# The role and regulation of angiogenesis during key stages of ovarian follicle development

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# DEDICATION

This Ph.D. thesis is dedicated to the father of the universe, an unending God, God Almighty, Chukwu Okike Abiama, and God of my ancestors for your grace, mercy, strength, power and guidance throughout this Ph.D. study in England, United Kingdom.

In addition, my Ph.D. thesis is dedicated to my late father, Mr Amadi Abraham to the fulfilment of my promise to him in my first year at University when l struggled in my studies after taking care of him in the hospital due to his illness. Your love for education was a great source of inspiration, aspiration and an imprint in me to make you proud as the best l am today.

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# ABSTRACT

Over the past five decades, there has been increasing concern over the decline in fertility of ruminants; a multifactorial problem with severe impacts on farmers' productivity and profitability. Central to reproduction is the adequate and timely function of the ovary. Adequate vascularity underpins many aspects of ovarian function and can be regulated by a range of interacting factors.

Firstly, this thesis investigated the role of maternal gestational protein restriction in regulating the development of blood vessels and germ cells in the sheep fetal ovary. This study modelled the potential effects of inadequate nutrition that dairy cows experience during early lactation at a time when they also become pregnant. Importantly, the number of oocytes is established by birth and any inadequacy could lead to premature ovarian failure.

The growth of the preovulatory follicle and its transition to the corpus luteum requires intense angiogenesis, which is critical to the tissue remodelling associated with luteinisation. This enables progesterone production to increase sufficiently to support a pregnancy. Insulin-like growth factor 1 (IGF1) and IGF2 are known to regulate multiple ovarian process and are key links between reproduction and metabolic status of the animal. Hypoxia regulates angiogenic factors via activation of hypoxia-inducible factor 1A (HIF1A) to establish a new vasculature in pathological situations, whilst the role of HIF1A in the intense endothelial cells (EC) proliferation that occurs during luteal development is poorly understood. Consequently, this thesis examined the regulation of angiogenesis during key stages of ovarian follicle development via the following studies: investigation of the effect of (1) the insulin-like growth factor system and (2) low oxygen concentration on EC network formation and progesterone production in a bovine luteinising follicular angiogenesis culture system.

In the first study, ewes were fed either a control (CP; n=7) or low protein diet (LP; n=8; 17.0 versus 8.7g crude protein) from conception to day 65 of gestation. After slaughter, fetal ovaries were subjected to histological (Haematoxylin and Eosin staining, Periodic-Acid Schiff staining) and immunohistochemical (OCT4, VASA, DAZL, Ki67, Caspase 3, CD31 expression) analysis. Mean fetal weight ($p>0.05$) was unaffected by diet, whilst protein restriction reduced fetal ovary weight ($p<0.05$) at day 65 of

gestation. The percentage area of cortex and germ cell density were unaffected by maternal diet ($p>0.05$), whilst low protein reduced the estimated total weight of germ cells ($p<0.05$), by approximately 30%. There was an abundance of OCT4, DAZL and VASA-positive germ cells in the fetal ovarian cortex, with OCT4 being the most abundantly expressed. There was no effect of maternal diet on the density, estimated weight, total volume, and total number of OCT4, DAZL, and VASA-positive cells ($p>0.05$). Maternal protein restriction did not affect germ cell proliferation or apoptosis or alter the ovarian vasculature ($p>0.05$).

In the second study, bovine luteinising follicular angiogenesis cultures were treated 1) with $LR^3$-IGF1 or IGF2 (10 or 100 ng/ml) in the absence and presence of FGF2+VEGFA (1 ng/ml), or 2) with IGF1 receptor inhibitor (picropodophyllin (PPP); 1 μM) in presence or absence of $LR^3$-IGF1, IGF2, or combined $LR^3$-IGF1+IGF2 (10 ng/ml), or 3) with luteinising hormone (5 or 50 ng/ml) in the presence or absence of $LR^3$-IGF1 (10 ng/ml) for 5 days. EC networks were quantified by von Willebrand factor immunohistochemistry. Progesterone production was analysed by ELISA and cell proliferation was determined by MTT assay. IGF1R expression was assessed in bovine ovarian tissue sections and cultured cells.

$LR^3$-IGF1 and IGF2 had limited effects on EC growth parameters, whilst PPP ($p<0.001$) markedly reduced EC growth parameters (by 60-70%). Cell proliferation was slightly increased (by 3-5%) by $LR^3$-IGF1 ($p<0.001$) and IGF2 ($p<0.05$) treatment. $LR^3$-IGF1 and IGF2 had small and variable effects on progesterone production, whilst PPP reduced progesterone concentration ($p<0.001$) with or without $LR^3$-IGF1 or IGF2 alone or in combination. LH (50 ng/ml) increased IGF1 concentrations ($p<0.001$). IGF1R was expressed in antral follicles, corpora lutea and cultured cells.

In the third study, bovine luteinising follicular angiogenesis cultures were incubated under control (20%) or low (3%) oxygen conditions. Cells were further cultured in the absence or presence of angiogenic-stimulation (FGF2+VEGFA; 1 ng/ml), with or without HIF1A inhibitor (echinomycin; 1 μM), and with or without PKA inhibitor (H89; 10 μM). EC growth parameters were quantified. Progesterone, lactate, and VEGFA concentrations were analysed in culture media by ELISA and cell proliferation determined by MTT assay. Western blotting was performed for expression analysis of steroidogenic proteins (STAR, CYP11A1 and HSD3B1), HIF1A and HIF1A

responsive proteins (PGK1, GLUT1, and BNIP3), and a marker of mural cells (ACTA2).

Low oxygen reduced EC growth parameters ($p<0.001$; by 35%) and progesterone production ($p<0.05$; by 35-55%). HIF1A inhibition via echinomycin treatment also reduced EC growth parameters ($p<0.001$; by 30%) and progesterone production ($p<0.01$; by 35-40%), in both 20% and 3% oxygen. Total cell proliferation, lactate production, ACTA2 expression and VEGFA concentrations were unaffected by oxygen conditions ($p>0.05$) but were decreased by echinomycin (at least $p<0.05$), in both 20% and 3% oxygen. This was particularly marked for VEGFA, where echinomycin reduced expression by 6-fold.

The steroidogenic proteins, HIF1A and HIF1A-responsive proteins and ACTA2 were expressed by cultured follicular cells. Low oxygen reduced STAR expression (32 kDa; $p<0.05$) but did not affect CYP11A1 or HSD3B1 expression ($p>0.05$). Echinomycin reduced HSD3B1 expression in 3% oxygen only ($p<0.05$), but did not affect STAR or CYP11A1 expression ($p>0.05$). HIF1A-responsive BNIP3 expression was reduced ($p<0.01$) and PGK1 tended to be reduced ($p=0.06$) by echinomycin treatment, in both 20% and 3% oxygen. In contrast, GLUT1 was unaffected by echinomycin ($p>0.05$). The reduction of the total EC network area by low oxygen was partially reversed by the addition of H89 ($p<0.05$). H89 reduced the number of branch points and degree of branching ($p<0.05$) but had no effect on the other EC network parameters. In addition, progesterone production was unaffected by H89 ($p>0.05$).

In summary, this thesis has shown the following: 1) maternal protein restriction had limited effect on fetal ovarian development in sheep; 2) Endogenous IGF1 and/or IGF2 production plays an important role in stimulating bovine follicular angiogenesis; 3) Low oxygen reduced progesterone production as expected, but contrary to our hypothesis low oxygen did not stimulate EC growth. Furthermore, many of the responses to HIF1A inhibition were observed at both 20% and 3% oxygen, suggesting that HIF1A may play a critical role in angiogenesis and steroidogenesis that is independent of oxygen environment. The negative effects of low oxygen on EC networks are possibly mediated in part by PKA signalling. In conclusion, this thesis further highlights the complex regulation of bovine follicular angiogenesis.

# PUBLISHED CONFERENCE ABSTRACTS

**Nwachukwu C. U.**, Robinson R. S., & Woad K. J., (2018). Effect of IGF2 on bovine luteinising follicular angiogenesis and progesterone production in vitro. 10th International Ruminant Reproduction Symposium (IRRS 2018). Foz Do Iguacu, Brazil. 16th–20th September 2018. http://www.irrs2018.org.

**Nwachukwu C. U.**, Woad K. J., & Robinson R. S., (2018). Effect of oxygen concentration and HIF1A activity on angiogenesis, progesterone production, proliferation in bovine luteinising follicular cells *in vitro*. Fertility 2018. ACC Liverpool. 4th-6th January 2018. http://www.fertilityconference.org.

**Nwachukwu C. U.**, Woad K. J., & Robinson R. S., (2017). Effect of hypoxia on angiogenesis and progesterone production in bovine luteinising follicular cells in vitro. 4th World Congress of Reproductive Biology (WCRB 2017). Okinawa, Japan. 27th–29th September 2017. http://www.wcrb2017.jp.

**Nwachukwu C. U.**, Woad K. J., & Robinson R. S., (2017). Effect of IGF1 on bovine luteal angiogenesis and progesterone production in vitro. 4th World Congress of Reproductive Biology (WCRB 2017). Okinawa, Japan. 27th–29th September 2017. http://www.wcrb2017.jp.

**Nwachukwu C. U.**, Woad K. J., Gardner D., & Robinson R. S., (2016). Effect of maternal dietary protein restriction on cell proliferation, apoptosis and vascularisation in the sheep fetal ovary at 65 days of gestation. National Ovarian Workshop: Fitzwilliam College, Cambridge, UK. 19th–21st September 2016. https://www.researchgate.net/publication/307577413.

**Nwachukwu C. U.**, Woad K. J., Gardner D., & Robinson R. S., (2016). Maternal dietary protein restriction had no adverse effect on fetal ovarian morphology and germ cell markers on day 65 of gestation in sheep. Society for Reproduction and Fertility Annual conference, Winchester, UK. 11–15th July 2016.
http://www.reproduction-abstracts.org/ra/0003/ra0003o025.htm.

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# Chapter 1: Review of Literature

## 1.1. General Introduction

Fertility in high producing dairy cows has been in decline over the past 30 years and is estimated to cost the United Kingdom (UK) economy £200 million per annum (Royal et al., 2000). Current conception rates in the UK dairy herd are less than 40% compared to first service conception rates of approximately 65% in the 1950's (Butler, 1998). Interestingly, other countries such as the United States of America (USA) (Lucy, 2001), Australia (Macmillan et al., 1996) and Ireland (Roche et al., 2000) have reported similar declines in reproductive efficiency. Thus, poor reproductive efficiency has become a global issue that has a significant negative impact on dairy farmers and the industry. A combination of better nutrition, genetic selection, proper physiology, and improved animal management, has increasingly improved milk yield per cow but has been associated with decreased of reproductive efficiency of high producing cattle (Walsh et al., 2011). This has been further compounded by the shift towards larger herds, which has been associated with reduced oestrous detection.

It is widely recognised that nutrition can affect fertility as well as the health, growth and production of dairy cattle (Kane et al., 2002, Fiske and Adams, 1985, Cordeiro et al., 2015). However, less is known about the effects of maternal nutrition during gestation on fertility and reproduction of the offspring (Thornton, 2010). The reproductive potential of a mammal has its origin in the fetus since the establishment of the ovarian reserve is completed by birth (Findlay et al., 2015). Thus, any stress (metabolic or other) has the potential to adversely affect the ovarian reserve, which could permanently reduce the fertility potential of the offspring. This situation could easily occur in the dairy cow since they will become pregnant at a time when they are producing large quantities of milk and are under metabolic stress. Recent evidence has clearly demonstrated that cattle with lower antral follicle counts (AFC) have decreased follicular and luteal function and most importantly, reduced fertility (Jimenez-Krassel et al., 2009, Sullivan et al., 2009, Mossa et al., 2012). Furthermore, maternal undernutrition has resulted in reduced AFC in the subsequent offspring (Sullivan et al., 2009, Mossa et al., 2013). In addition, the inclusion of fertility, breeding goal and strategic management might be used to prevent further deterioration of reproductive performance (Lucy, 2001, Norman et al., 2009).

A key component for optimal reproductive performance is the appropriate development of the ovarian follicle and its associated maturation of the oocyte (egg). The follicle is also responsible for production of oestradiol, which is in turn influenced by the gonadotrophins, follicle stimulating hormone (FSH) and luteinising hormone (LH). There is growing evidence that the vascularisation of the follicle and corpus luteum (CL) will influence their function. Thus, angiogenesis is a critical process for the establishment of a blood supply to enable folliculogenesis, ovulation and CL development and function (Robinson et al., 2009). For example, as the CL forms, thecal capillary sprouts start to migrate towards and grow into the folds of the stratum granulosum. The growth of new capillaries during luteal angiogenesis follows a series of events including changes in the basement membrane, migration, and proliferation of endothelial cells (EC) and development of capillary lumen (Plendl, 2000). This is controlled by a dramatic increase in the expression of fibroblast growth factor 2 (FGF2) and vascular endothelial growth factor (VEGFA) (Robinson et al., 2008, Laird et al., 2013). The development of this vascular bed is associated with increased luteal blood flow and plasma progesterone concentration (Acosta et al., 2003). However, follicular and luteal angiogenesis could be influenced by the metabolic status of the cow, which might explain poor follicular quality and luteal inadequacy in dairy cows. It is widely recognised that in the early post-partum period, dairy cows will enter a period of negative energy balance which is associated with low insulin-like growth factor (IGF) 1 concentrations. Intriguingly, IGF1 and IGF2 are pro-angiogenic growth factors and thereby influencing follicular and luteal angiogenesis and function.

The classic driver of angiogenesis is the lack of oxygen to that tissue, which leads to the up-regulation of the transcription factor hypoxia-induced factor (HIF) 1A. Then, HIF1A will stimulate the expression of a plethora of genes including VEGFA and other pro-angiogenic factors. This will, in turn, stimulate angiogenesis, the re-vascularisation of the tissue and increased blood and oxygen supply. It is believed that similar events occur during ovulation following the rupture of the follicle and disruption of blood supply to the tissue. However, relatively little is known about the role of low oxygen concentrations and HIF1A in the regulation of angiogenesis during the follicular-luteal transition. There is evidence that the LH surge as well as hypoxia can stimulate the HIF pathway in the follicle (van den Driesche et al., 2008). This thesis addresses the regulation of HIF1A in luteal

angiogenesis. Collectively, these studies will create a deeper understanding of the mechanisms behind subfertility that are essential to develop strategies to reverse this trend in declining fertility.

## 1.2. Ovarian structure and function in ruminants

### 1.2.1. The adult ovarian structure

The female reproductive system is comprised of a pair of ovaries, oviduct, uterus, cervix and vagina. The adult cow has two (almond-shaped) ovaries of 4-6 cm in length located in the abdominal cavity (Hafez and Hafez, 2000) and their size and shape vary with the stage of the reproductive cycle and age. The ovary has two principle functions: 1) production of the steroid hormones, namely oestradiol and progesterone, and 2) supporting oocyte development and its release (Durlinger et al., 2002, Hatzirodos et al., 2014). The ovary is the site of production and periodical release of female gametes at ovulation and is continually undergoing morphological changes such as follicular development and maturation, ovulation and corpus luteum (CL) formation and regression (Fig. 1.1).

The ovarian cortex is the outermost region of the ovary and is comprised of the *zona parenchymatosa* (sparsely vascularised with some larger blood vessels and a poorly defined boundary) and *tunica albuginea* (adjacent to surface epithelium that is sparsely populated with cells and very rich in collagen) (van Wezel and Rodgers, 1996). It is in the *zona parenchymatosa* that numerous follicles at various stages of folliculogenesis and any CL are located (Fig. 1.1). A connective tissue layer, tunica albuginea, lies immediately beneath the ovarian surface epithelium and adjacent to the cortex (König et al., 1988).

The main ovarian artery enters the hilus and divides into different twisted arteries penetrating the medulla of the ovary (the innermost region). This vascularised portion of the ovary is called the *zona vasculosa*, but it also accommodates a large quantity of lymphatic vessels, nerves and connective tissue (Plendl, 2000) (Fig 1.1). There is no clearly defined differentiation between the cortex and the medulla but follicles are not typically observed in the medulla (Plendl, 2000).

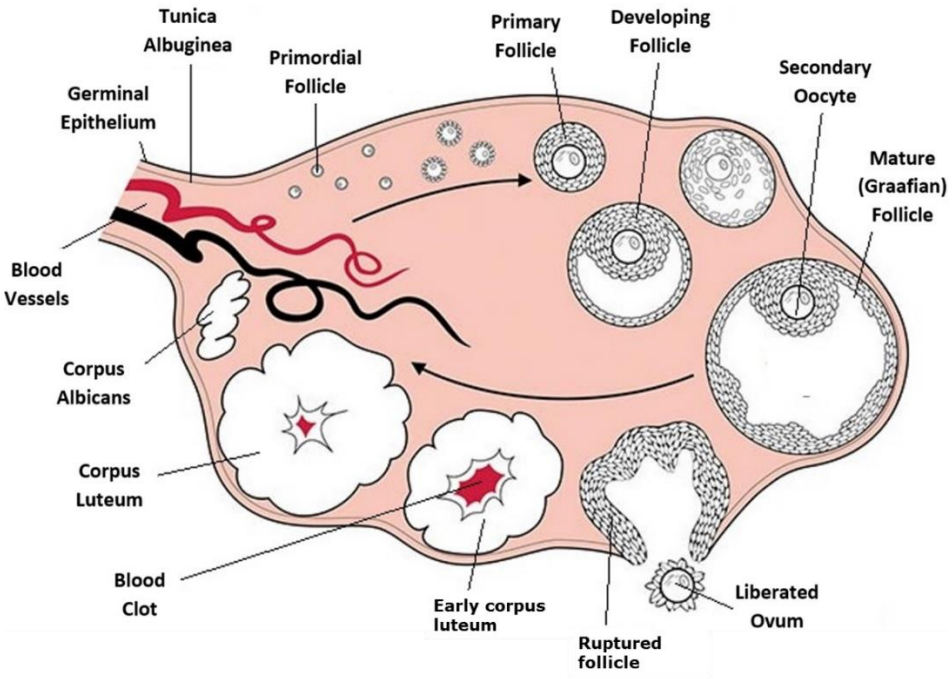



Fig. 1.1: The structure of the adult bovine ovary. The diagram describes the key stages of ovarian follicle and corpus luteum (CL) development in the cow. The ovary consists of an epithelial surface layer with an underlying connective tissue layer or tunica albuginea. The ovarian cortex houses follicles at the different stages of development from primordial to antral (Graafian) stages. The CL is also localised within the cortex region. The ovarian 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 development of the fetal ovary

The first research Chapter investigated the effects of maternal dietary restriction on fetal ovarian development in order to understand how such dietary treatments could affect the subsequent adult ovary and function. Therefore, the next section describes what is known about the formation of the ovary in the fetus.

The key events that take place during fetal ovarian development include: 1) movement of primordial germ cells (PGCs) from the yolk sac to the gonadal ridge; 2) the formation of the gonadal ridge; 3) extensive proliferation of germ cells; 4) migration of germ cells from the mesonephros; 5) formation of ovigerous cords containing the germ cells and pre-granulosa cells; 6) initiation of meiosis and the subsequent formation of primordial follicles; 7) significant loss of germ cells through atresia; and 8) the initial formation of antral follicles by birth (Juengel and Smith, 2014). These key events are described in detail in the following sections, with the focus on sheep and cattle and a comprehensive summary is shown in Table 1.1.

Table 1.1: Time sequence of the key developmental events during ovarian development in sheep and cattle. The information is derived from the following (Juengel et al., 2002, Sawyer et al., 2002, Burkhart et al., 2010, Garverick et al., 2010, Hummitzsch et al., 2013, Juengel and Smith, 2014, Smith et al., 2014).

| | **Day of gestation** | |
|---|---|---|
| **Event in fetal ovarian development** | **Sheep** | **Cattle** |
| Presence of the mesonephros and migration of the germ cells towards the gonadal ridges. | 18–23 | 18-31 |
| Evidence of gonadal ridges, and colonisation by mitotically active primordial germ cells. | 23–24 | 27–32 |
| Fetal ovarian differentiation. | 30 | 35 |
| Gonadal sex differentiation, initiation of steroidogenesis. | 32–35 | 39–45 |
| Evidence of ovigerous cords. | 38–45 | 60 |
| Meiotic initiation in primordial germ cells. | 55 | 75–80 |
| Apoptotic initiation in oogonia and primary oocytes. | 55–60 | 80–90 |
| Maximal number of germ cells in the gonad; first primordial follicles formed. | 75 | 90–100 |
| Majority of germ cells lost by atresia. | 90 | 130–140 |
| First primary follicles observed. | 90–100 | 150 |
| Ovigerous cords come close to surface epithelium. | 100 | 150 |
| Ovigerous cords mostly dissolved and most follicles formed. | 100–110 | 170 |
| Evidence of secondary follicles. Initiation of meiosis in germ cells completed | 120 | 195–200 |
| First small antral follicles observed. | 135 | 250 |
| **BIRTH** | 148–150 | 280-285 |
| Numerous preantral and antral follicles present. | 1–3 months | 1–6 months |

### 1.2.3. Germ cell migration and the formation of the gonadal ridge

The embryonic precursors of the gametes and the founder cells of the germline are called primordial germ cells (PGC) (Donovan and de Miguel, 2003). Around 5-6 weeks of gestation in humans, these cells are set aside to an extra-embryonic location, the yolk sac, and migrate by a combination of morphogenetic and self-propulsive movements along the hindgut to the growing gonads (known as the gonadal ridge) where they stay and proliferate in number (Wilhelm et al., 2007, Maheshwari and Fowler, 2008). In ruminants, the PGC migrate from the endoderm of the yolk sac to the developing hindgut and then extend through dorsal mesentery to the gonadal ridge around day 18–23 in sheep (Ledda et al., 2010) and day 18–31 in cattle (Wrobel and Suss, 1998). The PGCs will continue to proliferate as the migration to the gonadal ridge occurs (Fig. 1.2D). By day 23–24 of gestation in sheep, the coelomic epithelium of the mesonephros begins to thicken and shows the first evidence of the undifferentiated gonad (Juengel et al., 2002, Juengel and Smith, 2014), this occurs a little later around day 27–32 in cattle (Wrobel and Suss, 1998). Around the same time, the process of sexual differentiation occurs, and the gonadal structure develops. Also at this stage, the expression of octamer-binding transcription factor 4 (OCT4, POUF51) is predominant in germ cells but expression will cease when meiosis is initiated (Pesce et al., 1998) (Fig. 1.3).

The final number of germ cells will depend on the balance between the degree to which germ cells undergo mitosis and atresia. These events occur at different time points during fetal ovary development (Wrobel and Suss, 1998, Juengel et al., 2002). Namely, mitosis will predominate in early development, with the germ cells undergoing mitotic divisions with incomplete cytokinesis, producing interconnected oogonia within germ cell nests. As proliferation ceases, the germ cells (now known as oogonia) enlarge and develop into primary oocytes which commence meiosis 1 (around day 55 in sheep) and progress through leptotene, zygotene and pachytene before arresting at the diplotene of prophase 1 (Sawyer et al., 2002). At this stage, the first primary oocyte will have migrated and reached the ovarian medulla boundary (Sawyer et al., 2002). This coincides with the appearance of the first apoptotic germ cell (Reynaud and Driancourt, 2000). Generally, oocytes that not are covered by a single layer of pre-granulosa cells are the ones that are lost through apoptosis. It is believed that this

process will ensure that only the healthiest germ cells with optimal development and capability will remain. Once this stage is completed, the structure is called a primordial follicle. The vast majority of oocytes (>99%) will remain in a quiescent state of meiotic arrest and meiosis will only be reactivated at the time of ovulation (Depalo et al., 2003, Tingen et al., 2009).

In sheep, there is a substantial 13-fold increase in germ cell number between day 40 and 55 of gestation, with numbers estimated to peak around 1 million on day 75. Thereafter, marked germ cell atresia is observed, such that only 15% of those germ cells detected on day 75 are observed on day 90 (150,000) (Smith et al., 1993). On day 100, a second wave of germ cells will undergo apoptosis (Juengel et al., 2002) with the number of germ cells continuing to fall to around 65,000 on day 135, shortly before birth (Smith et al., 1993).

In cattle, similar rates of germ cell proliferation have been shown, with a phenomenal 20-fold increase in germ cell numbers between days 50 and 60 of gestation (Erickson, 1966). The maximal number of germ cells is observed on day 90-100 of gestation with an estimated number of around 2.7 million. Significant numbers of apoptotic germ cells can be observed by day 130-140 and the loss of oocytes continues until day 250 of gestation. By day 150, mitosis of germ cells is no longer observed (Burkhart et al., 2010, Garverick et al., 2010). Indeed, at any one time >25% of germ cells appear apoptotic, resulting in around 68,000 oocytes at birth (Juengel and Smith, 2014).

The molecular characterisation of the developing ovary has been extensively described using markers of the different germline stages and cell types within the ovary (Hummitzsch et al., 2013). These markers include OCT4, DAZL (Deleted in azoospermia) and VASA (DEAD-box) to identify the various germ cells undergoing development (PGC, oogonia, early/primary oocytes); FOXL2 (Forkhead box L2), cytokeratin 18, cytokeratin 19 and COUP-TF11 to detect somatic cells (Gonadal ridge epithelial-like; GREL) cells, granulosa cells, surface epithelium and stromal cells). This is summarised in Table 1.2.

Table 1.2: Molecular characterisation of different cell types in the developing fetal human ovary. The intensity of the expression is indicated as follows: - not expressed, -/+ inconsistently expressed, (+) weakly expressed, + expressed, ++ strongly expressed. GREL cells; gonadal ridge epithelial-like cells. Adapted from (Anderson et al., 2007, Hummitzsch et al., 2013).

| Marker | Primordial germ cells | Oogonia | Early / primary oocyte | Primary oocyte (in follicle) | GREL cells | Granulosa cells | Surface epithelium | Stromal cells |
|---|---|---|---|---|---|---|---|---|
| **OCT4** | ++ | -/+ | - | - | - | - | - | - |
| **DAZL** | - | ++ | + | - | - | - | - | - |
| **VASA** | - | -/+ | + | + | - | - | - | - |
| **FOXL2** | - | - | - | - | + | + | -/+ | (+) |
| **Cytokeratin 18** | - | - | - | - | +/++ | + | ++ | - |
| **Cytokeratin 19** | - | - | - | - | -/+++ | + | ++ | - |
| **COUP-TF11** | - | - | - | - | - | - | - | + |

### 1.2.4. Migration of cells from the mesonephros

The mesonephros is a transient, complex immature tissue that is formed during fetal development and contributes to both reproductive and renal structures. It contains many different cells types including stromal cells, endothelial cells (ECs) and epithelial cells related to the nephrons (Sawyer et al., 2002, Garverick et al., 2010, Burkhart et al., 2010). Originally, mesonephric cells were thought to be the source for the pre-granulosa cells of the primordial follicle (Sawyer et al., 2002, Hummitzsch et al., 2013, Burkhart et al., 2010, Garverick et al., 2010). On day 25 in the sheep, mesenchymal cells are observed moving into the developing ovary, closely followed by mesangial cells and then the epithelial cells. There is no distinct pattern of migration into the ovary but the mesangial and epithelial cells form a more organised structure by day 40 (Zamboni et al., 1979, Byskov, 1986). Cell migration from the mesonephros to the ovary appears to occur in a similar manner in sheep and cattle (Burkhart et al., 2010, Garverick et al., 2010). These migrating cell streams extend into the innermost part of the ovarian cortex, even into the medulla and are enclosed within the basement membrane (Fig. 1.2A&B). For example, the mesonephros was noticeable by day 45 with cell streams present in the ovarian medulla region by day 90. The flow of cells from the mesonephros continues until day 105 in sheep (Sawyer et al., 2002). The basement membrane that encloses the original mesonephros dissolves and the gonadal ridge is formed (Fig. 1.2C&D). Interestingly, steroidogenesis-related proteins, *CYP11A1, CYP17A1, CYP19A1* and *STAR* are first expressed between day 40 and 75 of gestation (Byskov and Lintern-Moore, 1973, McNatty et al., 2000).

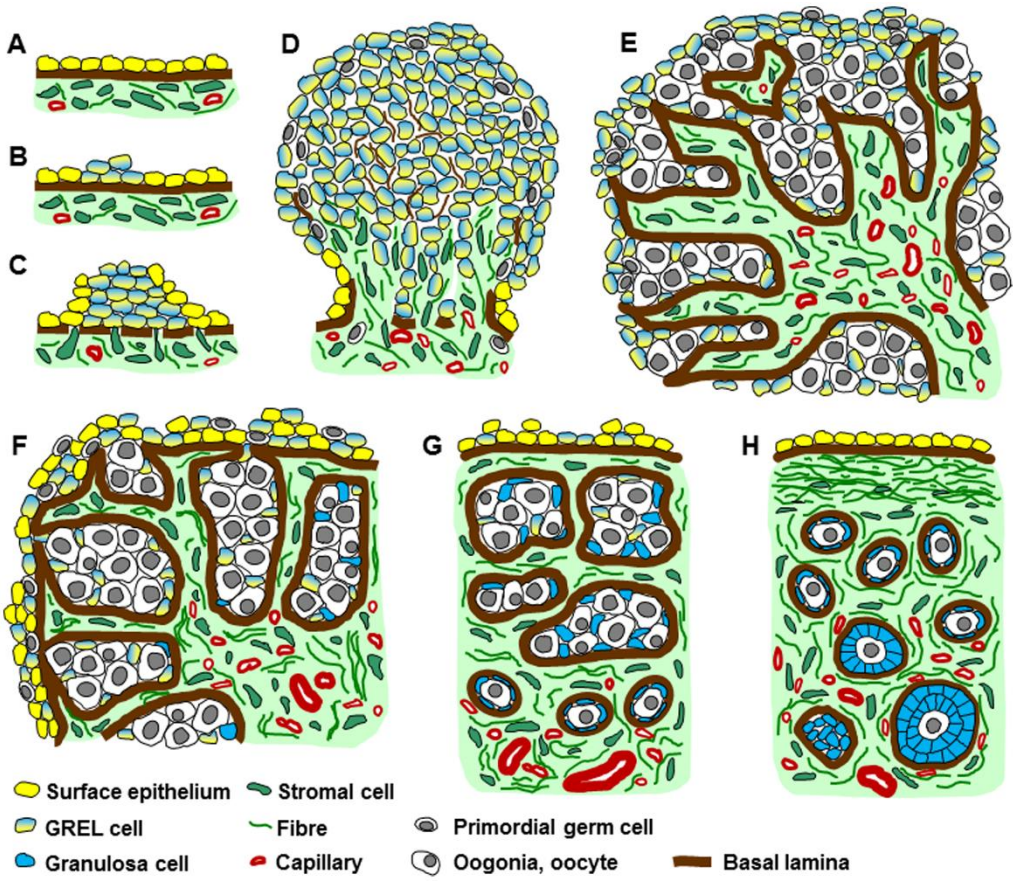



Fig. 1.2: The key events during fetal ovarian development. The fetal ovary begins to develop (A) at the site of the future gonadal ridge, (B-C) cells of the mesonephric surface epithelium begin to proliferate and differentiate, (D) proliferation continues and germ cells migrate in, (E-F) germ cells proliferate and ovigerous cords form around them, (G) primordial follicle formation begins, and (H) some primordial follicles commence development Adapted from (Hummitzsch et al., 2013, Smith et al., 2014).

## 1.2.5. Ovigerous cord formation

Ovigerous cords are irregular-shaped structures within the fetal ovary that contain the germ cells and their associated somatic pre-granulosa cells (Fig. 1.2F&G). Ovigerous cords are also called germ cell (egg) nests and display a tortuous pattern when the ovary is serially sectioned (Sawyer et al., 2002). More so, the ovigerous cords are tubular in structure and remain avascular.

On day 38 of gestation in the sheep, Sawyer et al., (2002) identified five different cell types in the fetal ovary: 1) Germ cells; 2) Mesothelial (ovarian surface epithelial); 3) Pre-granulosa (follicular); 4) ECs as part of the blood vessels; 5) Mesenchymal (stromal).

The developmental origin of pre-granulosa cells has been the subject of much debate. Namely, the stromal cells, epithelial cells and cells of the mesonephros have all been proposed to be the source of pre-granulosa cells (Byskov, 1986). In sheep, pre-granulosa cells were thought to be derived from the ovarian surface epithelium (Sawyer et al., 2002). However, recent evidence using detailed cell tracking in the bovine fetal ovary, has suggested rather that ovarian surface epithelial and granulosa cells share a common origin, termed gonadal ridge epithelial-like (GREL) cells. The origin of these GREL cells are from the gonadal ridge of the surface epithelium of the mesonephros (Fig. 1.2D&E).

By day 38 in the sheep fetal ovary, the association of germ cells within the ovigerous cords begins. Deposits of basal lamina are observed, which extend to become a continuous membrane, with the cords initially open to the surface. The distinct separation of ovigerous cords from the surrounding ovarian stroma occurs by day 45 in sheep and by day 75 in cattle (Garverick et al., 2010). The ovigerous cords start to disintegrate from around day 75 in sheep, as oocytes and their associated somatic cells separate from the ovigerous cord and start to form primordial follicles. At this point the oocyte is surrounded by flattened granulosa cells and a basal lamina (Sawyer et al., 2002).

The ovigerous cord maintains its basal lamina structural integrity until day 75 and 90 in sheep and cattle, however, the germ cells/oogonia continue to proliferate from the area near the ovarian surface epithelium. The germ cells then move from the cortex towards the medulla. The interface between the cortex and medulla is where primordial follicles appear first which happens from day 90 of gestation (Rae et al., 2001). Follicular formation is initiated at the medullary end of the ovigerous cords, thus forming a gradient of follicular development that extends from the ovarian surface. By day 90 in sheep, a distinct basal lamina under the ovarian surface epithelial cells separates the ovigerous cords from the ovarian surface. A small group of germ cells that do not participate in follicular formation have been found within the ovarian medulla and are known as medullary germ cells. These cells are associated with the rete cells and walls of blood vessels with no clear role or function (Sawyer et al., 2002).

There are several important aspects of follicular development in the fetus that remain to be elucidated: 1) the origin of pre-granulosa cells that become the somatic component of primordial follicles; 2) morphological changes that

follow the simultaneous regression of ovigerous cords, and 3) primordial follicle appearance. It is likely that adverse stresses on these events will have an impact on the number and quality of the oocytes/follicles formed. Moreover, how, when, and what exactly regulates these processes is still unclear.

## 1.2.6. Follicle formation in the fetus

The first primordial follicle is formed at the base of the ovigerous cords furthest from the ovarian surface and is observed on day 75 in sheep (Sawyer et al., 2002), and in the cow around day 90–100 (McNatty et al., 2000, Sawyer et al., 2002, Burkhart et al., 2010, Hummitzsch et al., 2013). During the formation of the follicle, increasing amounts of the basal lamina are synthesised and is henceforth discrete from the ovigerous cords. These processes appear to be regulated in part by ovarian stromal cells, which may act to segment the cord, resulting in small clusters of cells which go on to form follicles (Hummitzsch et al., 2013).

At this time, the size of the oocyte is around 20 to 50 µm while follicle size is approximately 30 to 65 µm. The number of granulosa cells within these follicles (classified as type 1/1a) ranges from 4 to 5, with the number of granulosa cells indicating the degree of developmental progress (Lundy et al., 1999). The increased number of granulosa cells associated with each oocyte increases the secretion of basal lamina leading to cord dissolution and follicle formation (Sawyer et al., 2002).

### 1.2.6.1. Molecular characterisation of primordial follicle formation

The gradient that is created during the formation of primordial follicles in ovigerous cords is accompanied by changes in the expression of germ cell maturation markers (Fig 1.3). Specifically, a maturing germ cell will progress from expressing OCT4 initially, to DAZL, and then to VASA [in mouse this is known as mouse vasa homolog, MVH] (Hummitzsch et al., 2015).

The transcription factor OCT4 is a critical mediator of the induction and maintenance of pluripotency in stem cells. OCT4 is highly expressed in early embryos, blastocysts and embryonic stem cells. In gonadal development, OCT4 is expressed by primordial germ cells (Fig. 1.3) within the gonadal ridge (Rosner et al., 1990, Guo et al., 2012) and is required for their survival (Kehler et al., 2004).

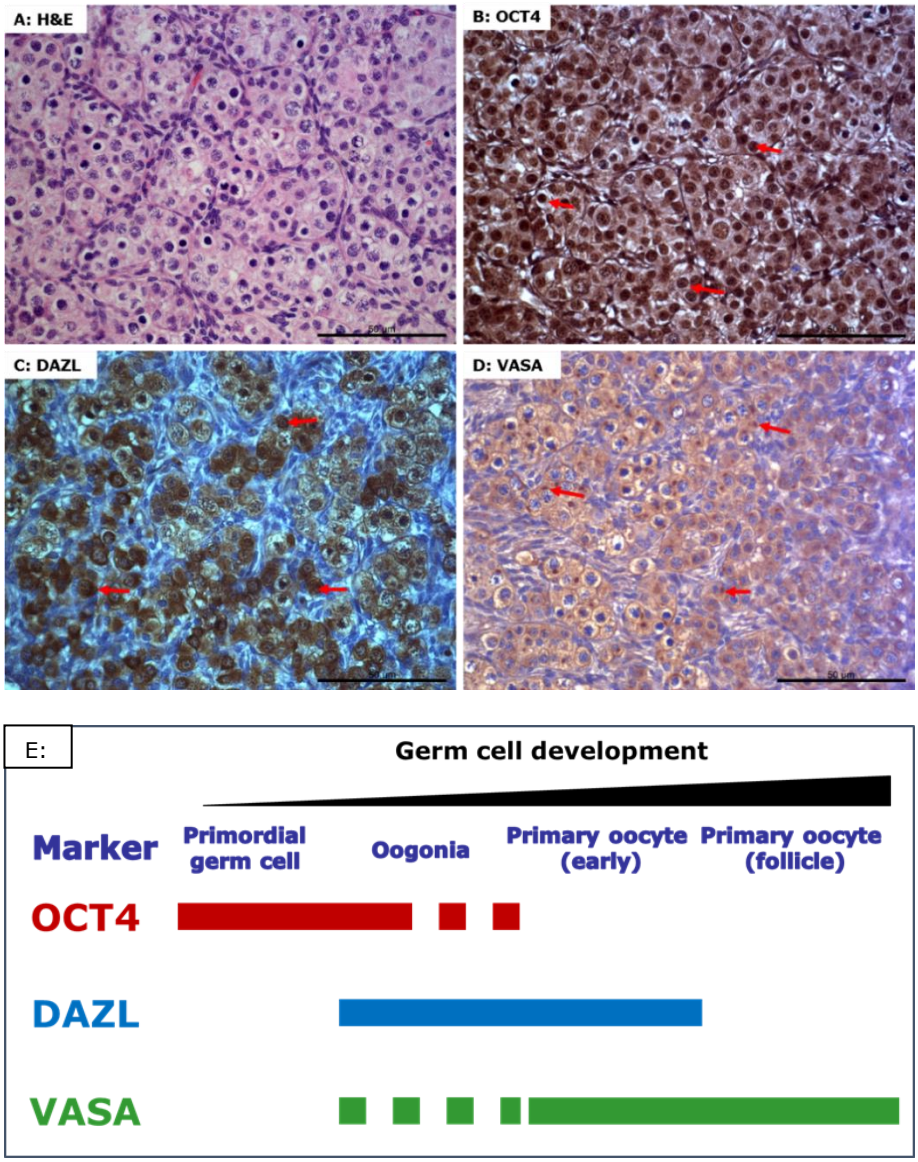



Fig. 1.3: Localization of germ cell markers in the sheep fetal ovary at day 65 of gestation. (A) Haematoxylin and eosin staining, (B) OCT4, (C) DAZL and (D) VASA immunostaining. The red arrows highlight the brown staining, indicating OCT4, DAZL and VASA-positive cells. (E) Schematic showing germ cell marker expression during fetal ovarian development. The images were taken from the Chapter 3 of this PhD thesis, while the schematic diagram was adapted from (Hummitzsch et al., 2013).

In the human fetal ovary, nuclear OCT4 expression was observed in most first trimester germ cells, while OCT4-positive germ cells in the second trimester were predominantly located in the cortical region of the ovary. This is where the most immature germ cells/oogonia are located. Moving towards the medulla, maturation progressed; with OCT4 expression much less frequent (Stoop et al., 2005, Anderson et al., 2007). OCT4 expression is

repressed after the oocyte enters meiotic prophase (Pesce et al., 1998) and is then re-expressed postnatally in growing oocytes (Sánchez and Smitz, 2012).

The DAZL gene family encodes three homologous germ cell-associated RNA-binding proteins (BOLL [BOULE], DAZL, and DAZ) that are critical for gametogenesis and oogenesis in different animal species (Zhang et al., 2008, Ruggiu et al., 1997). DAZL expression has been reported in mouse and human granulosa and theca cells albeit at lower levels (Ruggiu et al., 1997, Nishi et al., 1999, Pan et al., 2002).

In mouse fetal gonads, DAZL was detected from embryonic day 11.5 in post-migrating germ cells (Hackett et al., 2012) and in females DAZL expression increased at embryonic day 13.5 coinciding with the onset of meiosis (Seligman and Page, 1998). In the human fetal ovary, a similar increase in *DAZL* mRNA and protein was observed between 9–14 weeks of gestation (Ruggiu and Cooke, 2000, Anderson et al., 2007, He et al., 2013). During this period, DAZL underwent dynamic redistribution from the germ cell nucleus (RNA processing and storage) to the germ cell cytoplasm (Anderson et al., 2007, Reynolds and Cooke, 2005). DAZL expression is subsequently down-regulated during meiosis, but it is then upregulated in oocytes of newly formed primordial follicles (He et al., 2013). Functionally, DAZL is a key determinant of germ cell maturation, survival, development, and meiotic progression in rodents, human and other animal species.

Deletion or loss of DAZL expression leads to infertility (Ruggiu et al., 1997, Schrans-Stassen et al., 2001). It results in a reduction in post-migratory germ cell numbers (Haston et al., 2009), increased apoptosis of the post-migratory germ cells (Lin and Page, 2005), reduced expression of germ cell markers (Ruggiu et al., 1997) and delayed entry of oocytes into meiotic prophase (Saunders et al., 2003).

VASA is an RNA-binding protein (also known as DEAD box helicase 4 [DDX4]) with critical roles in germ-cell development (Raz, 2000). VASA is a RNA-helicase with broad ranging functions in RNA-dependant processes including the regulation of RNA binding, accessory protein interactions and subsequent target RNA translation (Mohr et al., 2002, Cordin et al., 2006). VASA protein expression is present in the germ cells of both sexes in sheep, cattle (Reding et al., 2010), horses (Kim et al., 2015), rhesus monkeys

(Hermann et al., 2007), pigs (Kim et al., 2013), mice (Tanaka et al., 2000), and humans (Castrillon et al., 2000).

In mice, MVH expression was first detected in primordial germ cells colonising the gonadal ridges on embryonic day 10.5–11.5 (Toyooka et al., 2000). In contrast in the human fetal ovary, VASA protein expression was not observed until the second trimester (Anderson et al., 2007). In the adult mouse ovary, MVH expression was oocyte-specific and was most abundant in primordial follicles, and appeared to decrease as maturation proceeded (Toyooka et al., 2000). Similarly, in the fetal human ovary, VASA expression was observed in oogonia and oocytes, with the greatest expression in primordial follicles (Stoop et al., 2005, Anderson et al., 2007).

#### 1.2.6.2. Primordial follicles at birth

Primordial follicles are maintained in a quiescent or resting state by factors that suppress follicle activation. Primordial follicle recruitment is a tightly regulated process controlled by multidirectional communication between the oocyte, granulosa cells, and surrounding somatic cells. This process is gonadotrophin-independent and relies on ovarian growth factors and signalling pathways such as members of the transforming growth factor (TGF) superfamily, vascular endothelial growth factor (VEGFA), fibroblast growth factor (FGF2), insulin-like growth factor (IGFs), platelet-derived growth factor (PDGF), epidermal growth factors (EGF), inhibin, activin, and cytokines (Fortune et al., 2000).

### 1.2.7. Folliculogenesis, ovulation and corpus luteum development

#### 1.2.7.1. Primary follicles

The transition from primordial to primary follicle is characterised by increased granulosa cell proliferation, a transition from flat to cuboidal granulosa cell morphology and an increased diameter of the oocyte (up to 75 µm in sheep) (Sawyer et al., 2002). This process will occur in cohorts with a group of primordial follicles initiated to leave the resting pool. This process is influenced by local paracrine signalling, however, its exact initiation remains unclear (Roy, 1994). Collectively, this leads to the development of one or two layers of granulosa cells such that the follicle can reach almost 0.1 mm in sheep (Sawyer et al., 2002) (Fig. 1.4). Primary follicles express FSH receptors at this stage and respond to FSH, but growth

remains gonadotrophin-independent (Findlay and Drummond, 1999). The primary follicle increases in size through the proliferation of the granulosa cells, which remain avascular and thus require diffusion of nutrients and oxygen from the local blood supply.

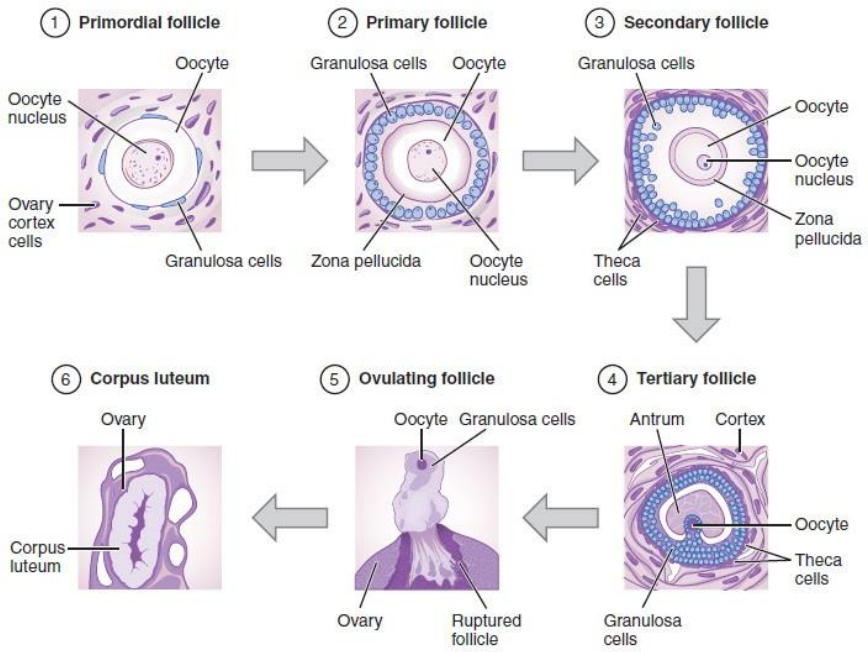



Fig. 1.4: A schematic diagram showing the key stages of ovarian follicular development: 1) primordial, 2) primary, 3) secondary (pre-antral) and 4) tertiary (antral or Graafian) follicles, 5) ovulation, leading to, 6) corpus luteum development. Adapted from (Findlay, 1995).

### 1.2.7.2. Secondary (pre-antral) follicles

The secondary follicle is characterised by two or more layers of granulosa cells and the absence of an antrum (fluid-filled cavity). The secondary follicle is approximately 0.2 mm in diameter, continues to grow independent of gonadotrophins, and remains dependent on intra-ovarian paracrine signals and oocyte-secreted factors for its growth promotion. The zona pellucida is a glycoprotein capsule, which forms around the oocyte at this time, separating the oocyte from the granulosa cells.

The key structural feature of a secondary follicle is that stroma-like theca cells are recruited (Fig. 1.4). The theca cells near to the basal lamina align close to each other as the theca layer differentiates into two layers; theca interna and theca externa. A network of capillary vessels also forms between the two thecal layers and begins to circulate blood to and from an individual

follicle (Fortune et al., 2000). In all, preantral follicle growth takes approximately 4-5 months. As the secondary follicle develops and once 6–12 layers of granulosa cells have appeared, fluid filled clefts begin to form (Lundy et al., 1999).

#### 1.2.7.3. Antral follicles

An antral follicle (also known as a Graafian or tertiary follicle) starts to form at 0.2–0.4 mm in diameter, growing up to 20 mm in cattle and will protrude from the ovarian surface. The development of the preovulatory follicle is associated with an increased density of vascularisation within the theca cell layer.

The steroidogenic cells in the theca interna are responsible for androgen production under the influence of LH. While the granulosa cells convert the thecal-derived androgens to oestradiol under the influence of FSH. Once the follicle reaches 4 mm in size, it becomes gonadotrophin-dependent and is under the control of FSH and/or LH signalling. The granulosa and theca cells continue to proliferate under the influence of LH and FSH stimulation and antrum increases in volume as the follicle develops.

***Recruitment and selection of the dominant follicle:*** The antral follicle has three major dynamic phases of development: recruitment, selection and dominance (Fig. 1.5). This is collectively referred to as a follicular wave and lasts 7-10 days. In ruminants, there are typically two or three consecutive follicular waves in each oestrous cycle (Fortune, 1988). Recruitment is where a cohort of small (antral) follicles start to develop, initiated by a FSH wave and are dependent on FSH for support. The growing antral follicles secretes both oestradiol and inhibin which negatively feedback on the anterior pituitary gland to suppress FSH release. The follicle that becomes the dominant follicle will shift from FSH to LH dependency as the granulosa cells acquire LH receptors and this follicle will diverge from the cohort. This dependence upon LH will persist through post-selection growth through to ovulation and is crucial for follicles to grow larger in size (Ginther et al., 2001, Mihm and Austin, 2002). The other non-selected follicles in the cohort will regress and undergo atresia. The dominant follicle will continue to secrete oestradiol, but if progesterone dominance (from the CL) is maintained, then gonadotropin releasing hormone (GnRH) pulsatility and levels will remain low and no LH surge or ovulation will occur. In this environment, the dominant follicle will regress after 4-6 days. Following the

regression of the first dominant follicle oestradiol levels will decrease, enabling levels of FSH to rise and a new follicular wave to start. This next follicular wave will either undergo atresia after recruitment and selection (3-wave cycle) or become the ovulatory follicular wave (2-wave cycle). Thus, depending on the species of animal, the end product of the antral follicular wave is to form a single dominant follicle (or several co-dominant follicles) that will become the pre-ovulatory follicle if progesterone concentrations are low.

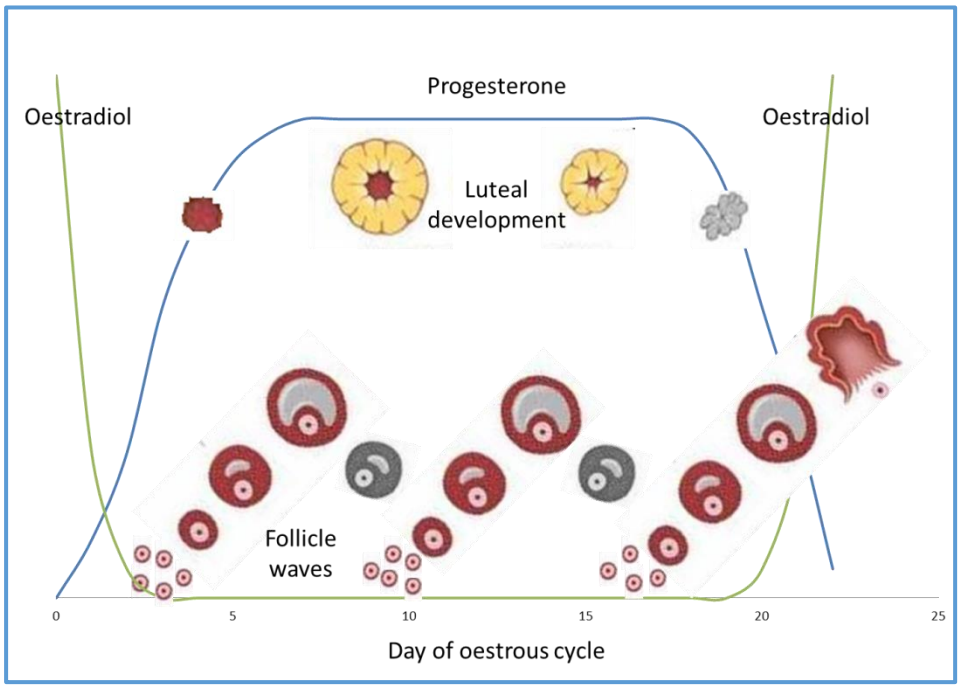



Fig. 1.5: Summary of the physiological and endocrinological events that take place from the recruitment of an antral follicle to luteal regression. These activities encompass the ovarian cycle (follicular and luteal development), and steroid hormone profiles from follicular to luteal phase in cattle.

***Final follicular development and ovulation:*** In the final stages of pre-ovulatory follicle development, the follicle will continue to grow (largely due to increased follicular fluid formation) and secrete increasing amounts of oestradiol. Consequently, a LH surge will be initiated leading to ovulation, and the release of the oocyte and antral fluid that results in the collapse of the follicle. At the same time, the basement membrane is degraded, enabling the development of new vessels from the pre-existing thecal vasculature. Not surprisingly, the follicular-luteal transition is an extremely dynamic process (Pate, 1996, Reynolds and Redmer, 1999, Niswender et al., 2000). For these events to occur, sufficient growth of blood vessels and establishment of blood supply is crucial (Reynolds and Redmer, 1999, Niswender et al., 2000).

#### 1.2.7.4. Luteal formation, development, and regression

When the pre-ovulatory follicle ovulates, a portion of blood vessels rupture causing haemorrhage and the resulting structure is named the corpus haemorrhagicum. During its formation, theca interna and the granulosa cells undergo luteinisation and differentiate into progesterone-secreting small and large luteal cells respectively. The CL contains a mixture of cell types in addition to the steroidogenic large and small luteal cells, including ECs, fibroblasts, smooth muscle cells and immune cells (Reynolds et al., 1994). To ensure normal growth and development, the different cell types must interact with each other in this complex tissue.

During the follicular-luteal transition period, the establishment of blood supply and the development of blood vessels are critical (Schams and Berisha, 2004). The tissue remodelling is intense with the angiogenic processes peaking between 2–3 days after ovulation (Reynolds et al., 2000). In fact, 85% of the proliferating cells at this time are of vascular origin. The rate of rapid growth of the early developing CL can be compared with aggressive and persistent tumour growth [i.e. around 0.5 g to >5 g within 5 days] and is associated with the increased metabolic needs of the increased steroidogenesis (Redmer and Reynolds, 1996). Blood flow reduces shortly after ovulation but gradually increases as the CL volume increases (Acosta et al., 2003). The vascularisation is such that every parenchymal cell in the mature CL is in direct contact with one or more capillaries (Redmer and Reynolds, 1996).

High progesterone production is essential to signal to the endometrium to prepare for pregnancy by secreting histotroph (to support the free-living embryo), suppressing luteolysis and preparing the endometrial lining for implantation. In the absence of pregnancy, the CL will undergo luteolysis and regress, between days 17 to 19 of the oestrous cycle in cattle. The CL will regress functionally by decreasing progesterone secretion. This allows another ovulatory surge of LH and oestrous cycle to commence. The CL will also undergo structural regression (destruction and removal of the luteal cells) to keep the ovary in proper proportion to the rest of the reproductive tract and enable the preovulatory follicle(s) to grow and subsequently ovulate.

In cattle, prostaglandin F2$\alpha$ (PGF2$\alpha$) is released in pulses from the endometrium, and causes luteal regression (Sawyer et al., 1990, Skarzynski et al., 2008). Morphologically, the regressing CL appears to undergo apoptosis, a highly regulated cell death process that eliminates distinct populations of cells by activating endonucleases within the cells (Arends et al., 1990, Juengel et al., 1993). The regressed CL will form a corpus albicans and remains visible for several subsequent cycles.

## 1.3. Hypothalamic-pituitary-ovarian axis

Throughout an animal's reproductive lifetime, the hypothalamus, pituitary gland and ovaries act in a complex coordinated endocrine manner to regulate multiple aspects of reproductive function (Fig. 1.6). The hypothalamic-pituitary-ovarian (HPO) axis is the primary regulator of the oestrous cycle. The oestrous cycle (interval between successive oestrus) lasts on average 21 (Grunert et al., 1973), and 17 days (Mellin and Busch, 1976) in cattle and sheep, respectively. The HPO axis drives the cyclical morphological and physiological changes in the ovaries, oestrus expression (sexual receptivity) and ovulation as well as preparing the genital tract for copulation, fertilisation and embryo implantation. This interplay involves in both negative and positive feedback mechanisms.

The hypothalamus releases gonadotrophin-releasing hormone (GnRH), which stimulates the anterior pituitary gland to release FSH and LH (Senger, 2003) (Fig.1.6). These gonadotrophins act on antral follicles stimulating them to produce oestradiol and inhibin as well their development. LH also triggers ovulation, and formation of the CL. A range of neuroendocrine factors – ovarian steroid hormones (progesterone and oestradiol),

neuromodulators (kisspeptin) and external factors (photoperiod and nutrition) controls the gonadotrophins and GnRH.

The hypothalamus contains GnRH neurons, which comprise two different hypothalamic nuclei: the surge and tonic centres. The surge centre is responsible for releasing GnRH in a surge-like manner leading to a LH surge and ultimately ovulation, while the tonic centre is responsible for the release of basal pulsatile levels of GnRH during the oestrous cycle. FSH and LH regulate follicular growth and development from the antral stage through to oocyte maturation and ovulation. However, FSH is particularly responsible for follicular recruitment and stimulates the production of oestradiol and inhibin as the follicle matures. As the follicle grows and approaches dominance, oestradiol and inhibin concentrations increase and thereby reduce FSH concentrations. This enables the selection of the dominant follicle, which is then dependent on LH for its support and further growth. In the absence of progesterone, this dominant follicle will respond to LH (and FSH) by increasing its oestradiol output. Once plasma oestradiol levels reach a threshold, a positive feedback mechanism will be created that result in a LH surge and ultimately the ovulation of the pre-ovulatory follicle. After ovulation, follicular cells differentiate under the influence of LH into luteal cells that form the CL. As the CL develops, it will secrete progesterone, which has a negative feedback on the hypothalamus that results in reduced FSH and LH pulsatility and suppression of oestrus (Fig. 1.6).

In the absence of pregnancy, the endometrial epithelial cells produce PGF2α, which causes luteolysis and rapidly reduces progesterone concentrations. This occurs on day 17 to 19 of the oestrous cycle in cattle. In response to falling progesterone concentrations, the anterior pituitary gland increases its secretion of gonadotrophins and a new cycle begins.

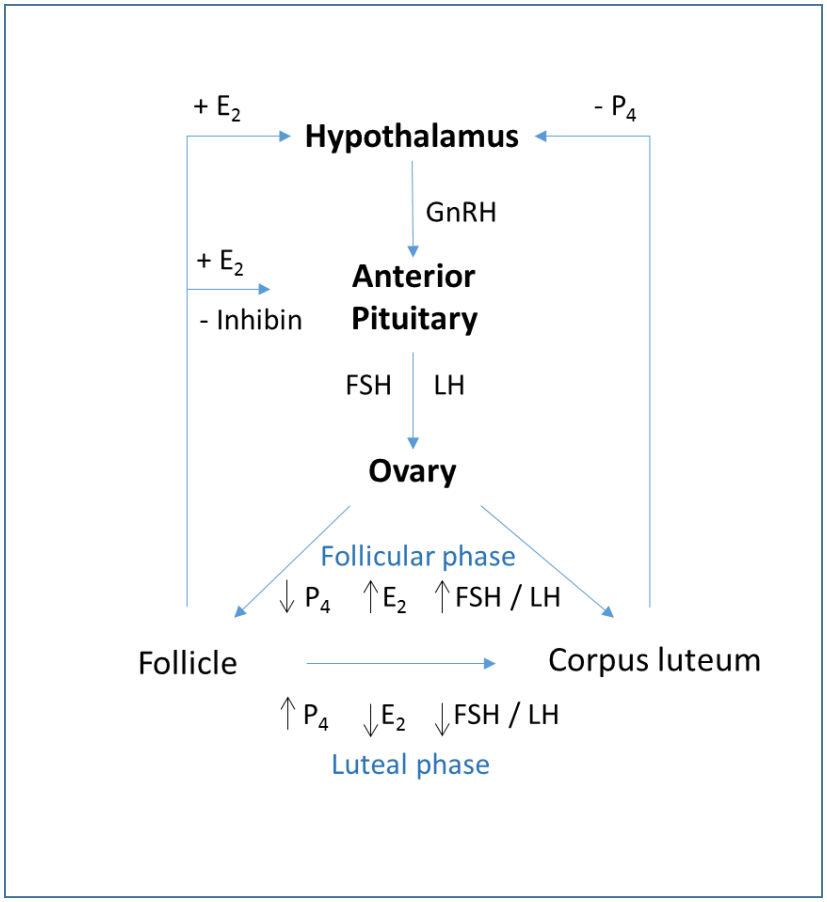



Fig. 1.6: The hypothalamic-pituitary-ovarian axis. The feedback mechanisms between the hypothalamus, anterior pituitary gland and ovary are represented; + positive feedback; - negative feedback. GnRH, gonadotrophin releasing hormone; P4, progesterone; E2, oestradiol; FSH, follicle stimulating hormone; LH, luteinising hormone.

## 1.3.1. Progesterone and oestradiol production

### 1.3.1.1. Oestradiol synthesis

The process of oestradiol production in the bovine pre-ovulatory follicle is described as the two-cell, two-gonadotrophin theory of oestradiol biosynthesis and is shown in Fig. 1.7. The precursor for all steroids is cholesterol, which is predominantly obtained from the circulation. Extracellular cholesterol is received in the form of high-density lipoprotein (HDL) or low-density lipoprotein (LDL) through their respective receptors located on the theca cell membrane (Fig. 1.7). There is also some *de novo* cholesterol biosynthesis from fatty acids in theca cells (Niswender, 2002).

The rate-limiting step of steroidogenesis is the transport of free cytoplasmic cholesterol to mitochondrial membranes by the protein steroidogenic acute regulatory protein (STAR) (Fig. 1.7). It is here that cytochrome P450 cholesterol side-chain cleavage (CYP11A1) enzyme is located which catalyses the conversion of cholesterol to pregnenolone. From the mitochondria, pregnenolone is transferred to the smooth endoplasmic reticulum where 3β-hydroxysteroid dehydrogenase (HSD3B1) acts to convert pregnenolone to progesterone (Reinhart et al., 1999, Stocco et al., 2001, Manna et al., 2003). Progesterone is converted to androstenedione through the actions of 17α-hydroxylase (CYP17A1) and 17, 20 lyase. Androgens are then secreted from the theca cells and diffuse freely through the basal lamina to the granulosa cells. Granulosa cells complete the synthesis of oestradiol through the activity of 17β-hydroxysteroid dehydrogenase (HSD17B) and P450 aromatase (CYP19A1) enzymes.

LH has numerous actions, via cAMP signalling, which result in the stimulation of steroidogenesis (Juengel et al., 1998, Clark et al., 2000, Stocco, 2001); it increases the number of intracellular organelles, such as mitochondria, smooth endoplasmic reticulum and lipid droplets. LH also stimulates the expression of the key steroidogenic proteins (e.g. STAR) in the theca cells. FSH (and LH) will stimulate oestradiol synthesis in a cAMP-dependent manner through the up-regulation of CYP19A and to a lesser extent HSD17B (Magoffin, 2005).

