both days in the LE. On day 13-14, this staining was predominantly localised to the apical surface. Scale bar = 50µm.

The expression profiles for different endometrial receptivity markers PGR, ESR, ITGA5/B1 and STC1 are summarised in Table 3.3.

Table 3.3: The expression of endometrial markers in the porcine endometrium on pregnancy days 3-5, 11-12 and 13-14. Scoring was based on a score of '-'no staining, '+' weak staining,'++' strong staining and nd= not determined.

Region	Marker	Day 3-5	Day 11-12	Day 13-14
Luminal epithelium	PGR	++	-	-
	ESR1	-	+	-
	STC1	+	++	++
	ITGA5/B1	nd	+	+
Glandular epithelium	PGR	+	++	++
	ESR1	-	+	+
	STC1	-	+	++
	ITGA5/B1	nd	+	+
Stroma	PGR	+	+	+
	ESR1	+	+	++
	STC1	+	+	+
	ITGA5/B1	nd	+	+

3.7 Discussion

The present study investigated the inter-communication between the endometrium, corpus luteum and embryo in the sow in the preparation of an environment for successful pregnancy. The principal focus was the effect of the number and developmental stage of embryos on this communication.

The mean number and total weight of the corpora lutea were similar in the animals that had a low or high number of embryos on days 3-5, 11-12 and 13-14 of pregnancy. In order to have numerous embryos, multiple ovulations are required. In this study, the mean number of ovulations was 28, however less than 50% of these led to an embryo. Moreover, the number of embryos did not appear to be influenced by the number of ovulations. This is in agreement with Bazer et al (1988) where Large White and prolific Chinese Meishan gilts were compared. In this study, the high number of corpora lutea in the Meishan breed was not associated with the number of embryos or total weight of corpus luteum.

Extending the lifespan of corpora lutea and the secretion of progesterone is essential for establishment of pregnancy and implantation (Thatcher et al., 1986, Geisert et al., 2014). Interestingly, in the present study on day 3-5 of pregnancy the total luteal content of progesterone was significantly higher in animals with a high number of embryos compared with those with a low number of embryos. However, by day 11-12 and 13-14 of pregnancy this difference was not observed. Moreover, there were no differences in progesterone per gram of endometrium tissue at all time-points despite total luteal progesterone increasing with time. This would suggest that the level of progesterone in the endometrium/embryo is experiencing does not vary that much. The observation on days 3-5 raises the intriguing possibility that in the early

embryo stages, the level of progesterone (or corpus luteum quality) affected the number of embryos. Equivalent observations have been made in the cow where the plasma concentration of progesterone in the immediate post-ovulatory period reflected subsequent pre-implantation embryonic development (Mann and Lamming, 2001). Similarly, the results from Chapter 2 clearly demonstrated that progesterone supplementation in early stage of embryo in ART women improved pregnancy rates.

In sows (as well as other mammals), the conceptus plays a crucial role in the establishment of pregnancy, by preparing the uterine endometrium for imminent implantation and pregnancy as well as maintaining the function of the corpus luteum (Morawska et al., 2012, Geisert et al., 2014). Oestradiol is the embryonic signal to its mother in the pig (Bazer et al., 1986). This study showed positive staining for ESR1 in the luminal epithelium on days 11-13 and 13-14 of pregnancy, albeit at low levels, indicating that embryonic oestradiol is able to signal to the endometrium. It has been reported that there were two phases of oestradiol secretion from the porcine embryo. Firstly, during elongation (approximately days 11-12) after which the level decreases on days 13-14 of pregnancy. However, after this, oestradiol secretion increases again from day 15 to 23 of pregnancy (Bazer et al., 1986, Waclawik, 2011). As expected oestradiol concentrations in the flush were numerically higher on day 11-12 than day 13-14. In addition, this study showed a low level of oestradiol in uterine flushes on day 3-5 of pregnancy. There is also evidence for secretion of oestradiol from rodent embryos during the pre-morula stage (Niemann and Elsaesser, 1987). The results from the present study showed that the oestradiol content in the uterine flush was not affected by the number of embryos on days 3-5 and 11-12 of pregnancy but was higher in animals that had filamentous embryos on day 11-12 of pregnancy. There was no result on day 13-14 of pregnancy regarding to the number

of embryo because all animals with low number of embryos were excluded due to presence of filamentous stage embryos. Oestradiol stimulates the maintenance of the corpus luteum by converting the secretion of prostaglandin F2α from endocrine to exocrine as well as increasing the secretion of the luteotrophic prostaglandin E2. Collectively this will maintain the corpus luteum function and progesterone secretion (Bazer, 2013). The optimal development of the corpus luteum is a key factor to determine appropriate progesterone secretion and conditions for pregnancy establishment (Watson and Fraser, 2003, De Andrea Ribeiro, 2007). Immunostaining for VWF revealed that the total vascularisation of the CL was similar on day 11-12 and 13-14 of pregnancy, and showed no association with progesterone content.

The progesterone receptor was intensively expressed in epithelial and stromal cells on day 3-5 of pregnancy then disappeared from luminal epithelial cells completely on pregnancy days 11-12 and 13-14. These immunostaining results are in agreement with previous studies in the pregnant gilt (Geisert et al., 1994) and sow (Sukjumlong et al., 2005). In this experiment, immunostaining of ESR1 varied across the different pregnancy days. Namely, there was no expression on day 3-5, but there was some positive expression in luminal and glandular epithelial cells and stromal cells on days 11-12 and 13-14 of pregnancy. It is unknown whether this upregulation is due to prolonged exposure to progesterone or oestradiol secretion from the embryo. Previously, it has been reported that *ESR1* mRNA is present in the endometrium on all days of the cycle with it maximal on day 10 and lowest on day 15. Similarly in pregnant sows, *ESR1* expression decreased gradually from day 10 to 18 (Geisert et al., 1993). Immunohistochemical studies have shown that ESR1 was detected in the luminal epithelium during early pregnancy (Sukjumlong et al., 2004,

Srisuwatanasagul, 2011). In this study, the antibody used to detect ESR1 was rabbit anti-human ESR1 rather than being porcine specific. This might have resulted in weaker binding of the antibody to the porcine ESR1 protein and thus reduced staining intensity.

The expression of stanniocalcin 1 (STC1) was clearly detected in the endometrium on pregnancy days 3-5, 11-12 and 13-14. The intensity of the immunostaining was greater on days 11-12 and 13-14 of pregnancy. Similarly, *STC1* mRNA has been shown to be abundantly expressed in the porcine endometrium (Ostrup et al., 2010, Samborski et al., 2013a, Samborski et al., 2013b, Lin et al., 2015). The endocrine regulation of STC1 has been investigated in the porcine endometrium during the oestrous cycle and pregnancy. It was shown that progesterone induced STC1 expression in the luminal epithelium during the cycle, which was further increased following oestrogen treatment (Song et al., 2009). This comes in agreement with the higher density of STC1 immunostaining on pregnancy days 11-12 and 13-14 in this study.

Integrins are transmembrane glycoproteins that consist of two subunits: (1) α subunits of which there are 18 different genes and (2) β subunit, which has nine different genes (Bowen et al., 1997). Integrins are in contact with the ECM and transfer cellular signals between endometrial epithelial cells and conceptus trophectoderm (Spencer and Bazer, 2004). Therefore, integrins play a fundamental role in mediating adhesion, migration and proliferation between cells during pregnancy (Rashev et al., 2005). Importantly, the temporal-spatial localisation of integrins has been considered as a marker of uterine receptivity and is associated with the time of successful implantation (Lessey et al., 1994). In the present study,

the expression of ITGA5/B1 was observed in luminal epithelial cells on pregnancy days 11-12 and 13-14 and supports a role in attachment/implantation. This expression was opposite to that for progesterone receptor which was absent from the luminal epithelium at this time. Thus, it is tempting to think that the removal of progesterone signalling in luminal epithelium stimulates the up-regulation of ITGA5/B1.

Establishment and successful implantation in the pig includes triggering many molecular signalling pathways in the corpus luteum, conceptus and endometrium. Certain levels of progesterone and oestradiol are required to synchronise this process. Based on these observations, the sex-steroid regulation of uterine receptivity markers (STC1, OPN and MUC1) in endometrial explants is investigated in next Chapter.

CHAPTER 4 : The effect of progesterone and oestradiol on stanniocalcin 1, osteopontin and mucin 1 expression in the *ex vivo* porcine endometrium

4.1 Introduction

The endometrium plays a central role in co-ordinating the mother's ability to respond to and support development of the conceptus and consequently it has attracted much research. In a highly harmonised manner, both oestradiol and progesterone are considered the principal regulators of its function. Oestradiol is classically associated with regulating the uterine environment to support spermatozoa transport. While progesterone acts on the uterus to influence the expression of several genes that regulate the establishment of pregnancy and conceptus attachment. In turn, paracrine secretions from the conceptus can further influence endometrial receptivity. Cytokines, growth factors, prostaglandins and particularly oestradiol are among the key factors influencing embryo-maternal communication (Ziecik et al., 2011, Bazer and Johnson, 2014, Szymanska and Blitek, 2016).

The circulatory level of progesterone can modulate endometrial function (Ashworth and Bazer, 1989). It is very widely accepted that increasing progesterone concentrations after ovulation are essential to prepare the endometrium for successful implantation in mammals (Glasier et al., 1994). For example, in the pig progesterone affected the ability of the embryo to implant by up-regulating the expression of genes such as prostaglandin-endoperoxide synthase-1 (PTGS1), microsomal prostaglandin E synthase-1 (mPGES-1) and vascular endothelial growth factor A (VEGFA) (Szymanska and Blitek, 2016).

Oestradiol is also essential for implantation and establishment of pregnancy in the pig (Bazer and Thatcher, 1977). The conceptus starts to secrete oestradiol when it starts to elongate. Intriguingly, if pregnant pigs are exposed to oestradiol prematurely (i.e. prior to elongation) then this leads to failure of implantation and loss of pregnancy. This was presumably due to the inappropriate upregulation of genes associated with attachment such that there is disruption of the attachment of embryo to the endometrium (Ross et al., 2007).

Recently, microarray studies in several mammalian species have shown that there is a unique pattern of gene expression at the time of implantation or uterine receptivity and these upregulated genes are common between different species. One of the genes that up-regulated is stanniocalcin 1 (STC1) (Ostrup et al., 2010, Samborski et al., 2013a, Samborski et al., 2013b, Lin et al., 2015). STC1 expression in the endometrium at the time of implantation appears to be under steroid control (Song et al., 2009) but it is unclear if progesterone and/or oestradiol directly regulate STC1. Generally, STC1 is considered a glycoprotein that has involvement in various physiological functions such as body fluid haemostasis (Turner et al., 2011, Yeung et al., 2012), and induction of angiogenesis processes (Law and Wong, 2013). It also has anti-inflammatory effects by reducing the inflammatory cascade, inhibits oxidative stress and inhibits anti-apoptotic pathways (Tang et al., 2014). STC1 also can inhibit macrophage function by attenuating intracellular calcium and thus decreasing their mobility (Kanellis et al., 2004).

Osteopontin (OPN) is one of the extracellular matrix molecules with a significant role in adhesion of embryo at the attachment time (Johnson et al., 2003, Johnson et al., 2014). Its secretion was induced in luminal epithelial cells of the endometrium

by oestrogen derived from the conceptus at the time of elongation time in the pig (White et al., 2005). Moreover, progesterone and conceptus factors induce OPN secretion from glandular epithelium after day 25 of pregnancy in sow (Bailey et al., 2010). Consequently, OPN concentrations increase dramatically from pregnancy day 25 to 85 (Garlow et al., 2002). OPN binds specific integrin subunits in luminal epithelium cells and trophectoderm. Thus, it is suggested that OPN promotes the migration of the trophectoderm and its attachment to endometrial epithelial cells at implantation (Erikson et al., 2009).

Mucin 1 (MUC1) can prevent interactions between cells and extracellular matrix thus the adhesion between embryo and endometrial luminal epithelium (Lagow et al., 1999). Several studies have indicated that there is a temporal loss or reduction of MUC1 at time of uterine receptivity in many species (Carson et al., 1998, Hoffman et al., 1998, Lagow et al., 1999, Meseguer et al., 2001, Carson et al., 2008). Furthermore, in the porcine endometrium, *MUC1* expression at attachment sites was lower than in the area between attachment sites (Ren et al., 2010). The current study tested the hypothesis that the endometrial expression of STC1, OPN and MUC1 are directly controlled by progesterone and oestradiol *in vitro*.

4.2 **Aims**

In order to address this hypothesis, there were three aims:

1) To characterise the association between *ex vivo* endometrial progesterone content and expression of STC1, OPN and MUC1.

2) To establish and characterise an endometrial explant system that was viable for at least 96 hours.

3) To determine the effects of different concentration of progesterone and oestradiol on the expression of stanniocalcin 1 (STC1), osteopontin (OPN) and mucin 1 (MUC1) in culture over time.

4.3 Material and methods

4.3.1 Experimental design

This experiment was conducted in two main parts (Fig 4.1):

1) *Ex vivo* study: Endometrial tissue was collected from pregnant sows (day 11-12, n=12). These were the same animals as those used in Chapter 3 and categorised according to endometrial progesterone content (described in Section 3.4.2.). STC1 protein expression was quantified by Western blot in endometrial tissue with the highest (mean=293 ng/g, n=3) and lowest (mean=95 ng/g, n=3) progesterone content.

2) *In vitro* study: Endometrial tissue was collected from pregnant sows on days 3-5 (n=2), 11-12 (n=3) and 13-14 (n=3). The day 3-5 pregnancy group was not included in the statistical analysis due to the low number of animals. Endometrial explant tissues (~100mg) were treated with (1) progesterone (0, 10 and 100 ng/ml) or (2) oestradiol (0, 0.1 and 1 ng/ml) in the absence or presence of 10 ng/ml progesterone in a 3 × 2 factorial design. The doses for progesterone and oestradiol were based on physiological levels and those used previously (Kempisty et al., 2015). Tissue was collected after 0, 48 and 96h of explant culture to determine the protein expression of STC1, OPN and MUC1 by Western blot. The expression of *STC1* mRNA was determined using RT-PCR in endometrium from sows on pregnancy days (5, 11 and 14) and during endometrial explant culture.

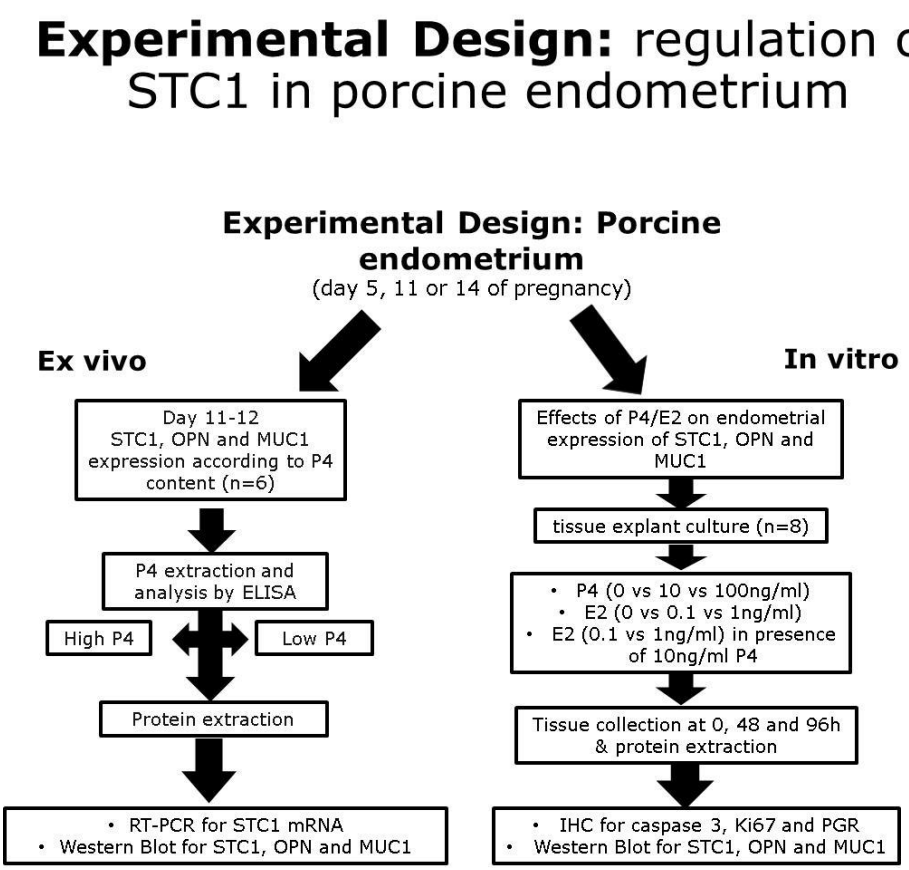


Figure 4.1: Schematic diagram showing the experimental design for the study investigating the effects of progesterone (P4) and oestradiol (E2) on STC1, OPN or MUC1 expression.

4.3.2 **Animals and tissue collection**

Porcine reproductive tracts were collected post mortem from a local slaughterhouse. For all 3 objectives, a total of 20 reproductive tracts were collected from pregnant pigs on specific days (day 5 [n=2], day 11-12 [n=15] and day 13-14 [n=3]). At the slaughterhouse, small strips of endometrium (~200mg) were collected from the folds where the embryo tends to attach. The endometrium was then placed in tubes containing 5 volumes of RNA later. Next, a small section from the same uterus (~20 cm in length) was ligated and removed from each horn, then transferred to the laboratory in PBS. For those used in explant tissue culture, the pieces of uterine horn were washed in PBS, then placed in a laminar flow hood and opened with a scalpel.

Endometrial strips were isolated from the opened uterus by careful dissection of the luminal mucosal layer and placed in pre-warmed (37°C) PBS. The endometrium then was cut with a sterile biopsy punch into small pieces (2mm in diameter) and placed into MCDB131 media (Sigma; Table 4.1).

4.3.3 **Endometrium tissue explant culture procedure**

Explant tissue culture was performed as previously described for the bovine endometrium (Mann, 2001) with some modifications. The endometrial biopsy punch tissue (diameter = 2mm and ~10 mg per punch) was placed on a metal grid in the middle of a 6-well plate, which was overlaid with a square piece of lens paper. A total of 10 endometrial punch pieces (~100mg) were placed in each well (Fig. 4.2). After that, 4.5 ml of media (MCBC131) was used to fill the well to a level that allowed air to go between the lens tissue and media. The tissue was cultured at 39˚C in a humidified incubator in 95% air, 5% carbon dioxide. The tissue was collected at 0, 48 and 96h and then either snap frozen and stored at -80^{o}C or fixed in Bouin's for 9h, prior to washing in 70% ethanol and then processed for paraffin embedding.

Table 4.1: Composition of supplemented media MCDB 131 as described previously in (Laird et al., 2013).

Supplement	Concentration
Human epidermal growth factor (Sigma)	5 ng/ml
Ascorbic acid (Sigma)	1 µg/ml
LR^3 insulin like growth factor-1 (Sigma)	20 ng/ml
Bovine serum albumin (Fisher Scientific)	0.1 %
Heparin (Sigma)	0.02 units/ml
Hydrocortisone (Sigma)	25 nM
Gentamycin sulphate, amphotericin (GA1000)	50 µg/ml
Luteinising hormone	5 ng /ml
ITS (insulin, transferrin and selenium; Sigma)	1×
Amphotericin B (Sigma)	50 ng/ml
Penicillin/Streptomycin (Sigma)	1×

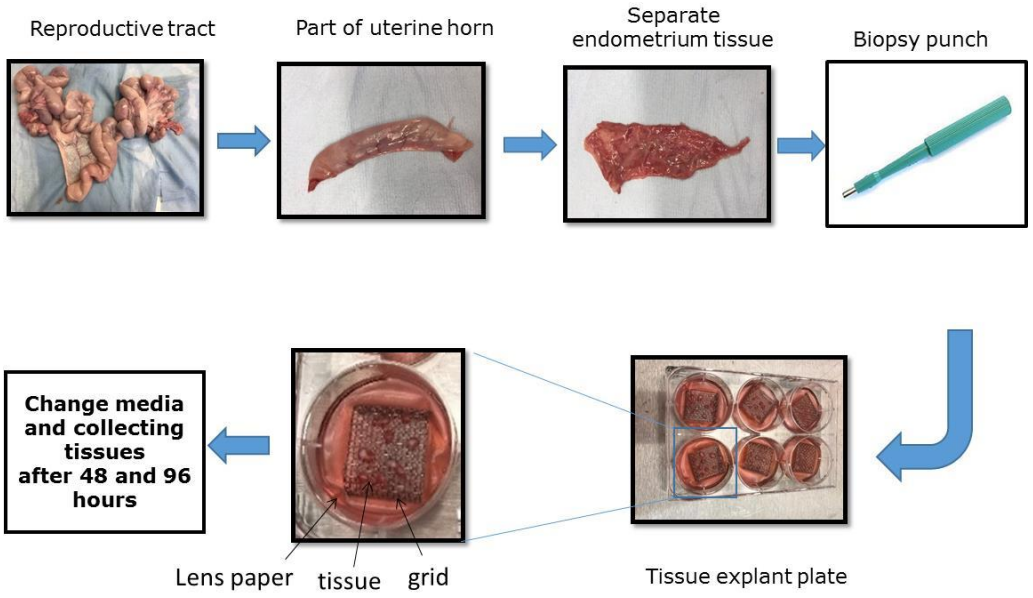


Figure 4.2: Schematic diagram of the endometrial explant tissue culture procedure.

4.3.4 **Immunohistochemistry**

Immunohistochemistry was performed to assess (1) the health status of endometrial explants (cell proliferation versus apoptosis) and (2) the presence of the progesterone receptor. The immunohistochemistry on paraffin-embedded endometrial explants was performed as described in Section 3.4.3. The antibodies used in this experiment are detailed in Table 4.2.

Table 4.2: Details of the antibodies used in endometrial explant immunohistochemistry.

Primary antibody	Supplier / Cat no	Blocking serum	Conc/dilution	Species
Cleaved caspase 3 [apoptosis]	Cell Signalling Technology / 9579	20% NGS	1:300	Rabbit anti human monoclonal
PGR	Leica Biosystems / 312	4% NHS	5µg/ml	Mouse anti human monoclonal
Ki67 [proliferation]	Vector VP-K452 (clone MM1)	4% NHS	1:100	Mouse anti human monoclonal

4.3.5 **Western Blotting**

Western blotting was performed to confirm the specificity of the primary antibodies and to quantify the expression of STC1, OPN and MUC1 protein in *ex vivo* and cultured endometrial explants over time. *Ex vivo* endometrial strips and explant tissues were collected, snap frozen and stored at -80ºC.

Sample preparation

Ex vivo endometrial tissue (~200 mg) or whole endometrial explant tissue (~100 mg) was defrosted on ice and placed in a MACS protein tube (Miltenyi Biotec). Cell extraction buffer (Fisher) containing complete protease inhibitors (Sigma) was added (5 volumes). The tissue was then homogenised using the gentle MACS dissociator on the protein program for a total of 5 times. MACS tubes were then placed on ice for 30min before centrifugation at 2000 g for 15min at 4ºC. Supernatant was collected and stored in aliquots at -80°C.

Protein determination

A Bradford assay was performed to determine the protein concentration of each sample. The standards were creating by serially diluting the top 2mg/ml bovine serum albumin (BSA) standard 1 in 2 in PBS. This gave a series of standards from 2000 to 7.8µg/ml. Standards or sample (10 µl) were added in duplicate to a 96 well plate (Nunc). PBS (10 µl) was added to the blank. Bradford solution (200 µl; Sigma) was added to each well, and then incubated on a plate shaker for 30min at RT. The absorbance was read at 595nm using an OPTIMA plate reader (FLUOstar OPTIMA, BMG-LABTECH, Germany). Protein concentration was calculated by plotting a

linear regression line of the standards and interpolating unknowns using Graph Pad Prism 7.

SDS polyacrylamide gel electrophoresis

Precast NuPAGE Bis-Tris 12% polyacrylamide gels (15 wells; Thermo Fisher) were used to give optimal separation for a wide range of molecular weight proteins. Samples of equal protein content (15 µg) were diluted in 2× Laemmli sample buffer (Bio-Rad) then heated at 100ºC for 10min and pulse centrifuged prior to loading. The gel was warmed to RT and all wells washed in MOPS running buffer (Thermo Fisher) then placed in the electrophoresis chamber (Surelock XCell, Thermo Fisher). After that, MOPS running buffer was added to fill the inner chamber to the top of the wells and halfway up the outer chamber. A molecular weight marker (Colour pre-stained, broad range; New England BioLabs) was added to the first well followed by the samples. The gel was electrophoresed at 200V (~100mA) for 60min.

Transfer and blotting

NuPAGE transfer buffer was prepared by adding 15 ml 20x TG buffer (Thermo Fisher) to 255ml dH_2O, which was then made up to 300ml with methanol. Then an appropriate size (8cm × 6.5cm) of PVDF membrane (BioRad) was cut, and activated in methanol for 10min before soaking in transfer buffer. Five blotting pads and two pieces of 3mm filter paper were soaked in transfer buffer for 20min and all air bubbles removed.

Once electrophoresis had finished, the gel was removed from the chamber using a flat gel knife to separate the two plates. Then the blotting sandwich was made from anode (+) to cathode (-) as follows:

- 2× blotting pads
- 3mm filter paper
- PVDF membrane;
- Gel
- 3mm filter paper
- 3× blotting pads

Then the blotting module was placed and locked tightly in the tank. Transfer buffer was added to the inner chamber to just above the top of the blotting sandwich, while the outer chamber was filled with cold distilled water. The tank was then placed in a cold room and run at 30V for 60min. Once the transfer was finished, the membrane was removed carefully and washed 3 times with PBS-T (Daya, 2009).

Immunoblotting

The PVDF membrane was blocked with 5% (w/v) milk in PBS-T for 1h on a rocker plate and then was incubated with primary antibody (Table 4.3) overnight at 4ºC on a roller. After that, the membrane was washed in PBS-T for 5 × 10min before adding the secondary antibody (Table 4.4) for 1h on a rocker plate at RT. The membrane was then washed with PBS-T for 5 × 10min. Following that, the membrane was rinsed with distilled water twice, before being covered with 2ml ECL prime (GE Healthcare) for 5min. Next, ECL prime was removed and cling film wrapped around the membrane, with all air bubbles removed.

Table 4.3: Details of the primary antibodies utilised for Western blotting.

Primary antibody	Dilution	Company
Rabbit anti-human MUC1	1/1000	Novus, NB1-60046,
Rabbit anti-human OPN	1/2000	Abcam, ab8448.
Rabbit anti-human STC1	1/500	Thermo Fisher, PA5-42104
Rabbit anti-human actin, beta	1/100000	GeneTex, GTX110564

Table 4.4: Detail of the secondary antibodies utilised for Western blotting.

Secondary antibody	Dilution	Company
Goat anti-rabbit HRP conjugate	1/40000	Sigma

Protein visualisation and quantification

Photic software and chemiluminescence exposure box were utilised to capture Western images. In dark conditions, the PVDF membrane was placed in the exposure box which was connected to a camera (Photex Camera, Photek HRPCS-3PSU, Germany) and, a gel image was acquired. The image was captured with a specific exposure that was stopped when a saturated band appeared.

Stripping of the PDVF membrane

The PVDF membrane was placed in PBS immediately after imaging. In order to remove any bound antibodies, the membrane was placed in stripping buffer (1.5% glycine, 0.1% SDS, 1% Tween20 pH2.2) for 2× 10min. This was followed by washing the membrane with PBS for 2× 10min, then with PBS-T for 2× 5min. Next, the membranes were blocked with milk before the second primary antibody was

added followed 5× 10min washes. This was followed by incubating with the appropriate secondary antibody.

Quantification of Western blot images

The captured image was imported into Image-J software (Wikimedia foundation Inc. USA). The intensity and area of each band of target protein and housekeeping protein (actin, beta [ACTB]) were quantified in all samples. Following quantification, each value for a target protein of interest was normalised to the value of ACTB for the same sample.

4.3.6 **RNA extraction and reverse transcription**

Extraction of RNA

Total RNA was extracted from *ex vivo* and explant tissues using an RNeasy Mini Kit (RNeasy® Plus Mini Kit, Qiagen, Germany). Using the manufacturer's protocol, 30mg of tissue was homogenised in 600µl of RLT buffer containing 1% β-mercaptoethanol using MACS RNA tubes in the gentle MACS dissociator (programme RNA 1). The lysate was centrifuged for 3min at maximum speed (18,000 r.p.m) and the supernatant was transferred to an Eppendorf tube to which an equal volume of 70% (v/v) ethanol was added and thoroughly mixed. Then, 650µl of this solution was loaded into the RNeasy spin column within a clean 2ml tube and centrifuged for 15sec at maximum g. The rest of the lysate was then loaded to the same RNeasy spin column and centrifuged for a further 15sec at maximum g. After that, the column was washed with 350µl RW1 buffer and centrifuged for 15sec at maximum g. Next, any DNA was removed by adding 80µl of DNA lysis solution (10µl of DNase + 70µl RDD buffer) and incubated for 10min at RT. This solution

was added to the column and centrifuged for 15sec at maximum g followed by two washes with 500µl RPE buffer. The first wash was centrifuged for 15sec at maximum g and the second one for 2min. The flow through was discarded after each step. Before eluting total RNA from column, the RPE residue was eliminated by transferring the column to a new tube and centrifuged at full speed for 1min. The total RNA was then eluted by adding 30µl RNase free water and centrifugation for 1min. Finally, 1.5µl of RNA was analysed using a NanoDrop8000 Spectrophotometer (Thermo Scientific, UK) and the remaining RNA was stored in aliquots at -80°C for cDNA synthesis. Absorbances at 230, 260 and 280nm were determined and appropriate ratios calculated to estimate RNA quality and quantity. All A260/A280 ratios were between 1.97-2.29, indicating suitable RNA quality.

Primer design

The primer pair to specifically amplify porcine STC1 was designed by Song et al. (2009). The particular primers were established on the porcine *STC1* mRNA (GenBank accession no. EU086592; size 500 bp, annealing temperature 58.6˚C) and were as follows: Forward, 5'-TCCAGATCCACATCTTCACG-3'; Reverse, 5'-CATCCGCTGGAAAGTAGAGC3'). Also, porcine GAPDH was used as a housekeeping gene to ensure successful cDNA synthesis (Forward: 5' CCAGGTTGTGTCCTGTGACT 3', reverse: 5' GAGCTTGACGAAGTGGTCGT 3'; size 96bp and annealing temperature is 60 °C). The two primers were diluted using RNase-free water to 10pmol/µl.