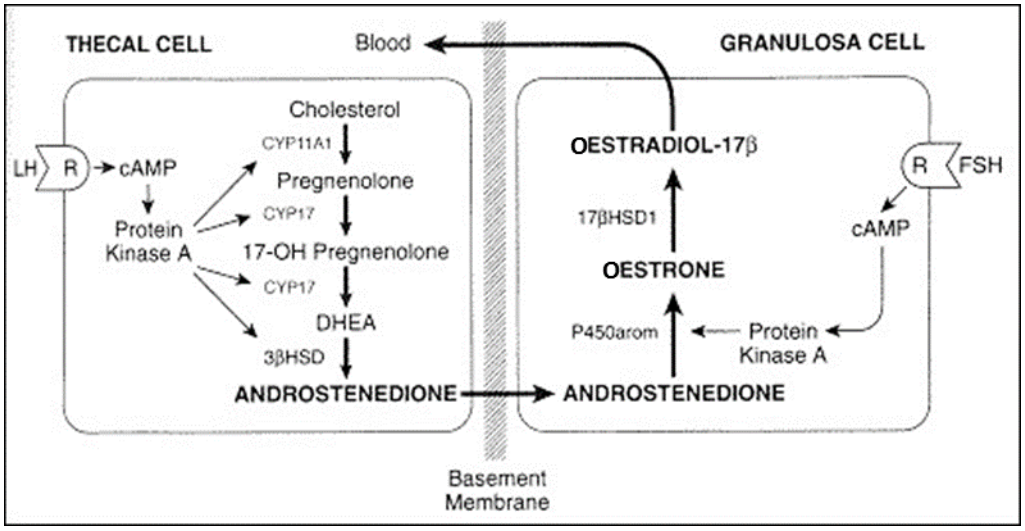



Fig. 1.7: The two-cell, two-gonadotrophin theory of oestradiol synthesis. The schematic diagram describes the process of oestradiol and progesterone production in the bovine preovulatory ovarian follicle. Adapted and modified from (Niswender, 2002, Magoffin, 2005, Walsh et al., 2012b).

### 1.3.1.2. Progesterone synthesis

The principal steroid hormone produced by the bovine CL is progesterone. Theca- and granulosa-derived cells differentiate to two distinct types of luteal cells that differ morphologically, physiologically and biochemically. These are referred to as small and large luteal cells respectively.

There are three sources of cholesterol that the cell utilises: 1) low-density lipoprotein, 2) high-density lipoprotein, and 3) hydrolysis of stored cholesterol esters by cholesterol esterase (Fig. 1.8). The free cholesterol is transported to mitochondria and then transferred from the outer to inner mitochondrial membrane (Wiltbank et al., 1993, Belfiore et al., 1994). This step is principally regulated by STAR (Clark et al., 1994) as well as the peripheral type benzodiazepine receptor (Papadopoulos et al., 1997). Cholesterol is then converted to pregnenolone by CYP11A1 and then transported out of mitochondria, and converted to progesterone by HSD3B1, which is present in smooth endoplasmic reticulum. Progesterone then diffuse out of the luteal cell (Fig. 1.8). The transport of cholesterol from the cytosol to the inner mitochondrial membrane is the major regulatory step in the production of progesterone.

Small luteal cells (theca-derived) contain LH receptors that respond to LH by increasing intracellular cAMP, which subsequently activates protein kinase A (PKA) and the synthesis of progesterone (Sugawara et al., 1995). Large

luteal cells are primarily of granulosa cell origin and secrete high basal progesterone concentrations, and do not respond to LH (Niswender et al.). The differences in the amount of progesterone secretion between the large and small luteal cells is mainly related to differences in the expression of STAR and the peripheral type benzodiazepine receptor (Fitz et al., 1982, Kallen et al., 1998). Conversely, activation of protein kinase C (PKC) inhibits progesterone synthesis in small and particularly large luteal cells. This is induced by $PGF_{2\alpha}$ binding to its FP receptor causing influx of calcium.

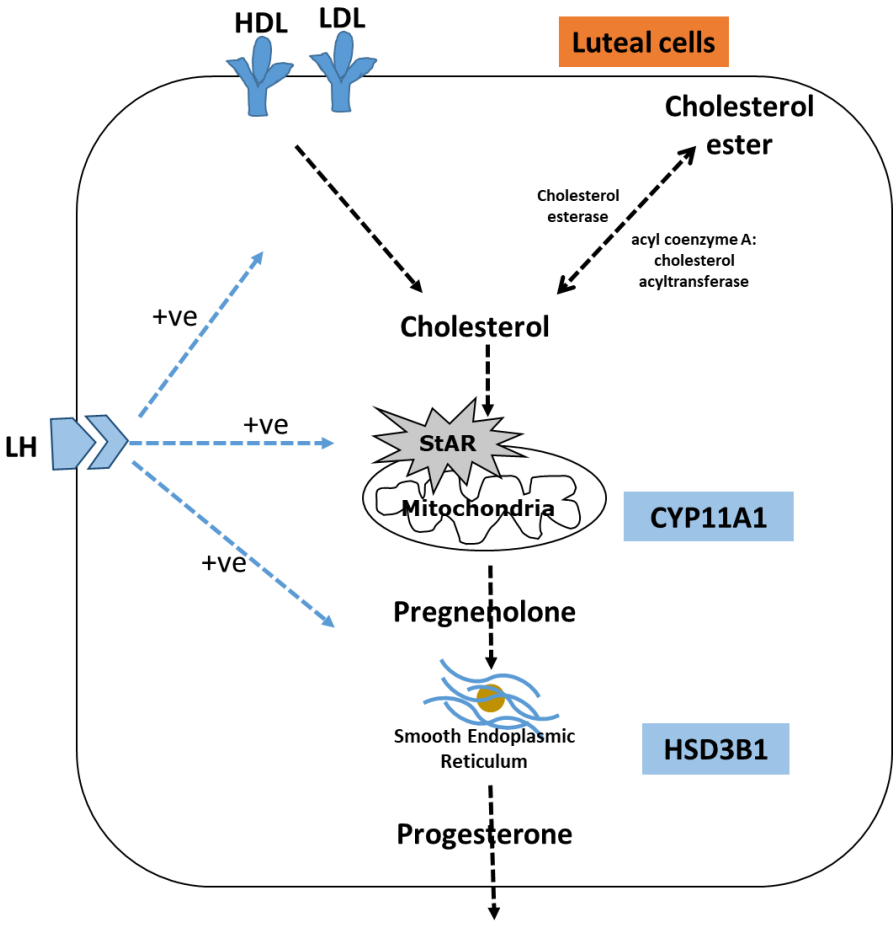



Fig. 1.8: The process of progesterone production and cholesterol biosynthesis in the bovine CL. LH signalling is only present on small luteal cells. Adapted and modified from (Niswender, 2002, Walsh et al., 2012b).

## 1.4. Nutritional effects on fetal development during pregnancy

The reproductive potential of mammals begins during the animal's fetal life. Fetal programming is used to describe a stimulus or insult caused during a critical period of fetal development that has long-lasting or permanent effects later in life including adulthood (Lucas, 1991). Fetal programming can be in response to hormonal, nutritional and/or metabolic challenges at any time during development. It can lead to altered cell proliferation, differentiation, and phenotype resulting in structural and/or functional modifications to the cells, tissues and organ systems (Sinclair et al., 2016). The concept of fetal programming was originally proposed by Prof David Barker in 1990s (Barker et al., 1993) and is referred to as the Barker hypothesis (Osmond et al., 1993, Langley and Jackson, 1994, Barker and Clark, 1997).

The concept that maternal nutrition at different stages of pregnancy can adversely influence the offspring's lifetime productivity, health and development has gained much interest and attention from epidemiological research in human populations. This has led to detailed experimental studies, in different animal models such as rodents and livestock (Greenwood et al., 2010). Additionally, there is growing evidence that maternal nutrition can induce epigenetic modifications in the fetal genome (Sinclair et al., 2016). Epigenetic changes could be described as the changes in the heritable gene expression without an alteration of the genetic code, such as DNA methylation and histone modification. The body system that has received the least attention has been the reproductive tract with limited studies investigating the effects of maternal nutrition on fertility-related parameters in the offspring. Therefore, there is a need to investigate the molecular mechanisms underlying the link between alterations to the intra-uterine environment and the short and long-term effects on the reproductive health of the offspring.

Intrauterine growth retardation (IUGR) is an impaired growth and development of the fetus during pregnancy. Consequently, IUGR is a massive problem in animal production as it influences the body composition, carcass quality, and impairs health. This will lead to a reduction in neonatal survival, growth, feed efficiency utilisation, and future production by the animals (Yates et al., 2018). Maternal nutrient constraints during pregnancy

reduce fetal growth in both humans and experimental animals (Wallace et al., 2004, Abu-Saad and Fraser, 2010, McKnight et al., 2011). Pregnancy may negatively influence maternal nutritional status because of increased uteroplacental blood flow, nutrient mobilisation, and transfer of nutrients from the dam to the fetus. Animal studies have revealed complex biological mechanisms responsible for IUGR (Wu et al., 2006). These mechanisms are corroborated by clinical observations and include: (1) reduced placental growth and vascularity; (2) impaired placental function (including the capacity for transport of amino acids and glucose); (3) oxidative stress in the placenta and conceptus; (4) reduced concentrations of haemoglobins (because of iron deficiency) for oxygen transport; (5) impaired cell signalling for regulation of protein synthesis in the placenta and fetus; and (6) impaired immune response of maternal/fetal placenta (Wu et al., 2004, Wu et al., 2006).

Maternal size and nutritional stressors are different maternal constraints that can affect the fetal growth and reproductive development during pregnancy (Redmer et al., 2004, Rhind, 2004, Wu et al., 2006, Gootwine et al., 2007, Kenyon, 2008, Gardner et al., 2009). Equally, the litter size can have a great influence on birth weight (Gardner et al., 2007). For example, singleton offspring are heavier at birth and at weaning than multiple fetuses (Morris and Kenyon, 2004, Gootwine et al., 2007, Kenyon, 2008). Multiple fetuses have higher nutrient demand and are more greatly affected by maternal constraints than singletons, hence, IUGR is prevalent in animals with multiple fetuses (Gootwine et al., 2007). Lighter dams carrying multiple fetuses (triplets and quartets) like pigs, rats and rabbits have smaller placentas, which effects the rate and capacity of maternal nutrient absorption and oxygen transfer to the fetuses (Gootwine et al., 2007) resulting in a higher probability of IUGR.

Maternal nutrient constraints are very common in animal production. Numerous factors affect nutrient requirements for livestock including breed, season, and physiological status. This can be caused by lack of feed availability, drought, poor pasture growth, overstocking of animals, breeding young dams, twin and triplet offspring, selection for increased milk production or breeding of livestock during poor pasture conditions (Wu et al., 2006, Reynolds et al., 2010).

### 1.4.1. Maternal influences on the fetus

The critical factor for fetal survival and health is an adequate nutrient and oxygen supply to the dam and this ability is dependent on her nutritional supply, body size, body composition, and metabolism. However, another important key factor is the composition of the diet in terms of protein, energy, fat, vitamin and mineral content (Robinson et al., 2006, Symonds et al., 2006, Wu et al., 2006, Symonds et al., 2010). The placenta is a unique organ of reproduction that helps in the exchange of nutrients, respiratory gases and excretory waste between the mother and offspring (Redmer et al., 2004). Thus, the developing fetus will be dependent on placental function, which is in turn dependent on maternal nutrition. The timing, duration and degree of nutritional insult may result in different effects on the growth and development of the fetus, neonatal survival and adulthood later in life (Vonnahme et al., 2003, Vonnahme et al., 2006, Gootwine et al., 2007, Quigley et al., 2008). It is only extreme maternal deprivation that will severely compromise pregnancy and leads to termination of the fetus (Bloomfield et al., 2003).

Birth weight is an indicator of fetal and dam's nutrition/health during pregnancy (Gardner et al., 2007). That said, birth weight is not a conclusive measure of the quality of fetal growth (Harding and Johnston, 1995, Wu et al., 2004) as there can be a period of catch-up growth or sensitive critical windows for particular organs that are affected by undernutrition (Bloomfield et al., 2006). Many studies have suggested that under or over-nutrition can specifically effect the total body weight as well as altering the ratios of the organ to fetal weights (Robinson et al., 1999, Vonnahme et al., 2003, Bloomfield et al., 2006, Quigley et al., 2008).

### 1.4.2. Effect of maternal nutrition during critical windows of pregnancy

The average gestation length of sheep and cattle is ~147 and 285 days respectively; and typically studies have split pregnancy up into 3 trimesters: early pregnancy (up to 50 days [sheep] and 95 days [cows]), mid-pregnancy (50-95 days [sheep] or 96-190 days [cows]) and late pregnancy (after 96 days [sheep] or 191 [cows]. This takes into account that throughout the gestation period, the growth pattern of the placenta and fetus differ (Gootwine et al., 2007, Kenyon, 2008). Pregnancy encompasses embryogenesis, fetal organogenesis, and placental development (Fig 1.9).

The nutrient requirements in this window are relatively small, but the metabolic activity and growth rate of the fetus is high (Robinson et al., 1999). In mid-pregnancy, the placenta will have reached its maximal size and there will be the maximal number of germ cells in the developing ovary (Schneider, 1996). The late pregnancy is often referred to as the fetal phase as it is characterised by maximal fetal growth and is associated with the highest nutritional demand (Symonds et al., 2006, Kenyon and Webby, 2007). There are long-term effects on the offspring following maternal nutritional restriction during all three critical periods (Fig. 1.9). For example, maternal nutrition restriction in embryonic, placenta and fetal stages of pregnancy can result in metabolic compromise, cardiovascular, renal and adipose tissue dysfunction (Fig. 1.9).

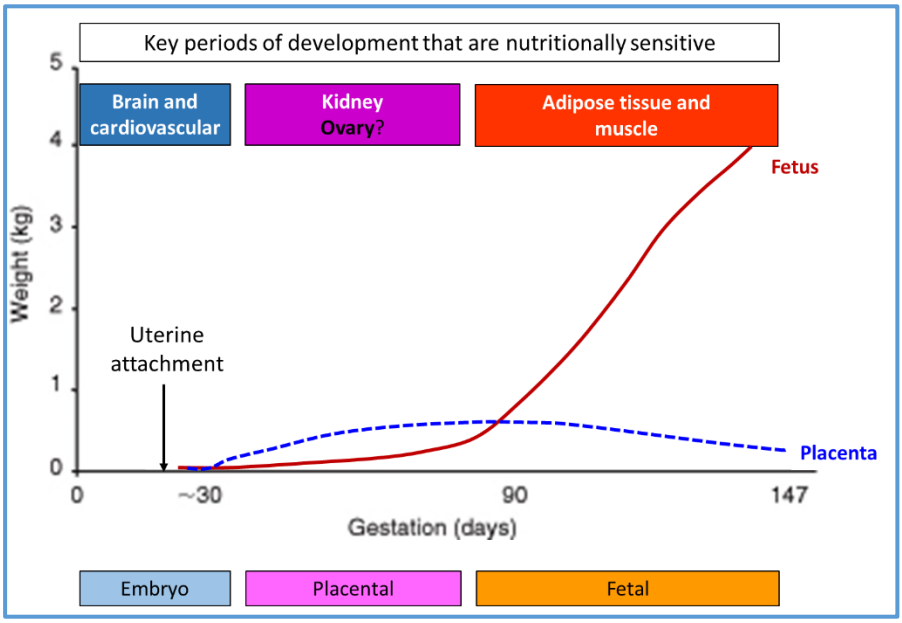



Fig. 1.9: Summary of long-term effects of maternal nutritional restriction during the critical developmental period on offspring. The key windows of placental and fetal organ development that are sensitive to nutrition during gestation in sheep are indicated. Adapted from (Symonds et al., 2006, Symonds et al., 2010).

During early pregnancy, the embryo's nutrient requirement is small but can be very specific in its temporal and qualitative requirements. This can result in subsequent altered fetal growth (Wu et al., 2004, Bloomfield et al., 2006). Indeed, any maternal nutrient manipulation during early pregnancy can lead to drastic changes in offspring live weight and body condition (Annett and

Carson, 2006). For the first 3 weeks of pregnancy, nutrients for the embryo are derived from uterine secretions that can reflect any changes in the nutritional environment of the dam (Symonds et al., 2006). Thereafter, nutrients are supplied via the placenta. Embryonic attachment occurs (~day 20), while placenta (maternal and fetal components) growth commences on ~day 30 (Symonds et al., 2010) with placentome number established by day 40 (Schneider, 1996). This is the time where the greatest proportion of cellular differentiation, organ formation and angiogenesis occur. Placentome number and size are nutritionally influenced (Heasman et al., 1999). The nutrient supply will also affect the local placental production of growth factors (e.g. IGF2) that will influence placental and fetal growth. High protein content during the early trimester reduced body weight of the adult male offspring but protein restriction either early or mid-trimester did not affect body weight or carcass fatness in male or female adults (Micke et al., 2011).

The major effects of nutritional challenges (under- or overnutrition) on fetoplacental growth and development appear to occur when the placenta is rapidly developing, i.e. day 40–80 of pregnancy in sheep (Robinson et al., 1999, Wu et al., 2006). Placental growth appears to be more sensitive to maternal feed restriction during the early pregnancy and fetal growth during the mid-pregnancy (McCrabb et al., 1992). It should be noted that there are studies where nutrient restriction during mid-pregnancy did not result in functional placental insufficiency and restricted fetal growth. It is feasible that these contradicting reports may relate to differences in dam breed, age, parity, maturity, size, body condition and nutritional status or the postnatal life of animals (Anthony et al., 1986, Vonnahme et al., 2006, Greenwood and Cafe, 2007). There are few studies in cattle and the majority of those have varied dietary protein content (i.e. 70 vs 240% recommended) during both early and mid-trimesters (Micke et al., 2010, Micke et al., 2011). For example, nutrition restriction at 55% and 50% of energy and protein NRC recommendations during second trimester in heifers were unaffected with respect to birth weight, postnatal growth and carcass characteristics (Long et al., 2010).

Late pregnancy is the time when most of the increase in fetal size takes place (Robinson et al., 1999, Symonds et al., 2006, Kenyon et al., 2007). Many important fetal growth factors (e.g. IGF2, insulin and thyroid hormones) are nutritionally regulated during this period (Bloomfield et al.,

2006). Insufficient maternal nutrition reduced the concentration of IGF1 and IGF2 in fetal circulation which decreased myogenic cell proliferation and formation of muscle fibres (Gonzalez et al., 2013).

### 1.4.3. Effects of maternal nutrient restriction on the fertility of the female offspring

Poor nutrition caused by inadequate, excess or imbalanced nutrient intake has been shown to adversely affect subsequent reproductive performance (e.g. delayed puberty, luteal inadequacy, reduced follicular reserve, reduced ovulation, and conception rates) (Smith and Somade, 1994, Ashworth et al., 2009, Scaramuzzi et al., 2011, Mossa et al., 2013). For example, maternal undernutrition during late pregnancy in ewes reduced fetal growth (Rhind et al., 1989) and the lambing rates in female offspring (Gunn et al., 1995). The sexual differentiation of both female and male offspring is almost completed by mid-pregnancy (Rhind, 2004). In addition, restricted in the dietary protein altered total number of oocytes, delayed ovarian development in fetal sheep (Rae et al., 2001), reduced ovulation rate in adult sheep (Rae et al., 2002), increased calving interval (Cushman et al., 2014), decreased antral follicle number in adult cattle (Mossa et al., 2013). More specifically delayed fetal ovarian development was observed on day 110 of gestation in restricted ewes (Rae et al., 2001). This was associated with decreased Ki67 (proliferation marker) in germ cells on day 65 of gestation in the maternal nutritionally restricted fetuses (Lea et al., 2006). In addition, pro-apoptotic, BAX expression in the primordial follicles was increased on day 110 gestation. This indicated increased loss of germ cells during the formation of follicles in nutrient-restricted animals (Lea et al., 2006). Since the number of oocytes is defined by birth, the potential of the animal's lifetime reproductive performance could be reduced. Thus, a consequence of poor fetal ovarian development could be premature ovarian failure. It is hypothesised that is, in part, due to improper development of the stromal compartment leading to the poor follicular formation (Robinson et al., 2006). These effects and the critical windows of maternal undernutrition on fetal ovarian development in sheep are summarises in Fig. 1.10.

In general, there are fewer studies investigating the effects of maternal dietary restriction on the offspring's subsequent reproductive performance. Some studies that have been conducted have showed a reduced number of antral follicles and anti-Mullerian hormone (AMH) concentrations in the adult

(Evans et al., 2012, Mossa et al., 2013). Importantly, female offspring with lower AMH concentration in their first year of age is a physiological indication of reduced or low follicular reserve (Evans et al., 2012).

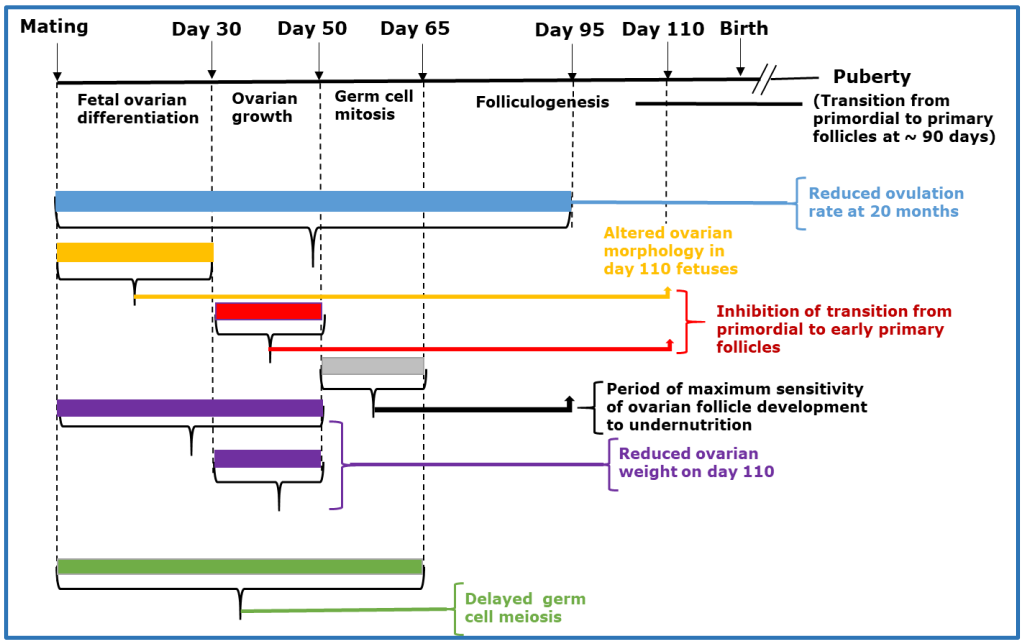



Fig. 1.10: Critical periods during gestation when maternal undernutrition has affected fetal ovarian development in sheep. Shaded areas refer to periods of contrasting maternal nutrition (0.5 vs 1.0 times maintenance) in discrete experiments. Adapted from Robinson et al. (2006).

### 1.4.4. Effects of protein restriction during gestation

Proteins, carbohydrates, fats, minerals and vitamins are key components in animal feeds that are required for a daily maintenance diet. These are important for growth, production and reproduction (Rinehart, 2008). Protein is typically the most expensive nutrient in a diet and is regularly deficient in an animal's daily ration (Burroughs et al., 1975, Jurgens, 2002). The primary aim of protein supplementation is to meet the amino acid requirement that is needed for *de novo* protein synthesis and tissue growth. Any protein supplement may contain true protein and/or non-protein nitrogen (e.g. urea) (Kellems and Church, 2002). However, the microbes in the sheep or cow's rumen will synthesise their amino acid requirements by converting non-protein nitrogen to ammonia to maintain microbial growth. Amino acids serve as building blocks for proteins and essential precursors for the synthesis of different physiological molecules–hormones, neurotransmitters, nitric oxide, creatine, glutathione, carnitine, and polyamines (Wu, 2009). For

the provision of protein to the fetus, there must be a balance of adequate essential and non-essential amino acids. A pregnant dam must consume a high-quality protein meet the daily protein requirement as most grains are deficient in the essential amino acids: lysine, methionine, threonine, and tryptophan (Lyman et al., 1956).

Maternal protein supplementation during pregnancy can increase body condition score and body weight of the cow at calving (Sanson et al., 1990, Bohnert et al., 2002). More importantly, maternal protein supplementation affected the development of calf reproductive capabilities (Martin et al., 2007). While protein malnourished rodents had a shorter lifespan in both males and females (Ozanne and Hales, 2004, Langley-Evans and Sculley, 2006). However, in sheep with a 50% restriction of protein intake (17 *vs.* 8.7 g crude protein/MJ metabolizable energy) from day 0 to 65, there was no effect on maternal amniotic or fetal plasma amino acid concentration but urea was reduced indicating reduced protein turnover (Dunford et al., 2014).

In rodents, maternal protein malnutrition affected various body systems in the offspring including higher blood pressure (Langley and Jackson, 1994), reduced glucose tolerance (Langley et al., 1994), lower birth weight (Simmons et al., 2001) and metabolic syndrome (McMillen and Robinson, 2005). Maternal protein restriction can affect fetus metabolism, insulin resistance, and obesity. Protein restriction resulted in increased postnatal growth catch-up (Hales et al., 1996, Zierath et al., 1996, Balasa et al., 2011). Maternal diet has affected placental factors in many species (Rutland et al., 2007). This is important as placental weight and vascularity are indicators of placental function and related to fetal growth.

The long-term effects of maternal protein restriction have not been fully elucidated in terms of the critical levels and time windows in relation to the germ cell development, postnatal growth and reproductive development of offspring even in their later life and generations to follow (Redmer et al., 2004). Moreover, there is little or no literature investigating how the vascularisation to the fetal ovary might be influenced by maternal nutrition restriction.

## 1.5. Insulin-like growth factor system

The insulin-like growth factor (IGF) system comprises of two protein ligands, IGF1 and IGF2, which can bind to two different cell-surface receptors, IGF type 1 receptor (IGF1R) and the mannose 6-phosphate/IGF type 2 receptor (M6P/IGF2R). They can be bound by a family of six high-affinity IGF binding proteins (IGFBP1 to 6) and their associated IGFBP degrading proteases (Fig 1.11; (LeRoith, 2003, Clemmons, 2007).

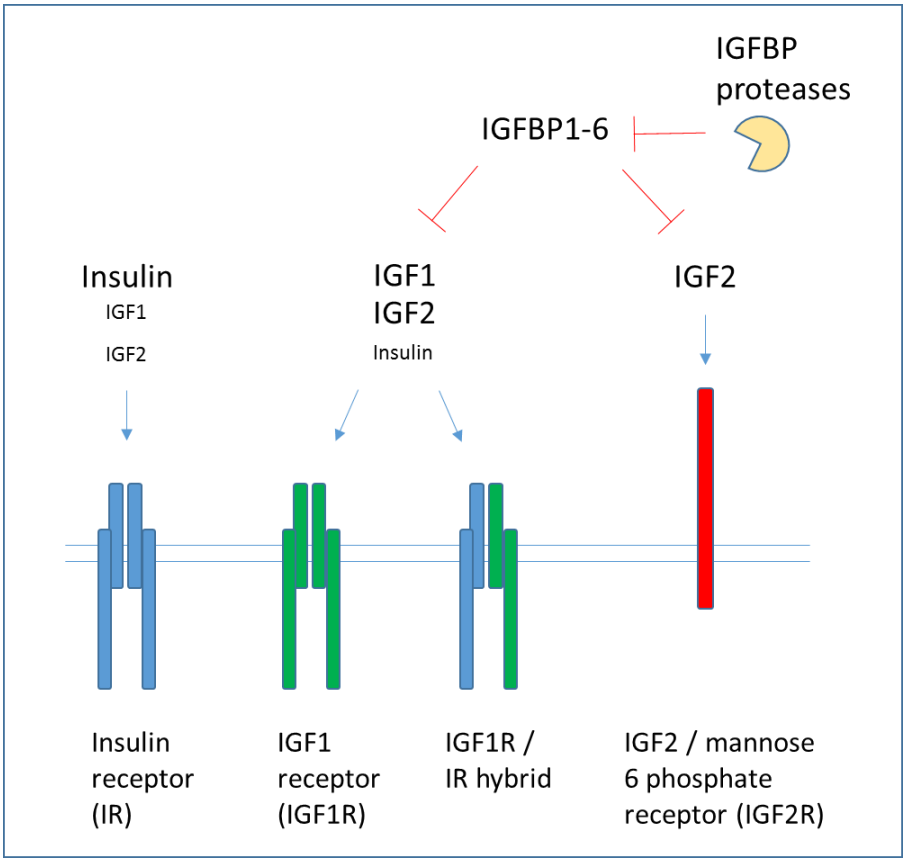



Fig. 1.11: The insulin and insulin-like growth factor (IGF) system. This comprises of insulin, IGF1 and 2 as the protein ligands that bind to the insulin receptor, IGF type 1 and 2 receptors and family of six IGF binding proteins IGFBP 1-6 and IGFBP degrading proteases.

### 1.5.1. The IGF ligands

Insulin-like growth factors 1 and 2, like insulin, are hormones that have multiple roles in regulating critical metabolic processes, cell proliferation, differentiation, migration, survival and apoptosis (Jones and Clemmons, 1995, Dupont and Holzenberger, 2003). Both IGF1 and IGF2 have insulin-

like metabolic effects, such as decreasing plasma glucose and increasing glucose uptake into cells. They can act in an endocrine, paracrine and/or autocrine manner to exert their effects on many different cells including hepatocytes, cardiac myocytes and ovarian steroidogenic cells (Lucy, 2011). Additionally, IGF1 and IGF2 are both expressed in the liver and in extrahepatic tissues (Clemmons, 2006, Kaplan and Cohen, 2007). The production of IGF1 and IGF2 is regulated at the transcriptional level, by RNA processing and translation as well as the proteolytic processing of the precursor protein (pre-pro IGF) into the final active IGF1 or IGF2 (Sussenbach et al., 1992).

#### 1.5.1.1. Insulin-like growth factor 1

Insulin-like growth factor 1 is a 70 amino acid single chain protein, cross-linked by 3 disulphide bridges and with a molecular weight of 7.6 kDa (Rinderknecht and Humbel, 1978b, Rinderknecht and Humbel, 1978a). IGF1 is synthesised in various tissues including the liver, skeletal muscle, bone, ovary and cartilage (Fig. 1.12). The primary site of IGF1 production is the liver from where it is secreted into the plasma and can be found at relatively high concentrations (150–400 ng/ml) in humans. Indeed, changes in plasma IGF1 concentrations pre-dominantly (80%) reflect changes in its synthesis and secretion from the liver (Yakar et al., 1999).

In plasma, IGF1 is principally protein-bound (by IGFBP3) with a small fraction (<1%) as free active IGF1 (Davis et al., 1992, Clemmons, 2007). The regulation of IGF1 expression is controlled by various factors but its production by the liver is principally regulated by growth hormone (GH) (also known as somatotropin; Fig. 1.12). GH increases IGF1 transcription (Davis et al., 1992) and might stimulate changes in the local production of IGFBPs (Campbell et al., 1991), resulting in increased plasma IGF1. Factors that regulate IGF1 concentration include GH levels but also the individual's genetic make-up, the time of day, age, sex, exercise status, stress levels, nutrition level, body mass index (BMI), disease state, race, and plasma oestrogen concentration (Juul et al., 1995, Blum and Schweizer, 2003, Friedrich et al., 2014).

Hepatic IGF1 expression is regulated by GH, which is in turn regulated by hypothalamic factors such as somatostatin (negative) and growth hormone releasing hormone (GHRH, positive; Fig. 1.12). During periods of metabolic stress (such as negative energy balance in early lactation), this

somatotrophic axis is disrupted. In this situation, liver GH receptor (GHR1A) expression is down-regulated, leading to decreased plasma IGF1 concentrations while GH continues to act on other tissues and have a tissue mobilisation effect (Lucy et al., 2001, Walters and Griffiths, 2009). Liver-derived IGF1 will principally act in an endocrine manner and thus provide a link between the metabolic status of the animal and growth promotion (Fenwick et al., 2008). IGF1 is also locally synthesised in other organs where it exerts autocrine and paracrine effects (Clemmons, 2007). In addition to its role in regulating metabolic status, there is also evidence of a role for IGF1 in multiple pathologies including tumourigenesis atherosclerosis, hypertension, restenosis, dysregulated angiogenesis and diabetic vascular disease (Delafontaine et al., 2004).

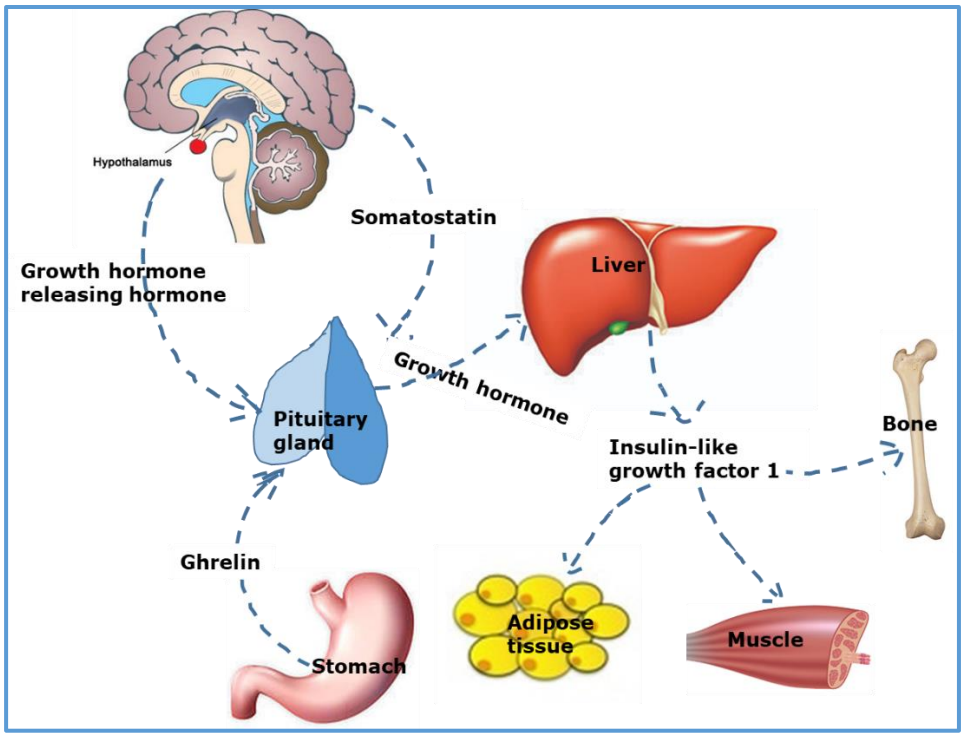



Fig. 1.12: The interplay between growth hormone and insulin-like growth factor 1 in different tissues and organs. Growth hormone-releasing hormone and somatostatin are secreted by the hypothalamus that stimulates/inhibits the release of GH from the pituitary gland. GH expression can be further increased by ghrelin release from the stomach. GH will then stimulate the hepatic production of IGF1 which stimulates adipose tissue, muscle and bone function. Adapted and modified from (Rozario et al., 2000).

#### 1.5.1.2. Insulin-like growth factor 2

There is a high degree of sequence homology between IGF2 and IGF1 (66% identical) but IGF2 is 3 amino acid shorter (67 amino acids) (Rotwein et al., 1986, Rotwein, 2018). IGF2 is considered the dominant IGF in the fetus and is critical for development and function of organs such as the brain, liver and kidney in the fetus. In the adult, plasma IGF2 concentrations are generally lower than IGF1 and are less GH-dependent compared to IGF1 (Iliev et al., 2014). In rodents, it has been suggeseted that IGF2 is a "prenatal growth factor" while IGF1 is a "postnatal growth factor" (Bach, 2015). However, this is an over-simplification, with local IGF2 expression being detected in several tissues in adults including the ovary and placenta and there are reports of circulating IGF2 levels being higher than IGF1 (Bennett et al., 1983, Gluckman and Ambler, 1993, Stewart et al., 1995). Importantly, the regulation and synthesis of IGF2 is different to that for IGF1 (Jones and Clemmons, 1995).

### 1.5.2. The IGF receptors

#### 1.5.2.1. The IGF tye 1 receptor

Major actions of IGF1 and IGF2 are exerted by high-affinity binding to IGF type 1 receptor (IGF1R) (LeRoith, 2003) that shares homology with the insulin receptor (48% identical) (Blundell et al., 1978, Rinderknecht and Humbel, 1978a). The IGF1R protein is a 1367 amino acid protein with a molecular weight of 130 kDa (Massague and Czech, 1982, Ullrich et al., 1986) The IGF1R, a tyrosine kinase receptor superfamily member and is composed of $\alpha$ subunits containing the IGF binding domain, that link to $\beta$ subunits which contain the tyrosine kinase catalytic activity domain. The IGF1R functions as a tetramer of 2 $\alpha$ and 2 $\beta$ subunits covalently linked by disulphide bonds (Heldin and Östman, 1996). IGF1R binds IGF2 with a 6-fold lower affinity than IGF1. It can also bind insulin but with 100-fold lower affinity (Federici et al., 1997, LeRoith, 2003).

The tyrosine kinase activity in the IGF1R $\beta$ subunits is activated by IGF1 or IGF2 binding (Jacobs et al., 1983, Rubin et al., 1983). The tyrosine kinase domains autophosphorylate specific tyrosine molecules on IGF1R that then act as the docking site for signalling proteins such as the insulin receptor substrate (IRS1-4) family. The recruited protein in turn will activate different

signalling cascades including the PI3-AKT-TOR and RAF-MAPK pathways that subsequently stimulate cell proliferation and survival (Sasaki et al., 1985).

#### 1.5.2.2. The IGF type 2 receptor

There is a second IGF receptor on the cell surface to which IGF2 (and to a much lesser extent IGF1) binds to, called the IGF type 2 receptor (IGF2R). This IGF2R is identical to the mannose-6-phosphate receptor. IGF2 binds to IGF2R with a lower affinity than to IGF1R and the IGF2R lacks tyrosine kinase activity (Brown et al., 2009). It was classically believed to be a mediator for the internalisation of IGF2 and thus targeting IGF2 for degradation. Consequently, IGF2R has been associated with having anti-proliferative and pro-apoptotic activities by reducing the interaction of IGF2 (or IGF1) with IGF1R (Hankins et al., 1996, O'gorman et al., 2002, Pollak, 2008).

### 1.5.3. IGF binding proteins

The actions of IGFs are further regulated by a family of six high-affinity binding proteins or IGFBPs (Firth and Baxter, 2002, Bach et al., 2005, Baxter, 2014). They all bind IGF1 and IGF2, but with slight differences in relative binding affinity and regulation. The IGFBPs share homology (~50%) with each other and are 25-45 kDa in molecular weight.

The structure of the IGFBP can be split into three domains (1) the conserved N-terminal region, which is cysteine-rich, contains multiple disulphide bonds, and is responsible for binding IGF; (2) the highly variable linker domain, which is the site of most post-translation modifications (glycosylation and phosphorylation). The linker domain contain sites for proteolysis by a number of specific IGFBP protease (Bach, 2015); (3) the conserved C-terminal domain that is cysteine-rich, which contains secondary IGF binding residues and binding site for other proteins such as heparin and acid labile subunit (ALS).

The local production of IGFBPs can be regulated by both IGF1 and IGF2 (Jones and Clemmons, 1995). The IGFBPs will determine the bioavailability and localisation of IGF to and within tissues. Thus, they can inhibit (by preventing IGF binding to IGF1R) or potentiate the biological activity of IGF1 or IGF2 (by prolonging IGF half-life/aiding presentation of IGF to the IGF1R). Plasma IGF1 and IGF2 are largely bound by IGFBPs, particularly IGFBP3. The IGFBPs are present in the circulation and extravascular fluids, will

prolong the half-life of the IGF and modulate their bioavailability activity (Le Roith et al., 2001). The tissue microenvironment can be dynamic with IGFBPs and IGF1R competing free IGFs. The binding affinities of IGFBPs are further altered by phosphorylation, partial proteolysis and adhesion to cell surface or extracellular matrix (LeRoith, 2003). The principal regulation of IGFBP is by proteolysis, which will augment or decrease their activity, adding a further layer of complexity to the IGF system. There is redundancy in the IGFBP family with multiple IGFBPs being expressed within the same tissue simultaneously (Bar et al., 1990). Furthermore, some IGFBPs display IGF-independent actions (Perks et al., 1999, Kim et al., 2004).

#### 1.5.3.1. IGFBP1

IGFBP1 is a 25 kDa protein that accounts for a very little proportion of the IGF1/IGF2 carrying capacity in the blood. It is unable to bind to the cell surface or extracellular matrix (Allard and Duan, 2018). IGFBP1 is synthesised in the liver and regulated by hormones and catabolic factors. For example, stress, hypoxia and starvation strongly induce hepatic IGFBP1 expression (Maures and Duan, 2002, Kajimura et al., 2006). In addition, the production of IGFBP1 is suppressed by insulin but is 4-5-fold increased during fasting (Busby et al., 1988). IGFBP1 has been shown to have IGF-independent actions (Mohan and Baylink, 2002).

#### 1.5.3.2. IGFBP2

IGFBP2 is a 31 kDa protein that binds to the cell surface or extracellular matrix (Allard and Duan, 2018). It is the second most abundant IGFBP in plasma but does not bind to ALS. IGFBP2 in serum is unsaturated and has an excess reservoir of binding capacity (Tönshoff et al., 2005, Clemmons, 2007). IGFBP2 expression is sensitive to changes in the energy balance status of the animal (Jones and Clemmons, 1995). IGFBP2 has been generally considered to bind the IGFs (Duan and Xu, 2005).

#### 1.5.3.3. IGFBP3

IGFBP3 is a 29 kDa protein, which has been shown to both promote and inhibit cellular growth by affecting the action of IGFs. Additionally, IGF-independent actions of IGFBP3 have been reported (Granata et al., 2004). IGFBP3 is the most abundant IGFBP in the blood with a high affinity for both IGF1 and IGF2 and accounts for 75–80% of the total carrying capacity. IGFBP3 binds either IGF1 or IGF2 in a 1:1 ratio. The binding of IGFs by

IGFBP3 will limit their bioavailability, but at the same time extend the plasma half-life of IGFs (Clemmons, 2007).

The principal tissue responsible for production of plasma IGFBP3 is the liver (Blum et al., 1993, Fenwick et al., 2008). The expression of IGFBP3 is regulated by metabolic status (Velazquez et al., 2008) with *IGFBP3* mRNA levels increased by insulin, IGF1 (Phillips et al. 1998) and GH (De Meyts et al., 1995). Presumably, this will enable a higher capacity to bind circulatory IGFs. Conversely, during severe catabolism, IGFBP3 is proteolytically degraded which results in a lower total IGF binding capacity thus reducing the growth-promotion by IGFs (Bereket et al., 1995). IGFBP3 concentration is decreased in blood during lactation (Davis et al., 1992).

#### 1.5.3.4. IGFBP4-6

IGFBP4 is a 24 kDa protein that does not bind to the cell surface as it lacks an Arg-Gly-Asp (RGD) sequence that enables binding to the extracellular matrix (Allard and Duan, 2018). IGFBP4 has been generally associated with binding the IGFs and inhibiting their action (Baxter, 2000, Duan and Xu, 2005).

IGFBP5 is a 29 kDa protein that binds to the cell surface/extracellular matrix and heparin. These particular structural features of IGFBP5 enable it to have important modulatory roles in cell survival, apoptosis and differentiation (Beattie et al., 2006).

IGFBP6 is a 34 kDa protein which has a major function in the regulation of IGF2 actions (Martin et al., 1994). IGFBP6 has a much greater affinity for IGF2 compared with IGF1 and generally has an inhibitory action. Thus, it is considered a "specific inhibitor" of IGF2 actions. Not surprisingly, IGFBP6 expression is associated with inhibition of growth *in vitro* and *in vivo* (Bach, 1999).

### 1.5.4. Role of the IGF system in ovarian function

#### 1.5.4.1. The expression of the IGF system during folliculogenesis and luteal development

IGF1 and IGF2 are potentially important intra-ovarian growth factors that are involved in the regulation of follicular development in many species (Zhou et al., 1997, Mazerbourg et al., 2003). IGF1 expression was increased during the transition of preantral follicles to gonadotrophin-dependent

follicular stage in cattle and rodents (Gong et al., 1991, Chase et al., 1998). IGF1 expression was observed using immunostaining in theca cells of bovine follicles while *IGF1* mRNA expression in granulosa cells and was further increased after selection (Schams et al., 1999). In contrast, IGF1 is not thought to be important in the development of ovarian follicles in humans. Moreover, IGF2 was more abundantly present in large dominant follicles than IGF1 (Dor et al., 1992). IGF1 mRNA and protein was not expressed in mouse follicles, but IGF2 was present (Rappolee et al., 1992). *IGF2* mRNA was localised to both bovine granulosa and theca cells with greater *IGF2* mRNA levels in theca cells of the dominant follicle than other follicles (Aad et al., 2013). Collectively, this would indicate that local IGF2 is more important than local IGF1 in respect to ovarian function.

IGF1R expression has been detected in most tissue and cell types. *IGF1R* mRNA was present in both theca and granulosa cells of dominant follicles in cows (Schams et al., 1999, Walsh et al., 2012a) and humans (Baumgarten et al., 2014). Locally produced IGF2 or systemic IGF exerts its effects through IGF1R present within multiple cell types within the follicle (Spicer et al., 2004, Spicer and Aad, 2007). The IGF bioavailability and IGFBP concentrations change during both growth and atresia of ovarian follicles. In ruminants, IGFBP1 expression was absent from the bovine ovary however, in human ovaries, *IGFBP1* mRNA expression was present in the granulosa cells of matured follicles (Schams et al., 1999). *IGFBP2* mRNA expression is present in rat granulosa cells (Liu et al., 1993). IGFBP6 was detected at very low levels in the granulosa cells of healthy and atretic antral follicles and was more abundant on the primate ovarian surface epithelium (Arraztoa et al., 2002).

In the bovine CL, *IGF1* mRNA expression was detected at low levels during the early luteal phase with increased expression observed in mid and late luteal phase. Utilising immunostaining, IGF was localised mainly in large and small luteal cells with some staining in the ECs (Schams et al., 1999). In contrast, IGF2 protein has also been localised to perivascular fibroblasts of large luteal blood vessels and pericytes of the capillaries (Amselgruber et al., 1994). Using *in situ* hybridization, *IGF2* mRNA expression was also localised to the steroidogenic luteal cells while *IGF1R* mRNA was widely observed in the large and small luteal cells (Woad et al., 2000). Similar approaches in the ovine CL, *IGFBP4* mRNA was found at low concentration (Perks and Wathes, 1996).

### 1.5.4.2. The activities of IGF1 and IGF2 during folliculogenesis

Both IGF1 and IGF2 can stimulate either granulosa cell proliferation or differentiation, depending on the stage of follicle development though IGF2 was present in greater concentrations in follicular fluid than IGF1 (Spicer et al., 1988). Indeed, *in vitro* studies showed that IGF1 was a potent stimulator of bovine granulosa cell differentiation by promoting the secretion of progesterone (Spicer and Aad, 2007). While in cyclic cows, exogenous IGF1 increased oestradiol concentrations in follicular fluid of small (2–5 mm) but not large (≥6 mm) follicles (Spicer et al., 2000). In sheep, IGF1 was a potent stimulator of follicle growth and oestradiol secretion by enhancing the action of gonadotrophins on steroidogenesis (Scaramuzzi et al., 1999). Increased IGF1 expression and/or bioavailability up-regulated the expression of FSH receptors (Zhou et al., 1997). While, in the rat thecal cells IGF1 increased *HSD3B1* and *LH receptor* mRNA expression (Magoffin and Weitsman, 1994). Similarly, IGF enhanced LH-induced androstenedione production by thecal cells mainly by increasing the numbers of LH receptors (Stewart et al., 1995, Campbell et al., 1998). There are reports of an interaction between FSH and IGF2, with IGF2 enhancing FSH-stimulated granulosa cell proliferation and steroidogenesis in cattle (Spicer and Aad, 2007) and human dominant follicles (Baumgarten et al., 2015).

IGF binding proteins (IGFBPs) can modulate the effects of IGF1 by transporting the ligand in circulation and extracellular fluids. *In vitro* studies with pig follicles, showed that IGF1 significantly stimulated IGFBPs production in a dose-dependent manner (Grimes and Hammond, 1992). The decreased *IGFBP2* and *IGFBP4* mRNA expression during follicular growth and in contrast increased expression during atresia, partly explains changes the intrafollicular levels of IGFs seen in many species. Furthermore, in *ex vivo* studies, there was a large decrease in *IGFBP2* and *IGFBP4* mRNA expression in granulosa cells during follicular growth in bovine (Armstrong et al., 1998), ovine (Besnard et al., 1996), porcine (Liu et al., 2000), but not in primate (Arraztoa et al., 2002), and human ovaries (Kwon et al., 2010). Furthermore, IGFBP2 and IGFBP4 intrafollicular levels were increased in atretic follicles in sheep (Monget and Monniaux, 1995).

### 1.5.4.3. The activities of IGF1 and IGF2 on luteal function

IGFs have a direct stimulatory effect on progesterone production and key expression of steroidogenic protein in luteal cells in many animal species

(Sauerwein et al., 1992, Silva et al., 2009). *In vitro*, IGF2 increased progesterone production in bovine luteal cells (Sauerwein et al., 1992), and pig granulosa cells where it increased CYP11A1 enzyme activity (Garmey et al., 1993). Moreover, there is a synergistic effect between IGF1 and LH on steroidogenesis in cows (Stewart et al., 1995) while in rodents, the key steroidogenic proteins (STAR, CYP11A1 and HSD3B1) mRNA and protein expression was increased in response of IGF1 stimulation (Magoffin and Weitsman, 1993).

## 1.6. The vascular system and angiogenesis

The vascular system constitutes a network of blood vessels that is crucial for all mammals, by supplying the body systems with oxygen, nutrients and hormones to grow and function adequately. It also aids elimination of metabolic products and carbon dioxide from the tissues and organs, for detoxification by the liver and excretion by the kidney (Flamme and Risau, 1992, Risau and Flamme, 1995). With respect to the ovary, the vascular system is vital to supply gonadotrophins and hormone precursors for steroidogenesis and then transport synthesised hormones to target sites (Molina and Molina, 2006). Arteries are the vessels that transport oxygenated blood from the lungs to the tissues while the exchange of oxygen, nutrients and waste products takes place in the thin-walled vessels of the capillary bed. The de-oxygenated blood is carried back to the heart through the veins and to the lungs where it becomes reoxygenated.

Blood vessels are comprised of an endothelium (a monolayer of ECs lining the lumen), a thin basal lamina and vascular smooth muscle cells. In the larger vessels (arteries and veins), a single or multiple layers of vascular smooth muscle cells surround and stabilise the vessels as well as enabling vessel contraction. In contrast, the microvessels are covered by occasional mural cells called pericytes, which are the same lineage as the smooth muscle cells and whose role is to promote vessel stabilisation (Gerhardt and Betsholtz, 2003).

### 1.6.1. Vasculogenesis

Vasculogenesis is the *de novo* process whereby the primitive vascular network is formed from mesoderm-derived precursor cells known as endothelial progenitor cells or angioblasts. Vasculogenesis involves three major steps; 1) hemangioblast stem cell and angioblast cell induction through FGF2 stimulation; 2) primordial vessel assembly mediated by VEGFA; 3) vasculogenesis to angiogenesis transition (Fig. 1.13) (Zygmunt et al., 2003).

In the embryo, a morphological feature known as a "Blood Island" is formed and consists of hemangioblasts. These are common precursors for both blood cells and blood vessels. The hemangioblasts in these blood networks differentiate to form an inner mass of hematopoietic stem cells and an outer layer of angioblasts, which eventually differentiate into endothelial progenitor cells (EPCs) (Huber et al., 2004). EPCs then develop into flattened ECs and join together to form small vascular structures that then fuse to form vessels. These small capillaries lengthen and interconnect, to form a primitive vascular network (Schoenwolf et al., 2014). This vascular network further develops throughout the embryo by angiogenesis and intercalation of new EPCs into the walls of existing blood vessels (Schoenwolf et al., 2014).

The development of hemangioblasts/angioblasts from mesodermal precursors is influenced by bone morphogenetic protein 4 (BMP4) and FGF2 (Faloon et al., 2000, Coutu and Galipeau, 2011), whereas VEGFA drives the differentiation of angioblasts into ECs (Fig. 1.13) (Drake et al., 2000, Poole et al., 2001).

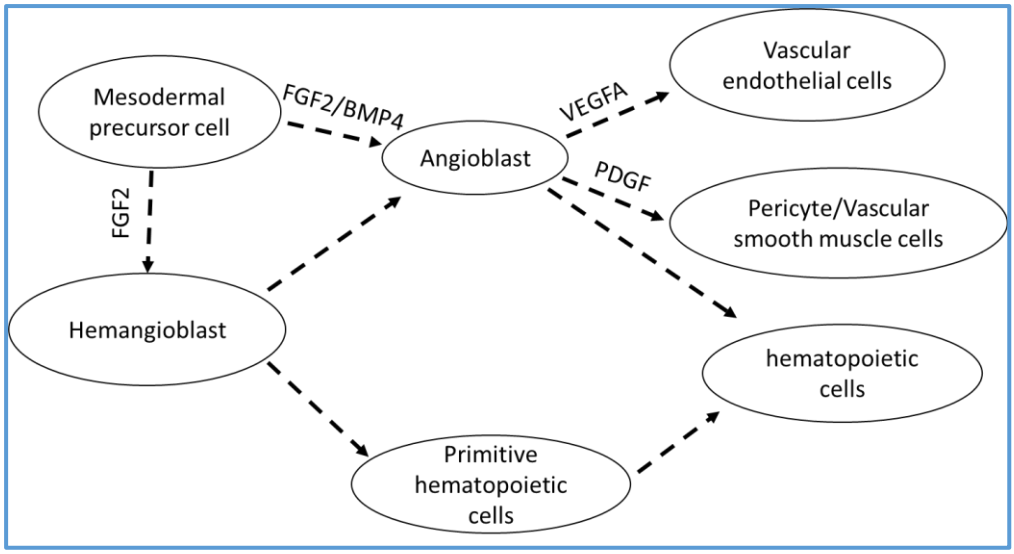



Fig. 1.13: The development of blood vessels by vasculogenesis. The mesodermal precursor cell (endothelial progenitor cells) will form a primitive vascular network by differentiating into hemangioblast stem cells and angioblast cells through FGF2 stimulation. VEGFA and PDGF stimulate angioblast differentiation into blood vascular EC and pericytes/vascular smooth muscle cells respectively. Hematopoietic cells are produced from both angioblast and primitive hematopoietic cells. Adapted and modified from (Nilsson, 2006).

## 1.6.2. Angiogenesis

Angiogenesis is the formation of new blood vessels from existing vasculature (Risau, 1997, Schams and Berisha, 2004). The angiogenic process starts with the targeted breakdown of the existing vasculature, through EC activation that enables ECs to form tip cells and sprout towards the angiogenic stimulation. The stalk EC will follow and undergo proliferation, which is followed by lumen formation, and maturation (Clapp et al., 2009). Angiogenesis forms a novel microcirculation, resulting in the vascularisation of formerly avascular tissues and organs (Burri and Djonov, 2002).

## 1.6.3. The process of angiogenesis

The initial angiogenic step involves the destabilisation of the existing vessel wall through protease action and resulting in vessel permeability. Next, sprouting of vessels as ECs differentiate into tip cells and migrate as the extracellular matrix (ECM) degrades. (Fig. 1.14). The cascade of events involved in the angiogenesis is initiated release of angiogenic growth factors (e.g. VEGFA) that bind to their respective receptors on ECs. This activates intracellular signal transduction pathways inducing EC tip cell formation and

sprouting (Fig. 1.14). These activated EC will migrate under the influence of the angiogenic factor concentration gradient towards the poorly vascularised tissue. VEGFA, at the same time, induces vascular permeability by reducing the formation of tight junctions. As the EC tip cells migrate they will deposit new ECM (e.g. fibronectin) along which the EC stalk cells can migrate and proliferate. In so doing, an elongated new vessel structure is formed. The next step is the formation of the lumen and re-establishment of the EC integrity via expression of tight junctions such as VE-cadherin. The final step is the stabilisation of the vasculature, which occurs through the recruitment of smooth muscle cells and pericytes (Fig. 1.14) (Robinson et al., 2009, Clapp et al., 2009). This latter process is principally regulated by the platelet-derived growth factor (PDGF) system.

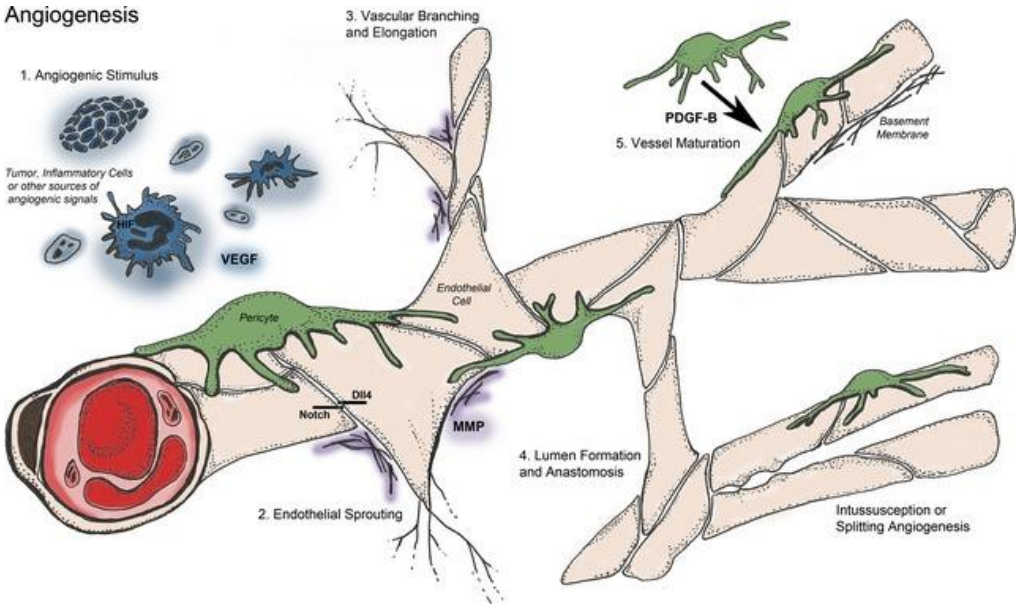



Fig. 1.14: The cascade of events involved in the angiogenesis process (1) Angiogenic factor production stimulation release growth factors – (VEGFA, NOTCH, DLL4, MMP) that bind to ECs and activate intracellular signal transduction pathways, (2) ECs are mobilized/activation and become incorporated into new blood vessels branching and elongation, (3) stimulating endothelial proliferation, migration, extracellular matrix remodeling (4) vascular tube formation and stabilisation of the vasculature occurs through the recruitment of smooth muscle cells and pericytes, (5) Vessel maturation by PDGFB activity (Logsdon et al., 2014). Available via license: CC BY 3.0

## 1.6.4. Vasculature and angiogenesis in the ovary

### 1.6.4.1. Follicular vasculature and angiogenesis

All primordial and primary follicles lack an independent capillary network and are dependent on vessels nearby in the ovarian stroma for oxygen, nutrients and hormone supply (Martelli et al., 2009). The transition from a primary to secondary follicle includes the acquisition of multiple avascular layers of granulosa cells. As follicles develop, they require the formation of an individual blood supply through the process of angiogenesis. As the follicle develops an antrum, the theca layer acquires a vascular sheath comprised of two capillary networks located in the theca interna and theca externa, respectively. The inner vascular sheath becomes flattened adjacent to the basement membrane that separates the avascular granulosa cell layer from the increasingly vascular theca (Stouffer et al., 2001). The acquisition of adequate vascular supply is considered a rate-limiting step in the selection and maturation of the dominant follicle programmed to ovulate (Stouffer et al., 2001). Indeed, spent media from pig granulosa cells collected from various follicle sizes revealed that spent media from small follicles enhanced EC sprouting, while that from larger follicle enhanced elongation of capillaries (Grasselli et al., 2003).

The capillary distribution occurs unevenly in different inner theca regions of the follicles (Jiang et al., 2003). While the vasculature is located in the theca layer, it is the granulosa layer where most angiogenic factors are produced, which then act on the theca layers (Plendl, 2000, Fraser and Wulff, 2001). In the large follicles, it appears that angiogenesis is less intense and prior to ovulation the vessels will vasodilate (Jiang et al., 2003). The physiological degeneration of the capillary beds results in follicular atresia caused by disrupted metabolic supply to the follicle. More importantly, the inhibition of angiogenesis leads to the attenuation of follicular growth, disruption of ovulation and severe detrimental effects on luteal development (Stouffer et al., 2001).

### 1.6.4.2. Luteal Angiogenesis

After ovulation, the basal membrane between the theca and granulosa layers breaks down, allowing the theca capillaries to sprout into the previously avascular granulosa layer. ECs comprise around 85% of the dividing cells in the early CL of primates (Stouffer et al., 2001), and sheep (Jablonka-Shariff et al., 1993). In the cow, EC proliferation reaches its peak at 2-3 days after

ovulation (Acosta et al., 2003). The period of intense angiogenesis can be 4-20-fold more intense than that of a tumour, and contributes to a rapid increase in size from 0.5 g to >5 g in 5 days in cattle (Stouffer et al., 2001). The establishment of a vascular system requires both mural cell recruitment as well as EC proliferation (Reynolds and Redmer, 1999, Fraser and Lunn, 2001). The recruitment of smooth muscle cells is regulated by ECs and helps to stabilise vessels and control their tone. In the smaller microvessels, pericytes ensheath capillaries to influence their function (Amselgruber et al., 1999, Redmer et al., 2001, Stouffer et al., 2001). The luteal vascularisation will be maximally developed by the mid-luteal phase (Watson and Fraser, 2003, Wulff et al., 2001a).

In luteolysis, there is a transient increase in luteal blood flow followed by the degeneration of both vasculature and steroidogenic cells (Hazzard and Stouffer, 2000). At the onset of CL regression, apoptosis occurs in the microvascular ECs (Davis et al., 2003) with the loss of tight junctions between ECs and disruption in the permeability barrier of the vascular wall. Consequently, the total volume of blood vessels decrease during early luteolysis in many species (Stouffer et al., 2001). During early luteolysis, the larger microvessels are maintained while there is dissolution of the smaller microvasculature that induces loss of luteal structure and function.

#### 1.6.4.3. Ovarian blood flow

Studies have shown that blood flow area and velocity in the preovulatory follicular wall were elevated after LH surge. These were correlated with the increase in plasma concentration of oestradiol-17β and the LH surge (Acosta et al., 2003, Acosta et al., 2004). After ovulation, luteal blood flow increases as the luteal volume and progesterone concentration increase (Herzog et al., 2011). The extensive vascularisation of the CL enables the highest blood flow per unit mass after ovulation (Reynolds and Redmer, 1999). Moreover, cows with lower mid-luteal phase progesterone concentrations had reduced luteal blood flow (Lüttgenau et al., 2011). Hence, it has been suggested that luteal blood flow is a better indicator of luteal function than luteal size (Matsui and Miyamoto, 2009, Herzog et al., 2010).

The high rate of blood flow in the CL depends on the composition and distribution of vascular smooth muscle and luteal blood vessels in the ovary (Wiltbank et al., 1990). The composition of the luteal structure is vascular endothelia cells (50%) and steroidogenic large and small luteal cells (30%)

(O'Shea et al., 1989). The pericytes around capillaries dilate and constrict to modulate blood flow and this can be influenced by nitric oxide (NO) production. Intriguingly, FGF2 and VEGFA regulate endothelial nitric oxide synthase (eNOS) expression in luteal pericytes (Beckman et al., 2006). It should be recognised that accurate measurement of luteal blood flow remains a great challenge in both livestock and humans. This challenge is related to the luteal microvasculature and the difficulty in detecting the extensive number of small blood capillaries (Beindorff et al., 2009).

### 1.6.5. The regulation of angiogenesis

The regulation of neovascularisation involves a fine balance between pro- and anti-angiogenic factors. Indeed, the formation of new vessels is downregulated in the normal state in most tissues (Skarzynski et al., 2013). Inappropriate growth factor stimulation can lead to undesirable and uncontrolled blood vessel growth, whilst the inhibition of vasculature formation impairs tissue remodelling or wound healing (Carmeliet et al., 2009). Numerous stimulatory and inhibitory angiogenic factors have been identified and are described in Table 1.3 and 1.4 respectively.