Reverse Transcription (cDNA Synthesis)

According to the company protocol, cDNA was synthesised from 1µg RNA (up to 11µl) and adding the reagents used (Table 4.5).

Table 4.5: Preparation of the reagents for cDNA synthesis procedure

Reagent	Volume
RNA (1µg)	Up to 11 µl
Random hexamers primers	1µl
dNTP (10 mM)	1µl
RNase-free H_2O	Made up to13 µl

For each sample, two tubes were prepared (1) for cDNA synthesis and (2) negative control to detect if genomic DNA was present, whereby reverse transcriptase was omitted. The mixture was heated to 65°C for 5min and then incubated on ice for at least 1min. After that, the second master-mix was prepared (Table 4.6).

Table 4.6: Preparation of the Master Mix for cDNA synthesis for a single sample.

Reagent	Volume
5× first-strand buffer	4 µl
0.1 M DTT	1 µl
RNasin	1 µl

Next, 1µl Superscript III reverse transcriptase (Invitrogen, USA) was added to each positive tube and 1µl of H_2O to each negative control. Tubes were centrifuged and 6µl of master mix was added and mixed gently by pipetting. The tube was incubated at 25°C for 5min before incubation at 50°C for 60min. To inactivate the reaction, the samples were heated at 70°C for 15min. Finally, cDNA was diluted with 20 µl H_2O and stored at – 20°C.

Polymerase chain reaction (PCR)

The reagents for the polymerase chain reaction were prepared by adding the reagents listed in Table 4.7 into a PCR tube.

Table 4.7: The reagents used for the PCR (New England BioLabs)

Reagent	Volume (per sample)
10x PCR buffer	2.5µl
$MgCl_2$	0.125µl
10 mM dNTPs	0.5µl
10 µM Forward primer	0.5µl
10 µM Reverse primer	0.5µl
Template /H_2O	1µl
Taq polymerase	0.125µl
Nuclease free water	19.875µl
Total	**25 µl**

The PCR tubes were placed in a PCR machine (2720 Thermal Cycler, AB Applied Biosystems, Singapore) and the following cycling conditions used: 95°C for 5min, then 35 cycles of denaturation at 95°C for 30sec for, annealing at 58°C for 30sec and then extension at 72°C for 1min. This was followed by a final extension at 72°C for 10min.

Amplification was confirmed by agarose gel electrophoresis. To 5µl of each sample, 1µl of 6x DNA loading dye (Thermo Fisher Scientific) was added. The DNA ladder was prepared by adding 1µl of 100bp DNA ladder (New England BioLabs) with 1µl of 6× DNA loading dye (New England BioLabs) and 4µl of H_2O.

A 2% (w/v) agarose gel was prepared by adding 2 g agarose (Sigma) to 100ml of 1× Tris-acetate-EDTA (TAE). Then, the mixture was heated in a microwave for 4min. 5µl Nancy 520 (1µg/ml Nancy–520, Sigma, UK) was added to the dissolved agarose mixture, and then poured into a casting tray and left to solidify. The samples were

loaded onto the gel, which was run at constant voltage (90 V) for 90min, after which the bands were directly visualised on an ultraviolet trans-illuminator (Image Quant 300, Germany).

4.3.7 Statistical analysis

Data were checked for normality and homogeneity of variance by using residual plots and Levene's test respectively in Genstat prior to statistical analysis. Where necessary, the data were logged transformed.

1) *Ex vivo* study: The STC1 protein expression in the sows with low or high endometrial progesterone content were compared using a t-test.
2) *In vitro* study: A randomised block two-way ANOVA in Genstat 17th Edition (VSNi, Hemel Hempstead, UK.) was used to investigate the effect of progesterone (0, 10, 100ng/ml) on protein expression over time (48h vs 96h). The data were blocked by culture number (animal), which paralleled the layout used in the Western blot. This minimised any inter-blot and inter-culture variations enabling any treatment effects to be analysed within a set. Student-Newman-Keuls post-hoc multiple comparison tests were performed to determine where any differences lay. The effect of oestradiol (0, 0.1, 1ng/ml) in the presence or absence of progesterone (0, 10ng/ml) was analysed by two-way ANOVA. In this analysis, each time point (48h and 96h) was analysed separately. For each two-way ANOVA, the main effects and their interaction were considered. Only significant interaction are reported for clarity purposes.

4.4 Results

4.4.1 Establishment and characterisation of endometrial explant tissue culture system

The histological appearance of the endometrial biopsy explants was similar at all 3 time points (0, 48 and 96h) in culture (Fig. 4.3). In each cross-section, there was a moderate density of broadly circular rings of epithelial cells that resembled the glandular epithelium. Surrounding these, there were loosely-packed stromal cells. Based on histological appearance, there were few blood vessels/endothelial cells observed within the explant. There was retention of luminal epithelium in the explant, though the amount varied between sections.

In order to determine the validity of the procedure and viability of the explanted tissue, both caspase 3 (apoptotic marker) and Ki67 (proliferation marker) immunolocalisation were performed in the endometrial explants before and after culture (Fig. 4.3). Positive caspase 3 staining was detected at low levels in both epithelial and stromal cells. In both cell types, visibly there was a similar amount at times 0, 48h and 96h (Fig 4.3A, B and C respectively). Positive Ki67 staining was present in the nucleus of some stromal cells across the tissue at 0, 48h and 96h. There was very limited Ki67 staining in the epithelial cells. The control sections were blank (Fig 4.3 D and H).

The expression of the progesterone receptor in the endometrial explants was assessed to confirm its continued expression *in vitro* over time. At 48h, there was extensive and intense staining of PGR in the "glandular" epithelium. The staining was localised exclusively to the nucleus. PGR expression was also detected in the stromal cells but was absent from any luminal epithelium that was present (Fig 4.4A). At

96h, PGR expression was still detected in the “glandular” epithelium and stromal cells, albeit at lower levels. The control section was devoid of any staining.

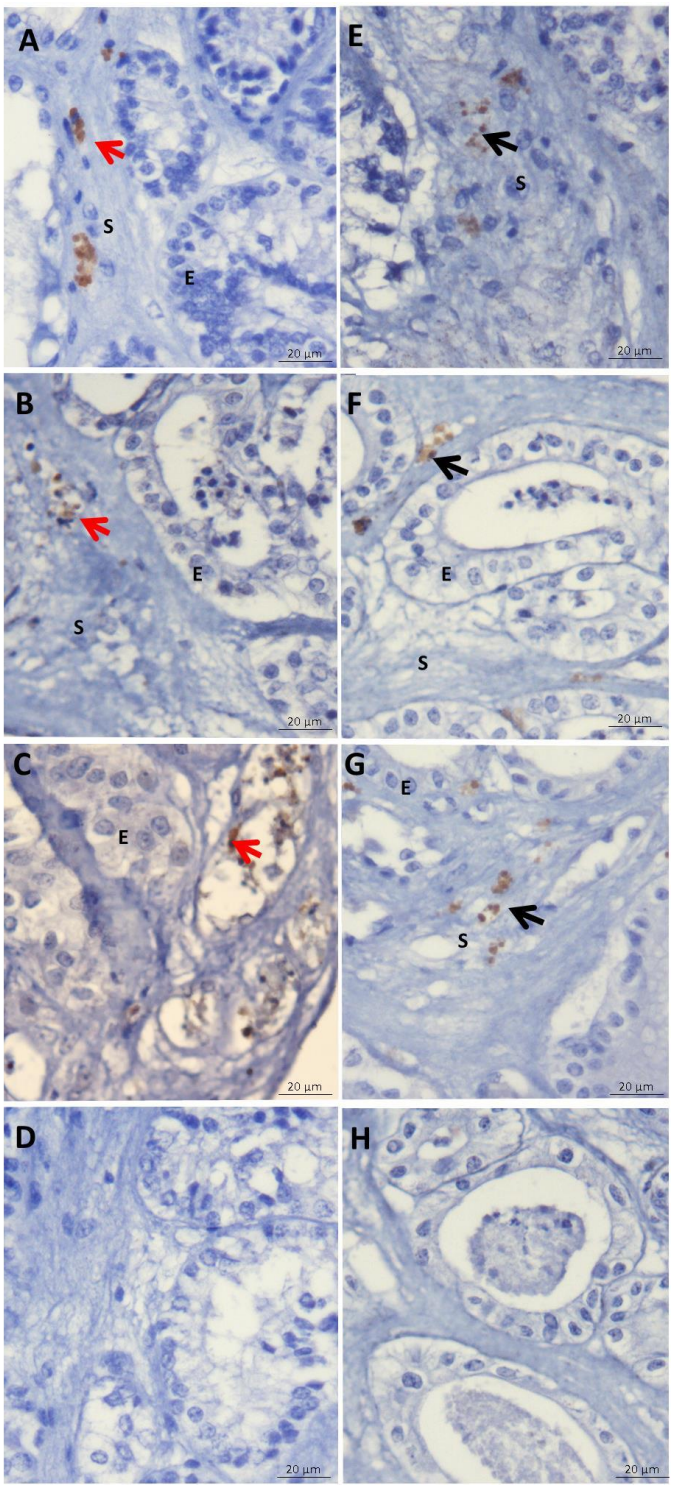


Figure 4.3: Immunohistochemistry showing expression of caspase 3 and Ki67 in endometrium tissue during culture. Caspase 3 staining (red arrows) is shown in (A): before explant culture (t=0h); (B): after 48h; (C): after 96h with the control section shown in (D). Ki67 staining (black arrows) is shown in (E): before explant culture (t=0h); (F): after 48h; (G): after 96h and the control section is shown in (H). S=stromal cells, E=glandular epithelial cells. The magnification was at 400x and the scale bar= 20µm.

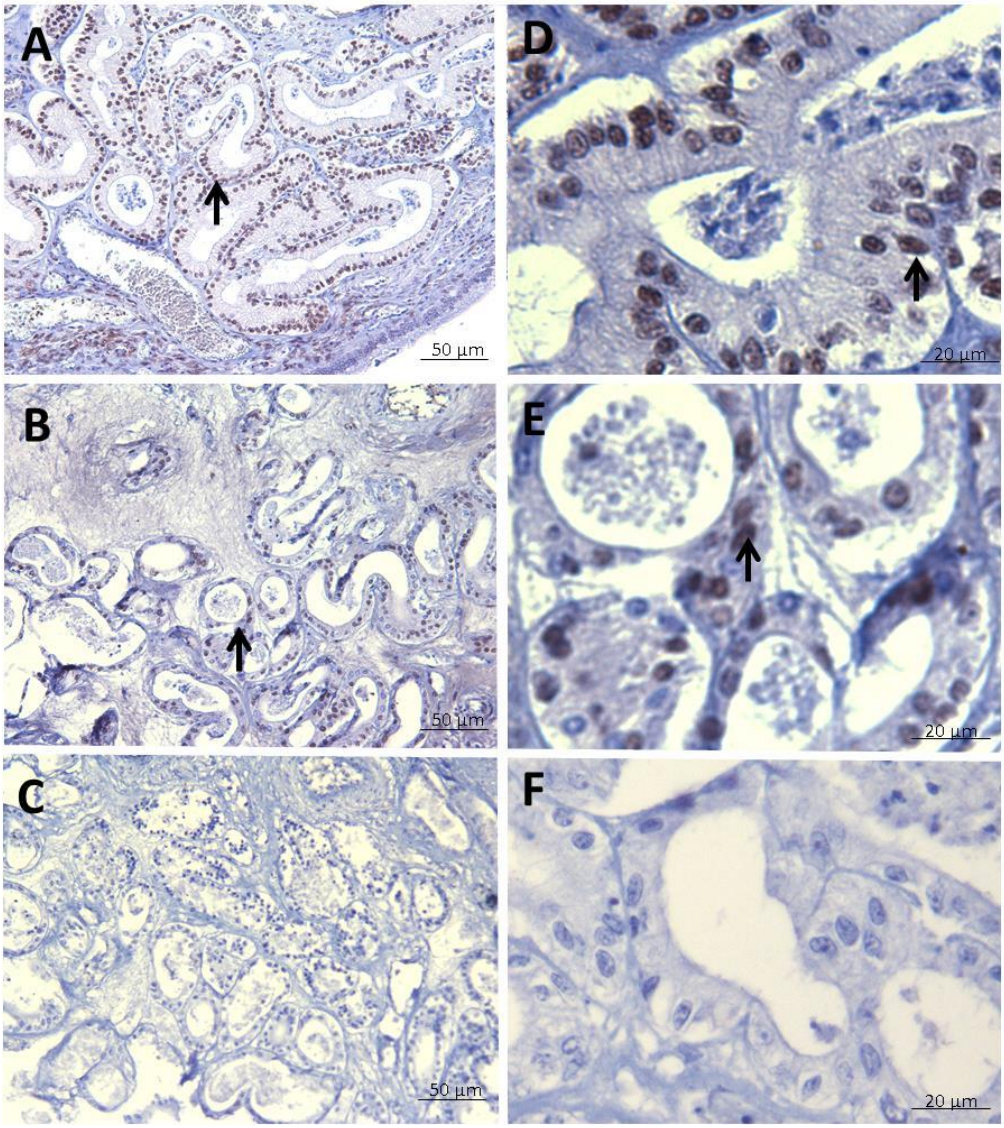


Figure 4.4: Immunohistochemistry showing the expression of progesterone receptor (PGR, black arrows) in endometrium explants during culture. Representative images show positive PGR staining (A & D): after 48h and (B & E) after 96h in culture. The negative immunohistochemical control is shown in (C & F). The scale bar represents 50 & 20µm.

4.4.2 **Association between progesterone content and *ex vivo* endometrium STC1 protein expression**

The pregnant sows on day 11-12 group were split into two groups based on their endometrial progesterone content: group 1 (n=3), that had low progesterone content (mean = 95 ng/g), and group 2 (n=3), that had high progesterone content (mean = 293 ng/g). Western blot analysis showed that there were two bands present for STC1 protein (Fig 4.5A). There was a dominant band detected at 25kD and one with lower abundance at 54kD. A single band was detected at 42kD for the housekeeping protein, actin beta (ACTB) that was expressed at similar levels in all samples (Fig 4.5A). Quantification of the intensity of both STC1 bands showed that STC1 protein expression in the endometrium was similar in the presence of either low or high progesterone content ($p>0.05$: Fig.4.5B).

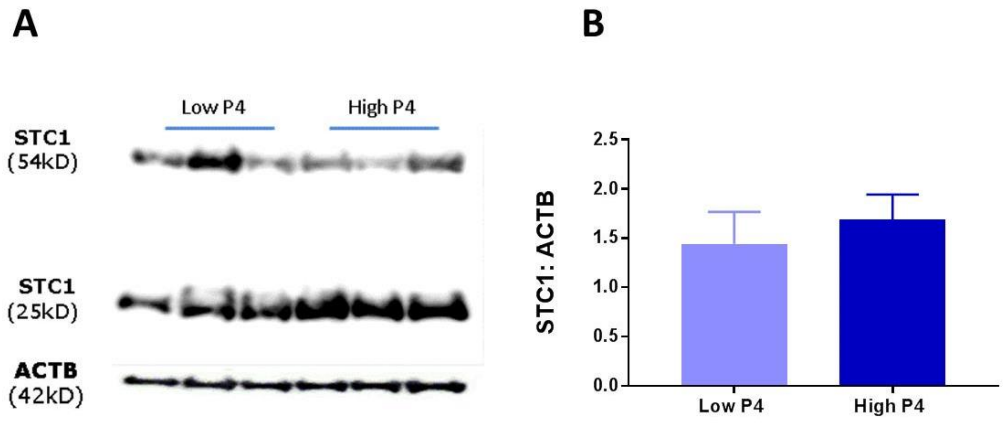


Figure 4.5: The expression of stanniocalcin 1 (STC1) protein in the day 11-12 pregnant endometrium *ex vivo*. (A) Western blot analysis of STC1 showed two bands (25 and 54kD). (B): The quantification of STC1 normalised to actin, beta [ACTB] in animals with low or high endometrial progesterone (P4) content. The values are mean (n=3 animals) + SEM.

4.4.3 Effects of progesterone and oestradiol treatment on stanniocalcin 1 expression in endometrial explant culture

To further investigate the presence of the two STC1 bands, a Western blot was performed on recombinant human STC1. This revealed two bands at 30kD and 60kD. At the same time, protein samples from porcine endometrium (at 0h) were run on the Western blot and also revealed two similar bands but at 25kD and 54kD. Porcine and human STC1 proteins are both 247 amino acids in length with 94% homology and are thus expected to have a similar molecular weight. In the same Western blot, samples from endometrial explants cultured for 48h revealed a single band at 54kD (Fig. 4.6).

Figure 4.7 shows the effect of culture time on STC1 protein expression from endometrium collected on different pregnancy days (11-12 and 13-14). The expression of STC1 varied over culture time ($p<0.001$) and there was a day of pregnancy × time interaction ($p<0.01$; Fig. 4.7). Namely, the expression of STC1 protein declined with time in culture but this decline appeared greater in explants from day 11-12 of pregnancy.

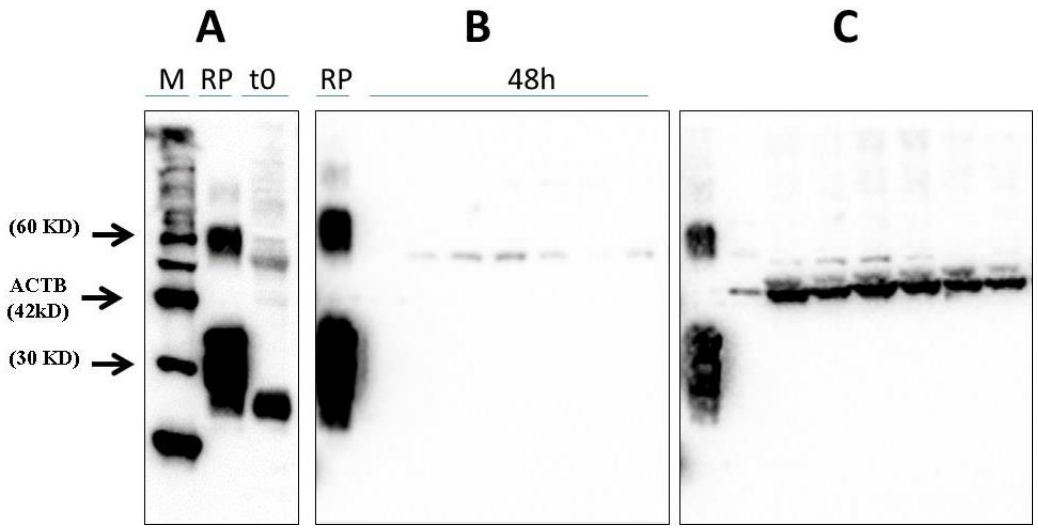


Figure 4.6: Characterisation of Western blot for stanniocalcin 1 (STC1). (A) shows the marker (M), the protein weight in kD for the human STC1 recombinant protein (RP) and tissue collected just before explant culture (t=0). While (B) shows the endometrial explant collected after 48h in culture. (C) shows the housekeeping gene actin beta (ACTB). After the membrane was stripped.

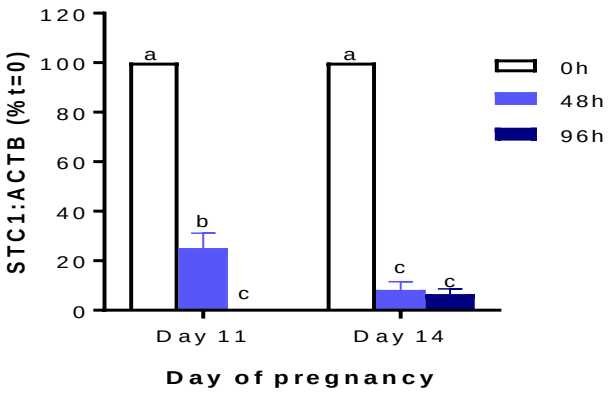


Figure 4.7: The quantification of stanniocalcin 1 (STC1) protein expression in endometrial explants over time. The endometrial explants were collected at 0, 48 and 96h. The expression of STC1 (normalised to actin beta [ACTB]) was different according to the time in culture ($p<0.001$ with a>b>c; $p<0.05$) and pregnancy day*time ($p<0.01$). The values are mean (n=6 cultures) + SEM.

In the next experiment, the effect of progesterone and oestradiol on endometrial explants was investigated. Again, STC1 was expressed at 0 and 48h in explants from day 11-12 pregnant endometrium but was absent after 96h in culture (Fig. 4.8A). In contrast, STC1 expression in the endometrium from day 14 of pregnancy was present at all time points but was greater at 0h ($p<0.01$, Fig. 4.9A).

Quantification showed that treating endometrial explants with progesterone had no significant effect on STC1 protein expression after 48h in culture ($p>0.05$; Fig 4.8A, B). A similar effect was observed whether day 11-12 ($p>0.05$; Fig 4.8B) or day 14 ($p>0.05$; Fig 4.9B) pregnant endometrium tissue was cultured. In the day 14 pregnant endometrial explants, STC1 expression was maintained until 96h but again there was no effect of progesterone treatment ($p>0.05$; Fig 4.9B,C).

The treatment of day 11-12 pregnant endometrial explants with oestradiol (0, 0.1 and 1 ng/ml), had no effect on STC1 expression in the absence ($p>0.05$; Fig 4.8) or presence of 10 ng/ml of progesterone ($p>0.05$; Fig. 4.8). There was a similar outcome with endometrial explants from day 14 of pregnancy after 48 h in culture ($p>0.05$; Fig 4.9D). Furthermore, at 96h of culture, oestradiol treatment had no effect on STC1 expression ($p<0.05$; Fig 4.9E).

Figure 4.10 represents STC1 expression in endometrium explants from day 5 of pregnancy. There was no statistical analysis for this day due to the low number of animals (n=2). However, a single band was detected for STC1 at 54kD in all samples after 48h of culture and this band appeared to be much reduced after 96h.

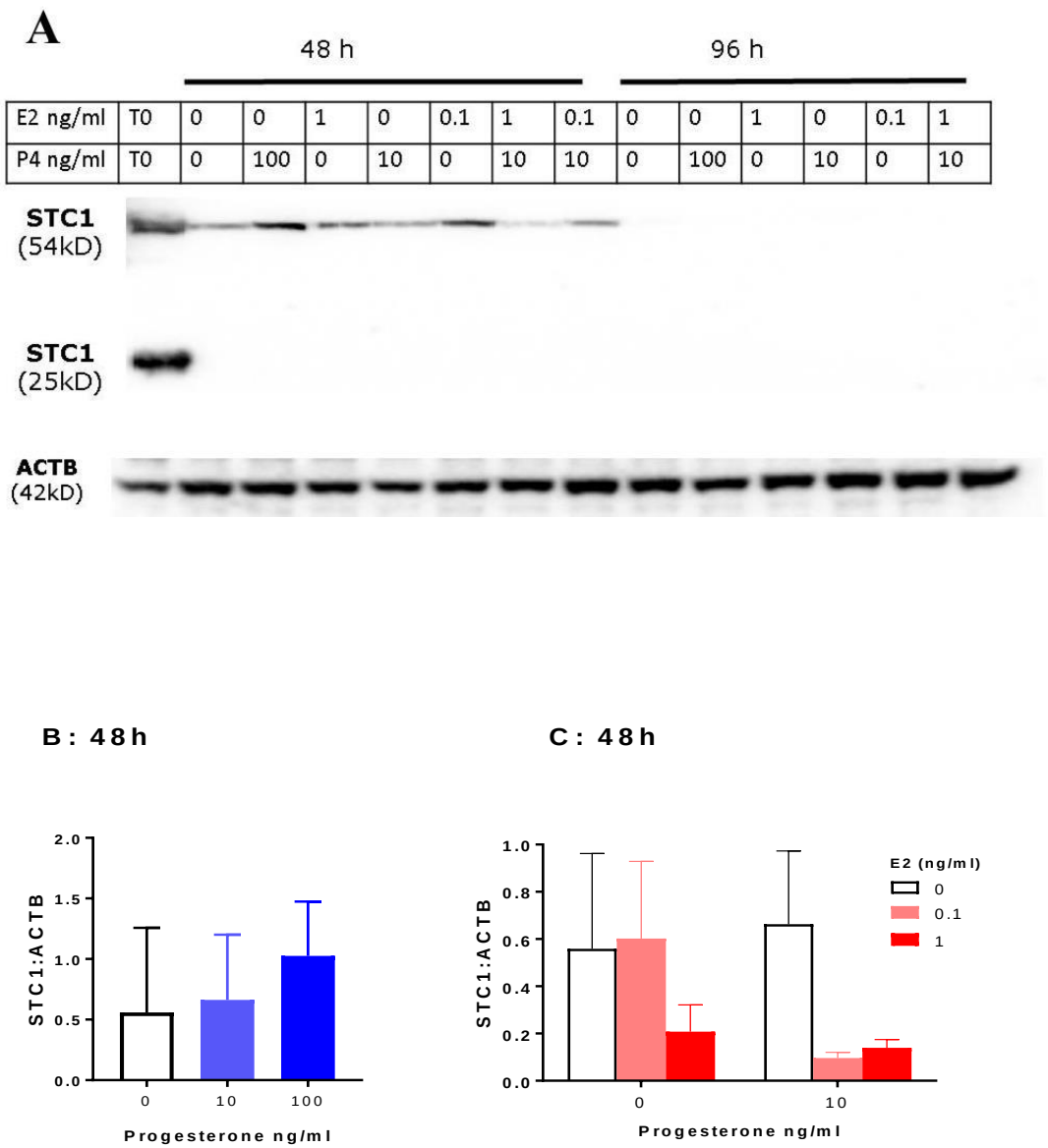


Figure 4.8: The expression of stanniocalcin 1 (STC1) protein in the day 11-12 pregnant endometrial explant culture. (A) Shows a representative Western blot for STC1 with treated samples. There was a double band (25 and 54kD) at time 0h while a single band (54kD) in explant tissue cultures. (B): There was no effect of progesterone on the expression of STC1 (normalised to actin beta [ACTB]) at 48h of culture (n=3 cultures). (C): There was no effect of oestradiol on the expression of STC1 in endometrial explants in the presence and absence of progesterone (n=3 cultures). Data are mean + SEM.

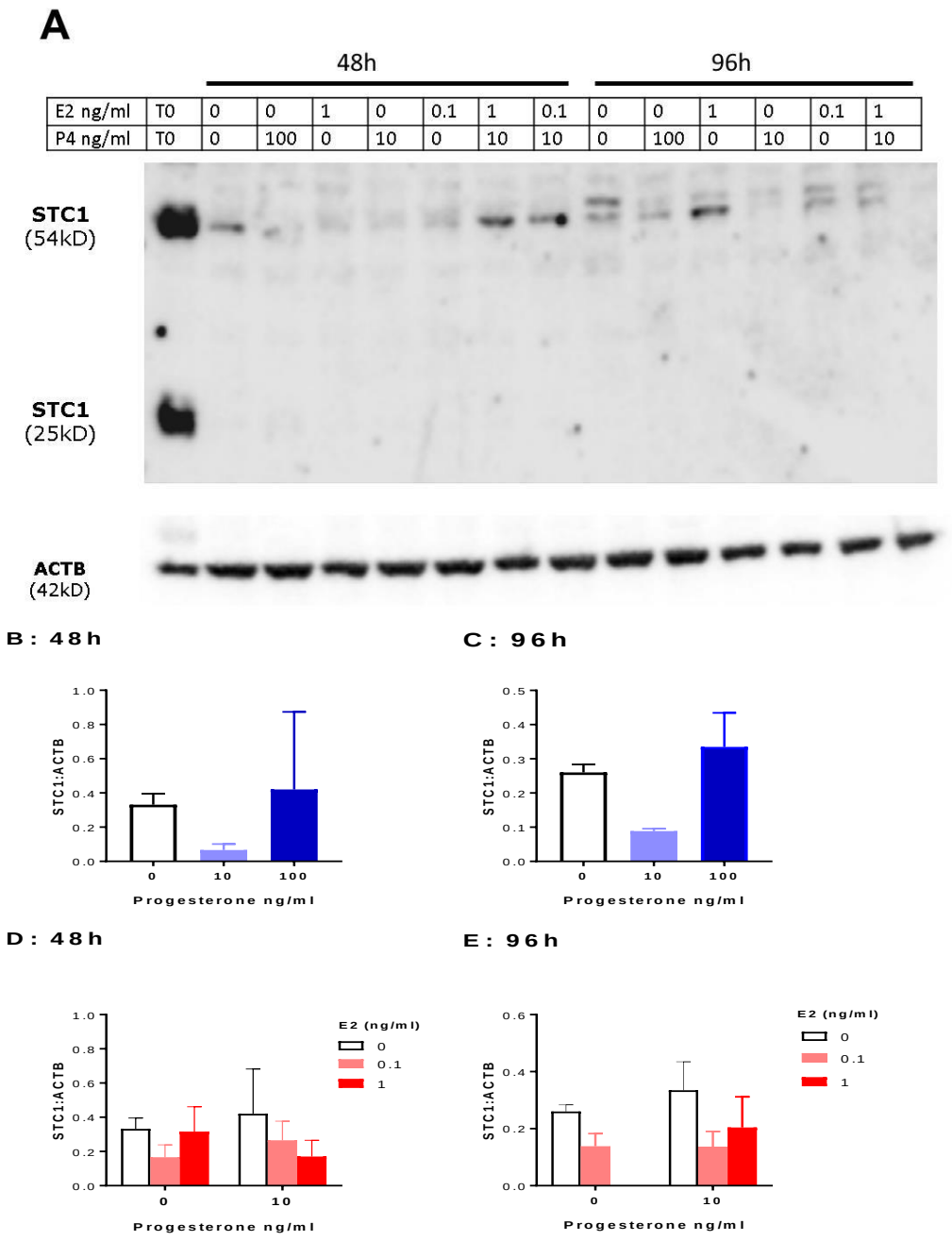


Figure 4.9: The expression of stanniocalcin 1 (STC1) protein in endometrial explants from days 13-14 of pregnancy during culture. (A) shows a representative Western blot for STC1 with treated samples Western blot analysis showed two bands (25 and 54kD) at time 0h but single band (54kD) in explants at 48h and 96h. (B, C): The effect of progesterone on the expression of STC1 (normalised to actin beta [ACTB]) during explant tissue culture at 48 h and 96h. (D, E): The effect of oestradiol on STC1 expression in explants at 48 and 96 hours. There were no effects of progesterone or oestradiol treatment. The data are mean (n=3 cultures) + SEM.