Table 1.3: Key pro-angiogenic factors detected in the ovary. EC: Endothelial cell. ECM: Extracellular Matrix.

| Angiogenic Factors | Isoforms | Receptors | Biochemical properties | Functions | References |
|---|---|---|---|---|---|
| Vascular endothelial growth factor A (VEGFA) | VEGFA121, VEGFA145, VEGFA165, VEGFA189, VEGFA206. | VEGFR1 (FLT1), VEGFR2 (KDR), VEGFR3 (FLT4) | Binds to heparin and ECM (except VEGFA121) VEGFA121, 145 and 165 are secreted and soluble while VEGFA189, 206 are cell-associated | Vascular permeability, detachment of pericytes, degradation of the basement membrane.<br>EC proliferation, migration, and tube formation. | (Ferrara et al., 2003, Robinson et al., 2009) |
| Fibroblast growth factor 2 (FGF2, basic FGF) | 18 kDa (cytoplasmic), 22–34 kDa (nuclear) | FGFR1-4 and their isoforms (tyrosine kinase activity) | Heparin-binding | EC sprout formation, proliferation, migration and tube formation. Pericytes proliferation and migration. | (Presta et al., 2005, Woad et al., 2012, Laird et al., 2013) |
| Platelet-derived growth factor (PDGF) | PDGFA,<br>PDGFB | PDGFRA, PDGFRB (both tyrosine kinase receptors | Dimerisation required for activity PDGF-AA, BB (homodimers) | Pericytes proliferation and migration. Involved in EC tip cell formation. | (Fredriksson et al., 2004) |
| Angiopoietin | ANGPT1,<br>ANGPT2 | Tie2 (tyrosine kinase receptor) ANGPT2 is an endogenous antagonist | - | Detachment of pericytes and basement membrane degradation. Maturation, EC lumen acquisition, re-attachment of pericytes and vessel maintenance. | (Maisonpierre et al., 1997, Augustin et al., 2009) |
| Epidermal growth factor (EGF) | EGF | EGFR: intracellular tyrosine kinase. | Heparin binding peptide | EC proliferation and differentiation. | (Schneider and Yarden, 2014) |

Table 1.4: Anti-angiogenic factors detected in the ovary. EC: Endothelial cell. ECM: Extracellular Matrix.

| **Angiogenic Factors** | **Isoforms** | **Receptors** | **Biochemical properties** | **Functions** | **References** |
|---|---|---|---|---|---|
| Vascular endothelial growth factor A (VEGFA, b isoform) | VEGFA121b, VEGFA145b, VEGFA165b, VEGFA189 VEGFA206b | Inhibitory action in VEGFR1 and VEGFR2 | Binds to heparin and ECM (except VEGFA121b) VEGFA121b, 145b and 165b are secreted and soluble while VEGFA189b, 206b are cell-associated | Inhibits EC proliferation, migration, and tube formation. | (Glass et al., 2006, Robinson et al., 2009) |
| Thrombospondin | TSP1 and TSP2 | CD36 Integrin-associated protein (IAP, CD47) | High molecular weight glycoprotein Heparin binding ECM associated | TSP1: induces endothelial apoptosis and destabilises EC contacts.<br>TSP2: inhibits EC migration, modulates cell adhesion and antichemotactic. | (Armstrong and Bornstein, 2003) |
| Transforming growth factor–β (TGFB) | TGFB1, TGFB2, TGFB3. | TGFRB1, TGFBR2 | Non-heparin binding peptide (dimers) | Cellular inhibition and differentiation. Stimulates pericytes differentiation, ovarian angiogenic morphogenesis, Extracellular matrix deposition. | (Nasu et al., 2005) |

#### 1.6.5.1. Vascular endothelial growth factor A

Vascular endothelial growth factor A (VEGFA) is considered the pro-primary angiogenic growth factor responsible for regulating both physiological and pathological angiogenesis. VEGFA is expressed in many different tissues and cell types, including the ovary (Augustin, 2000, Hazzard and Stouffer, 2000). Indeed, there was embryonic lethality in VEGFA knockout mice (Ferrara and Davis-Smyth, 1997, Ferrara, 2005). VEGFA is a potent and specific stimulator of vasodilation, promotes vascular EC proliferation, migration and differentiation resulting in the formation of new capillaries and immature vessels (Neeman et al., 2007). In addition, VEGFA increased vascular permeability of capillaries and post capillary venules (Stouffer et al., 2001, Redmer et al., 2001).

In the ovary, VEGFA controls the various EC functions through paracrine and/or autocrine mechanisms. VEGFA may influence primordial follicle activation and development, since VEGFA neutralisation resulted in primordial follicle loss without impact on other preantral follicles (Roberts et al., 2007). *In vivo*, follicular development and steroidogenic function are promoted by VEGFA (Zimmermann et al., 2002, Celik-Ozenci et al., 2003, Roberts et al., 2007). While short-term inhibition of angiogenesis with an anti-VEGFA blocking antibody interfered with normal development and function of dominant follicles in rhesus monkey (Zimmermann et al., 2001). Equally, VEGFA neutralisation or VEGF receptor inhibition also inhibited ovulation in human corpus luteum (Fraser et al., 2005). These treatments were associated with suppression of follicular angiogenesis, decreased thecal proliferation and EC area. There was also decreased granulosa cell proliferation and poor development of the ovulatory follicle. Consequently, ovulation was suppressed and there was an increased rate of follicular atresia (Wulff et al., 2002).

In the ovary, all VEGFA isoforms as well as the VEGFR1 and VEGFR2 have been detected (Stouffer et al., 2001). In cattle, *VEGFA* mRNA and protein was temporally expressed during follicular growth and development (Greenaway et al., 2005). Namely, granulosa cells of both primordial and primary follicles were VEGFA-negative, while the pre-antral follicles were weakly positive for VEGFA but its expression was high in the granulosa cells of larger antral follicles (Greenaway et al., 2005). Similarly, in pigs, VEGFA expression increased with follicle size and diameter (Ferrara et al., 2003). The theca interna cells of developing follicles also immunostained intensely

for VEGFA suggesting that VEGFA might be responsible for the formation of the primitive capillary network in the early stage of antral follicle development (Macchiarelli et al., 1993). In non-human primates, *VEGFA* mRNA was also absent from primordial, and primary preantral follicles but appeared when the vascular network was formed in the theca layer of secondary follicles (Field et al., 2014). Moreover, it was suggested that VEGFA acting through VEGFR2 controls the recruitment-selection process of follicles in the non-human primates (Zimmermann et al., 2002). *In vitro*, VEGFA production was increased by human chorionic gonadotrophin (hCG) treatment while VEGFA promoted granulosa cell survival (Christenson and Stouffer, 1997, Wang et al., 2002).

Luteal angiogenesis is critically regulated by VEGFA, with VEGFA neutralisation suppressing luteal EC proliferation and vascularisation that resulted in diminished steroidogenic function (Wulff et al., 2001b). The greatest expression of VEGFA and VEGFR2 were observed during early CL development, with their declining expression as the luteal phase progressed and minimal levels being present during luteal regression (Abramovich et al., 2009). VEGFA was localised to EC and steroidogenic cells while VEGFR1 and VEGFR2 were mostly localised in luteal vasculature in cattle (Hünigen et al., 2008).

In many tissues, VEGFA expression is principally induced by exposure to hypoxic conditions and down-regulated by normoxic condition (Ferrara, 2005). Indeed, in luteal cells, hypoxia was a primary regulator of VEGFA expression (Jiang et al., 2011a). This aspect is discussed further in Section 1.7. *VEGFA* mRNA and protein expression was stimulated by LH in primate granulosa cells (Christenson and Stouffer, 1997) and VEGFA was increased in follicular fluid after the LH surge (Hazzard et al., 1999). In bovine and primate granulosa cells, LH acted synergistically with IGF1 to stimulate VEGFA secretion (Martinez-Chequer et al., 2003, Schams et al., 2001).

#### 1.6.5.2. Fibroblast growth factor 2

Fibroblast growth factor 2 (FGF2) is also an important regulator of ovarian angiogenesis (Berisha et al., 2000, Berisha et al., 2004). It is a potent EC mitogen (Gospodarowicz et al., 1985, Miyamoto et al., 1992) that regulates steroidogenesis, cell differentiation and neovascularisation (Fraser and Lunn, 2001, Jiang et al., 2011b). Furthermore, local neutralisation of FGF2 in the bovine CL resulted in reduced CL size and progesterone (Yamashita

et al., 2008). *FGF2* mRNA was abundant in the theca cells of rat preovulatory follicles (Koos and Olson, 1989). While, in bovine follicles before the LH surge, FGF2 protein was localised to the cytoplasm of thecal ECs and pericytes but was absent from granulosa cells (Stirling et al., 1991, Berisha et al., 2006). Interestingly, after the LH surge FGF2 localisation shifted to the nucleus of most granulosa cells, before returning to the ECs in the developing CL. Furthermore, *FGF2* mRNA and protein expression were increased by LH (Berisha et al., 2006, Laird et al., 2013).

FGF2 plays a vital role in early luteinisation in the cow (Robinson et al., 2007). FGF2 protein was highly expressed in the early bovine CL (day 1-2) and its expression decreased rapidly after the follicular-luteal transition (Robinson et al., 2007). Using an *in vitro* luteinising follicular angiogenesis culture system, EC network formation was promoted by FGF2 and was critically dependent on FGFR1 (Laird et al., 2013). In the bovine CL, FGF2 appears to be particularly important for promoting EC sprouting and branching (Woad et al., 2012).

FGF2 signals through FGFR1 and FGFR2, with FGFR1 being highly expressed in both the follicle and CL (Schams and Berisha, 2004). FGFR1 was localised to ECs at all stages of follicular and luteal development but was particularly abundant in the larger microvessels. In contrast, FGFR2 was present at low levels in luteal steroidogenic cells at all stages. Bovine luteal ECs in culture were stimulated to grow in response to FGF2 and VEGFA (Robinson et al., 2009, Woad et al., 2009). Inhibition of FGFR1 signalling reduced the total area and number of EC networks by 94% compared to the controls, while VEGFR2 inhibition resulted in a smaller 60% reduction in the total area of EC networks in a bovine *in vitro* culture system (Woad et al., 2009). FGFR1 inhibition was effective at multiple time points, suggesting that FGF2 promotes endothelial sprouting, vascular branching, and EC proliferation in a dose-dependent manner in the preovulatory follicle (Laird et al., 2013) and CL (Woad et al., 2012).

### 1.6.6. The role of the IGF system in angiogenesis

IGF1 has also been shown to promote EC migration (Grant et al., 1987) and the formation of EC vessel-like tubes when grown on ECM (Nicosia et al., 1994, Papapetropoulos et al., 1997). IGF1 and IGF2 are mitogenic and anti-apoptotic factors for vascular smooth muscle cells and ECs (Arnqvist, 2008).

Given, these activities, it is not surprising that IGF1/IGF2 are involved in early vascular development. It is believed that this is an indirect action by inducing VEGFA synthesis (Wang et al., 1998, Hoeben et al., 2004, Kim et al., 2012). In human umbilical vein ECs (HUVEC), most IGF1 actions are mediated through IGF1R while IGF2 probably acts more through the insulin receptor (Bid et al., 2012). The action of the IGFs in regulating angiogenesis as expected are further influenced by the IGFBPs. Notably, IGFBP2 increased angiogenesis via transcriptional regulation of VEGFA (Azar et al., 2011) while the knockdown of IGFBP2 resulted in impaired vessel formation (Wood et al., 2005). Also, IGFBP2 increased EC migration *in vivo* through a IGF1-IGF1R dependent pathway (Png et al., 2012).

In contrast, the effects of IGFBP3 are conflicting with reports of IGFBP3 increasing EC apoptosis and antagonising VEGFA action (Granata et al., 2004). Conversely, in HUVECs, IGFBP3 was anti-apoptotic and positively regulated angiogenesis through its interaction with IGF1R signalling by up-regulation of proangiogenic genes, such as VEGFA and matrix metalloproteinase 2 (MMP2) (Granata et al., 2007).

IGFBP4 has generally been associated with an inhibitory effect on angiogenesis (Moreno et al., 2006). For example, IGFBP4 inhibited EC tube formation induced by IGF1 as well as those induced by VEGFA, and FGF2 suggesting that IGFBP4 effects might be IGF-independent as well. IGF1-independent actions of IGFBP4 have been demonstrated in other cell systems (Perks et al., 1999, Singh et al., 1994). Furthermore, IGFBP4 may bind to as yet unidentified endothelial receptor capable of inhibiting pro-angiogenic signalling pathways (Moreno et al., 2006). In addition, IGFBP4 inhibited angiogenesis induced by IGF1 and FGF2 but not VEGFA and this inhibition depended on p38MAPK signalling pathways (Contois et al., 2012). Furthermore, recombinant IGFBP4 blocked the potent effects of IGF1 on EC sprouting, while the overexpression of IGFBP4 suppressed tube formation by HUVECs (Gealekman et al., 2014). Similarly, IGFBP5 inhibited VEGFA-induced proliferation, invasion, and tube formation of HUVECs, while the overexpression of IGFBP5 prevented the migration and tube formation of ECs both *in vitro* and *in vivo* (Rho et al., 2008).

## 1.7. Hypoxia and its role in regulating angiogenesis

Hypoxia is a reduction in the oxygen level in the local tissue microenvironment. At the cellular and molecular level, hypoxia can occur

due to an imbalance between the supply and demand of oxygen. Hypoxia occurs in both pathological and physiological environments of organ (ovary), tissue (follicle, CL) and cells type (endothelial) (Lee et al., 2004, Chen et al., 2009). In mammals, a constant and regular supply of oxygen via the vasculature is absolutely vital for tissue development and homeostasis (Semenza, 2007). Thus, if there is a disrupted supply of oxygen (i.e. inadequate vasculature) or increased demand (i.e. increased tissue metabolic rate) then hypoxia will ensue (Carmeliet, 2005). Decreased oxygen delivery causes vascular dysfunction and pathologies (Carmeliet, 2005).

In biological systems, hypoxia is a relative term, as the normal physiological oxygen concentration ($pO_2$) varies from tissue to tissue (Table 1.5). Namely, arterial blood has normal $pO_2$ of 13%, while $pO_2$ in myocardium is nearer 10% with most highly perfused tissues lower still at ~5%. Most reports have indicated that the ovary is near to 5% with no definite percentage has been determined due to the cyclical nature of events that occur in the ovary (Carreau et al., 2011). At the other end of the scale, poorly perfused tissue such as cartilage and bone marrow, are often below 1% oxygen (Stockwell, 1991, Braun et al., 2001, Parmar et al., 2007). A range of tissues and their $pO_2$ are shown in Table 1.5. Often, low oxygen and its molecular sequelae play a critical role in the development of the pathogenesis of many diseases, with the classic situation being tumourigenesis. The process that monitors oxygen homeostasis is through the regulation of hypoxia-inducible factor (HIF) activity; a transcription factor responsible for activation of multiple genes to counteract the hypoxic conditions.

Table 1.5: Estimated Oxygen concentration in different tissues.

| **Body tissue** | **Oxygen level** | | **Reference** |
|---|---|---|---|
| | mmHg | % | |
| Atmospheric oxygen | 150-160 | 20-21 | (Bradley et al., 1978, Broxmeyer et al., 1985) |
| Heart | 72 | 10 | (Roy et al., 2003) |
| Arterial blood | 100 | 13.2 | (Kety and Schmidt, 1948) |
| Lung | ~43 | 5.6 | (Le et al., 2006, Ivanovic, 2009) |
| Liver | ~40 | 5-6 | (Wölfle and Jungermann, 1985, Jungermann and Kietzmann, 1997, Brooks et al., 2004) |
| Kidney | ~72 | 9-11 | (Brezis and Rosen, 1995, Welch et al., 2001) |
| Oviduct and Uterus | - | 2-8 | (Fischer and Bavister, 1993) |
| Ovarian follicle | - | 1-5 | (Van Blerkom et al., 1997, e Paula et al., 2008, Redding et al., 2008) |
| Brain | ~34 | 4-5 | (Whalen et al., 1970, Meixensberger et al., 1993) |
| Muscle | ~29 | 3.5-4.5 | (Kiaer and Kristensen, 1988, Boekstegers et al., 1990) |

### 1.7.1. Hypoxia-inducible factor

#### HIF1A and B subunits

Hypoxia-inducible factor (HIF) is a heterodimeric basic helix-loop-helix transcriptional factor comprising of HIF1A and HIF1B subunits (Wang and Semenza, 1995, Semenza et al., 1996, Chen et al., 2009). HIF1B is also known as aryl hydrocarbon receptor nuclear translocator (ARNT). The activity and expression of HIF1A depends on the cellular oxygen concentration while HIF1B is constitutively expressed in the cell nucleus and its activity is not affected by hypoxia (Jiang et al., 1996, Wenger, 2002, Bruick, 2003).

#### HIF1A isoforms and structures

HIF1A, like its homologues HIF2A and HIF3A contains an oxygen-dependent degradation domain (ODD) (Huang et al., 1999) that is regulated by prolyl hydroxylation (PHD) (Maxwell et al., 1999, Cockman et al., 2000, Kamura et al., 2000). This then interacts with von Hippel-Lindau (pVHL) (Tanimoto et al., 2000, Jaakkola et al., 2001) and E3 ubiquitin ligase complex that targets HIF1A for proteasomal degradation under normal oxygen condition (Salceda and Caro, 1997, Berra et al., 2001). In contrast, HIF1A protein will be protected and rapidly accumulate under hypoxia. The stabilised HIF1A protein will translocate from the cytoplasm to the nucleus where it dimerises with HIF1B and the HIF complex to become transcriptionally active (Wang and Semenza, 1995). The expression of HIF2A and HIF3A is more restricted than HIF1A which is expressed ubiquitously under hypoxia (Chen et al., 2009). HIF1A activates the expression of genes through binding to hypoxia response elements (HRE) with the core sequence 5'-RCGTG-3' located in the enhancer and promoter regions (Wang and Semenza, 1995, Dunwoodie, 2009). It is the basic helix-loop-helix motifs in HIF1A that binds to the HRE (Aragonés et al., 2009, Pasanen et al., 2010). In the presence of oxygen, an asparagine residue in the C-terminal transactivation domain of HIF1A is hydroxylated by HIF asparaginyl hydroxylase, and this posttranslational modification essentially blocks the recruitment of transcriptional coactivators (Mahon et al., 2001, Wenger and Katschinski, 2005). HIFs bind to HREs of over 200 genes of which about 70 have been extensively studied (Laughner et al., 2001).

## 1.7.2. The target genes and processes of HIF1A

The expression of several pro- and anti-angiogenic factors are modulated the hypoxic microenvironment thereby creating a mechanism by which angiogenesis is controlled (Chen et al., 2009). Some target genes for HIF action increase oxygen transport in the blood by promoting red blood cell maturation (e.g. erythropoietin (EPO), transferrin), angiogenesis (e.g. VEGFA, endothelin 1), availability of glucose to maintain metabolic activity (i.e. glucose transporters or regulatory glycolytic enzymes), and cell death (apoptotic Bcl-2 protein, BNIP3). The functions and roles of the HIF1 target genes are as follows:

### 1.7.2.1. Stimulation of angiogenesis

VEGFA is a hypoxia-inducible gene that is a key player in physiological and pathological angiogenesis. VEGFA expression is regulated by a variety of stimuli including hypoxia, nitric oxide (NO) and other growth factors (Klagsbrun and D'Amore, 1996). In most tissues, hypoxia induces the synthesis of VEGFA thereby controlling angiogenesis and the local blood flow of the microenvironment (Kimura et al., 2001). VEGFA mediates physiological angiogenesis and increases the permeability of blood vessels (Ferrara et al., 2003). VEGFA will then act on ECs 1) to increase vascular permeability and 2) to activate ECs such that they have a "tip" cell phenotype and migrate towards the region of hypoxia. Consequently, new vasculature is formed and tissue then is supplied to adequate oxygen and hypoxia wanes and VEGFA expression is switched off (Thibeault et al., 2010).

### 1.7.2.2. Glucose metabolism

Glycolysis provides two molecules of ATP for each glucose molecule, whereas in the tricarboxylic acid (TCA) cycle and oxidative phosphorylation provides an additional 36 molecules. However, oxidative phosphorylation requires oxygen and hence under hypoxic microenvironment, cells switch to the oxygen-independent metabolic pathways where they use glycolysis as a primary mechanism of ATP production. The end product of glycolysis is pyruvate which is converted to lactate by lactate dehydrogenase (LDH). This conversion enables the recycling of $NAD^+$ and thus the continuation of glycolysis. Enhanced lactate production will lead to a decrease in cellular pH potentially limiting the source of ATP despite sufficient glucose supply.

HIF1 regulates the expression of several key regulatory enzymes in the glycolytic pathway, as well as glucose transporters (GLUT1 and GLUT3) that mediate cellular glucose uptake (Wenger, 2002, Rankin and Giaccia, 2008, Behrooz and Ismail-Beigi, 1997). This will increase glucose uptake and thus supply of glucose for glycolysis. Furthermore, hypoxia induces the expression of phosphoglycerate kinase 1 (PGK1) (Firth et al., 1994, Semenza et al., 1996, Chen et al., 2001). Hypoxia also induces the expression of lactate dehydrogenase. Collectively, these proteins promote cellular adaptation to reduced oxygen availability (Wenger, 2002).

#### 1.7.2.3. Cell death (apoptosis)

HIF1 plays a role in regulating tissue homeostasis under conditions of chronic hypoxia by promoting cell apoptosis (Carmeliet et al., 1998). Specifically, the gene encoding the pro-death factor Nip3 (BNIP3) is induced in response to hypoxia by HIF1 complex binding to the located within its promoter (Bruick, 2000). Hypoxic conditions increased HIF1, BNIP3 and Caspase 3 expression in bovine luteal cells *in vitro* (Nishimura et al., 2006, Nishimura et al., 2008, Nishimura et al., 2018). The induction of apoptosis by hypoxia was partially responsible for the decreased progesterone production in these cells. Furthermore, *BNIP3* mRNA expression was higher in bovine granulosa cells under hypoxia (3%), while granulosa cells of the large follicles (>10 mm) had higher *BNIP3* mRNA and protein levels than small follicles (2–8 mm) (Nishimura et al., 2018). Also, *BNIP3* mRNA and protein expression in the CL was maximal during the early luteal stage indicating that hypoxia might be present at this stage.

#### 1.7.2.4. Cell growth (proliferation and survival)

In granulosa cells, contrary to expectations rapid cell proliferation occurs under relatively low oxygen condition during follicular development. Additionally, mathematical modelling has revealed that intrafollicular oxygen conditions are hypoxic (Redding et al., 2008) with follicular fluid having $pO_2$ concentration of 1.3-5.5% and 5.5% (Van Blerkom et al., 1997). In bovine granulosa cells, low oxygen (5%) increased cell proliferation and upregulated HIF1A associated genes (Shiratsuki et al., 2016). In contrast, bovine granulosa cell proliferation was reduced under 5% oxygen when cells were cultured at a higher temperature (Zeebaree et al., 2018). In bovine luteal cells, hypoxia increased cell proliferation in the early luteal phase suggesting

that hypoxic conditions could promote CL formation (Klipper et al., 2010, Jiang et al., 2011a).

## 1.7.3. Hypoxia in ovarian physiology

This next section explores the role of hypoxia in regulating ovarian physiology further, with focus on its role in follicular and luteal development.

### 1.7.3.1. Changes in hypoxia during follicular development

It is feasible that hypoxia plays a pivotal role in regulating follicular angiogenesis process through stimulation of VEGFA expression in granulosa cells. Indeed, oxygen concentrations in the follicular fluid of larger antral follicles are lower than that in small antral follicles in cows (Paula et al., 2008), pigs (Basini et al., 2004) and women (Redding et al., 2008). Moreover, hypoxia increased VEGFA expression in swine granulosa cells without impairing cell growth and proliferation (Basini et al., 2004). In primates, nuclear HIF1A immunostaining was largely absent in growing pre-antral and antral follicles but there was marked up-regulation of nuclear HIF1A in the granulosa cells of preovulatory follicles (Duncan et al., 2008). While, the inhibition of VEGFA *in vivo* up-regulated luteal nuclear HIF1A immunostaining in a time-dependent manner presumably by inducing hypoxia. Their results also indicated that follicular HIF1A was up-regulated by both hypoxia and hCG *in vivo* and *in vitro* (Duncan et al., 2008).

There is also potential involvement of hypoxia and HIF1 in the regulation of steroidogenesis. Under hypoxic conditions (1–5% oxygen), progesterone production was reduced in rat (Koos and Feiertag, 1989) and porcine granulosa cells (Basini et al., 2004) as well as bovine luteal cells (Nishimura et al., 2006, Jiang et al., 2011a). This has been associated with decreased *CYP11A1* mRNA and activity (Nishimura et al., 2006). However, it is unclear whether hypoxia inhibits also aromatase activity in granulosa cells in bovine.

There is an interaction between LH/cAMP signalling and hypoxia in the regulation of HIF1A expression in granulosa cells. Namely, the induction of hypoxia with cobalt chloride elevated HIF1A protein level in human granulosa cells. Furthermore, LH treatment combined with cobalt chloride synergistically augmented the level of HIF1A protein. These findings suggest that HIF1A protein level is regulated transcriptionally by LH and post-translationally through protein stabilisation under hypoxic conditions (Yalu et al., 2015). In mouse granulosa cells transfected with a mutated HRE in

the VEGFA gene promoter, it was demonstrated that FSH and LH-induced VEGFA expression *in vitro* was ablated. This finding clearly supports a gonadotrophin mediated, HIF-dependent mechanism for VEGFA production (Rico et al., 2014).

#### 1.7.3.2. Hypoxia during ovulation and in the early luteal phase

In the immediate post-ovulatory collapsed follicle, the blood supply is disrupted and thus there is likely to be hypoxia. Indeed, there is accumulation, nuclear localisation and activity of HIF1 proteins after ovulation (Boonyaprakob et al., 2005, Duncan et al., 2008, Tam et al., 2010). In contrast, HIF1A and HIF1B have not been found in the CL at the later stages of the luteal phase (Nishimura and Okuda, 2010, Jiang et al., 2011a). Similarly, in the human CL, nuclear HIF1A protein in granulosa–lutein cells was highest during luteal formation and absent in functional CL (van den Driesche et al., 2008). In further agreement, induction of HIF1A and HIF2A was found in the mouse ovary following hCG-induced ovulation (Kim et al., 2009). Moreover, echinomycin (a small molecular inhibitor of HIF1), blocked ovulation in mice via binding to HRE indicating that HIF signalling is essential for ovulation as well as regulating the expression of VEGFA (Kim et al., 2009).

There are conflicting results on the effects of oxygen concentrations on HIF1A expression in luteal-derived cells in culture. For example, culturing bovine or murine luteal cell in hypoxic conditions (2–3% oxygen) increased HIF1A and VEGFA expression (Nishimura and Okuda, 2010, Tam et al., 2010). In contrast, bovine luteal ECs under normoxic (20% oxygen) and hypoxic (1% oxygen) conditions had similar expression of *HIF1A* and *VEGFA* mRNA (Tscheudschilsuren et al., 2002). An explanation for the lack of hypoxic effect is that HIF1A regulation often occurs at the protein rather than transcription level (Salceda et al., 1997). Some evidence has shown that LH signalling can directly upregulate HIF1A in granulosa-lutein cells (van den Driesche et al., 2008, Tam et al., 2010).

## 1.8. Aim of the Ph.D. thesis

The central aim of this thesis was to elucidate the role and regulation of angiogenesis during key stages of ovarian development and function. This thesis has utilised two different models to study ovarian angiogenesis: 1) an *ex vivo* model of the fetal sheep ovary and 2) a physiologically relevant *in vitro* follicular luteinising angiogenesis system.

The sheep fetal ovary model was designed to mimic the potential effects of maternal protein restriction that occur during early lactation in dairy cows that are pregnant. The key endpoints, which assessed the fetal ovarian morphology and development, were germ cell numbers and degree of development, germ cell proliferation and apoptosis and vascularisation to and within the fetal ovary.

The *in vitro* luteinising follicular angiogenesis system modelled the changes that occur during ovulation and the follicular-luteal transition. The principle endpoints of this system are the degree of EC network formation and its pattern and the production of progesterone. This system was used to evaluate the effects of the IGF system and different oxygen concentrations.

### 1.8.1. Hypothesis

It is widely perceived that inadequate vascularisation of the follicle and/or CL will lead to reduced ovarian function and thus fertility. Based on this concept, the overarching hypothesis was that ovarian and follicular-luteal angiogenesis are regulated by nutrition and its metabolic signals as well as being stimulated by hypoxia.

### 1.8.2. Objectives of the experimental chapters

In order to test this hypothesis, three experimental chapters were conducted with the following objectives:

1.) To determine the effects of maternal dietary protein restriction on the development of the fetal sheep ovary and its vascularisation.
2.) To investigate the role that the activation of IGF1R by IGF1 and/or IGF2 has on the ability of bovine luteinising follicular cells to develop EC networks and produce progesterone.
3.) To examine the impact of reduced oxygen concentration on the degree of EC network formation and progesterone production in bovine luteinising follicular cells.

# Chapter 2: Materials and methods

All methods that are shared between several experimental chapters are detailed herein. Experimental chapters with independent methods not used elsewhere are detailed in the appropriate chapter. General laboratory consumables were sourced from Fisher Scientific, Loughborough, United Kingdom (UK) or Sigma-Aldrich, Poole, UK.

## 2.1. Bovine luteinising follicular angiogenesis culture system

An *in vitro* culture system was used to mimic the follicular-luteal transition phase and to investigate the regulation of angiogenesis and steroidogenesis, as previously validated (Robinson et al., 2008, Woad et al., 2012, Laird et al., 2013).

### 2.1.1. Preparation of coverslips and fibronectin coating

Coverslips (circular, 19 mm diameter and 0.25 mm thick) were pre-soaked in 70% ethanol (v/v) for at least 1 hour, air-dried for 1 hour and transferred to a 12-well plate (Sigma-Aldrich, Poole, UK) ready for culture. Fibronectin (500 µl, 20 µg/ml; Sigma-Aldrich, Poole, UK) was added per well and incubated at 39ºC for 4 hours, thereafter the fibronectin was removed and allowed to air-dry in the incubator at 39ºC overnight. The following day, the wells were washed in 1 ml of sterile water and equilibrated with 1 ml of MCDB 131 media (Sigma-Aldrich, Poole, UK) and left in the incubator at 39ºC until required (maximum 3 days).

### 2.1.2. Media supplements and preparation

MCDB 131 media (Sigma-Aldrich, Poole, UK) is a specialised endothelial cell (EC) media and was prepared according to the manufacturer's instructions (Table 2.1). It is important that in some of the specific experiments individual reagent(s) were omitted or replaced (see respective Chapters for details). Insulin (Fisher Scientific, Loughborough, UK), transferrin (Sigma-Aldrich, Poole, UK) and selenium (Sigma-Aldrich, Poole, UK) were added individually at final concentrations of 100 ng/ml, 5 ng/ml and 5.5 µg/ml respectively. To the supplemented media, 0.5% (v/v) fetal calf serum (FCS; Sigma-Aldrich, Poole, UK) was added for the first 18 hours only and thereafter the FCS was omitted. Thus, for the majority of the culture period, the cells were in serum-

free media with 1 mg/ml BSA used as the protein source (Laird et al., 2013). The addition of FCS for the first 18 hours helped to maintain a confluent monolayer until the end of culture.

Table 2.1: Composition of MCDB 131 endothelial cell media (500 ml)

| **Supplement** | **Final concentration** |
|---|---|
| $LR^3$ insulin like growth factor-1* | 20 ng/ml |
| Luteinising hormone (AFP11743B: Torrance, CA, USA) | 5 ng /ml |
| ITS or added individually insulin, transferrin and selenium* | 1× |
| Human epidermal growth factor* | 5 ng/ml |
| Ascorbic acid* | 1 µg/ml |
| Bovine serum albumin (Fisher Scientific) | 0.1% |
| Heparin* | 0.02 units/ml |
| Hydrocortisone* | 25 nM |
| Gentamycin sulphate* | 50 µg/ml |
| Amphotericin B* | 50 ng/ml |
| Penicillin/Streptomycin* | 1× |

* Sourced from Sigma-Aldrich, Poole, UK

### 2.1.3. Bovine ovary collection and isolation of follicular cells

The harvesting of follicular cells (granulosa and theca cells) from large bovine follicles and subsequent culture procedures were based on previously published methods (Robinson et al., 2008, Laird et al., 2013). Bovine ovaries were collected from the local abattoir and transported to the laboratory in warmed PBS. Unpaired ovaries were visually inspected and selected (n=10-12) for dissection based on the presence of large antral follicles (10–16 mm diameter) with clear fluid and visible vascularisation. Ovaries were sprayed with 70% (v/v) industrial methylated spirits (IMS) and rinsed in sterile PBS. The culture procedures henceforth were performed under aseptic conditions throughout to minimise risk of contamination. All glassware and surgical instruments were autoclaved prior to culture and all cell culture media were sterile filtered through a 0.22 µm filter.

For each culture, 10–12 large antral follicles were dissected out using a mixture of sharp and blunt dissection. Follicular fluid was aspirated from each follicle using a 10 ml syringe and discarded. The aspirated follicles were then hemisected, and an inoculation loop was used to gently scrape the granulosa cells into 20 ml of M199 media (Sigma-Aldrich, Poole, UK) with 25 µg/ml DNase type I (Sigma-Aldrich, Poole, UK). Clumps of granulosa cells were minimised through quick processing. After collection into a 50 ml falcon tube, the granulosa cells were centrifuged at 300 g for 10 minutes and the cell pellet was washed with MCDB 131 media. Then the supernatant was discarded and the cell pellet re-suspended into 10 ml of MCDB 131 media. This was repeated 2 further times, pipetted by pastette and filtered through a 70 µm cell strainer. The cells were then centrifuged (300 g, 10 minutes) and the final cell pellet was resuspended in 2 ml of MCDB 131 with 2% FCS before cell counting.

Theca shells were gently teased from the follicular wall and then digested with 20 ml M199 media containing 5 mg/ml collagenase type I (Sigma-Aldrich, Poole, UK), 1 mg/ml hyaluronidase type IS (Sigma-Aldrich, Poole, UK) and 25 µg/ml DNase type I (Sigma-Aldrich, Poole, UK) for 30-45 minutes at 37ºC in a water bath. After the dissociation period, the digestion was stopped by adding 1 ml of FCS, pipetted and cells were sieved through a 70 µm cell strainer for clump removal. The dispersed theca cells were centrifuged (300 g, 10 minutes), after which the supernatant was discarded

and the cell pellet re-suspended into 10 ml of MCDB 131 media. Any red blood cells were lysed by adding 5 volumes of lysis buffer (155 mM ammonium chloride, 10 mM potassium bicarbonate and 0.01% (w/v) EDTA) for 5 minutes. The cells were then immediately centrifuged at 300 g for 10 minutes and the subsequent cell pellet washed in MCDB 131 media. Then the supernatant was discarded and the cell pellet re-suspended into 10 ml of MCDB 131 media. This was repeated 2 further times. The final cell pellet was resuspended in 2 ml of MCDB 131 with 2% FCS before cell counting.

Cell number and viability was assessed using a haemocytometer and trypan blue solution (Sigma-Aldrich Poole, UK) added in a 1:1 ratio (Johnson et al., 2013) under 40× magnification. A typical cell yield was considered to be around 2-4 × $10^6$ cells/ml (theca) and 3-5 × $10^6$ cells/ml (granulosa) with viability of around 70–80% (theca) and 30-40% (granulosa). Granulosa cells were diluted to 3 × $10^5$ viable cells/ml and theca cells to 1 × $10^5$ viable cells/ml. Then, 1ml of each cell type was plated into each well of the fibronectin coated 12-well plate (final density of 4 × $10^5$ cells per well).

The dispersed luteinising granulosa and theca cells were cultured at 39ºC in a humidified incubator in 5% $CO_2$/95% air for up to 5 days. After the first 18 hours, the cells were washed with 1 ml of sterile PBS to remove any cell debris and unattached cells. Then, fresh serum-free MCDB 131 media were replaced and appropriate treatments initiated according to each experimental design. The culture was monitored daily to observe cell density and morphology and confirm the absence of microbial contamination. On day 3 and 5 of culture, the spent media were collected (and replaced). The collected spent media were stored at -20ºC until analysis, to assess the production of progesterone over time thus confirming the progress of luteinisation. On day 5 of culture, the cells were fixed in cold acetone: methanol (1:1) at 4ºC for 10 minutes, then washed twice in PBS for 5 minutes each, prior to immunocytochemical assessment.

## 2.2. Immunostaining

### 2.2.1. Bovine ovarian tissues

Adult bovine ovarian tissue sections from ovarian cortex cross section (n=3), large antral follicles (10-20 mm, n=3), day 5 corpus luteum (n=3) and day 8 corpus luteum (n=2) were microtomed at 10 µm by Mrs Amanda Robinson, University of Nottingham. Two sections were mounted onto poly-L-lysine

microscope slides and then baked dry at 50ºC. Two slides from each of the above samples were used for the immunolocalisation of IGF1R (see Table 2.2 and Section 2.2.3).

### 2.2.2. Colourimetric immunocytochemistry

The expression of several target proteins were investigated using fixed cells following periods in culture; primary antibodies are detailed in Table 2.2. Endothelial cells were immunostained for von Willebrand factor (VWF) as described previously (Robinson et al., 2008, Laird et al., 2013) with minor modifications. Mural cells (pericytes) were immunolocalized with alpha-smooth muscle actin (ACTA2) immunocytochemistry (Skalli et al., 1989). The expression of IGF1R and HIF1A was immunolocalised according to the company's datasheets.

### 2.2.3. Standard immunohistochemistry or immunocytochemistry protocols

Immunohistochemistry of ovarian tissue sections and immunocytochemistry of cultured cells used similar general protocols, detailed below.

The ovarian sections were dewaxed in xylene (2 x 5 minutes) then rehydrated in decreasing concentrations of ethanol (100%, 95%, and 70%) for 2 minutes each and then washed in PBS (2 × 5 minutes).

Antigen retrieval was performed by immersing the tissue slides in pre-warmed 10 mM citrate buffer (pH 6.0), microwaving (900 W microwave) on a medium control for 2 × 5 minutes and then allowed to cool for 30 minutes to recover antibody-binding sites. The slides then were washed in PBS for 2 × 5 minutes. These steps were omitted when staining cultured cells.

Endogenous peroxidase activity (in sections or cells) was blocked using 3% (v/v) hydrogen peroxide in methanol for 10 minutes, followed by washing in PBS (2 × 5 minutes). Non-specific binding was blocked by incubating with the appropriate blocking solution (Table 2.2) in PBS for 30 minutes at room temperature (RT). After this, excess protein blocking solution was tipped off from the slides/12-well plate without washing. Slides/wells were then incubated with the appropriate primary antibody (Table 2.2) for 2 hours at RT and then washed with PBS solution (2 × 5 minutes). Both immunohistochemistry and immunocytochemistry negative control

slides/wells were incubated with the relevant IgG at equivalent concentration to the primary antibody being tested.

Afterwards, slides/wells were incubated with the appropriate biotinylated secondary antibody diluted in PBS according to the manufacturer's instructions (Vectastain Elite, ABC system, Vector Laboratories, Peterborough, UK) at RT for 30 minutes. Tissue sections/wells were then washed twice in PBS for 5 minutes. Afterwards, the avidin-biotin complex (prepared 30 minutes prior to application; Vector Laboratories, Peterborough, UK) was added and incubated for 30 minutes. After the incubation, tissue sections/wells were washed twice in PBS for 5 minutes. Diaminobenzidine tetrahydrochloride (DAB) substrate (Vector Laboratories, Peterborough, UK) was prepared according to the manufacturer's recommendations and incubated for 5-10 minutes. Tissue sections/wells were then rinsed with slow running tap water for 5-10 minutes.

The tissue sections/cells were then counterstained in Harris' haematoxylin for 20 seconds, rinsed in slowing running tap water for 5 minutes and 1% (v/v) ammoniated water added for 30 seconds. The tissue sections/cells were then dehydrated in increasing concentrations of ethanol (70%, 95%, and 100%) for 2 minutes each. Coverslips were then removed from each well and dipped in xylene (2 x 20 seconds) before mounting onto uncoated microscope slides using DPX mounting medium (Fisher Scientific). Tissue sections were dipped twice in xylene (2 x 5 minutes), before mounting in DPX mounting medium.

### 2.2.4. Image capture for bovine ovarian tissues and cultured follicular cells

The immunostained fixed cultured follicular cells and bovine ovarian tissues were captured under 5X, 20X and 40X magnification using a light microscope (DM500B, Leica Microsystems, Germany) and camera (DFC350FX, Leica Microsystems, Germany). Image analysis was conducted for cultured follicular cells using Image ProPlus 6.3 (Media Cybernetics, Marlow, UK). The bovine ovarian tissue images were used as picture representation of each ovarian tissue. The images were not analysed but were used for the immunolocation of the specific protein in the bovine ovarian tissues.

Table 2.2: Details of the antibodies used in immunohistochemistry and immunocytochemistry

| **Antigen** | **Source** | **Host Species** | **Blocking solution** | **Final concentration** | **Secondary antibody** | **Sequence identity with bovine (%)** |
|---|---|---|---|---|---|---|
| Human VWF | Dako (A0082) | Rabbit, polyclonal | 20% NGS | 5 µg/ml (in 2% NGS) | Goat anti-rabbit | 81 |
| Human IGF1R | Novus (NBP1-77680) | Rabbit, polyclonal | 20% NGS | 25 µg/ml (in PBS) | Goat anti-rabbit | 100 |
| Human ACTA2 | Sigma-Aldrich (A2547) | Mouse, monoclonal | 4% NHS | 5 µg/ml (in PBS) | Horse anti-mouse | 100 |
| Human HIF1A | ThermoFisher (H1alpha67) | Mouse, monoclonal | 4% NHS | 25 µg/ml (in PBS) | Horse anti-mouse | 94 |

NGS, Normal goat serum, NHS, Normal horse serum

## 2.2.5. Dual immunofluorescent detection

Dual immunofluorescent detection of HIF1A or IGF1R plus isolectin B4 (endothelial cell (EC) marker) was conducted to determine whether HIF1A or IGF1R were localised to EC in the culture system. The luteinising follicular cells were cultured and fixed as previously described (Section 2.1.3).

The cultured fixed follicular cells were washed twice in tris-buffered saline (TBS) for 5 minutes and then blocked in 4% NHS for 30 minutes. The primary antibodies: 20 µg/ml mouse anti-human HIF1A (Thermo Fisher, Loughborough, UK) or 20 µg/ml mouse anti-human IGF1 receptor (Novus) were added to the wells with 25 µg/ml biotinylated *Griffonia (Bandeiraea) simplicifolia* isolectin B4 (Vector Laboratories, Peterborough, UK).

The primary antibodies/isolectin B4 were incubated in a humidified chamber for 2 hours at RT. Thereafter, the cells were washed twice in TBS for 5 minutes before adding 20 µg/ml Texas-Red anti-mouse IgG (Vector Laboratories, Peterborough, UK) and avidin DCS, fluorescein labelled (20 µg/ml, Vector Laboratories, Peterborough, UK) for 1 hour at room temperature. Afterwards, the cells were washed twice in TBS for 5 minutes, the coverslips were removed and 3 drops of Vectashield with DAPI (Vector Laboratories, Peterborough, UK) was added before mounting on a slide. This was then sealed with nail varnish and stored for 2–3 hours at -20°C before image capture and analysis.

Several images were taken under the 40× objective lens using a Leica DM 5000 B fluorescence microscope, equipped with a Leica DFC 350 fx camera (Leica Microsystem) for each coverslip.

## 2.2.6. Image analysis of von Willebrand factor staining

The VWF immunostaining was quantified as previously described (Robinson et al., 2008). Wells were visualised under 5× magnification using a Leica-DM4000B microscope (Leica Microsystems, Germany). According to each experiment, two wells per treatment were selected for image analysis. A total of 12 random fields of view were captured from across each coverslip.

The images were analysed using Image ProPlus 6.3 (Media Cybernetics, Marlow, UK) as follows. Firstly, the images were further processed by tiling into 2 × 2 tiles, resulting in three tiled images being analysed per coverslip. The areas of brown VWF-positive staining were then roughly drawn round using the kidney-shaped "area of interest" tool and these were selected for

further analysis. The specific brown VWF staining was then selected using the "colour select tool", using either manual colour cube (1 × 1 pixel) tab to manually select positive staining or manually adjusting the "histogram" tab until the appropriate staining was identified. Next, the selected area of staining was "measured" with the minimal area set at 500 $\mu m^2$. The selection was then "smoothed" and endpoints (area, perimeter, count) recorded for each tile. An EC network was defined as a group of >3 EC in close proximity to each other (i.e. a cluster). The count was the number of EC networks. This was repeated for each tile and the mean area, perimeter and number (count) of EC networks were determined.

In order to determine the number of branch points, the selected area of staining was extracted from the image and then sorted according to size onto a light grey background. Then any dark objects were automatically detected and from which a black and white mask was created. From this mask image, white areas were automatically detected and the "filters" tool used to dilate (11×11 circle) the selected area. The branch/end (with 50% threshold and triple/4+branches) tool was then used to determine the number of branch points in the particular image. The degree of branching was calculated by dividing the number of branch points by the number (count) of EC networks. The mean area of each EC network was calculated by dividing the total area of EC by the number (count) of EC networks. Each coverslip was analysed separately and incorporated into the statistical analysis as individual replicates.

## 2.3. Assays

A number of different assays were performed on the spent media (collected on day 3 and/or 5) and cells from the luteinising follicular angiogenesis culture system. The concentration of progesterone, IGF1 and VEGFA were determined by ELISA, while cell proliferation and lactate concentrations were determined by MTT or L-Lactate Assay.

### 2.3.1. Determination of progesterone production *in vitro*

The progesterone concentration of spent media were determined using an in-house ELISA as previously described (Claycomb et al., 1998). The first step was to prepare the key reagents as follows:

*Progesterone standards:* Progesterone (1 mg; Sigma-Aldrich, Poole, UK) was carefully weighed into a glass tube and 10 ml of ethanol added. This created

a stock solution of 100 µg/ml that was stored at -20ºC. The stock was then diluted 1:1000 in PBS to give a 100 ng/ml solution, which was then serially diluted (1 in 2) in ELISA buffer (PBS containing 0.1% BSA, pH 7; see Appendix) to yield a range of standards (0.195 – 50 ng/ml).

*Progesterone HRP conjugate:* Ultrapure water (250 µl) was added to the progesterone HRP conjugate (Sigma-Aldrich, Poole, UK) which was vortexed and allowed to reconstitute for 30 minutes. This was then aliquoted (10 µl) and stored at -20ºC until use. To each aliquot, 990 µl of ELISA buffer was added to give a 1:100 congugate that was stored at 4ºC for up to 1 month. Prior to analysis, the conjugate was diluted to a working concentration of 1:12,500 by adding 40 µl of 1:100 conjugate to 5 ml of ELISA buffer.

*Progesterone antiserum:* Ultrapure water (400 µl) was added to the progesterone antiserum (Sigma-Aldrich, Poole, UK), vortexed and allowed to reconstitute for 30 minutes. The solution was then diluted 1:50 in a coating buffer (0.05 M sodium bicarbonate buffer, pH 9.6; see Appendix), aliquoted into 80 µl and stored at -20ºC until use. From each aliquot, 62.5 µl was added to 15 ml of coating buffer and mixed thoroughly before coating (final dilution of anti-serum, 1 in 12,000).

#### 2.3.1.1. Coating of ELISA plates

Progesterone antiserum (50 µl; 12,000 dilution) was added to all wells of a 96 well-plate (Maxisorp, Nunc), except for 3 non-specific binding (NSB) wells. The plates were gently shaken to ensure even spread of the progesterone antiserum across the well, then sealed with plate sealer and stored for at least 2 nights at 4ºC before use.

#### 2.3.1.2. Progesterone ELISA procedure

The antiserum-coated ELISA plate was washed 5 times with wash buffer (0.15 M NaCl plus 0.05% (v/v) Tween 20). Next, ELISA blocking buffer (PBS plus 1% BSA; see Appendix) was added to each well and incubated for 1 hour at RT on a plate shaker. The ELISA plate was washed 5 times with wash buffer before adding 50 µl of ELISA buffer to the 3 NSB wells. Then in triplicate, each progesterone standard (0–50 ng/ml) was added to the appropriate wells. The samples were diluted (1:100-200 fold depending on day of collection) in ELISA buffer prior to adding (50 µl) to the appropriate well of the ELISA plate in triplicate. Following this, 50 µl of 1:12,500 progesterone HRP conjugate was added to all wells and the ELISA plate

incubated on a plate shaker at RT. After 2 hours the plate was washed 5 times with wash buffer then thoroughly blotted dry. Then, 50 µl of 3, 3', 5, 5'-tetramethylbenzidine (1-step TMB, Thermo Fisher, Loughborough, UK) substrate was added to each well and incubated at RT on a plate shaker for 30 minutes. Sulphuric acid (2 M) was added to all wells to stop colour development. Optical density (OD) was then measured on an OPTIMA plate reader (FLUOstar OPTIMA, BMG-LABTECH, Germany) at a wavelength of 450 nm.

The mean OD for the NSB wells was subtracted from each OD sample/standard reading and then the sample/standard OD reading was expressed as a percentage of the OD for 0 ng/ml progesterone standard (total binding, TB). The % Binding/Total Binding (%B/TB) for each progesterone standard was plotted against the progesterone concentration and a 4-parameter logistic line fitted using GraphPad Prism (Fig. 2.1). The progesterone concentration of the sample was interpolated from this curve. The triplicate values were averaged for each culture well. Each experimental study was analysed in a single assay and the intra-assay coefficient of variation for each experiment ranged from 11 to 14%.

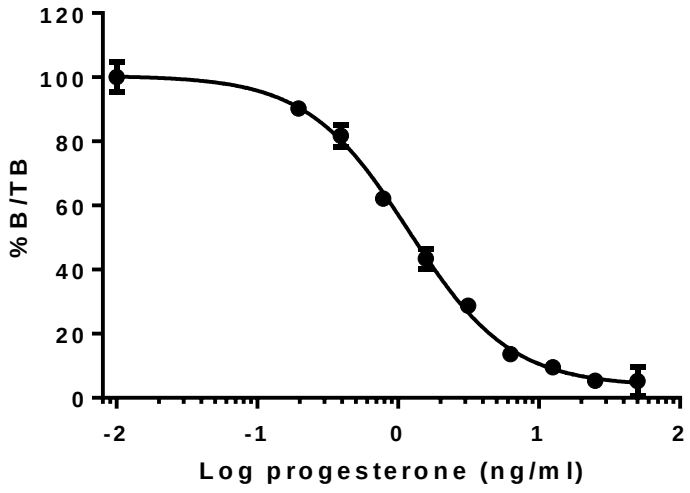



Fig. 2.1: A representative standard curve for the quantification of progesterone production in culture by ELISA. %B/TB is % Binding/Total Binding.

## 2.3.2. Determination of IGF1 production *in vitro*

The IGF1 concentration in the spent media was determined using a human IGF1 DuoSet ELISA that was performed according to the manufacturer's procedure (R&D Systems, Abingdon, UK). IGF1 concentrations were measured in triplicate, in undiluted spent media, with no pre-treatment of samples. The ELISA was designed against human IGF1, however, there is 100% homology with bovine IGF1. There is no cross-reactivity with IGF2 at 50 ng/ml. The following reagents were prepared before analysis:

*Mouse anti-human IGF1 capture antibody*: The antibody vial was reconstituted in 0.5 ml of PBS and then diluted in PBS without carrier protein to a stock concentration of 480 µg/ml. This was further diluted 1 in 120 to a working concentration of 4 µg/ml.

*Biotinylated goat anti-human IGF1 detection antibody:* The detection antibody vial was reconstituted with 1.0 ml of reagent diluent, which was then diluted further (1 in 60) to a working concentration of 0.15 µg/ml.

*Recombinant human IGF1 standards:* The IGF1 standard vial was reconstituted in 0.5 ml of distilled water (110 ng/ml) which was diluted 1 in 55 in distilled water to yield the top IGF1 standard of 2 ng/ml. This was then serially diluted on a seven point standard curve from 2000-31.2 pg/ml IGF1.

*Streptavidin-HRP conjugate:* Each vial contained 2.0 ml of streptavidin conjugated to horseradish-peroxidase. The working concentration was prepared by adding 300 µl of Streptavidin-HRP to 11.7 ml of Reagent Diluent.

### 2.3.2.1. Coating of ELISA plates

Each well in the 96-well plate (Nunc: MaxiSorp) was coated with 100 µl of 4 µg/ml human IGF1 capture antibody diluted in PBS. The plate was then sealed and incubated at RT overnight. The following morning, the plates were washed thrice with 250 µl of 0.05% Tween 20 in PBS and then blotted completely dry on paper towel. The plates were then blocked with 300 µl/well of Reagent Diluent and incubated for 1 hour at RT. After incubation, the plates were washed thrice with 250 µl of 0.05% Tween 20 in PBS.

### 2.3.2.2. IGF1 ELISA procedure

The assay was performed at RT. Reconstituted human IGF1 standards (0-2000 pg/ml) and neat spent media samples were added to the coated 96-

well plates (100 µl/well) in triplicate and incubated for 2 hours at RT. Next, the wells were washed thrice with 250 µl of 0.05% Tween 20 in PBS. After the washes, 100 µl of biotinylated goat anti-human IGF1 detection antibody was added to each well and incubated for 2 hours at RT. Next, the wells were washed thrice with 250 µl of 0.05% Tween 20 in PBS. Then, 100 µl/well streptavidin conjugated horseradish-peroxidase was added, and incubated for 20 minutes at RT after which the wells were washed thrice in 400 µl of 0.05% Tween 20 in PBS. Thereafter, 100 µl/well of substrate solution (R&D Systems, Abingdon, UK) was added and incubated for 30 minutes. The reaction was stopped by adding 50 µl/well of 2M sulphuric acid.

Optical density was measured on an OPTIMA microplate reader (FLUOstar OPTIMA, BMG-LABTECH, Germany) at a wavelength of 570 nm. A linear regression line was fitted through the standards and this was used to determine the unknowns (samples). The limit of detection was 50 pg/ml and the mean intra-assay %CV was 9.8%.

## 2.3.3. Determination of VEGFA production *in vitro*

The concentration of VEGFA in spent media was determined using a bovine specific VEGFA ELISA set (GenWay, United States of America (USA)) according to the manufacturer's recommendations. The reagents were prepared as follows:

*Bovine VEGFA detection antibody*: Bovine VEGFA detection antibody was reconstituted in 500 µl reagent diluent and then further diluted in 11.5 ml reagent diluent.

*Bovine VEGFA standards:* The stock VEGFA standard was reconstituted in 750 µl standard and sample diluent to a concentration of 30 ng/ml. Serial dilution (1 in 2) of this top standard was prepared by mixing 250 µl standard with 250 µl standard and sample diluent and repeating until a final concentration of 0.23 ng/ml.

*Streptavidin-HRP conjugate:* Streptavidin-HRP (500 µl) was diluted in 11.5 ml Reagent Diluent.

### 2.3.3.1. VEGFA ELISA procedure

The 96-well plates were purchased pre-coated with VEGFA antibody and stored at 4ºC. Plates were prepared for use by removing the liquid and blot-

drying on paper towel. The VEGFA standards and spent media samples were added (100 µl) to the appropriate well in duplicate, then the plate was sealed and incubated at RT for 1 hour. All the samples were assayed on a single 96-well plate. The plates were washed four times in 250 µl Wash Buffer and gently tapped to blot dry. Next, 100 µl of bovine VEGFA detection antibody was added to each well, and incubated at RT for 1 hour. Next the plates were repeatedly washed four times in 250 µl Wash Buffer and blotted dry. Then, 100 µl of Streptavidin-HRP Working Solution was added to each well and incubated for 30 minutes. The plate was then emptied and the wells washed 4 times with the 250 µl Wash Buffer and blotted dry. Then, 100 µl of TMB substrate solution was added to each well and the plate was incubated in the dark at RT for 30 minutes. The reaction was stopped by adding 100 µl of Stop Solution to each well.

Optical density was measured at 450 nm on an OPTIMA microplate reader (FLUOstar OPTIMA BMG-LABTECH, Germany). The VEGFA standards were logged and plotted using GraphPad Prism with a 4-parameter logistic regression fitted. The limit of detection was 0.25 ng/ml and the intra-assay %CV was 16.0%.

### 2.3.4. Determination of lactate production *in vitro*

Lactate concentrations were determined by Amplite colourimetric L-lactic acid (L-lactate) assay kit (AAT Bioquest Inc., Sunnyvale, California, USA). Lactate concentration was measured as the formation of nicotinamide adenine dinucleotide (NADH) from $NAD^+$. The reagents were prepared as follows:

*L-lactate assay solution:* NAD stock solution was dissolved in 100 µl of sterile distilled water, which created a 100× NAD stock solution. Next, 5 ml of L-lactate assay buffer was added to the bottle. To this, 50 µl of NAD stock solution (100×) was then added into the "Enzyme mix" bottle and mixed thoroughly.

*L-lactate standards:* L-lactate stock (100 mM) was diluted in PBS buffer to generate 1 mM L-lactate standard solution. This L-lactate standard solution was then serially diluted (1 in 3) to create a range of L-lactate standards from 0 to 1000 µM (0, 1.37, 4.12, 12.4, 37.0, 111, 333 and 1000 µM).

#### 2.3.4.1. L-Lactate assay procedure

Each L-lactate standard (50 µl) and spent media sample was added in duplicate to the appropriate wells of a standard 96-well microplate (flat-bottomed, Costar). For the assay blank, 50 µl L-lactate assay solution was added. Next, 50 µl of L-lactate assay solution was added to each well and then incubated at RT for 2 hours, protected from light.

Optical density was measured on an OPTIMA microplate reader (FLUOstar OPTIMA BMG-LABTECH, Germany) at a wavelength of 405 nm. The colour development in the assay blanks was subtracted from the OD values for the standard/samples. The log lactate concentrations were plotted against OD values and a 4-parameter logistic regression line fitted using GraphPad Prism. The lactate concentration of the sample was interpolated and the mean of the duplicates calculated. The intra-assay %CV was 15.9% and all samples were analysed in a single assay.

### 2.3.5. Determination of cell proliferation rate

Luteinising follicular cell proliferation/growth was measured using MTT cell proliferation assay kit (Cayman Chemical, USA) according to the manufacturer's instructions and as used previously (Cory et al., 1991, Berridge et al., 2005). The bovine luteinising follicular cells were cultured in 96-well fibronectin-coated plates; 100 µl granulosa cells ($1.5 \times 10^5$ viable cells/ml) plus 100 µl theca cells ($0.5 \times 10^5$ viable cells/ml) were plated (final density of $2 \times 10^4$ cells/well per well) in 6 replicate wells. The reagents were prepared as described below:

*Assay buffer:* The cell-based assay buffer tablet was dissolved in 100 ml of sterile distilled water and was stored at RT.

*MTT reagent solution:* The MTT reagent was dissolved in 5 ml of assay buffer, which was then vortexed.

*Crystal dissolving solution:* Immediately before use, a vial of crystal dissolving, sodium dodecyl sulphate (SDS) powder was dissolved in crystal dissolving, hydrochloride solution and thoroughly mixed to prepare the crystal dissolving solution.

#### 2.3.5.1 Cell proliferation assay procedure

Bovine luteinising follicular cells were cultured in MCDB 131 media for 48 hours before the MTT assay was performed. MTT reagent solution (10 µl) was added to the media in each well. The plate was gently mixed for 1 minute on a shaker and the cells were left to incubate for 4 hours at 39ºC in the humidified incubator. Next, 100 µl of crystal dissolving solution was added to each well and incubated for 18 hours at 39ºC in the humidified incubator as the solution dissolved the formazan crystals producing a purple solution.

The reaction solution in the 96-well plate was measured on an OPTIMA microplate reader (FLUOstar OPTIMA BMG-LABTECH, Germany) at a wavelength of 570 nm. Mean ± SEM values of the raw absorbance were plotted.

## 2.4. Western blotting

Western blotting was performed to evaluate the specificity of the primary antibodies and to quantify the expression of several proteins (Table 2.3-2.4) in the cultured bovine luteinising follicular cells: steroidogenic acute regulatory (STAR), P450 cholesterol side chain cleavage (CYP11A1), 3β-hydroxysteroid dehydrogenase (HSD3B1), smooth muscle actin (ACTA2), insulin-like growth factor 2 (IGF2), Histone cluster 3 H3 (HIST3H3), beta actin (ACTB), apoptotic BCL-2 protein-interacting protein 3 (BNIP3), glucose transporters 1 (GLUT1), hypoxia-inducible factor 1A (HIF1A), and phosphoglycerate kinase 1 (PGK1).

Table 2.3: The primary antibodies used for western blotting analysis.

| Target antigen | Supplier: ID | Dilution | Host / clonality | Sequence identity[1] % |
|---|---|---|---|---|
| *Steroidogenesis related* | | | | |
| Steroidogenic acute regulatory protein (STAR) | Abcam: ab96637 | 1:2000 | rabbit / polyclonal | 86 |
| P450 cholesterol side chain cleavage (CYP11A1) | Abcam: ab75497 | 1:1000 | rabbit / polyclonal | 68 |
| 3β-hydroxysteroid dehydrogenase (HSD3B1) | Dr Richard Parker | 1:800 | rabbit / polyclonal | NA |
| *Mural cell marker* | | | | |
| α-smooth muscle actin (ACTA2) | Sigma-Aldrich: A2547 | 1:1000 | mouse / monoclonal | 100 |
| *Insulin-like growth factor system* | | | | |
| Insulin-like growth factor 2 (IGF2) | Merck: S1F2: 05-166 | 1:2000 | mouse / monoclonal | 97 |
| *Hypoxia-regulated proteins* | | | | |
| BCL-2 protein-interacting protein 3 (BNIP3) | Novus: NB100-1543 | 1:500 | mouse / monoclonal | 95 |
| Glucose transporter 1 (GLUT1) | Sigma-Aldrich: (Clone SP168) | 1:200 | mouse / monoclonal | |
| Hypoxia-inducible factor 1α (HIF1A) | ThermoFisher: H1alpha67 | 1:500 | mouse / monoclonal | 94 |
| Phosphoglycerate kinase 1 (PGK1) | Novus: NBP1-55154 | 1:500 | rabbit / polyclonal | 98 |
| *Housekeeping proteins* | | | | |
| Histone H3 (HIST3H3) | Abcam: ab1791 | 1:30000 | rabbit / polyclonal | 100 |
| Actin, beta (ACTB) | GeneTex: GTX110564 | 1:50000 | rabbit / polyclonal | 100 |

[1]Amino acid identity against bovine sequence using BLAST; NA unknown.

Table 2.4: The secondary antibodies used for western blotting analysis.

| Secondary antibody | Dilution | Supplier: ID number |
|---|---|---|
| Goat anti-rabbit HRP conjugate | 1/80000 in blocking solution | Sigma-Aldrich: A9169 |
| Sheep anti-mouse HRP conjugate | 1/50000 in blocking solution | GE Healthcare: NXA931 |

### 2.4.1. Collection of cultured luteinising follicular cells and protein extraction

On day 5 of culture, the media was collected and to each well 300 µl of ice-cold 0.1 M Tris-HCl pH 7.4 with complete protease inhibitor (Sigma-Aldrich, Poole, UK) was added to the luteinising follicular cells. For the HIF1A western blotting analysis, 5 µg/ml of reconstituted MG-132 (Selleckchem, USA) was also added to inhibit proteasome activity and thus preserve protein stability. The cells were scraped off using the end of the pipette and/or cell scraper. Then, the detached cells were pipetted up and down, then placed in a microcentrifuge tube and centrifuged at 13000 g for 10 minutes. The supernatant was removed and the pellet resuspended with 100 µl of ice-cold complete cell extraction buffer (ThermoFisher, USA) with protease inhibitor cocktail (plus MG-132 as appropriate). The cells were incubated on ice for 30 minutes with regular vortexing. The lysed cells were then centrifuged at 13,000 g for 10 minutes. The lysate was collected in a clean microcentrifuge tube, aliquoted and then stored at -80ºC freezer until further analysis. The cell debris was discarded.

### 2.4.2. Bradford Assay for protein determination

Protein concentrations were determined by Bradford protein assay. Bovine serum albumin (BSA, Sigma-Aldrich, Poole, UK) was used to create standards by serially diluting (1 in 2) from 2 mg/ml to 7.8 µg/ml. The standards and diluted samples (10 µl) were added in duplicate and triplicate respectively to a 96 well plate (Nunc). PBS (10 µl) was added as the blank. Bradford solution (200 µl; Sigma-Aldrich, Poole, UK) was added to each well and then incubated on a plate shaker for 30 minutes at RT. The absorbance was read at 595 nm using a plate reader (FLUOstar OPTIMA BMG-LABTECH, Germany). Protein concentration was calculated by plotting a linear

regression line of the standards and interpolating unknowns using Microsoft Excel.

### 2.4.3. Protein preparation for western blotting

Protein samples (20 µg) were prepared by pipetting out the required volume and adding 4x Laemmli sample buffer (ThermoFisher, USA) containing 5% β-mercaptoethanol. The samples were then heated at 100$^{O}$C for 10 minutes on a heat block. The samples were then pulse centrifuged prior to loading. Molecular markers were loaded untreated directly onto the precast NuPAGE gel (details below).

### 2.4.4. SDS polyacrylamide gel electrophoresis

Protein samples were then subjected to SDS-PAGE according to the method described (Laemmli, 1970). Firstly, 800 ml of 1× 3-(N-morpholino) propane sulfonic acid (MOPS)-SDS running buffer (ThermoFisher, USA) was prepared and added to the electrophoresis chamber. A 12-well precast NuPAGE gel (12% Bis-Tris: Fisher Scientific) was removed from its comb, the wells washed 3-times with 1× MOPS-SDS running buffer and allowed to warm at RT. The gel was then placed into a SureLock X Cell II electrophoresis chamber (Thermo Fisher, Loughbrough, UK) and 200 ml of 1× MOPS-SDS running buffer was added to the inner chamber. Pre-stained molecular weight markers (15 µl; Blue Protein Standard Broad Range New England Biolabs or Precision Plus Protein All Blue standards, BioRad, Watford, UK) were loaded into the first and last wells. The prepared protein samples were loaded into the wells alongside a positive control sample where appropriate. The outer chamber was then filled with MOPS running buffer and the gel was left to run at 200 V for 50 minutes.

### 2.4.5. Gel transfer and blotting

NuPAGE transfer buffer was prepared from 15 ml 20× TG buffer (Thermo Fisher, Loughbrough, UK) plus 255 ml distilled water and 30 ml methanol. The PVDF membrane (BioRad, Watford, UK) was cut to size, bottom left hand corner cut for easy identification and then activated in methanol for 10 minutes before soaking in transfer buffer for at least 5 minutes. Blotting pads (5 per gel) were pre-soaked in transfer buffer for 20 minutes and then squeezed to ensure removal of air bubbles. Two pieces of filter paper (3 mm thickness) were cut to the same size as the PVDF membrane and soaked in transfer buffer.

At the completion of electrophoresis, the gel was removed from the chamber, placed on a flat surface and separated from the plate using a gel knife. The top of the gel was sliced off, the gel was removed and the PVDF membrane placed on top. The membrane and gel were then sandwiched between filter paper and blotting pads within the blotting module. Then the blotting sandwich was made from anode (+) to cathode (-) as follows: 2× blotting pads, 3 mm filter paper, PVDF membrane, gel, 3 mm filter paper, 2× blotting pads. The module was placed within the tank and the inner chamber filled with transfer buffer and locked tightly. The outer chamber was filled with cold distilled water. The tank was placed in a cold room (4°C) and run at 30 V for 1 hour. Once the transfer was completed, the membrane was removed and stained with Ponceau S to confirm the transfer, then rinsed 3 times with PBS-T (0.1% (v/v) Tween 20 in PBS).

## 2.4.6. Immunoblotting

The PBS-T solution was prepared along with a blocking solution (5% milk in PBS-T). The PVDF membrane was washed for 1 hour in blocking solution at RT on a shaking plate. The membrane was then incubated with primary antibody, as detailed in Table 2.3, in blocking solution overnight at 4°C on a shaking plate. The following morning, the primary antibody was poured off and the membrane washed in PBS-T for 5 x 10 minutes. The membrane was then incubated with secondary antibody for 1 hour in blocking solution (Table 2.4). The membrane was then washed in PBS-T for 5 x 10 minutes and rinsed in distilled water twice. ECL prime (GE Healthcare, UK) solution was applied for 5 minutes, wrapped in cling film and exposed to X-ray hyperfilm. The X-ray film was then developed using the SRX-101A developer (Konica Minolta, UK) or chemiluminescence sensitivity matching X-ray film (BioRad, Watford, UK).

## 2.4.7. Stripping the PVDF membrane

The PVDF membrane was placed in PBS after blotting and stored at 4°C until ready to strip. Membranes were then incubated in a mild stripping buffer (see Appendix) at RT for 10 minutes, this step was then repeated using fresh buffer. Membranes were then incubated for 2 x 10 minutes in PBS, followed by incubation in PBS-T for 2 x 5 minutes. ECL prime was added and a film was developed to check that stripping had been successful. Membranes were washed in PBS-T for 10 minutes and then re-probed.

# Chapter 3: The effect of maternal protein restriction on fetal ovary structure and development in sheep

## 3.1. Introduction

The progressive decline in the fertility of dairy cows has severely affected herds' reproductive performance, productivity and profitability across the globe (Gardner et al., 1998, Royal et al., 2000, Butler, 2003, Robinson et al., 2006). For example, conception rates (percentage of animals pregnant at first service) have fallen from 55.6% in 1975-1982 to 39.7% in 1995-8 (Royal et al., 2000) and 40% (1998-2008) (Hudson and Green, 2018). This decline in fertility is a multifactorial problem that is associated with factors relating to genetics, physiology, nutrition and animal management (Macmillan et al., 1996, Butler, 1998, Lucy, 2001, Roche et al., 2000, Walsh et al., 2011). It is hoped that the inclusion of fertility strategies, breeding goals and strategic management will help to prevent further deterioration of reproductive performance (Lucy, 2001, Norman et al., 2009). However, a deeper understanding of the mechanisms behind subfertility is essential to reverse this trend in declining fertility.

The reproductive capacity of an offspring is determined during fetal ovary development with the number of oocytes principally determined by birth (Zuckerman, 1951, Block, 1952). The various morphological, physiological, and endocrinological events involved in fetal ovary development before birth are; a) movement of the primordial germ cells from the yolk sac to the gonadal ridge; b) formation of the gonadal ridge; c) extensive germ cell proliferation; d) migration of germ cells from the mesonephros; e) formation of ovigerous cords containing the germ cells and pre-granulosa cells; f) initiation of meiosis and the subsequent formation of primordial follicles; and g) significant loss of germ cells through apoptosis (Juengel et al., 2002, Sawyer et al., 2002, Hummitzsch et al., 2013, Hummitzsch et al., 2014). It is likely that adverse maternal stress and/or nutrition restriction during these events will have an impact on the subsequent quality and quantity of the oocytes/follicles formed. Moreover, how, when, and what exactly controls these processes is still unclear.

Within ruminant livestock production systems, periods of nutrient inadequacy provide critical production challenges. In addition, fetal growth restriction resulting from disturbed maternal undernutrition has been associated with negative impacts upon developing tissues, metabolism, growth efficiency, and body composition of various farm animals (Robinson et al., 2006, Caton et al., 2007, Symonds et al., 2010). Effects of maternal dietary restriction during pregnancy can affect the offspring, resulting in long-term effects in the adult (Symonds et al., 2006, Symonds et al., 2010). Understanding the effect of maternal nutrition during specific critical windows during gestation is important to our understanding of subsequent fertility and reproduction of sheep and cattle (Thornton, 2010).

During gestation, adequate nutrition to the fetus is critically important for its metabolic activity, organ growth, development and functionality (Redmer et al., 2004). In general, poor nutrition has been associated with adversely affected reproductive performance in sheep and cattle. This has been observed at different stages of reproductive development and manifests as delayed puberty, reduced ovulation, low conception rates, fetal losses, long post-partum anoestrus, poor lactation, high pre/postnatal mortality, morbidity and poor neonatal performance (Smith and Somade, 1994, Ashworth et al., 2009, Scaramuzzi et al., 2011). Moreover, maternal undernutrition resulted in lower total oocyte number and delayed germ cell maturation in the fetal sheep ovary (Rae et al., 2001). In the adult offspring, maternal dietary restriction reduced ovulation rate (Rae et al., 2002), led to raised FSH, and reduced both AMH levels and antral follicle count (AFC) (Sullivan et al., 2009, Mossa et al., 2013).

Recent evidence has clearly demonstrated that cattle with lower AFC have decreased follicular and luteal function leading to reduced fertility (Jimenez-Krassel et al., 2009, Sullivan et al., 2009, Mossa et al., 2012). Inadequate maternal nutrition in sheep affected fetal ovary formation and development in the near-term fetus through delayed folliculogenesis (Borwick et al., 1996, Rae et al., 2001, Lea et al., 2006, Mossa et al., 2013). This is likely to have resulted from reduced cellular proliferation, increased expression of pro-apoptotic markers and reduced vascularisation in the fetal ovary (Lea et al., 2006, Andrade et al., 2013).

Several pluripotency markers have been used to differentiate between the different stages of germ cell development that occur in the fetal ovary. These include octamer binding transcription factor 4 (OCT4), deleted azoospermia-

like (DAZL), and VASA (Dead Box: *DDX4/MVH*) (Anderson et al., 2007, Hummitzsch et al., 2013) (Fig 3.1).

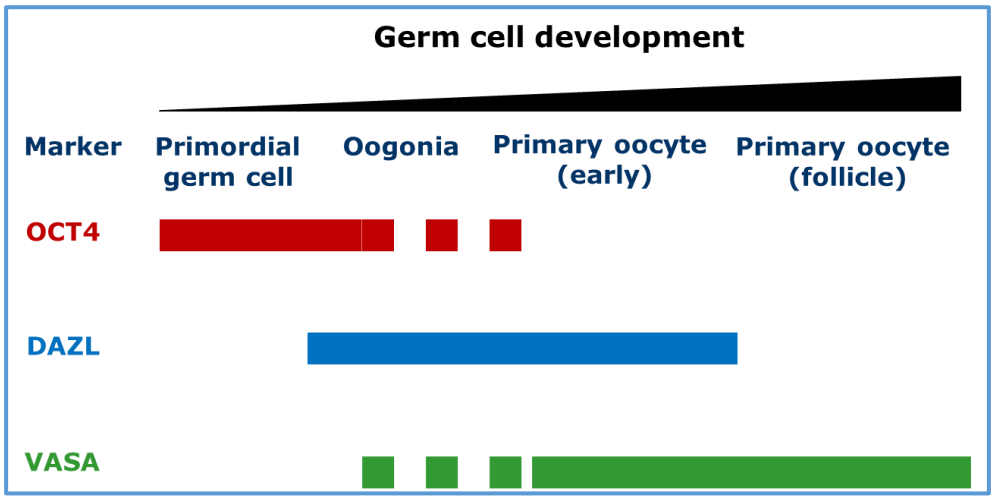



Fig. 3.1: Germ cell marker expression during fetal ovarian development.

### 3.1.1. Hypothesis

This study tested the hypothesis that maternal protein restriction would adversely affect germ cell development and the vascularisation of the fetal sheep ovary at 65 days of gestation.

### 3.1.2. Objectives

The objectives were to examine;

1) Fetal ovarian morphology and development by immunolocalising specific markers of germ cell development;
2) Determining the rate of proliferation and apoptosis; and
3) Determining the degree of vascularisation to and within the whole fetal ovary.

## 3.2. Materials and methods

### 3.2.1. Animal study design and fetal ovary collection

The Institutional Research and Ethics Committee (University of Nottingham, Sutton Bonington Campus) approved the animal study, which was performed in accordance with the United Kingdom (UK) Animals (Scientific Procedures) Act, 1986. The study design is summarised in Fig. 3.2.

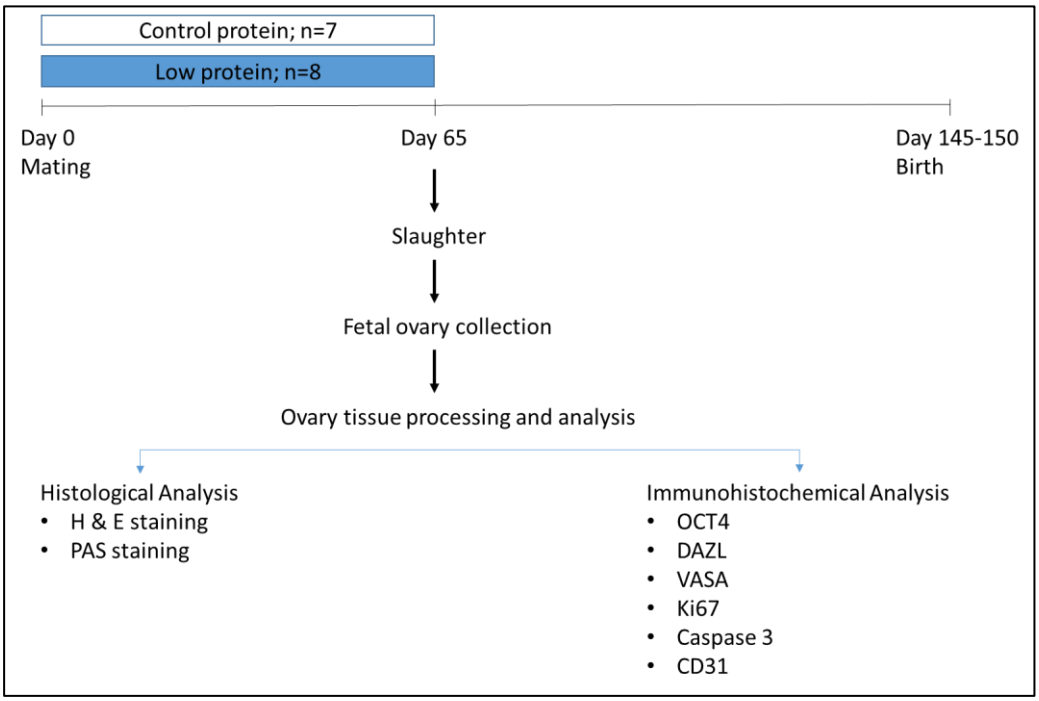



Fig. 3.2: Experimental study design: the role of gestational maternal diet on offspring ovarian structure and development.

The principal investigator for this study was Prof David Gardner (University of Nottingham), who alongside his co-researchers were responsible for the experimental design, care of the animals and collection of fetuses that were used in this study. Dr. Bob Robinson (University of Nottingham) was responsible for the dissection and collection of the fetal ovaries. The samples were collected in January 2011.

Scottish Blackface ewes (n=31) carrying twins (all ewes carrying singletons or triplets were excluded) were randomly assigned to groups and fed either a control diet providing adequate protein (180 g $kg^{-1}$ crude protein), or a low protein diet (80 g $kg^{-1}$ crude protein). Both diets were made isocaloric on a dry matter, as-fed with an effective protein level of either 17.0 g crude

protein/MJ metabolizable energy (control protein; CP, n=7) or 8.7 g crude protein/MJ metabolizable energy (low protein: LP, n=8), further detailed in Table 3.1. The diet was formulated by Dr. Stewart Rhind, Macaulay Institute, Aberdeen and as previously described (Dunford et al., 2014).

Table 3.1: Energy and protein composition of the experimental diets.

| **Diet** | **Protein** ($g.kg^{-1}$ crude protein) | **Energy** ($MJ.kg^{-1}$ DM) | **Protein:energy ratio** ($g.kg.MJ\ DM^{-1}$) |
|---|---|---|---|
| Control protein | 180 | 10.6 | 17.0 |
| Low protein | 80 | 9.2 | 8.7 |

DM, dry matter.

The formulated diet was fed from conception to day 65 of gestation at which point the sheep were euthanised by barbiturate overdose, slaughtered and fetal tissues collected. One twin (male or female) from each pair of fetuses was semi-randomly assigned for further processing. The other twin was used for creation of corrosion casts of that twin's vasculature. This meant that the gonads were collected from 15 females and 16 male fetuses. The fetal testes were not utilised in this study and males were excluded from further analysis. Both fetal ovaries (left and right) were collected, weighed, and recorded. One ovary was frozen immediately after collection and stored at -80°C. The other ovary was fixed in Bouin's for 6 hours, then paraffin-embedded according to standard procedures and used for future histological and immunohistochemical analysis as detailed in Fig. 3.2 and Table 3.2.

Table 3.2: Paraffin embedding protocol for processing fetal sheep ovary tissue.

| Step | Chemical | Time period |
|---|---|---|
| 1 | Bouin's fixative | 6 hours |
| 2 | 70% ethanol | 1 hour |
| 3 | 80% ethanol | 1 hour |
| 4 | 95% ethanol | 1 hour |
| 5 | 100% ethanol | 1 hour |
| 6 | 100% ethanol | 1 hour |
| 7 | 100% ethanol | 1 hour |
| 8 | Toluene | 1 hour |
| 9 | Toluene | 1 hour |
| 10 | Molten paraffin wax (60°C) | 1 hour |
| 11 | Molten paraffin wax (60°C) | 1 hour 30 minutes |

The fixed and embedded fetal ovaries (n=15) were assigned a unique block number that was different to the ewe's identity, to allow blinded processing and analysis (Table 3.3). The tissue blocks (n=15; control, n=7; low protein, n=8) were trimmed at 20 µm and serially sectioned at 5 µm throughout (RM 2255 microtome, Leica Microsystems, Germany). Two sequential tissue sections were mounted onto a poly-L-lysine coated glass slide (Fisher Scientific, Loughborough, UK) and transferred to a heated plate (Hot plate, HI 1220, Leica, Germany) at 36°C for 30 minutes to remove any air bubbles. The tissue slides were then transferred to a slide rack and incubated overnight at 45°C for adequate adhesion to the poly-L-lysine glass slides.

The size of ovaries varied between animals and hence the total number of sections for each animal was different (Table 3.3). The slides selected for histological and immunohistochemical analysis are stated in Appendix.

Table 3.3: Ovarian tissue blocks and sections prepared from each fetal sheep ovary, following maternal dietary treatment.

| Ovarian tissue block number | Dietary treatment | Total number of sections |
|---|---|---|
| N110090 | **Control** | 340 |
| N110093 | **Control** | 204 |
| N110095 | **Control** | 254 |
| N110097 | **Control** | 200 |
| N110102 | **Control** | 302 |
| N110110 | **Control** | 240 |
| N110111 | **Control** | 216 |
| N110088 | **Low** | 294 |
| N110096 | **Low** | 266 |
| N110098 | **Low** | 216 |
| N110104 | **Low** | 202 |
| N110108 | **Low** | 246 |
| N110109 | **Low** | 198 |
| N110134 | **Low** | 104 |
| N110135 | **Low** | 292 |

Control, control protein. Low, low protein.

## 3.2.2. Histological analysis

### 3.2.2.1. Haematoxylin and Eosin staining

Haematoxylin and eosin (H&E) staining was performed to enable general histological and morphological assessment of the fetal ovaries and identification of germ cells within the ovigerous cords (or egg nests, as previously described (McNatty et al., 2000, Sawyer et al., 2002). For clarity, this study will use the term "germ cell" to encompass primordial germ cells (PGC), oogonia and primary oocytes.

H&E staining was performed using every 20th slide throughout the whole fetal ovary (n=15). The ovarian tissue sections were dewaxed in xylene twice for 5 minutes, rehydrated in ethanol (100%, 95%, and 70%) for 5 minutes each, and washed in phosphate-buffered saline (PBS; pH 7.4; Fisher Scientific, Loughborough, UK) for 5 minutes.

To stain the basophilic structures (e.g. nucleus) blue-purple, sections were placed in Harris' haematoxylin for 5 minutes and then rinsed in running tap water until the water became clear. The sections were then dipped in 1% (v/v) acidified industrial methylated spirits (IMS) twice, rinsed in running tap water and dipped twice in an ammoniated water to "blue" the stain. This was followed by counterstaining with eosin for 5 minutes and washing in running tap water until the water became clear.

The sections were then dehydrated in a series of ascending ethanol (70%, 95%, and 100% v/v) and dehydrated in xylene (2 x 5 minutes). Finally, the slides were mounted in DPX mountant with a glass coverslip and then allowed to dry overnight.

### 3.2.2.2 Periodic acid-Schiff staining

The periodic acid-Schiff (PAS) staining technique was used to demonstrate the presence of carbohydrate-dense regions within tissue sections (e.g. basement membranes surrounding the ovigerous cords and vasculature within the fetal ovary) (Reynolds and Redmer, 1992). The periodic acid oxidises the carbohydrate macromolecules, producing aldehyde groups that condense with Schiff's reagent to form a magenta colouration. PAS staining was performed using every 20th slide throughout the whole fetal ovary (n=15).

The ovarian tissue sections were dewaxed in xylene twice for 5 minutes and then rehydrated in ethanol (100%, 95%, and 70%) for 5 minutes each and washed twice in PBS for 5 minutes. The tissue sections were oxidised in 1% (v/v) periodic acid solution (Merck Millipore, Watford, UK) for 5 minutes and rinsed in deionised water for 5 minutes. Schiff's reagent (Merck Millipore, Watford, UK) was added for 15 minutes, and rinsed in running water for 3 minutes. The tissue sections were then counterstained in Harris' haematoxylin for 2 minutes, rinsed in running water, dehydrated in ethanol (70%, 95%, and 100%) for 2 minutes each, and dehydrated twice in xylene for 5 minutes. Finally, the slides were mounted in DPX mountant with a glass coverslip and then allowed to dry overnight.

## 3.3. Immunohistochemical analysis

Immunolocalisation studies were performed in fetal sheep ovaries to identify and quantify germ cells (OCT4, DAZL, and VASA), cell proliferation (Ki67), cell apoptosis (Caspase 3) and vascular endothelial cells (CD31).

The details of the markers and the specific antibodies used are shown in Table 3.4. Negative control tissues had an equivalent concentration of rabbit IgG or mouse IgG (Sigma-Aldrich, Poole, UK) to the primary antibody being applied in each experiment. All immunohistochemical protocols required some optimisation. The principle steps that were optimised were; (1) concentration of the primary antibody to ensure appropriate staining and (2), length of the DAB substrate step in order to reduce the background colour. The final antibody concentrations are shown in Table 3.4.

The ovarian tissue sections were immunostained using the details of antibodies listed in Table 3.4 and following the immunohistochemistry protocol as detailed in Section 2.2.3.

Table 3.4: Details of antibodies used for immunohistochemistry analysis.

| Antigen | Function/Marker | Source | Host Species | Sequence identity with ovine (%) | Dilution Factor | Final concentration | Time of DAB substrate step |
|---|---|---|---|---|---|---|---|
| Human OCT4 | Transcription factor in pluripotent cells (e.g. PGCs) | Abcam (ab19857) | Rabbit, polyclonal | 91 | 1:250 | 4 µg/ml | 20 – 40 seconds |
| Human DAZL | RNA binding protein expressed in oogonia | Abcam (ab34139) | Rabbit, Polyclonal | 98 | 1:250 | 4 µg/ml | 20 – 40 seconds |
| Human VASA | Transcription factor of dead-box gene | Abcam (ab13840) | Rabbit, polyclonal | 96 | 1:300 | 3.3 µg/ml | 20 – 40 seconds |
| Human Ki67 | Cellular proliferation | Dako, clone MM1 (VP-K452) | Mouse, monoclonal | 99 | 1:80 | 6 µg/ml | 20 – 40 seconds |
| Human Caspase 3 | Cellular apoptosis | Cell Signalling (#9579) | Rabbit, polyclonal | 100 | 1:200 | 3 µg/ml | 10 – 15 minutes |
| Human CD31 | Vascular endothelial cells | Abbiotec (ABIN1582260) | Rabbit, polyclonal | 85 | 1:300 | 3.3 µg/ml | 5 minutes |

#### 3.3.1.1. Germ cell markers

Different germ cell markers were used to identify the various stage of germ cell development (Fig 3.1). In brief, OCT4 is a pluripotency marker for PGC/oogonia identification (Anderson et al., 2007, Hummitzsch et al., 2013); DAZL detects PGC that have differentiated into early oogonia (Anderson et al., 2007, Hummitzsch et al., 2013); VASA is expressed in the cytoplasm of early oogonia/primary oocytes (Anderson et al., 2007, Hummitzsch et al., 2013).

#### 3.3.1.2. Cell proliferation marker: Ki67

Immunohistochemistry for Ki67 was performed to assess cell proliferation in the fetal ovary. The immunohistochemistry protocol was as described in Section 2.2.3 except for the following change: Immunoreaction was visualized by incubating DAB solution for 20–40 seconds and haematoxylin incubation shortened to 20 seconds. In the subsequent image analysis, only cells with a germ cell phenotype (i.e. within the egg nest) were used to determine the germ cell proliferation index.

#### 3.3.1.3. Cell apoptosis marker: Caspase 3

Caspase 3 immunohistochemistry was used to assess the degree of apoptosis (cellular death) in the fetal ovary. The immunohistochemistry protocol was as for Ki67 except for the following change: Immunoreaction was visualized by incubating DAB solution for 10–15 minutes and haematoxylin incubation was 20 seconds. Again, only cells with a germ cell morphological appearance were identified to determine the germ cell apoptotic index.

#### 3.3.1.4. Vascular endothelial cell marker: CD31

CD31 (PECAM1) is a marker of vascular EC and was used to detect the vascularisation of both the cortical and medullary regions of the fetal ovary. The immunohistochemistry protocol was as described in Section 2.2.3 except for the following change: Immunoreaction was visualized by incubating in DAB solution for 5 minutes.

## 3.4. Image analysis

All images were captured using a light microscope (DM500B, Leica Microsystems, Germany) and camera (DFC350FX, Leica Microsystems, Germany). Image analysis was conducted using Image ProPlus 6.3 (Media Cybernetics, Marlow, UK) and all data were recorded and processed in Microsoft Excel spreadsheets (Microsoft Office, 2013).

All processed sections from each fetal sheep ovary were evaluated by image analysis. The data generated from both the histological and immunohistochemical analysis were used to morphologically characterise the fetal ovary with the data converted into 3 principle parameters: area ($\mu m^2$), volume ($mm^3$) and weight (mg).

For each H&E, PAS, OCT4, DAZL, VASA, Ki67, Caspase 3 and CD31 stained section, four images were captured randomly across the section under a 40× magnification objective. This was repeated for the adjacent section on that slide as well as for each slide stained. This meant that H&E, PAS, OCT4, DAZL, VASA, Ki67, Caspase 3 and CD31 stained images were typically collected for each endpoint. An example of images captured for H&E and DAZL staining is shown in Fig. 3.3B and C.

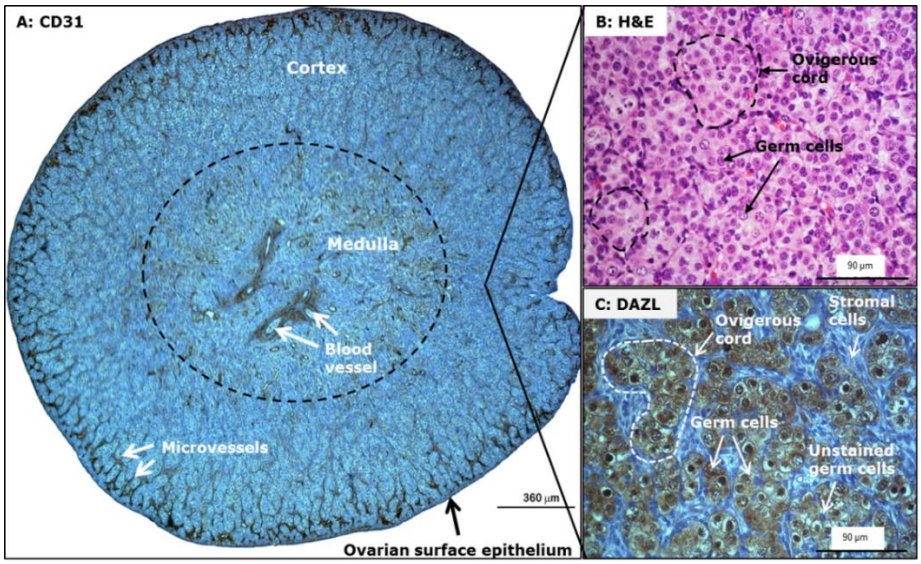



Fig. 3.3: Histological analysis of the sheep fetal ovary. Photomicrograph images of a sheep fetal ovary at day 65 of gestation (A) immunostained for CD31, (B) stained with H&E, and (C) immunostained for DAZL. Low magnification (5x) image (A) showing the ovarian surface epithelium, cortex, medulla (as indicated by black dashed line) microvessels and blood vessels (arrows). High magnification (40x) images (B, C) highlighting an area of the cortex region after H&E staining and DAZL immunostaining (brown). Dotted lines (white) indicate the ovigerous cords; germ cells, unstained germ cells, and stromal cells (arrows). The scale bars are 360 µm (A) and 90 µm (B, C).

### 3.4.1. Quantification of the total area of each section and percentage area occupied by cortex

Images of CD31 immunostained sections were captured under 5× objective and stitched to form a composite image which captured the whole fetal ovary cross section (Fig 3.3A). CD31 immunostained sections were used as this showed the clearest demarcation between the medulla and cortex regions (Fig 3.3A). Next, the area of the whole section and medulla was measured by careful drawing around the section edge and medulla-cortex demarcation, respectively. This was repeated for each CD31-stained section. From this, the area and proportion occupied by cortex was calculated (Equation 1). This information was required to estimate the total number of germ cells present in the whole ovary.

$$\%\ \boldsymbol{area\ occupied\ by\ cortex} = \frac{total\ section\ area - area\ of\ medulla}{total\ section\ area} \qquad \text{(Eq. 1)}$$

The total ovarian volume (μm$^3$) was calculated as follows (Equation 2):

$$\boldsymbol{Total\ ovarian\ volume} = Mean\ area\ of\ section\ (measured) \times thickness\ of\ section\ (5\mu m) \times number\ of\ sections\ per\ fetal\ ovary \quad \text{(Eq. 2)}$$

This volume (μm$^3$) was converted to mm$^3$ by dividing by $10^9$. The total medulla volume was calculated in a similar manner and this was subtracted from the total ovarian volume to yield the estimated total cortex volume.

### 3.4.2. Estimating germ cell counts: Haematoxylin and Eosin staining

Images obtained from Haematoxylin and Eosin staining (H&E) staining were used to count the germ cells within the ovigerous cords. Germ cells within ovigerous cords were identified based on their histological appearance and were manually counted using the pointer function (Image ProPlus 6.3). The mean germ cell number (count) for each section was calculated from the average of the 4 fields of view. The germ cell density was calculated by dividing the count by the area of the field of view (78742 μm$^2$).

Next, the estimated total germ cell weight and total number of germ cells were calculated. This required several calculations and assumptions, which are described below. Firstly, the mean area of each germ cell was estimated by measuring the diameter of multiple germ cells (n>100) across several animals from both dietary groups. The mean germ cell diameter was 14.2 μm. The first assumption was that each germ cell was circular in shape. Using the formula for the area of a circle = $\pi r^2$, the mean area of each germ cell was calculated to be 158.4 μm$^2$.

The % area occupied by germ cells was then calculated as follows (Equation 3):

$$\%\ \boldsymbol{area\ occupied\ by\ germ\ cells} = \frac{count \times germ\ cell\ area\ (158.4\ \mu m^2)}{field\ of\ view\ area\ (78742 \mu m^2)} \quad \text{(Eq. 3)}$$

The % area occupied by germ cell was then utilised to estimate the total weight of germ cells in both fetal ovaries as shown in Equation 4. The germ cell weight was calculated as a different measure of the total number of germ cells / ovarian reserve. The benefit of this endpoint is that fetal ovarian weights were directly measured whereas the ovarian volume was estimated. This calculation (Equation 4), though, assumed that the germ cells were only present in the cortex and that the cortex was distributed evenly throughout

the ovary. It also assumed that both right and left ovaries had similar morphological characteristics.

$$\textbf{Total germ cell weight} = \%\ area\ occupied\ by\ \text{germ cells}\ (Eq.3) \times \%\ cortex\ (Eq.1) \times total\ fetal\ ovary\ weight\ (mg) \quad \text{(Eq. 4)}$$

The estimated total number of germ cells in a single ovary was calculated (Equation 5). In order to perform this calculation, it was necessary to assume that a germ cell was spherical in shape and the volume was calculated as $\frac{4}{3}\pi r^3$ (1499 μm$^3$). This number was multiplied by 2 to obtain the total number of germ cells in both ovaries.

$$\textbf{Total number of germ cells} = \frac{Volume\ of\ cortex\ (\mu m^3) \times \%\ area\ occupied\ by\ germ\ cells\ (Eq\ 3)}{Volume\ of\ a\ single\ germ\ cell\ (1499\ \mu m^3)} \quad \text{(Eq. 5)}$$

## 3.4.3. Quantification of Periodic acid-Schiff stain

The periodic acid-Schiff (PAS) stain was visually identifiable as a magenta colouration. The PAS stain associated with the ovigerous cords was manually selected using the "Area of Interest" tool in Image ProPlus 6.3 and then counted using the count/size tab. Image ProPlus was then used to determine the area and percentage area of staining. The latter endpoint was used to calculate the estimated weight of PAS$^{+ve}$ tissue (basement membrane) as follows (Equation 6):

$$\textbf{Estimated weight of PAS positive tissue} = \%\ area\ of\ PAS\ staining \times total\ fetal\ ovary\ weight\ (mg) \quad \text{(Eq. 6)}$$

## 3.4.4. OCT4, DAZL and VASA positive cells

OCT4$^{+ve}$, DAZL$^{+ve}$ and VASA$^{+ve}$ cells within the ovigerous cords were manually counted using the pointer tool (Image ProPlus). For each marker the mean count was calculated as the average of four fields of view from each fetal ovary section. This count was repeated for all sections across the whole ovary from which an overall mean count was calculated. This was used to calculate the density of OCT4$^{+ve}$ cells, estimated weight of OCT4$^{+ve}$ cells (Equation 4), and estimated total number of OCT4$^{+ve}$ cells (Equation 5) in the sheep fetal ovary. This was repeated for DAZL and VASA. The mean number of OCT4$^{+ve}$, DAZL$^{+ve}$, and VASA$^{+ve}$ cells for a given ovary were used to determine the following ratios: OCT4:DAZL; OCT4:VASA and DAZL:VASA.

### 3.4.5. Proliferation index (Ki67 positive and Ki67 negative cell counts)

The number of $Ki67^{+ve}$ and $Ki67^{-ve}$ germ cells in the ovigerous cords were manually counted using Image ProPlus 6.3. The mean number of $Ki67^{+ve}$ cells was calculated as the average of four fields of view. The proliferation index was calculated as the proportion of all germ cells which were $Ki67^{+ve}$.

### 3.4.6. Apoptotic index (Caspase 3 staining)

Caspase 3 staining was used to determine the apoptotic index of germ cells within the fetal ovary. The number of Caspase $3^{+ve}$ germ cells and total number of germ cells in the ovigerous cords were manually counted using Image ProPlus 6.3. The mean number of Caspase $3^{+ve}$ cells was calculated as the average of four fields of view. The apoptotic index was calculated as the proportion of germ cells which were Caspase $3^{+ve}$.

### 3.4.7. Degree of vascularisation

The CD31 staining was performed to assess two principle components of vascularisation of the fetal ovary: 1) the degree of microvascularisation in the ovarian cortex, the location of the developing egg nests and 2) the area occupied by the larger blood vessels within the ovarian medulla (Fig. 3.3).

1) *Degree of microvascularisation in the cortex:* Four fields of view were captured (under 40× objective) for each section, for this analysis. An "Area of Interest" trace was drawn around the inner edge of the microvasculature in the cortex through to the ovarian surface and this encapsulated area selected. The colour select tool in Image ProPlus was then used to identify the brown colour (CD31 staining). Next, Image ProPlus determined the % area of CD31 staining within this area. This was repeated for each section, from which an overall mean was calculated.

2) *Size and dimensions of blood vessels in the medulla*: The whole medulla region was captured under 40× Objective. The blood vessels were identified by histological appearance and CD31 positive staining. A pointer tool in Image ProPlus was used to draw around the lumen of each vessel stained by CD31. Then, Image ProPlus determined the number of vessel cross sections, lumen area and perimeter. This was

repeated for each CD31-stained section, from which an overall mean was calculated.

## 3.5. Statistical analysis

All the data were checked for normality and heterogeneity of variance before statistical analysis. GenStat was used to analyse the statistical significance ($p<0.05$) of differences. All data were subjected to Student's (unpaired) t-test using GenStat; GraphPad Prism 6.3 (GraphPad Prism software, California, United States of America (USA)) was used to plot graphs. The density of OCT4, VASA and DAZL positive germ cells within each ovary were compared using paired t-tests. Data values were expressed as mean ± SEM.

## 3.6. Results

### 3.6.1. The effect of maternal dietary protein restriction on fetal and ovarian weight

Mean fetal weight at day 65 of gestation was not different between the two dietary groups ($p>0.05$; Fig. 3.4A). However, fetal ovary weight was affected by diet ($p<0.05$; Fig. 3.4B), with maternal protein restriction leading to reduced fetal ovary weight (20±1.2 mg) versus the control protein (26±2.6 mg) diet. Total cortex area and percentage area of cortex were unaffected by maternal diet ($p>0.05$; Fig. 3.4C-D).

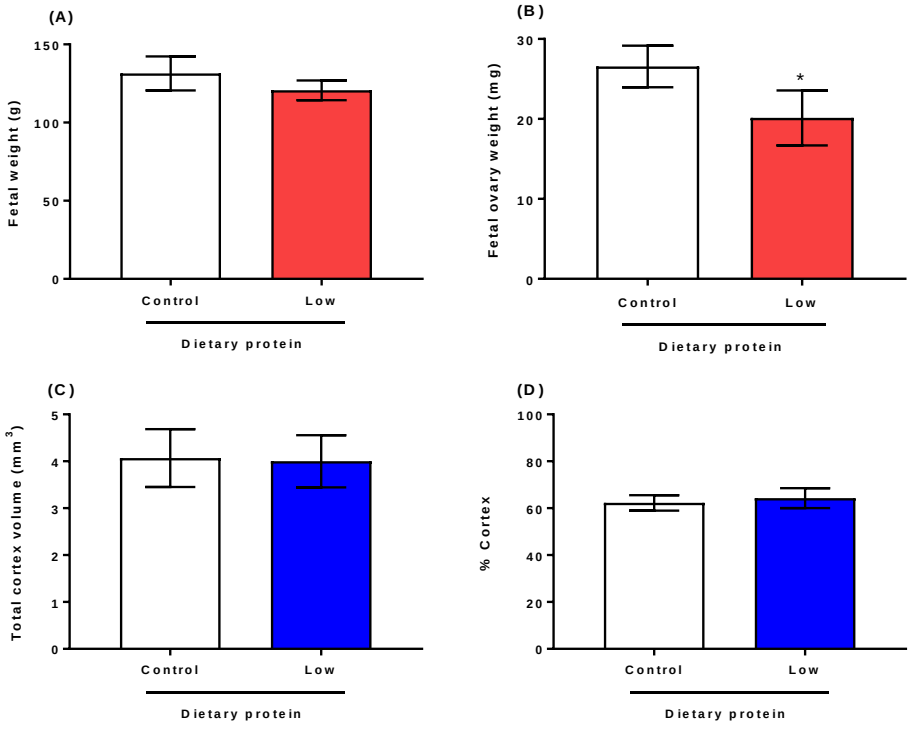



Fig. 3.4: The effect of maternal dietary protein restriction on fetal weight, fetal ovary weight, total cortex volume and percentage area of cortex, of sheep on day 65 of gestation. (A) fetal weight; (B) fetal ovary weight (paired); (C) total cortex volume; (D) percentage area of cortex. Data are mean ± SEM. Significance is indicated by an asterisk ($p<0.05$). Fetal ovarian weight was decreased in the low protein dietary group (n=8) compared with control group (n=7).

Haematoxylin and eosin (H&E) staining was performed to determine the gross ovarian morphology and germ cell count. The germ cells were identified as near circular structures with a centrally located nucleus and were larger than other cells within the fetal ovary. Germ cells were seen in clusters (egg nests) with a surrounding basement membrane (Fig. 3.5). However, there were no obvious differences in the gross morphology of the egg nests or the ovary as a whole between the different diets (Fig. 3.5A&B).

The mean germ cell count (across both diets) was 287 per field of view representing ~60% of the field of view. This is presented as germ cell density (Fig 3.5C). There was no statistical difference in germ cell density between the diets ($p>0.05$). The estimated total weight of germ cells was significantly reduced by approximately 30% in the low protein group compared with control protein group ($p<0.05$) (Fig 3.5D). While, the pattern was similar, there was no effect of diet on the estimated total volume of germ cells or estimated total number of germ cells ($p>0.05$; Fig. 3.5E&F).

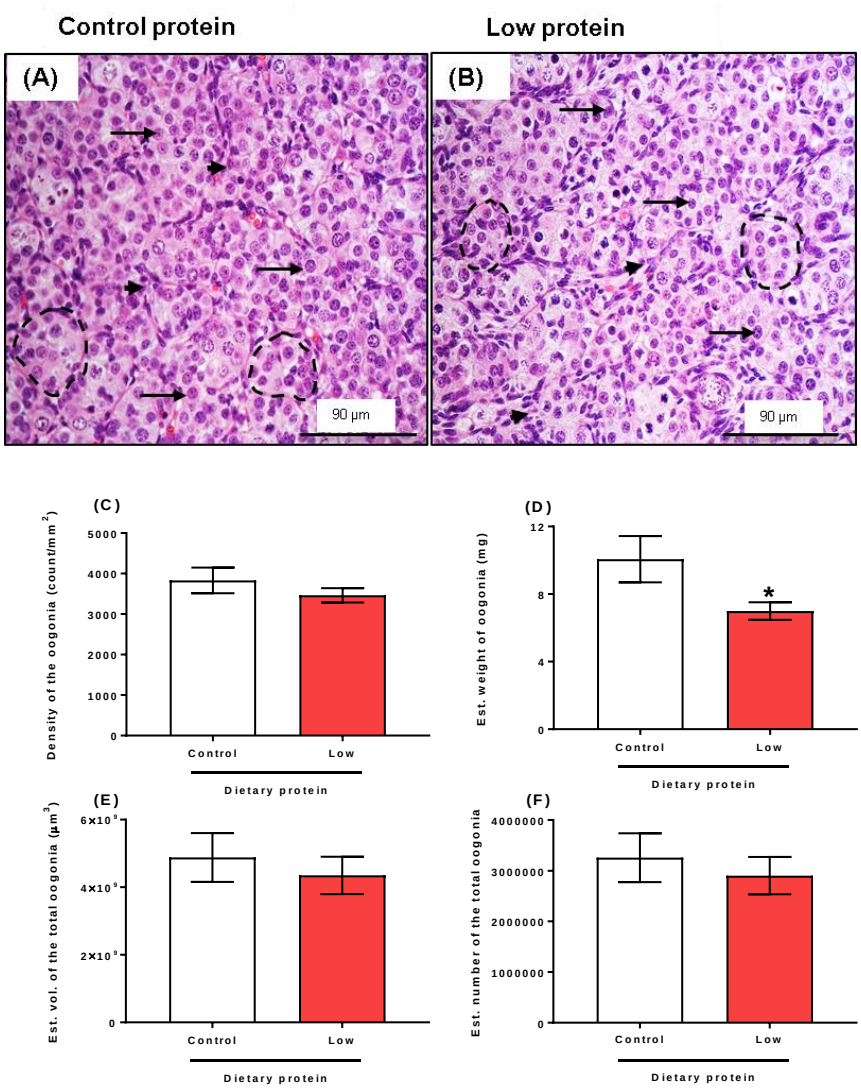



Fig. 3.5: The effect of maternal dietary protein restriction on germ cell morphology in the day 65 fetal sheep ovary as determined by haematoxylin and eosin (H&E) staining. Representative images of H&E stained sections of fetal ovaries following (A) control and (B) low protein diets. Germ cells (arrows) were organised into ovigerous cords enveloped by basement membrane (dashed line) and stromal cells (arrowheads); scale bars=90 µm. Graphs show (C) density (count/mm$^2$), (D) estimated total weight (mg), (E) estimated total volume (µm$^3$), and (F) estimated total number of germ cells. Significance is indicated by an asterisk ($p<0.05$). Data are mean ± SEM. The estimated total weight of germ cells was decreased after maternal protein restriction (n=8) versus control (n=7).

## 3.6.2. Periodic acid-Schiff Staining

Periodic acid-Schiff (PAS) staining was performed to localise the basement membrane, which was evident associated with vascular components and particularly intense in the envelopes that surround a cohort of developing germ cells within the ovarian cortex (Fig. 3.6). The pattern of PAS staining was similar between the two different dietary groups (Fig. 3.6A&B).

There was no effect of diet on the percentage of PAS staining, whilst the estimated weight of basement membrane tended to be reduced by the low protein diet treatment versus control (p=0.09) (Fig. 3.6C&D).

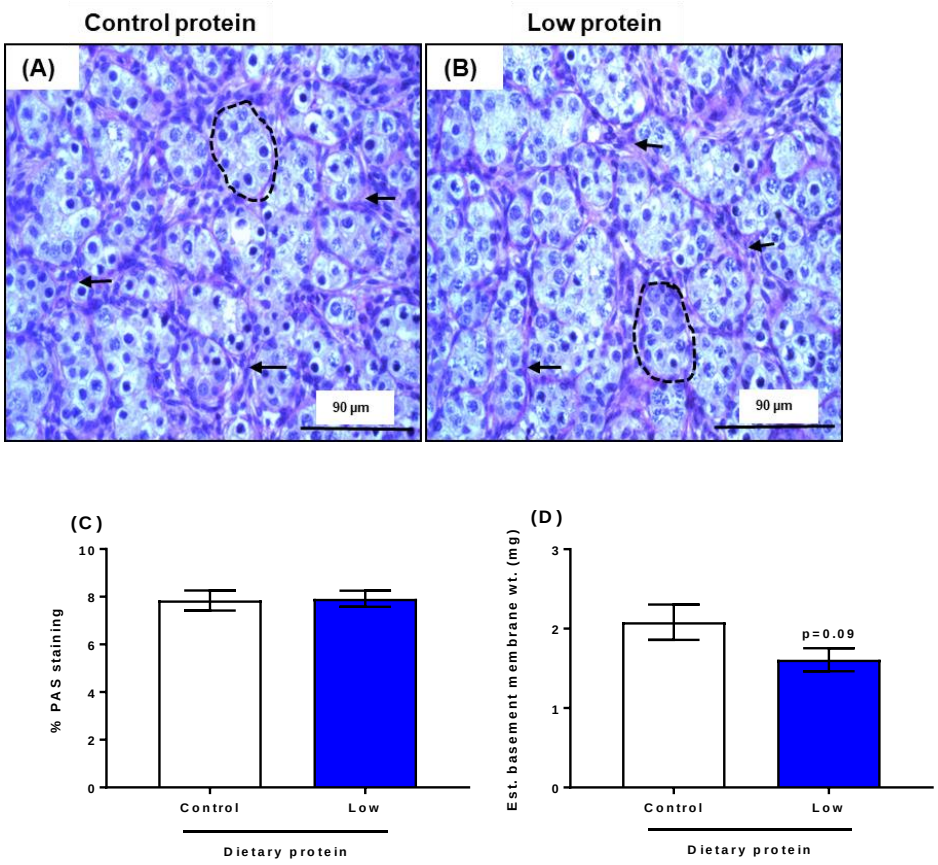



Fig. 3.6: The effect of maternal dietary protein restriction on basement membrane morphology in the day 65 fetal sheep ovary as determined by PAS staining. PAS staining (magenta) was associated with the basement membrane surrounding ovigerous cords (dotted line and black arrows). Representative images of fetal ovaries from sheep fed (A) control protein (n=7), and (B) low protein (n=8) diets. Graphs show (C) percentage PAS staining and (D) estimated weight of the basement membrane. Data are mean ± SEM. The scale bars are 90 µm. The estimated basement membrane weight tended to be reduced by low protein (p=0.09).

### 3.6.3. OCT4 immunostaining

The effect of maternal dietary protein restriction on germ cell development in the day 65 fetal sheep ovary was determined by OCT4 staining (Fig. 3.7). OCT4-positive staining was observed in the nuclei of germ cells, predominantly localised in the cortex area proximal to the ovarian surface epithelium. There was no visual difference in nuclear staining between the different diet groups. Staining was absent in the presence of rabbit IgG (Fig. 3.7C).

Furthermore, OCT4 was expressed in germ cells of differing sizes, including smaller and larger germ cells. At the OSE, the germ cells were numerous, whilst towards the medulla germ cells were fewer but larger in size.

There was no effect of diet on the density (count/$mm^2$), estimated weight (mg), estimated total volume ($\mu m^3$), or estimated total number of OCT4-positive cells in the fetal sheep ovaries at day 65 of gestation ($p>0.05$) (Fig. 3.7D-G).

Pre-granulosa cells (OCT4 negative) were also observed within the ovigerous cords, with up to one or two cells present per cord cross section. Pre-granulosa cells were small and oval-shaped in appearance, and were closely associated with the germ cells.

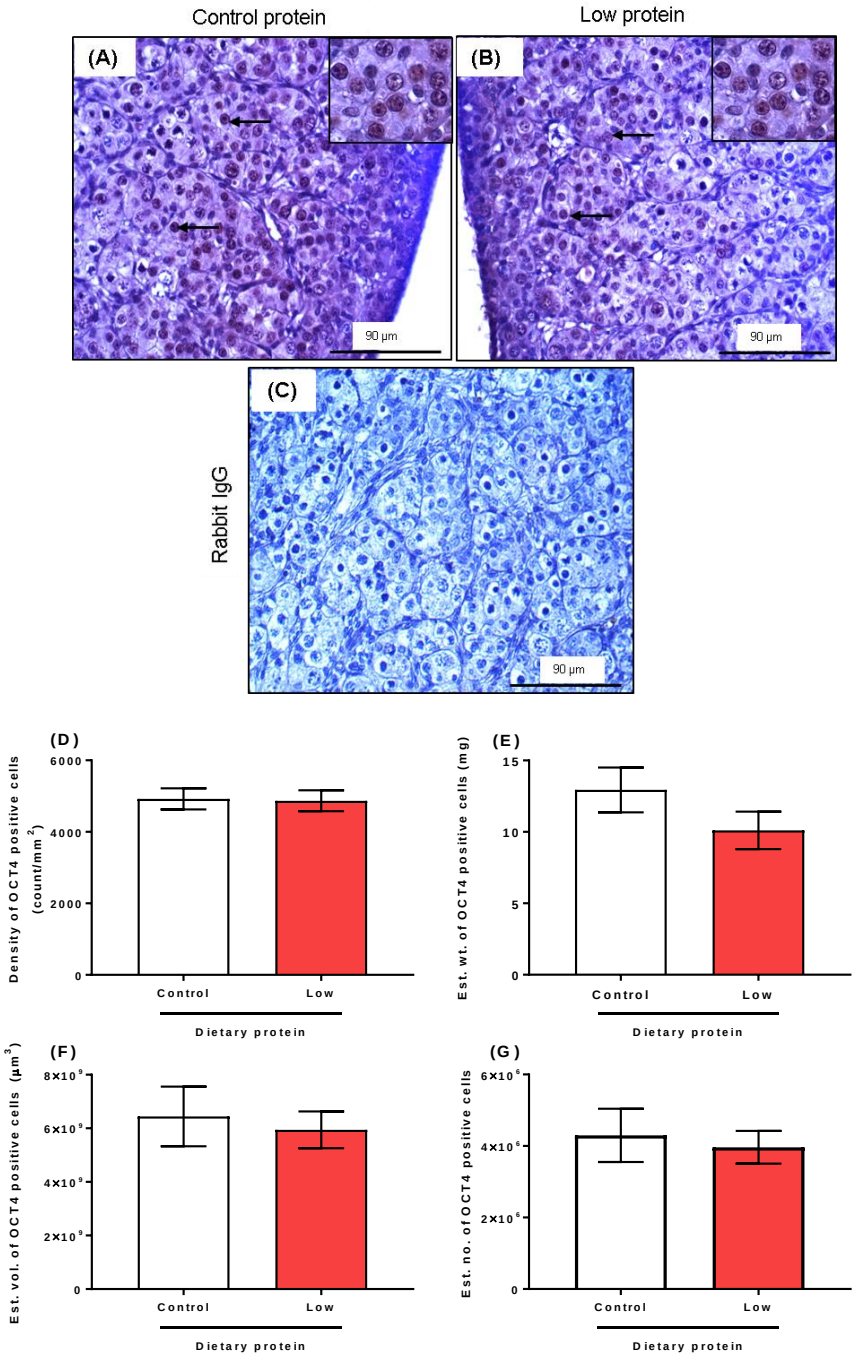



Fig. 3.7: The effect of maternal dietary protein restriction on OCT4-positive germ cell development in the day 65 fetal sheep ovary. Representative images of fetal ovaries from sheep fed (A) control protein (n=7); (B) low protein (n=8) diets. (C) shows the absence of staining in the presence of control rabbit IgG. Graphs show (D) density, (E) estimated weight (F) estimated volume and (G) estimated number of OCT4-positive cells. Photomicrographs show germ cells (arrows) organised into ovigerous cords enveloped by basement membrane. There was no effect of diet. Scale bars=90 µm. Data are mean ± SEM.

### 3.6.4. DAZL immunostaining

DAZL staining was localised differently to germ cells within the ovigerous cords (Fig. 3.8) and was observed to be both nuclear (germ cells undergoing meiosis (oocytes)) and cytoplasmic (oogonia). However, the staining of these germ cells were measured both together. The nuclear staining was more intense and the nuclei appeared more condensed. Not all germ cells within a cross section of an ovigerous cord exhibited the same degree of staining.

A small proportion of germ cells were unstained (DAZL negative cells), however there was no clear difference in their diameter, position relative to the cortex or morphology that appeared associated with their lack of staining versus neighbouring germ cells, which were positively stained. The control rabbit IgG sections displayed no brown staining (Fig.3.8C).

There was no effect of diet ($p>0.05$) on the density (count/$mm^2$), estimated weight (mg), estimated total volume ($\mu m^3$), and estimated total number of DAZL-positive cells in the day 65 sheep fetal ovary (Fig. 3.8D-G).

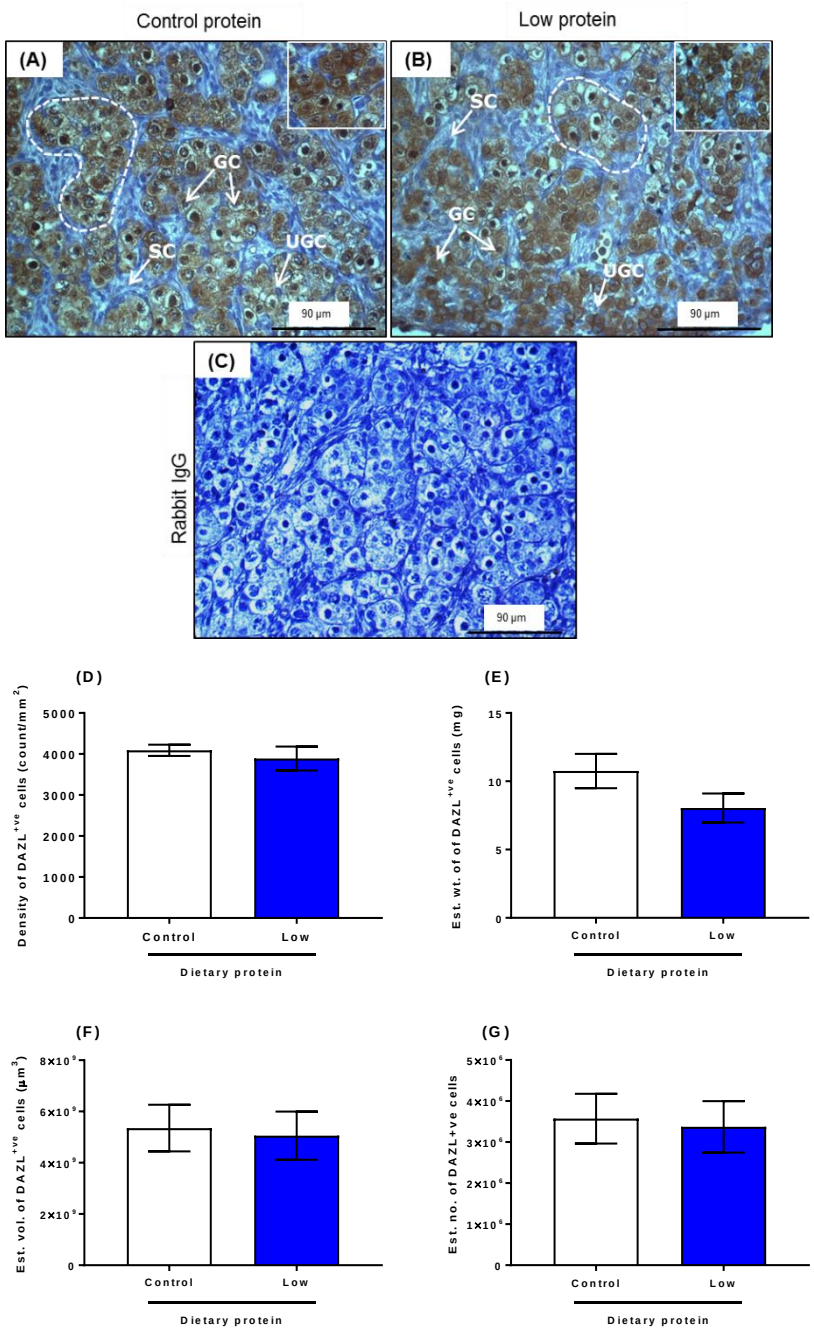



Fig. 3.8: The effect of maternal dietary protein restriction on DAZL-positive germ cell development in the day 65 fetal sheep ovary. Representative images of fetal ovaries from sheep fed (A) control protein (n=7); (B) low protein (n=8) diets. (C) shows the absence of staining with control rabbit IgG. Graphs show (D) density, (E) estimated weight, (F) estimated volume and (G) estimated number of DAZL-positive cells. Photomicrographs show germ cells (GC) organised into ovigerous cords (dashed line). DAZL negative germ cells were also observed (UGC) plus stromal cells (SC). Insets show higher magnification. Scale bars=90 µm. Data are mean ± SEM. There was no effect of diet.

### 3.6.5. VASA immunostaining

VASA protein was detected in the cytoplasm of germ cells (Fig. 3.9). The intensity of the VASA staining was greater in the cytoplasm of larger germ cells within the ovigerous cord than in the smaller germ cells. The majority of the germ cells were VASA-positive within a particular ovigerous cord. In several germ cells, a distinct compact perinuclear structure exhibited accentuated VASA immunostaining. The control rabbit IgG sections displayed no brown staining (Fig.3.9C).

There was no effect of diet ($p>0.05$) on the density (count/$mm^2$), estimated weight (mg), estimated total volume ($\mu m^3$), and estimated total number of VASA-positive cells in the sheep fetal ovary at day 65 of gestation (Fig. 3.9D-G).

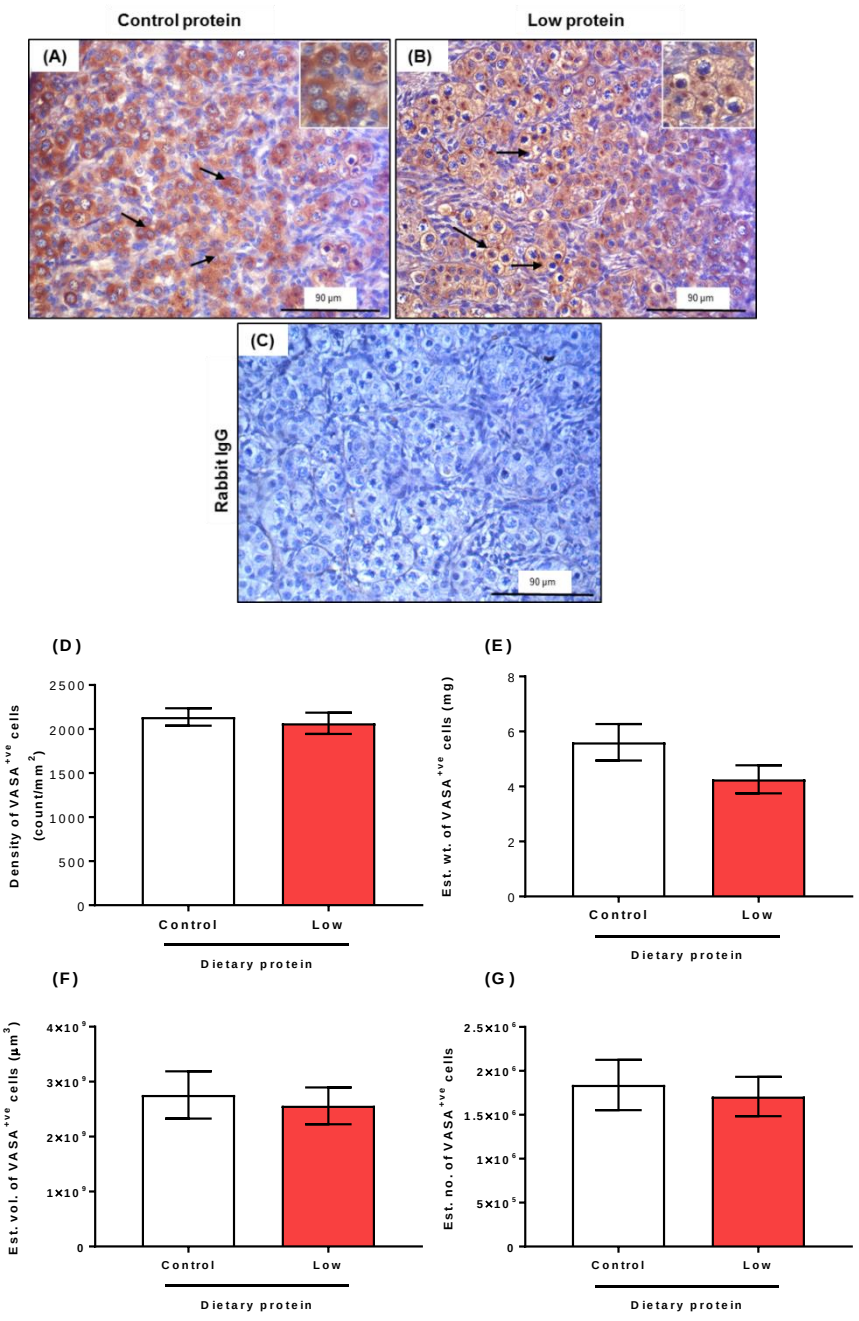



Fig. 3.9: The effect of maternal dietary protein restriction on VASA-positive germ cell development in the day 65 fetal sheep ovary. Representative images of fetal ovaries from sheep fed (A) control protein (n=7); (B) low protein (n=8) diets. (C) shows the absence of staining in the presence of control rabbit IgG. Graphs show (D) density, (E) estimated weight (F) estimated volume and (G) estimated number of VASA-positive cells. Photomicrographs show germ cells (arrows) organised into ovigerous cords. VASA negative germ cells were also observed. Insets show higher magnification. Scale bars=90 µm. Data are mean ± SEM. There was no effect of diet.

### 3.6.6. Ratio of OCT4, DAZL, and VASA immunostaining

There was an abundance of OCT4, DAZL and VASA-positive cells in the fetal ovarian cortex on day 65 of gestation (Fig. 3.10), with the greatest density of germ cells expressing OCT4 ($p<0.001$). The numbers of OCT4, DAZL, and VASA-positive cells within the ovigerous cords were similar in both dietary groups ($p>0.05$).