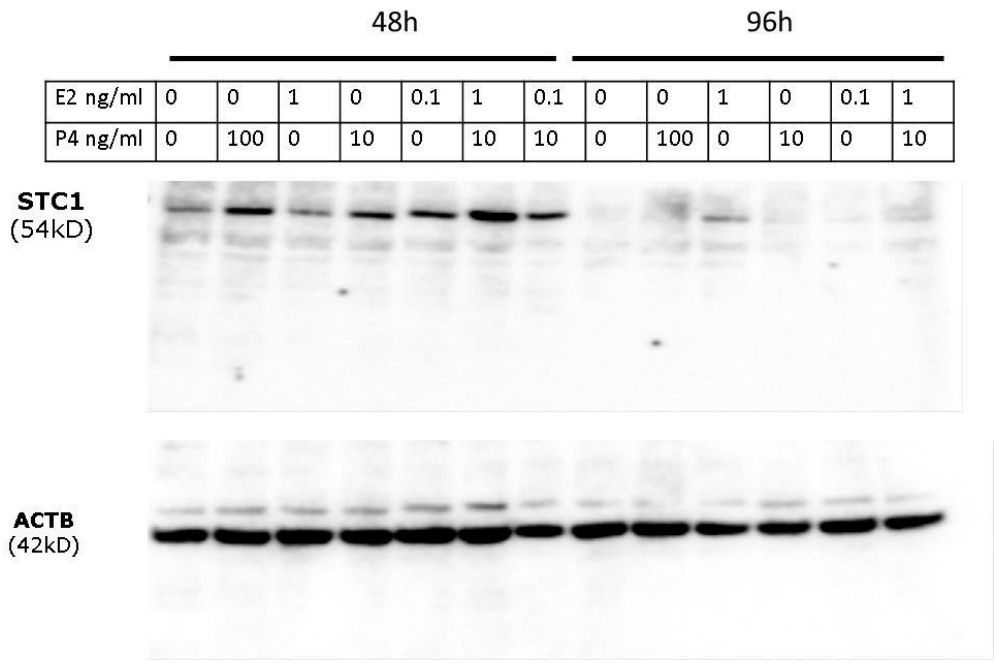


Figure 4.10: The expression of stanniocalcin 1 (STC1) protein in endometrial explants from day 3-5 of pregnancy during culture. (A) Shows a representative Western blot for STC1. Western blot analysis showed 0h but single band (54kD) during explant tissue culture at 48h and 96h.

4.4.4 Expression of *stanniocalcin 1* mRNA in the porcine endometrium

Initially, it was important to confirm that *STC1* mRNA was expressed in the porcine endometrium during pregnancy and that the PCR primers resulted in amplification. Indeed PCR reactions with the STC1 primer pair yielded a strong single band at the expected at size of 500 base pairs. This band was observed in the endometrial cDNA of all animals on day 5, 11 and 14 of pregnancy (Fig. 4.11). The negative control lane was blank, demonstrating a lack of contamination. In addition, samples without reverse transcriptase had no amplification, demonstrating a lack of genomic DNA carryover in the original RNA extractions. In order to determine if *STC1* mRNA was expressed during culture, PCR for STC1 was performed. An appropriately sized band was observed at all time-points (0, 48h and 96h). This indicated that *STC1*

mRNA expression was present throughout the culture window (Fig.4.12). Negative control was blank.

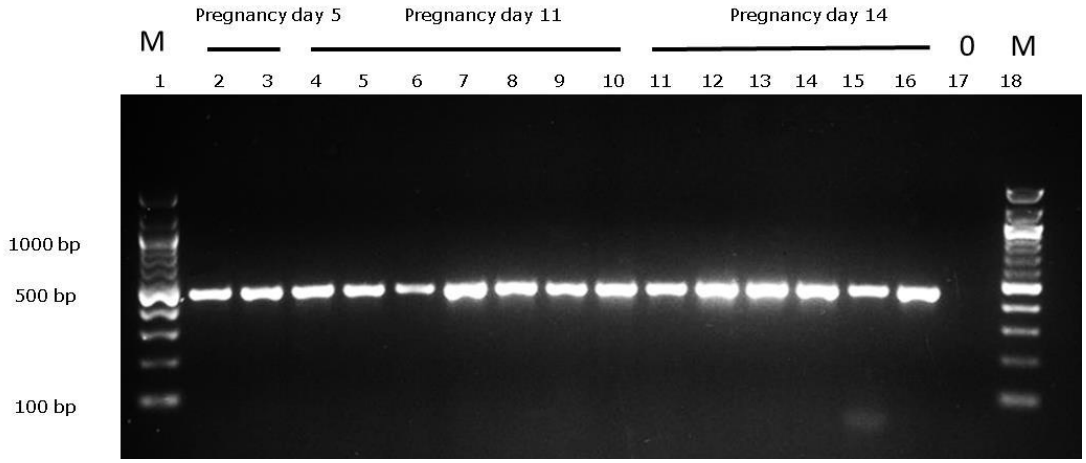


Figure 4.11: Representative PCR reactions on a agarose gel showing the expression of *STC1* mRNA in porcine endometrium on different days of pregnancy as follows: 1 and 18 =Markers (M); lanes 2 and 3 =pregnancy day 5; lanes 4 – 10 = pregnancy day 11; lanes 11 – 16 = pregnancy day 14; lane 17= PCR negative control. bp= base pair.

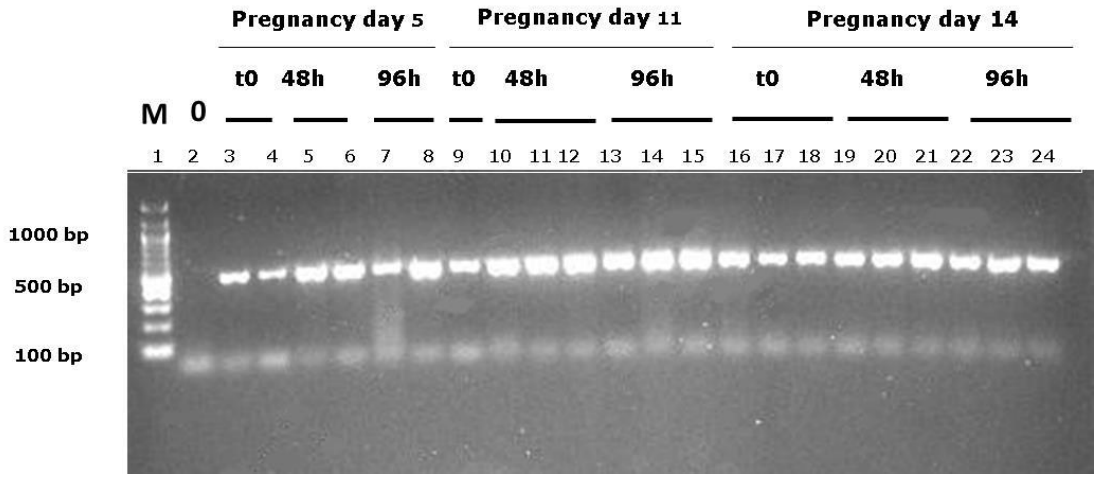


Figure 4.12: Representative PCR reactions showing the expression of STC1 mRNA in porcine endometrium explants during culture. The lanes are as follows 1=Marker (M), 2= PCR negative control; 3-8 = explants from day 5 pregnant animals at t=0 (lanes 3,4); 48h (lanes 5,6) and 96h (lanes 7,8); explants from day 11-12 pregnant animals at t=0 (lane 9); 48h (lanes 10,11,12) and 96h (lanes 13,14,15); and 16-24= explants from day 14 pregnant animals at t=0 (lanes 16-18); 48h (lanes 19-21) and 96h (lanes 22-24). bp= base pair.

4.4.5 **Effects of progesterone and oestradiol on the expression of osteopontin (OPN) in the porcine endometrium**

Western blot showed that there were three different prominent bands for OPN at 34, 39 and 55kD in *ex vivo* and explant tissue culture (Fig 4.13A and 4.14). The band at 55kD appeared to be a doublet. In the *ex vivo* study, there were no statistical differences ($p>0.05$) in OPN expression in endometrium when grouped according to low *versus* high progesterone content (Fig. 4.13B). In the endometrial explants, OPN expression of each band was similar ($p>0.05$) at each culture time point. Quantification showed that there was no effect of either progesterone or oestradiol treatment and its interaction on OPN expression in culture ($p>0.05$).

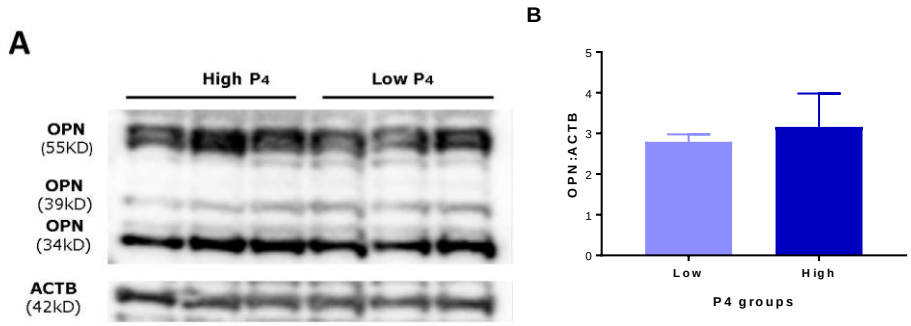


Figure 4.13: The expression of osteopontin (OPN) protein in the endometrium (pregnancy day 11-12) *ex vivo*. (A) Western blot analysis of OPN showed three bands (34, 39 and 55kD). (B) The quantification of OPN (55kD) normalised to actin beta [ACTB] in animals with low or high progesterone (P4) endometrial content. There was no significant effect of progesterone content. The values are mean (n=3 animals) + SEM.

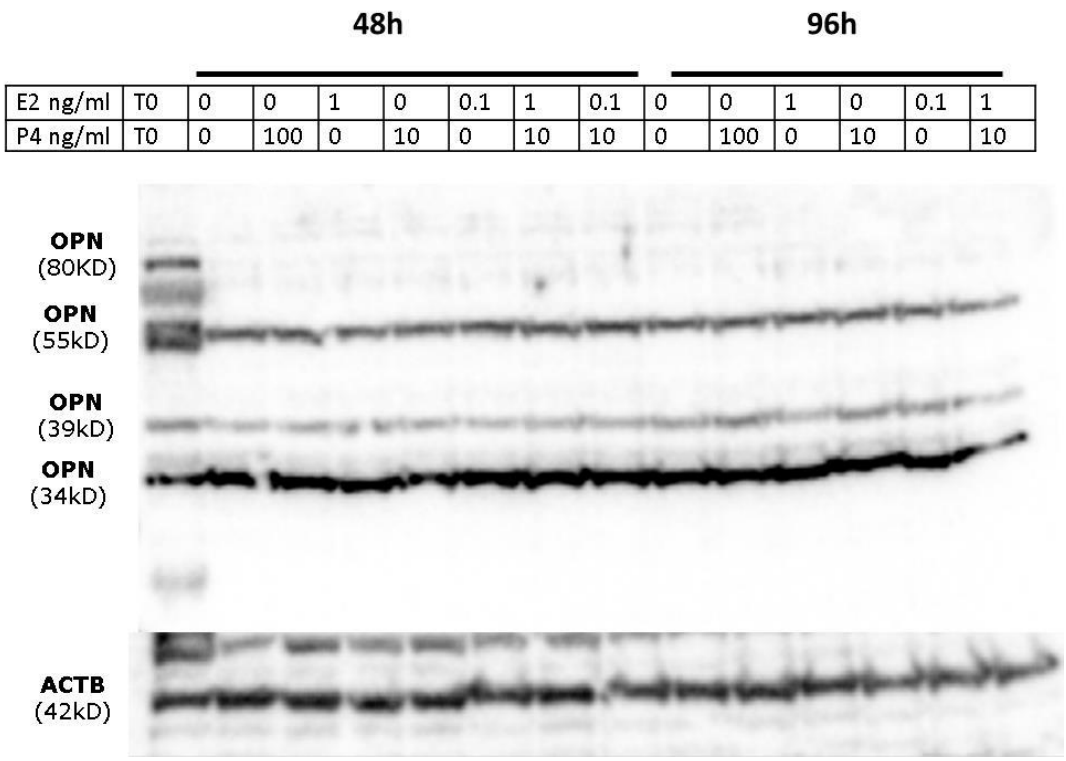


Figure 4.14: The expression of osteopontin (OPN) protein in the endometrium explants during culture. A representative Western blot for OPN with treated samples showing multiple bands (34, 39 and 55kD) at all culture times. The band for actin beta [ACTB] = 42kD is also shown.

4.4.6 **Effects of progesterone and oestradiol on the expression of mucin 1 (MUC1) in the porcine endometrium**

Western blotting detected two relatively faint bands of 21 and 55kD for mucin 1 (MUC1) in endometrium tissue from day 11-12 pregnant sows, grouped by low (95 ng/g) and high (293 ng/g) progesterone content (Fig. 4.15). There was a tendency for MUC1 to be greater in the high progesterone group but this failed to reach significance (p=0.07). In the endometrial explants, no MUC1 was detected at either 48 or 96h in culture (Fig 4.16). In comparison, MUC1 was detected in *ex vivo* endometrial at time 0h, and the housekeeper protein ACTB was present in all samples at all time-points.

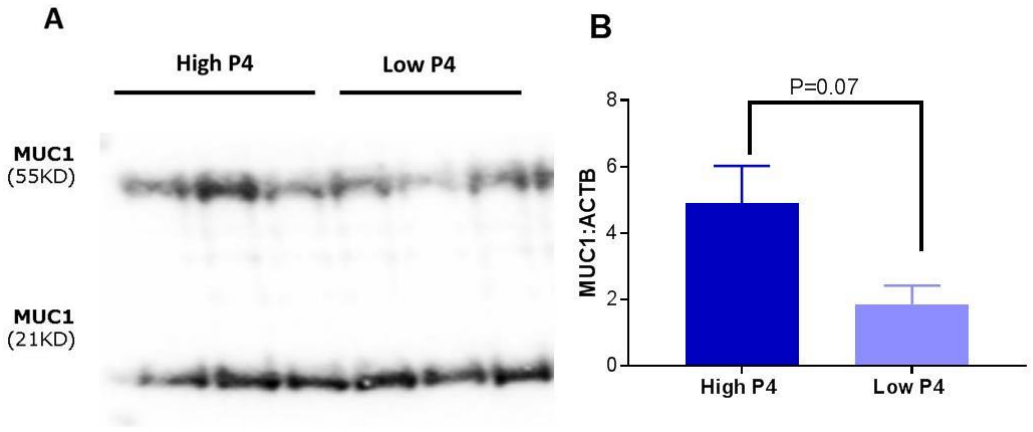


Figure 4.15: The expression of mucin 1 (MUC1) protein in the endometrium (pregnancy day 11-12) *ex vivo*. (A) Western blot analysis of MUC1 showed two bands (21and 55kD). (B) The quantification of MUC1 (55kD) normalised to actin, beta [ACTB] in animals with low or high progesterone (P4) endometrial content. The data are mean (n=3 animals) + SEM.

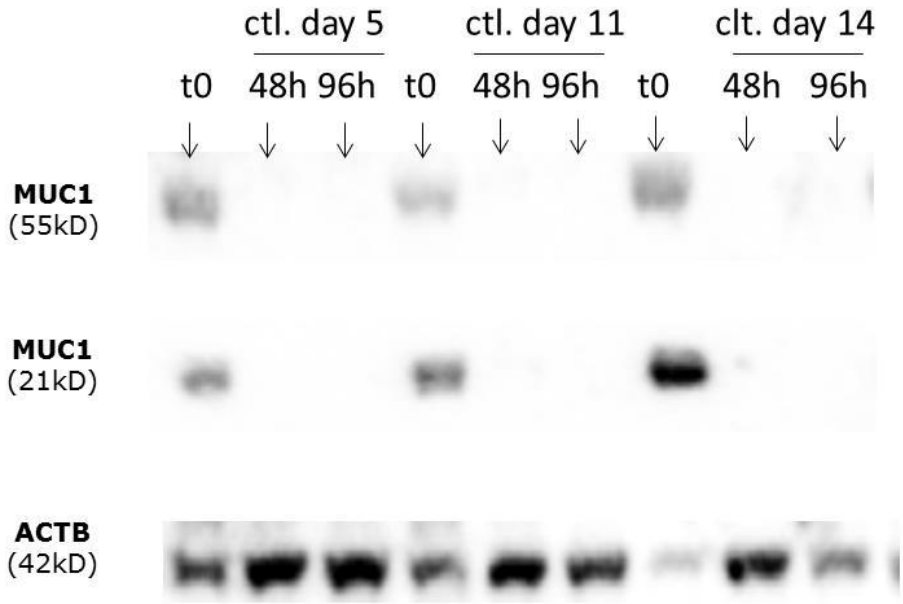


Figure 4.16: The expression of mucin 1 (MUC1) protein in endometrium explants over time. A representative Western blot for MUC1 at time 0 (t0), control at 48h (clt.48h) and control at 96h (clt. 96h) of experiment. MUC1= mucin 21 and 55kD. No band for MUC1 was detected in the endometrial explant cultures.

4.5 **Discussion**

In order to test the hypothesis that the expression of key endometrial receptivity markers was influenced by progesterone and oestradiol levels, a culture method was developed in which the porcine endometrium *ex vivo* maintained its histological integrity and viability.

Different parameters were used to monitor the viability of endometrium tissue cultured using the *in vitro* explant procedure. These have shown that (1) the tissue remained histologically intact; (2) there were minimal changes in caspase 3 expression which suggests no increase in apoptosis; (3) Ki67 was expressed at 48h and 96h suggesting that proliferation was still occurring and that the tissue was healthy; (4) the presence of the progesterone receptor after 96h suggests that potential mechanisms are in place for progesterone to exert an effect. Others have demonstrated the viability of endometrium in similar explant culture systems (after 96h) in the cow (Mann, 2001). The potential of the system was confirmed when progesterone and oestradiol treatment affected the secretion of prostaglandin F2α in the bovine endometrium (Mann, 2001).

STC1 mRNA and protein was expressed by endometrial tissue in culture. Two STC1 protein bands at 25 and 54kD were detected and their expression declined with time in culture. The predicted molecular weight for porcine STC1 with no post-translation modification is 27kD. STC1 band was detected at a similar weight to the weight of recombinant human STC1. Thus, we have a high confidence that the 25kD band is porcine STC1 protein. Moreover, previous studies detected STC1 protein at 25kD in uterine flushes from pregnant sows (Song et al., 2009) and in ovine endometrium

tissue (Song et al., 2006). Ovine STC1 is the same length as porcine STC1 with 96% identity.

This study showed that the expression of STC1 protein declined significantly over time in culture, to be largely undetectable by 96h. This might suggest that critical stimulatory signals are not maintained in culture or that some form of inhibition is released in culture. The lack of stimulation in response to progesterone and oestradiol might suggest that synthesis of STC1 protein requires factors other than progesterone and/or oestrogen *in vitro*. Previous *in vivo* results concluded that *STC1* mRNA expression in the luminal epithelium requires progesterone and this expression was further increased by oestradiol. Oestradiol was also important in the down-regulation of *STC1* mRNA expression in luminal epithelium cells observed by day 25 of pregnancy (Song et al., 2009). In the current study, the endometrial tissue was cut into pieces that kept the original structure of endometrium but that might affect the percentage of luminal epithelial cells in a whole tissue. This is not in case in this experiment, because tissues at all experiment times (0h, 48h and 96h) undergo the same physical conditions (10 pieces for 2 mm each) also STC1 protein was expressed in stroma cells in the immunohistochemistry study (Chapter 3). On the other hand, both doses of progesterone (10 and 100 ng/ml) did not show differences. This result is in agreement with previous study in equine endometrium explant culture. *STC1 mRNA* was not effected by steroid hormones when the endometrium tissue was cultured with 0.01, 0.1, 1, 10 or 100ng/ml of E2 and 10ng/ml of P4 (Kikuchi et al., 2011). This might indicate that STC1 expression needs more than progesterone and oestrogen to alter its expression. This might be an additional factor or that a specific physiological condition is required to mimic the peri-implantation endometrium.

STC1 mRNA increased in the endometrium around the time of implantation in ewes (Song et al., 2006) and mice (Stasko et al., 2001). Moreover, in pseudopregnant pigs, STC1 expression in luminal epithelial cells was induced following exposure to progesterone and this expression was further increased by oestradiol treatment (Song et al., 2009). In contrast, the current study did not find evidence to support the hypothesis that STC1 protein expression was regulated by progesterone. No significant differences in STC1 protein levels were observed in animals with high *versus* low endometrial progesterone content. It could be that a threshold level of progesterone is required to stimulate STC1 and once that threshold is reached progesterone has no further effect. In addition, the treatment of endometrial explants *in vitro* with progesterone did not increase STC1 protein. This lack of progesterone stimulation cannot be as a result of the loss of PGR receptor expression as they were maintained over time in culture.

Oestradiol had no effect on STC1 expression *in vitro,* however it had been hypothesised that conceptus-derived oestradiol would stimulate STC1 expression. In the current study, there was significant variation between cultures in the effects of oestradiol, which might partly explain the lack of significance. However, the overall pattern was that STC1 expression appeared to be reduced under oestradiol treatment. While, as explained above, in an *in vivo* study by Song et al (2009) oestradiol alone did not induce *STC1* mRNA in ovariectomised pig, but it increased the stimulatory effects of progesterone.

In this study, we showed that OPN was expressed during culture but that its expression was not affected by time or progesterone or oestradiol treatment. There were 3 main bands for OPN on Western blot analysis. In the absence of post-

translational modification porcine OPN is predicted to be 34kD but is predicted to be 44kD following post-translational modification. There are several protease cleavage sites within OPN and has been shown to be 32kD when cleaved. A 64kD has been detected in humans (Santos-Ribeiro et al., 2014). Previously, OPN protein was detected in porcine uterine secretions as three bands at 70, 45 and 32kD (Bailey et al., 2010) which is broadly similar to that observed in this study.

OPN is expressed in the porcine luminal epithelium on day 12 of pregnancy, and is regulated by progesterone and affected by conceptus oestrogen. Bailey et al (2010) hypothesised that a servomechanism including serial effects of several hormones and cytokines, are required for the secretion of OPN in glandular epithelium cells. The majority of the previous studies utilised *ex vivo* tissue. In the present study, the level of OPN expression was steady at all experimental time points (0h, 48h and 96h) and in all control and treated wells. This might indicate that OPN is omnipresent at a moderate level during the pre- and implantation period.

This study demonstrated that MUC1 protein expression was detected in *ex vivo* endometrium but not at any time point in culture. The expression of MUC1 in endometrium tissue was not significantly different between low and high progesterone groups. MUC1 is considered an effective inhibitor of cells binding to each other or cells binding with extracellular matrix (Gendler and Spicer, 1995). In the pig, MUC1 expression decreases significantly in attachment sites at time of implantation (Ren et al., 2010). Specifically, they showed that MUC1 protein was mainly localised to the luminal epithelium and the staining was positive on day 13 of pregnancy. Our study did not show any expression of MUC1 protein in any samples of explant tissue in culture. This might be because the biopsy samples

included from all endometrium tissue (glandular, luminal epithelia, stroma cells) and thus the luminal epithelium component is diluted out or does not maintain its integrity during culture. Alternatively, there is a factor present in the live animal that is missing in culture such that MUC1 expression is not maintained.

In conclusion, a porcine endometrial explant culture system has been developed which maintains cellular integrity, viability and progesterone receptor expression over several days. It provides a useful system to investigate the regulation of receptivity markers, although the precise mechanisms remain to be fully understood.

CHAPTER 5 Establishment and regulation of a porcine endometrial angiogenesis culture system *in vitro*

5.1 **Introduction**

In mammals, implantation of the embryo into the endometrium is crucial for the establishment of pregnancy. Thus, a successful implantation requires a fully receptive endometrium and the appropriate synchronisation between embryo and endometrium. Throughout the period of endometrial receptivity, the endometrium undergoes a sequence of gene expression changes in response to hormonal changes and the presence of the conceptus (Bergh and Navot, 1992, Chen et al., 2017). Additionally, in preparation for pregnancy and placenta formation, there are changes in the vascularisation of the endometrium at the embryo - endometrium interface. Therefore, adequate blood supply to the endometrium is essential at the time of pregnancy, and inappropriate uterine vascularisation might lead to unsuccessful implantation.

Angiogenesis is the development of new vascular structures from pre-existing blood vessels through proliferation, elongation and sprouting of endothelial cells (Eilken and Adams, 2010, Woad and Robinson, 2016). Several different cell types, growth factors and extracellular matrix substances can control angiogenesis and the formation of blood vessels in the endometrium (Torry et al., 2007). It is the embryonic trophoblast cells that attach to the endometrium and will eventually form the placenta. Furthermore, the conceptus produces and secretes angiogenic growth factors, that might play a crucial role in stimulating angiogenesis at this time (Cross et al., 2002). Specifically, the porcine conceptus potentially stimulates angiogenesis through the secretion of vascular endothelial growth factor A(VEGFA) and interferons (IFNs) (Geisert et al., 2014). Uterine natural killer cells (uNK) have been

shown to influence the process of uterine angiogenesis through the secretion of cytokines and angiogenic growth factors, in humans (Trundley and Moffett, 2004, Chen et al., 2017) and pigs, (Linton et al., 2008).

Steroid hormones may modulate the expression of genes critical to endometrial angiogenesis such as stimulating the secretion of VEGFA (Barkhem et al., 1998). In ovariectomised mice, progesterone-induced endometrial angiogenesis and endothelial cell proliferation was partly mediated through VEGFA (Walter et al., 2005). An important consideration in the pig is that oestradiol, as part of maternal recognition of pregnancy, is secreted by the embryo before implantation (Bazer et al., 1986, Waclawik, 2011). Oestradiol can stimulate angiogenesis via regulating the expression and secretion of endometrial VEGFA in the pig (Albrecht et al., 2003).

Prostaglandins (PGs) contribute to endometrial remodelling during the establishment of pregnancy and both prostaglandins PGE2 and PGF2α are synthesised at the time of implantation in pigs (Waclawik, 2011). PGF2α is a luteolytic factor and exogenous PGF2α treatment will terminate pregnancy. However, the concentration of PGF2α in the porcine uterine lumen increases dramatically around implantation (Weems et al., 2006). There is some evidence that prostaglandins affect angiogenesis in cancer tissues and *in vitro* culture models of angiogenesis. For example, in coronary venular endothelial cells (CVEC) culture, endothelial cell migration and capillary formation was stimulated by PGE2 through activation of the FGF receptor 1 (FGFR1) (Finetti et al., 2008). PGF2α also has pro-angiogenic roles through the transactivation of the epidermal growth factor receptor (EGFR) (Sales et al., 2005). Moreover, studies have also indicated that PGE2 and PGF2α up-regulate the

expression of VEGFA and FGF2 and their receptors in porcine endometrium and in CVEC cells (Finetti et al., 2008, Kaczmarek et al., 2008, Kaczynski et al., 2016).

Stanniocalcin (STC1) is thought to be an important regulator of early pregnancy in the pig with STC1 expression upregulated in the peri-implantation porcine endometrium (Song et al., 2006). Intriguingly, STC1 stimulated angiogenesis via the activation of VEGF/VEGFR2 pathway in tumour tissues (Yeung et al., 2012, Law and Wong, 2013). Based on this it is tempting to speculate that STC1 regulates endometrial angiogenesis and hence implantation.

At the time of implantation, many genes are upregulated and these are likely to influence the implantation and placentation processes. However, there is limited understanding of the link between these upregulated genes and their role in regulating the angiogenesis process during the establishment of pregnancy.

This chapter will focus on the regulation of angiogenesis during the window of implantation by investigating the effects of the conceptus and endometrial-secreted factors on endometrial angiogenesis *in vitro*.

Hypothesis

Stanniocalcin 1 (STC1), prostaglandin F2alpha (PGF2α), prostaglandin E2 (PGE2) and pregnant uterine flushes will stimulate endometrial angiogenesis *in vitro*.

Aims

In order to address this hypothesis, the aims of this chapter were as follows:

1) Establish a novel culture system to enable the investigation of angiogenesis in the porcine endometrium;
2) Elucidate the effects of STC1, PGF2α, PGE2 and pregnant uterine flushes on the formation of endothelial cell networks *in vitro*.

5.2 Material and methods

5.2.1 Animals

Porcine reproductive tracts were obtained from a local abattoir and transported to the laboratory in PBS at ambient temperature. Non-pregnant porcine uterine tracts were selected that were in the luteal phase, as based on the presence of corpora lutea on the ovaries (Fig. 5.1).

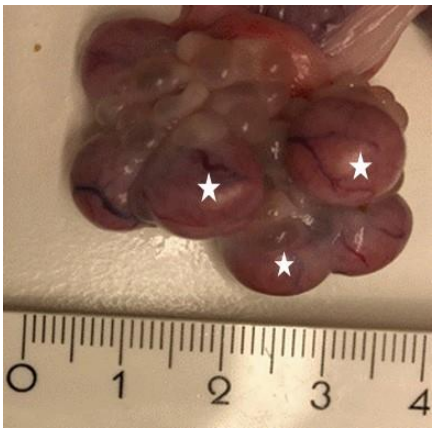


Figure 5.1: Porcine ovary (from gilt) in the luteal phase of the oestrous cycle as indicated by numerous corpora lutea (asterisk).

5.2.2 Endometrial angiogenesis culture system

Coating of coverslips

Coverslips (circular, 19 mm diameter, and 0.25 mm thick) were placed in 70% (v/v) ethanol for >1h, then placed on a petri dish to dry for 1h before being transferred to a 12-well plate under aseptic conditions. Fibronectin (500µl; 10µg/ml) was added to each well and incubated for 4h in an incubator at 39°C. The fibronectin was then removed and the coverslips left to dry at 39°C overnight. On the culture day, the wells were washed once with 1ml of sterile distilled water, and then 1ml endothelial cell media was added. Plates were then incubated at 39°C until cell plating.