OCT4-positive cells were marginally more abundant than DAZL ($p<0.001$), which, in turn, was 2-fold more abundant than VASA-positive cells ($p<0.001$). This would suggest that germ cells were at different stages of development on day 65 of gestation with most cells in the earlier stages of oogenesis (Fig. 3.10A-C). However, the ratios between the different germ cell markers were the same across the different diets ($p>0.05$; Fig. 3.10). There was no evidence that on day 65 of gestation the gross fetal ovarian structure or number of germ cells were influenced by dietary protein restriction in sheep.

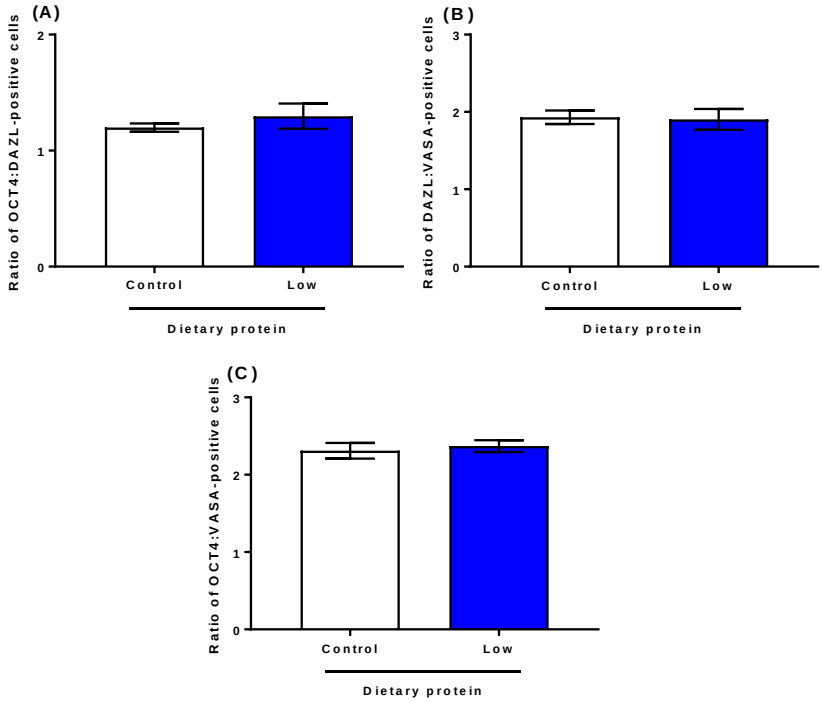



Fig 3.10: The effect of maternal protein restriction on the ratio of germ cell markers (OCT4, DAZL, VASA-positive cells) in the fetal sheep ovary. The graphs show the ratios of (A) OCT: DAZL-positive cells, (B) DAZL: VASA-positive cells and (C) OCT: VASA-positive cells. The dietary treatments were control protein; n=7 and low protein; n=8. Data are mean ± SEM. There was no effect of maternal diet.

## 3.6.7. Cell proliferation and apoptosis

Ki67 immunostaining was performed to assess the proliferation rate in the fetal ovary, with only germ cells within the egg nest being quantified in order to determine the germ cell proliferation index (Fig. 3.11A&B). There was extensive nuclear expression of Ki67 in the ovarian cortex, particularly in the region nearest the ovarian surface epithelium (Fig. 3.11A&B). The control mouse IgG sections displayed no brown staining (Fig. 3.11C). There was extensive proliferation across all sections with proliferation indices of 25-30%, indicating that proliferation was high at this time. However, there was no effect of diet on the proliferation index ($p>0.05$; Fig. 3.11G).

Caspase 3 staining was used to assess the degree of apoptosis in the sheep fetal ovary (Fig. 3.11D&E). The pro-apoptotic regulatory protein Caspase 3 was immunolocalised to a small proportion of germ cells (Fig. 3.11D&E).

Caspase 3 staining was not germ cell specific, but, only those cells within the egg nests were used to determine the germ cell apoptotic index. Caspase 3-positive cells were present at a low abundance with a mean apoptotic index of 3.5% (control) and 4.3% (low protein). The control mouse IgG sections displayed no brown staining (Fig. 3.11F). There was no effect of diet on the apoptotic index ($p>0.05$) (Fig. 3.11H).

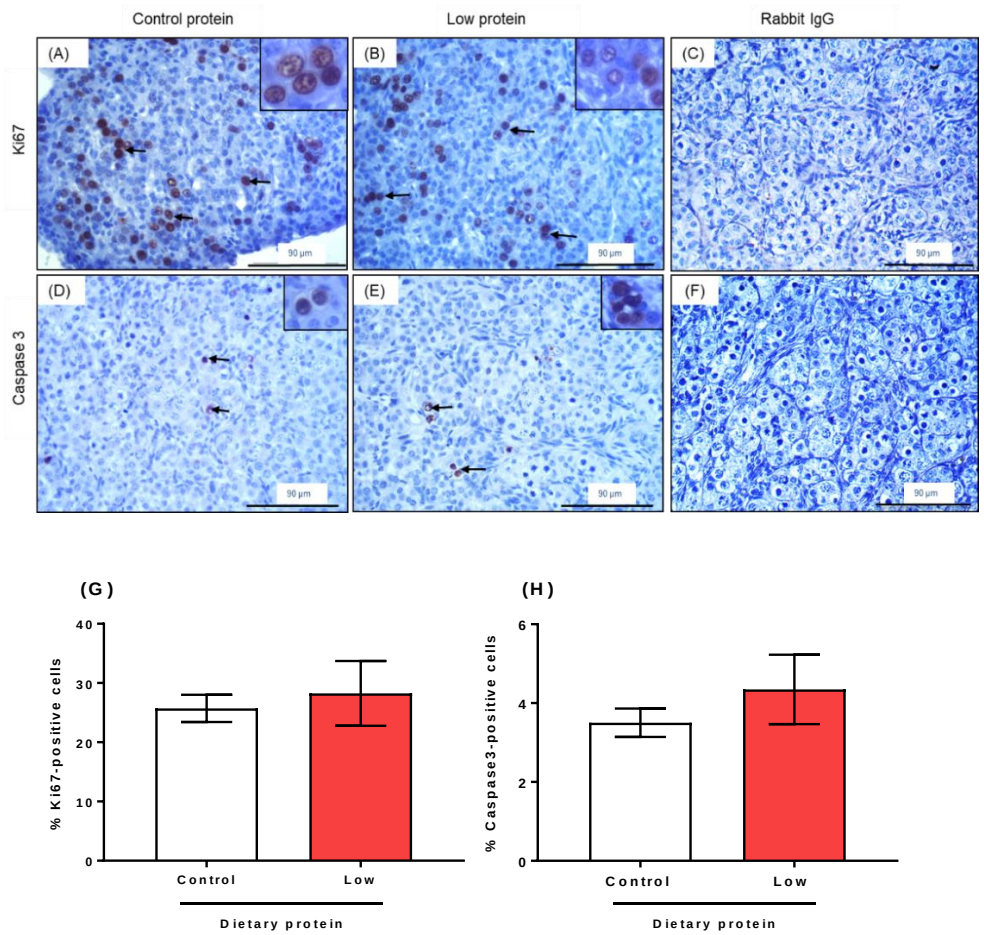



Fig. 3.11 The effect of maternal dietary protein restriction on cell proliferation and apoptosis in the day 65 fetal sheep ovary as determined by Ki67 (A-C) and Caspase 3 staining (D–F). Representative images of fetal ovaries from sheep fed (A, D) control protein (n=7); (B, E) low protein (n=8) diets. (C, F) show the absence of staining in the presence of control rabbit IgG. Photomicrographs show Ki67-positive or Caspase 3-positive cells as indicated by arrows. Insets show images at a higher magnification. Scale bars=90 µm. Graphs show percentage of (G) Ki67 positive germ cells (proliferation index) and (H) Caspase 3-positive cells (apoptotic index). Data are mean ± SEM. There was no effect of diet.

### 3.6.8. Degree of vascularisation of the fetal ovary as determined by CD31 immunohistochemistry

CD31 immunostaining was used to determine the degree of vascularisation in the day 65 sheep fetal ovary. The vascularisation analysis was split into two principle components; (1) microvasculature in the ovarian cortex and (2) the larger blood vessels within the ovarian medulla (Fig. 3.12A). The numerous capillaries within the ovarian cortex were long and thin and thread-like, suggestive of a complex microvasculature (Fig. 3.12B&C). Whilst the blood vessels within the medulla (Fig. 3.13) were fewer in number, but much greater in size, with a clear lumen and a more extensive vessel wall, indicating larger arterioles and / or venules and larger capillaries (Fig. 3.13A&B). It should be noted that the CD31 immunohistochemistry also stained the vascular wall of the larger blood vessels.

There was no obvious difference in the pattern of vascularisation between dietary groups. Indeed, the microvascularised area and percentage microvascularised area of the cortex were not significantly different between the dietary groups ($p>0.05$; Fig. 3.12D&E). The average number, lumen area and perimeter of the blood vessels within the medulla were not altered by maternal diet ($p>0.05$; Fig. 3.13).

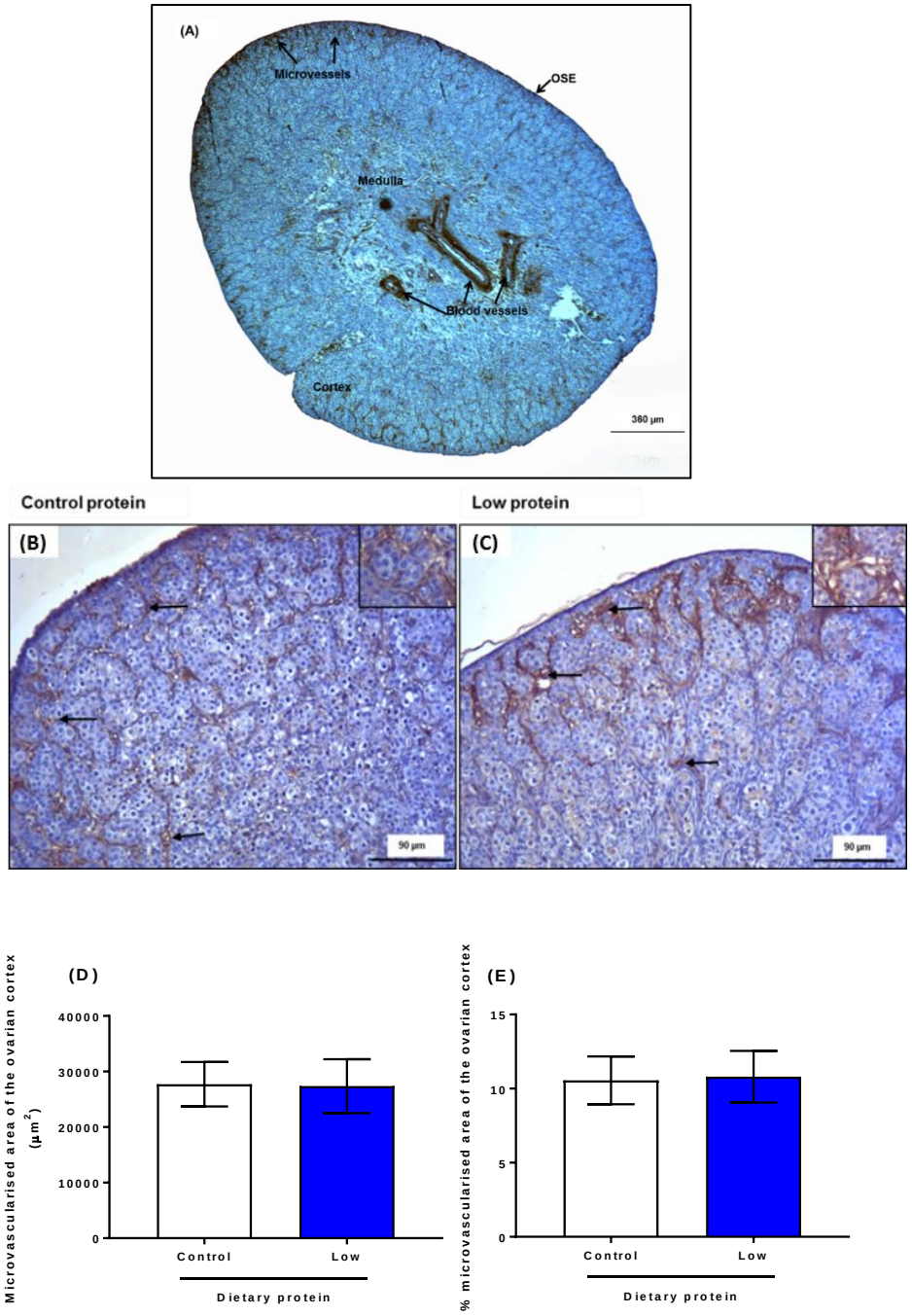



Fig. 3.12: The effect of maternal dietary protein restriction on the vascularisation of the day 65 fetal sheep ovary, as determined by CD31 immunostaining. (A) Low magnification (5x) image to highlight the cortex and medulla regions. Sheep were fed (B) control protein (n=7) and (C) low protein (n=8) diets; microvessels (arrows) are shown. Graphs show (D) the microvascularised area and (E) the percentage microvascularised area of the cortex. There was no effect of diet. Data are mean ± SEM. The scale bars are 360 µm (A) and 90 µm (B, C).

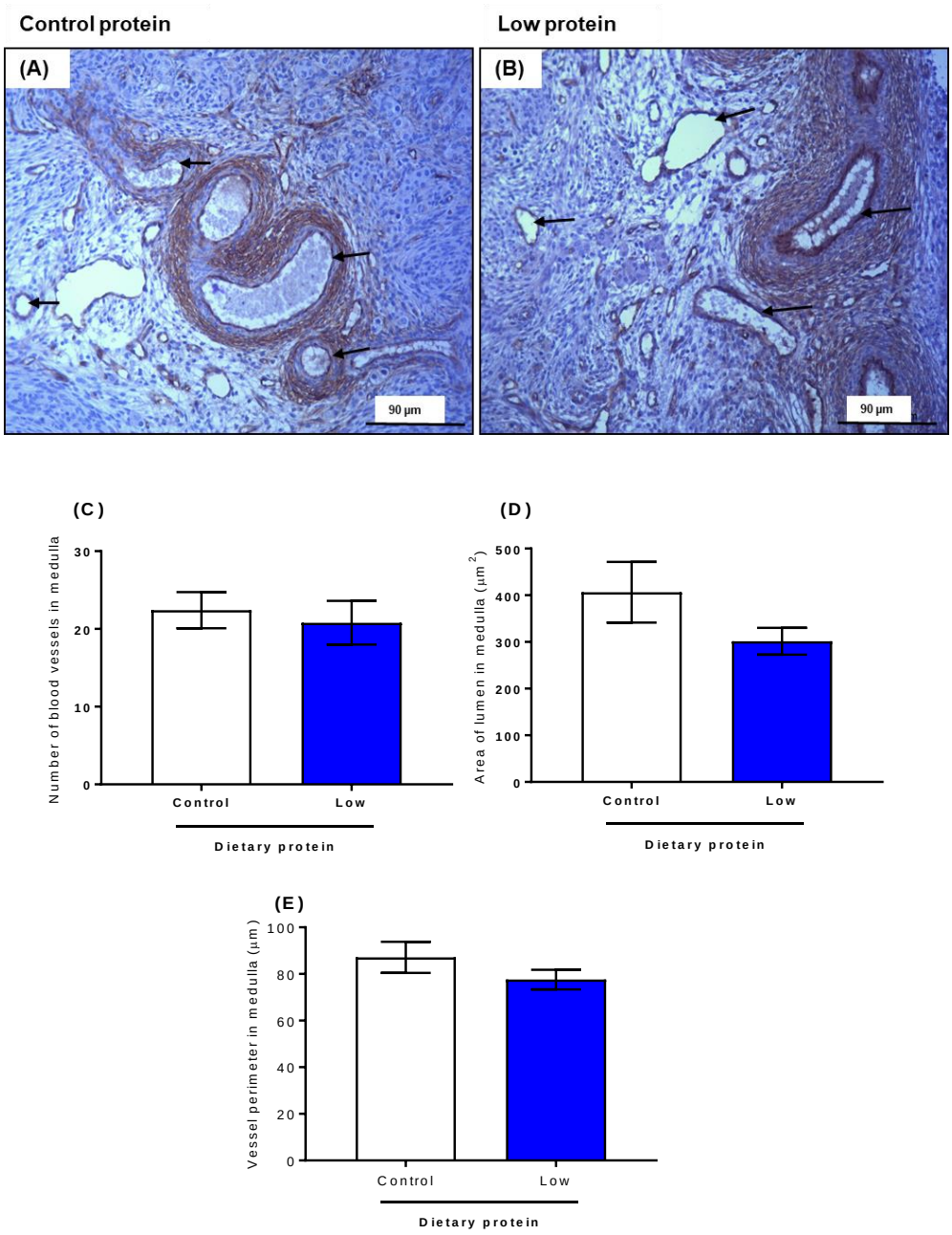



Fig. 3.13: The effect of maternal dietary protein restriction on blood vessel structure in the fetal ovarian medulla. Representative images of fetal ovaries from sheep fed (A) control protein (n=7); (B) low protein (n=8) diets. Photomicrographs show CD31-positive endothelial cells lining the lumen of blood vessels (arrows) alongside staining of the outer vascular wall. Scale bars are 90 µm. Graphs show (C) number, (D) area of lumen (E) and perimeter of blood vessels in the medulla. Data are mean ± SEM. There was no effect of diet.

## 3.7. Discussion

This study investigated whether maternal gestational protein restriction adversely affects the structure and development of the sheep fetal ovary on day 65 post-conception. In the present study, maternal protein restriction did not alter fetal weight on day 65 of gestation. This agrees with previous studies where fetal weight (on day 70 of gestation) was similarly unaffected by a 30% maternal protein restriction (Nishina et al., 2003). Other studies with a moderate global nutrient restriction during early-mid gestation have also shown no effect on fetal weight in sheep (Heasman et al., 1999, Rae et al., 2001). In contrast, total nutrient restriction of ewes has also been used to model intrauterine growth restriction (Morrison, 2008) and a 50% total nutrient restriction in early pregnancy resulted in markedly smaller fetuses on day 78 of gestation (Vonnahme et al., 2003).

The whole fetus weight is a crude measure of the development and function of individual organs. Fetal ovary weight was reduced by approximately 30% in response to maternal protein restriction. In contrast, other organs from the same fetuses showed no effect of diet on the relative size of organs such as the heart, kidney and liver (Dunford et al., 2014). Our study was based on a 50% reduction in protein intake (17 *vs.* 8.7 g crude protein/MJ metabolisable energy) from conception to day 65 of gestation. In contrast to current findings, maternal undernutrition (100% vs 50% energy requirement) did not alter mean fetal ovarian mass at day 60 or 110 of gestation (Rae et al., 2001). Whilst in some cases underfeeding counter-intuitively led to increased fetal ovarian weight (Borwick et al., 1996, Osgerby et al., 2002).

*Germ cell development*

Haematoxylin and eosin staining revealed that the overall morphology and pattern of germ cell development in each fetal ovary corresponded closely with those of other studies from around the same period of gestation in sheep (McNatty et al., 1994, Sawyer et al., 2002). At day 65 of gestation, numerous germ cells were observed in cohorts. These so-called egg nests or ovigerous cords were especially prominent in the cortical region and were observed from near to the ovarian surface epithelium as well as towards the medulla region of the fetal ovary. Furthermore, at this time point, the germ cells within the ovigerous cords were separated from the stromal cells, which

agreed with previous observations (McNatty et al., 2000, Juengel and Smith, 2014). Periodic acid-Schiff staining clearly identified the basement membrane surrounding the cohorts of germ cells, revealing the distinctive features of fetal ovarian morphology (Sawyer et al., 2002).

The histological analyses revealed no morphological differences in response to dietary treatment. In addition, germ cell density, estimated total volume and estimated total number were unaffected by maternal dietary constraints. Similarly, the percentage of PAS staining and the estimated weight of basement membrane were not altered by control versus low protein diets.

In this study, the estimated total weight of germ cells was reduced by maternal protein restriction. This could indicate that there were fewer germ cells in the whole ovary, presumably due to decreased fetal ovary weight/size in the sheep that had been fed a protein-restricted diet. However, when the total number of germ cells were estimated were not affected by diet. The reason for this discrepancy is not known but it is feasible that slight differences in the processing which meant that ovary and weight were not correlated as expected ($r^2$=0.45, n=14, P>0.05). The restricted maternal dietary protein also did not affect the mean fetal germ cell density, or total volume versus controls. This might suggest that germ cell development is not sensitive to nutritional intake during the first trimester (day 65 gestation) in the sheep. Whereas, in other studies, maternal undernutrition (50% energy) similarly did not alter the total number of germ cells at day 65, but did decrease the number of germ cells entering meiosis. In well-nourished fetuses this stage had already been completed, suggesting that undernutrition caused a delay in fetal ovarian development (Rae et al., 2001). Maternal undernutrition (50% energy) also led to increased fetal oogonia concentrations and percentage of germ cells still entering meiosis on day 62 of gestation (Borwick et al., 1996), that was associated with a similar delay in meiosis.

The present study did not quantify meiosis, however, the reduction in the fetal ovary and estimated germ cell weights might indicate a delay in ovarian follicular development in the low protein group animals. OCT4, DAZL and VASA were used as germ cell markers, to further characterise the relative development of the germ cells and whether they were affected by maternal protein diet. All 3 germ cell markers were detected in the cortical region

where germ cells are found. Whilst the immunolocalisation of OCT4, DAZL and VASA share similarities to each other and also some overlap, they do exhibit distinct patterns of expression, with predominant expression changing over time in germ cell development from OCT4 to DAZL to VASA-positive (Anderson et al., 2007, Hummitzsch et al., 2013, Hummitzsch et al., 2014). OCT4 was expressed in all size of germ cells. DAZL/VASA were more abundantly expressed in the germ cells that were larger within the ovigerous cord. Near the OSE, the germ cells were more numerous than towards the medulla. The germ cells found near to the medullary region appeared larger in size. At day 65 of gestation, the germ cells were mostly OCT4-positive cells, which indicated that the germ cells were either primordial germ cells or oogonia.

The density of OCT4, DAZL or VASA-positive cells, their estimated weight, volume and number were unaffected by maternal diet. This demonstrates that germ cells from a number of developmental stages are present on day 65 gestation, with that likely to include primordial germ cells, oogonia and primary oocytes (Fig. 3.1). Based on histological classification (Lundy et al., 1999), no primordial follicles were observed. This is in agreement with previous reports (Rae et al., 2001, Sawyer et al., 2002). However, maternal protein restriction did not seem to affect the developmental progress of germ cells, since the germ cell marker ratios were not altered in response to low protein.

*Proliferation and apoptosis*

Ki67 immunostaining was performed to assess the degree of cell proliferation in the fetal ovary at 65 day gestation. Approximately 25-30% of germ cells were Ki67 positive, indicating that significant cell proliferation was occurring. Germ cell proliferation has been previously observed at this time in fetal sheep ovaries, and continues through to day 90-100 (Sawyer et al., 2002). Indeed, mitosis predominates up to day 75 in sheep when germ cell numbers peak, before a wave of germ cell atresia begins (Smith et al., 1993). In the current study, maternal protein restriction did not affect Ki67 protein expression. Our findings disagree with previous findings, whereby restricted maternal feed intake (50%) from mating to day 65 decreased fetal germ cell expression of Ki67 versus controls (100% intake) (Lea et al., 2006). It is not clear whether this reflects a different fetal

response to reduced intake versus reduced protein, or rather a technical difference based on the experimental approaches to analysis.

Apoptosis is a distinct, organised form of cell death which has an important role in ovarian development. Morphologically, apoptotic cells exhibit cell shrinkage, membrane alterations, DNA fragmentation and the formation of apoptotic bodies. Caspase 3 is the common effector of apoptosis and was immunolocalised to the fetal ovaries in the current study. Apoptotic germ cell death was observed in the fetal ovaries at the same time as other germ cells were undergoing mitotic cell proliferation. The apoptotic index was approximately 4% and maternal protein restriction did not affect Caspase 3 expression at day 65 of gestation. Apoptosis is thought to account for the loss of more than 75% of fetal germ cells, between day 75 and 90 in sheep (Smith et al., 1993) and has been shown to vary according to maternal diet (Lea et al., 2006). However, pro-apoptotic Bax expression was not affected by maternal diet to day 65 (Lea et al., 2006).

*Ovarian vascularisation*

Blood vessels are initially formed in the fetus via vasculogenesis (Bussolino et al., 1997). Indeed, vascularisation was evident in both cortical and medullary region of the fetal ovaries at day 65 of gestation. The vasculature in the cortical and medullary regions of the ovary differed in structure. Namely, in the cortex it was much more a microvasculature while in the medulla region it was capillary and/or defined blood vessels, in agreement with previous observations (Kikuta et al., 1991, Cursiefen et al., 1998, Kang et al., 2001). The development of primary oocytes within the ovigerous cords and subsequent formation of primordial follicle are all dependent on the blood supply to the medulla and then the cortex. This is necessary for the delivery of oxygen, nutrients and removal of waste products (McFee and Cupp, 2013).

The degree of CD31 staining in the microvascularised cortex was not different between dietary groups. Similarly, the number, area and perimeter of the larger blood vessels in the medulla were unaffected by dietary protein restriction. Collectively, this would suggest that the supply of nutrients and oxygen to the developing germ cells would be similar in both the control and low protein groups. Furthermore, since the vasculature was unaffected at this stage, the inference is that the blood supply would have been

maintained, during the next critical stage of oogenesis and folliculogenesis, namely formation of primordial follicles. Intriguingly, in the same fetuses the microvasculature (identified using CD34 immunohistochemistry) of the fetal kidney was adversely affected by the low protein diet. However, the macrovasculature as measured by corrosion casts was not affected (Dunford et al., 2014). At this stage of gestation, the kidney is much larger in size and it was feasible to measure the vasculature using vascular corrosion casts (Dunford et al., 2014). This approach should be considered in future ovarian experiments. That said, it is feasible that the ovarian vascular development is less sensitive to dietary protein restriction than the fetal kidney. Combined with the limited or lack of effect of the low protein diet on germ cell numbers, density and development, it is possible that there is potentially a mechanism whereby the ovary is protected to a certain extent from metabolic stresses. This might be necessary to protect the animal's potential for reproduction. While this is an interesting hypothesis, it would be essential to first investigate the effects of this dietary regime on later stages of fetal (i.e. day 110 and birth) and subsequent ovarian development in the neonate and adult (i.e. puberty, antral follicle counts and AMH levels).

One limitation of the present study was that it did not fully determine the number of pre-granulosa (GREL) cells in the fetal ovary. It is feasible that these GREL cells, critical for the formation of the primordial follicle and maintenance of the germline, are more sensitive to metabolic challenges. Attempts were made to localise these cells using GREL specific markers (e.g. FOXL2). However, there was considerable background and non-specific staining in the immunohistochemistry and thus this was not pursued further. Another limitation was the assumptions in calculation in the study. The calculations were based from the mathematical formula on the unit measurement of volume, weight and area. This assumption might have challenges in terms of under or overestimation of the numbers of germ cells within the sheep fetal ovary.

*Conclusion*

In conclusion, the fetal ovarian weight was adversely affected by dietary protein restriction. However, more detailed morphological and histological analysis revealed that there was limited evidence that on day 65 of gestation the ovarian structure or number of germ cells were influenced by maternal dietary protein restriction in sheep. The observations were contrary to our

expectation, as similar maternal protein restricted diets in sheep have adversely affected several aspects fetal ovarian development at later stages of gestation. In addition, the maternal protein restriction did not affect proliferation or apoptotic indices, or the degree of vascularisation in the day 65 fetal ovine ovary. This would suggest that the initial proliferation of germ cells and formation of the fetal ovary was not sensitive to maternal dietary protein restriction.

The study re-iterated the presence of germ cells at different stages of development i.e. primordial germ cells, oogonia through to primary oocytes as indicated by OCT4, DAZL and VASA immunostaining on day 65 of gestation. However, no primordial follicles were observed at this stage. Furthermore, the proliferation index was greater than the apoptotic index indicating that the loss of germ cell had only just begun. It is feasible that the next "apoptotic-dominant" phase would be more sensitive to dietary challenges.

The original plan for the thesis was to extend this study to investigate the potential underlying molecular mechanisms for any changes to the germ cell numbers. This would have involved RNAseq, bioinformatic and qPCR analysis on the other frozen ovary from each animal. However, given the largely non-significant findings it was decided to move the project onto investigating the regulation of the vasculature development and angiogenesis in the follicular-luteal transition.

# Chapter 4: The effect of the IGF system on endothelial cell network formation and progesterone synthesis in a bovine luteinising follicular angiogenesis culture system

## 4.1. Introduction

Rapid luteal development and extensive steroidogenesis are crucial for conception and thus reproductive efficiency. Hence, inadequate function of the CL is considered a major cause of poor embryo development and early embryonic mortality in dairy cows (Boland et al., 2001, Evans and Walsh, 2011). The follicle-luteal transition is an extremely dynamic process involving numerous morphological and biochemical changes that occur after the luteinising hormone (LH) surge (Pate, 1996, Reynolds and Redmer, 1999, Niswender et al., 2000). For these remodelling events to occur, sufficient growth of blood vessels and establishment of blood supply is crucial (Reynolds and Redmer, 1999, Niswender et al., 2000). Furthermore, the rate of progesterone production has been correlated to the ovarian blood flow and vascularisation (Fraser and Lunn, 2001, Acosta et al., 2004, Schams and Berisha, 2004).

Numerous pro- and anti-angiogenic regulators control the rate and process of angiogenesis in the follicle and CL. The major stimulators of angiogenesis are believed to be vascular endothelial growth factor A (VEGFA), fibroblast growth factor 2 (FGF2), and platelet derived growth factors (PDGFs) (Gospodarowicz et al., 1985, Ferrara and Davis-Smyth, 1997, Spicer and Aad, 2007). Conversely, thrombospondin, angiostatin and transforming growth factor B are considered important anti-angiogenic factors.

VEGFA and FGF2 are potent stimulators of angiogenesis in both follicular and luteal cells *in vitro* (Robinson et al., 2007, Woad et al., 2009, Laird et al., 2013). In the pre-ovulatory follicle, FGF2 protein was expressed in the theca layer (cytoplasm of EC and pericytes) (Reynolds et al., 2000) while *VEGFA* mRNA and protein were localised in both theca interna and granulosa cells. Furthermore, VEGFA concentration increased as the follicle developed in cows (Berisha et al., 2000). After ovulation, *VEGFA* mRNA and protein as

well as FGF2 protein were abundantly expressed in the early bovine CL (Schams et al., 1994, Berisha et al., 2000). Moreover, local neutralization of FGF2 or VEGFA suppressed luteal growth and progesterone secretion during the early luteal phase in cattle (Yamashita et al., 2008). This is supported by studies in rats, where administration of a VEGF receptor inhibitor reduced CL number, luteal vascularisation and inhibited progesterone production (Ferrara et al., 1998). Collectively, these studies demonstrated the critical importance of FGF2 and VEGFA for adequate luteal vascularisation and function. However, several other important pro-angiogenic factors such as IGF1 and IGF2 are likely to play an important modulatory role in the follicle-luteal transition. This might be particularly relevant in the post-partum dairy cow, where IGF1 and IGF2 are nutritionally/metabolically regulated.

The insulin-like growth factor (IGF) system comprises of two ligands, IGF1 and IGF2 which bind to two different cell-surface receptors, IGF type 1 receptor (IGF1R) and the IGF type 2 receptor (IGF2R). Their action is further regulated via a family of six high-affinity IGF binding proteins (IGFBP1-6) and their assiociated IGFBP degrading proteases (LeRoith, 2003, Clemmons, 2007). The regulation of IGF1 expression is controlled by various factors such as nutrition, and plasma oestrogen concentration, but its production by the liver is principally regulated by growth hormone (GH) (Friedrich et al., 2014). GH increases plasma IGF1 concentrations and might stimulate production of IGFBP3 by the liver (Davis et al., 1992). Thus, systemic IGF1 concentrations can reflect the metabolic status of an animal. In contrast, plasma IGF2 concentration are much lower than IGF1 and are less GH-dependent in adults (Iliev et al., 2014). However, in the ovary including the CL, there is extensive local production of IGF2 (Woad et al., 2000). Both IGF1 and IGF2 have insulin-like metabolic effects such as decreasing plasma glucose and increasing glucose uptake into cells (LeRoith and Yakar, 2007, Pollak, 2008). They can act in an endocrine, paracrine and/or autocrine manner and exert their effects on many different organs, tissues and cells including hepatocytes, cardiac myocytes and ovarian steroidogenic cells (Turgeon et al., 2006, Bach, 2015).

Insulin-like growth factors 1 and 2, like insulin, are pleiotropic hormones that have multiple roles in regulating critical metabolic processes, cell proliferation, differentiation, migration, survival and apoptosis. IGF1 is also an intra-ovarian growth factor that is involved in the regulation of follicular

development (Webb and Campbell, 2007). IGF1 was expressed by theca interstitial cells of bovine follicles throughout folliculogenesis while in granulosa cells *IGF1* mRNA expression in the dominant follicle increased after selection (Schams et al., 1999). *IGF2* mRNA was localised to both granulosa and theca cells with greater *IGF2* mRNA levels in theca cells of the dominant follicles than other follicles (Aad et al., 2013).

Both IGF1 and IGF2 exert their action through high-affinity binding to the tyrosine kinase receptor, IGF1R that binds IGF1 with higher affinity than IGF2 (Jones and Clemmons, 1995, Federici et al., 1997, LeRoith, 2003). *IGF1R* mRNA is present in both theca and granulosa cells of dominant follicles (Schams et al., 1999). Acting through IGF1R, both IGF1 and IGF2 stimulate steroid production by follicular and luteal cells in many species (Silva et al., 2009). For example, in cyclic cows, exogenous IGF1 increased oestradiol concentrations in follicular fluid of small (2–5 mm) but not large (≥6 mm) follicles (Spicer et al., 2000). While, IGF1 enhanced LH-induced androstenedione production in bovine theca cells by increasing the numbers of LH receptors (Stewart et al., 1995, Campbell et al., 1998). In luteal cells, IGF1 increased STAR protein expression and consequently had stimulatory effects on progesterone production (Devoto et al., 1999, Sekar et al., 2000). IGF1 also stimulated granulosa cell survival (Armstrong et al., 2000, Schams et al., 2002, Spicer and Aad, 2007), proliferation and differentiation (Spicer and Aad, 2007) in cows. IGF2 also promoted granulosa cell proliferation and differentiation and was present in follicular fluid at a greater concentration than IGF1 (Spicer et al., 1988). There have been reports of FSH and IGF2 interaction, with IGF2 enhancing FSH-stimulated granulosa cell proliferation and steroidogenesis in cattle and human dominant follicles (Spicer and Aad, 2007, Baumgarten et al., 2015).

In circulation, IGFBPs can modulate the effects of IGF1 by transporting and/or binding the IGFs to extracellular fluids. In cattle, low intra-follicular IGFBP4 levels are necessary for a follicle to attain FSH independence and reduce the inhibitory effect that IGFBP4 has on steroidogenesis (Mihm et al., 2000). Indeed, the addition of IGFBP4 to theca and granulosa cells inhibited the gonadotrophin-stimulation of steroid synthesis (Putowski et al., 1997, Mason et al., 1998). In many species (e.g. cows, sheep, pigs, and mares), intra-follicular levels of IGFBP2 and IGFBP4 greatly decrease as the follicle

approaches the pre-ovulatory stage (Besnard et al., 1996, Armstrong et al., 1998, Liu et al., 2000, Mazerbourg et al., 2003).

In the CL, *IGF1* mRNA expression was lower during the early luteal phase than the mid and late luteal phases. Using *in situ* hybridisation, *IGF2* mRNA was expressed by steroidogenic luteal cells while *IGF1R* mRNA was widely and abundantly observed in the large and small luteal cells and albeit to lesser extent in EC (Woad et al., 2000). Using immunohistochemistry, IGF1 was localised mainly to large and small luteal cells with staining also evident in EC (Schams et al., 1999). In contrast, IGF2 protein was also localised in perivascular cells of large blood vessels and capillary pericytes (Amselgruber et al., 1994).

In other tissues, strong IGF1R immunoreactivity was present in EC and pericytes of newly formed arteries and veins (Bar and Boes, 1984, Li et al., 2005). While transient IGF1 immunoreactivity was evident in the vascular cells during angiogenesis (King et al., 1985, Hansson et al., 1989). IGF1 potentially could promote angiogenesis by increasing the expression and secretion of VEGFA in granulosa and luteal cells when they were treated alone or with LH (Schams et al., 2001). This agreed with similar studies using rabbit cornea and retina models where IGF1 induced angiogenesis and EC proliferation (Grant et al., 1993). Furthermore, inhibition of IGF1R signalling in embryonic stem cells with picropodophyllin (PPP) reduced the formation of IGF-induced precursor EC (Piecewicz et al., 2012). Collectively, this suggests that there is an active IGF system in the follicle and corpus luteum and that IGF1 and/or IGF2 have the potential to stimulate angiogenesis. However, there is very limited information on the role of IGF1 and/or IGF2 in controlling angiogenesis in the bovine ovary.

### 4.1.1. Aim and hypothesis

This Chapter evaluated the effects of the IGF system on EC network formation, progesterone production and cell proliferation in bovine luteinising follicular cells *in vitro*. The hypothesis was that IGF1 and/or IGF2, acting through IGF1 receptor, will promote EC network formation and progesterone production in bovine luteinising follicular cells.

### 4.1.2. Studies

1.) To determine the effect of IGF1 and/or IGF2 treatment on EC network formation, progesterone production and cell proliferation in bovine luteinising follicular cells.
2.) To evaluate the endogenous production of IGF1 and IGF2 in culture and to assess the effect of LH on IGF1 production.
3.) To assess the effect of inhibition of IGF1R signalling on EC network formation and progesterone production of bovine luteinising follicular cells.
4.) To evaluate the effect of endogenous IGF1 sequestration with IGFBP4 on EC network formation and progesterone production of bovine luteinising follicular cells.

## 4.2. Materials and Methods

Luteinising follicular cells were harvested from large bovine follicles (>10 mm) as previously described (see Section 2.1.3) and plated onto fibronectin-coated 12 well plates (see Section 2.1.1) with $3x10^5$ granulosa cells plus $1x10^5$ theca cells to the final density of $4x10^5$ cells/well. Cells were cultured in supplemented MDCB 131 media (see Section 2.1.2 for preparation) with the following important alterations: 1) $LR^3$-IGF1 was omitted from the supplemented MDCB 131 media; 2) Insulin, transferrin and selenium were added individually rather than as a single supplement to enable insulin to be added at 100 ng/ml, rather than 10 µg/ml and thus minimising the potential stimulation of IGF1R by insulin. 3) For Study 2, LH was omitted from the supplemented MDCB 131 media, but was added as a treatment according to experimental design (0, 5 or 50 ng/ml).

### 4.2.1. Study 1: Effect of IGF1 or IGF2 on endothelial cell network formation, progesterone production and cell proliferation in bovine luteinising follicular cells

The hypothesis was that IGF1 and/or IGF2 would promote EC network formation and progesterone production in bovine luteinising follicular cells. This study assessed the following endpoints:

a) Endothelial cell (EC) network formation as determined by von Willebrand factor immunocytochemistry and image analysis.
b) Progesterone production as determined by ELISA.
c) Degree of cell proliferation as analysed by MTT assay.

Bovine luteinising follicular cells (n=4 cultures) were cultured for 5 days and on day 1 were treated with either control medium (no $LR^3$-IGF1 or IGF2 treatment) or two different doses of either $LR^3$-IGF1 or IGF2 (10 and 100 ng/ml) with or without angiogenic-stimulation (FGF2+VEGFA; both 1 ng/ml) (Fig. 4.1). These doses of IGF1/IGF2 were based on those used previously and those found in circulation (Spicer and Aad, 2007). Each treatment was performed in duplicate. The addition of bovine FGF2 (Abcam: ab129471) and bovine VEGFA (Abcam: ab88201) was based on the three-to-four fold increase in EC network formation observed in luteal culture systems (Robinson et al., 2008, Woad et al., 2012).

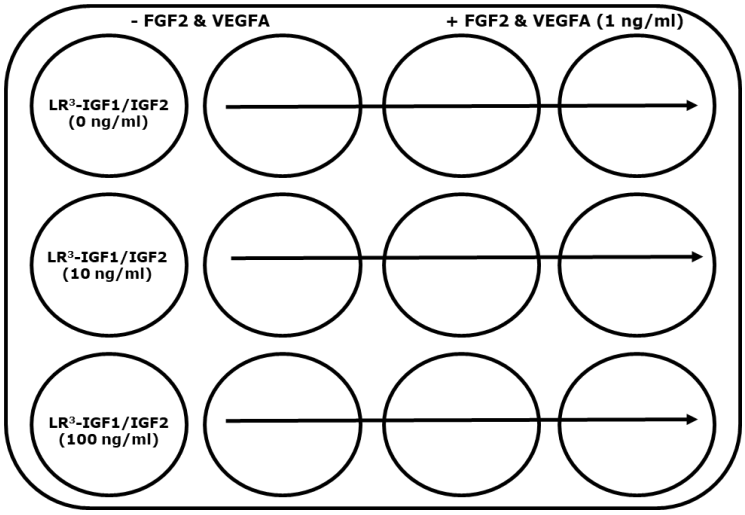



Fig. 4.1: The plate layout for study 1, which investigated the effects of $LR^3$-IGF1 and IGF2 on endothelial cell network formation and progesterone production in bovine luteinising follicular cells.

Spent media was collected on days 3 and 5 and replaced with fresh supplemented MDCB 131 media. The collected media (from days 3 and 5) were stored at -20°C. The days 3 and 5 were selected to assess the progesterone concentration and its production rate over time, thus indicating luteinisation. Progesterone analysis was performed in triplicate by ELISA (Section 2.3) following 100–200-fold dilution of samples in PBS. At the end of culture (day 5), the cells were fixed in ice-cold acetone: methanol (1:1) for immunocytochemistry analysis. The EC networks were quantified by von Willebrand factor immunocytochemistry and image analysis as detailed in Section 2.2.3.

In parallel, bovine luteinising follicular cells (n=5 cultures) were prepared for the assessment of viable cell growth by utilising a MTT assay (Cayman Chemical, United States of America (USA)). The bovine luteinising follicular cells were treated with control, $LR^3$-IGF1, IGF2, or combined $LR^3$-IGF1 + IGF2 (10 ng/ml); each treatment was performed in six replicates.

### 4.2.2. Study 2: The expression of the IGF system in luteinising follicular cells and bovine ovarian tissues

This study had the following objectives:

a) To quantify the endogenous production of IGF1 and assess the effect of LH on IGF1 production by ELISA.
b) To assess the endogenous production of IGF2 by western blot.
c) To immunolocalise the IGF1R in paraffin-embedded bovine follicles and corpus luteum.
d) To immunolocalise the IGF1R in cultured luteinising follicular cells.
e) To co-localise the IGF1R and isolectin B4 (an EC marker) in culture by dual immunofluorescence.

Bovine luteinising follicular cells (n=8 cultures) were treated with different doses of luteinising hormone (0, 5 or 50 ng/ml) (AFP11743B; biopotency 1.06 × oLH NIDDK-I-2; a gift from Dr A. F. Parlow, National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), Torrance, CA, USA) (Fig 4.2). Each treatment was performed in triplicate. In this study, the supplemented MCDB 131 media had LH, $LR^3$-IGF1 and ITS replaced by individual components with LH added as a treatment at 0, 5 or 50 ng/ml.

In the first 4 cultures, the spent media were collected on days 3 and 5 to determine IGF1 concentrations by ELISA (Section 2.3.2). IGF2 quantification was not feasible as there is no specific bovine IGF2 ELISA commercially available. On day 5 of culture, the cells were collected and lysed for IGF2 protein quantification by western blot and dot blot (Section 2.7.1).

In the second 4 cultures, the cells were cultured as per design but on day 5, the cells were fixed in ice-cold acetone: methanol. The first 2 wells were then immunostained for IGF1 receptor (Novus: NBP1-77680; Section 2.2.3) and the third well for IGF1R and isolectin B4 (EC marker) co-localisation by dual immunofluorescence (Section 2.2.4).

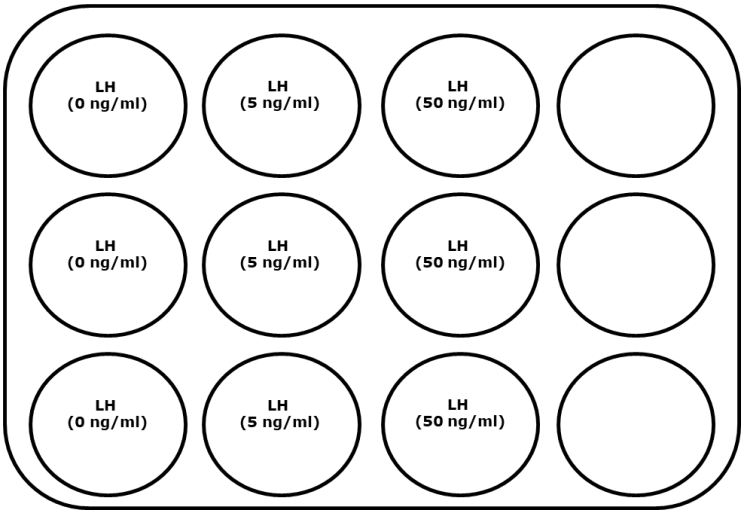



Fig. 4.2: The plate layout for study 2, which investigated the effect of luteinising hormone (LH) on endogenous production of IGF1 and IGF2 in bovine luteinising follicular cells.

IGF1R was also immunolocalised in sections of adult bovine ovarian tissues (dissected large antral follicles (n=3) and day 5-8 corpus luteum (n=5)) as described in Section 2.2.3 and Table 2.2.

### 4.2.3. Study 3: The effect of inhibiting IGF1R signalling on endothelial cell network formation and progesterone production of bovine luteinising follicular cells

The hypothesis was that inhibition of IGF1R signalling would block the action of endogenously produced IGFs and thereby decrease EC network formation and progesterone production in bovine luteinising follicular cells.

Bovine luteinising follicular cells (n=4 cultures) were treated with a specific IGF1 receptor inhibitor (1 μM; picropodophyllin (PPP); S7668; Selleckchem, USA) in the presence or absence of $LR^3$-IGF1, IGF2, or combined $LR^3$-IGF1+IGF2 (10 ng/ml) (Fig. 4.3); each treatment was performed in duplicate. Picropodophyllin was selected based on its specific inhibition of IGF1R tyrosine kinase signalling with no known effects on other tyrosine kinase receptors. The concentration of PPP was based on reported $IC_{50}$ values and doses used in similar culture systems (Larsson and Axelson, 2007, Bieghs et al., 2014).

Picropodophyllin was initially dissolved in dimethyl sulfoxide (DMSO), with the final DMSO concentration in each treatment being 0.1%. Thus, the control wells were treated with 0.1% DMSO. The cells were treated throughout culture with the experiment terminated on day 5. Spent media were collected on days 3 and 5 of culture and analysed for progesterone production (Section 2.3.1). On day 5 of culture, the cells were fixed in ice-cold acetone: methanol and the EC network formation was quantified by VWF immunohistochemistry and image analysis (Section 2.2.3).

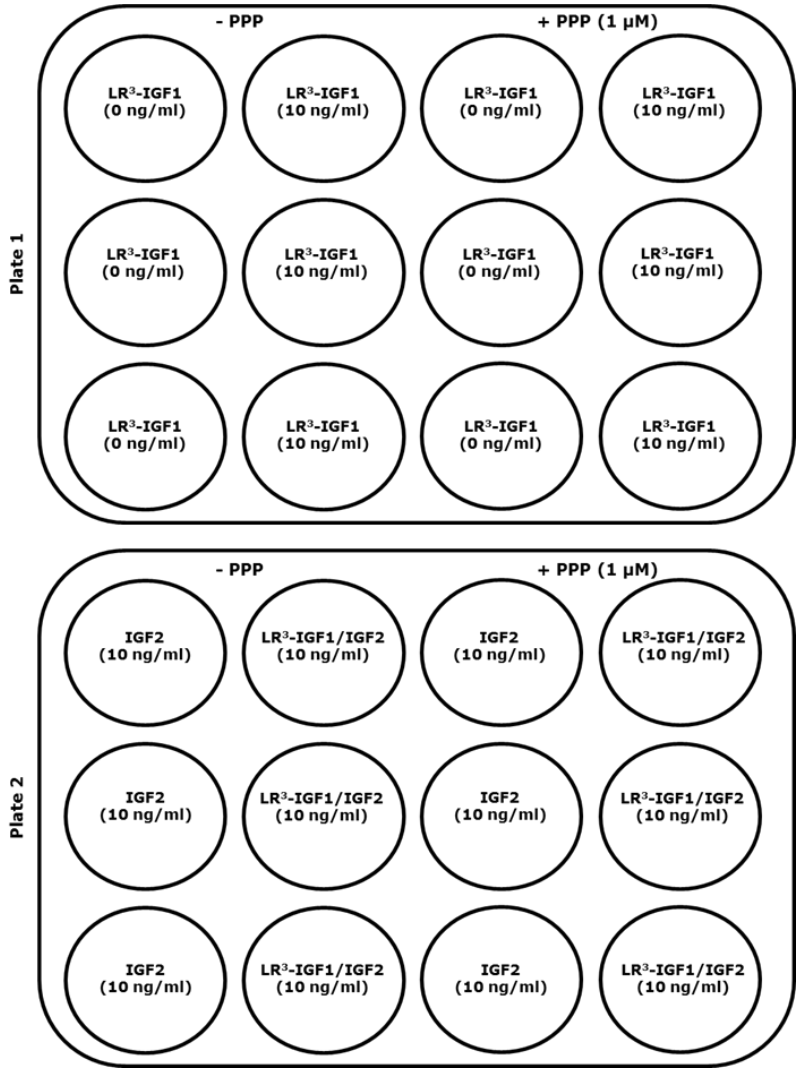



Fig. 4.3: The plate layout for Study 3, which investigated the effect of inhibition of IGF1R signalling on endothelial cell network formation and progesterone production in bovine luteinising follicular cells. Specific IGF1 receptor inhibitor (picropodophyllin (PPP): 1 µM).

### 4.2.4. Study 4: The effect of IGFBP4 on endothelial cell network formation and progesterone production of the bovine luteinising follicular cells

The hypothesis tested was that IGFBP4 would sequester endogenously produced IGF and thus reduce EC network formation and progesterone production in bovine luteinising follicular cells. While, $LR^3$-IGF1, which is not bound by IGFBP4 will stimulate EC network formation and progesterone production when cells are co-treated with IGFBP4.

Bovine luteinising follicular cells (n=4 cultures) were treated throughout culture with 100 ng/ml IGF binding protein 4 (human IGFBP4: Pepro-Tech: 350-05B) in the presence or absence of human $LR^3$-IGF1 (10 ng/ml) (Fig. 4.4); each treatment was performed in triplicate. Spent media were collected on days 3 and 5 of cultures for progesterone analysis (Section 2.3.1). On day 5 of culture, cells were fixed in ice-cold acetone: methanol for immunocytochemical analysis of EC network formation by VWF immunocytochemistry and image analysis (Section 2.2.3).

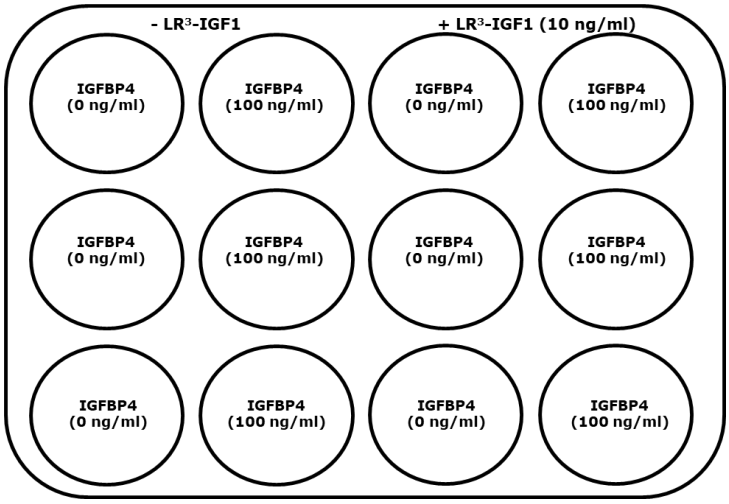



Fig. 4.4: The plate layout for Study 4 which investigated the effect of insulin-like growth factor binding protein 4 (IGFBP4) on endothelial cell network formation and progesterone production in bovine luteinising follicular cells.

## 4.2.5. Immunostaining

### 4.2.5.1. Immunocytochemistry

Cultured luteinising follicular cells were immunostained for VWF and IGF1R as detailed in Section 2.2.3 and Table 2.2. The primary antibodies were polyclonal rabbit anti-human VWF (Dako: A0082) and polyclonal rabbit anti-IGF1 receptor (Novus: NBPI-77680).

### 4.2.5.2. Immunofluorescence

The IGF1R and isolectin B4 (EC marker) were co-localised in cultured follicular cells by dual immunofluorescence, according to Section 2.2.4 using mouse anti-human IGF1 receptor (Novus) and biotinylated *Griffonia (Bandeiraea) simplicifolia* isolectin B4 (Vector).

## 4.2.6. ELISA and MTT assays

### 4.2.6.1. Quantification of progesterone production

The progesterone production by luteinising follicular cells was evaluated in the spent media from individual wells on day 3 and 5 of culture; these were stored at -20°C until assay (Section 2.3.1). The samples from each culture were assayed in triplicate on a single plate and mean intra-assay CV was 11-14% for each study.

### 4.2.6.2. Quantification of IGF1 concentration

IGF1 concentration in spent media was evaluated using a human IGF1 DuoSet ELISA, according to the manufacturer's procedure (R&D Systems: DY291-05) and as described in Section 2.3.2. Human and bovine IGF1 are identical in amino acid sequence. The samples from each culture were assayed on a single plate and mean intra-assay CV was 9.8%. The limit of detection was 50 pg/ml.

### 4.2.6.3. MTT Cell Proliferation Assay

Bovine luteinising follicular cells were treated with control, $LR^3$-IGF1, IGF2, or combined $LR^3$-IGF1 and IGF2 (10 ng/ml). To determine the degree to which cells had proliferated, a MTT cell proliferation assay was conducted. The MTT assay kit (Cayman Chemicals) was performed as according to the manufacturer's instructions (Section 2.3.5).

### 4.2.7. Western blotting

The cultured follicular cells were collected and lysed for IGF2 protein quantitation via western blot and dot blot, using the methods described in Section 2.4.1. Following protein extraction from lysed cells, protein concentrations were determined using a Bradford assay. Based on this protein (20 µg) was loaded onto a mini-gel (western blot) or activated PVDF membrane (dot blot) for IGF2 protein analysis. Dot blots were performed to eliminate any potential confounding issues associated with gel electrophoresis or protein transfer steps. ACTB protein was used to confirm transfer which was expressed at similar levels in all samples. The intensity of IGF2 protein expression was not quantified due to its low level of expression.

### 4.2.8. Statistical analysis

Data were checked for normality using residual plots and for homogeneity of variance with Levene's test; where appropriate, data were log transformed. For all experiments, $p<0.05$ was declared as significant and all experimental data values are presented as mean ± SEM from replicated experiments. Each experiment was replicated four or more times, and within each experiment, there were duplicate or triplicate culture wells.

For the EC network parameters (i.e. total EC network area, number of branch points) and cell proliferation, the data were converted to % of mean for the control wells from that particular culture. The statistical approach used was a randomised-block ANOVA using Genstat 18$^{th}$ Edition (Hemel Hempstead, United Kingdom (UK)). For each endpoint, the data was blocked by culture and the particular well nested within. The exact factors depended on the factorial design of each study. For Study 1, a 3 × 2 factorial design was used with the different doses of LR$^3$-IGF1 or IGF2 and presence or absence of angiogenic-stimulation used as factors. For Study 3, a 4 [control, LR$^3$-IGF1, IGF2 or combined IGFs] × 2 [control or PPP] factorial design was performed. For Study 4, a 2 × 2 factorial design was conducted with LR$^3$-IGF1 and IGFBP4 treatment used as the factors. The main effects and their interaction were included in each statistical model.

For the progesterone production analysis, data were log transformed. The day of culture was initially kept in the factorial design. However, as predicted

there was a large effect of culture day and thus the data from days 3 and 5 were analysed independently with the same factorial design as for EC network data (i.e. 3 × 2 factorial design: $LR^3$-IGF1 [0, 10 or 100] × absence/presence of FGF2+VEGFA).

The effect of LH on IGF1 concentrations (Study 2) was analysed as a one-way randomised-block ANOVA with the LH dose used as the factor. The data were blocked by culture and well. A Student's Newman Keuls (SNK) multiple comparisons test was used to determine which doses of LH were different.

## 4.3. Results

### 4.3.1. Study 1: Effect of IGF1 or IGF2 on endothelial cell network formation, progesterone production and cell proliferation in bovine luteinising follicular cells

#### 4.3.1.1. Effects of IGF1

In the control wells, endothelial cells (EC) were closely associated with each other and were largely organised into structures with a network-like appearance. These EC networks (a group of closely apposed ECs) were often elongated and tubule-like in appearance, with some complex multiple branching points extending from the main body of EC. The vast majority of the EC formed into a network-like pattern. However, some EC were observed in small islands with limited branching away from the central body of cells. Other cell types (presumably steroidogenic cells, pericytes, and smooth muscle cells) were more numerous and present alongside the EC (Fig. 4.5A). The growth and development of the EC networks appeared to be slightly greater under angiogenic-stimulated conditions (FGF2+VEGFA; Fig 4.5D-F). These organised, intricate EC networks were formed at all doses of $LR^3$-IGF1 treatment in the presence and absence of angiogenic stimulation (Fig. 4.5B-F). There was a similar pattern of EC networks, in terms of number, shape and size, across all $LR^3$-IGF1 treatments.

The addition of FGF2+VEGFA increased the number, total area, and perimeter of the EC networks ($p<0.001$), and number of branch points ($p<0.05$) by 30% (Fig. 4.6A-C). However, the mean area of each EC network ($p=0.07$) and degree of branching ($p=0.05$) only tended to increase in response to FGF2+VEGFA treatment (Fig. 4.6D and F). $LR^3$-IGF1 treatment at either dose had no effect on the number, total area, or perimeter ($p>0.05$) of EC networks (Fig. 4.6A-C). Equally, the mean area of each EC network, total number of branch points or degree of branching (per EC network) versus control, were unaffected by $LR^3$-IGF1 treatment irrespective of FGF2+VEGFA treatment ($p>0.05$; Fig. 4.6). In addition, there was no $LR^3$-IGF1 by FGF2+VEGFA treatment interaction ($p>0.05$).

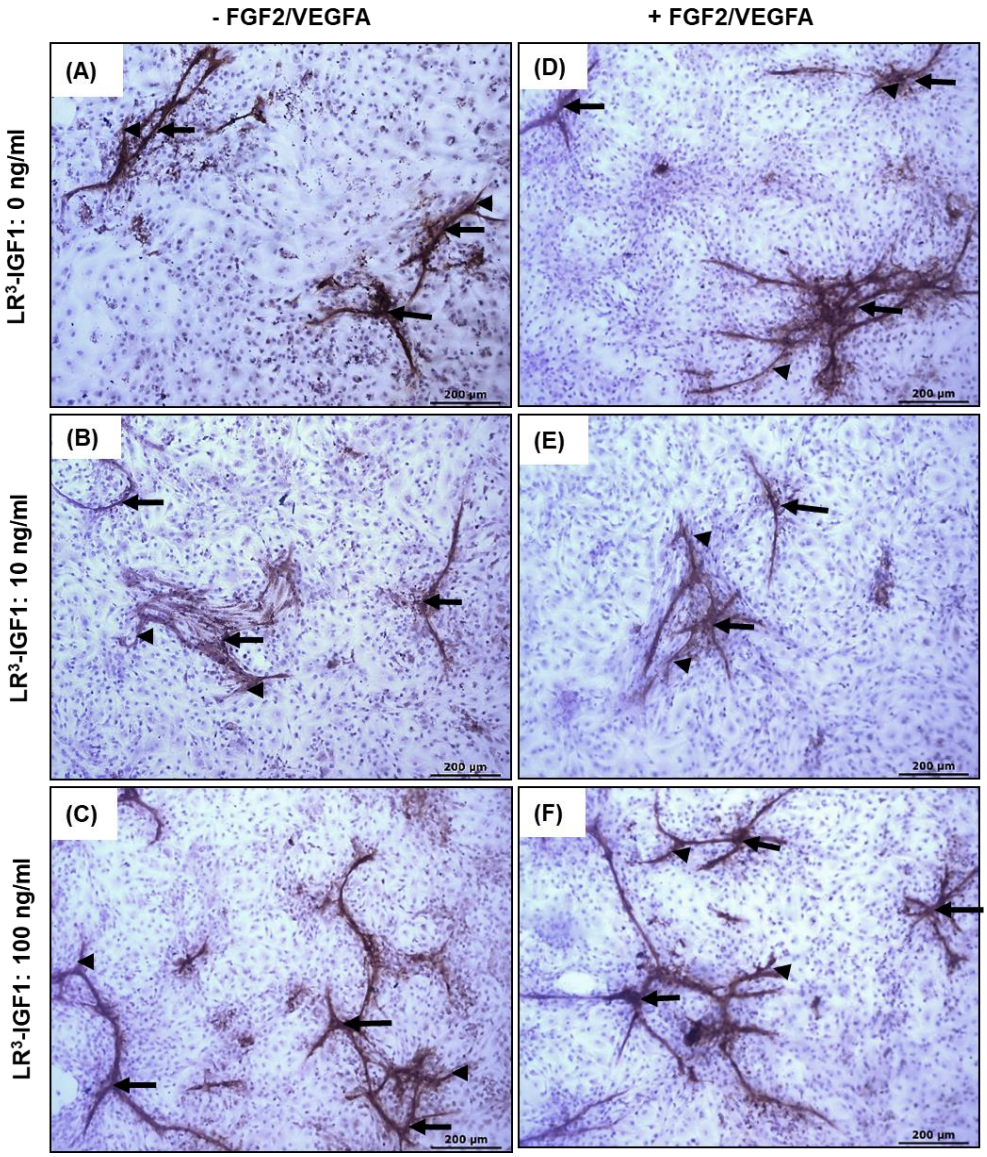



Fig. 4.5: The effect of $LR^3$-IGF1 on EC network formation in the absence (A-C) and presence (D-F) of FGF2+VEGFA-stimulation in bovine luteinising follicular cells. Cells were treated with either 0 (A, D), 10 (B, E) or 100 ng/ml $LR^3$-IGF1 (C, F). The EC networks were identified with von Willebrand factor immunocytochemistry as indicated by the positive brown staining (arrows), with branches indicated by arrow heads. Scale bars represent 200 µm.

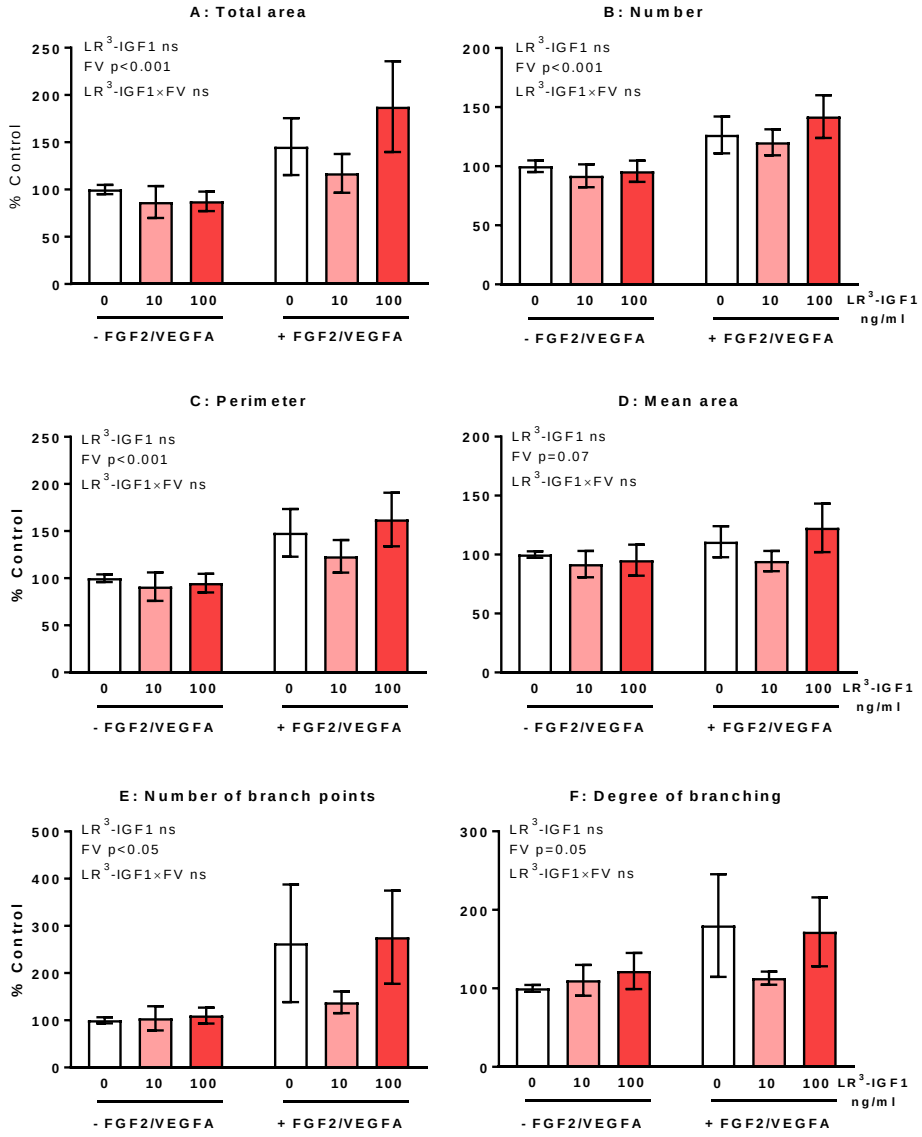



Fig. 4.6: Effect of $LR^3$-IGF1 on EC network formation in the presence and absence of FGF2+VEGFA (FV) in bovine luteinising follicular cells. $LR^3$-IGF1 was added at 0, 10 or 100 ng/ml in both absence and presence of FGF2+VEGFA (1 ng/ml). The effects of treatment are shown on (A) total area of EC networks, (B) number of EC networks, (C) perimeter of EC networks, (D) mean area of each EC network, (E) number of branch points and (F) degree of branching. $LR^3$-IGF1 treatment had no effect ($p>0.05$) on number, total area, or perimeter of EC networks, whilst the addition of FGF2+VEGFA increased ($p<0.001$) these endpoints by 30%. The data are mean ± SEM; n=4 cultures.

There was a significant day effect on progesterone production ($p<0.001$) with progesterone concentrations in the spent media 2.5-fold greater ($p<0.001$) on day 5 than day 3 (Fig. 4.7). Progesterone production was unaffected by $LR^3$-IGF1 treatment ($p>0.05$) irrespective of FGF2+VEGFA treatment (Fig. 4.7). The addition of angiogenic-stimulation (FGF2+VEGFA) tended to slightly decrease progesterone concentration ($p=0.05$) on day 3 but had no effect on day 5 ($p>0.05$). On either day, there was no $LR^3$-IGF1 by FGF2+VEGFA treatment interaction ($p>0.05$) (Fig. 4.7).

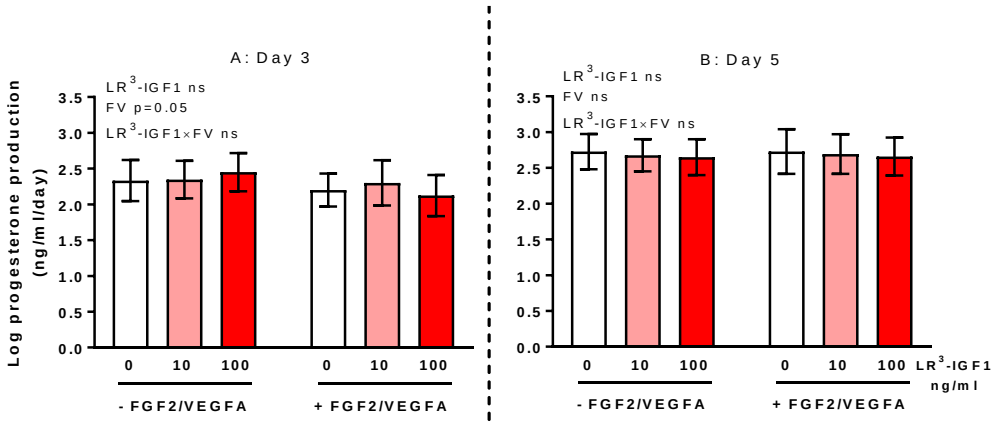



Fig. 4.7: Effect of $LR^3$-IGF1 on progesterone production by bovine luteinising follicular cells on (A) day 3 and (B) day 5 of culture in the presence and absence of FGF2+VEGFA (FV). Progesterone production (ng/ml/day) increased between day 3 and 5 ($p<0.001$), whilst $LR^3$-IGF1 treatment ($p>0.05$) had no effect on progesterone production. Addition of FGF2+VEGFA tended ($p=0.05$) to decrease progesterone concentration on day 3 but not on day 5. The data are mean ± SEM; n=4 cultures.

### 4.3.1.2. Effects of IGF2

Highly organised, intricate EC networks were observed at all concentrations of IGF2 in the presence and absence of FGF2+VEGFA treatment (Fig. 4.8). In this study, there appeared to be a similar pattern and degree of EC network formation in the presence (Fig. 4.8A-C) and absence (Fig. 4.8D-F) of angiogenic stimulation (FGF2+VEGFA).

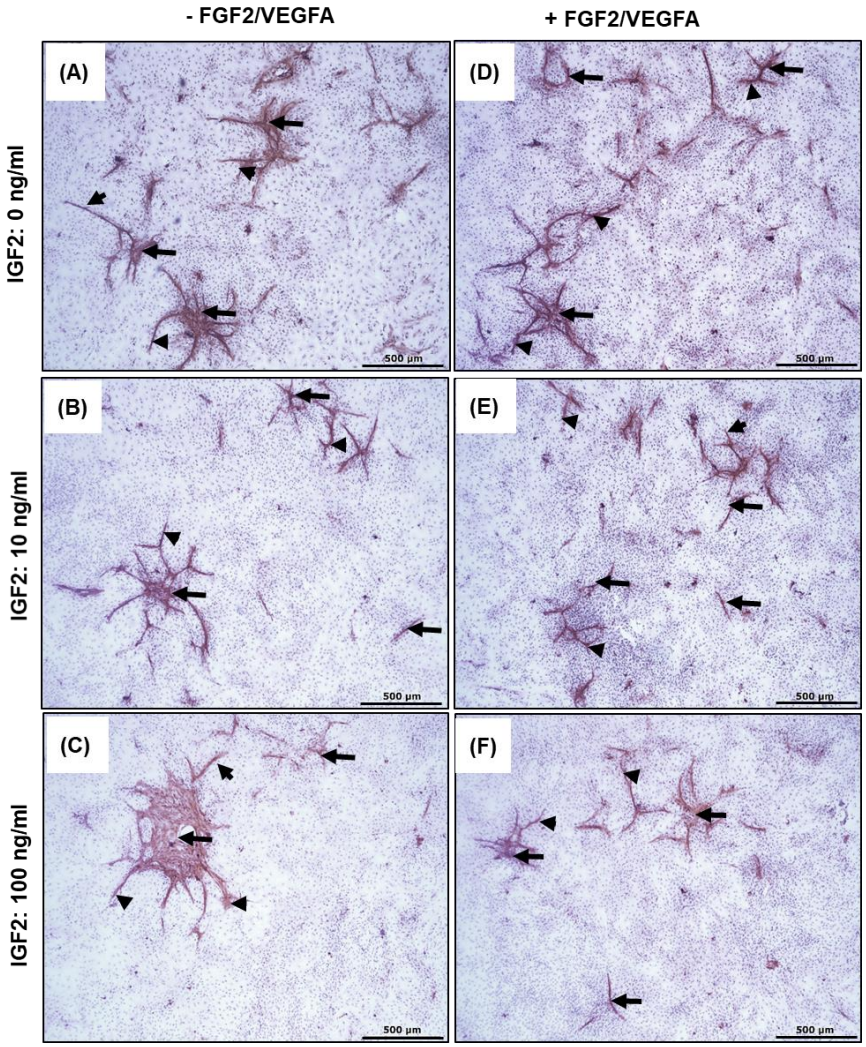



Fig. 4.8: The effect of IGF2 on EC network formation in the absence (A-C) and presence (D-F) of FGF2+VEGFA, in bovine luteinising follicular cells. Cells were treated with either 0 (A, D), 10 (B, E) or 100 ng/ml IGF2 (C, F). The EC networks were identified by von Willebrand factor immunocytochemistry and are indicated by the positive brown staining (arrows). The branches are indicated by arrowheads. Scale bars represent 500 µm.

The addition of angiogenic-stimulation (FGF2+VEGFA) failed to have an effect ($p>0.05$) on the degree of EC network formation (Fig. 4.9). However, IGF2 treatment decreased the total area of EC networks ($p<0.05$) and tended to decrease the number of EC networks ($p=0.08$), perimeter of EC networks ($p=0.07$) and number of branch points ($p=0.09$) versus control (Fig. 4.9A-C and E). There was no IGF2 by FGF2+VEGFA treatment interaction ($p>0.05$; Fig. 4.9).

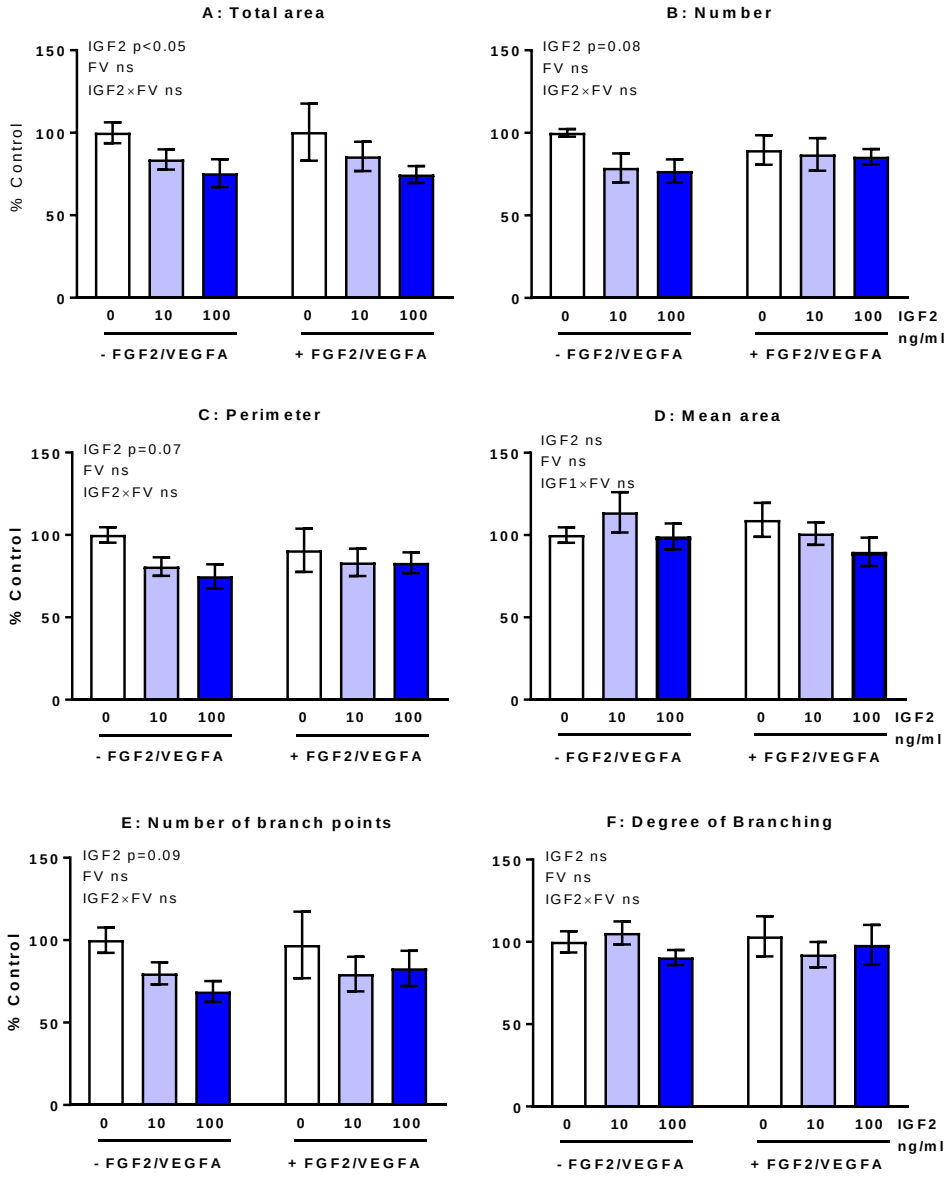



Fig. 4.9: Effect of IGF2 on EC network formation in the presence and absence of FGF2+VEGFA (FV) in bovine luteinising follicular cells. IGF2 was added at 0, 10 or 100 ng/ml in presence or absence of FGF2+VEGFA (1 ng/ml). The effects of treatment are shown on (A) total area of EC networks, (B) number of EC networks, (C) perimeter of EC networks, (D) mean area of each EC network, (E) number of branch points and (F) degree of branching. Treatment with IGF2 decreased the total area of EC networks ($p<0.05$) and tended to decrease the number, perimeter of EC networks and number of branch points. In this experiment, FGF2+VEGFA treatment ($p>0.05$) had no effect EC network formation. The data are mean ± SEM; n=4 cultures.

The concentration of progesterone in the spent media was 2.1-fold greater ($p<0.001$) on day 5 than 3 of culture. On both days, progesterone production was unaffected by treatment with IGF2 ($p>0.05$) in the presence or absence of FGF2+VEGFA treatment (Fig. 4.10). Treatment with FGF2+VEGFA tended to decrease progesterone concentration ($p=0.09$) on day 5. There was no IGF2 by FGF2+VEGFA interaction ($p>0.05$; Fig. 4.10).

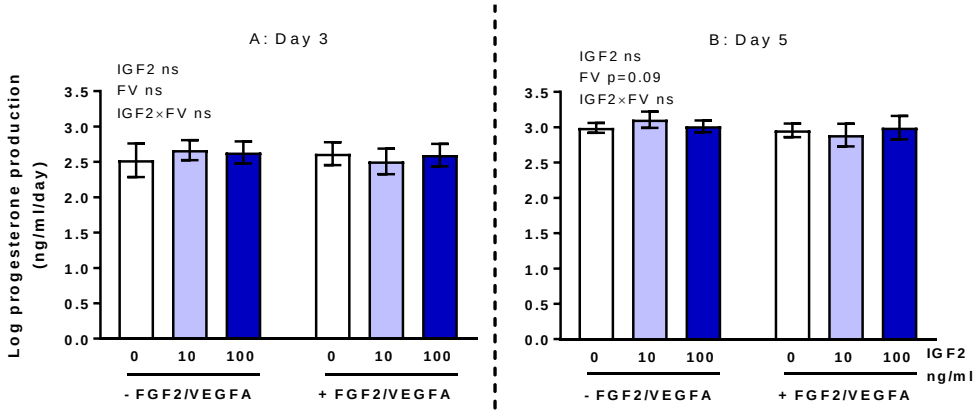



Fig. 4.10: Effect of IGF2 on progesterone production by bovine luteinising follicular cells on day 3 (A) and day 5 (B) of culture in the presence and absence of FGF2+VEGFA (FV). Progesterone production (ng/ml/day) was increased by day of culture ($p<0.001$), however IGF2 treatment ($p>0.05$) had no effect on progesterone production. The data are mean ± SEM from 4 cultures.

$LR^3$-IGF1 ($p<0.001$) and IGF2 ($p<0.05$) treatment affected the proliferation of bovine luteinising follicular cells (Fig. 4.11). Namely, the number of viable cells was marginally increased by treatment with $LR^3$-IGF1 ($p<0.001$; 5% increase) and IGF2 (3%; $p<0.05$). However, there was no interaction between $LR^3$-IGF1 and IGF2 treatments (Fig. 4.11).

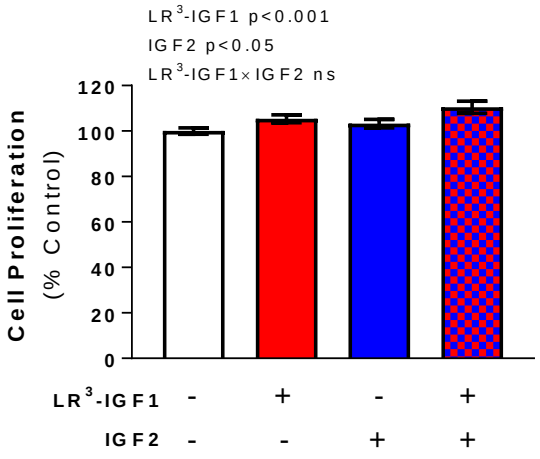



Fig. 4.11: Effect of $LR^3$-IGF1 and IGF2 on the proliferation of bovine luteinising follicular cells. The data are expressed as a percentage of the control cells. Both $LR^3$-IGF1 and IGF2 treatment increased cell proliferation. The data are mean ± SEM; n=5 cultures.

## 4.3.2. Study 2: The expression of the IGF system in luteinising follicular cells and bovine ovarian tissues

It was important to determine whether there was endogenous production of IGF1 and/or IGF2 in the culture system that could explain the lack of IGF treatment effect. Additionally, it was vital to confirm the presence of IGF1R given the lack of response to IGF1 or 2, in terms of angiogenesis or progesterone production. For this study, no exogenous IGF1, $LR^3$-IGF1 or IGF2 treatments were added. IGF1 was detected in the spent media of luteinising follicular cells at 80-120 pg/ml (Fig. 4.12). The levels of IGF1 were lower on day 5 versus day 3 of culture ($p<0.001$) while LH at 50 ng/ml (not 5 ng/ml) increased IGF1 concentrations compared with control by 20% on day 3 ($p<0.001$) and by 24% on day 5 ($p<0.001$; Fig 4.12).

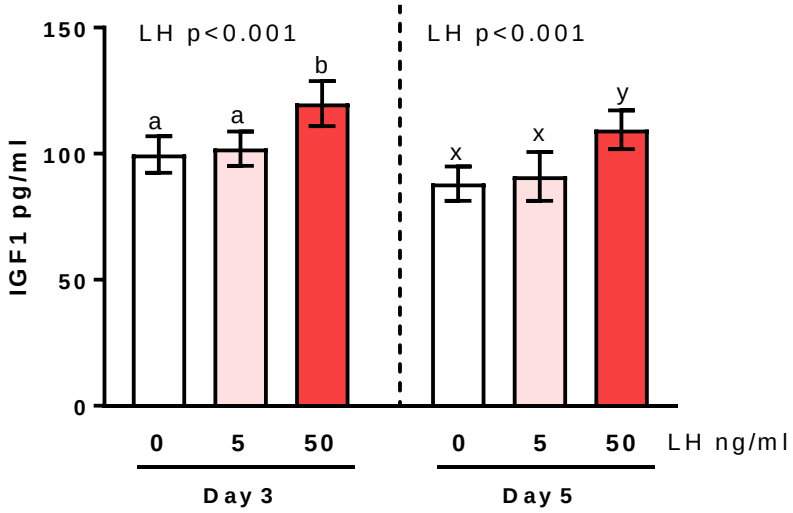



Fig. 4.12: The endogenous IGF1 production by bovine luteinising follicular cells, and the effect of LH treatment. LH treatment (50 ng/ml) increased IGF1 concentration on both day 3 and day 5 of culture. The data are mean ± SEM; n=4 cultures; a<b, x<y, $p<0.05$

In the cell lysates from the same experiment as above, IGF2 protein (7.5 kDa) was not detected by western blotting using a specific anti-rat IGF2 antibody. However, there was a clear single band for beta-actin (ACTB) at 42 kDa (Fig. 4.13) on the same blot after stripping and then re-probing to confirm protein transfer. Further western blots were performed with increased amounts of protein loaded (50 µg) and running the gel for a shorter time (see Section 2.4.3). However, no bands at 7.5 kDa were detected. Thus, it was decided that dot blots would be performed to avoid any potential confounding issues such as inappropriate denaturation, gel electrophoresis or transfer. In the dot blot, recombinant human IGF2 (rhIGF2, the same as used in Study 1) was detected within 125–500 ng. Furthermore, the intensity of the dots increased when more rhIGF2 was loaded. No dots were observed in the presence of the negative protein control (1 µg BSA; Fig. 4.13). Faint dots were present in the cell lysates from control and LH-treated cells, which was similar in 3 different culture experiments. This indicated that there is a low level of endogenous production of IGF2 by bovine luteinising follicular cells (Fig. 4.13).

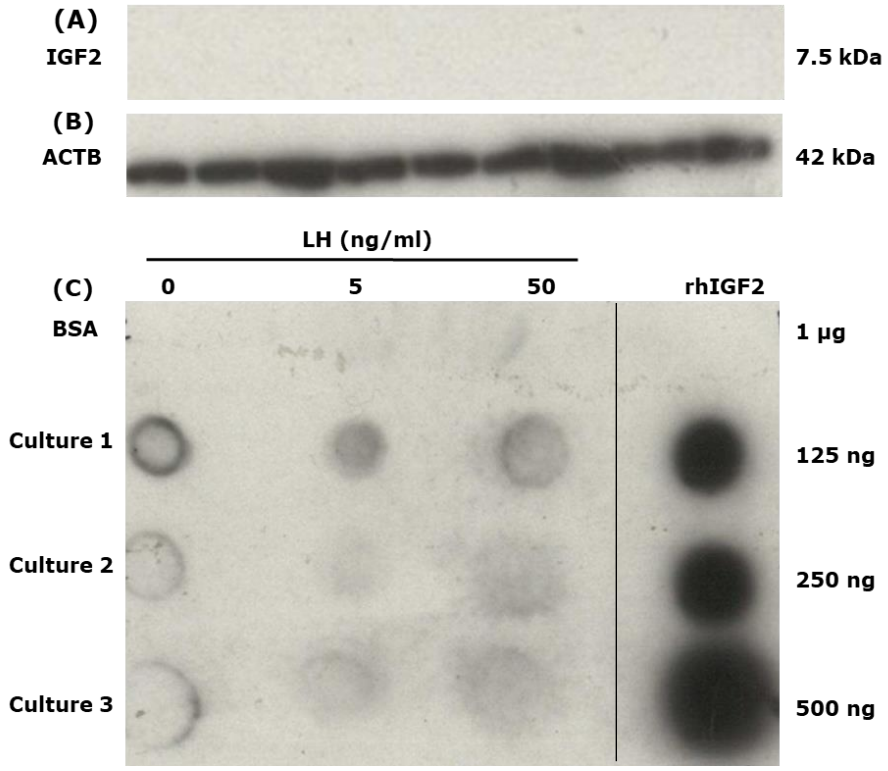



Fig. 4.13: Expression of IGF2 protein in bovine luteinising follicular cells. A) western blot for IGF2 using specific anti-bovine IGF2 antibody. In each well, 20 µg protein was loaded from cells treated with control or LH (5 or 50 ng/ml). No band was detected at the predicted molecular weight (7.5 kDa). B) Confirmation of protein transfer by re-probing the blot for beta-actin (ACTB) where a single band (42 kDa) was present. C) Recombinant human IGF2 protein (rhIGF2) was detected when loaded at 125, 250 or 500 ng, while the negative control (1 µg bovine serum albumin, BSA) was blank. In cell lysates (20 µg protein), a faint dot was detected in cells treated with control and LH in 3 different cultures.

Next, the spatial expression of IGF1R protein in bovine ovarian follicles and CL as well as in cultured luteinising follicular cells was investigated by immunohistochemistry and immunocytochemistry. In pre-antral follicles, IGF1R was present in the oocyte, granulosa cells and EC within the theca layer (Fig 4.14A). In antral follicles, IGF1R protein expression was also detected in the granulosa cells, particularly in those cells adjacent to the basement membrane (Fig. 4.14B). To a lesser extent, IGF1R was present in the EC within the theca layer of pre-antral follicles and antral follicles (Fig. 4.14C). There was faint staining in the negative control sections (Fig 4.14D). No quantification of staining was performed since the objective was to localise IGF1R protein expression.

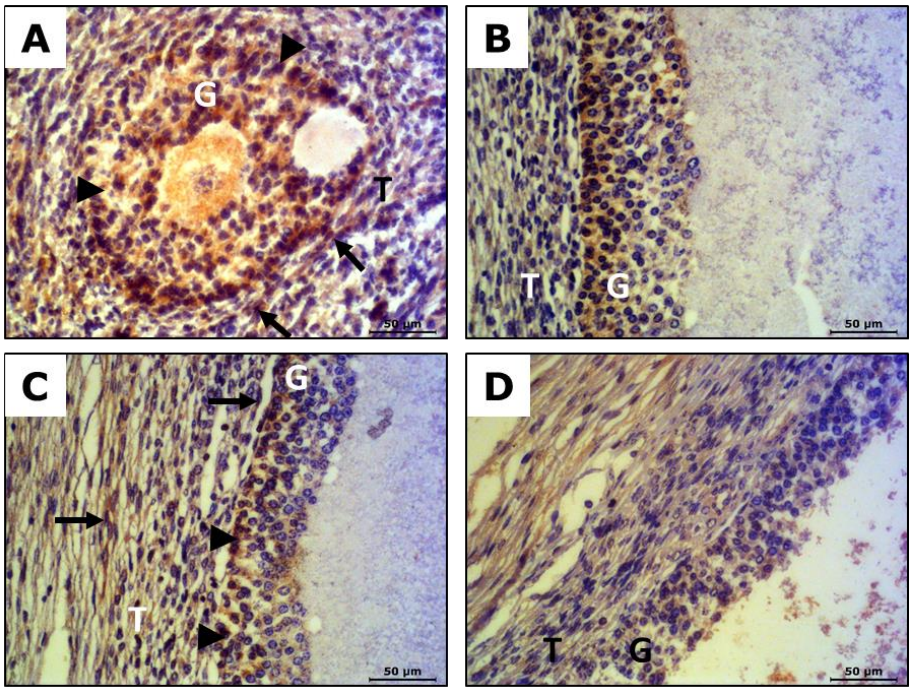



Fig. 4.14: Immunohistochemical localisation of IGF1R protein in bovine ovarian follicles. Representative images showing positive immunostaining of IGF1R (A-C) in follicles (antral and pre-antral). This was localised to steroidogenic cells (arrowheads) of the granulosa cells and to a lesser extent theca endothelial cells (arrows). Staining was weak following incubation with rabbit IgG (negative control) (D). Scale bars are 50 µm. T, Theca cells, G, Granulosa cells.

In the CL, IGF1R immunostaining was present in the cytoplasm of steroidogenic cells in both early (day 5) and mid CL (day 8) (Fig. 4.15A and C). The intensity of the staining was variable across each section and between sections. There was evidence that IGF1R was localised to micro-capillary EC of both the day 5 and 8 CL (Fig. 4.15A and C). There was some weak staining present in the negative control slides (equivalent rabbit IgG concentration) but this was at a lower intensity (Fig 4.15B and D).

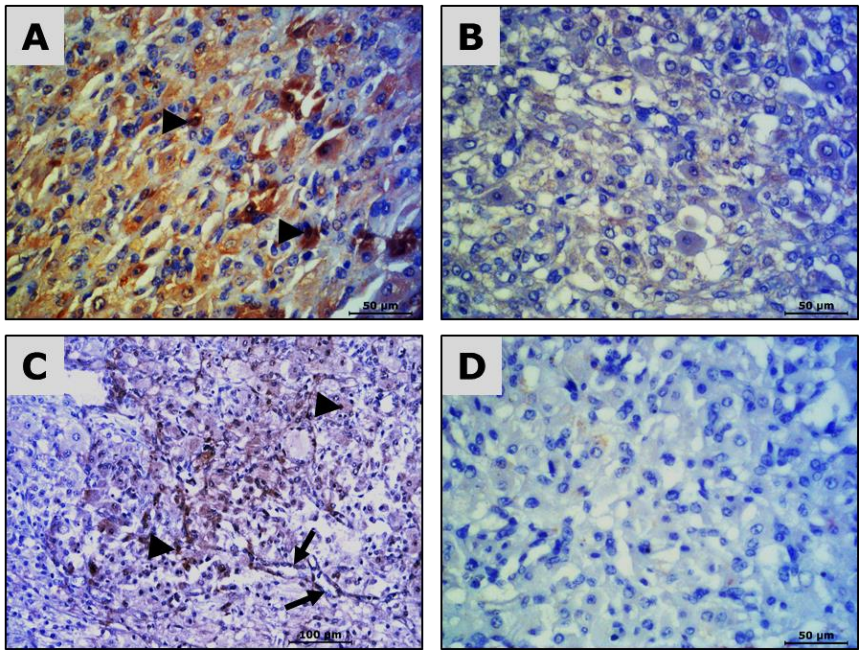



Fig. 4.15: Immunohistochemical localisation of IGF1R protein in the bovine corpus luteum. Representative images from (A-B) day 5 and (C-D) day 8 of the oestrous cycle are shown. Staining was absent following incubation with rabbit IgG (negative control) (B, D). The arrowheads point to the brown staining in steroidogenic cells (large and small luteal cells) and arrows pointing to endothelial cell staining. Scale bars are 50 - 100 µm.

The next step was to investigate the localisation of IGF1R protein in cultured bovine luteinising follicular cells (Fig. 4.16). There was moderate expression of IGF1R in numerous cells across each coverslip with staining appearing more intense in the areas where the cell density was greatest. These areas are often associated with the formation of EC networks. There appeared to be some weak nuclear staining, which was not expected (Fig. 4.16A-C). The rabbit IgG control showed no evidence of brown positive staining (Fig. 4.16D).

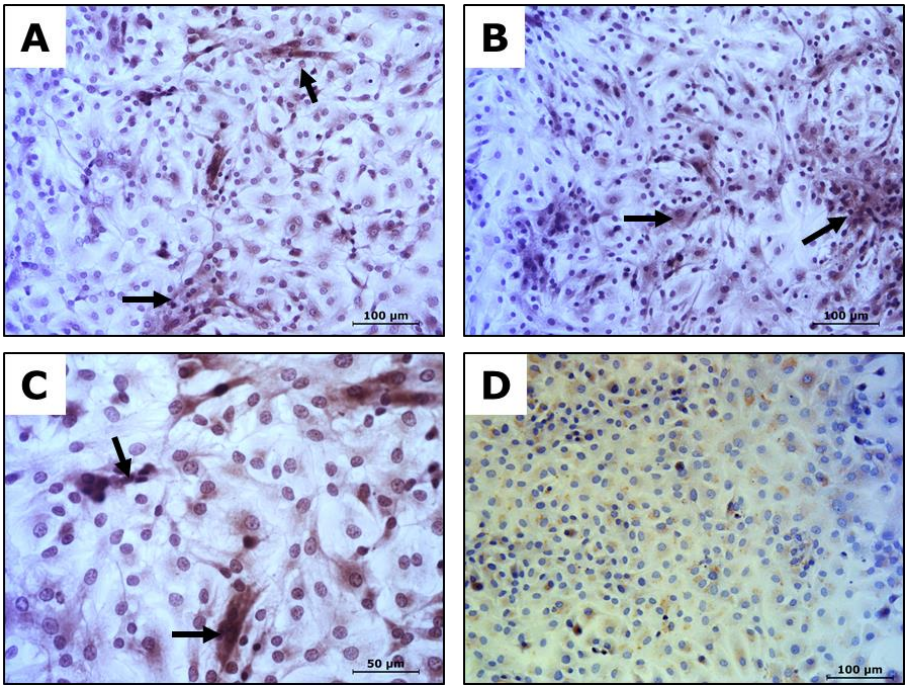



Fig. 4.16: Immunohistochemical localisation of IGF1R protein in bovine luteinising follicular cells. Representative images are shown for (A-C) IGF1R protein and (D) Rabbit IgG control. There was moderate immunostaining for IGF1R protein in numerous cells across the whole well. There was particularly intense staining present in the more cell-dense regions, however the staining did not appear to be associated with a particular cell phenotype. The rabbit IgG control showed no evidence of brown staining. Scale bars are 50 - 100 µm.

In order to identify whether the IGF1R protein was localised to the EC, dual immunofluorescence was conducted with IGF1R immunocytochemistry and isolectin B4 (Fig. 4.17). There was very clear staining of the EC with a network-like appearance. However, there was no positive staining for IGF1R and due to limited resources. It has not been possible to confirm presence of IGF1R in EC.

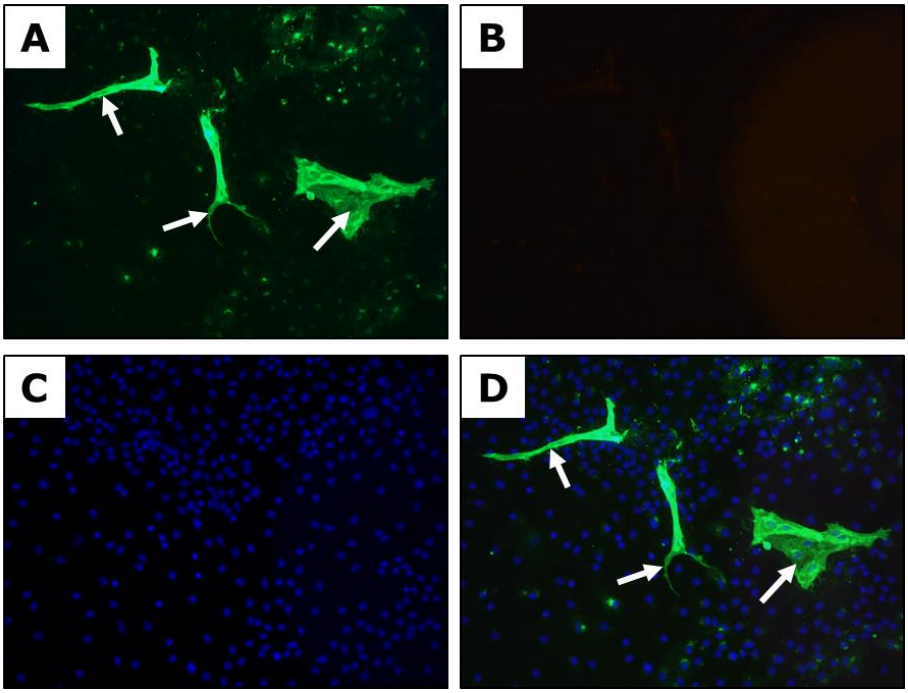



Fig. 4.17: Dual immunofluorescence of IGF1R protein and isolectin B4 co-localisation in the bovine luteinising follicular cells. The bovine luteinising follicular cells were immunostained with (A) isolectin B4 (green), (B) IGF1R (red), and (C) counterstained with DAPI (Blue). The three images are merged in (D) to show any co-localisation. Isolectin-positive endothelial cells were evident with typical network-like appearance, however no red IGF1R was detected.

### 4.3.3. Study 3: The effect of inhibiting IGF1R signalling on endothelial cell network formation and progesterone production of bovine luteinising follicular cells

This study investigated the effects of specifically inhibiting the activation of IGF1R (the receptor through which both IGF1 and IGF2 act) on EC network formation. It investigated the effects of IGF1R blockade in the presence and absence of exogenously added $LR^3$-IGF1, IGF2 and combined $LR^3$-IGF1 and IGF2.

In all treatments, cells covered the whole surface of the well by day 5 of culture, however there appeared to be reduced density of cells in the IGF1R inhibitor-treated wells. Highly organised, intricate EC networks were formed in control wells in the absence and presence of IGF1R inhibitor (PPP; Fig. 4.18). However, it was clearly evident that in the PPP-treated wells, there were fewer EC networks and those that were present were scantier and with fewer branches extending from the central EC island body. The size and appearance of the EC networks appeared to be similar in the IGF-treated wells to versus control in both the absence and presence of PPP treatment.

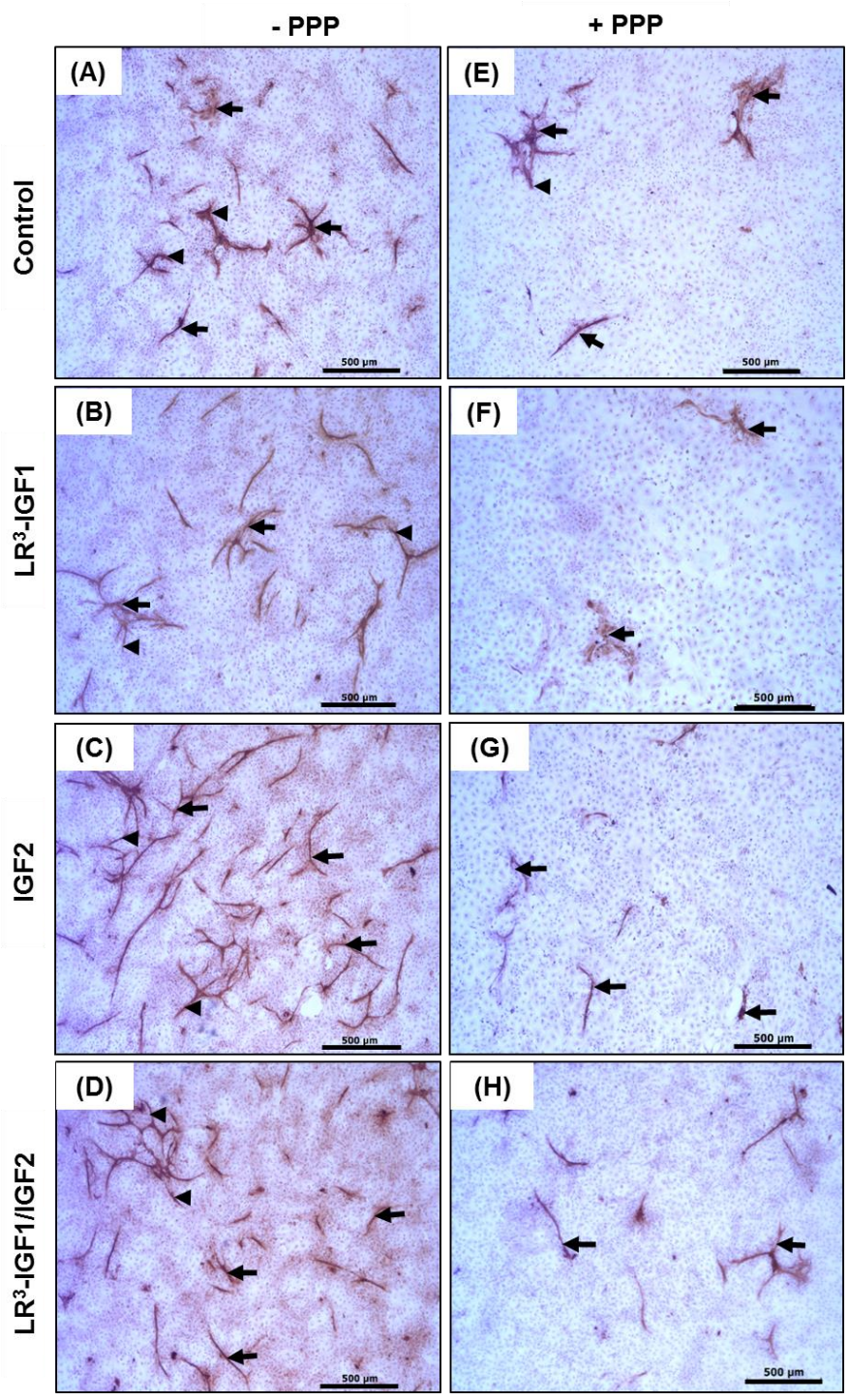



Fig. 4.18: Effect of inhibition of IGF1R signalling on endothelial cell (EC) network formation in bovine luteinising follicular cells. Representative images show EC network formation in the absence (A-D) and presence of (E-H) the IGF1R inhibitor, picropodophyllin (PPP). Cells were treated with either control (A, E), 10 ng/ml $LR^3$-IGF1 (B, F), 10 ng/ml IGF2 (C, G), or 10 ng/ml $LR^3$-IGF1+IGF2 (D, H). The EC networks were immunostained with von Willebrand factor as indicated by the positive (brown) staining (arrows) and the branches (arrowheads) of the EC networks. The scale bars were 500 µm.

Treatment with PPP ($p<0.001$) had drastic effects on most of the EC network endpoints measured. Namely, PPP treatment ($p<0.001$) reduced the number, total area, perimeter, mean area of each EC network, and number of branch points by 60-70% versus untreated wells (Fig. 4.19). This reduction was observed in both the presence and absence of $LR^3$-IGF1, IGF2 and combined $LR^3$-IGF1+IGF2 treatments (Fig. 4.19). However, PPP treatment had no effect on the degree of branching within each EC network (Fig. 4.19F).

There was no effect of $LR^3$-IGF1 or IGF2 treatment on most of the EC network endpoints that were measured; the number ($p>0.05$), total area ($p>0.05$), perimeter ($p>0.05$), mean area of the EC networks ($p>0.05$), and number of branch points ($p>0.05$) were not different from controls. However, IGF2 reduced the degree of branching in the presence and absence of PPP ($p<0.01$; Fig. 4.19F). There were no $LR^3$-IGF1 by IGF2 interactions ($p>0.05$; Fig. 4.19).

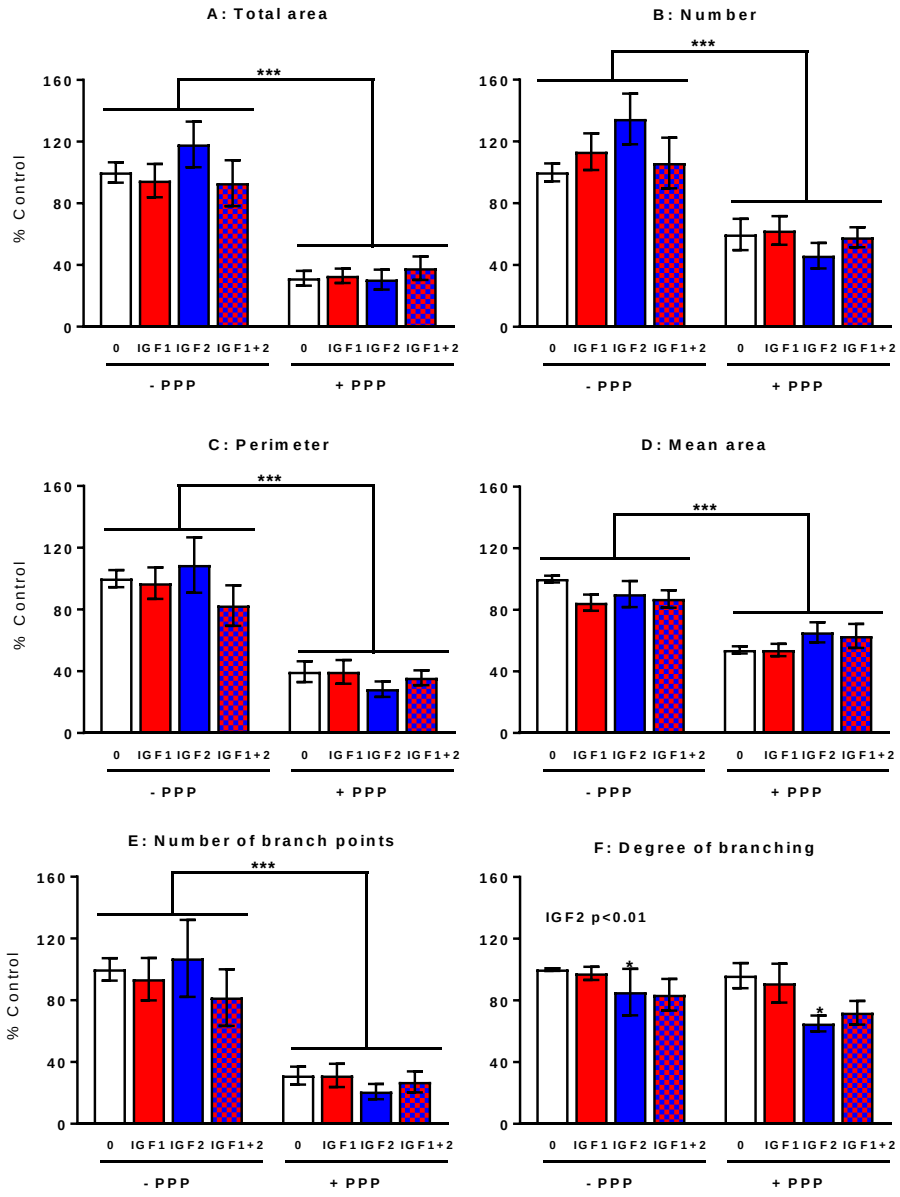



Fig. 4.19: Effect of inhibiting IGF1R signalling on endothelial cell (EC) network formation in control and IGF-treated bovine luteinising follicular cells. PPP (1 μM) was added in the presence or absence of LR3-IGF1, IGF2, or combined $LR^3$-IGF1+IGF2 (10 ng/ml). The effects of treatment are shown on (A) total area of EC networks, (B) number of EC networks, (C) perimeter of EC networks, (D) mean area of each EC network, (E) number of branch points and (F) degree of branching. PPP treatment reduced all EC network endpoints (except degree of branching) by 60-70%. Treatment with $LR^3$-IGF1 or IGF2 had no effect on the majority of EC network parameter, except for degree of branching which was reduced by IGF2 ($p<0.01$). The data values are mean ± SEM from 4 cultures. ***, $p<0.001$ vs no PPP treatment.

There was a significant effect of culture day on progesterone production ($p<0.001$), with progesterone concentrations 2.7-fold higher ($p<0.001$) on day 5 than day 3 of culture (Fig. 4.20). Thus, progesterone production on each culture day was analysed separately. PPP decreased progesterone production on day 3 ($p<0.05$) and 5 of culture ($p<0.001$; Fig. 4.20). This effect was observed in control and IGF-treated wells.

On day 3 of culture, $LR^3$-IGF1 treatment ($p<0.05$) increased progesterone production by 11% in both the absence and presence of PPP while IGF2 had no effect on progesterone production ($p>0.05$; Fig. 4.20A). There were no $LR^3$-IGF1 or IGF2 by PPP treatment interactions ($p>0.05$; Fig. 4.20A).

On day 5 of culture, $LR^3$-IGF1 had no effect but IGF2 ($p<0.05$) decreased progesterone production by 12%. Furthermore, PPP treatment ($p<0.001$) reduced progesterone production by 30% (Fig 4.20B). In addition, there was a $LR^3$-IGF1 by IGF2 interaction ($p<0.05$). This revealed that $LR^3$-IGF1 increased progesterone production in the absence of IGF2 but reduced progesterone production in its presence ($p<0.05$; Fig. 4.20B).

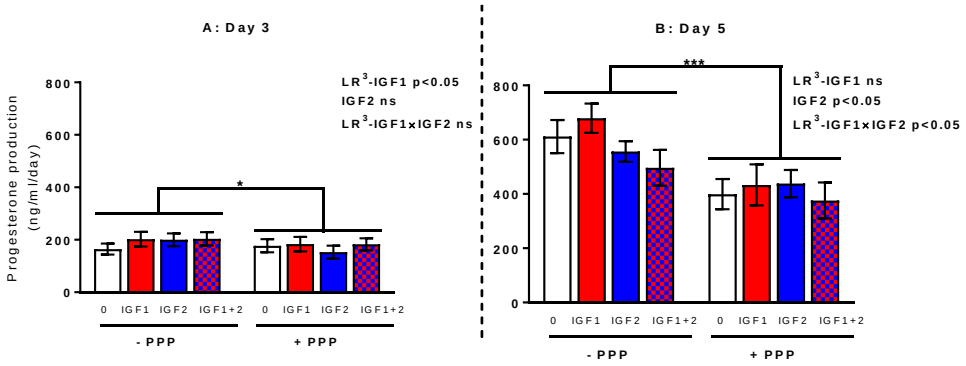



Fig. 4.20: Effect of inhibiting IGF1R signalling on progesterone production in control and IGF-treated bovine luteinising follicular cells on (A) day 3 and (B) day 5 of culture. Progesterone production was affected by day of culture ($p<0.001$) with nearly 3-fold higher production on day 5. PPP decreased progesterone production on day 3 (*; $p<0.05$) and 5 (***; $p<0.001$). The effects of the IGF1 or IGF2 and their interaction are indicated on the graphs. The data values are mean ± SEM (n=4 cultures).

### 4.3.4. Study 4: Effect of IGFBP4 on endothelial cell network formation and progesterone production of the bovine luteinising follicular cells

This experiment was designed such that endogenous production of IGF1 or IGF2 would be sequestered and thus inhibited by the addition of IGFBP4. This IGFBP is considered to have a predominant inhibitory action (Mihm et al., 2000, Zhou et al., 2003). Then, in this scenario, the effects of $LR^3$-IGF1, which is not bound by IGFBPs, on luteal EC network formation, would be determined. In this study, moderately sized EC networks were formed in control wells and these appeared similar in terms of size, number and shape in cells treated with $LR^3$-IGF1, IGFBP4 and the $LR^3$-IGF1/IGFBP4 combination (Fig. 4.21).

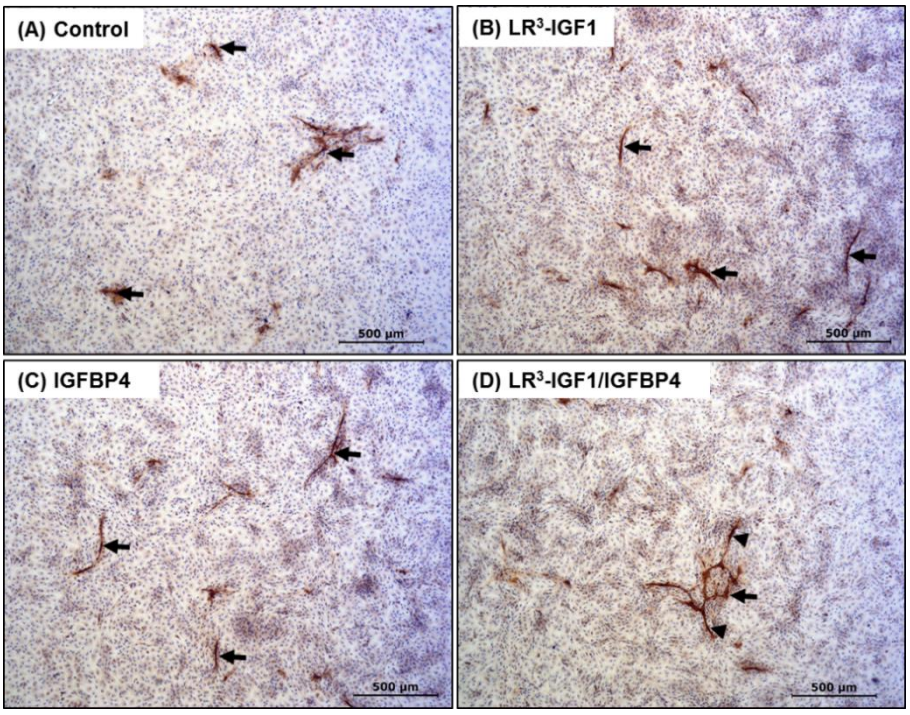



Fig. 4.21: Effect of insulin-like growth factor binding protein 4 (IGFBP4) on formation of endothelial cell (EC) networks in control and $LR^3$-IGF1-treated bovine luteinising follicular cells. Representative images of EC networks from (A) control, (B) $LR^3$-IGF1, 10 ng/ml (C) IGFBP4, 100 ng/ml and (D) combined $LR^3$-IGF1/IGFBP4 treatment are shown. The EC networks were identified using von Willebrand factor immunocytochemistry with positive (brown) staining indicated by arrows. The extension of EC that formed a branch from the EC network are indicated by arrowheads. The scale bars are 500 µm.

IGFBP4 treatment significantly reduced the number of EC networks ($p<0.05$; Fig. 4.22A) but had no effect on the other EC network endpoints (total area, perimeter and mean area of each EC network, total number of branch points and degree of branching) (all $p>0.05$; Fig. 4.22). Conversely, $LR^3$-IGF1 treatment ($p<0.05$: Fig. 4.22F) increased the degree of branching within each EC network but $LR^3$-IGF1 treatment had no effect on the other parameters analysed ($p>0.05$; Fig. 4.22). Contrary to the experimental design, there were no $LR^3$-IGF1 by IGFBP4 interactions ($p>0.05$; Fig. 4.22) on all EC network endpoints.

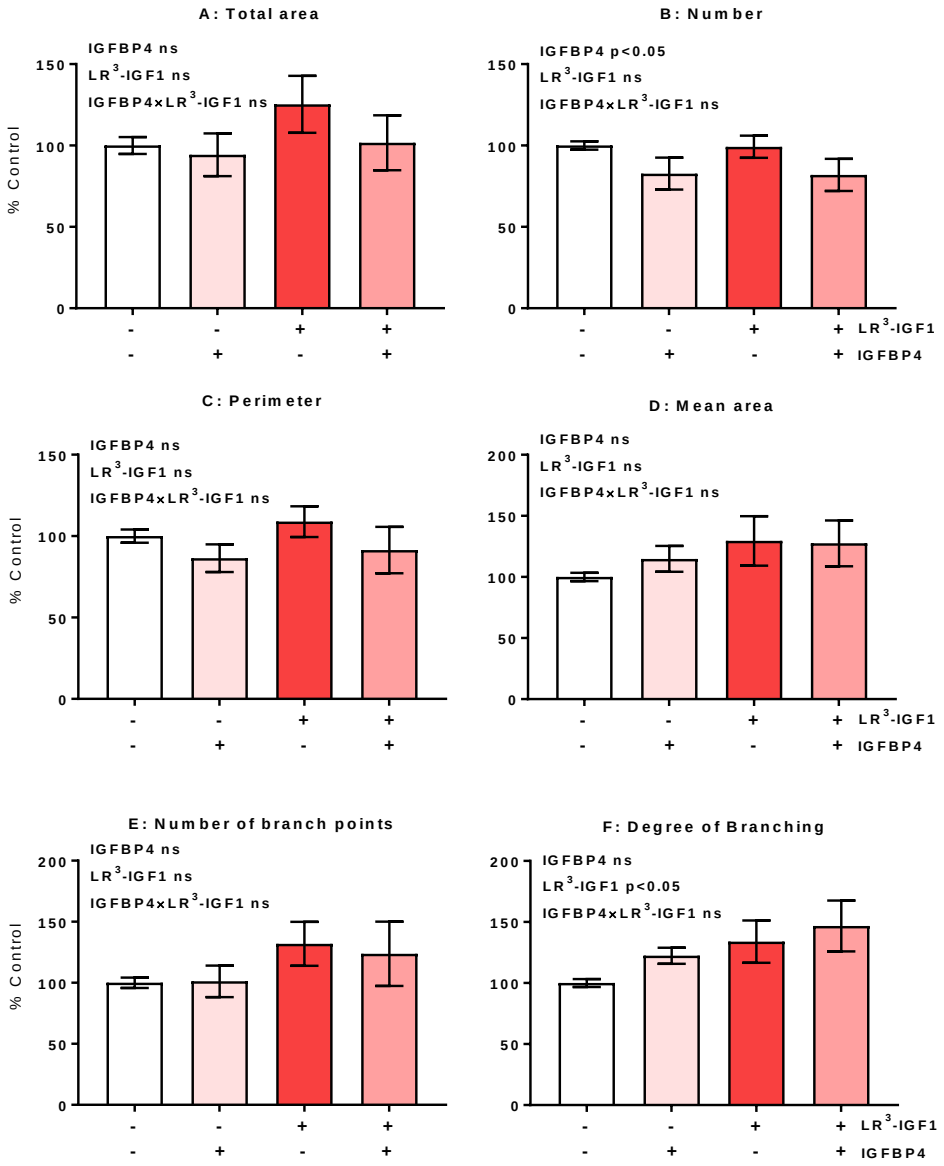



Fig. 4.22: Effect of insulin-like growth factor binding protein 4 (IGFBP4) on the quantification of EC networks in control and $LR^3$-IGF1-treated bovine luteinising follicular cells. The effects of treatment are shown on (A) total area of EC networks, (B) number of EC networks, (C) perimeter of EC networks, (D) mean area of each EC network, (E) number of branch points, and (F) degree of branching. Cells were treated in a 2 × 2 factorial design with $LR^3$-IGF1 (0 or 10 ng/ml) and IGFBP4 (0 or 100 ng/ml). The effects of $LR^3$-IGF1 and IGFBP4 and their interaction are indicated on the graph. The data values are mean ± SEM from 4 cultures.

There was a significant effect of time in culture on progesterone production ($p<0.001$), with its concentration 2.1-fold greater ($p<0.001$) on day 5 than day 3 (Fig. 4.23). $LR^3$-IGF1 treatment decreased progesterone production by 13% on day 3 of culture ($p<0.01$) and by 21% on day 5 ($p<0.01$; Fig. 4.23). IGFBP4 treatment did not affect progesterone production ($p>0.05$) on either culture day and there were no interactions between $LR^3$-IGF1 and IGFBP4 treatments ($p>0.05$; Fig 4.23).

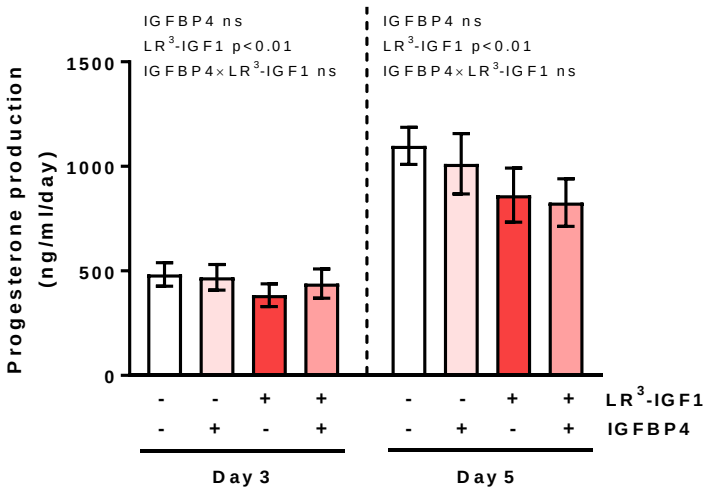



Fig. 4.23: Effect of insulin-like growth factor binding protein 4 (IGFBP4) on progesterone production in control and $LR^3$-IGF1 treated bovine luteinising follicular cells. The effects of $LR^3$-IGF1 and IGFBP4 and their interaction are indicated on the graph. The data values are mean ± SEM from 4 cultures.

## 4.4. Discussion

This Chapter assessed the overarching hypothesis that IGF1 and/or IGF2 acting through IGF1R will promote EC network formation and progesterone production in bovine luteinising follicular cells. This hypothesis was tested using an *in vitro* culture system that mimics the follicular-luteal transition in which EC networks develop. For the first time, the involvement of IGF1R signalling in the stimulation of angiogenesis and progesterone production in luteinising follicular cells has been investigated. However, the addition of exogenous $LR^3$-IGF1 or IGF2 failed to stimulate EC network formation or progesterone production. This is possibly due to the endogenous production of IGF1 (and possibly IGF2) by the cultured cells activating IGF1R. To further investigate the role of endogenous IGFs, a novel approach was employed by which these were sequestered using IGFBP4. However, minimal effects of IGFBP4 were detected and further work is required to fully elucidate the role of IGFs in stimulating angiogenesis.

The central component to all these experiments was the luteinising follicular angiogenesis culture system in which follicular (granulosa and theca) cells were co-cultured, allowed to luteinise and with the endothelial cells forming networks that resemble a capillary bed. As expected, the EC isolated from the theca layer consistently formed into networks with tubule-like branches that extended from the EC network. Furthermore, the formation of these EC networks was further stimulated by the addition of pro-angiogenic growth factors (FGF2 and VEGFA) albeit to limited extent. This agrees with the original study that described the culture system (Laird et al., 2013). Importantly, in this culture system, steroidogenic cells were maintained and underwent luteinisation as demonstrated by the increased progesterone production over time.

*Effects of IGF1*

In three different studies, $LR^3$-IGF1 had limited effects on the formation of EC networks. The one exception was in the third study where $LR^3$-IGF1 increased the degree of branching within an EC network. In this series of experiments, the dose of IGF1, presence of exogenous pro-angiogenic factors, inclusion of insulin in the media and sequestration of endogenous IGFs have been considered and the outcome was contrary to the original hypothesis. Furthermore, it disagrees with previous findings in other

systems where IGF1 dose-dependently stimulated the migration of bovine carotid artery EC (Nakao-Hayashi et al., 1992, Piecewicz et al., 2012). In the present study, two doses of $LR^3$-IGF1 treatment (10 & 100 ng/ml) were included. These were based on plasma concentrations of IGF1 that are present in cows during periods of negative and positive energy balance (Kobayashi et al., 1999, Fenwick et al., 2008). Moreover, the dose of $LR^3$-IGF1 used was 100-1000 fold higher than the mean detectable concentration of endogenously produced IGF1 (0.1 ng/ml) in the spent media. Additionally, $LR^3$-IGF1 at 10 ng/ml stimulated overall cell proliferation as determined by MTT assay. This observation is supported in other studies where the treatment of granulosa cells (from large follicles) with IGF1 and IGF2 at 100 ng/ml increased cell numbers by 1.8- and 1.6-fold over controls respectively (Nakao-Hayashi et al., 1992, Spicer and Aad, 2007). Collectively, this indicates that the dose(s) chosen had the potential to cause an effect.

Previous studies showed that IGF1 treatment combined with FGF2 and VEGFA promoted endothelial cell differentiation in embryonic stem cells (Sun et al., 2009). Thus, it was important to treat the follicular cells with IGF1 under basal and angiogenic-stimulated conditions. Indeed, it was observed that angiogenic-stimulation (FGF2 and VEGFA) increased the number, total area, perimeter of the EC networks, and number of branch points in the luteinising follicular cells by 30%. However, there was no interaction with IGF1 or IGF2, which indicates there was no synergistic or additive effect between these growth factors.

In the present study, the concentration of insulin was importantly reduced to 100 ng/ml instead of 10 µg/ml present in ITS supplement. The insulin concentration was reduced to minimise insulin-induced activation of IGF1R signalling. However, even with the reduced insulin concentration, it is still feasible that insulin might still bind to IGF1R leading to partial activation and this needs to further investigated using receptor binding assays. It is also feasible that insulin could activate insulin receptor signalling which could mimic the effects of IGF1R activation. Indeed, insulin stimulated cell proliferation and survival via the insulin receptor and consequently increased steroidogenesis in bovine granulosa and theca cells (Spicer et al., 1993, Spicer and Chamberlain, 1998, Spicer et al., 2002). Further studies are required to investigate the effects of insulin and insulin receptor activation in this culture system.

The present study clearly demonstrated that IGF1R activation was important for luteal angiogenesis and progesterone production. Thus, the logical next step was to determine whether IGF1R stimulation was due to exogenous production of IGFs. Our experimental approach was to sequester endogenously produced IGFs with 100 ng/ml IGFBP4. This IGFBP4 dose suppressed IGF-induced cell proliferation (Mohan et al., 1995) and is ~300-fold greater in terms of molarity than the level of IGF1 detected in the spent media (3.6 nM [IGFBP4] vs 12 pM [IGF1]). The other reason for selecting IGFBP4 was because it is principally associated with inhibitory actions on the bovine ovary. For example, *IGFBP4* mRNA is strongly expressed in atretric follicles and *IGFBP4* mRNA expression is down-regulated as the follicle approaches its pre-ovulatory size (Armstrong et al., 1998). In addition, intra-follicular levels of IGFBP4 were increased in follicles undergoing atresia in both cattle and sheep (Monget and Monniaux, 1995, Mazerbourg et al., 2003). Moreover, IGFBP4 inhibited gonadotropin-stimulated steroid synthesis in theca and granulosa cells (Putowski et al., 1997, Mason et al., 1998). However, in the present study IGFBP4 had minimal effects on EC network formation and progesterone production, which was contrary to expectations. The exception was that IGBP4 treatment significantly reduced the number of EC networks. The reasons for this lack of effect are unclear but could be due to the proteolytic inactivation of IGFBP4 by production of IGFBP proteases in luteinising follicular cells. Equally, the IGFBP4 dose could be insufficient despite being in molar excess.