Isolation of endometrial cells for culture

A reproductive tract was washed briefly in 70% ethanol (v/v) and a selected piece of uterine horn was cut away and subsequently handled aseptically. The piece of uterine horn was opened and 10 thin strips (<3mm) were dissected from the endometrium, and placed in sterile, warm 1 x PBS, before being roughly chopped with scissors. The tissue pieces were then placed in a conical flask containing 10 ml sterile-filtered Medium 199 containing 5 mg/ml collagenase 1A (C2674, Sigma), 1 mg/ml hyaluronidase (H3056; Sigma) and 25 µg/ml DNase I (D5025; Sigma). This was then incubated in a shaking water bath for 45min at 37˚C. After that, the dissociation solution/tissue was poured into a 50 ml falcon tube to allow the tissue to settle. The dissociation solution was then pipetted off through a 70µm filter into a new falcon tube. Fresh dissociation solution (10ml) was added to the remaining tissue, which was then incubated for a further 45min at 37˚C. Meanwhile, the filtered solution was centrifuged at 300 g for 5min, after which the supernatant was removed and the cell

pellet suspended in culture media. After the second dissociation, tissue was allowed to settle and before being filtered as before. Isolated cells from both dissociations were then combined together and centrifuged at 300 g for 5min.

The supernatant was removed and 5 volumes of red blood cell lysis buffer (155 mM NH_4Cl; 10 mM $KHCO_3$ and 0.1 mM EDTA) were added to the cells and incubated for 3-5min. Cells were immediately centrifuged at 300 g for 5min and washed twice with EC media (Lonza EGM-2 or MCDB 131 [Sigma] based, according to the experimental design see section 5.3.2) with a centrifugation at 300 g for 5min after each wash. After the final centrifugation, the cell pellet was re-suspended with 5 ml of EC media then the number of viable cells (as determined by Trypan Blue dye) counted using a haemocytometer.

Cells were diluted to 1x 10^5 per ml in EC media and from this, 2 ml were seeded in each well of the fibronectin-coated 12-well plate (or according to the experimental design). The cells were cultured in a humidified 5% CO_2 / 95% air at 39°C. In order to remove any dead cells and erythrocytes completely, the endometrial cell monolayers were washed twice in culture medium after 2 and 24h in culture. Thereafter, the medium was changed on day 3 and 5 of culture. At the end of culture (typically day 5) the cells on the coverslips were either collected or fixed for immunocytochemical analysis. Fixation and permeabilisation of cells was performed by adding ice-cold methanol:acetone (1:1) for 5min, at 4˚C, then washed twice with PBS.

Endothelial cell media and its supplements

Two different base culture media were used: (1) specialised endothelial cell media (EGM-2, Lonza) and (2) serum-free growth factor supplemented MCDB 131 media (Sigma). The Lonza EGM-2 media wassupplemented as described in Table 5.1 and was prepared as per manufacturer's instructions (Table 5.1). The MCDB 131 was prepared as follows: 11.6g of powdered medium was dissolved in 950 ml of autoclaved distilled water. To this, sodium bicarbonate (1.18g) was added and stirred gently before adding distilled water to make up to a final volume of 1 litre. The media was then filter sterilised through a 0.22µm filter. These two types of media were used separately. The Lonza EGM-2 was used when optimising the initial culture conditions, while the MCDB 131 media was used for the experimental treatments. Table 5.1 shows the concentration of the different supplements in each medium.

Table 5.1: Composition of the supplements added to the different endothelial cell media (Lonza EGM-2 and MCDB 131).

Supplement	EGM-2	MCDB 131
Human epidermal growth factor	0.5ml*	5 ng/ml
Ascorbic acid	0.5 ml*	1 µg/ml
LR³ insulin like growth factor-1	0.5 ml*	20 ng/ml
Fetal bovine serum	2% (v/v)	-
Bovine serum albumin	-	0.1% (w/v)
Heparin	0.5 ml*	10 unit/ml
Hydrocortisone	0.5 ml*	25 nM
VEGFA	1 ng/ml	1 ng/ml
FGF2	1 ng/ml	1 ng/ml
LH	5 ng /ml	5 ng /ml
Insulin	10 µg/ml#	10 µg/ml#
Transferrin	5.5 µg/ml#	5.5 µg/ml#
Selenium	5 ng/ml#	5 ng/ml#
Gentamycin sulphate,	0.5 ml*	-
Gentamycin sulphate	-	50 µg/ml
Amphotericin B	-	50 ng/ml
Penicillin	100 units/ml	100 units/ml
Streptomycin	0.1mg/ml	0.1mg/ml

*The concentration of these supplements is proprietary, but has stated the volume added to 500 ml. #Added together as a 100x ITS supplement.

5.2.3 Immunohistochemistry

Fixation and Blocking

After fixation in ice-cold acetone:methanol, all immunolocalisation studies utilised the ABC method in combination with the horseradish peroxidase - 3,3'-diaminobenzidine (DAB) detection system. To prevent endogenous peroxidase from being visualised, coverslips were incubated with 3% (v/v) hydrogen peroxide in methanol for 10min at RT. Then wells were washed twice with 1 x PBS for 5min. To prevent antibody binding to non-target sites, the cells were blocked with 20%

(v/v) normal goat serum (NGS) or 4% (v/v) normal horse serum (NHS) according to the type of secondary antibody for 30min at RT.

Primary antibodies

Primary antibodies were added (300 µl) to each well and incubated in a humidified chamber for 2 hours at RT (Table 5.2). After that, the coverslips were washed twice with 1 x PBS for 5min each.

Table 5.2: Antibodies and experimental conditions for immunohistochemistry

Primary antibody [supplier, catalogue number]	**Final concentration** (dilution factor)	**Diluent**	**Secondary antibody**
Rabbit anti-human von Willebrand factor [Dako, A0082]	5 µg/ml (1:1000)	2% NGS (in PBS)	Biotinylated goat anti-rabbit
Mouse anti-human Progesterone receptor [Leica, NCL-L-PGR-312]	5 ng/ml (1:50)	1 x PBS	Biotinylated horse anti-mouse
Mouse anti-human Vimentin [Leica, NCL-L-VIM-V9,]	5 ng/ml (1:100)	1 x PBS	Biotinylated horse anti-mouse
Mouse Anti-Human Cytokeratin [Dako, M0821]	5 µg/ml (1:50)	1 x PBS	Biotinylated horse anti-mouse
Mouse anti-human alpha smooth muscle Actin [Abcam, ab7817]	5 µg/ml (1:80)	1 x PBS	Biotinylated horse anti-mouse

Secondary antibody

The appropriate secondary antibodies (Table 5.2) were prepared according to the Vector ABC Elite method as follows: biotinylated goat anti-rabbit IgG was diluted 1:250 with 2% (v/v) NGS in 1 x PBS or biotinylated horse anti-mouse was diluted 1:250 in NHS in 1 x PBS. Secondary antibody was added and then coverslips were incubated for 30min at RT. After that coverslips were washed twice with 1 x PBS for 5min each.

The avidin-biotin complex

The avidin-biotin complex was pre-prepared 30min before addition as per the manufacturer's instruction (Vector), and subsequently incubated for 30min at RT. Then coverslips were washed twice with 1 x PBS for 5min each.

Detection of antigen

Antigen-antibody binding was visualised by using 3, 3'-diaminobenzidine tetrahydrochloride (DAB; Vector). DAB solution (300 µl; prepared as per instructions) was added to each well and incubated for 1-5min, and then the wells were rinsed under tap water for 10min to stop any further colour development.

Counterstaining and mounting

Coverslips were counterstained with haematoxylin for 30 sec then washed with running tap water for 5min, before dehydration in a series of increasing ethanol concentrations (70%, 95% and 100%) for 2min each. Then the coverslips were removed from the plate and placed in xylene for 20 seconds, then in the fresh xylene

for another 20 seconds. The coverslips were then mounted with DPX mounting medium (Raymond A Lamb, East Sussex, UK) onto glass slides.

***Image acquisition*.**

Representative images were acquired to allow comparison of the staining obtained with the different antibodies across different time points. Representative images (12 pictures) were taken randomly across each whole coverslip under bright field with a Leica DM 4000 B microscope (Leica Microsystem (UK) Ltd, Milton Keynes, UK), fitted with a Qlmaging Micropublisher 5.0 RTV colour camera (Qlmaging (UK) Ltd, St. Helens, Merseyside, UK).

5.2.4 Dual immunofluorescence staining of von Willebrand factor and cytokeratin

To determine the temporal and spatial pattern of epithelial and endothelial cell growth and characterise the development of specific structures (e.g. endometrial gland-like structures and endothelial cell networks), VWF antibody (endothelial cell marker) was co-localised with cytokeratin antibody (epithelial cell marker) in the endometrial angiogenesis culture system.

Coverslips were collected after 2, 12, 24, 48, 72, 120 and 168 hours of culture (two coverslips per time point). Acetone: methanol (1:1) was used to fix the coverslips for 5min, followed by washing twice with 1 x PBS for 5min each. Cells were then permeabilised with 0.5% triton in 1 x PBS for 1 hour, then washed with 1 x PBS (2x 5min). Non-specific binding was blocked with 2% bovine serum albumin (BSA) in 1 x PBS for 1 hour.

The first primary antibody, rabbit anti-human VWF (Dako) was diluted 1:1000 in 20% NGS. It was incubated for 2 hours at RT and then the coverslips were washed with 1 x PBS (3x 5min). The secondary antibody, FITC anti-rabbit IgG (20 μg/ml Vector, diluted in 2% NGS) was applied to the cells and incubated for 1h at RT. Then the cells were washed with 1 x PBS (2x 5min). The second primary antibody monoclonal mouse anti-human cytokeratin (Dako; diluted 1:50 in PBS) was applied for 2 hours at RT before washing in 1 x PBS (3x 5min). The secondary antibody, Texas Red anti-mouse IgG (20 μg/ml Vector, diluted in 2% NHS) was added to the coverslips and incubated at RT for 1 hour. The coverslips were washed as previously before adding Vectashield with DAPI (Vector; 3 drops) to the coverslips for mounting. This was then sealed with nail varnish and stored at -20ºC for 2-3 hours. Twelve images were captured for each coverslip under the 40X objective using a Leica DM 5000 B florescence microscope, equipped with a Leica DFC 350fx camera (Leica Microsystem (UK), Ltd, Milton Keynes, UK).

5.2.5 Image analysis of von Willebrand factor immunohistochemistry

The quantification of von Willebrand factor (VWF) staining was established according to that previously published (Robinson et al. 2008). For each treatment in each culture, two wells were selected for analysis. Images were captured from a total of 12 fields of view per well randomly across the coverslips under 5x objective using a Leica-DM4000B microscope (Leica Microsystems Ltd., Milton Keynes, UK) and Qlmaging Micropublisher 5.0 RTV colour camera (Qlmaging (UK) Ltd., St Helens, UK). The images were analysed by Image Pro-Plus 6.5 (Media Cybernetics, Wokingham, UK). Four captured images were tiled to create a composite image (total 3 tiles per coverslip). The brown areas of positive staining for VWF had two

different appearances according to the shape of the group of cells and features as follows: (1) branch-like or thread-like network areas and (2) unbranched clusters of cells forming an island (no network appearance). The latter type were not analysed due to the variation in the appearance between cultures. However, these brown areas with a branch-like and thread-like appearance representing endothelial cell (EC) networks were selected using the manual colour cube in the colour selection tool. The highlighted brown areas were smoothed, then any holes in the staining were removed and any VWF staining area <100 μm^2 was excluded. Then, the total area of VWF network staining, total number of endothelial cell networks, the number of endothelial cells networks, and the perimeter of positive VWF were recorded. Automated measurement within the Image ProPlus software was used to determine the number of branch points that were present within the EC networks. The degree of sprouting was calculated by dividing the total number of branch points by the total number of EC networks (Woad et al., 2012, Masri et al., 2007).

5.2.6 **Statistical analysis**

Data were checked for normality and homogeneity of variance by using residual plots and Levene's test respectively in Genstat prior to statistical analysis. Data were log transformed where appropriate. A randomised block one-way ANOVA in GenStat 17th Edition (VSNi Hemel Hempstead, UK.) was used, to investigate the effect of treatment (adding angiogenesis factors (VEGFA and FGF2). STC1; PGE2; PGF2α and uterine flushes) on the different parameters of EC network formation. Each treatment was analysed separately and added as the factor to the fixed model on the response variable (total area of endothelial cell networks, area of individual EC networks and branch points). The culture was used as the block. Separate analysis

was performed to look at the effects of treatments under basal or angiogenic stimulated conditions. For all experiments $p<0.05$ was considered as significant and all data are presented as mean + SEM.

5.3 Establishment of a novel culture system to investigate angiogenesis and gland formation in the porcine endometrium.

5.3.1 Objectives

In order to establish a culture system that could investigate angiogenesis in the porcine endometrium the following objectives were developed:

1) Establish a methodology that isolated different cell types (in particular endothelial cells) from endometrial tissue.

2) Characterise the growth of endothelial cells alongside other cell types (stromal, epithelial and smooth muscle cells) within the culture system

3) Confirm the presence of progesterone receptor expression in the endometrial culture system.

4) Compare the extent and pattern of endothelial cell growth in the presence of either specialised endothelial cell medium (Lonza) or serum-free growth-factor supplemented MCDB 131 media (Sigma).

5) Investigate endothelial and epithelial cell growth over time, using a detailed time course (2h-5d).

5.3.2 Experimental design

1) Optimisation of culture conditions

Endometrial cells (as described in Section 5.2.2) that were enzymatically dispersed from porcine endometrium (in the luteal phase) were plated onto fibronectin-coated coverslips [200,000 cells; 12-well plate]. Cells were grown in specialised endothelial cell medium (EGM-2 from Lonza), 2 ml per well, under angiogenic-stimulated

conditions (VEGF and FGF2; both 1ng/ml). The cells were washed after 18h and replaced with fresh media. Thereafter, media was collected and replaced with fresh media every two days. These conditions (number of cells, dispersion procedure and culture media) were based on those described for bovine luteal angiogenesis cultures (Robinson et al., 2008).

In this culture procedure, the isolated endometrial cells grouped and aggregated to each other and large gaps formed between the aggregates in the initial culture. Thus, in order to minimise this, an additional cell washing step was applied and comparisons made between washing after 1h, 2h or 18h from seeding. Additionally, the effect of the number of plated cells was tested ($1x10^5$ vs $2x10^5$ per ml of media).

2) Comparison between EGM-2 and MCDB 131 media

This experiment examined whether MCDB 131 media (Sigma) was able to maintain the formation of EC networks to the same extent as the proprietary endothelial cell growth media (EGM-2, Lonza). The effects of washing cells 1 or 2h after seeding cells were studied with each type of media independently.

Endometrial cells (n=3 cultures) were plated in 12 well plates under angiogenic stimulated conditions (FGF2 and VEGFA; both 1ng/ml) in either EGM-2 media or MCDB 131 media (described in Section 5.2.2). Cells were washed after 1 or 2h. Media was subsequently changed after 18 hrs then every 2 days. On days 3 or 5 of culture, cells were fixed (see Section 5.2.2) by acetone:methanol. Next, VWF, PGR, cytokeratin, vimentin and SMA immunohistochemistry were performed.

3) The development of endothelial cell networks and endometrial glands–like structures over time

Endometrial cells (n=4 cultures) were seeded in 12-well plates. Cells were grown in supplemented MCDB 131 media. Cells were fixed after 2h, 12h, 1d, 3d, 5d and 7d in culture using acetone: methanol (1:1) as described previously. This experiment aimed to study the timeline of EC network formation in the endometrial cell culture. In addition, based on the previous culture experiment, the development of endometrial gland-like structures was also monitored. At each time point, 2 wells were fixed for immunohistochemistry to detect VWF (endothelial cells) and 2 wells fixed for cytokeratin (epithelial cells). In addition, a uterine cross section was used as a positive control for cytokeratin antibody and to confirm the specificity of the cytokeratin antibody as an epithelial cell marker.

5.3.3 Results

5.3.3.1 Optimising culture conditions

The initial experiments demonstrated that isolated endometrial cells were successfully dissociated with a high level of viability (typically 50-70%) before plating. In culture, the cells appeared to proliferate and could be grown for 7 days before becoming confluent. Under an inverted microscope, it was clear that there were cells of mixed phenotypes present throughout the culture.

The tissue culture procedure was subsequently optimised using different washing times after seeding. The first washing time was 18 hours after plating as performed in bovine previous luteal angiogenesis studies. At 18 hours (Fig 5.2 A, B and C), most cells were densely aggregated together and with large holes observed among them on day 5 of culture. Thus, two more washing steps were used to adjust this

protocol; namely 1 and 2 hours after plating. Washing cells after 2 hours, led to monolayer growth with endothelial cells (EC) forming small but distinct networks between other endometrial cell types (Fig. 5.2 D, E and F). In contrast, washing cells 1h after plating assisted to remove dead cells and resulted in the growth of a monolayer of cells with a small number of cells staining positively for VWF. The EC formed more linear structures with more limited branching (Fig.5.2 G, H and I). Comparison of different cell densities at plating (experimental range 1x 10^5 and 2x 10^5 cells per ml) did not affect the degree to which the EC networks formed.

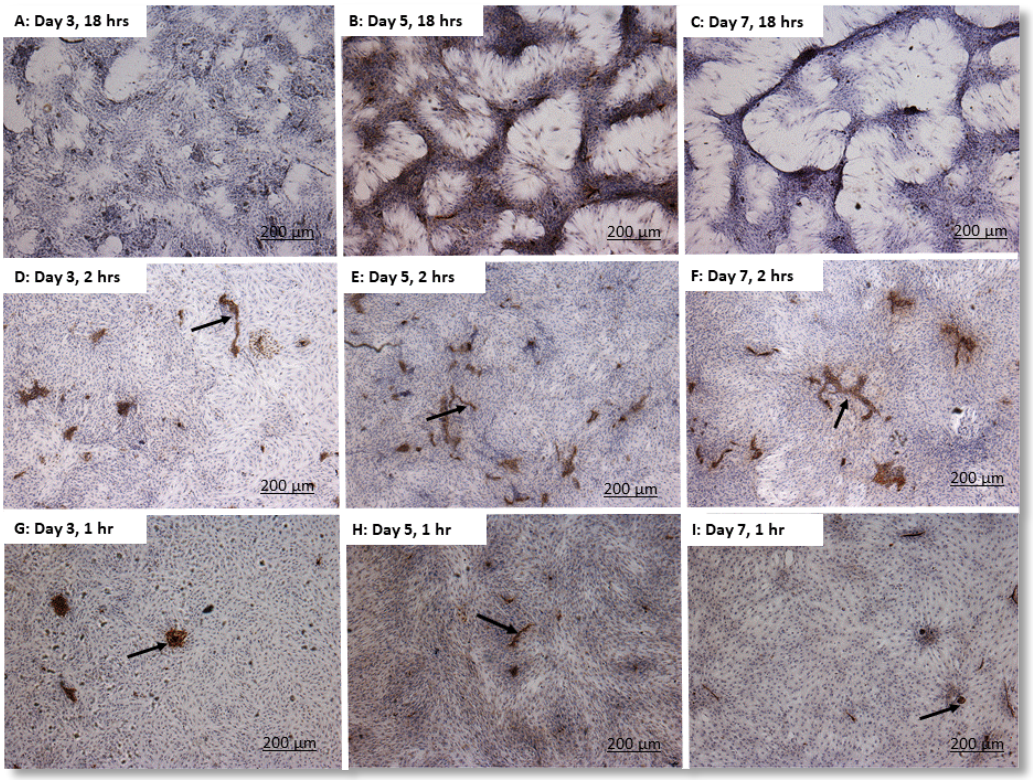


Figure 5.2: Optimisation of endometrial angiogenesis culture system: effect of the time of washing on formation of endothelial cell networks. Endothelial cells were detected by immunohistochemistry for VWF (brown). Pictures (A, B and C): show when cells were washed after 18h then fixed on day 3, 5 and 7 of culture respectively. Pictures (D, E and F): show when cells were washed after 2 h and then fixed on day 3, 5 and 7 of culture respectively. Pictures (G, H and I): after cells were washed after 1 h and then fixed on day 3, 5 and 7 of culture respectively. Washing cells after 2 hours appeared to maximally support the growth of endothelial cell networks. All cells were cultured in EGM-2 media (Lonza) with angiogenic factors (1 ng/ml FGF2 and 1 ng/ml VEGFA) added. Areas of EC network formation are marked (arrow). Scale bar =200 µm.

5.3.3.2 **Optimising culture conditions; comparison of Lonza (EGM-2) and MCDB 131 media**

On day 3 of culture, the cells had formed a monolayer that contained multiple cell types/phenotypes. Endothelial cells (EC) grew closely associated together and formed islands (~ 25 cells) which then apparently transformed into networks by culture day 5 in each treatment. EC networks typically had a central body and sometimes formed irregular EC structures. The degree and pattern of endothelial cell

networks that formed with EGM-2 and MCDB 131 media were similar (Fig. 5.3). Immunostaining of VWF demonstrated that endothelial cell islands were observed with both media and had a similar appearance throughout the culture. By day 3, each island (30-50 cells) that formed was roughly circular and contained 5-8 cells across the island (Fig. 5.3 A and B). By day 5 of culture, most of the endothelial cell clusters were similar in size to day 3 but had transferred to more specialised structures with the presence of endothelial cell branches and sprouting away from the cluster (Fig. 5.3 C and D). The degree of branching in the different EC networks visually appeared to be greater in these cells grown in supplemented MCDB 131 media. While this was not quantified, it was decided to utilise MCDB 131 as the basal media for further experiments.

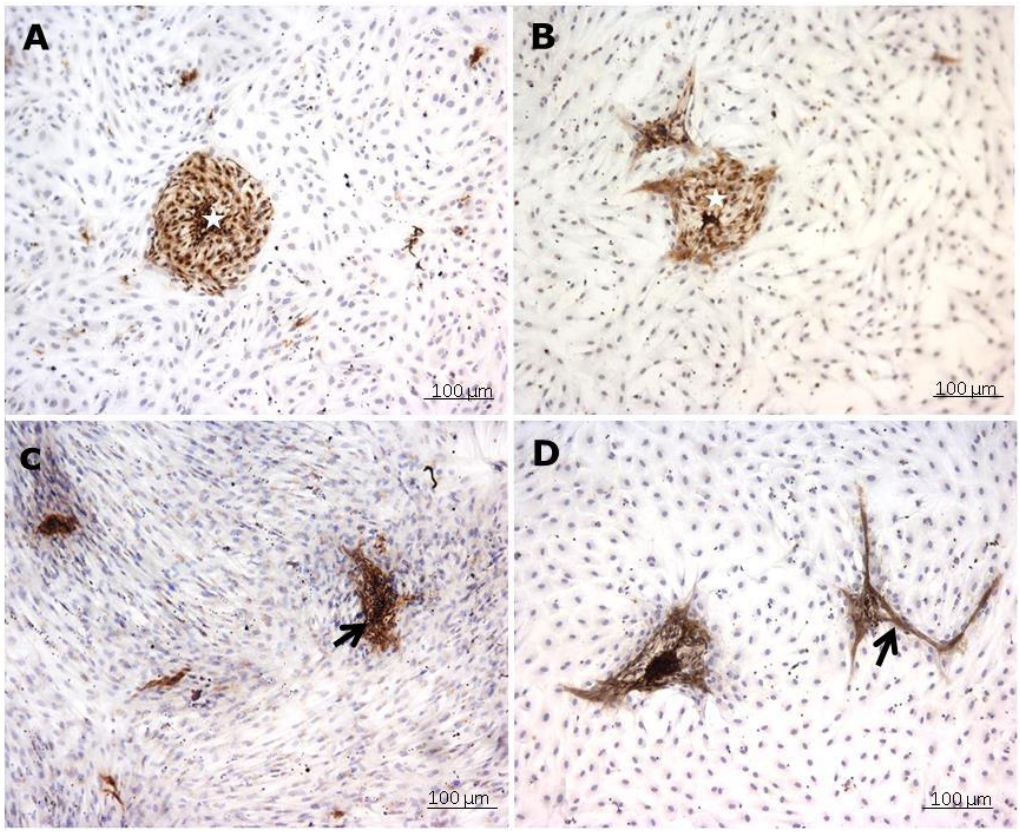


Figure 5.3: Optimisation of endometrial angiogenesis culture system: effect of the media (Lonza EGM-2 vs MCDB 131) on formation of endothelial cell networks. The immunohistochemical localisation of VWF as a marker of endothelial cells (EC; brown) was performed. The images are from (A): EGM-2 on culture day 3; (B): MCDB 131 on culture day 3; (C): EGM-2 on culture day 5, (D): MCDB 131 on culture day 5. Visually the in-house MCDB 131 media had slightly higher endothelial cell networks compared with the proprietary Lonza EGM-2 media in this procedure. Angiogenic factors (1 ng/ml FGF2 and 1 ng/ml VEGFA) were added to both media. Arrows show EC networks, while white asterisks show EC islands. Scale bar =100 µm.

5.3.3.3 **Expression of progesterone receptor and smooth muscle actin in endometrial cell culture**

Progesterone receptor (PGR) was immunolocalised to the cell nucleus in multiple cell types throughout the culture period (namely culture days 3, 5 and 7; Fig. 5.4A, B and C). Visually, most of the cells were positive for PGR on days 3 and 5 of culture but the degree of expression appeared to decrease on day 7 of culture.

Smooth muscle actin (SMA) was clearly detected in a proportion of cells in the endometrial cell culture on days 3, 5 and 7 (Fig. 5.4 D, E and F). The SMA-positive cells appeared to have at least 2 different phenotypes: (1) flattened, narrow cells with intense SMA staining and (2) those with a larger diameter and with projections that made contact with other cells. However, there were large areas of cells that were devoid of any SMA staining and these often had the appearance of an epithelial or endothelial cell island. It was also noticed that SMA-positive cells appeared to be in close contact/association with these epithelial / endothelial-like cell islands.

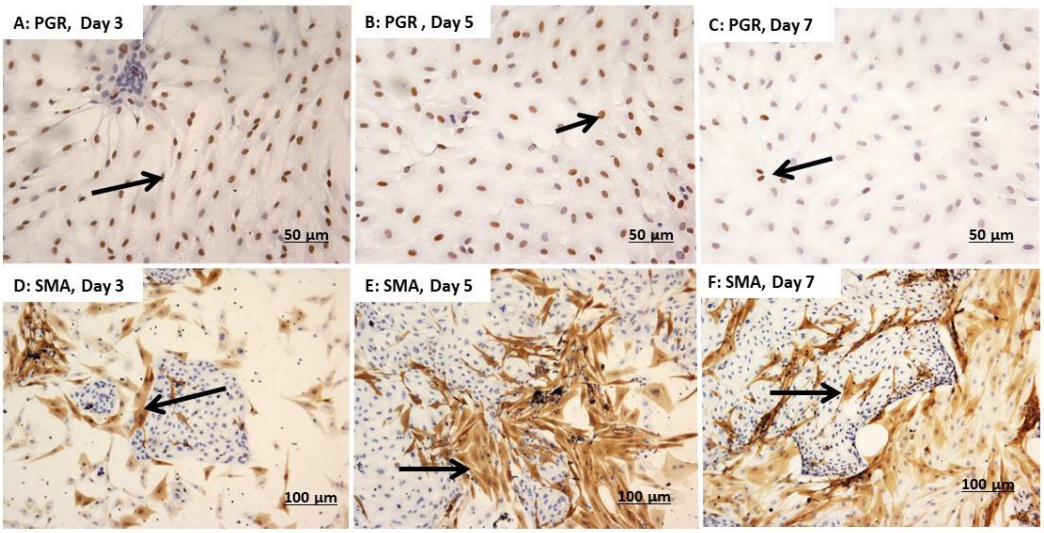


Figure 5.4: Immunohistochemical localisation of progesterone receptors (PGR) and smooth muscle actin (SMA) in endometrial cell culture. Images shown are from (A): PGR on day 3; (B): PGR on day 5; (C): PGR on day 7; (D): SMA on day 3; (E): SMA on day 5 and (F) SMA on day 7. Cells were cultured in Lonza EGM-2 media in the presence of FGF2 and VEGFA. Positive staining is indicated by the arrows. Scale bar = 50 or 100 µm as indicated.

5.3.3.4 **Development of von Willebrand factor-positive endothelial cells over time**

Immunostaining for VWF was used to detect endothelial cells, throughout culture, from the earliest time point (2 hours) until day 7 of culture. Two hours after seeding, endothelial cells were detected as isolated individual cells. By day 2 of culture, endothelial cells were identified in small and compact islands of cells (10 to 15 cells) (Fig. 5.5 A and B), and they had the classic cobblestone appearance. By day 3 of culture, these EC islands were larger in size and often more than one EC island was visible in a single field of view under 20x magnification (Fig. 5.5 C). Occasionally, endothelial cells were detected by day 3 of culture within an EC island. These were then surrounded by other VWF-negative endometrial cells (Fig. 5.5 D). On day 5 of culture, the endometrial cells had formed a confluent monolayer. The endothelial cells were typically present within with a network of specialised cells. Typically, each EC network had a central base (presumably the original EC island) from which branches were developed to form the network. The number of branches varied between the different EC networks within the same culture. Other cell types covered a greater area of the coverslip than previously and their density was increased over time. The number and appearance of the branched EC networks was broadly similar on day 5 and 7 of culture (Fig. 5.5 E and F).