*Effects of IGF2*

This chapter also investigated the effect of IGF2 on EC network formation in the presence and absence of angiogenic-stimulated conditions. In the luteinising follicular cells, EC networks were formed in both basal and angiogenic-stimulated conditions with a similar pattern and appearance. Surprisingly, the results showed that IGF2 treatment decreased (e.g. total EC area) and tended to decrease a number of the EC network parameters (e.g. number of networks, number of branch points), which was contrary to the hypothesis. Furthermore, our results disagreed with previous findings where IGF2 increased the proliferation of bovine retinal capillary endothelial cells and pericytes as well as bovine aortic endothelial cells (King et al., 1985, Hansson et al., 1989). The reasons for the slight reduction in EC network formation are unknown. It is feasible that in this multi-cell type

culture system, IGF2 selectively promoted the proliferation of non-endothelial cell types (i.e. smooth muscle cells) and/or the hypertrophy of steroidogenic cells. Consequently, the space for EC growth and/or development was more limited leading to reduced EC network formation. Indeed, in the present study, IGF2 marginally increased the overall cell proliferation but which cell type(s) were affected was unclear.

It was decided to detect IGF2 by western blot since there were no commercial bovine specific IGF2 ELISA and bovine and human IGF2 are not 100% identical. Unfortunately, no IGF2 was detected in the cell lysate by western blot using an anti-rat IGF2 antibody that was 97% homologous between rat and bovine IGF2 amino acid sequences. Next, a dot blot was performed in which recombinant human IGF2 was detected but only faint dots for bovine IGF2 were observed. This would indicate that if IGF2 is expressed in the luteinising follicular cells it was in relatively low amounts. Further studies should be conducted to investigate the *IGF2* mRNA expression of bovine follicular cells using real time PCR.

*The effects of IGF1R activation*

Collectively, these initial studies indicated that IGFs might not play an important role in stimulating EC network formation and progesterone production. Thus, it was important to investigate the effects of specifically blocking IGF1R signalling with a specific tyrosine kinase inhibitor. Picropodophyllin (PPP) was selected based on its specific inhibition of IGF1R signalling in EC networks in other systems (Larsson and Axelson, 2007, Bieghs et al., 2014). Importantly, there was a marked reduction (60-70%) of EC network formation with PPP treatment compared to controls. PPP reduced number, total area, perimeter, mean area of the EC networks, number of branch points and degree of branching in bovine luteinising follicular cells. These effects of PPP were observed whether follicular cells were not treated with IGFs or treated with $LR^3$-IGF1, IGF2, or both $LR^3$-IGF1 and IGF2. This clearly indicated that IGF1R signalling is important for angiogenesis in bovine luteinising follicular cells, and endogenous production of IGF1 and/or IGF2 might be important mediators of follicular-luteal angiogenesis. This study agrees with previous finding where the inhibition of IG1R signalling significantly reduced the formation of IGF-induced endothelial precursor cells (Piecewicz et al., 2012).

To further explore whether IGF1R activation induced EC network formation by acting directly on endothelial cells or indirectly via steroidogenic cells, immunohistochemical localisation of IGF1R protein was performed. IGF1R expression was immunolocalised to steroidogenic cells of large antral follicles, early and mid-CL. There was also some evidence that IGF1R was detected in endothelial cells of some follicles/CL though dual-localisation was not performed. This agreed with previous observations using *in situ* hybridisation where *IGF1R* mRNA was abundantly expressed in the bovine follicle and corpus luteum predominantly in the steroidogenic cells with evidence of expression in endothelial cells (Schams et al., 1999, Woad et al., 2000). In luteinising follicular cells, IGF1R expression was particularly evident in regions where cells were in higher density and these areas often contain endothelial cells. The attempts to co-localise IGF1R to EC using dual immunofluorescence were unfortunately unsuccessful. This failure might be due to the incorrect primary antibody concentration or fluorophore-conjugated secondary antibody being too low to give a strong signal. The low signal might require addition of extra steps in the protocol such as a saponin/antigen retrieval method or including an extra amplification steps with the secondary antibody. A more suitable alternative might be to use a different IGF1R antibody.

*Progesterone production: effect of IGF1 and IGF2*

Progesterone production significantly increased over time in culture, which indicated that the cells were progressively undergoing luteinisation and/or proliferating. However, there was varied effect of IGF1 and IGF2 treatment on progesterone production with some studies showing small increases, others no effects and one study a small decrease. Thus, the present study does not agree with other reports that demonstrated IGF1 and IGF2 stimulated progesterone production in follicular and luteal cells across many animal species (Scaramuzzi et al., 1999, Spicer and Aad, 2007, Silva et al., 2009). Additionally, our findings did not agree with the reports of synergetic interactions between gonadotrophin and IGFs in promoting granulosa cell proliferation and steroidogenesis in cattle and humans (Spicer and Aad, 2007, Baumgarten et al., 2015). The reason for this discrepancy is unknown but it is feasible that in the present study, the steroidogenic cells are undergoing luteinisation and steroidogenesis at near their maximal capacity such that there is minimal potential for this to be increased. Indeed, Laird

et al., (2013) showed that LH only increased progesterone production by 15% in the same culture system. This is perhaps because there are a number of stimulants that can induce luteinisation such as fibronectin coating, addition of 5 ng/ml LH and the presence of other exogenous growth factors (e.g. epidermal growth factor). Alternatively, previous *in vitro* studies on pig follicles have shown that IGF1 stimulated production of IGFBPs in a dose-manner (Grimes and Hammond, 1992) which in turn could sequester any exogenously added IGF1 or IGF2.

Progesterone production, though, was clearly decreased by PPP and the degree of this suppression increased with time in culture. Specifically, by day 5 of culture, progesterone concentrations were 60% lower in cells treated with PPP. Thus, endogenous IGF1R signalling is involved in the promotion of progesterone production. This could be further explored by elucidating its interplay with the insulin receptor, which is also known to promote steroidogenesis. It is important that the effects of IGF1R inhibition on the expression of STAR, CYP11A1 and HSD3B1 by western blot were analysed with particular emphasis on comparing the PPP effects on day 3 versus day 5. Equally, it would useful to determine the effects of IGF1R inhibition on intracellular levels of cAMP, expression of LHCGR and the uptake of cholesterol as a mechanism by which IGFs promote steroidogenesis.

The limitation of this Chapter was that there was no or limited effect of IGFBP4 treatment on endothelial cell network formation or progesterone production. The design of this experiment was to add sufficient IGBP4 to sequester all endogenously produced IGF1/2. However, this was not confirmed in the present study and the lack of significant effect makes it difficult to draw any definite conclusion from this experiment. The obvious next step would be to measure IGF1 concentrations in the spent media with and without stripping IGFBPs. This could confirm to what extent the endogenous produced IGFs were sequester.

Another limitation is the utilisation of a mixed cell type culture system of endothelial cells, steroidogenic cells, and pericytes. While this is more realistic of the *in vivo* situation it does make it difficult to determine the exact mechanism/cell type by which the IGFs are exerting their actions. Unfortunately, it was not feasible localise the IGF1R *ex vivo* and *in vitro* which would assist in dissecting the mechanism of action. Equally, the

cellular source of endogenously produced IGF1 or IGF2 was not determined. This was hampered by not having a bovine-specific IGF2 antibody for western or immunohistochemistry analysis. That said, based on the homology between rat and bovine, the anti-rat IGF2 should have cross-reacted.

*Conclusion*

In conclusion, exogenously added IGF1 and IGF2 had minimal effects on luteinising follicular angiogenesis and progesterone production, but IGF1 receptor signalling was important for the development of EC networks and progesterone production in luteinising follicular cells. This implies that there was endogenous activation of IGF1R and that IGF1 production by luteinising follicular cells was increased in a LH-responsive manner. Conversely, there was limited evidence of IGF2 production. Importantly, IGF1R was present in antral follicles, early corpus luteum and luteinising follicular cells in culture. Moreover, there was some but not conclusive evidence that IGF1R was expressed in endothelial cells indicating that IGFs could directly stimulate endothelial cell activity. The sequestration of endogenously produced IGFs with IGFBP4 had no effect on EC network formation and progesterone synthesis, though further work is required to confirm sequestration. An alternative approach would be to knock-down IGF1 and IGF2 synthesis by using small RNA inhibitors.

Looking at the bigger picture, inadequate nutrition and metabolic imbalance down-regulates reproductive function in ruminant animals. This will particularly effect metabolic hormones including growth hormone, insulin and IGF1. Indeed, these hormones have been associated with reduced fertility. The effect of negative energy balance (NEB) is observed through reduced CL weight, decreased progesterone concentration in blood and/or milk of lactating cows. There is strong evidence that NEB affects serum IGF1 concentrations and that this might affect follicular development, the follicle-luteal transition and subsequent luteal function (Spicer et al., 2000). More so, studies have shown that insulin and IGF1 concentrations are reduced by the dietary restriction and negative energy balance of the peripartum period (Diskin et al., 2003). Indeed, reduced serum IGF1 concentrations during luteal development suppressed luteal growth and progesterone synthesis in heifers (Yung et al., 1996). Combining these observations with the present studies, then reduced IGF1R stimulation (as a consequence of lower IGF1

levels) could lead to reduced luteal angiogenesis in the follicle-luteal transition. The consequence of this would be reduced luteal development and function and emphasises the importance of the IGF system in the ovulatory period.

# Chapter 5: The effect of oxygen concentration on endothelial cell network formation and progesterone production in a bovine luteinising follicular angiogenesis culture system.

## 5.1. Introduction

All metabolic processes including ATP production are profoundly affected by the supply of oxygen which in turn is influenced by the degree of vascularisation within that tissue (Carreau et al., 2011). Indeed, in the ovary, oxygen concentration/supply influences the development of follicles. During the pre-antral phase, there is the development of blood capillaries within the theca layer while the granulosa layer remains avascular (Feng et al., 2017). Therefore, oxygen has to diffuse from these blood capillaries through the basement membrane to reach the multi-layered granulosa cells and oocyte. Consequently, granulosa cells of large antral follicles are exposed to relatively low oxygen concentrations (Huey et al., 1999) and not surprisingly, follicular fluid oxygen concentrations decrease as the antral follicle reaches its pre-ovulatory size (Fischer et al., 1992, Van Blerkom et al., 1997, Basini et al., 2004).

Indeed, mathematical models have predicted that the mean dissolved oxygen level is 11-51 mmHg (~1.5-6.7%) in the pre-ovulatory follicle of humans (Redding et al., 2008) which is potentially lower than observed in other similar tissues. The lower physiological oxygen concentration ($pO_2$) is associated with decreased pH and increased $pCO_2$ in the follicular fluid. This could indicate that follicular cells would have a proportionally higher anaerobic respiration rate. Additionally, in mice, there is an increase in glucose consumption and lactate accumulation in follicles after hCG treatment (Hillensjö, 1976, Hillier et al., 1985, Harris et al., 2007). The increased formation of lactate further indicates that follicular metabolism is anaerobic. Indeed, lactate concentration in preovulatory follicular fluid was positively correlated with follicular diameter (Gull et al., 1999). Furthermore, lactate can act as an effective signalling molecule that induces granulosa cell differentiation (Baufeld and Vanselow, 2018).

Oxygen is essential for cellular growth, metabolism and survival but it is feasible that follicular development is adapted to a certain extent to occur in relatively reduced oxygen concentrations. In addition, several factors might affect the concentration of oxygen in the follicle which include follicular size, degree of vascularisation and blood supply (Van Blerkom et al., 1997). After ovulation, the CL is much more metabolically active to meet the huge steroidogenesis demands and is consequently likely to be more sensitive to low oxygen concentrations. Furthermore, the ovulatory process is associated with increased vascular permeability and disruption of the established follicular vasculature.

Hypoxia describes a reduction in oxygen below the typical physiological oxygen concentration present in a given tissue and broadly speaking cells in 1-5% dissolved oxygen levels are considered to be under hypoxic conditions. Importantly, hypoxia is known to promote pathological disorders including the formation of new blood vessel in tumours. Cellular responses to hypoxia are largely mediated by the oxygen-regulated transcriptional activator, hypoxia-inducible factor 1 (HIF1) (Wang et al., 1995).

HIF1 is a transcriptional factor that is formed by the heterodimerisation of HIF1A and HIF1B subunits (Semenza et al., 1996, Chen et al., 2009). The activity and expression of HIF1A is profoundly influenced by cellular oxygen concentration while HIF1B is constitutively expressed in the cell nucleus (Wenger, 2002, Bruick, 2003). HIF1A protein is degraded under normal oxygen conditions but accumulates rapidly in hypoxia. Increased HIF1A levels enable it to translocate from the cytoplasm to the nucleus where it dimerises with HIF1B to form HIF1A complex and become transcriptionally active (Wang and Semenza, 1995). HIF1 up-regulates the expression of genes responsible for increased oxygen transport, angiogenesis via VEGFA expression, glucose metabolism, cell proliferation and programmed cell death.

Low oxygen has been reported to contribute to the stimulation of vascularisation that is important during follicular development, ovulation and corpus luteum development. HIF1A has a role in follicular and early luteal development in pigs (Basini et al., 2004) and primates (Duncan et al., 2008). In the primate ovary, nuclear HIF1A immunostaining was largely absent from preantral and antral follicles, but there was marked up-regulation of nuclear HIF1A in granulosa cells at ovulation that persisted into the early CL.

A reduction in luteal vasculature via VEGFA inhibition resulted in up-regulation of luteal nuclear HIF1A staining (Duncan et al., 2008).

Hypoxia, through activation of HIF1A, up-regulates pro-angiogenic factors to stimulate a new vasculature and may regulate the intense proliferation of endothelial cells during luteal development (Chen et al., 2009). In most tissues, hypoxia induces the expression of proteins such as VEGFA thereby controlling the local blood supply to the microenvironment (Kimura et al., 2001). VEGFA mediates physiological angiogenesis and increases the permeability of blood vessels (Ferrara et al., 2003). It has been proposed that HIF1A expression is regulated via the interaction between cAMP signalling and hypoxia in human granulosa cells and bovine luteinised granulosa cells (Yalu et al., 2015). Namely, HIF1A protein expression was increased by chemical-induced hypoxia and further increased by LH and cAMP treatment. Conversely hypoxia has been shown to be the main regulator of VEGFA expression rather than gonadotrophins in primary cultures of luteal cells from human (Tesone et al., 2005) and non-human primates (Tropea et al., 2006). *HIF1A* mRNA and protein was upregulated by FSH treatment in mouse granulosa cells from dominant follicles (Zhou et al., 2017).

LH and FSH increased *VEGFA* mRNA expression in mice granulosa cells in a HIF1A-mediated manner (Rico et al., 2014). Steroidogenesis and angiogenesis are influenced by hypoxic conditions during corpus luteum formation (Nishimura and Okuda, 2010). Hypoxia decreases progesterone production that might be in part due to hypoxia inducing apoptosis. In addition, hypoxia downregulated the expression of the key steroidogenic proteins STAR, CYP11A1 and HSD3B1, which would explain the decreased progesterone production (Jiang et al., 2011a). At the end of the luteal phase, the deficiency of oxygen caused by decreased blood supply to the bovine CL is suggested to be a major factor in the progression of functional and structural luteolysis (Nishimura et al., 2006, Nishimura et al., 2008, Nishimura and Okuda, 2010). However, the role of hypoxia, HIF1A and protein kinase A (PKA) signalling in the regulation of angiogenesis and steroidogenesis during the follicular-luteal transition remains less clear.

### 5.1.1. Aim

To evaluate the effects of low oxygen concentration on endothelial cell (EC) network formation and progesterone production in bovine luteinising follicular cells and to investigate the underlying mechanisms involved in causing these effects.

### 5.1.2. Objectives

1.) To evaluate the effect of low oxygen concentration on EC network formation and progesterone production in bovine luteinising follicular cells.
2.) To determine the role of HIF1A in mediating EC network formation and progesterone production in bovine luteinising follicular cells and its interaction with low oxygen concentrations.
3.) To investigate the role of PKA signalling in mediating EC network formation and progesterone production in bovine luteinising follicular cells and its interaction with low oxygen concentrations.

## 5.2. Study 1: The effect of low oxygen concentration on endothelial cell network formation and progesterone production in bovine luteinising follicular cells

The hypothesis tested was that low oxygen concentrations will promote endothelial cell (EC) network formation but will decrease progesterone production in bovine luteinising follicular cells.

### 5.2.1. Experimental design

Follicular cells were harvested from large (>10 mm) bovine follicles as described (Section 2.1.3) and plated onto fibronectin-coated 12-well plates as detailed (Section 2.1.1) at $3x10^5$ granulosa cells/well plus $1x10^5$ theca cells/well. The cells were cultured in supplemented serum-free MDCB 131 media as described (Section 2.1.2) in different incubators (Sanyo MCO-19M $CO_2$ and $O_2/CO_2$ incubators, United Kingdom (UK)) under high (20%) or low (3%) oxygen conditions. The cells (n=4 cultures) were grown in the absence and presence of angiogenic-stimulation (FGF2+VEGFA, both 1 ng/ml). Each treatment was performed in duplicate. The cells were cultured for 5 days, with the spent media collected on days 3 and 5 of culture and then stored at -20$^o$C until analysis. At the end of culture, the cells were fixed in ice-cold acetone: methanol (1:1) for immunostaining. The EC network formation in the fixed cells was evaluated using VWF immunocytochemistry (polyclonal rabbit anti-human VWF; Dako: A0082) followed by image analysis as detailed (Section 2.2.3 and Table 2.2). While the progesterone concentrations in spent media (diluted 100-200 fold) were determined by ELISA as described (Section 2.3.1).

In an additional 3 cultures, bovine luteinising follicular cells were cultured under 20% or 3% oxygen conditions without angiogenic stimulation for 5 days. The cells were fixed at the end of culture to localise vascular-associated mural pericytes using $\alpha$-smooth muscle actin (ACTA2) immunocytochemistry and hypoxia-inducible factor 1A (HIF1A) protein using immunocytochemistry and immunofluorescence; as described (Section 2.2.3 and Table 2.2). The primary antibodies used were monoclonal mouse anti-human smooth muscle actin (A2547: Sigma-Aldrich, Poole, UK) and monoclonal mouse anti-human HIF1A (ThermoFisher Scientific: H1alpha67).

There was no quantification of α-smooth muscle actin (ACTA2) and hypoxia-inducible factor 1A (HIF1A) protein staining since the objective was to confirm the localisation of ACTA2 and HIF1A proteins in cultured follicular cells.

### 5.2.2. Statistical analysis

Data were checked for normality using residual plots and for homogeneity of variance with Levene's test; where appropriate, data were log transformed. The EC network endpoints were converted to % of the mean control wells for each culture. All data were shown as mean ± SEM from replicated experiments. Statistical analysis was performed using Genstat 18$^{th}$ edition (Hemel Hempstead, UK). A two-way randomised block ANOVA was performed for the 2 (20% *vs* 3% oxygen) × 2 (absence *vs* presence of angiogenic stimulation) factorial design. In each case, the data were blocked by culture with the individual wells nested within. For the progesterone production analysis there was a large effect of day, thus the data were analysed by each day of collection separately (day 3 and day 5).

### 5.2.3. Results: The effect of low oxygen concentration on endothelial cell network formation

On day 5 of culture, extensive organised EC networks were formed in both oxygen microenvironments in both the absence and presence of angiogenic stimulation (FGF2+VEGFA) (Fig. 5.1). There were numerous branch points from the EC networks (group of ECs in cluster). The EC formed elongated and tubule-like structures with some complex multiple branching between points extending from the endothelial cell cluster. It was clear that non-endothelial cells (such as steroidogenic and mural cells) had also proliferated in culture (Fig. 5.1). Under angiogenic-stimulation, the EC networks appeared to be greater in number and size under 20% oxygen (Fig. 5.1A and C) compared to 3% oxygen (Fig. 5.1B and D).

Angiogenic-stimulation with FGF2+VEGFA increased the total area ($p<0.05$), the number ($p<0.05$) and perimeter ($p<0.01$) of EC networks by approximately 30% (Fig. 5.2A-C) in both 3% and 20% oxygen conditions. Contrary to expectations, under low oxygen conditions there was an approximate 35% reduction in the total EC area ($p<0.001$), perimeter of EC staining ($p<0.001$) and mean area of the EC networks ($p<0.001$) (Fig.5.2).

The total number of branch points ($p<0.05$) and degree of branching ($p<0.05$) within each EC network were also reduced (Fig. 5.2). However, there was no effect of oxygen concentration on the number of EC networks ($p>0.05$). In addition, there was no interaction between oxygen concentration and angiogenic stimulation treatment for all endpoints measured ($p>0.05$; Fig. 5.2).

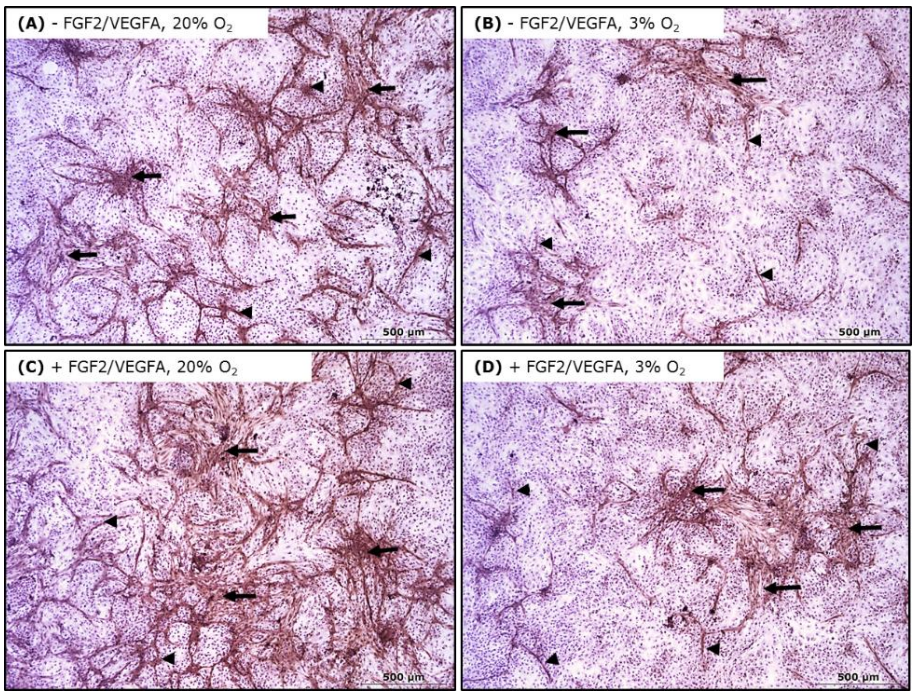



Fig. 5.1: The effect of low oxygen concentration on EC network formation in the absence (A & B) and presence (C & D) of angiogenic (FGF2+VEGFA) stimulation in bovine luteinising follicular cells. Cells were cultured under high (20%; A,C) or low (3%; B,D) oxygen conditions. The EC networks were immunostained for von Willebrand factor as indicated by brown staining (arrows), with branches identified by arrow heads. Scale bars represent 500 µm, n=4 cultures.

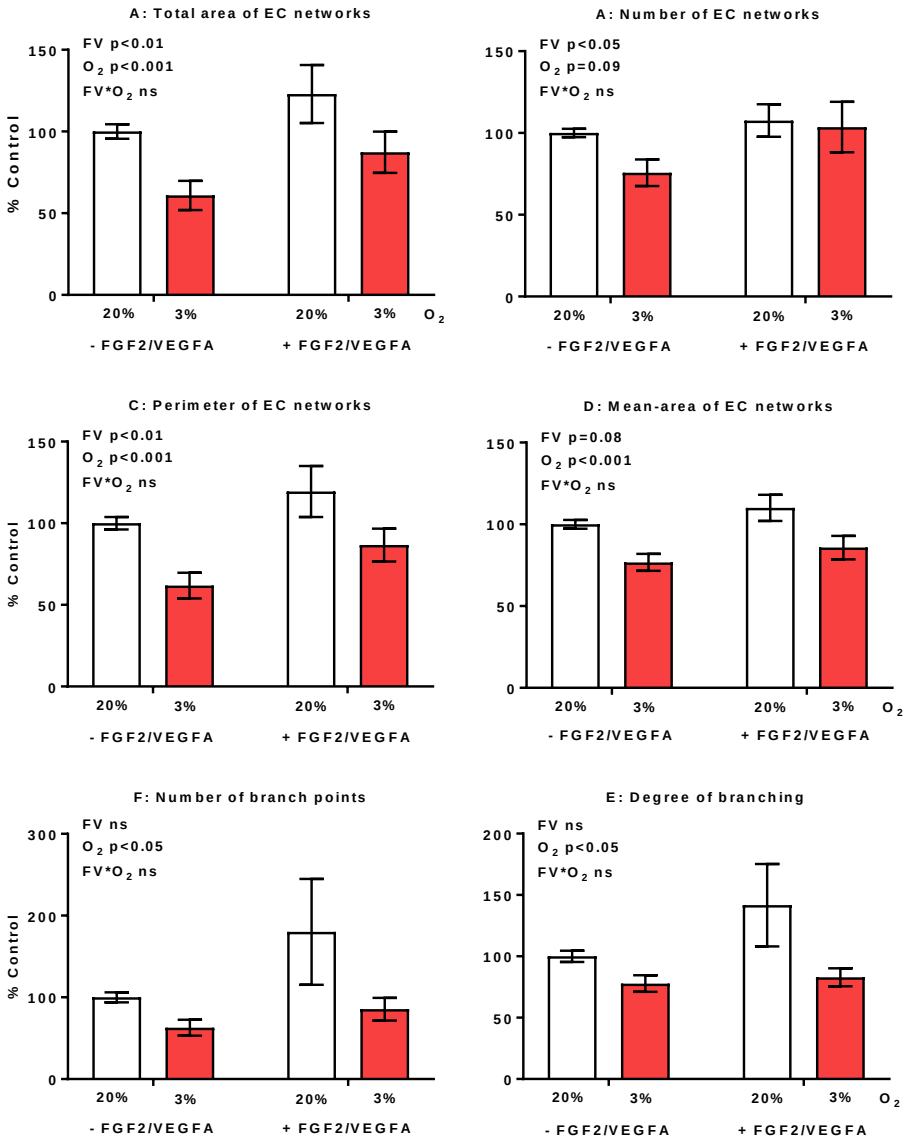



Fig. 5.2: The effect of different oxygen concentrations on EC network formation in the absence and presence of angiogenic (FGF2+VEGFA; FV) stimulation in bovine luteinising follicular cells. The endpoints measured were (A) total area of EC networks, (B) number of EC networks, (C) perimeter of EC networks, (D) mean area of each EC networks, (E) total number of branch points, and (F) degree of branching within an EC networks. The main effects of angiogenic stimulation (FV) and oxygen concentration ($O_2$) as well as the interaction are indicated on each graph. The data are mean ± SEM, n=4 cultures.

## 5.2.4. Results: The effect of low oxygen concentration on progesterone production

There was a significant effect of time in culture on progesterone production ($p<0.001$) with the progesterone concentration being 3.5-fold greater ($p<0.001$) on day 5 than day 3 (Fig. 5.3). Progesterone production was reduced under low oxygen conditions on both day 3 ($p<0.05$; ~35%) and 5 ($p<0.001$; ~55%) of culture. There was no effect of treatment with angiogenic stimulation ($p>0.05$) on progesterone production (Fig. 5.3). Furthermore, there was no interaction between oxygen concentration and angiogenic stimulation ($p>0.05$) on progesterone production (Fig. 5.3).

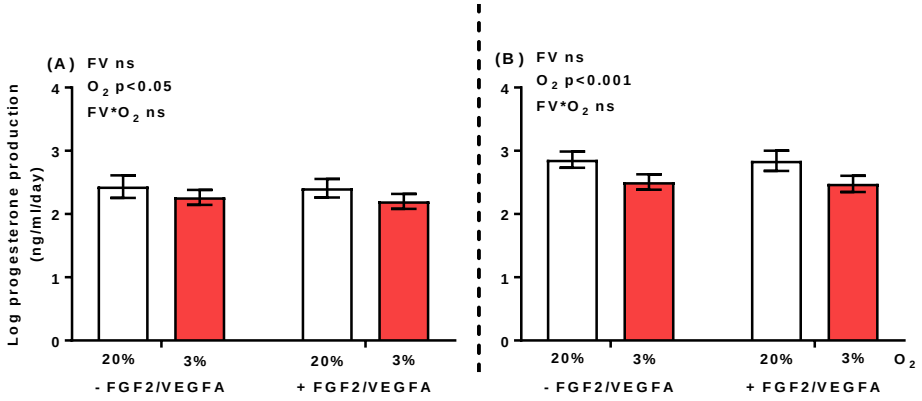



Fig. 5.3: The effect of low oxygen concentration on progesterone production in the absence and presence of angiogenic (FGF2+VEGFA; FV) stimulation in bovine luteinising follicular cells on (A) day 3 and (B) day 5 of culture. Progesterone production was reduced by low oxygen on day 3 ($p<0.05$) and 5 ($p<0.001$) of culture. The data are mean ± SEM, n=4 cultures.

## 5.2.5. Results: The effect of low oxygen concentration on ACTA2 expression

In order to further characterise the *in vitro* effects of low oxygen conditions on the vascular cells, mural cells (pericytes) were identified using ACTA2 protein as a marker. There was extensive staining of ACTA2 protein in the cultured bovine luteinising follicular cells (Fig. 5.4). The mural cells appeared to be broad, elongated, and largely clustered together. Moreover, the degree of ACTA2 immunostaining present across the well appeared to be greater in area occupied in 20% oxygen (Fig. 5.4A) compared to 3% oxygen (Fig.

5.4B). However, in 3% $O_2$ those ACTA2-positive cells often had more intense ACTA2 staining. The mouse IgG immunocytochemistry control showed no evidence of positive staining (Fig. 5.4C).

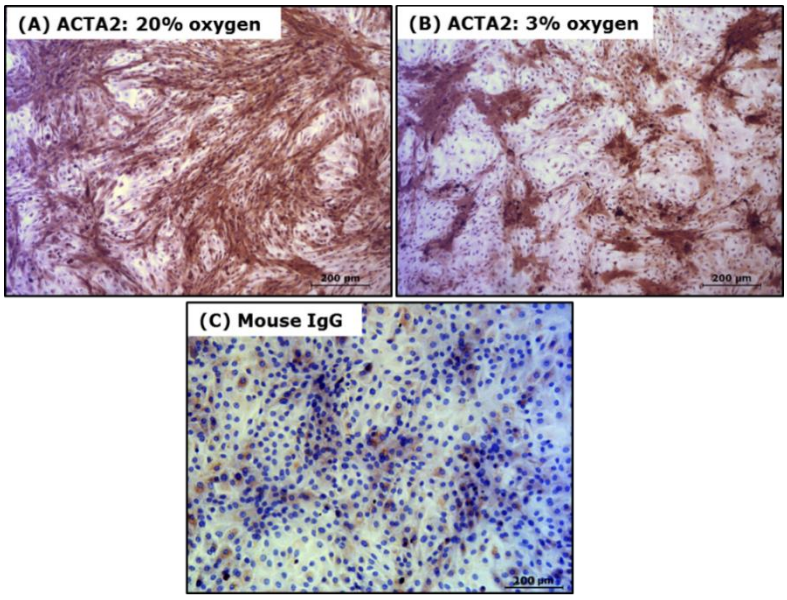



Fig. 5.4: Immunolocalisation of α-smooth muscle actin (ACTA2) in cultured bovine luteinising follicular cells under different oxygen concentrations. Representative images are shown for ACTA2 immunostaining of cells under (A) 20% oxygen concentration, (B) 3% oxygen concentration, and (C) mouse IgG control. The ACTA2 immunostaining appeared more abundant in 20% oxygen compared to 3% oxygen. The mouse IgG control showed no evidence of brown staining. Scale bar is 100-200 μm.

## 5.2.6. Results: The effect of low oxygen concentration on the localisation of HIF1A

The initial results showed that the formation of EC networks was reduced when cells were cultured under low oxygen conditions. Thus, it was important to determine the expression and localisation of HIF1A in the culture system and determine the effect of low oxygen concentration on its expression. Thus, HIF1A immunocytochemistry was performed in bovine luteinising follicular cells that were cultured under both 20% and 3% oxygen. HIF1A protein was present in a small proportion of cells cultured in both 20% and 3% oxygen (Fig. 5.5A and C). The staining intensity varied from weak to moderate and was either perinuclear or nuclear. The proportion of HIF1A-

positive cells appeared similar in both oxygen concentrations. Additionally, HIF1A did not appear to be localised to any particular cell type. Importantly, mouse IgG control had limited evidence of positive brown staining in both oxygen conditions (Fig. 5.5B and D).

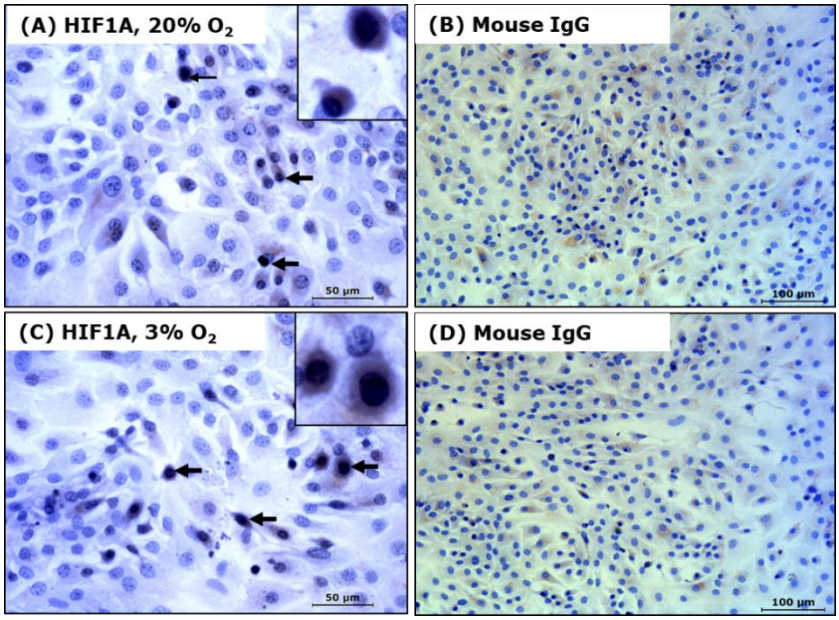



Fig. 5.5: Immunohistochemical localisation of hypoxia-inducible factor 1A (HIF1A) protein in bovine luteinising follicular cells. Representative images are shown for (A) HIF1A protein in cells cultured under 20% oxygen with the mouse IgG control shown in (B); (C) HIF1A protein in cells cultured under 3% oxygen with the mouse IgG control shown in (D). HIF1A was detected in cells cultured in both 20% and 3% oxygen and is indicated by arrows. The image insets show the nuclear staining for HIF1A under magnification. The mouse IgG controls had weak generalised staining across the section. Scale bar is 50 - 100 µm.

## 5.3. Study 2: The effect of HIF1A inhibition on endothelial cell network formation and progesterone production in bovine luteinising follicular cells.

There was evidence of HIF1A activity in luteinising follicular cells irrespective of oxygen concentration. This could, in part, explain the lack of stimulation of EC network formation in low oxygen conditions observed in study 1.

This experiment investigated this aspect further by testing the hypothesis that HIF1A signalling plays an important role in the stimulation of EC network formation and progesterone production in bovine luteinising follicular cells.

The specific objectives were;

a) To evaluate the effect of HIF1A inhibition on EC network formation as determined by VWF immunohistochemistry and image analysis.
b) To assess the effect of HIF1A inhibition on progesterone production determined by ELISA and expression of steroidogenic proteins involved in progesterone synthesis analysed by western blot.
c) To determine cellular proliferation using a MTT cell assay and cellular metabolic activity by measuring lactate concentrations.
d) To evaluate the expression of HIF1A target proteins using western blotting (e.g. BNIP3) and ELISA (VEGFA).

### 5.3.1. Experimental design

#### 5.3.1.1. EC network formation and progesterone production

Bovine luteinising follicular cells (n=4 cultures) were cultured in high (20%) or low (3%) oxygen conditions and treated with control or a specific HIF1A inhibitor (echinomycin; Calbiochem; 1 μM). Echinomycin is a DNA-binding agent that binds to the hypoxia response element (HRE) site within the promoters of HIF1A target genes (Kong et al., 2005). The concentration of HIF1A inhibitor used was based on its $EC_{50}$ value and doses previously used (Park et al., 2008, Kim et al., 2009). Echinomycin was initially dissolved in dimethyl sulfoxide (DMSO), with the final DMSO concentration in each treatment of 0.1%. Therefore, the control wells were also supplemented with 0.1% DMSO. Each treatment was performed in duplicate. The spent media was collected on days 3 and 5 of culture, then stored at -20$^{o}$C until

progesterone analysis. On day 5, the cells were fixed with ice-cold acetone: methanol for VWF immunocytochemistry and image analysis.

#### 5.3.1.2. Western blotting, lactate and VEGFA analysis

In an additional 4 cultures, bovine follicular cells were cultured and treated as described (Section 5.3.1). The spent media was collected on days 3 and 5 of culture and were analysed for lactate (anaerobic rate indicator) and VEGFA (HIF1A responsive protein) concentration. At the end of the culture (day 5), the cells were harvested in 0.1 M ice-cold Tris-HCl Buffer containing protease inhibitor (Sigma-Aldrich, Poole, UK). For the HIF1A western blotting analysis, 5 µg/ml of reconstituted MG-132 (Selleckchem, United States of America (USA)) was also added to inhibit proteasome activity and thus preserve protein stability. The cells were then lysed and proteins extracted. The protein concentration in the lysate was determined by Bradford assay, with 20 µg loaded onto a mini-gel. Next, western blotting was performed to determine the expression of the following proteins: steroidogenic proteins (STAR, CYP11A1, and HSD3B1), HIF1A and HIF1A responsive proteins (HIF1A, PGK1, GLUT1, and BNIP3), mural cells (ACTA2) and normalised to the expression of ACTB or HIST3H3 (Section 2.4.3). Protein expression was measured by quantifying the intensity of each protein band using ImageJ (National Institutes of Health, USA). The protein expression was normalised against the intensity of either ACTB or HISTH3 protein (housekeeping protein) band. The housekeeping protein was selected based a different molecular weight to the target protein.

#### 5.3.1.3. Cell proliferation (MTT assay)

Bovine luteinising follicular cells (n=5 cultures) were cultured on fibronectin-coated 96-well plates under high (20%) or low (3%) oxygen concentrations. The cells were treated with either control (0.1% DMSO) or echinomycin (1 µM, HIF1A inhibitor). The cells were grown for 3 days and the number of viable cells determined using a MTT cell proliferation assay (Section 2.3.5).

### 5.3.2. Statistical analysis

For *EC network analysis*, the endpoints from the image analysis (e.g. total EC area, number of branch points) were expressed as a percentage of the control (no echinomycin, 20% oxygen) for that particular culture. Significance was determined using a two-way randomised block ANOVA,

with the data blocked by culture number and the cells/wells nested within the culture block. The factors were oxygen (20% vs 3%) and echinomycin (absence vs presence).

In the *western blot analysis,* the intensity of each band was quantified using ImageJ and then normalised to the expression of ACTB or HIST3H3 before statistical analysis. The data was then analysed by a two-way randomised block ANOVA as described above.

In the *progesterone, lactate and VEGFA analysis*, the data was checked for normal distribution and homogeneity of variance and was log transformed where necessary. Initially, a three-way randomised block ANOVA was employed including day of culture as one of the factors. However, since there was a large effect of day, the data was analysed by two-way randomised block ANOVA on each day of culture separately.

In *cell proliferation analysis*, the absorbance was expressed as % control (no echinomycin, 20% oxygen) and then analysed by a two-way randomised block ANOVA.

### 5.3.3. Results: The effect of HIF1A inhibition on endothelial cell network formation.

On day 5 of culture, organised EC networks were formed in all treatments and oxygen concentrations, albeit to different degrees (Fig. 5.6). On closer examination, the degree of EC network formation appeared less when cells were cultured in 3% oxygen and treated with echinomycin (Fig. 5.6). In particular, there were fewer EC networks and number of branch points ($p<0.05$) (Fig. 5.6).

The total area of EC networks was clearly reduced by both echinomycin treatment (~30%, $p<0.001$), and oxygen concentration (~30%, $p<0.001$ Fig.5.7A). However, there was no echinomycin × oxygen interaction which showed an additive effect between echinomycin and 3% oxygen (~60% reduction). Similarly, echinomycin treatment and oxygen concentration reduced both the number of EC networks ($p<0.01$, Fig.5.7B) and the perimeter of the EC networks ($p<0.01$; Fig.5.7C) by a similar extent as observed for the total area. Collectively, this indicated that the effect of echinomycin was the same in both 20% and 3% oxygen. In contrast, the size of each EC network (mean area) was not affected by either echinomycin treatment or oxygen concentration ($p>0.05$, Fig.5.7D). In regards to the amount of branching, the total number of branch points were reduced by both echinomycin treatment ($p<0.01$) and oxygen concentration ($p<0.01$; Fig.5.7E). However, the degree of branching within a particular EC network was unaffected by echinomycin treatment and oxygen concentration and in addition there was no significant interaction (Fig.5.7F).

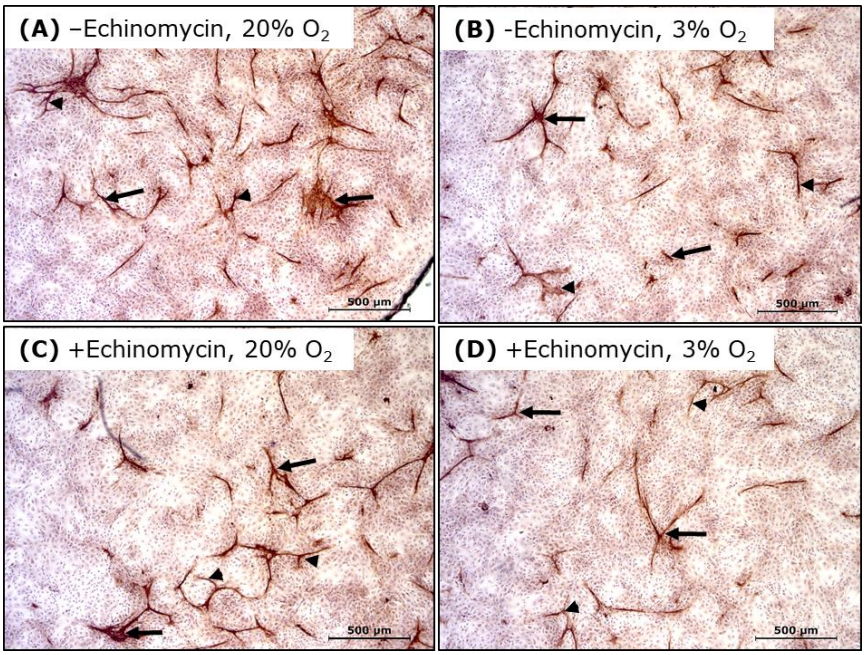



Fig. 5.6: The effect of HIF1A inhibition on EC network formation in bovine luteinising follicular cells cultured in different oxygen (20% vs 3%) concentrations. EC networks were identified by von Willebrand factor immunostaining and are shown by brown staining (arrows) with elongated branches shown by arrowheads. Representative images are shown for cells cultured in 20% oxygen (A,C) or 3% oxygen (B,D) and treated with either control (A,B) or echinomycin (1 µM, HIF1A inhibitor, C,D). The EC networks were elongated and tubule-like in appearance, with some complex multiple branching points extending from the EC networks. Scale bars represent 500 µm, n=4 cultures.

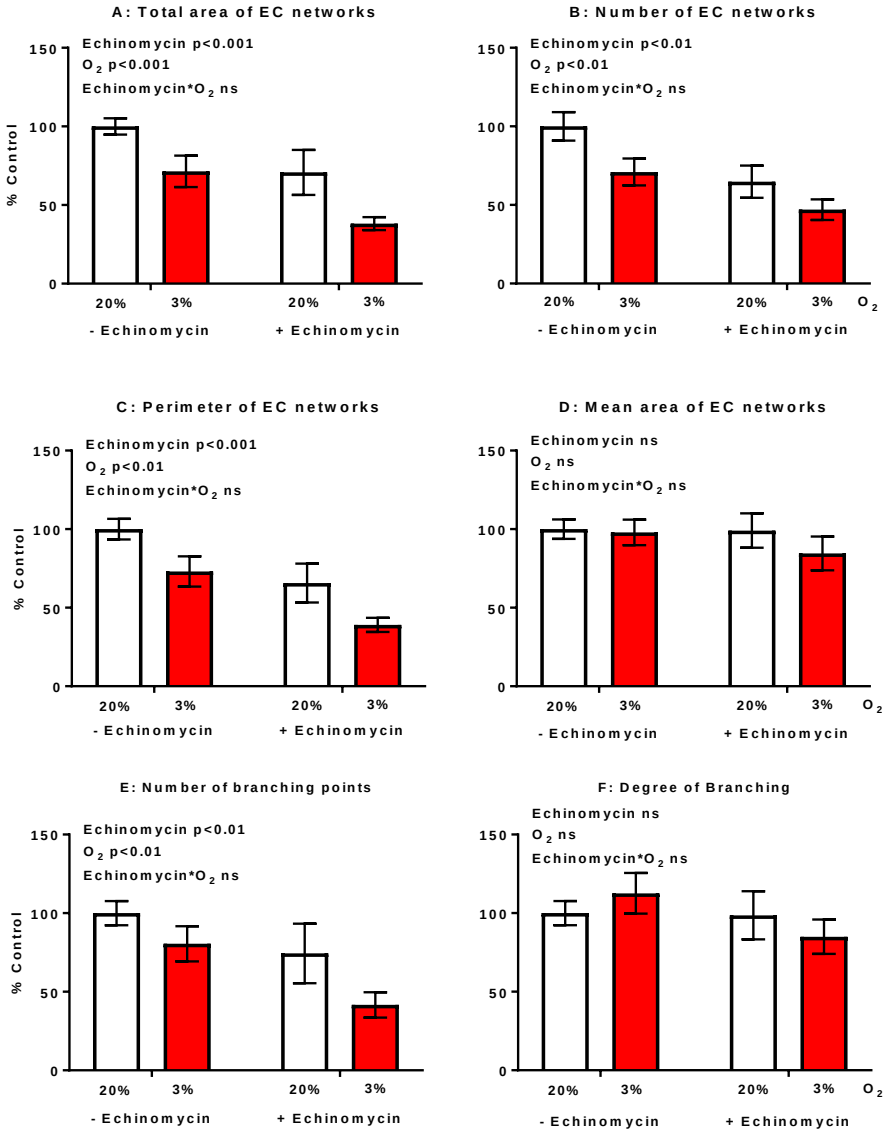



Fig. 5.7: The effect of HIF1A inhibition on the quantification of EC network formation in bovine luteinising follicular cells cultured in different oxygen (20% vs 3%) concentrations. The cells were treated with either control or echinomycin (1 μM, HIF1A inhibitor). The endpoints were: (A) total area of EC networks, (B) number of EC networks, (C) perimeter of EC networks, (D) mean area of each EC networks, (E) number of branch points and (F) degree of branching within an EC networks. The statistical effects of oxygen concentration, echinomycin and their interaction are indicated on each graph. The data are mean ± SEM, n=4 cultures.

### 5.3.4. Results: The effect of HIF1A inhibition on progesterone production.

There was a significant effect of time in culture on progesterone production ($p<0.001$) with progesterone concentrations 3.2-fold higher on day 5 than day 3 (Fig. 5.8). Consequently, each day of culture was analysed individually. On day 3 of culture, echinomycin treatment ($p<0.01$) and 3% oxygen ($p<0.05$) decreased progesterone production by 40% and 38% respectively (Fig. 5.8A). On day 5, there was a similar effect of echinomycin treatment ($p<0.01$) reducing progesterone production by 35%. However, on this day progesterone production was unaffected by 3% oxygen ($p>0.05$; Fig. 5.8B). There was no interaction between echinomycin treatment and oxygen concentrations on either day 3 or 5 of culture (Fig. 5.8).

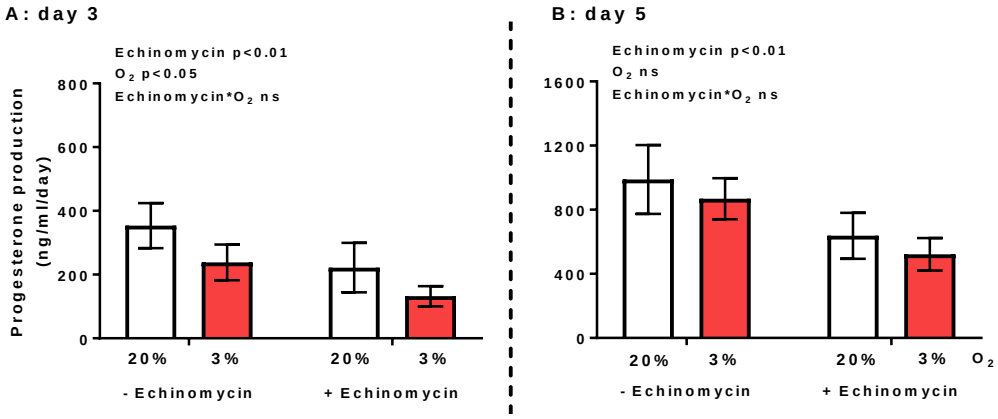



Fig. 5.8: The effect of HIF1A inhibition on progesterone production in bovine luteinising follicular cells cultured in different oxygen (20% vs 3%) concentrations. The cells were treated with either control (DMSO; - echinomycin) or with HIF1A inhibitor; + echinomycin (1 μM). The production of progesterone increased from (A) day 3 to (B) day 5 of culture ($p<0.001$). Progesterone production ($p<0.01$) was reduced by echinomycin treatment and oxygen concentration on day 3 of culture. On day 5, echinomycin treatment ($p<0.01$) but not oxygen concentration reduced progesterone production. The data are mean ± SEM, n=4 cultures.

### 5.3.5. Results: The effect of HIF1A inhibition on steroidogenic protein expression

The expression of STAR protein in the bovine luteinising follicular cells was detected as two bands at 25 and 32 kDa (Fig. 5.9A). The predicted molecular weight for STAR is 32 kDa, however a lower molecular weight band has been detected at 25-26 kDa (Stocco et al., 2001). Both STAR bands were quantified, and analysis revealed that echinomycin had no effect on STAR 25 and 32 kDa ($p>0.05$: Fig. 5.9B-C). However, low oxygen concentration decreased 32 kDa STAR protein ($p<0.05$: Fig. 5.9C) and tended to decrease 25 kDa STAR protein ($p=0.09$: Fig. 5.9B). There were no echinomycin × oxygen interactions for either STAR isoform ($p>0.05$: Fig. 5.9B-C).

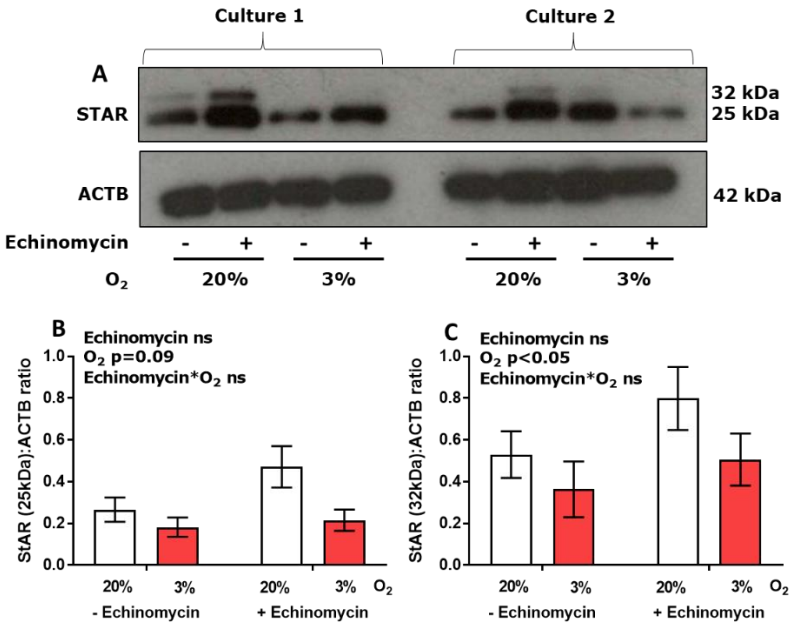



Fig. 5.9: The effect of HIF1A inhibition on steroidogenic acute regulatory protein (STAR) protein expression in bovine luteinising follicular cells cultured in different oxygen (20% vs 3%) concentrations. The cells were treated with control (DMSO) or HIF1A inhibitor, echinomycin (1 μM). (A) shows a representative western blot with a double band for STAR (25 and 32 kDa) and a single band for actin, beta (ACTB, 42 kDa) which was consistently expressed across the different samples. The quantification of (B) 25 kDa STAR and (C) 32 kDa STAR protein expression normalised to ACTB is shown. The effects of echinomycin, oxygen concentration and their interaction are indicated on the graph. The data are mean ± SEM, n=4 cultures.

CYP11A1 protein was detected as a single band at its expected molecular weight of 60 kDa (Fig. 5.10A). CYP11A1 protein expression was unaffected by echinomycin treatment ($p>0.05$) and oxygen concentration ($p>0.05$; Fig. 5.10B). There was no interaction ($p>0.05$) between echinomycin treatment and oxygen concentration (Fig. 5.10B).

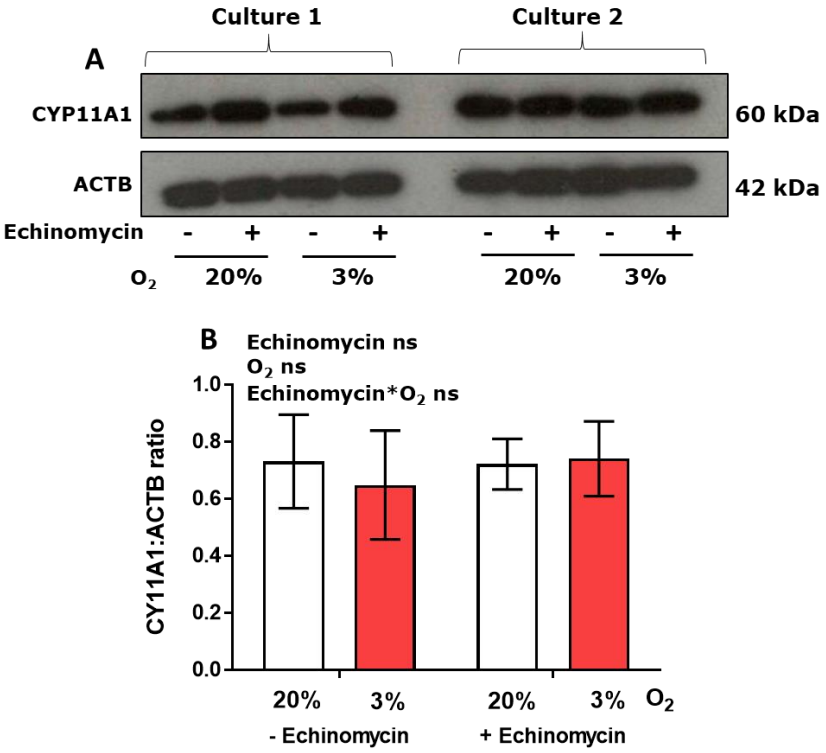



Fig. 5.10: The effect of HIF1A inhibition on P450 cholesterol side-chain cleavage (CYP11A1) protein expression in bovine luteinising follicular cells cultured in different oxygen (20% vs 3%) concentration. The cells were treated with control (DMSO) or echinomycin (1 μM). (A) shows a representative western blot with a single band for CYP11A1 (60 kDa) and actin, beta (ACTB, 42 kDa) which was consistently expressed across the different samples. (B) shows the quantification of CYP11A1 protein expression normalised to ACTB. The effect of echinomycin, oxygen concentration and their interaction is indicated on the graph. The data are mean ± SEM, n=4 cultures.

HSD3B1 protein was detected as a single band at its expected weight of 44 kDa by western blot (Fig.5.11A). HSD3B1 protein expression was unaffected by either echinomycin treatment ($p>0.05$) or oxygen concentration ($p>0.05$; Fig.5.11B). There was an interaction ($p<0.05$) between echinomycin treatment and oxygen concentration (Fig. 5.11B). This showed that HSD3B1 protein expression was reduced by echinomycin treatment in 3% oxygen but not in 20% oxygen (Fig. 5.11B).

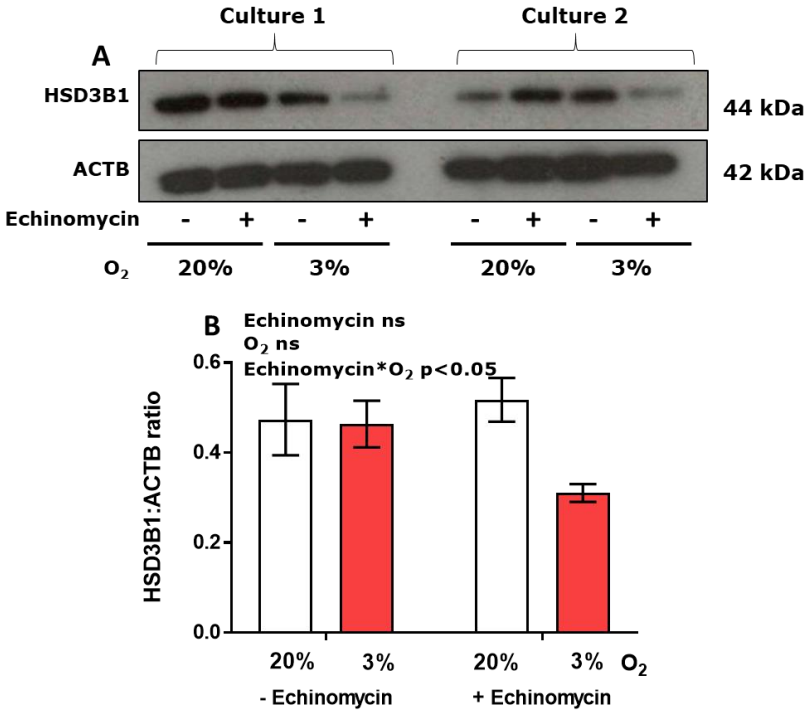



Fig. 5.11: The effect of HIF1A inhibition on 3-βhydroxysteroid dehydrogenase (HSD3B1) protein expression in bovine luteinising follicular cells cultured in different oxygen (20% vs 3%) concentrations. The cells were treated with control (DMSO) or echinomycin (1 μM). (A) shows a representative western blot with a single band for HSD3B1 (44 kDa) and actin, beta (ACTB, 42 kDa) which was consistently expressed across the different samples. (B) shows the quantification of HSD3B1 protein expression normalised to ACTB. The effects of echinomycin, oxygen concentration and their interaction are indicated on the graph. The data are mean ± SEM, n=4 cultures.

## 5.3.6. Results: The effect of HIF1A inhibition on ACTA2 protein expression

To quantify the growth of mural cells in the culture system, ACTA2 protein expression was analysed by western blotting. ACTA2 protein was detected as a single band at its expected molecular weight (42 kDa; Fig. 5.12A). ACTA2 protein expression was decreased 2-fold by echinomycin treatment ($p<0.05$), but was not affected by oxygen concentration ($p>0.05$; Fig.5.12B). There was no interaction ($p>0.05$) between echinomycin treatment and oxygen concentrations (Fig. 5.12B).

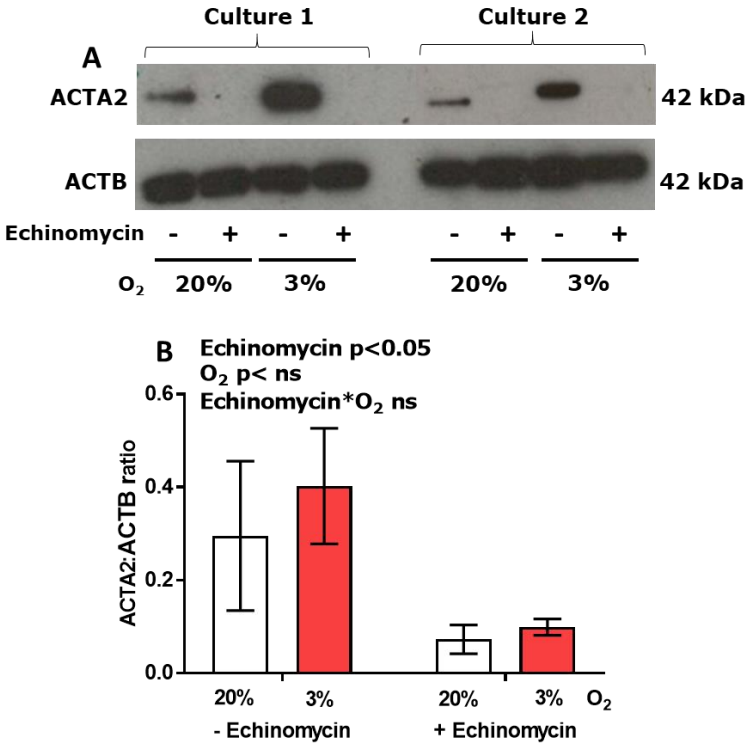



Fig. 5.12: The effect of HIF1A inhibition on smooth muscle actin (ACTA2) protein expression in bovine luteinising follicular cells cultured in different oxygen (20% vs 3%) concentrations. The cells were treated with control (DMSO) or echinomycin (1 μM). (A) shows a representative western blot with a single band for ACTA2 (42 kDa) and ACTB (42 kDa). The housekeeping protein, ACTB was consistently expressed across the different samples. (B) shows the quantification of ACTA2 protein expression normalised to ACTB. The effect of echinomycin, oxygen concentration and their interaction are indicated on the graph. The data are mean ± SEM, n=4 cultures.

### 5.3.7. Results: The effect of HIF1A inhibition on expression of HIF1A and HIF regulated proteins

To determine whether low oxygen concentration increased the expression of HIF1A protein, western blots were performed. HIF1A protein was detected as a single band at its predicted molecular weight (Fig. 5.13). However, despite the addition of the proteasome inhibitor MG-132, HIF1A protein was only detected in 2 out of the 4 cultures. HIF1A is known to be readily degraded within cells and during processing. This is likely to have occurred in this situation. Consequently, quantification and statistical analysis was not feasible. In the 2 cultures where HIF1A was detected, HIF1A protein appeared to be marginally increased in 3% oxygen in the absence of echinomycin. There was though a marked reduction in HIF1A levels in cells treated with echinomycin particularly in 3% oxygen. Thus, it was decided to measure the expression of HIF1A target genes, which are more stable as a proxy for HIF1A activity.

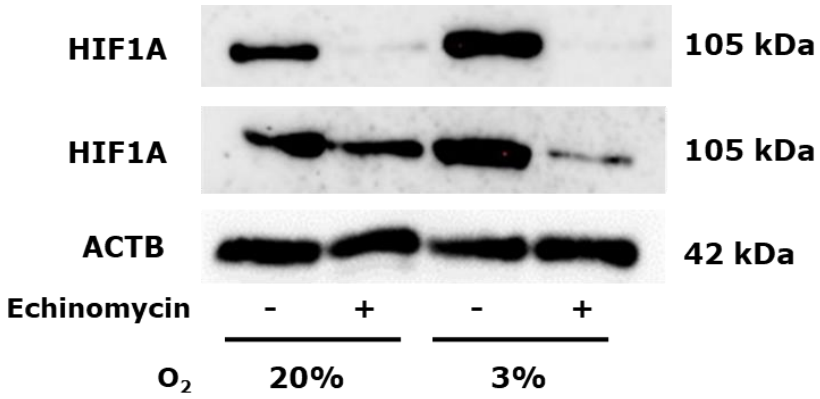



Fig. 5.13: The expression of hypoxia-inducible factor 1A (HIF1A) in bovine luteinising follicular cells cultured under 20% or 3% oxygen concentration. The cells were treated with either control (-) or echinomycin (+). In 2 cultures, HIF1A protein was detected as a single band at 105 kDa and ACTB 42 kDa, however, no band was detected in the other 2 cultures. The housekeeping protein, ACTB was used for normalisation and was consistently expressed across the samples.