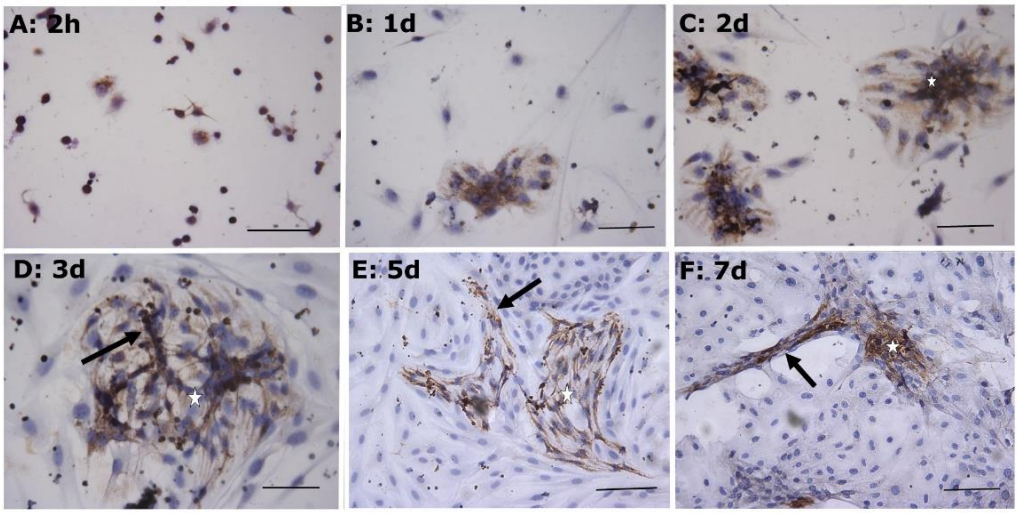


Figure 5.5: Time-course of endothelial cell (EC) network formation in primary porcine endometrial cultures. EC were immunostained for von Willebrand factor (VWF) after (A): 2h with EC appearing as isolated cells; (B): 1 day where there was the formation of EC islands of 5-8 cells; (C): 2 days at which point EC islands of 20-30 cells were present; (D): 3 days when EC started to sprout and form branches; (E): 5 days, where EC networks had started to form a capillary network-like appearance. This was similar on (F) day 7 of culture. Arrows indicate EC sprouting while asterisks show EC islands. Scale bar 20-50µm as indicated.

5.3.3.5 Development of cytokeratin-positive epithelial cells over time

In order to confirm the localisation of the cytokeratin antibody to epithelial cells, immunohistochemistry on porcine uterine cross section was performed. Cytokeratin-positive epithelial cells were detected in the epithelial cells that lined the uterine lumen as well as those within the endometrial glandular epithelium (Fig. 5.6 E). The stroma or endothelial cells showed no positive staining for cytokeratin.

In culture, epithelial cells were also detected and were identified as small cells with a round nucleus and were principally present as an irregular shaped island (Fig 5.6.A). These cells appeared to be negative for VWF, SMA and vimentin (Fig. 5.6 pictures A, B and C respectively) and positive for cytokeratin (CK) immunostaining (Fig. 5.6 D). However, dual staining was not performed to confirm this observation.

Figure 5.7 illustrates the development of cytokeratin-positive epithelial cells over time. Two hours after seeding, the epithelial cells were detected in differently sized groups of cells or as individual cells. After day 1 of culture, groups of cells appeared to have further increased in size (Fig 5.7 A and B). By the end of day 2, epithelial cells islands were substantially greater in size and formed a gland-like shape, with more than 10 layers of cells surrounding a central hole (Fig. 5.7 C). Epithelial cell islands continued to increase in size after day 5 and 7 and eventually occupied a large proportion of a field of view in some wells under 5× magnification (Fig. 5.7 D and E). In order to further characterise the epithelial and endothelial cell islands in culture, dual fluorescent immunohistochemistry was performed. In this immunohistochemistry, epithelial (CK; red) and endothelial (VWF, green) cells were present throughout the culture period, largely as separate islands (Fig. 5.8). At 2h after seeding, the epithelial cells had formed into a group, while the endothelial cells

were largely still separated. The islands of CK+ve cells were generally larger than those present for VWF+ve endothelial cells.

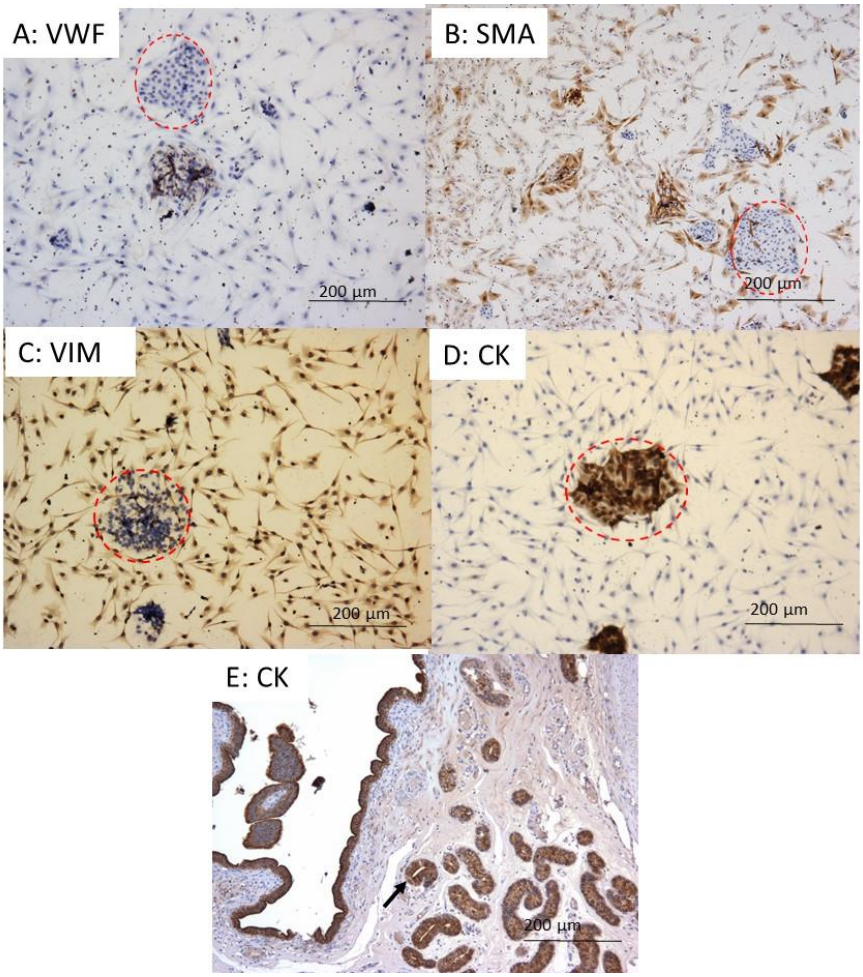


Figure 5.6: The presence of different cell types in the endometrial angiogenesis culture system. Representative images are shown as (A): von Willebrand factor (VWF), (B): Smooth muscle actin (SMA), (C): Vimentin (VIM) and (D): cytokeratin (CK). Based on the pattern of CK-positive cell island, presumptive epithelial islands are shown in the other images by a red-dashed line. The serum free supplemented MCDB 131 media was used in this experiment. Additionally, E: shows CK immunohistochemistry in a cross section of uterine horn where the positive staining was present in luminal and glandular epithelium only (arrows indicates epithelial cells islands). Scale bar 200 µm.

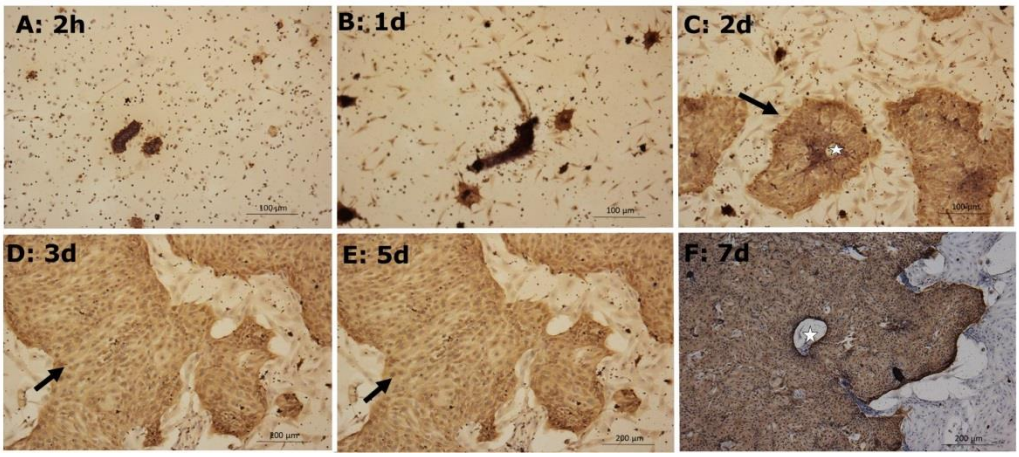


Figure 5.7: Time-course of epithelial cell development in the porcine endometrial angiogenesis culture system. Epithelial cells were stained by immunohistochemistry for cytokeratin antibody (CK). Representative images are shown from the following culture time-points: (A) 2h; (B) day 1; (C) day 2; (D) day 3; (E) day 5; (F) day 7. Arrows indicate the localisation of epithelial cells within islands while asterisks indicate holes in the centre of CK+ve islands. Scale bar = 100 or 200 µm.

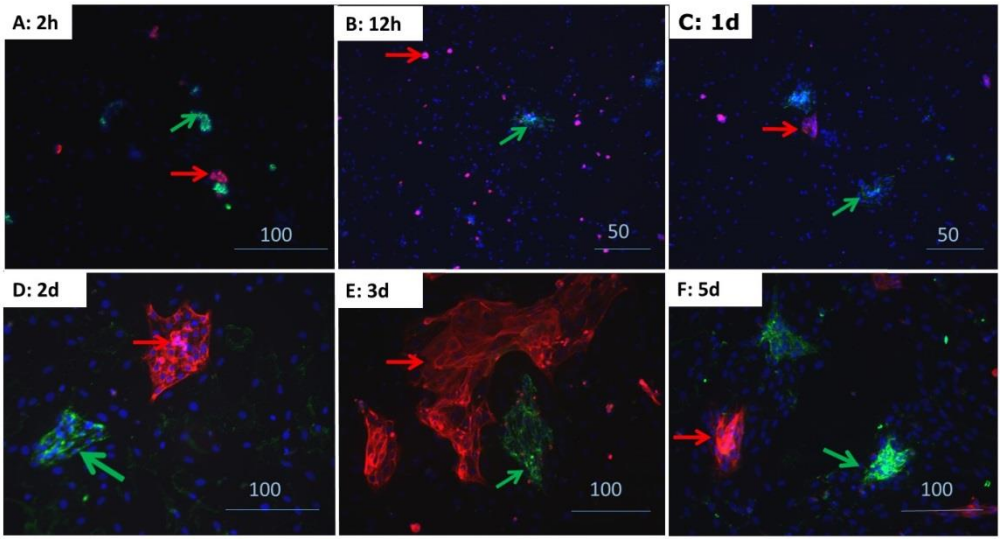


Figure 5.8: The time-course of epithelial and endothelial cell growth in a porcine endometrial angiogenesis culture system. The cells were stained with florescent immunohistochemistry for VWF (green colour) and CK (red colour). Representative images from the following culture time-points are shown as: (A): 2h; (B): 12h; (C): day 1; (D): day 2; (E): day 2 and (F): day 5. Scale bar = 50-100 µm as indicated.

5.3.4 Discussion

This experiment has established a novel porcine endometrial cell culture system and demonstrated that endothelial cells derived from the porcine endometrium form networks in culture, alongside other endometrial stromal and epithelial cells.

Previous studies have isolated luminal epithelial, glandular epithelial and stromal cells from pig endometrium (Zhang et al., 1991) or employed co-culture with both luminal epithelial and stromal cells (Blitek et al., 2011). Others have developed *in vitro* models using immortalised cell lines from porcine endometrial cells (Wang et al., 2000) or isolated endometrial endothelial cells (Czarzasta et al., 2018). The present study is the first to describe the isolation and development in culture of endothelial cell networks from porcine endometrial tissue alongside other endometrial cells types (stromal and epithelial cells).

The heterogeneous types of cells provided monolayer growth that enabled the formation of endothelial cells networks. Moreover, the establishment of the culture system was performed with defined media based on MCDB 131 media as well as the undefined proprietary Lonza EGM-2 that was utilised previously in a luteal angiogenesis culture system (Robinson et al., 2008).

Cell growth was improved by the addition of a washing step 1 or 2 hours after plating. This was performed to remove any dead cells and debris present after enzymatic isolation and thus creating more space for the viable cells to grow. It may have also removed other live cells (stroma and epithelial cells), therefore increasing the proportion of endothelial cells and favouring their growth. Washing after 2 hours resulted in more extensive endothelial cell growth than after 1h and this may have

been the result of better recovery from any damage caused during the isolation process. This step has been used in other endothelial cell isolation protocols (Relou et al., 1998) and it has been suggested that endothelial cells adhere to the fibronectin coating quicker and with a greater affinity than other cell types. Hence, this has been employed to enrich the proportion of endothelial cells at plating. The current observations would support this.

There was broadly similar degree of EC network formation when cells were grown in supplemented MCDB 131 media and Lonza EGM-2 media. Based on this observation, it was decided to use supplemented MCDB 131 media for the following reasons: (1) the MCDB 131 media was serum-free thus eliminating the addition of unknown factors; (2) the supplements added to the MCDB 131 were at a defined concentration and source and (3) the degree of branching points appeared slightly greater in MCDB 131 media. This media has also been utilised and characterised in a follicular angiogenesis culture system (Laird et al., 2013).

The culture system contained several factors in the medium that were not individually investigated to assess their effects on the culture. However, vascular endothelial growth factor A (VEGFA) is one of the angiogenic factors that plays key roles in regulating tumour (Kim et al., 1993) and luteal angiogenesis (Robinson et al., 2009, Woad and Robinson, 2016). The importance of VEGFA was clearly demonstrated when angiogenesis endothelial precursor cells did not differentiate into endothelial cell, in VEGFR2 deficient mice (Demir et al., 2010). VEGFA is incorporated in many *in vitro* angiogenesis culture system based on its positive effects on endothelial cell proliferation and migration (Robinson et al., 2008). Though often overlooked, FGF2 also has vital roles in angiogenesis. For example,

in bovine luteinising culture, the degree of endothelial cell branching was increased by FGF2 (Laird et al., 2013). Also, it can enhance the effectiveness of VEGFA in culture (Woad and Robinson, 2016). The effects of FGF2 and VEGFA on porcine endometrial angiogenesis were investigated in the next section.

Epidermal growth factor (EGF) and insulin like growth factor 1 (IGF1) are added to many commercial angiogenesis kits. For example, these were added to EGM-2 media in an *in vitro* co-culture system using human umbilical vein endothelial cells (HUVECs) with endometrial cells (Canosa et al., 2017). While, in *ex vivo* tissue both the pig endometrium and conceptus secrete IGF1 at the time of implantation (Waclawik, 2011). In a bladder carcinoma cell line, EGF increased the production of VEGFA (Perrotte et al., 1999, van Cruijsen et al., 2005). Therefore, considering the physiological nature of endothelial cells in endometrial tissue, adding these factors appears to support to growing endothelial cells networks *in vitro*.

The endometrial angiogenesis culture was developed with luteinising hormone (LH) added. LH has been used in other angiogenesis systems due to its stimulatory effects on angiogenesis and relevance to progesterone production, for example, in culture system for follicular and luteal angiogenesis (Laird et al., 2013), in primate ovulatory follicles (Trau et al., 2015). Moreover, VEGFA activity in the endometrium can be modulated by human chorionic gonadotrophin (HCG) (Licht et al., 2001) and LHCGR are present in the porcine endometrium including within the vasculature (Ziecik et al., 2005).

It should be noted that this culture method also has some limitation. The principal one is that it did not start with a fixed number of endothelial cells for each culture nor is it known exactly how many endothelial cells are present. This aspect is limited

by being unable to accurately isolate endothelial cells from the mixed cell population after dispersion. The addition of the washing step 2h after plating should have enhanced the proportion of endothelial cells present. A key feature of the culture system is that endothelial cells grow in close proximity to other cell types, closely mimicking the natural interaction of cells. In this regard, the current culture system was able to sustain multiple cell types that formed alongside endothelial cells that developed into network-like structures.

In addition to supporting endothelial cell growth, the current *in vitro* system resulted in significant epithelial cell growth. Of particular note was the formation of uterine glandular-like structures, where epithelial cells formed into multiple cell islands with a broadly circular appearance. Intriguingly, these cell islands by day 5-7 of culture had a central hole. It is tempting to speculate that this central hole was the equivalent to the gland lumen. To the best of my knowledge, this is the first report of such structures. The formation of gland-like structures means that the media components support their formation as well as the EC networks. It is feasible that EGF and IGF1 are important for gland-like formation since EGF and IGF1 induced proliferation of epithelial cells (Eritja et al., 2010). Thus, this culture system will help to establish an experimental design that assesses the angiogenesis in endometrium with the same population of endometrial cells. The next step was to utilise this novel culture system to determine the effects of different factors on the formation of EC networks *in vitro*.

5.4 Effects of STC1, PGE2, PGF2α and pregnant uterine flushing media on *in vitro* endometrial angiogenesis

5.4.1 Objectives

1) Determine the effects of STC1, PGF2α and PGE2 on endometrial angiogenesis *in vitro*.

2) Identify the effects of uterine flushes media from pregnant sows on endometrial angiogenesis *in vitro*.

5.4.2 Material and methods

5.4.2.1 Endometrial angiogenesis cultures

Porcine reproductive tracts (post-pubertal gilts were used in this experiment) were collected at abattoir and endometrial cells were prepared as previously described (5.2.1). The endometrial cells were diluted to 1 x 10^5 viable cells per ml in MCDB 131 media and 2 ml was added to each well of a 12 well plate containing fibronectin-coated coverslips. Cells were grown for 5 days in a humidified incubator at 39ºC in 5% CO_2 and 95% air. The cells were washed after 2 hours and the media were changed on days 1 and 3 of culture. The treatments (STC1, PGF2α, PGE2 or uterine flushing media from day 11-12 or 13-14 pregnant sows) were added with media on day 1 and 3 of culture (Fig. 5.9). All treatments were conducted in the presence or absence of FGF2 and VEGFA (1 ng/ml each).

5.4.2.2 Treatments

Recombinant human STC1 protein (Source BioScience Nottingham, UK) was diluted with distilled water and added to the media at a final concentration of 0, 5 and 50 ng/ml (Fig. 5. 9A). PGF2α and PGE2 (Cayman Chemical) were dissolved in

100% ethanol, and then were added to the media at a final concentration of 10 and 100 ng/ml for each (Fig. 5. 9B and C). The final concentration of ethanol was 0.1% (in 100ng/ml wells) and equivalent ethanol concentrations were added to the untreated (control) wells.

Finally, uterine flushes were collected from day 11-12 (pooled from 5 animals) or day 13-14 (pooled from 5 animals) of pregnancy. These were concentrated and the protein concentration determined using a Bradford assay. The uterine flushing was added at either 200µg/ml or 20µg/ml protein. The control wells for these uterine flushing treatments were treated with bovine serum albumin (BSA) at 200 or 20µg/ml (in addition to the 1mg/ml BSA that was already present in the media). This was conducted to account for any effects of adding extra protein supplement to the cells (Fig. 5.9 D and E). The concentration of BSA in the basal media was 1mg/ml (see section 5.4.2.3 for further details).

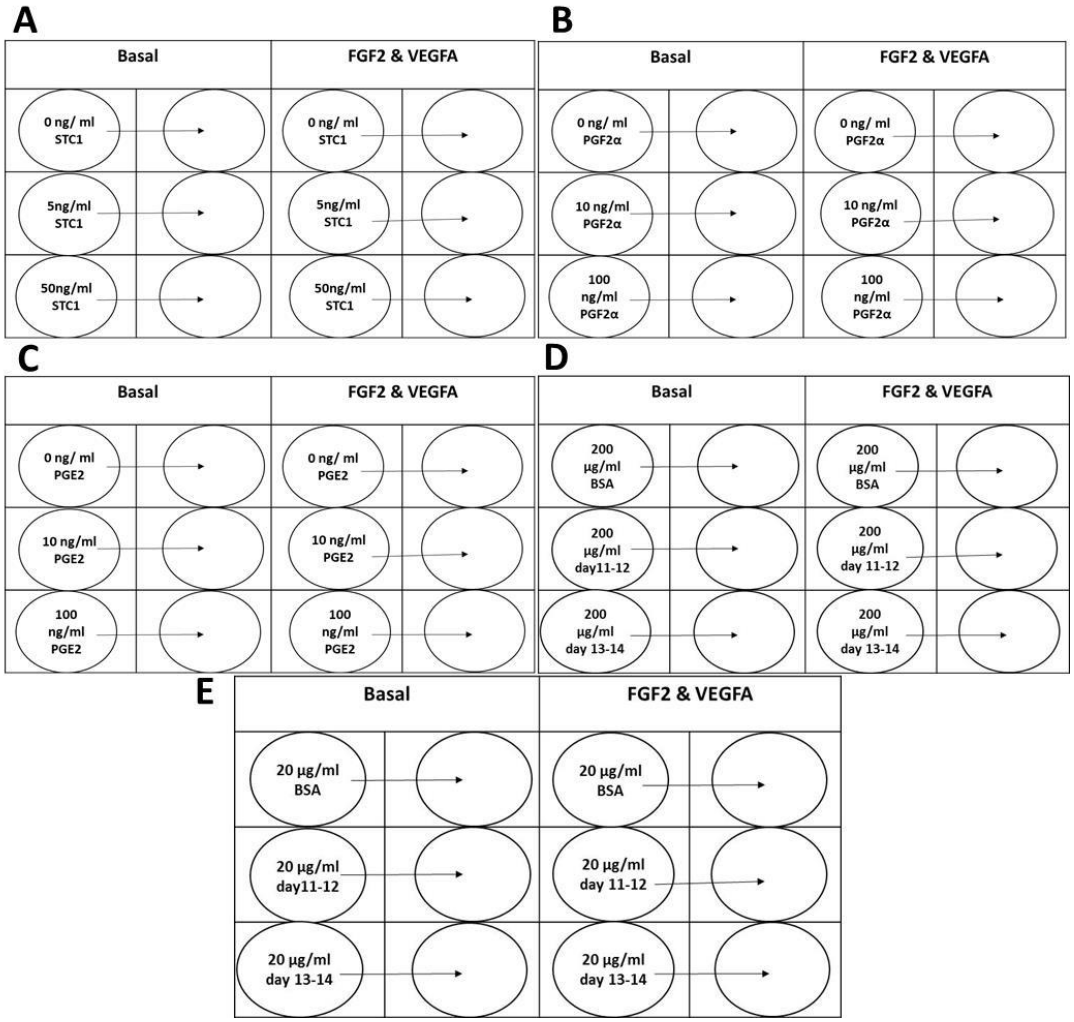


Figure 5.9: Plate layout for endometrial angiogenesis experiment. Cells were treated under basal and angiogenic stimulated conditions (1ng/ml FGF2 and 1ng/ml VEGFA). Cells were treated with (A) 0, 5 and 50 ng/ml STC1; (B) 0, 10, and 100 ng/ml PGF2α; (C) 0, 10, and 100 ng/ml PGE2. (D) Cells were treated with 200 µg/ml protein from uterine flushing media from pregnancy day 11-12 and 13-14. (E) Cells were treated with 20 µg/ml protein from uterine flushing media from pregnancy day 11-12 and 13-14.

5.4.2.3 **Preparation of uterine flushing media for cell culture treatments**

The uterine flushes were collected at the time of embryo collection (as described in Chapter 3) by flushing each uterine horn twice with 20ml of 1% (v/v) fetal bovine serum in phosphate buffer solution (PBS). The flushing media from each animal was individually clarified by centrifugation (3000g for 10min) and the supernatant aliquoted (5ml), and frozen at -80ºC until used. The supernatant (2 ml each) from 5 animals from either day 11-12 or 13-14 of pregnancy were pooled together. From this, 10ml of flushing media was concentrated down to 1ml using ultracel YM-3 Centricon centrifugal cellulose membrane filter device (3kD molecular weight cut off). Each filter was filled with 2 ml of flushing media, then centrifuged at 5000 g for 5 hours at 4ºC (Avanti J-E centrifuge, Beckman.) Then, the concentrated flushing media was collected and sterilised through a 0.22µm filter (Fig 5.10). Total protein concentration of each sample was then assessed by Bradford assay (Sigma) and used to supplement endometrial angiogenesis cultures at 20 or 200 µg/ml total protein.

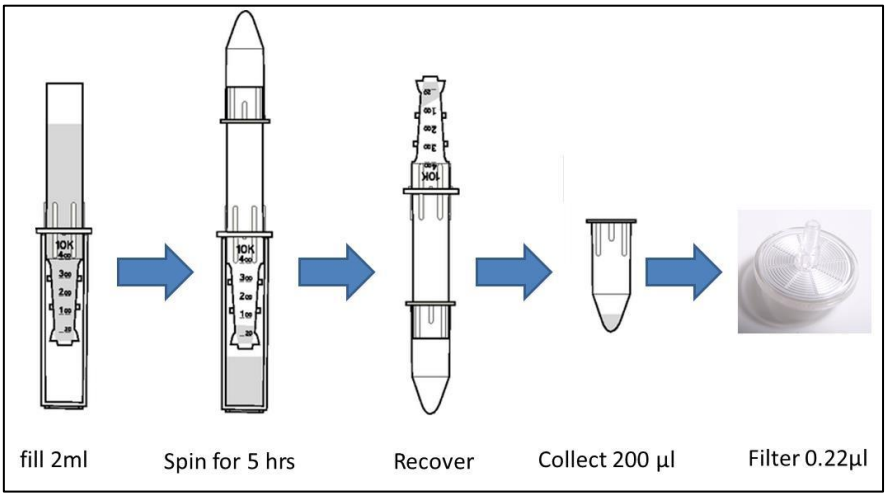


Figure 5.10: The processing and preparation of uterine flushing media.

5.4.3 **Results**

5.4.4 **The effect of angiogenic factors, FGF2 and VEGFA, on porcine endometrial angiogenesis**

On day 5 of culture, moderate endothelial cells (EC) networks were produced in the presence and absence of angiogenic factors, FGF2 and VEGFA. Both total area and perimeter of EC networks were significantly greater ($p<0.05$) by approximately 40% under angiogenic-stimulated compared with basal conditions. FGF2 and VEGFA also increased the number of EC networks ($p<0.05$) and total number of branch points ($p<0.05$; data not shown), whilst the number of branch points per endothelial network was not significantly different ($p>0.05$) (Fig 5.11).

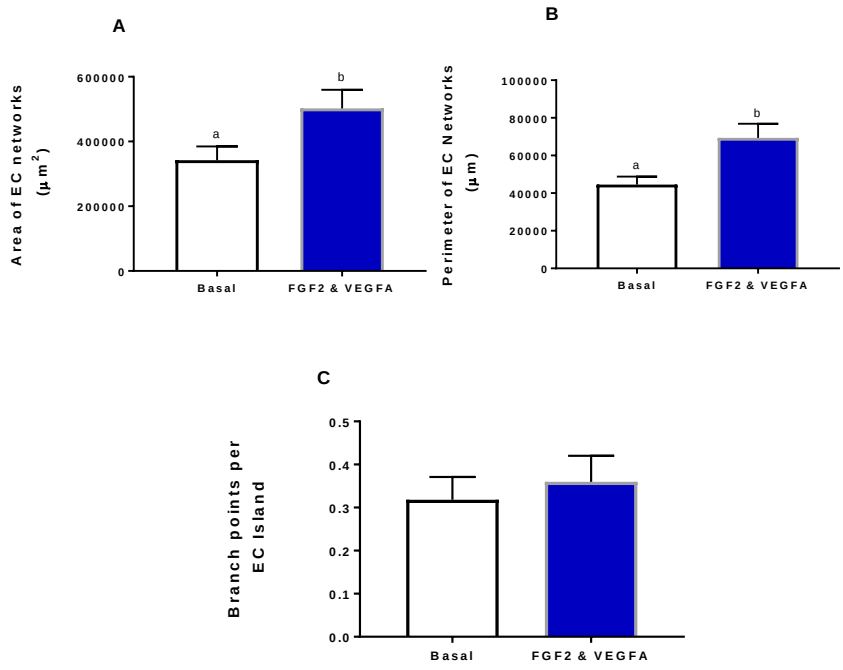


Figure 5.11: Effect of angiogenic factors (FGF2 and VEGFA) on the development of endothelial cell (EC) networks on day 5 of endometrial culture versus basal conditions. (A) Total area of EC networks; (B) Perimeter of EC networks; (C) Branch points per EC network. In the presence of angiogenic factors, both area and perimeter were increased. Significant differences between treatment groups ($p<0.05$) are indicated by different letters; a < b. Values are mean (n= 12 cultures) +SEM.

5.4.4.1 **Effect of stanniocalcin 1 on porcine endometrial angiogenesis**

Endothelial cell networks were observed in all wells to different extents (Fig 5.12 and 5.13). The networks were principally linear in nature, but there were examples where these appeared to have linked together, particularly in the stanniocalcin (STC1) treated wells. STC1 treatment increased EC networks in a dose-independent manner, in both the absence and presence of FGF2 and VEGFA. STC1 (5 and 50 ng/ml) increased total EC network area by 2.2-fold ($p<0.001$) and increased EC network perimeter by 2.3-fold in the absence ($p<0.001$) and 1.8-fold in the presence ($p<0.001$) of FGF2 and VEGFA. STC1 did not affect the degree of branching within an EC network ($p>0.05$) in either the presence or absence of FGF2 and VEGFA (Fig. 5.14).

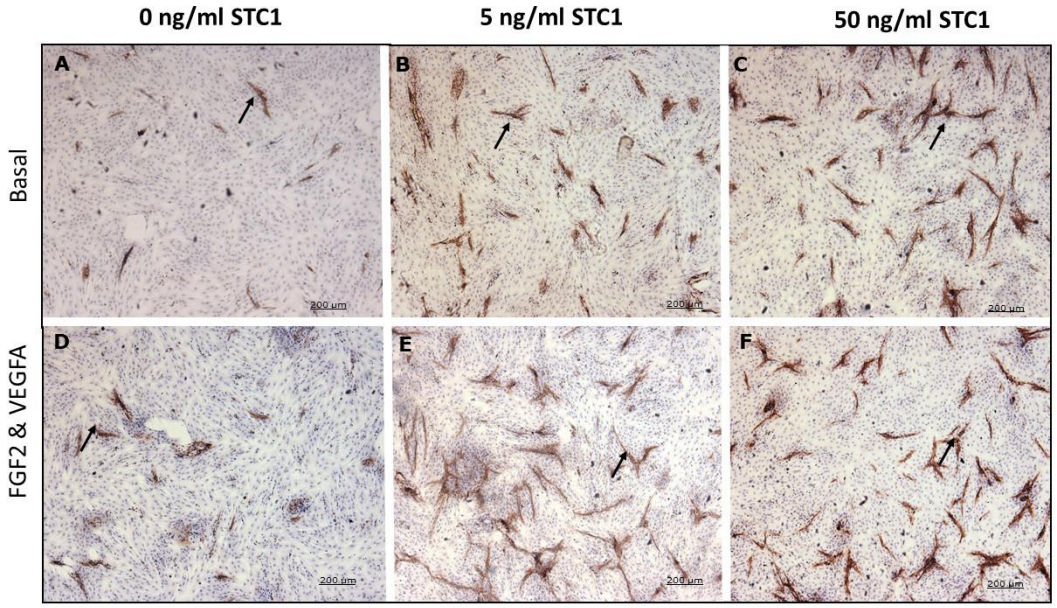


Figure 5.12: Effect of stanniocalcin (STC1) on the development of endothelial cell (EC) networks on day 5 of culture. Cells were cultured under basal conditions (A-C) or angiogenic-stimulated conditions with FGF2 and VEGFA (D-F). STC1 was added at 0ng/ml (A, D), 5ng/ml (B, E) or 50ng/ml (C, F). Endothelial cells formed highly organised networks in the presence of STC1, in both basal and angiogenic-stimulated conditions. Arrows indicate EC networks. Scale bars represent 200µm.

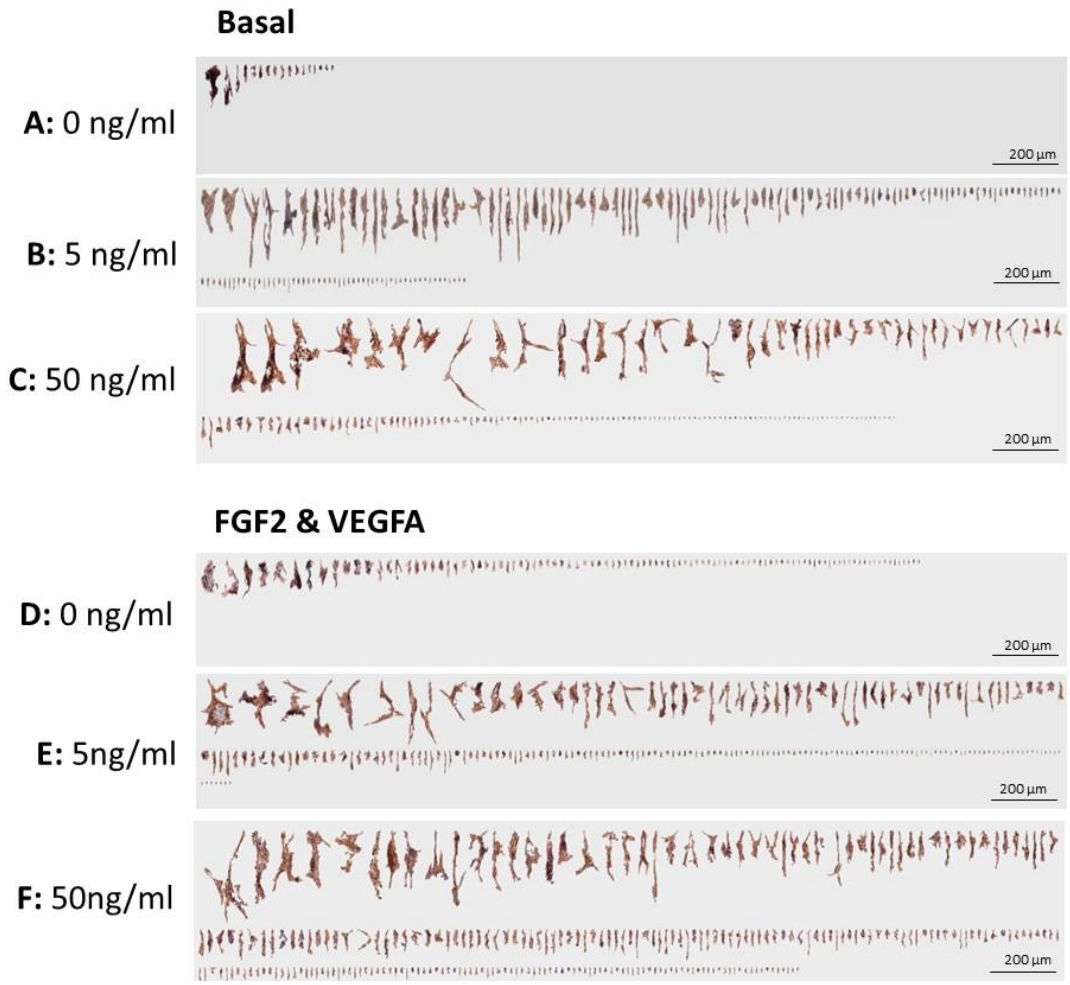


Figure 5.13: Effect of stanniocalcin (STC1) on the development of endothelial cell (EC) networks on day 5 of angiogenesis culture. Sorted by area. Cells were cultured under basal conditions (A-C) or supplemented with FGF2 and VEGFA (D-F). STC1 was added at 0ng/ml (A, D), 5ng/ml (B, E) or 50ng/ml (C, F).

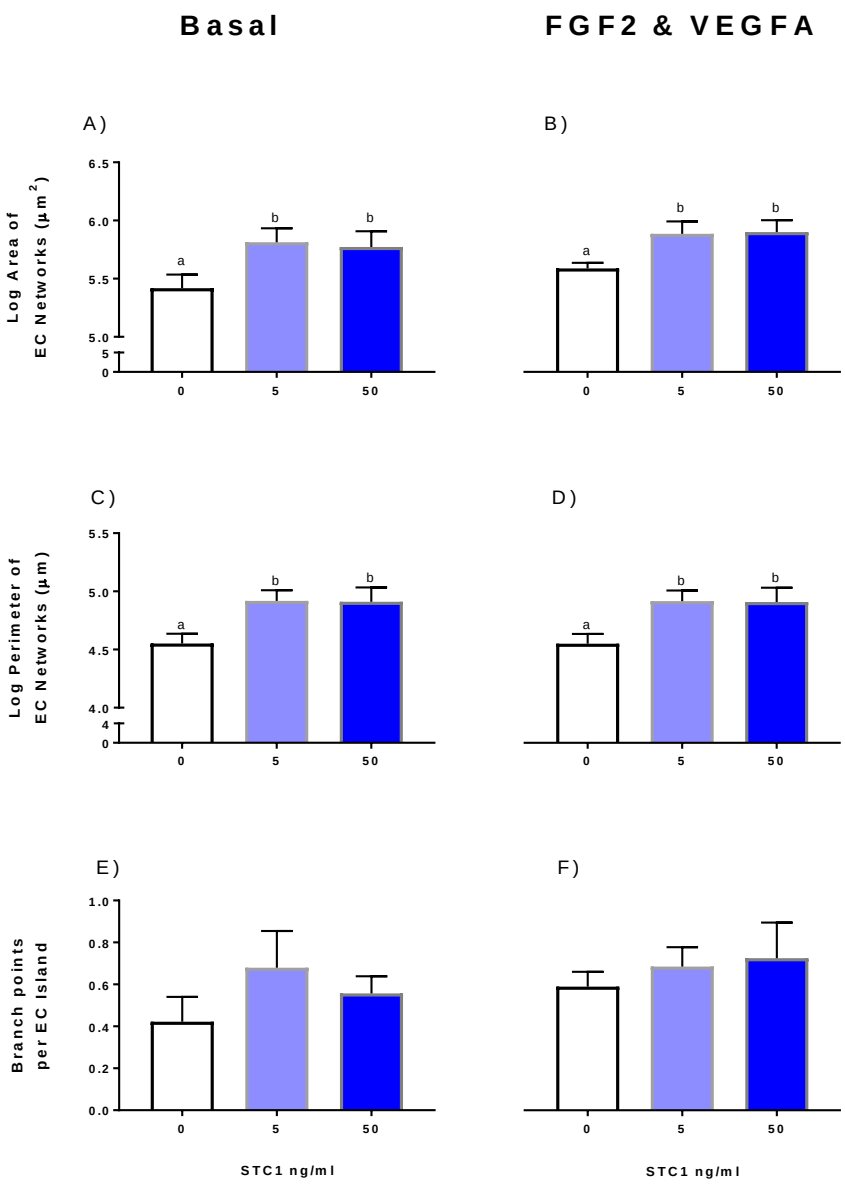


Figure 5.14: Effect of stanniocalcin (STC1) on the development of different endothelial cell (EC) network endpoints on day 5 of culture. Cells were cultured under basal conditions (A, C, E) or supplemented with FGF2 and VEGFA (B, D, F). STC1 was added at 0, 5 or 50ng/ml. The data are mean + SEM from 4 cultures. Significant differences between treatment groups ($p<0.05$) are indicated by different letters; a < b.

5.4.4.2 **The effect of prostaglandin E2 (PGE2) on porcine endometrial angiogenesis**

After 5 days in culture, endothelial cells (EC) networks were observed in all treatment groups (PGE2 0, 10 and 100ng/ml) and in both basal and angiogenic-stimulated conditions (Fig. 5.15 and 5.16).

Treatment with 10ng/ml PGE2 increased the total area of EC networks in the absence of FGF2 and VEGFA ($p<0.05$), but not their presence ($p>0.05$). Conversely, neither 10 nor 100ng/ml PGE2 had any effect on the perimeter of EC networks or the number of branches per EC, irrespective of FGF2 and VEGFA treatment (Fig. 5.17).

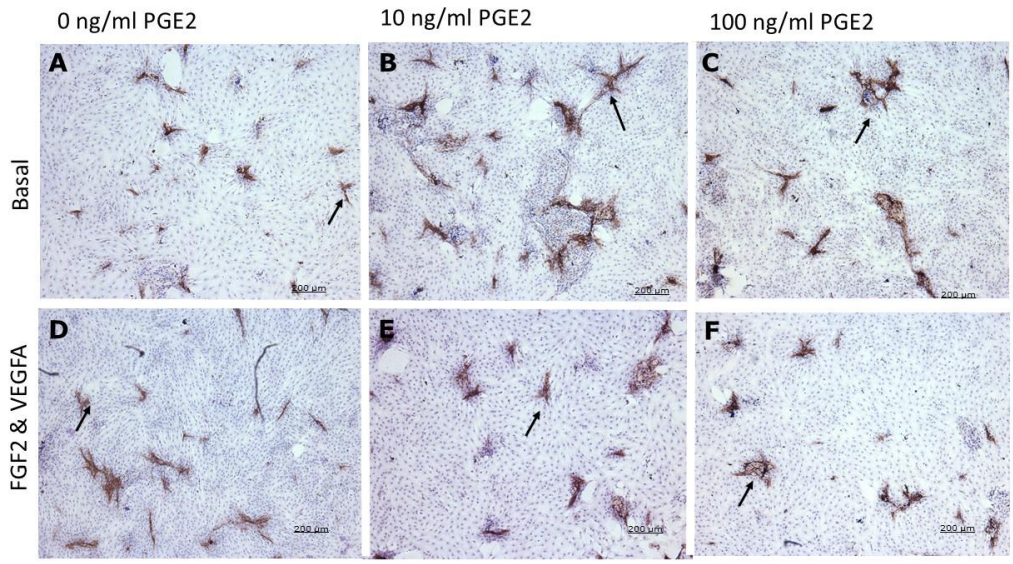


Figure 5.15: Effect of prostaglandin E2 (PGE2) on the development of endothelial cell (EC) networks on day 5 of culture. Cells were cultured under basal conditions (A-C) or supplemented with FGF2 and VEGFA (D-F). PGE2 was added at 0ng/ml (A, D), 10ng/ml (B, E) or 100ng/ml (C, F) Arrows indicate EC networks. Scale bars represent 200µm.

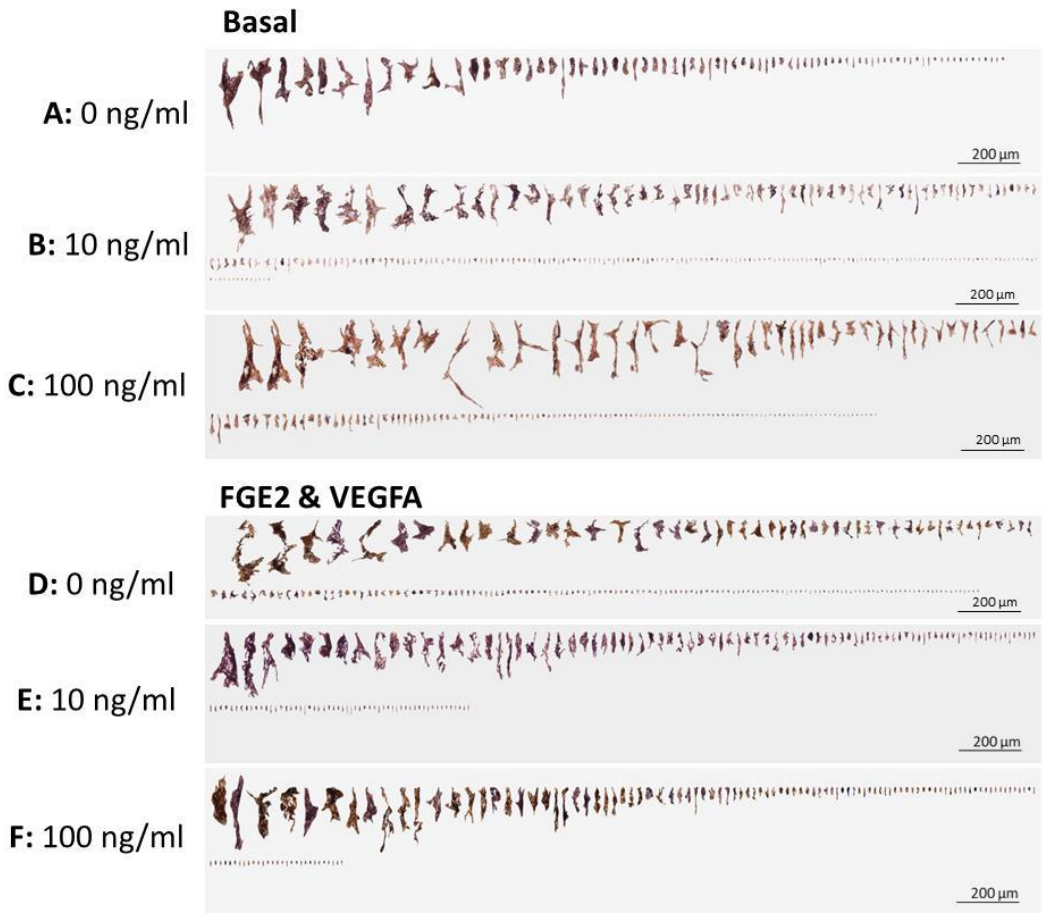


Figure 5.16: Effect of prostaglandin E2 (PGE2) on the development of endothelial cell (EC) networks on day 5 of angiogenesis culture. Sorted by area. Cells were cultured under basal conditions (A-C) or supplemented with FGF2 and VEGFA (D-F). PGE2 was added at 0ng/ml (A, D), 10ng/ml (B, E) or 100ng/ml (C, F).

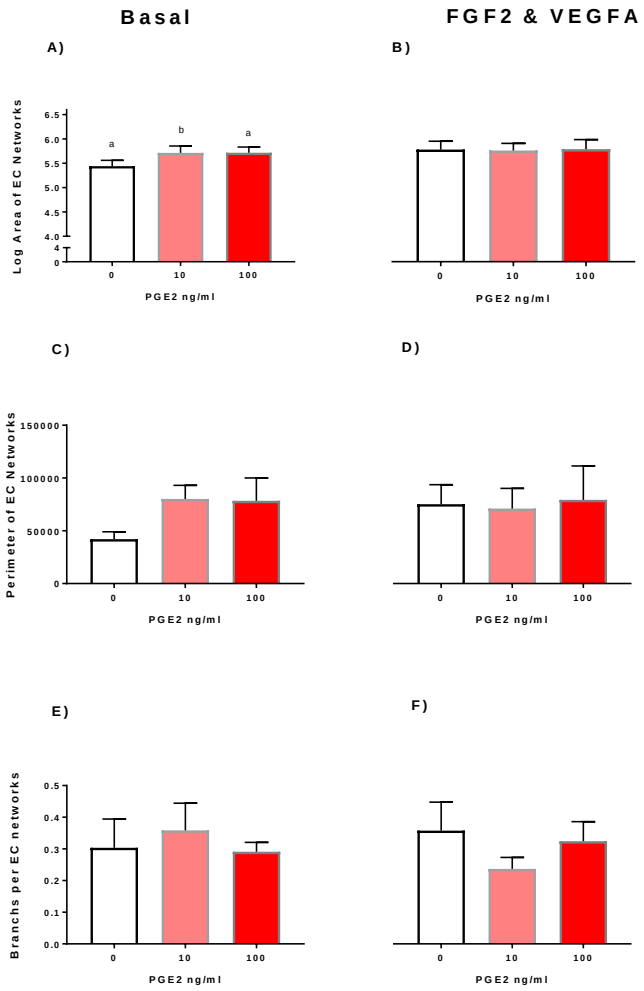


Figure 5.17: Effect of prostaglandin E2 (PGE2) on the development of endothelial cell (EC) network endpoints on day 5 of culture. Cells were cultured under basal conditions (A, C, E) or supplemented with FGF2 and VEGFA (B, D, F). PGE2 was added at 0, 10 or 100ng/ml. Data are mean + SEM from 4 cultures. Significant differences between treatment groups ($p<0.05$) are indicated by different letters; a < b.

5.4.4.3 **The effects of PGF2α on porcine endometrial angiogenesis**

Endometrial endothelial cell (EC) network formation was not significantly altered by treatment with PGF2α (0, 10, 100 ng/ml). Patterns of EC networks appeared visually similar in all treatment groups in both basal and angiogenic conditions (Fig. 5.18 and 5.19).

There were no statistical effects of PGF2α (Fig. 5.20); total area ($p>0.05$), perimeter ($p>0.05$), branch points per endothelial network ($p>0.05$) and number of branch points in culture ($p>0.05$) under basal and angiogenic-stimulated conditions.

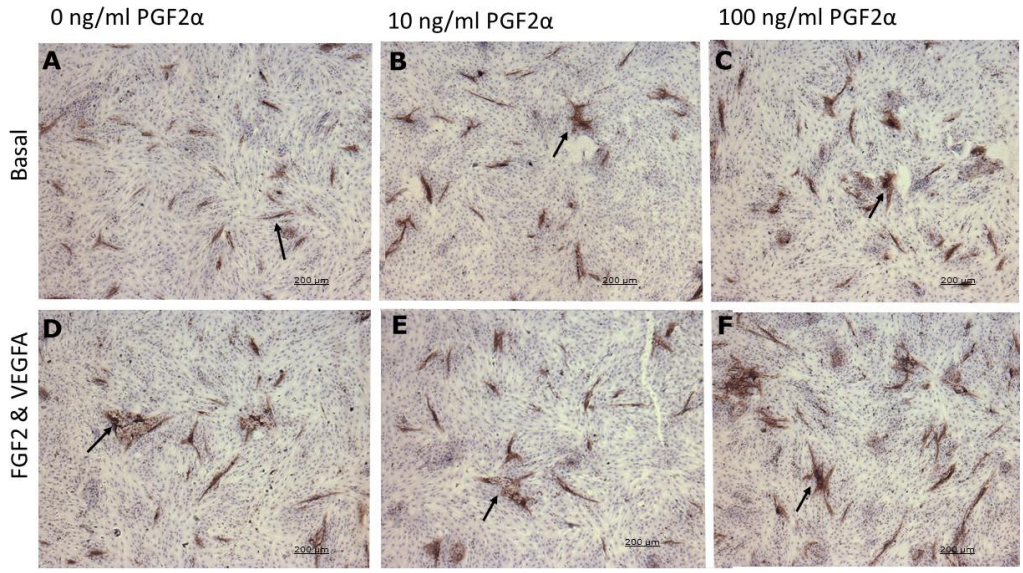


Figure 5.18: Effect of prostaglandin F2α (PGF2α) on the development of endothelial cell (EC) networks on day 5 of culture. Cells were cultured under basal conditions (A-C) or supplemented with FGF2 and VEGFA (D-F). PGF2α was added at 0ng/ml (A, D), 10ng/ml (B, E) or 100ng/ml (G, F). Arrows indicate EC networks. Scale bars represent 200µm.

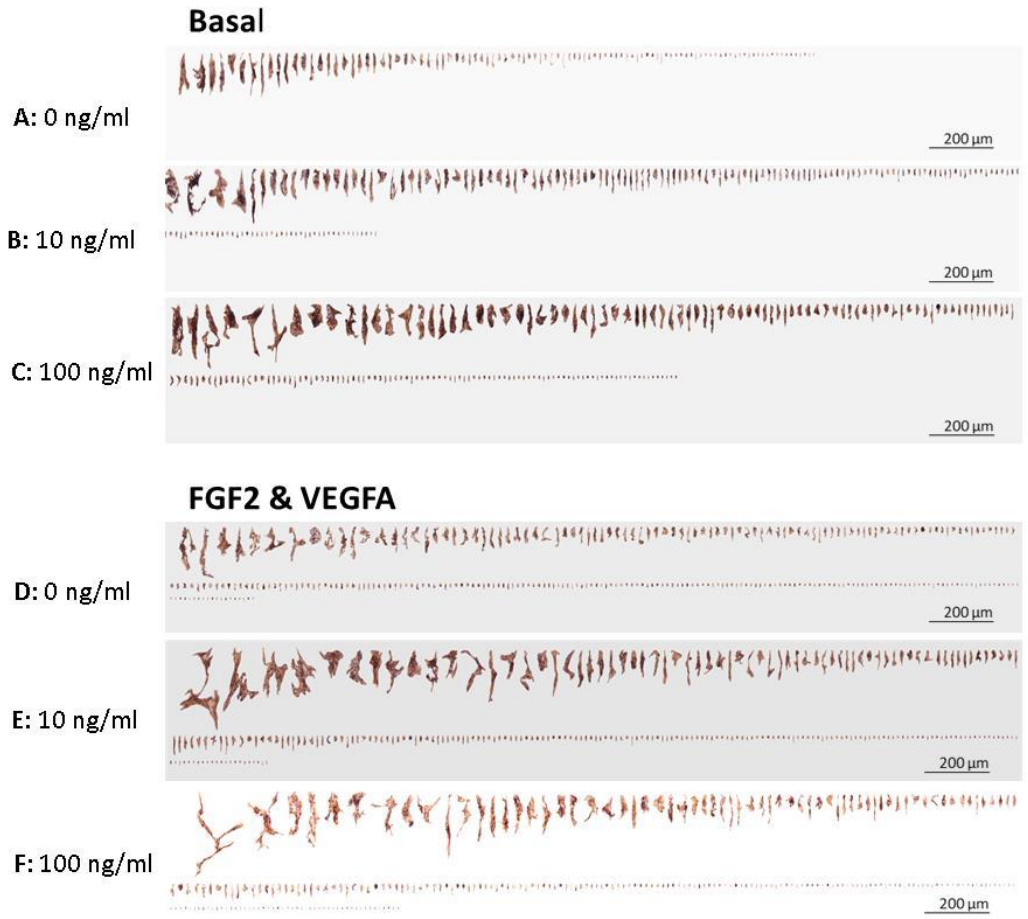


Figure 5.19: Effect of prostaglandin F2α (PGF2α) on the development of endothelial cell (EC) networks sorted by area. Cells were cultured under basal conditions (A-C) or supplemented with FGF2 and VEGFA (D-F). PGF2α was added at 0ng/ml (A, D), 10ng/ml (B, E) or 100ng/ml (C, F).

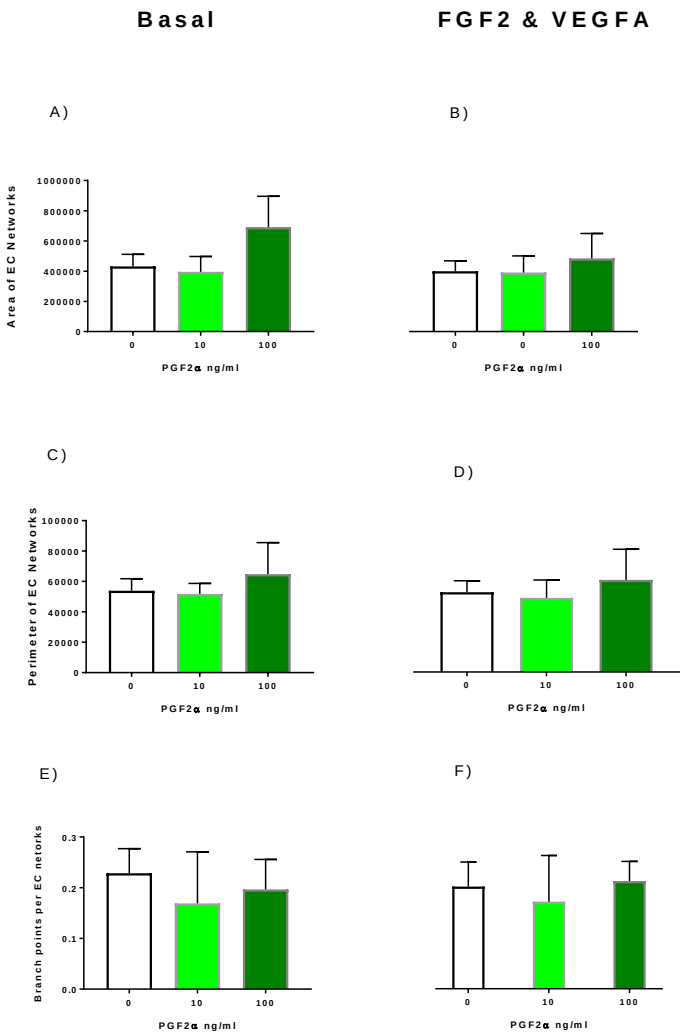


Figure 5.20: Effect of prostaglandin F2α (PGF2α) on the different endothelial cell (EC) network endpoints on day 5 of angiogenesis culture. Cells were cultured under basal conditions (A, C, E) or supplemented with FGF2 and VEGFA (B, D, F). PGF2α was added at 0, 10 or 100ng/ml. Data are mean + SEM (n=4 cultures). No significant treatment effects were detected.

5.4.4.4 **The effects of uterine flushing medium on porcine endometrial angiogenesis**

The addition of bovine serum albumin (BSA) at equivalent concentrations (to uterine flushes treatment) had little effect on endothelial cells (EC) networks parameters. However, under basal conditions only, 20µg/ml BSA increased EC network perimeter versus media alone ($p<0.05$) by 62% (Fig. 5.21). In contrast, the total area of EC network and branch points per network were unaffected by the addition of 20µg/ml BSA, irrespective of FGF2 and VEGFA treatment ($p>0.05$). Equally, BSA at 200µg/ml had no significant effect on EC networks in either basal or angiogenic conditions versus control media (Fig. 5.22).

It was evident visually, that uterine flushing media from pregnancy day 11-12 or 13-14 had marked adverse effects on the ability of endothelial cells to form networks and that occurred under both basal and angiogenic conditions. However, this effect was dose-dependent. Namely, in the initial experiments, the highest protein concentration was used and adding 200µg/ml flushings media from either day 11-12 or 13-14 of pregnancy, completely blocked EC formation under both basal and angiogenic-stimulated conditions (Fig. 5.23 and 5.24). Since no endothelial cell networks were formed in the cells treated with uterine flushings, there was no requirement for image or statistical analysis.

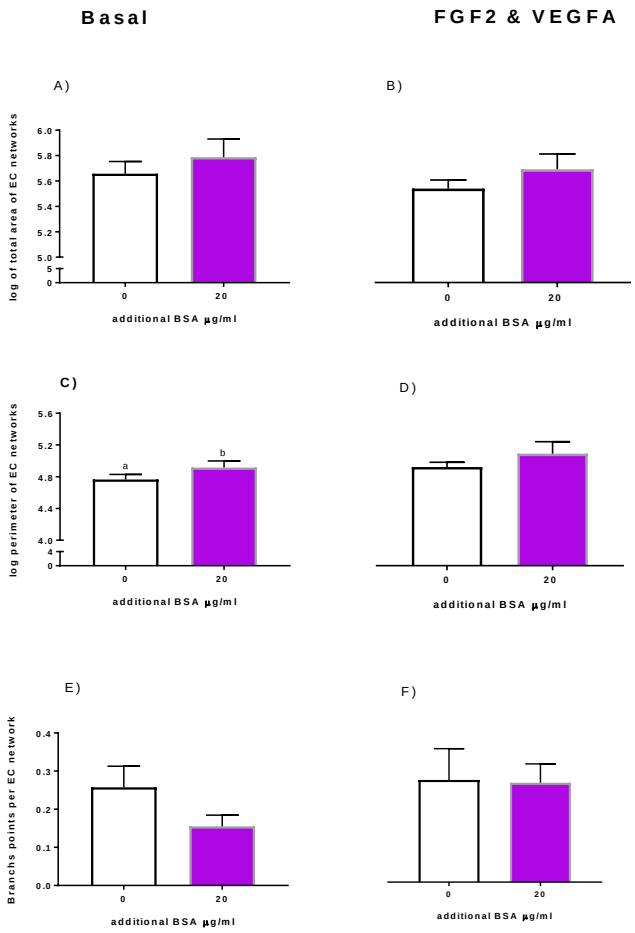


Figure 5.21: Effect of treating cells with additional bovine serum albumin (BSA) on the development of endothelial cell (EC) networks on day 5 of culture. Cells were cultured under basal conditions (A, C, E) or supplemented with FGF2 and VEGFA (B, D, F). BSA was added at 0 or 20µg/ml. Data are mean + SEM from 4 cultures. a<b; p<0.05.

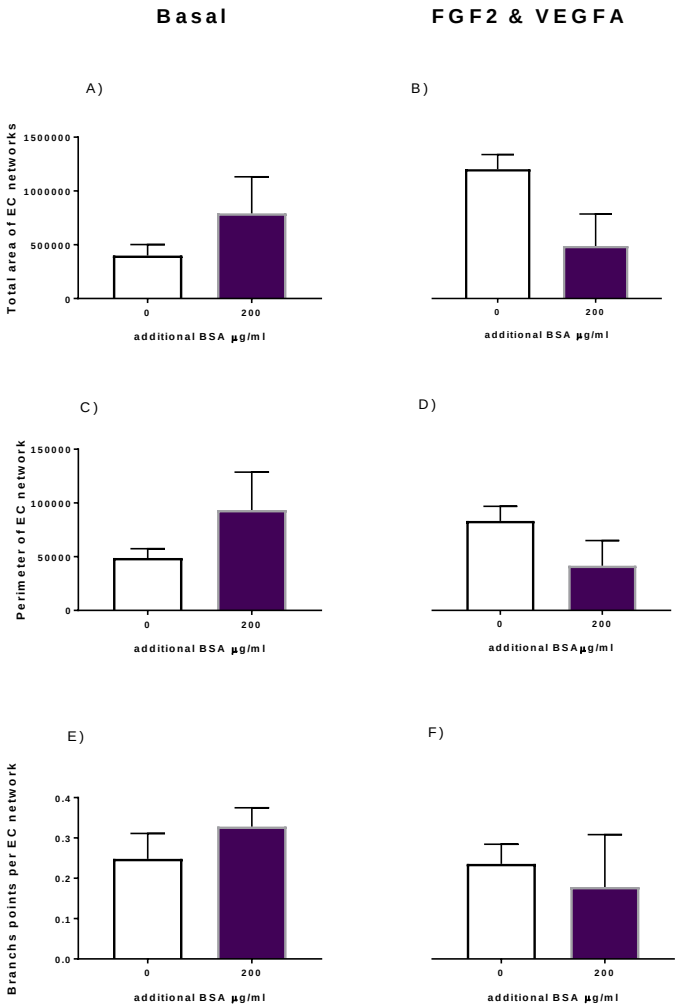


Figure 5.22: Effect of treating endothelial cells (EC) with additional bovine serum albumin (200 µg/ml BSA) on the development of endothelial cell (EC) networks on day 5 of culture. Cells were cultured under basal conditions (A, C, E) or supplemented with FGE2 and VEGFA (B, D, F). Data are mean + SEM from 4 cultures. No significant treatment effects were found.

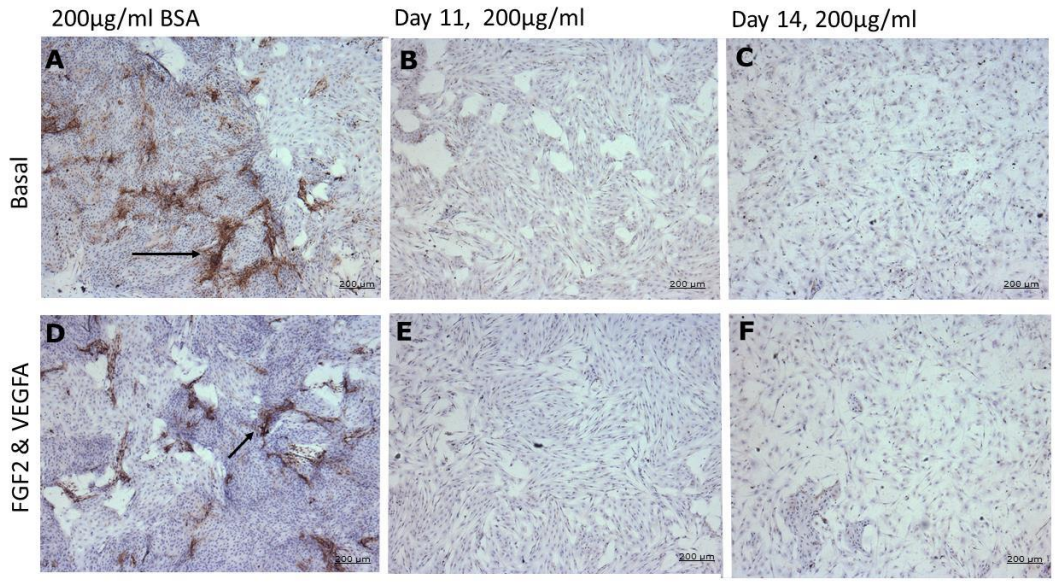


Figure 5.23: Effect of uterine flushing (200µg/ml total protein) on the development of endothelial cell (EC) networks on day 5 of culture. Cells were cultured under basal conditions (A-C) or supplemented with FGF2 and VEGFA (D-F). The control consisted of an additional 200µg/ml of bovine serum albumin (BSA) (A, D); flushing media from day 11-12 of pregnancy (B, E) and flushing media from day 13-14 of pregnancy (C, F). Arrows indicate presence of EC networks. Scale bars represent 200µm.

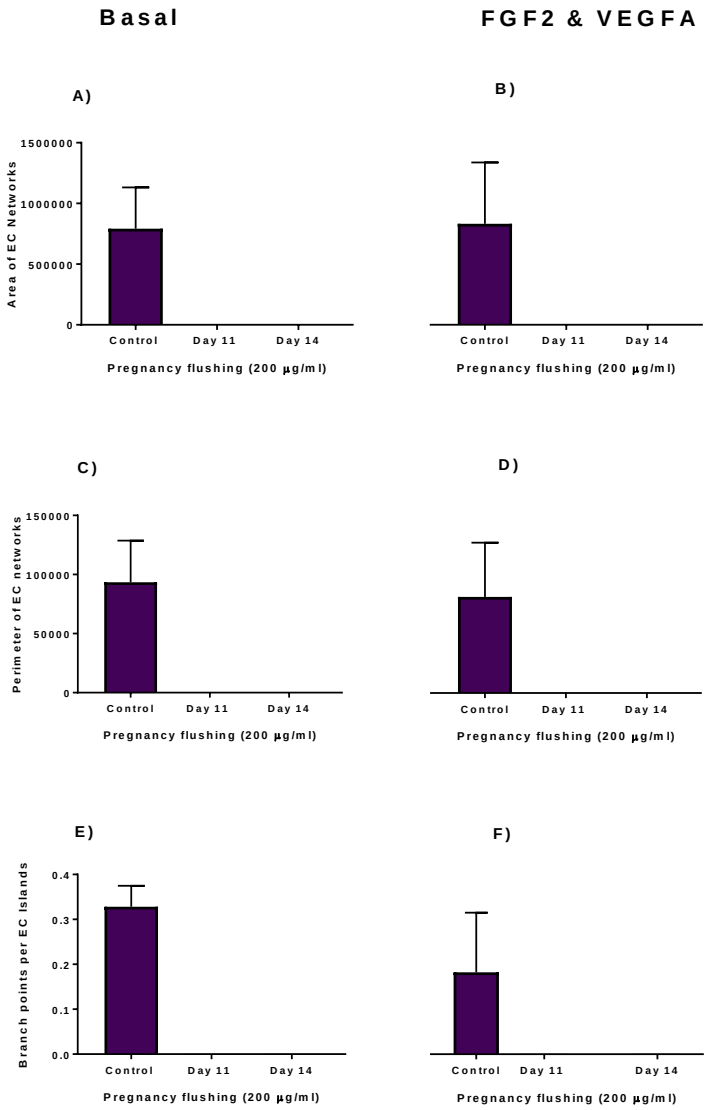


Figure 5.24: Effect of uterine flushing media (total protein 200µg/ml) from different days of pregnancy on the development of endothelial cell (EC) networks endpoints. Cells were cultured under basal conditions (A, C, E) or supplemented with FGF2 and VEGFA (B, D, F). Control media contained additional 200µg/ml of bovine serum albumin (BSA), and flushing media was at equivalent concentration from pregnancy day 11-12 or 13-14. Data are mean + SEM from 3 cultures.

Based on these dramatic results, it was decided to use a lower protein concentration. Endothelial cell networks were observed following treatment with 20µg/ml flushing media from either day of pregnancy (Fig. 5.25 and 5.26). Treatment with day 11-12 pregnant uterine flush did not significantly affect EC networks versus controls ($p>0.05$). In contrast, EC growth was much reduced by uterine flushes from day 13-

14 of pregnancy in the presence and absence of FGF2/VEGFA, compared to 20 µg/ml BSA ($p<0.05$).

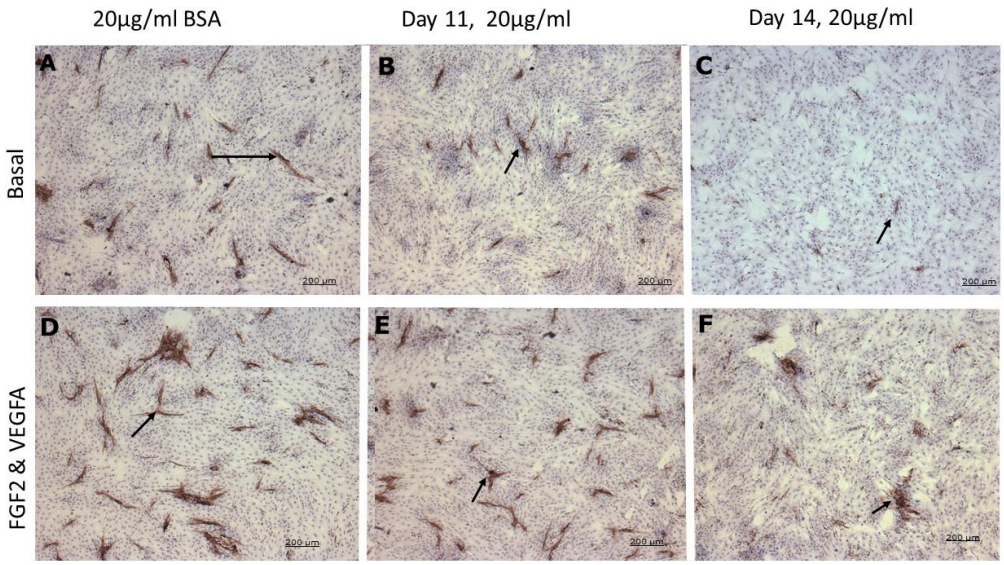


Figure 5.25: Effect of uterine flushing (20µg/ml total protein) on the development of endothelial cell (EC) networks on day 5 of culture. Cells were cultured under basal conditions (A-C) or supplemented with FGF2 and VEGFA (D-F). Control media contained an additional 20µg/ml of bovine serum albumin (BSA) (A, D); flushing media from day 11-12 of pregnancy (B, E) and flushing media from day 13-14 of pregnancy (C, F). Arrows indicate EC networks. Scale bars represent 200µm.

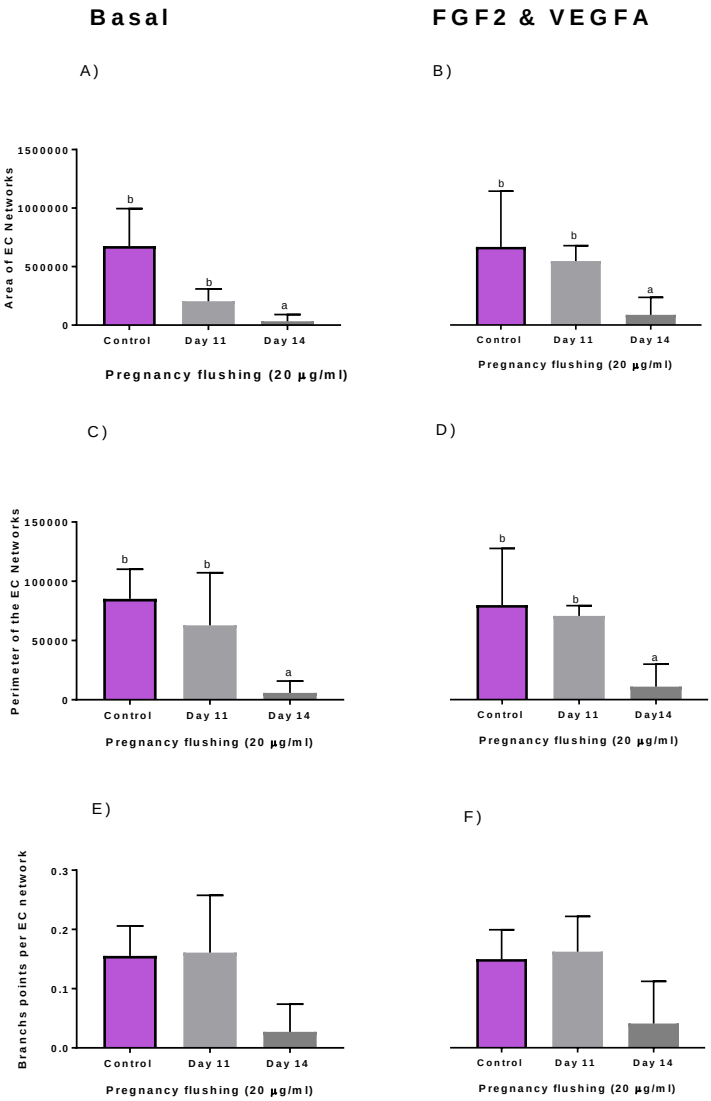


Figure 5.26: Effect of pregnant uterine flushing (20µg/ml total protein) on the development of endothelial cell (EC) network endpoints. Cells were cultured under basal conditions (A, C, E) or supplemented with FGF2 and VEGFA (B, D, F). Control media contained an additional 20µg/ml of bovine serum albumin (BSA), and flushing media was from pregnancy days 11 or 14. Data are mean + SEM. a<b; $p<0.05$, from 3 cultures.

5.4.5 **Discussion**

This study investigated the regulation of endometrial angiogenesis, by factors known to be critical to implantation. The addition of FGF2 and VEGFA increased endometrial endothelial cell network area and perimeter. These factors are considered the principal regulators of normal and abnormal angiogenesis in many tissues (Ferrara et al., 2003).

In the porcine endometrium, FGF2 protein is abundantly expressed during the peri-implantation period (days 10-14), and localised to luminal, stromal and glandular cells (Gupta et al., 1996, Welter et al., 2004). FGF2 expression correlated with the onset of angiogenesis at implantation sites during early placentation in the monkey (Liu et al., 2005). FGF2 was also shown to enhance blastocyst attachment in the mouse (Taniguchi et al., 1998) and increased endometrial receptivity in women (Paiva et al., 2011).

VEGFA increased angiogenesis in the endometrium of rodents (Walter et al., 2005) and primates (Sengupta et al., 2007). While, VEGFR blockade during early murine pregnancy reduced decidual angiogenesis and disrupted pregnancy (Douglas et al., 2009). In the pig, endometrial VEGFA expression correlated with vascularity changes during gestation (Vonnahme et al., 2001) with increased expression around the times of ovulation and early pregnancy. This was suggestive of roles in receptivity and implantation (Kaczmarek et al., 2008).

In the present study, endometrial EC network formation was observed in both the absence and presence of FGF2 and VEGFA. Therefore, whilst the addition of angiogenic factors increased the formation of EC networks, it was not obligatory for

angiogenesis in this culture system. Indeed, the addition of FGF2 and VEGFA only modestly stimulated angiogenesis by 1.46 fold. This may be because the heterogeneous population of endometrial cells endogenously produced pro-angiogenic factors such as FGF2 and / or VEGFA *in vitro.* Supplementation with 1ng/ml FGF2 and VEGFA may have little impact therefore, if high concentrations in the media were already present. In a bovine model of follicular angiogenesis, endogenous VEGFA was abundantly produced, with concentrations ranging from 0.5-26ng/ml (personal communication, Dr RS Robinson 2017).

Several microarray and candidate gene PCR studies have investigated gene expression during the window of implantation (WOI), or at preimplantation period. Stanniocalcin 1 (STC1) was one of the genes that highly expressed in the porcine endometrium (Ostrup et al., 2010, Samborski et al., 2013a, Samborski et al., 2013b, Su et al., 2014, Lin et al., 2015). Indeed, STC1 was highly upregulated at the time of implantation suggesting it has a role in preparing endometrium and establishment of pregnancy but its exact role has not yet been uncovered.

This experiment for the first time has demonstrated that STC1 was a potent stimulator of endometrial endothelial cell networks. Stanniocalcin 1 increased the area and perimeter of endothelial networks by around 2-fold, independent of the addition of exogenous FGF2 and VEGFA. Stanniocalcin 1 has been implicated as a regulator of endothelial cell function (Chakraborty et al., 2007) and is highly expressed during tumour-associated angiogenesis (Gerritsen et al., 2002). Its expression is upregulated by FGF2 and VEGFA in human endothelial cells (Holmes and Zachary, 2008). STC1 promoted gastric cancer endothelial cell tube formation *in vitro* in part by increasing VEGFA expression (He et al., 2011). Thus, STC1 may

have stimulated angiogenesis indirectly by inducing VEGFA expression or activity. Similarly, in human umbilical vein endothelial cells, STC1 overexpression stimulated cell proliferation, migration, tube formation and sprouting, and these pro-angiogenic responses were mediated by upregulation of VEGFA / VEGFR2 expression and signalling (Law and Wong, 2013).

This would indicate a positive feedback loop between VEGFA and STC1 (Law and Wong, 2013). However, in the present study, STC1-induced angiogenesis was not dependent on the presence of exogenous angiogenic factors and was not further enhanced by the addition of FGF2 and VEGFA. In light of the lack of any additive /synergistic response to STC1 in the presence of FGF2 and VEGFA in this system any positive feedback therefore remains equivocal.

The role of stanniocalcin in angiogenesis is further supported by their interactions with other angiogenic factors. STC1 modulated angiogenic responses to hepatocyte growth factor, where it may act to stabilise immature blood vessels (Zlot et al., 2003) and STC2 affected sprouting via angiopoietins (Law and Wong, 2013). The mechanism by which STC1 increased endometrial angiogenesis warrants further investigation i.e. to study the effects of STC1 in the presence of a VEGFR2 inhibitor in order to determine whether STC1 is acting via VEGFA signalling or a different mechanism.

In the current study angiogenic factors (VEGFA and FGF2) alone or with STC1 did not alter the degree of endothelial cell sprouting. This is in contrast to previous reports in other systems (Ferrara, 2000, Gerhardt et al., 2003, Law and Wong, 2013). It is unclear whether this reflects the absence of appropriate stimulation or whether this is an inherent property of the culture system. *In vivo*, angiogenesis in the uterus

occurs by elongation during the proliferative phase while the development of the capillary plexus occurs by intussusception during both proliferative and secretory phases. Immunohistochemistry studies do not show any sprouting mechanism of endothelial cells in human endometrium tissue in contrast the sprouting biomarker (ITGA5/B3) was detected in blood vessels that already exist (Asahara et al., 1999).

Both prostaglandin E2 (PGE2) and prostaglandin F2α (PGF2α) are secreted by the porcine endometrium and conceptus at the time of attachment (Waclawik et al., 2017). The current study showed that 10ng/ml PGE2 increased the total area of endothelial cell networks versus control, but only in the absence of angiogenic factors. That might suggest that PGE2 acts to stimulate EC network formation by acting through FGF2 and/or VEGFA–induced mechanism. Additionally, there were dose-dependent effects of PGE2 on the angiogenesis in endometrium tissue.

In tumours, increased prostaglandin endoperoxide synthase (COX-2) expression and elevated prostaglandin levels have been associated with cancer progression. Furthermore, COX2-derived PGE2 promotes angiogenesis by stimulating the production of angiogenic factors, including VEGFA (Tsujii et al., 1998). A similar pro-angiogenic response has been observed in reproductive tissues. For example, PGE2 stimulated the release of VEGFA by porcine luteal cells (Kowalczyk et al., 2008) and endometrial cells *in vitro* (Kaczmarek et al., 2008).

In contrast, PGF2α did not affect the formation of endothelial cell networks in the current study. This is despite a pro-angiogenic role being previously suggested in reproductive tract cancers, whereby PGF2α increased VEGFA expression and signalling in endometrial adenocarcinoma cells and explants (Sales et al., 2005). Activation of the prostaglandin F2α receptor also increased FGF2 signalling through

FGFR1 in adenocarcinoma cells, leading to endothelial cell network formation (Keightley et al., 2010). In addition, PGF2α also stimulated angiogenic pathways in endometrial cells of women with endometriosis (Rakhila et al., 2015). Previous studies in porcine endometrial explants have also demonstrated PGF2α-stimulated VEGFA expression and the resultant conditioned media induced endometrial endothelial cell proliferation (Kaczynski et al., 2016).

It has been proposed that the autocrine/paracrine effects of PGF2α on angiogenesis are influenced by the effects of conceptus-derived oestradiol, which upregulated endometrial PGF2α receptors (PTGFR) (Kaczynski et al., 2016). That might explain why PGF2α did not influence the angiogenesis in the current study since non-pregnant tissue was utilised. Consequently, PTGFR expression might be reduced and thus PGF2α was unable to exert any effect, also, it might indicate that PGFα action is mediated by conceptus secretion at the time of implantation.

The most unexpected result of this study was the dramatic negative effects of pregnant uterine flushing on endometrial angiogenesis *in vitro*. Endothelial cell network formation was completely absent in the presence of flushing at 200µg/ml protein from pregnancy days 11 or 14. This is likely to mean that the factor(s) responsible were present at both time points. Moreover, when the uterine flush protein concentration was reduced by 10-fold (20 µg/ml) then EC network formation was still decreased by the day 14 pregnant flushing. However, day 11-12 pregnant uterine flushings had no significant effect on EC network formation.

The pig conceptus undergoes considerable morphological change during early pregnancy (days 10-14), developing rapidly from small spherical (2mm) to elongated and filamentous forms (up to 1000mm). Around the time of elongation, blastocyst-

derived oestradiol increases and this coincides with increased uterine secretory activity (Geisert et al., 1982). Uterine secretions are thought to provide an environment that supports embryo growth, particularly in species with non-invasive trophoblast and diffuse placentation, and form an important part of the embryo-maternal interaction (Roberts and Bazer, 1988).

Pregnant uterine flushings contain increased oestradiol, prostaglandins, calcium, fructose, proteins (including uteroferrin and plasminogen activator) and certain amino acids versus non-pregnant flushings (Roberts and Bazer, 1988, Bazer et al., 2015, Steinhauser et al., 2016). In addition, there is increased release of numerous growth factors and cytokines, such as EGF, IGF1, FGF7, VEGFA, IL6, TGFB and LIF (Geisert et al., 2014).

Uterine flushings are therefore a complex mixture of components, including factors with expected pro-angiogenic functions, such as VEGFA. Equally, there is extensive development of the vasculature in the endometrium during early pregnancy. This is in marked contrast to the total absence of endothelial cell network formation observed herein and this was an unexpected outcome. Some anti-angiogenic factors have been identified in the uterine flushed from pigs such as MMP (Lenhart et al., 2002) and interferons (Joyce et al., 2007).

The absence of endothelial cell growth might suggest that either pro-angiogenic factors were not present in the flushings (at collection and / or after preparation) or their pro-angiogenic function was not maintained. Furthermore, pregnant flushings blocked angiogenesis even in the presence of exogenous FGF2 and VEGFA, suggesting that there was an inhibition of angiogenesis rather than just reduced stimulation. The absence of angiogenesis might therefore reflect altered receptor (e.g.

VEGFR) expression or signalling *in vitro* in response to treatment, or increased presence of inhibitors (such as specific binding proteins, e.g. IGFBPs) or anti-angiogenic factors in the uterine flushes (e.g. transforming growth factor beta TGFB and interferon gamma IFNG).

Alternatively, the physical process of flushing may have concentrated or released deleterious factors from the endometrium, which have blocked angiogenesis at the level of the endothelial cell, or via supporting cells such as pericytes. In addition, matrix metalloproteinases (MMPs) can have opposing effect on the angiogenesis depending on the local environment. MMPs are required for degradation of basement membrane of the vessels to remodel the extracellular matrix (ECM) to allow migration of endothelial cells and invasion into the surrounding tissue. However, MMPs contribute more to angiogenesis than just degrading ECM components. MMP1, 3 and 9 increased angiogenesis by assisting in the removal of pericytes from vessels undergoing angiogenesis. However, MMPs can adversely affect angiogenesis by releasing inhibitors of angiogenesis. This is mediated through cleavage of factors such as collagen and plasminogen and by cleaving ligand-binding domains of cell receptors (Rundhaug, 2005). Moreover, MMP2 and 9 were increased in uterine fluid of IVF patients with recurrent implantation failure (Inagaki et al., 2003). Both pregnancy days 11-12 and 13-14 are during the time of peak conceptus oestradiol (Bazer et al., 1986). As mentioned previously (Chapter 3), oestradiol concentration was greater on pregnancy day 11-12 than day 13-14. MMPs are generally thought to be upregulated by oestradiol and downregulated by progesterone in the endometrium. In this experiment, endometrial tissue was collected from non-pregnant animals in the luteal phase while uterine flushing was collected from pregnant animals. This means the progesterone:oestradiol ratio is

different in the cycling luteal phase animal compared to the pregnant animal during preimplantation time. Therefore, it would be interesting to investigate the effect of pregnant uterine flushing on pre-implantation pregnant endometrium in future studies.

Angiogenesis in uterus is a complicated process, which is regulated (in) directly by many factors such as progesterone and oestradiol, also the conceptus and its secretions have an important role in blood vessel formation. In order to investigate this, a novel culture system has been developed and validated. In this system, endothelial cells form networks alongside epithelial gland-like structures. Importantly, EC network formation was promoted by FGF2/VEGFA treatment. According to the results of this chapter, both STC1 and PGE2, to a lesser extent, stimulated angiogenesis in endometrial culture. Contrary to expectations, there was downregulation of angiogenesis in endometrial culture (from non-pregnant sows) by pregnant uterine flushes.

CHAPTER 6 General Discussion

Establishment of pregnancy is the consequence of mutual dialogue between the blastocyst and the receptive endometrium. Although the outline of the communication between endometrial cells and the embryo has been recognised, an understanding of the exact process has not been fully characterised yet. However, an enhanced understanding of the molecular pathways that control uterine-blastocyst interaction has a clinical importance and might introduce further strategies to avoid failure of implantation and increase pregnancy outcome. Thus, the overarching hypothesis of this thesis was that the timing of uterine receptivity is related to the presence of the embryo and the production of steroids by the corpus luteum.

Numerous studies have indicated that, the non-synchronisation between the transfer of the embryo and uterine receptivity is the main cause of implantation failure (Tay and Lenton, 2002, Devoto et al., 2009, Lawrenz and Fatemi, 2018). Therefore, the main themes in this study were to determine the most successful progesterone supplementation regimen, with particular emphasis on the time of supplementation and the route of administration, for luteal phase support in women undergoing ART cycles and to apply an animal model to improve our understanding of the communication between embryo and uterus during the peri-implantation time.

This study has demonstrated that progestogen supplementation human in IVF/ICSI cycles is essential. In addition, this study has demonstrated that the start time of supplementation (oocyte retrieval-embryo transfer) and the route of progestogen (IM is better than vaginal) have an important effect on pregnancy outcome in stimulated cycles. It is clear that the progesterone hormone profile is different between natural and stimulated cycles (Fig.6.1). Successful pregnancy requires synchrony between a

fully functional receptive uterus and a competent embryo at blastocyst stage (Simón et al., 2000). Maximum uterine receptivity (the window of implantation) is a limited period of time that is induced by adequate and timely exposure to oestradiol and progesterone. It lasts around 48 hours and occurs between days 20-24 in a natural cycle and generally, it is a constant period (7-10 days after LH surge or hCG injection in IVF/ICSI cycle) in all women except those with recurrent implantation failure in IVF/ICSI cycles (Lawrenz and Fatemi, 2017). In a natural cycle, a single dominant follicle normally develops and the levels of FSH decline as a result of negative feedback mechanisms between the ovary, hypothalamus and pituitary (Fleming and Jenkins, 2010). While in stimulated cycles, multiple follicles develop in response to treatment with high daily concentrations of gonadotrophins. As long as the daily dosage of gonadotrophin is unchanged, multiple dominant follicles are achieved in stimulated cycles (Kyrou et al., 2012). Consequently, progesterone concentrations increase ranging from 0.8ng/ml in natural cycle to 2.0ng/ml in a stimulated cycle during the late follicular phase (Givens et al., 1994). Thus, uterine receptivity appears more advanced in stimulated cycles than in natural cycles (Fig. 6.1B). Endometrial tissue biopsies at oocyte retrieval of a stimulated cycle show mature endometrial glands combined with decreased expression of progesterone and oestrogen receptors and reduced glandular and stromal proliferation index, compared with the endometrial profile of the natural cycle (Lawrenz and Fatemi, 2017, Lawrenz and Fatemi, 2018). Therefore, progesterone supplementation is used to extend the window of implantation and achieve pregnancy maintenance. Many studies have aimed to determine the implantation window by manipulating the endocrine profile of the pre- and peri-implantation period (Sharma and Kumar, 2012) and correlating

hormonal levels with bimolecular and morphological changes with uterine receptivity.

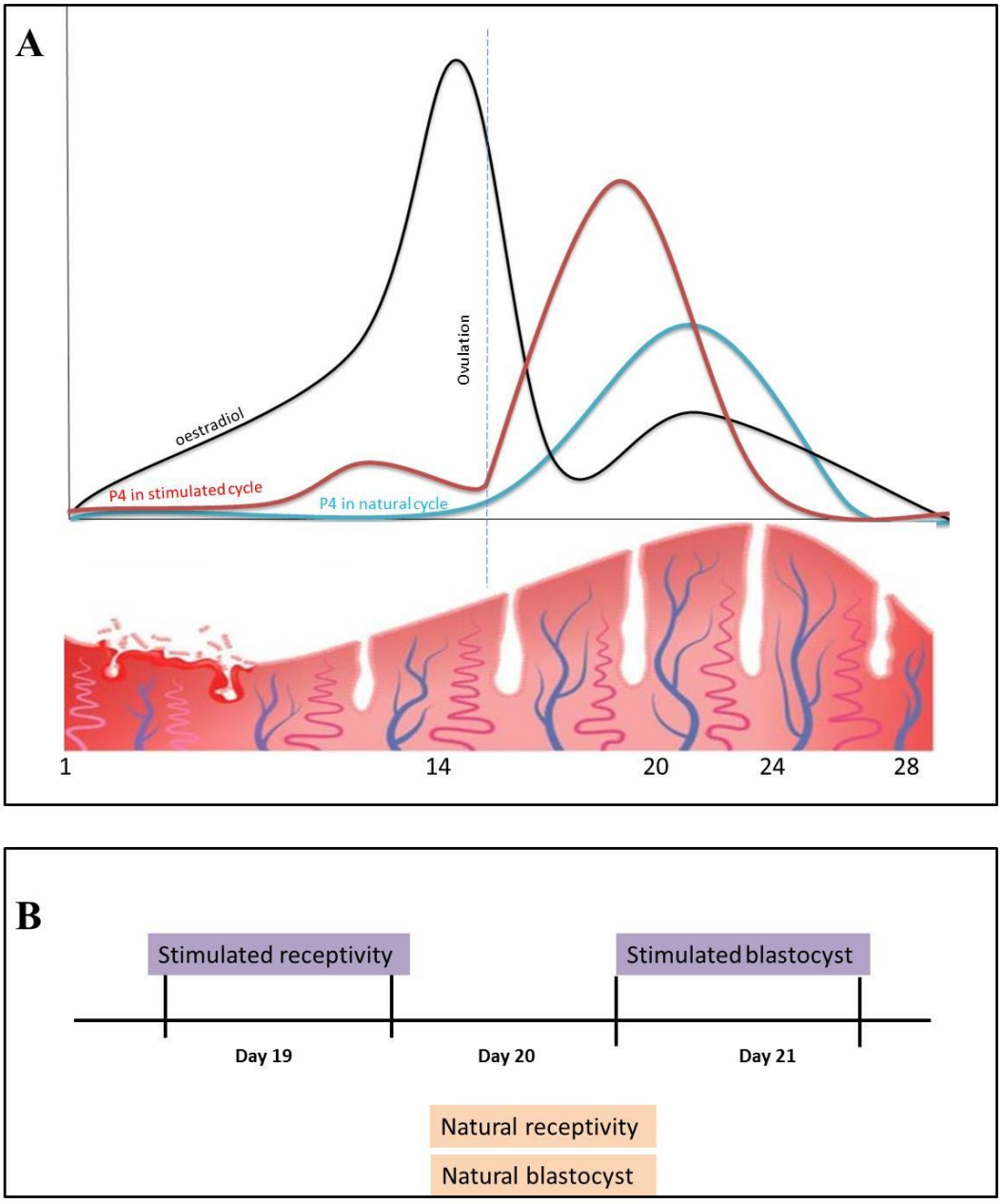


Figure 6.1: Progesterone (P4) and oestradiol profile in a stimulated human cycle. (A) Shows an elevated of progesterone in stimulated cycle and (B) Shows the mis-synchronisation between receptive uterus and blastocyst in stimulated cycle.

In stimulated cycles, several gonadotrophin regimes are used to stimulate follicles (Heger et al., 2012). Elevated oestradiol and progesterone concentration before oocyte retrieval and changes in the ratio of oestradiol to progesterone might result in

irregular endometrial profiles, in terms of growth factors levels and expression of cell adhesion molecules in the endometrium (Macklon and Fauser, 2000). Integrin A5/B3 (Sueoka et al., 1997) and pinopodes (Martel et al., 1991) have been investigated as endometrial receptivity markers. Integrin expression is specifically high in luminal and glandular epithelial cells at optimal uterine receptivity (Lessey et al., 1994). Implantation failures after IVF and embryo transfer are associated with reduced expression of integrin A5/B3 in luminal and glandular epithelial tissue (Tei et al., 2003). Moreover, pinopode formation is affected after ovarian stimulation treatment in IVF/ICSI cycles. In stimulated cycles, the formation of pinopodes is positively associated with progesterone concentration at the end of follicular phase. The quantity and life span of pinopodes was not affected by the ovarian stimulation treatment, while the time of pinopode formation was earlier than in a natural cycle by 1-2 days (Nikas et al., 1999). In natural cycles, there is an innate synchrony between the window of implantation and the blastocyst, while in a stimulated cycle, embryonic development is likely delayed by almost one day. In addition, uterine receptivity is usually advanced in stimulated cycles, therefore the asynchrony between blastocyst and uterus is the main cause for the low pregnancy rate in stimulated cycles (Nikas, 1999).

Progesterone concentration after oocyte retrieval is much higher in stimulated cycles than natural cycles. Progesterone reaches 15ng/ml in mid-luteal phase in natural cycles (Deichert et al., 1986), while in stimulated cycles, when hCG is used for oocyte maturation it reaches around 30-80ng/ml (Humaidan et al., 2010). Then, in a stimulated cycle progesterone concentration starts to decline rapidly day 5 after hCG injection (Connell et al., 2015). Therefore, progesterone supplementation is needed

to cover this premature decline, and the time of starting treatment as we have shown is a critical factor for a successful pregnancy.

Moreover, this study (chapter 2) indicated the preference of the intramuscular route of progestogen compared with the vaginal route, in terms of clinical pregnancy rate. Serum progesterone concentrations are markedly higher after luteal support via the intramuscular route rather than the vaginal route (29 and 4.8 ng/ml, respectively), while the progesterone content in the endometrium was higher by the vaginal route than the intramuscular route (1.05 and 0.43 ng/mg protein, respectively) (Cicinelli et al., 2000). Therefore, the benefit to pregnancy achieved by the intramuscular route might be due to its systemic effects on hypothalamus and because its action more closely mimics endogenous ovarian-derived progesterone. Therefore, this study highly recommends the supplementation of progestogen via the IM route, starting after 1-1.5 days after oocyte retrieval.

It is clear from the current study that the start time is very important to optimise pregnancy rate in women. It was found that starting progestogen supplementation between oocyte retrieval and embryo transfer was optimal. However, given the enhanced and premature progesterone levels in women with stimulated cycles adding additional progestogen at this time seems counter-intuitive. Clearly, the underlying mechanisms require further investigation.

This study used the pig as a model to study the communication between the endometrium and embryo during the peri-implantation time. With the exception of the mode of implantation (non-invasive, central in pigs vs invasive, interstitial) their mechanisms of uterine receptivity are remarkably similar. The study (chapter 3) indicated that the receptivity profile for the sow’s endometrium was similar to that

reported in women. Expression of progesterone receptors in the luminal epithelium was very high in the endometrium from animals on pregnancy day 3-5 then disappeared in the luminal epithelium at pregnancy days 11-14, when progesterone was high in the endometrium tissue. Moreover, integrin A5/B1 was expressed in the endometrial luminal epithelium at days 11-14 of pregnancy (Fig. 6.2).

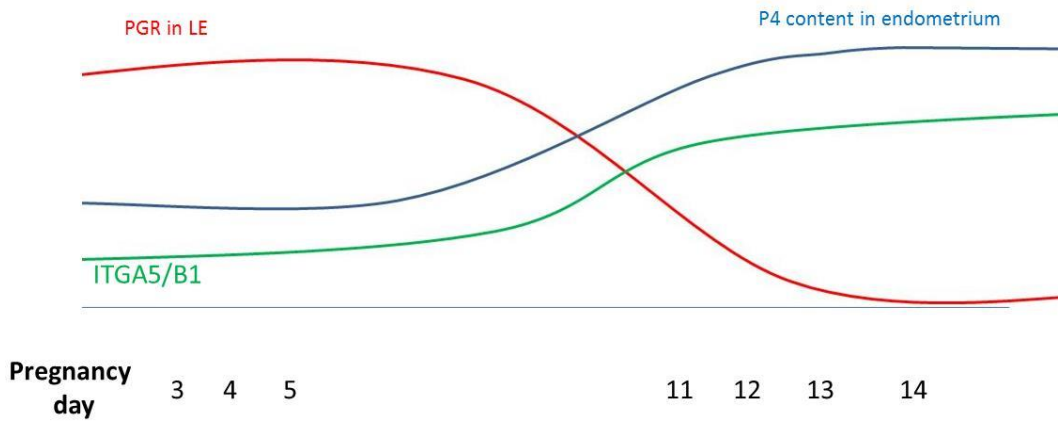


Figure 6.2: Uterine receptivity profile in sows. Progesterone receptors (PGR) were expressed in luminal epithelial cells (LE) at days 3 to 5 of pregnancy then disappeared at days 11 to 14. While, integrin (ITGA5/B1) was expressed at days 11-14 in luminal epithelial cells.

Uterine receptivity is mainly controlled by progesterone and oestradiol, in the pig. Initiation of angiogenesis is considered one of the signs of uterine receptivity and progesterone increased endometrial vascularity in the absence of additional ovarian or placental factors (Bailey et al., 2010). Moreover, progesterone and oestradiol increased angiogenesis in the endometrium by increasing fibroblast growth factor 7 (Ka et al., 2007), vascular endothelial growth factor A (Welter et al., 2003) and fibroblast growth factor 2 expression in the porcine endometrium (Welter et al., 2004). Stanniocalcin 1 is considered as an angiogenic stimulator in tumour and endothelial cell lines (He et al., 2011, Law and Wong, 2013). *STC1* mRNA was expressed in the porcine endometrium and this expression increased dramatically in

pregnancy. Both progesterone and oestrogen increased *STC1* mRNA expression in luminal epithelial cells *in vivo* (Song et al., 2009). However, this study was unable to demonstrate a direct effect of progesterone and oestradiol on STC1 expression in the porcine endometrium *in vitro.* The current study hypothesised that progesterone and/or oestradiol regulate angiogenesis via altering stanniocalcin 1 expression (Chapter 4 and 5). However, there was very limited evidence in the present study to support that progesterone or oestradiol directly regulated STC1 protein expression *in vitro*. *STC1* mRNA and STC1 protein were expressed in the *ex vivo* endometrium at peri-implantation time but STC1 levels were unrelated to progesterone or oestradiol content (Chapter 3 and 4). This is in agreement with previous study on equine which has a similar implantation method (Kikuchi et al., 2011). While *in vivo* study showed that STC1 expression was under steroid hormones (Song et al., 2009). In explant tissue culture, the expression of stanniocalcin 1 was reduced significantly over the time in culture. This would indicate the requirement for an endogenous factor(s) to maintain its expression. Progesterone and oestradiol had no significant effects on the expression of stanniocalcin 1 in endometrium. Stanniocalcin 1 significantly increased endothelial cell network formation in endometrial cell cultures. However, given the potent effect of STC1 at stimulating angiogenesis and its prime localisation to the luminal epithelium and in endometrium tissue in general (Chapter 3), this would indicate that STC1 directs neovascularisation toward the luminal epithelium and hence near to the forming placenta.

It is highly likely the overarching endocrine control of endometrial receptivity is by steroid hormones and the loss of PGR expression in the luminal epithelium is an important trigger. The presence of an embryo(s) is likely to further programme the endometrial receptivity to enable implantation and pregnancy establishment.

However, in the present study, there were limited associations between luteal and endometrial progesterone content and embryo number or oestradiol content in the uterine flushing. The only significant finding was that total luteal progesterone was greater in sows with more embryos present on day 3-5. This might reflect the quality of the pre-ovulatory follicle / oocyte and its ability to develop into a CL / embryo. This association though was not observed at later time-points and might indicate that molecular and cellular peri-attachment changes are more subtly regulated. For example, Rosario and his colleagues (2005) had shown that the embryo, before attachment time, increased endometrial angiogenesis and decidual reaction in the primate endometrium.

Thus it was unexpected and contrary to expectations that the uterine flush media at 200 µg/ml total protein from day 11-12 or 13-14 pregnant sow blocked the formation of endothelial cell networks. When the protein concentration was reduced to 20µg/ml, there was a different response for the day 11-12 and day 13-14 pregnant uterine flushes. This would indicate that there is an "anti"-angiogenic protein released by the porcine embryo and that its production is increased as the embryo develops. The porcine trophoblast produces interferon gamma (IFNG) between days 12 and 20 of pregnancy and is the major interferon produced (Murphy et al., 2009). Intriguingly, it has been shown that IFNG is an angiogenesis regulatory factor in several systems (e.g. Deng et al. (2014) and Galvão et al. (2013)). Thus, it is tempting to speculate that the factor present in the uterine flushes that suppressed endometrial angiogenesis *in vitro* could be by IFNG. Clearly, the next step would be to confirm the presence of IFNG in the flushing and then block its action with a specific receptor inhibitor or IFNG-specific antibody. It has been reported that IFNG expression in the term human placenta was elevated in women who underwent IVF compared with placenta

from natural pregnancies. Indeed, the potential roles of embyrotoxic cytokines (such as IFNG) in embryonic loss in ART women has gathered momentum and certainly warrants further investigation (Robertson et al., 2018). In another way, the expected increase in IFNG in uterine flushes might stimulate a specific mechanism that suppresses angiogenesis during the attachment phase. Moreover, Huang et al. (2002) stated that STAT1 (transcription factor induced by interferons) negatively regulated the angiogenesis and stopped tumour growth and metastasis. Therefore, it can be hypothesised that this is a mechanism by which angiogenesis was suppressed in endometrial cells treated with uterine flushing media.

In summary, this thesis has demonstrated that the highest pregnancy rates in women undergoing ART were achieved when progestogen was supplemented 1-1.5 days after oocyte retrieval with respect to timing and administered intramuscularly with respect to route. This is different to that currently recommended in infertility clinics and emphasises the importance of re-evaluating current practise. Utilising a porcine endometrial receptivity model, STC1 has been identified as a potent pro-angiogenic factor, in fact more efficacious than FGF2/VEGFA. It is likely that inadequate STC1 would affect endometrial receptivity and thus pregnancy establishment.

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APPENDICES

Appendix 1: Database of the articles that used progestogen supplementation in IVF/ICSI cycles with fresh embryo transfer

No	Reference	Year	Region	Route	Duration (weeks)	Time	Days after OoR	E_2	No. of Women	No. of Pregnancy	No of live births	No. of study groups
1	Abate et al 1996	1996	Europe	IM	3-10	OoR-ET	1-1.5	No	40	14	9	2
				Vaginal					40	15	10	
2	Abate et al 1999	1996	Europe	Control	3	OoR-ET	1-1.5	No	43	14	-	2
				IM					43	8		
3	Abate, Perino et al 1999b	1997	Europe	Control	3-10	OoR-ET	1-1.5	No	52	18	-	3
				IM					52	10		
				Vaginal					52	4		
4	Abu-Musa et al 2008	2006	Middle East	Vaginal	3-10	OoR	0	No	62	24	-	2
				Mixed		OoR-ET	1-1.5		63	22		
5	Aghahosseini et al 2011	2008	Middle East	Vaginal	3-10	NA	NA	No	55	6	-	2
									53	7		
6	Aghsa, Rahmanpour et al. 2012	2012	Middle East	Rectal	3-10	OoR	0	No	75	26	-	2
				Vaginal					75	32		
7	Artini et al., 1995	1993	Europe	Control	3	OoR-ET	1-1.5	No	44	4	-	3
				IM					44	6		
				Vaginal					44	7		
8	Bahceci and Ulug, 2008	2006	Middle East	IM(2)	NA	OoR	0	No	912	462	-	4
				Vaginal(2)					1110	512		

No	Reference	Year	Region	Route	Duration (weeks)	Time	Days after OoR	E_2	No. of Women	No. of Pregnancy	No of live births	No. of study groups
9	Baker et al., 2014a	2009	USA	SC	3-10	OoR	0	No	392	167	161	2
				Vaginal					390	181	168	
10	Baruffi et al 2003	2002	South America	Vaginal	NA	OoR	0	No	51	14	-	2
						ET	2-2.5		52	15		
11	Belaisch-Allart et al 1987	1985	Europe	Control	3	OoR	0	No	143	16	-	2
				Oral					136	20		
12	Ben-Nun, I., et al. 1990	1990	Middle East	IM	3	Before OoR	No	No	44	18	-	2
				Control					40	9		
13	Bergh and Lindenberg, 2012	2006	Europe	Vaginal(2)	3	OoR-ET	2-2.5	No	1983	679	580	2
14	Buvat et al., 1990	1988	Europe	Oral(4)	3	OoR-ET	1-1.5	No	161	26	-	4
15	Ceyhan et al., 2008	2006	Middle East	Vaginal	3-10	OoR	0	No	21	13	-	2
								Yes	23	13		
16	Chakravarty et al., 2005	2002	Asia Pacific	Oral	3	ET	No	No	79	19	19	2
				Vaginal					351	80	80	
17	Chantilis et al., 1999	1997	USA	IM	NA	OoR	0	No	99	44	36	2
				Vaginal					92	34	32	
18	Check et al., 1991	1990	USA	IM	3-10	Before OoR	No	No	68	6	1	2
						OoR	0		59	12	1	
19	Costabile et al., 2001	1997	Europe	IM(2)	3-10	OoR	0	No	300	132	-	2
20	dal Prato, Bianchi et al. 2008	2001	Europe	IM	3-10	OoR	0	No	138	45	36	1
				Vaginal					247	87	73	2
21	Damario et al., 1999	1997	USA	IM	3-10	OoR	0	No	227	120	112	2
				Vaginal					44	16	15	
22	Drakakis et al., 2007	2004	Europe	Mixed	3	OoR	0	Yes	39	13	-	2
								No	38	5		

No	Reference	Year	Region	Route	Duration (weeks)	Time	Days after OoR	E_2	No. of Women	No. of Pregnancy	No of live births	No. of study groups
23	Elgindy, El-Haieg et al. 2010	2004	Middle East	IM	3-10	ET	No	Yes(2)	180	74	-	3
								No	90	27		
24	Engmann, DiLuigi et al. 2008a	2006	UK	IM	3-10	OoR-ET	1-1.5	Yes	84	42	-	2
								No	82	52		
25	Engmann, DiLuigi et al. 2008b	2006	UK	IM	3-10	OoR-ET	1-1.5	Yes	30	17	-	2
								No	29	15		
26	Farhi et al., 2000	1997	Middle East	Mixed	NA	OoR-ET	1-1.5	Yes(2)	71	12	-	4
								No(2)	214	69		
27	Fatemi et al., 2006	2004	Europe	Vaginal	3-10	OoR	1-1.5	Yes	92	30	-	2
								No	90	26		
28	Feinberg et al., 2013	2007	USA	Mixed	3-10	ET	No	No	169	94	71	2
				Vaginal					512	240	208	
29	Fanchin et al., 2001	2000	Europe	Vaginal(2)	3	OoR	0	No	43	18	-	2
						ET	2-2.5		41	12		
30	Friedler et al., 1999	1997	Middle East	Oral	3	After ET	0	No	30	10	-	2
				Vaginal					34	16		
31	Fujiwara, 2015	2012	Asia	Vaginal(2)	3-10	OoR-ET	1-1.5	No	90	20	-	2
32	Ganesh et al., 2011	2009	Asia	Oral	3-10	ET	No	No	422	121	-	3
				Vaginal(2)					941	242		
33	Gao et al 2017	2015	Asia	IM(2)	3-10	OoR-ET	1-1.5	No	94	52	43	2
						OoR	0	No	103	53	48	

No	Reference	Year	Region	Route	Duration (weeks)	Time	Days after OoR	E_2	No. of Women	No. of Pregnancy	No of live births	No. of study groups
34	Geber et al. 2007	2001	Middle East	Vaginal(2)	3-10	OoR-ET	1-1.5	No	244	98	-	2
35	Germond M et al 2002	2000	Europe	Vaginal(2)	3-10	OoR	0	No	114	25	24	2
36	Gorkemli et al. 2004	2001	Middle East	Vaginal(2)	3-10	OoR	0	No	188	58	-	2
37	Goudge et al., 2010	2010	USA	IM(2)	3	ET	03-Apr	No	51	32	25	2
					3-10	OoR	0		46	29	24	
38	Gun et al 2016	2013	Middle East	Vaginal(2)	3-10	OoR	0	No	177	43	-	2
39	Haydardedeoglu et al., 2012	2009	Middle East	Vaginal(2)	3-10	After ET	No	No	271	98	-	2
40	Ho, Chen et al., 2008	2008	Asia	IM	NA	OoR-ET	2-2.5	Yes	77	27	-	2
				Vaginal					67	38		
41	Hurd et al. 1996	1992	USA	Control	Control	Control	control	No	35	1	-	2
				Vaginal	3-10	ET	2-2.5		44	8		
42	Iliodromiti et al., 2013a	2011	Multi-centre	Vaginal	3-10	OoR	0	Yes	275	115	-	3
43	Iliodromiti et al., 2013b	2010	UK	Mixed	3-10	OoR	0	Yes	363	109	108	2
				Vaginal	3			No	257	77	75	
44	Ismail et al., 2016	NA	USA	Vaginal	3-10	OoR	0	No	220	91	-	2
45	Iwase et al., 2008	1999	Asia	IM	3	ET	2-2.5	No	20	5	4	2
				Oral					20	4	3	

No	Reference	Year	Region	Route	Duration (weeks)	Time	Days after OoR	E_2	No. of Women	No. of Pregnancy	No of live births	No. of study groups
46	Jabara et al., 2009	NA	USA	IM	NA	OoR	0	No	122	53	38	2
				Vaginal					170	89	63	
47	Kahraman et al., 2010	2010	Middle East	IM	3-10	OoR-ET	1-1.5	No	217	119	-	2
				Vaginal					209	111		
48	Khan et al., 2009	2005	USA	IM	3-10	OoR	0	No	200	203	-	4
				Vaginal(3)		OoR-ET	1-1.5	Yes	40	20		
49	Khrouf et al., 2016	NA	Asia	Rectal	NA	ET	2-2.5	No	58	11	-	3
				Vaginal(2)					128	30		
50	Kleinstein, 2005	1999	Europe	Vaginal(2)	3-10	ET	03-Apr	No	430	102	-	2
51	Kohls et al., 2012	2009	Europe	Vaginal(2)	3-10	OoR	0	No	220	60	-	2
52	Kolibianakis et al., 2005	2003	Europe	Vaginal(2)	3-10	OoR-ET	1-1.5	Yes	92	71	-	2
53	Kuivasaari, et al. 2005	1996	Europe	Vaginal(3)	3-10	OoR-ET	NA	No	347	110	73	3
54	Kupferminc et al., 1990	1988	Middle East	Control	Control	Control	Control	No	51	14	-	2
				Oral	3-10	ET	2-2.5		54	16		
55	Kwon et al., 2013	2013	Asia	Vaginal	3-10	OoR	0	Yes	54	26	-	2
								No	54	20		
56	Leeton et al., 1985	1983	NA	IM(2)	3	ET	NA	No	106	24	-	3
				Control	control	Control	control		80	12		
57	Lewin et al., 1994	1994	Middle East	IM	3	ET	NA	No	50	14	11	2
								Yes	50	13	10	
58	Licciardi et al., 1999	1997	USA	IM	NA	OoR-ET	1-1.5	No	19	11	-	2
				Oral					24	11		

No	Reference	Year	Region	Route	Duration (weeks)	Time	Days after OoR	E_2	No. of Women	No. of Pregnancy	No of live births	No. of study groups
59	Lin et al., 2013	2010	Asia	IM(4)	3	OoR	0	No	167	101	89	4
								No	32	15	14	
								Yes	171	87	77	
								Yes	31	16	11	
60	Lockwood et al. 2014	2009-2010	Europe	SC	3-10	OoR	0	No	319	93	91	2
				Control	Control	Control	Control		321	100	98	
61	Ludwig and Diedrich, 2001a	1999	Europe	Vaginal(2)	NA	OoR-ET	NA	Yes	191	45	-	2
62	Ludwig and Diedrich, 2001b	NA	Europe	Vaginal(2)	3-10	OoR-ET	NA	No	126	31	-	2
63	Lukaszuk et al 2005	2002	Europe	Vaginal	NA	OoR	0	No	78	18	-	1
								Yes	146	62		2
64	Michnova et al 2017	NA	Europe	Vaginal	3-10	OoR	2-2.5	No	53	29	28	2
						OoR-ET			47	25	20	
65	Mitwally, Diamond et al. 2010	2005	USA	Vaginal	3-10	OoR-ET	1-1.5	No	399	210	-	2
				IM					145	71		
66	Mochtar et al 2006	1993-1997	Europe	Vaginal	3	Before-OoR	No	No	130	30	26	3
						OoR	0		127	37	26	
						ET	03-Apr		128	36	27	
67	Moini et al., 2011a	2008	Middle East	Vaginal	3-10	OoR-ET	1-1.5	Yes	47	23	-	2
								No	51	19		
68	Moini et al., 2011b	2008	Middle East	IM(2)	3-10	OoR	0	No	103	31	31	3
				Vaginal					50	17	17	
69	Papaleo, Quaranta et al. 2010	2007	Europe	IM	3-10	OoR	0	No	86	27	21	2
				Vaginal					86	17	11	
70	Papankolaou et al 2011	2006	Europe	Vaginal	3-10	OoR-ET	1-1.5	No	31	4	-	2

No	Reference	Year	Region	Route	Duration (weeks)	Time	Days after OoR	E_2	No. of Women	No. of Pregnancy	No of live births	No. of study groups
71	Perino et al 1997	1994	Europe	IM	3	OoR-ET	1-1.5	No	150	69	66	2
				Vaginal					150	41	33	
72	Pouly et al., 1996	1994	Europe	Oral	3-10	NA	NA	No	144	36	32	2
				Vaginal					139	40	32	
73	Proctor, Hurst et al. 2006	1999	USA	IM	3	OoR	0	No	237	116	-	2
					3-10				121	56		
74	Propst, Hill et al. 2001	1998	USA	IM	3-10	OoR-ET	1-1.5	No	99	48	39	2
				Vaginal					102	31	25	
75	Saharkhiz et al., 2015	2015	NA	Oral	3-10	OoR	0	No	96	30	-	2
76	Salehpour et al., 2013	2012	Middle East	Oral	3-10	OoR	0	No	40	10	-	2
				Vaginal					40	13		
77	Schoolcraft, Hesla et al. 2000	1997	USA	IM	3-10	OoR-ET	22-2.5	No	46	28	23	2
				Vaginal					43	26	23	
78	Serna et al., 2008	2006	Europe	Vaginal	3-10	OoR-ET	1-1.5	No	81	34	-	2
								Yes	79	33		
79	Silverberg, Vaughn et al., 2012	2008	USA	IM	NA	OoR-ET	2-2.5	Yes	302	194	137	2
				Vaginal				Yes	172	122	89	
80	Simunic et al.2007	2004	Europe	Vaginal(2)	3-10	OoR	0	No	266	85	-	2
81	Smitz et al., 1993	1991	Europe	Vaginal	3-10	Before-OoR	No	No	195	57	47	2
								Yes	138	54	44	
82	Smitz et al., 1992	1991	Europe	IM	3-10	Before-OoR	No	Yes	131	35	-	2
				Vaginal				Yes	131	44		
83	Sofuoglu et al., 2015	2013	Middle East	Vaginal(2)	3-10	OoR	0	No	463	103	-	2

No	Reference	Year	Region	Route	Duration (weeks)	Time	Days after OoR	E_2	No. of Women	No. of Pregnancy	No of live births	No. of study groups
84	Sohn et al., 1999	1993	USA	IM	3	Before-OoR	No	No	140	18	-	2
						OoR	0		142	35		
85	Stadtmauer, Silverberg et al. 2013	2008	USA	Vaginal(2)	3-10	OoR-ET	1-1.5	No	1297	617	-	2
86	tomic et al 2015	2012	Europe	Oral	3-10	OoR	0	No	415	117	-	2
				Vaginal					416	126		
87	Tonguc, Var et al. 2011	2008	Middle East	Vaginal	3	OoR	0	Yes(3)	285	99	-	3
88	Tourrnaye et al 2017	2013	Europe	Vaginal	3-10	OoR	0	No	492	195	170	2
				Oral					475	169	142	
89	Unfer et al., 2004a	2001	Europe	Vaginal	3-10	OoR	0	No	361	134	-	2
				IM					373	87		
90	Unfer et al., 2004b	2001	Europe	IM	3-10	OoR	0	Yes	155	61	-	2
								No	129	27		
91	Wang et al., 2009	2004	Asia	Vaginal(3)	3	OoR	0	No	460	237	-	2
92	Williams, Oehninger et al., 2001	2000	USA	Vaginal	3-10	After ET	No	No	67	30	-	2
						ET	03-Apr		59	36		
93	Yanushpolsky, Hurwitz et al., 2008	2001	USA	IM	3-10	OoR-ET	2-2.5	No	110	51	-	2
				Vaginal					105	50		
94	Yanushpolsky, Hurwitz et al., 2010	2001	USA	IM	3-10	OoR-ET	2-2.5	No	201	85	85	2
				Vaginal					206	93	93	

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Appendix 2: Database of the progestogen supplementation articles in which frozen embryo transfer was used.

No	Reference	Year	Region	Route	No. of Women	No. of clinical pregnancy	No. of study groups
1	Alsbjerg, Polyzos et al. 2013	2006	Europe	Vaginal	346	141	2
2	Bjuresten, Landgren et al. 2011	2009	Europe	Control	216	45	2
				Vaginal	219	96	
3	Chang, Han et al. 2011	2007	Asia Pacific	Vaginal	648	248	3
4	Eftekhar, Rahsepar et al. 2013	2011	Middle East	Control	51	14	2
				IM	51	17	
5	Feinberg et al. 2013	2007	USA	Vaginal	98	23	2
				Mixed	96	46	
6	Haddad, Saguan et al. 2007	2003	USA	Vaginal	211	59	2
				IM	199	76	
7	Kim, Lee et al. 2014	2009	Asia Pacific	Control	83	29	2
				Vaginal	145	59	
8	Kyrou, Fatemi et al. 2010	2006	Europe	Vaginal	243	56	2
				Control	209	44	
9	Lelaidier et al. 1992	1990	Europe	Vaginal	122	13	2
10	Lightman, Kol et al. 1999	1997	Middle East	IM	170	27	2
				Vaginal	184	31	
11	Shapiro et al.2014	2010	USA	Vaginal	238	144	2
				IM	682	421	
12	Yu, Ma et al. 2014	2010	Asia Pacific	Vaginal (2)	535	221	2
13	Zarei et al	2017	ME	Oral	100		2

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Appendix 3: Data base of the Progesterone supplementation in intrauterine insemination (IUI)

No	Reference	Year	Region	Formulation of Luteal Phase	Ovarian Stimulation	No. of Women	No. of Pregnancy	No. of Studies
1	Erdem, Erdem et al. 2009	2007	Middle east	CONTROL	FSH	105	25	2
				P4		109	43	
2	Kyrou, Fatemi et al. 2010	2008	Europe	CONTROL	Aromatase inhibitor	196	17	2
				P4		204	19	
3	Montville, Khabbaz et al. 2010	2002	USA	CONTROL	letrozole	31	0	4
				P4		38	8	
				P4	clomiphene citrate	91	11	
				P4		124	19	
4	Maher 2011	2008	Middle east	P4	FSH	136	25	2
				P4		132	39	
5	Miralpeix, González-Comadran et al. 2014	2012	Asia Pacific	P4	clomiphene citrate	188	23	2
				P4		201	36	
6	Yazici, Savas et al. 2013	2011	Middle east	P4	FSH	55	9	2
				P4		55	10	

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