The first HIF1A responsive protein evaluated was BNIP3. A single band at the expected molecular weight of 25 kDa was detected for BNIP3 in bovine luteinising follicular cells (Fig.5.14A). BNIP3 protein expression was markedly reduced by echinomycin treatment ($p<0.05$), but was not affected by oxygen conditions ($p>0.05$; Fig.5.14B). There was no interaction ($p>0.05$) between echinomycin treatment and oxygen concentration (Fig. 5.14B).

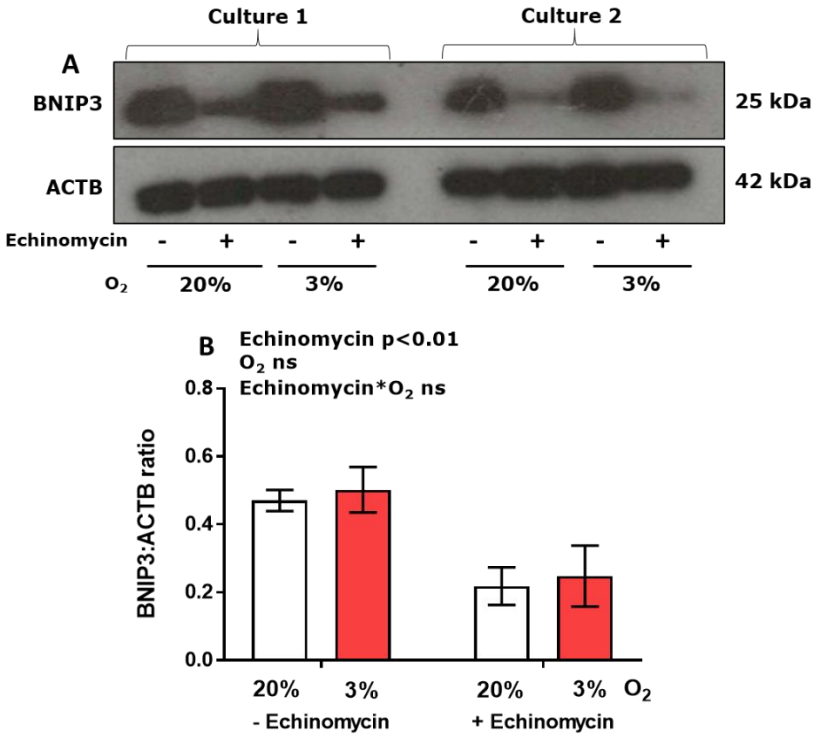



Fig. 5.14: The effect of HIF1A inhibition on BNIP3 protein expression in bovine luteinising follicular cells cultured in 20% and 3% oxygen concentration. The cells were treated with control (DMSO) or echinomycin (1 μM). (A) shows a representative western blot with a single band for BNIP3 (25 kDa) and ACTB (42 kDa). The housekeeping protein, ACTB was consistently expressed across the different samples. (B) shows the quantification of BNIP3 protein expression normalised to ACTB. The effects of echinomycin, oxygen concentration and their interaction are indicated on the graph. The data are mean ± SEM, n=4 cultures.

For GLUT1 analysis, a single, faint band was detected at 43 kDa. This molecular weight was below the published size of 50 kDa (Fig. 5.15A). Despite the low intensity of the band, quantification was performed. GLUT1 protein expression tended to be reduced by oxygen concentration ($p=0.08$) but was not affected by echinomycin treatment ($p>0.05$; Fig.5.15B). There was no interaction ($p>0.05$) between echinomycin treatment and oxygen concentration (Fig. 5.15B).

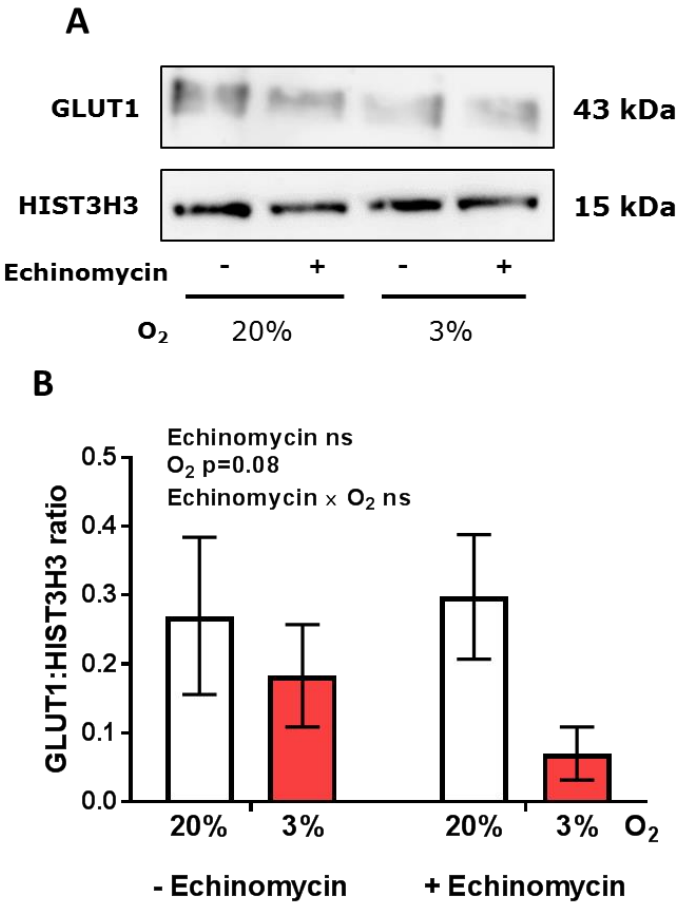



Fig. 5.15: The effect of HIF1A inhibition on GLUT1 protein expression in bovine luteinising follicular cells cultured in 20% and 3% oxygen concentrations. The cells were treated with control (DMSO) or echinomycin (1 μM). (A) shows a representative western blot with a single band for GLUT1 (43 kDa) and HIST3H3 (15 kDa). The housekeeping protein, HIST3H3 was consistently expressed across the different samples. (B) Shows the quantification of GLUT1 protein expression normalised to HIST3H3. The effects of echinomycin, oxygen concentration and their interaction are indicated on the graph. The data are mean ± SEM, n=4 cultures.

The western blot analysis for PGK1 protein revealed a single band at 75 kDa which was greater than the expected molecular weight (45 kDa; Fig.5.16A). The western blot was repeated several times and the single band was present at the same molecular weight each time. The expression of PGK1 protein was significantly reduced in the 3% oxygen ($p<0.01$) and tended to be reduced by echinomycin treatment ($p=0.06$; Fig.5.16B). There was no interaction ($p>0.05$) between echinomycin treatment and oxygen concentration (Fig. 5.16B).

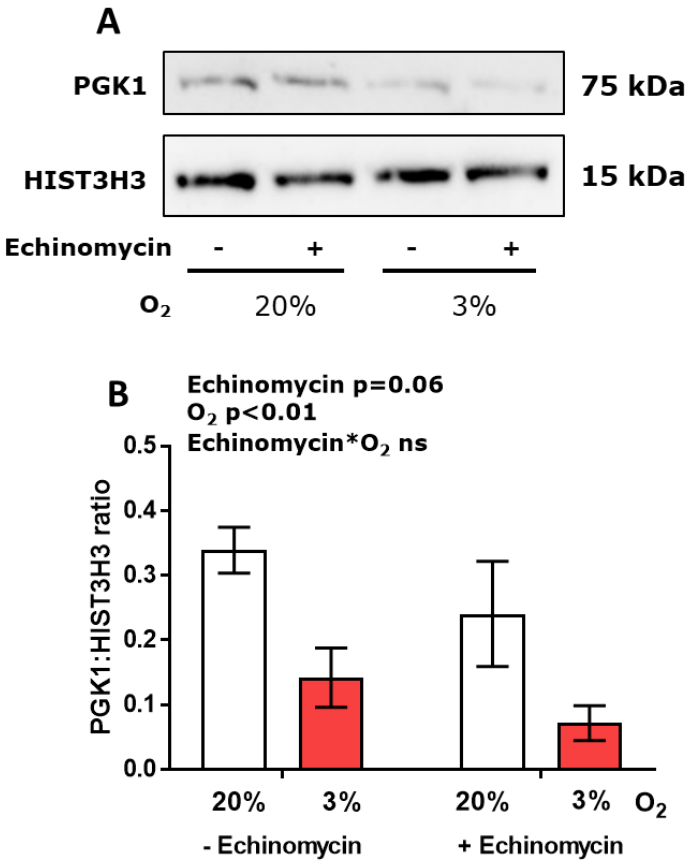



Fig. 5.16: The effect of HIF1A inhibition on PGK1 protein expression in bovine luteinising follicular cells cultured in 20% and 3% oxygen concentration. The cells were treated with control (DMSO) or echinomycin (1 μM). (A) shows a representative western blot with a single band for PGK1 (75 kDa) and HIST3H3 (15 kDa). The housekeeping protein, HIST3H3 was consistently expressed across the different samples. (B) shows the quantification of PGK1 protein expression normalised to HIST3H3. The effects of echinomycin, oxygen concentration and their interaction are indicated on the graph. The data are mean ± SEM, n=4 cultures.

The next target was VEGFA, which was measured in the spent media by ELISA. There was a significant effect of time in culture on VEGFA production with VEGFA concentration 6-fold greater ($p<0.001$) on day 5 than day 3 (Fig. 5.17). Echinomycin treatment markedly decreased VEGFA production on day 3 ($p<0.001$) and day 5 ($p<0.001$) by around 75%. The effect of echinomycin was observed in both 3% and 20% oxygen. However, on both days, VEGFA production was unaffected by oxygen concentration ($p>0.05$; Fig. 5.17) and there was no echinomycin × oxygen interaction ($p>0.05$; Fig. 5.17).

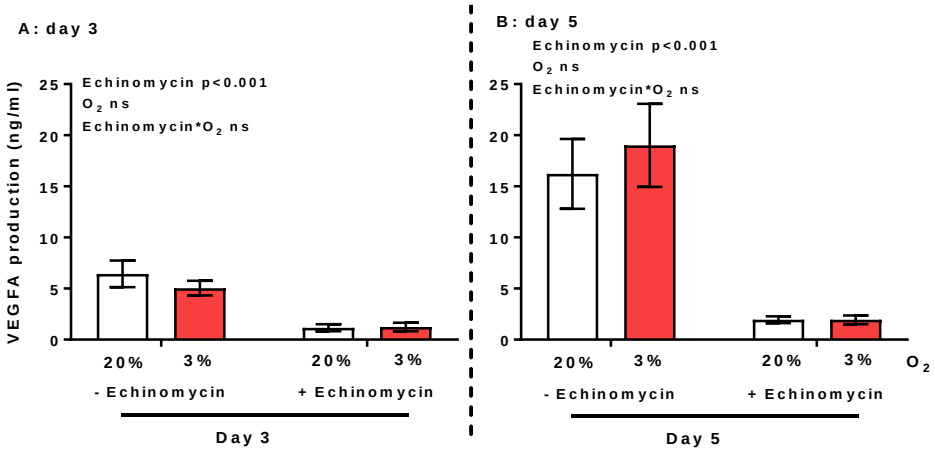



Fig. 5.17: The effect of HIF1A inhibition on VEGFA levels in bovine luteinising follicular cells in 20% and 3% oxygen. The cells were treated with control (-echinomycin) or echinomycin (1 μM). Spent media was collected on day 3 (A) and 5 (B) of culture and were analysed for VEGFA by ELISA. There was a significant effect of time in culture ($p<0.001$) with VEGFA concentration greater on day 5. The effects of echinomycin, oxygen concentration and their interaction are indicated on the graph. The data are mean ± SEM, n=4 cultures.

## 5.3.8. Results: The effect of HIF1A inhibition on cell proliferation in luteinising follicular cells cultured in different oxygen concentrations

There was no effect of oxygen concentration on the proliferation of bovine luteinising follicular cells on day 3 of culture. This was in agreement with the observed similar levels of confluence at the end of culture in 3% and 20% oxygen. However, cell proliferation was decreased in echinomycin treatment by 20% ($p<0.001$; Fig. 5.18). The effect of echinomycin was similar in 3% and 20% oxygen. There was no interaction between echinomycin × oxygen ($p>0.05$; Fig. 5.18).

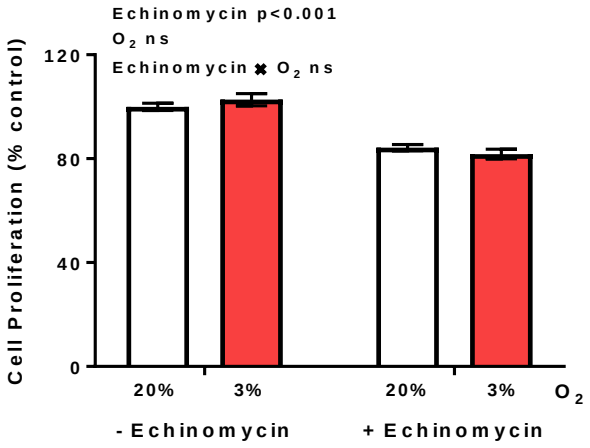



Fig. 5.18: The effect of HIF1A inhibition on cell proliferation in bovine luteinising follicular cells cultured in 20% and 3% oxygen. The cells were treated with control (- echinomycin) or echinomycin (1 μM). Cell proliferation was determined by MTT assay on day 3 of culture. The effect of echinomycin, oxygen concentration and their interaction is indicated on the graph. The data are mean ± SEM, n=5 cultures.

## 5.3.9. Results: The effect of HIF1A inhibition on lactate levels

There was a significant effect of time in culture on lactate concentrations ($p<0.001$) with the lactate levels across all treatments approximately 50% greater on day 5 than day 3 (Fig. 5.19A and B). On day 3, lactate concentration was decreased by echinomycin treatment ($p<0.001$; Fig. 5.19A). There was also an echinomycin × oxygen interaction ($p<0.01$) which showed that in 20% oxygen, echinomycin treatment decreased lactate production, but echinomycin had no effect in 3% oxygen (Fig.5.19A). On day 5 of culture, there were no effects of echinomycin, oxygen concentration or an interaction ($p>0.05$; Fig. 5.19B).

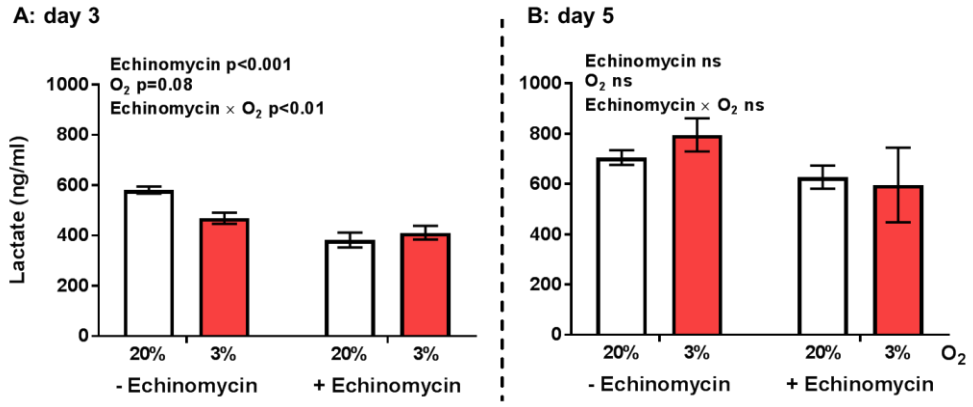



Fig. 5.19: The effect of HIF1A inhibition on lactate levels in bovine luteinising follicular cells in 20% and 3% oxygen. The cells were treated with control (-echinomycin) or echinomycin (1 μM). Spent media was collected on day 3 (A) and 5 (B) of culture and analysed for lactate using an enzymatic assay. There was a significant effect of time in culture ($p<0.001$) with lactate concentrations greater on day 5. The effects of echinomycin, oxygen concentration and their interaction are indicated on the graph. The data are mean ± SEM, n=3 cultures.

## 5.4. Study 3: Effect of PKA inhibition on endothelial cell network formation and progesterone production in bovine luteinising follicular cells.

There is evidence that gonadotrophins increase HIF1A activity in follicular cells by increasing intracellular cAMP concentrations and activating protein kinase A (PKA) signalling (Jiang et al., 2011a, Yalu et al., 2015, Kowalewski et al., 2015). Thus, this experiment investigated the effects of inhibiting PKA on HIF1A responsive genes and the potential subsequent effects on EC network formation and progesterone production.

The hypothesis was that PKA inhibition would decrease HIF1A protein and thereby decrease the formation of EC network.

The specific objectives were;

a) To evaluate the EC network formation using von Willebrand factor immunohistochemistry and image analysis.
b) To determine the progesterone production using ELISA and the expression of steroidogenic proteins by western blotting.
c) To evaluate the expression of HIF1A-responsive proteins using western blotting.
d) To assess the cell growth using a MTT cell proliferation assay.

### 5.4.1. Experimental design

#### 5.4.1.1. EC network formation and progesterone production

Bovine luteinising follicular cells were cultured in high (20%) or low (3%) oxygen concentration (n=3 cultures) in the absence or presence of the PKA inhibitor, H89 (10 μM; Calbiochem) throughout the culture. H89 is a selective, competitive and potent inhibitor of protein kinase A that was used at 10 μM in alignment with previous studies (Sakumoto et al., 2000, Chin and Abayasekara, 2004). H89 was initially dissolved in DMSO with the final concentration of DMSO being 0.1%. Thus, 0.1% (v/v) DMSO was added to the control wells. The cells were cultured for 5 days with the spent media collected on day 3 and 5 of culture for progesterone analysis. At the end of culture (day 5), the cells were fixed with ice-cold acetone:methanol and then

VWF immunocytochemistry was performed. The degree of EC network formation was determined by image analysis.

#### 5.4.1.2. Protein extraction and western blotting

In an additional 4 cultures, the cells were treated in the same manner as described in Section 5.4.1. On day 5 of culture, the cells were scraped off and processed for protein extraction and western blot analysis. The target proteins were 1) the HIF1A-responsive protein BNIP3; 2) proteins associated with progesterone synthesis (STAR, CYP11A1, HSD3B1); and 3) mural cell marker, ACTA2.

#### 5.4.1.3. Cell proliferation

Bovine luteinising follicular cells (n=5 cultures) were cultured on fibronectin coated 96-well plates under high (20%) or low (3%) oxygen concentrations. The cells were then treated with either control (DMSO) or H89 (10 μM, PKA inhibitor) for 3 days. Each treatment was performed in 6 replicates. At the end of culture, the number of viable cells were determined using a MTT cell proliferation assay (Section 2.3.5).

### 5.4.2. Statistical analysis

For *EC network analysis*, the endpoints from the image analysis (e.g. total EC area, number of branch points) were expressed as a percentage of the control (no H89, 20% oxygen) for that particular culture. Significance was determined using a two-way randomised block ANOVA with data blocked by culture. The factors were oxygen (20% vs 3%) and H89 (absence vs presence).

For the *progesterone analysis*, the data did not require transformation as the untransformed values were normally distributed and exhibited homogeneity of variance. Initially, a three-way randomised block ANOVA was performed including day of culture as one of the factors. However, since there was a large effect of day, the data was then analysed on each day of culture separately.

For the *western blot analysis,* the band intensity was quantified using ImageJ and then normalised to the expression of ACTB or HIST3H3. The data was then analysed by a two-way randomised block ANOVA as described above.

For *cell proliferation analysis*, the absorbance was expressed as % control (no H89, 20% oxygen) and then analysed by two-way randomised block ANOVA.

### 5.4.3. Results: The effect of PKA inhibition on endothelial cell network formation

In all conditions and treatments, organised and intricate EC networks were formed (Fig. 5.20A-D). These ranged in size from a few cells to large, highly branched structures that appeared to have merged with other networks of endothelial cells. The larger EC networks were elongated and tubule-like in appearance. Additionally, the pattern of VWF staining appeared similar in both in the absence (Fig. 5.20A-B) and presence (Fig. 5.20C-D) of H89 treatment. The degree of EC network formation appeared to be more similar in both 20% and 3% oxygen than previous experiments (Fig. 5.20A-D). There was a similar level of cell confluence and coverage across the whole well with large areas of non-EC cells indicating the growth of other cell types including steroidogenic cells.

Total area of EC networks was unaffected by both H89 treatment ($p>0.05$) and oxygen concentration ($p>0.05$; Fig. 5.21A) however, there was a significant H89 × oxygen concentration interaction ($p<0.05$; Fig. 5.21A). This showed in the absence of H89, total area of EC networks was decreased by ~35% when cells were cultured in 3% oxygen. In contrast, in the presence of H89, the total area of EC networks were similar in 3% and 20% oxygen (Fig. 5.21A).

The number and perimeter of EC networks were unaffected by H89 treatment ($p>0.05$) and oxygen concentration ($p>0.05$) and furthermore, there was no H89 × oxygen concentration interaction ($p>0.05$; Fig. 5.21B-C). There was a trend for the mean area of each EC network to be reduced by H89 treatment ($p=0.06$; Fig. 5.21D) but this endpoint was unaffected by oxygen concentration. H89 treatment reduced both the total number of branch points ($p<0.05$; Fig. 5.21E) and degree of branching within an EC network ($p<0.01$; Fig. 5.21F). However, oxygen concentration had no effect on number of branch points and degree of branching (Fig.5.21E-F).

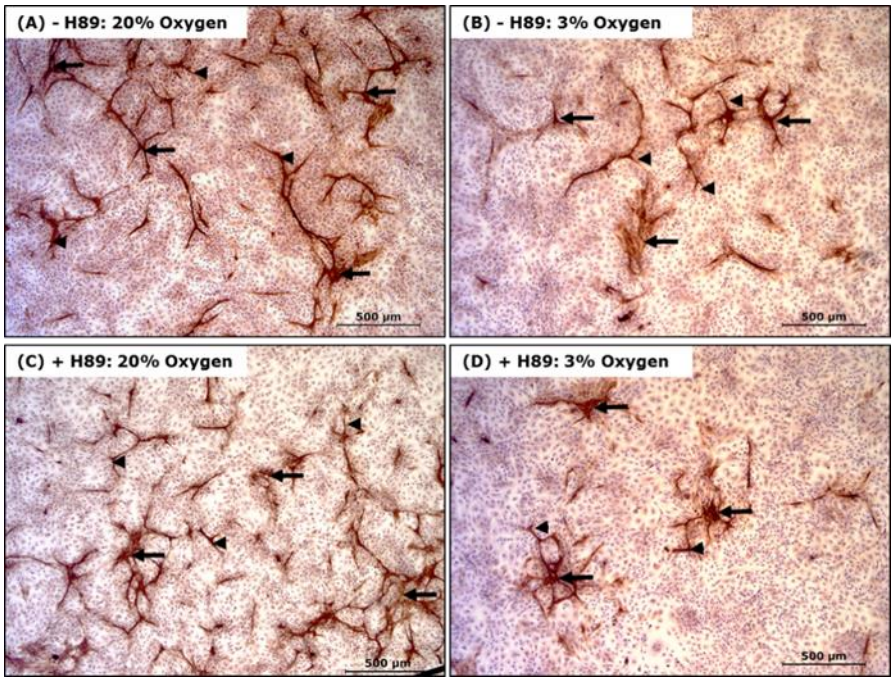



Fig. 5.20: The effect of PKA inhibition on EC network formation in bovine luteinising follicular cells cultured in different oxygen (20% vs 3%) concentrations. EC networks were identified by von Willebrand factor immunostaining and are shown by brown staining (arrows) with elongated branches shown by arrowheads. Representative images are shown for cells cultured in 20% oxygen (A,C) or 3% oxygen (B,D) and treated with either control (A,B) or H89 (10 µM, PKA inhibitor, C,D). The EC networks were elongated and tubule-like in appearance, with some complex multiple branching points extending from the EC networks. Scale bars represent 500 µm, n=3 cultures.

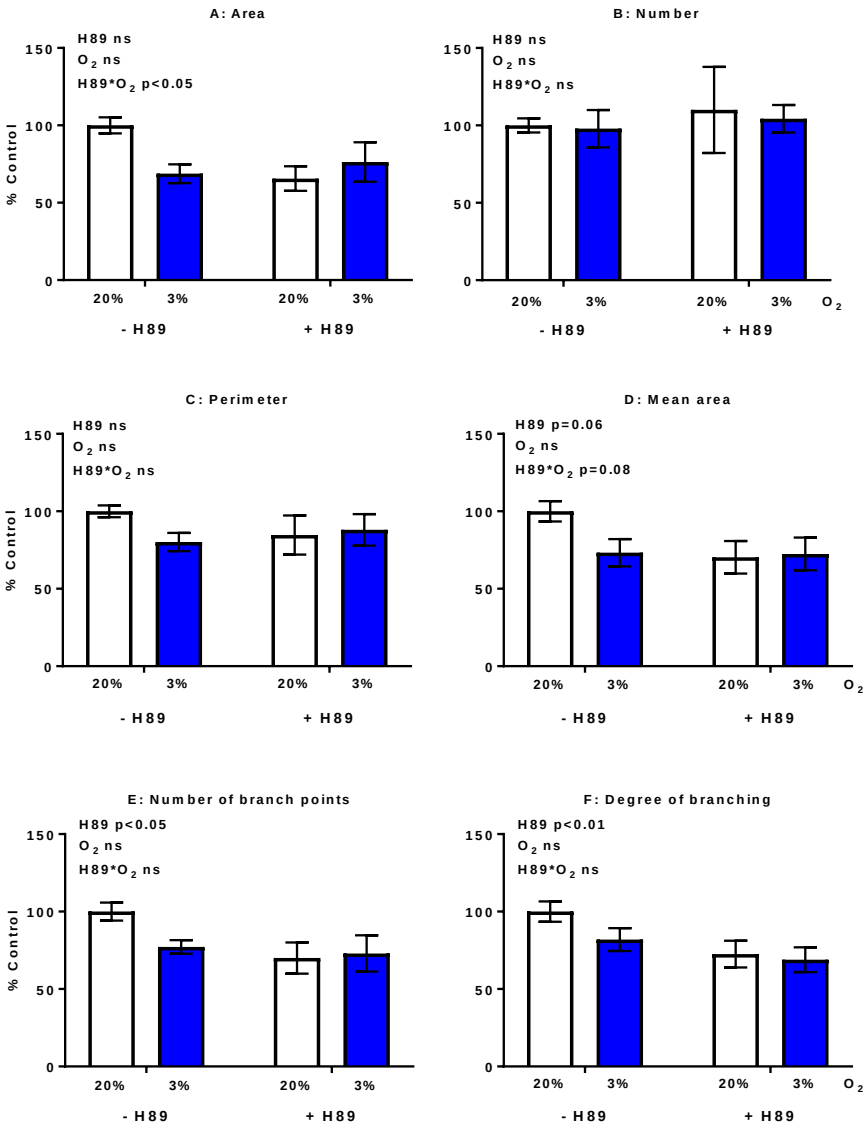



Fig. 5.21: The effect of PKA inhibition on the quantification of EC network formation in bovine luteinising follicular cells cultured in different oxygen (20% vs 3%) concentrations. The cells were treated with either control or H89 (10 μM, PKA inhibitor). The endpoints were (A) total area of EC networks, (B) number of EC networks, (C) total perimeter of EC networks, (D) mean area of each EC network, (E) total number of branch points and (F) degree of branching within an EC network. The statistical effects of oxygen concentration, H89 and their interaction are indicated on each graph. The data are mean ± SEM, n=3 cultures.

### 5.4.4. Results: The effect of PKA inhibition on progesterone production

There was a significant effect of time in culture on progesterone production ($p<0.001$) with progesterone concentrations being 3.1-fold greater on day 5 than 3 (Fig. 5.22). Consequently, progesterone production was analysed separately on day 3 and 5 of culture. Progesterone production was unaffected by H89 treatment ($p>0.05$) on both day 3 and 5 of culture (Fig. 5.22). On day 3 of culture, while progesterone concentrations were numerically lower in 3% oxygen this difference was not significant ($p>0.05$) partly due to the varied effect (Fig. 5.22A). On day 5 of culture, 3% oxygen decreased progesterone production ($p<0.05$). More importantly, there was a H89 × oxygen concentration interaction ($p<0.05$) in which progesterone production was reduced by H89 treatment in 20% oxygen but had no effect in 3% oxygen (Fig. 5.22B).

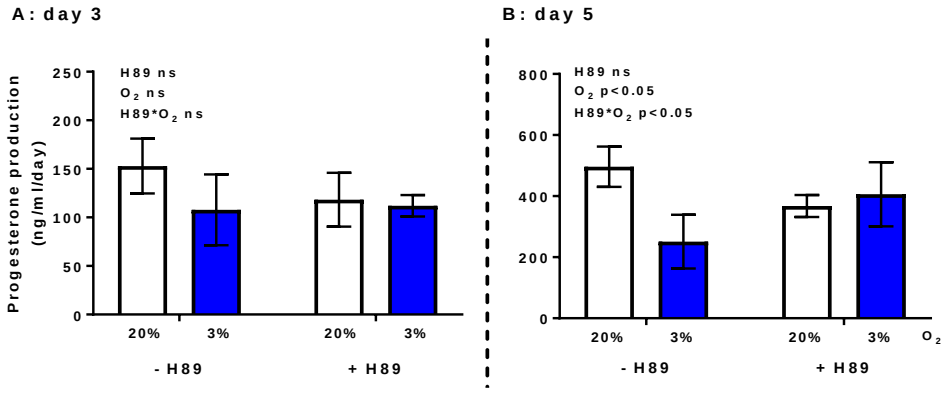



Fig. 5.22: The effect of PKA inhibition on progesterone production in bovine luteinising follicular cells cultured in different oxygen (20% vs 3%) concentrations. The cells were treated with either control (DMSO) or with the PKA inhibitor, H89 (10 μM). The production of progesterone increased from (A) day 3 to (B) day 5 of culture ($p<0.001$). Progesterone production ($p>0.05$) was unaffected by H89 treatment and oxygen concentration on day 3 of culture. On day 5, there was a H89 × oxygen concentration interaction ($p<0.05$) in which progesterone production was decreased in 3% oxygen in the absence but not in the presence of H89. The data are mean ± SEM, n=3 cultures.

### 5.4.5. Results: The effect of PKA inhibition on the expression of steroidogenic proteins

In bovine luteinising follicular cells, STAR protein was detected as a double band (25 and 32 kDa; Fig. 5.23A). Quantification of STAR expression showed that H89 treatment had no effect on the 25 kDa or 32 kDa STAR protein expression ($p>0.05$, Fig. 5.23B-C). However, low oxygen concentration decreased the level of STAR protein (25 kDa and 32 kDa; $p<0.05$: Fig. 5.23B-C). There was no interaction between H89 treatment and oxygen concentration on STAR protein ($p>0.05$: Fig. 5.23B-C).

CYP11A1 (P450scc) protein was detected as a single band at its expected molecular weight of 60 kDa in the bovine luteinising follicular cells (Fig.5.24A). The quantification of CYP11A1 protein expression revealed that it was unaffected by H89 treatment ($p>0.05$), oxygen concentration ($p>0.05$) and that there was no H89 × oxygen concentration interaction ($p>0.05$; Fig. 5.24B).

Western blot for 3β-hydroxysteroid dehydrogenase (HSD3B1) showed a single band at 44 kDa (Fig.5.25A). HSD3B1 protein was unaffected by H89 treatment and oxygen concentration ($p>0.05$; Fig.5.25B). There was no interaction ($p>0.05$) between H89 treatment and oxygen concentration (Fig. 5.25B).

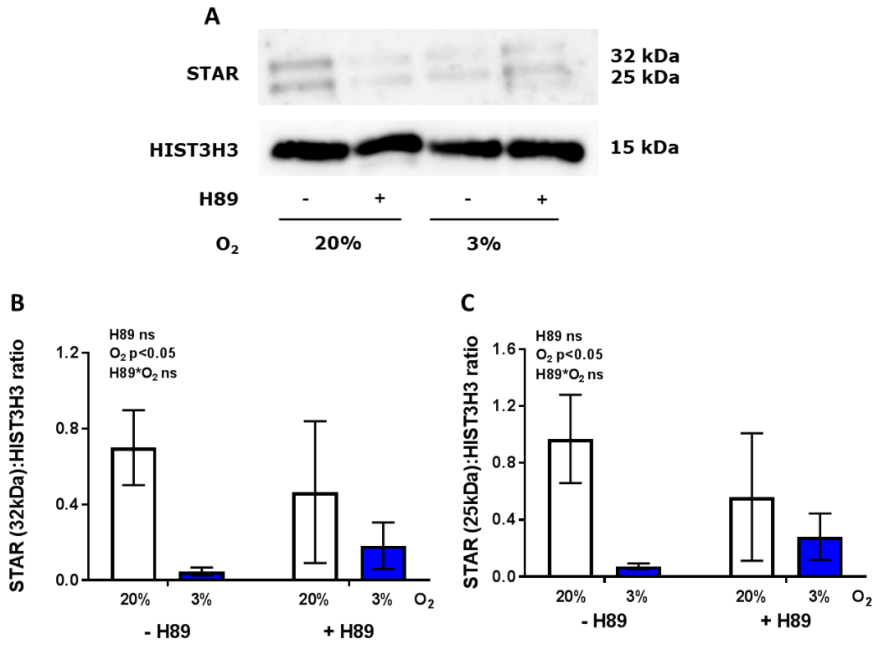



Fig. 5.23: The effect of PKA inhibition on steroidogenic acute regulatory protein (STAR) protein expression in bovine luteinising follicular cells cultured in different oxygen (20% vs 3%) concentrations. The cells were treated with control (DMSO) or a PKA inhibitor (H89, 10 μM). (A) shows representative western blot with a double band for STAR (25 and 32 kDa) and a single band for histone 3 H3 (HIST3H3, 15 kDa) which was consistently expressed across the different samples. The quantification of (B) 25 kDa STAR and (C) 32 kDa STAR protein expression normalised to HIST3H3 are shown. The effects of H89, oxygen concentration and their interaction are indicated on the graph. The data are mean ± SEM, n=4 cultures.

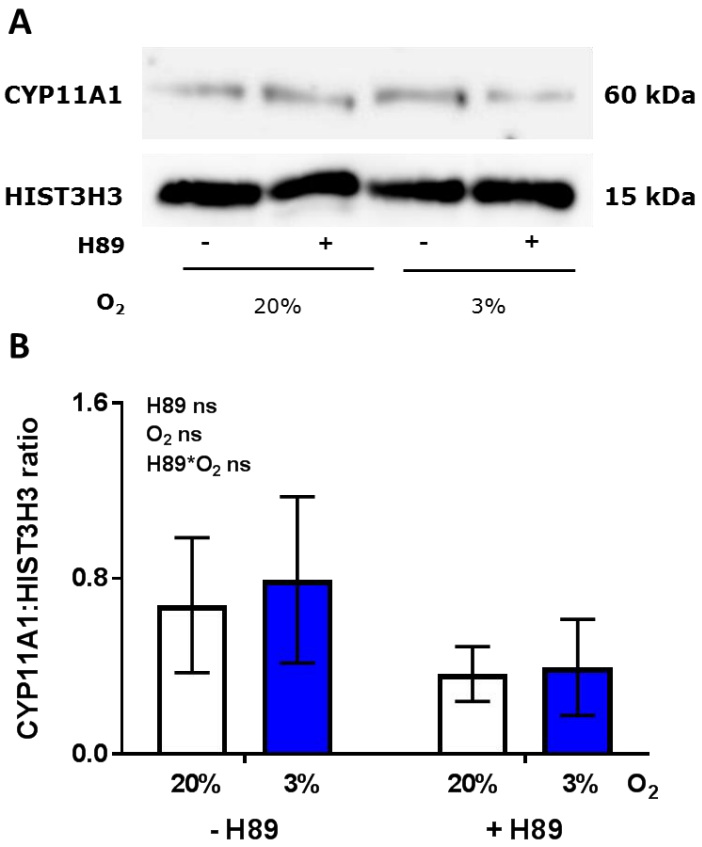



Fig. 5.24: The effect of PKA inhibition on CYP11A1 (P450 cholesterol side-chain cleavage) protein expression in bovine luteinising follicular cells cultured in different oxygen (20% vs 3%) concentrations. The cells were treated with control (DMSO) or a PKA inhibitor (H89, 10 μM). (A) shows a representative western blot with a single band for CYP11A1 (60 kDa) and histone 3 H3 (HIST3H3, 15 kDa). The housekeeping protein, HIST3H3 was consistently expressed across the different samples. (B) shows the quantification of CYP11A1 protein expression normalised to HIST3H3. The effects of H89, oxygen concentration and their interaction are indicated on the graph. The data are mean ± SEM, n=4 cultures.

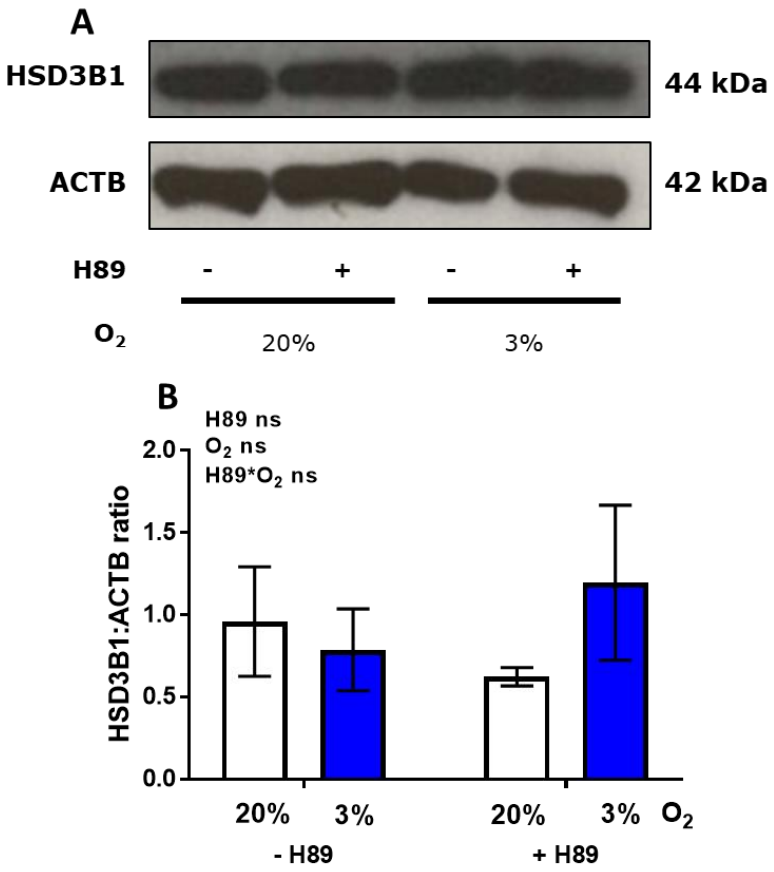



Fig. 5.25: The effect of PKA inhibition on 3β-hydroxysteroid dehydrogenase (HSD3B1) protein expression in bovine luteinising follicular cells cultured in different oxygen (20% vs 3%) concentration. The cells were treated with control (DMSO) or a PKA inhibitor (H89, 10μM). (A) shows a representative western blot with a single band for HSD3B1 (44 kDa) and ACTB (42 kDa). The housekeeping protein, ACTB was consistently expressed across the different samples. (B) shows the quantification of HSD3B1 protein expression normalised to ACTB. The effects of H89, oxygen concentration and their interaction are indicated on the graph. The data are mean ± SEM, n=4 cultures.

## 5.4.6. Results: The effect of PKA inhibition on the expression of the HIF-regulated protein BNIP3

The HIF1-responsive protein, BNIP3 was expressed as a single band at 25 kDa in bovine luteinising follicular cells (Fig.5.26A). Quantification of BNIP3 protein (normalised to ACTB) showed that H89 treatment had no effect on BNIP3 expression ($p>0.05$, Fig. 5.26B), but BNIP3 expression was increased in cells cultured in low (3%) oxygen ($p<0.05$, Fig. 5.26B). There was no interaction between H89 treatment and oxygen concentration on the expression of BNIP3 protein ($p>0.05$, Fig. 5.26B).

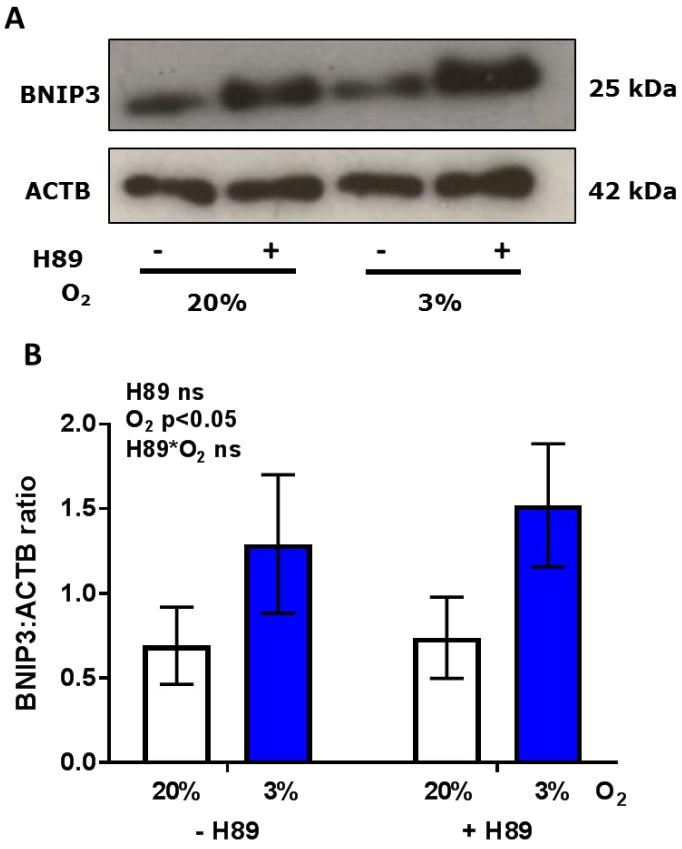



Fig. 5.26: The effect of PKA inhibition on BNIP3 protein expression in bovine luteinising follicular cells cultured in different oxygen (20% vs 3%) concentrations. The cells were treated with control (DMSO) or a PKA inhibitor (H89, 10 μM). (A) shows a representative western blot with a single band for BNIP3 (25 kDa) and ACTB (42 kDa). The housekeeping protein, ACTB was consistently expressed across the different samples. (B) shows the quantification of BNIP3 protein expression normalised to ACTB. The effects of H89, oxygen concentration and their interaction are indicated on the graph. The data are mean ± SEM, n=4 cultures.

## 5.4.7. Results: The effect of PKA inhibition on ACTA2 protein expression

In bovine luteinising follicular cells, ACTA2 protein was expressed as a single band at 42 kDa (Fig.5.27A). Quantification of the ACTA2 protein expression showed that H89 treatment had no effect on ACTA2 expression ($p>0.05$; Fig. 5.27B). There was a trend for ACTA2 protein expression to be increased in low oxygen but this was not significant ($p=0.06$, Fig. 5.27B). There was no interaction between H89 treatment and oxygen concentration on ACTA2 protein expression ($p>0.05$: Fig. 5.27B).

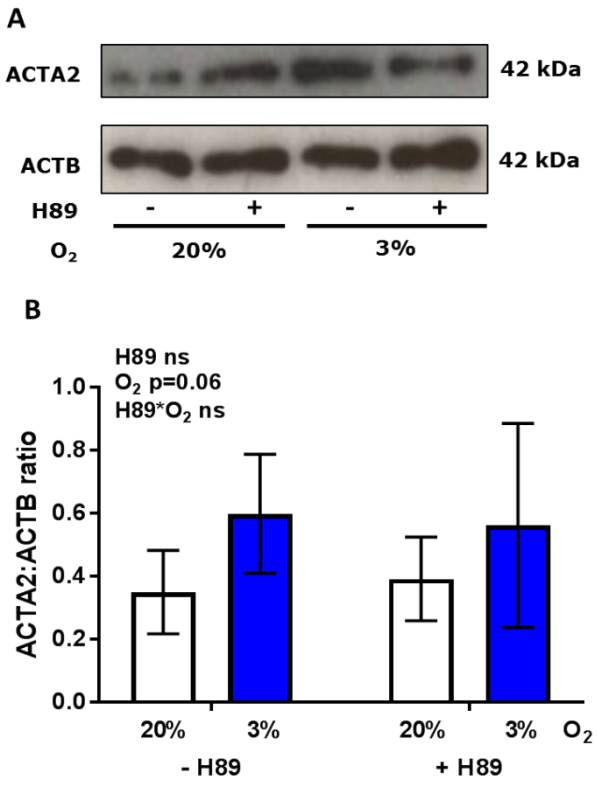



Fig. 5.27: The effect of PKA inhibition on smooth muscle actin (ACTA2) protein expression in bovine luteinising follicular cells cultured in different oxygen (20% vs 3%) concentrations. The cells were treated with control (DMSO) or a PKA inhibitor (H89, 10 μM). (A) shows a representative western blot with a single band for ACTA2 (42 kDa) and ACTB (42 kDa). The housekeeping protein, ACTB was consistently expressed across the different samples. (B) shows the quantification of ACTA2 protein expression normalised to ACTB. The effects of H89, oxygen concentration and their interaction are indicated on the graph. The data are mean ± SEM, n=4 cultures.

## 5.4.8. Results: The effect of PKA inhibition on cell proliferation

Cell proliferation was increased by H89 treatment by approximately 10% in both 20% and 3% oxygen ($p<0.01$; Fig. 5.28). In this experiment, there was no effect of oxygen concentration on cell proliferation and there was no H89 × oxygen interaction ($p>0.05$; Fig. 5.28).

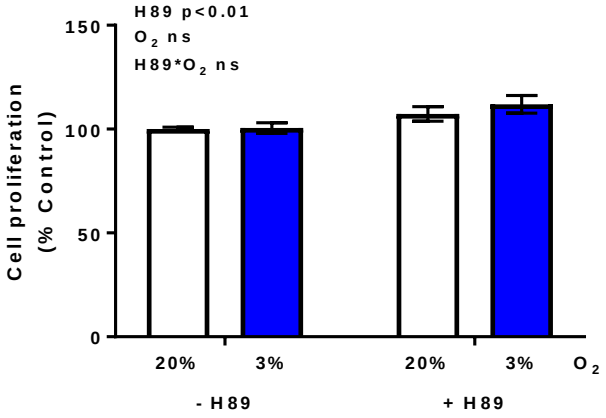



Fig. 5.28: The effect of PKA inhibition on cell proliferation of bovine luteinising follicular cells in different oxygen (20% vs 3%) concentrations. The cells were treated with control (DMSO) or a PKA inhibitor (H89, 10 μM). Cell proliferation was determined by MTT assay and the data normalised to the control for that culture (20%, no H89). H89 increased cell proliferation ($p<0.01$) while oxygen had no effect. The data are mean ± SEM, n=4 cultures.

## 5.5. Discussion

Oxygen is vital to maintaining tissue and cellular metabolic homeostasis. An extensive, organised vasculature is required for the delivery of oxygenated blood to the tissue, especially in those tissues that are highly metabolically active such as the CL. Thus, this Chapter evaluated the effect of low oxygen concentrations on the formation of EC networks and progesterone production and the underlying mechanisms in bovine luteinising follicular cells.

For the first time, this Chapter showed that in bovine luteinising follicular cells, the total area, perimeter, mean area of the EC networks, number of branch points were consistently reduced by low (3%) oxygen. In addition, progesterone production by luteinising follicular cells was decreased by up to 55% when cultured in 3% oxygen. In the second study, the HIF1A inhibitor, echinomycin profoundly inhibited the formation of EC networks and this effect was observed in both 3% and 20% oxygen. This would indicate that HIF1A signalling was central to the promotion of EC network formation irrespective of oxygen concentration. In addition, the expression of the HIF1A-induced proteins, VEGFA and BNIP3 were decreased by echinomycin but were unaffected by oxygen concentration. Echinomycin also decreased progesterone production in both 20% and 3% oxygen, which in part might be due to reduced expression of HSD3B1. In the final experiment, there was a PKA inhibitor (H89) by oxygen concentration interaction on the total area of EC networks. This indicated that the suppressive effect of low oxygen on follicular angiogenesis *in vitro* was blocked by inhibition of PKA signalling. A similar H89 by oxygen interaction was observed on progesterone production, although the expression of the key steroidogenic proteins was unaffected.

*Performance of the culture system*

Importantly, in the initial study, the luteinising follicular cells responded to angiogenic stimulation by increasing the total area of EC network formation. This result agrees with previous findings utilising the same culture system (Laird et al., 2013), and corroborates observations from an analogous luteal angiogenesis culture system where FGF2+VEGFA (1 ng/ml) stimulated endothelial cell network development (Robinson et al., 2008). Additionally, in the present study, the adverse effect of low oxygen concentration on EC network formation was observed in both basal and angiogenic-stimulated

conditions. Furthermore, progesterone production increased with time indicating that the steroidogenic cells were luteinising and functioning as expected. Collectively, these results provide evidence that the culture system was operating in a physiologically relevant manner.

*Low oxygen and its adverse effect on endothelial cell networks*

The underlying hypothesis for this chapter was that low oxygen concentrations would up-regulate HIF1A and subsequently stimulate the angiogenic processes. However, contrary to expectations, luteinising follicular cells under low oxygen had reduced formation of organised EC networks. This occurred in the presence or absence of angiogenic-stimulation. Furthermore, with only a few exceptions all quantified EC network endpoints were adversely affected. The reason for this outcome is unclear and there are no reports describing low oxygen concentrations reducing angiogenesis *in vitro* or *in vivo*. One explanation is that overall cell activity and proliferation were reduced by the long-term culture of the cells in low oxygen leading to reduced capability to produce ATP. However, this is unlikely since the cell proliferation as determined by MTT assay was similar in 20% and 3% oxygen. Furthermore, the cell confluence at the end of culture was similar and progesterone production increased over time in both oxygen concentrations.

An alternative explanation is that low oxygen concentrations altered the function/phenotype of mural cells, which in turn had an adverse effect on EC network formation. It has been estimated that during the follicular-luteal transition, pericytes occupy 15% of luteal structure (O'Shea et al., 1989, Christenson and Stouffer, 1997) with pericytes forming a major part of microvessels and assisting with their stabilisation and maturation (Armulik et al., 2005, Laird et al., 2013). However, mural cells/pericytes have a high degree of plasticity in their phenotype, ranging from those that deposit ECM and co-ordinate the migration of EC (activated pericytes) through to those regulating contractile function (vascular smooth muscle) (Amselgruber et al., 1999, Stallcup, 2013). In these different roles, they can have either a stimulatory or an inhibitory effect on EC migration, proliferation and function. In the present culture system, mural cells are often seen in close proximity to the EC networks (Laird et al., 2013). Thus, in this chapter, ACTA2 protein was used to localise and quantify mural cells in 20% and 3% oxygen. The quantification of ACTA2 protein by western blot indicated that

ACTA2 expression levels were unaffected by low oxygen concentration in one study but tended to increase in another study. The housekeeping protein, ACTB was used to normalise protein expression in most experiments. However, where the antibodies (test and control) detected bands of similar molecular weight, the blot was stripped. After stripping, the blot was exposed to ECL to confirm that stripping had been achieved. After which the blot was probed in alternation with rabbit polyclonal and mouse monoclonal antibodies to avoid cross-reactivity and duplication of bands on the membrane.

Immunocytochemical localisation revealed that mural cells were located amongst and near to the EC networks in both oxygen concentrations. However, the pattern of staining appeared to be different, with fewer ACTA2-positive cells present in 3% oxygen but the ACTA2 staining appear to be more intense in those that were present. Vascular smooth muscle cells have been shown to have more intense ACTA2-staining than pericytes (Bergers and Song, 2005, Verbeek et al., 1994, Chasseigneaux et al., 2018). Based on this, it is feasible that the low oxygen has stimulated the differentiation of pericytes towards a vascular smooth muscle cell phenotype. There are few reports investigating the effect of hypoxia on pericytes differentiation but this concept is supported by Schneider et al. (2015) where the cell-to-cell interactions between endothelial cells and pericytes in a co-culture system were affected by hypoxia. Furthermore, hypoxia increased SMA expression and differentiation of pericytes into smooth muscle-like cells (Wang et al., 1999). Clearly, considerable further investigation is required to determine the effects of hypoxia on the mural cells and their subsequent effects on EC network formation.

The reduction in EC network formation in low oxygen could be because HIF1A expression was up-regulated in luteinising follicular cells in 20% oxygen as well as under more hypoxic conditions (i.e. 3% oxygen). In multiple cell systems, it is widely recognised that HIF signalling is a primary driver of angiogenesis acting at least, in part, by increasing VEGFA expression (Tsuzuki et al., 2012). Unfortunately, it was not possible to reliably and consistently determine HIF1A expression in luteinising follicular cells. This is likely to be due to the instability of HIF1A protein (Alig et al., 2015). However, in those cultures in which HIF1A was detected, it was present in both 20% and 3% oxygen at relatively similar levels. This

observation confirmed previous findings in bovine luteal cells (Nishimura and Okuda, 2010, Jiang et al., 2011a). Moreover, the expression of HIF1A responsive proteins (i.e. BNIP3 and VEGFA) was unaffected by oxygen concentrations, while the other HIF-regulated protein that were investigated were either decreased in 3% oxygen (PGK1) or tended to be decreased (GLUT1). Collectively, this would indicate that HIF1A expression and its activity was abundant in 20% oxygen and that it was not further increased in low oxygen conditions. While, this opposes the conventional regulation of HIF1A, there is evidence to explain this observation. Firstly, HIF1A expression was up-regulated by hCG in human luteinising granulosa cells (van den Driesche et al., 2008) and in the present study 5 ng/ml LH was added to the MCDB 131 endothelial cell media. Secondly, low oxygen stimulated EC cord formation in HUVECs, when cells were grown on growth factor-poor matrigel but not when grown on growth-factor rich matrigel (Calvani et al., 2006). Again, while FGF2 and VEGFA were omitted from the supplemented MCDB 131 media, it did contain numerous other growth factors (IGF1, EGF, insulin) and there was an abundance of VEGFA in the spent media from the luteinising follicular cells (5-20 ng/ml VEGFA). Therefore, it is not very surprising that low oxygen did not appear to increase HIF1A activity given the growth factor availability. However, it was important to investigate the role of HIF1A in regulating EC network formation. Thus, the effects of treating cells with echinomycin, a recognised HIF1A inhibitor, on EC network formation were determined.

*HIF signalling and its effect on* endothelial cell *networks*

In the second study, organised EC networks were formed in both 20% and 3% oxygen in the presence or absence of echinomycin treatment. Image analysis showed that echinomycin treatment reduced the total area, number and perimeter of EC networks as well as the total number of branch points by 30-40%. This effect agrees with the reduced vascularisation of the CL in mice treated with echinomycin (Kim et al., 2009). Interestingly, in the present study, there were no echinomycin by oxygen interactions, which indicated that the effect of echinomycin occurred irrespective of oxygen concentration. The vast majority of previous studies show that echinomycin treatment reverses the effect of hypoxia (Fadhillah et al., 2017, Calvani et al., 2006). This reason for this discrepancy is unclear but could be related to the relatively high expression of HIF1A in 20% and 3% oxygen conditions.

Indeed, echinomycin treatment reduced HIF1A protein expression in the 2 cultures where it was detected, as well as decreasing the levels of HIF1A-responsive proteins such as BNIP3 and VEGFA. Moreover, these effects were observed in both oxygen concentrations. The down-regulation of VEGFA protein was particularly marked (75% reduction). The inhibitory action of echinomycin on VEGFA expression has been widely reported (Tsuzuki et al., 2012, Alig et al., 2015). This not only confirms that in the present study VEGFA was a HIF1A-responsive protein in bovine luteinising follicular cells but could also explain the reduced formation of EC networks with echinomycin treatment. Indeed, Laird et al. (2013) speculated that the high endogenous production of VEGFA was the reason for the lack of effect in response to exogenous VEGFA treatment. Thus, it would be interesting to determine if the addition of VEGFA reversed the negative effects of echinomycin on the development of EC networks. An alternative, but related explanation is that overall cell proliferation was 20% lower with echinomycin treatment, which cell type was affected is unknown, however. It is also feasible that echinomycin reduced the proliferation of mural cells since ACTA2 expression was 2-fold reduced by echinomycin. The loss of activated pericytes, which are known to co-ordinate and promote the angiogenic process, would also explain the reduced EC network formation.

An important observation from this study is that, in the presence of echinomycin, the total area, number and perimeter of EC networks were reduced to a greater extent when cells were cultured in 3% oxygen. The original assumption was that low oxygen concentrations would increase HIF1A activity and this would be the mechanism by which low oxygen exerted its effects. However, if EC network formation was still decreased by low oxygen when HIF1A was blocked, then the low oxygen must at least, in part, be exerting its action in a HIF1A-independent manner.

There are some reports of hypoxia acting through non-HIF-dependent pathways. As already mentioned, oxygen deprivation has profound effects on cell metabolism, and hence the cell's response is to activate HIF to mitigate these adverse effects. Chronic hypoxia will though ultimately lead to energy starvation and the stimulation of the adenosine monophosphate activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) pathways. These are the principle HIF-independent pathways (Simon et al., 2008). The activation of AMPK and mTOR pathways will further

increase if hypoxia continues (Liu et al., 2006). AMPK will then phosphorylate and inhibit eukaryotic initiation factor 2 (eIF2), a protein critically important for the translation of mRNA. The stimulation of mTOR also down-regulates protein synthesis through multiple mechanisms including regulating the availability of cellular eIF4E (Simon et al., 2008). That said, the current literature suggests that inhibition of protein synthesis is not a global cellular event and that some proteins (typically pro-angiogenic factors such as VEGFA and FGF2) are preferentially translated (Blais et al., 2006). The exact effect the activation of these pathways has cell type-dependent, with cell growth unaffected in some cell types (Liu et al., 2006). For example, embryonic stem cells have enhanced proliferation rates in hypoxic conditions (Simon et al., 2008).

AMPK plays a central role in regulating a cell's energy homeostasis, largely by activating glucose uptake and fatty acid oxidation. This combined with inhibition of protein synthesis will hopefully restore the cell's energy status back to normal (Lee et al., 2015). Contrary to expectations, lactate production (an indicator of anaerobic respiration) was unaffected by low oxygen. An important next step would be to measure the cellular levels of AMP, ADP and ATP under hypoxic conditions to clarify if the luteinising follicular cells were energy depleted. If the cell's energy status was unaffected, then AMPK might be acting in an alternative manner. Over recent years, there has been increasing interest in use of AMPK inhibitors (e.g. compound C) or activators (e.g. metformin) to regulate angiogenesis for various diseases (Gao et al., 2018). Intriguingly, AMPK inhibitors increased VEGFA production, and *in vitro* growth of pre-antral and antral follicle that was associated with enhanced angiogenesis (Lu et al., 2017). Metformin decreased ovarian endothelial cell area in a rat polycystic ovarian syndrome model (Di Pietro et al., 2015). There are though reports of opposing effects where metformin promoted proliferation and migration of HUVECs (Esfahanian et al., 2012). This indicates that the role of AMPK in regulating angiogenesis is complex and that is likely to be dose and culture system dependent. Equally, it would be interesting to determine the effects of differing doses of AMPK activator/inhibitor treatments on EC network formation in our physiologically relevant culture system.

*The reduction of progesterone production in low oxygen*

The production of progesterone was decreased when cells were cultured in 3% oxygen. This pattern was generally consistent across three different studies, although it was not always significant on both days of culture. Similarly, bovine follicular cells that were allowed to luteinise had reduced steroidogenic output when cultured under low oxygen conditions (Fadhillah et al., 2017, Baddela et al., 2018). Also, hypoxic conditions reduced progesterone production in rat (Koos and Feiertag, 1989), pig (Basini et al., 2004) and bovine luteal cells (Nishimura et al., 2006, Jiang et al., 2011a).

Overall, there was no effect of low oxygen concentration on cell proliferation, thus the decreased progesterone production is less likely to be due to fewer steroidogenic cells (assuming all cell types proliferate at similar rates in both oxygen concentrations). To explore the underlying mechanism behind the reduction of progesterone production by low oxygen further, western blotting was employed to assess the expression of key steroidogenic protein.

STAR is the key to rate-limiting step of steroidogenesis and regulates the transport of cholesterol to the inner mitochondria membrane and its expression is profoundly increased during luteinisation (Bao and Garverick, 1998, LaVoie et al., 2004). Thus progesterone production is likely to be sensitive to changes in the expression of STAR. Western blotting revealed two bands for STAR at 25 kDa and 32 kDa. This has been reported previously in bovine luteal cells (Rekawiecki et al., 2005, Jiang et al., 2011a) and it is likely that the smaller STAR isoform represents the processed, active form. In the current study, 3% oxygen generally down-regulated protein expression of both STAR isoforms and this would at least in part explain the decreased progesterone production. This is supported by observations that 1% oxygen decreased STAR protein expression and progesterone production. However, these results disagree with previous findings where chemical-induced hypoxia (with cobalt chloride) increased progesterone production and STAR expression (Fadhillah et al., 2017). While in comparison to 20% oxygen, STAR protein was increased in 10% but not 5% oxygen (Fadhillah et al., 2014). Collectively, these results suggest that the exact effect of low oxygen on progesterone production might be influenced by the degree of hypoxia. In contrast, expression of CYP11A1 and HSD3B1 proteins was unaffected by 3% oxygen concentration. A similar lack of effect of oxygen concentration on CYP11A1 and HSD3B1 expression has been previously reported (Fadhillah et al., 2014, Fadhillah et al., 2017).

An alternative explanation is that low oxygen concentrations decreased PGK1 and tended to decrease GLUT1 expression that would lead to reduced glucose utilisation and potentially glycolysis. Furthermore, lactate production tended to be reduced in low oxygen concentrations on day 3 of culture indicating possible decreased production of pyruvate/glycolysis. These effects were contrary to expectations as hypoxia is more typically associated with increased capability to generate ATP via glycolysis. That said, since progesterone production is an energy consuming process, if glycolysis was reduced then this could affect progesterone production. Equally, low oxygen concentrations had no effect on cell proliferation, which would indicate the energy status of the cells was not that limiting.

*Inhibition of HIF signalling and progesterone production*

In the second study, it was clear that echinomycin treatment decreased progesterone production by luteinising follicular cells on day 3 and 5 of culture. Though, it should be noted that in echinomycin-treated cells progesterone production still increased over time. Similarly, echinomycin reduced progesterone production in chemically induced hypoxic bovine luteal cells (Fadhillah et al., 2017). *In vivo*, echinomycin treatment also reduced plasma progesterone concentrations in pseudo pregnant rats but this could have been an indirect effect of decreasing luteal VEGFA levels (Pan et al., 2015). To further investigate the reduction of progesterone production by echinomycin, western blotting was employed to assess the expression of key steroidogenic proteins. In contrast to previous findings, echinomycin had no effect on STAR and CYP11A1 protein expression (Fadhillah et al., 2017). However, there was an oxygen concentration by echinomycin interaction on HSD3B1 protein levels. This indicated that in the presence of echinomycin, HSD3B1 protein was reduced by low oxygen concentration. This suggests that HIF1A up-regulates HSD3B1 expression while low oxygen concentrations down-regulate HSD31B expression in a HIF1A-independent manner. The mechanism by which this occurs is unknown.

*PKA inhibition: effect on* endothelial cell *network formation and progesterone production*

In the final study, the effects of PKA inhibition in different oxygen concentrations were investigated. This was based on reports that gonadotrophins increased HIF1A activity in follicular cells by increasing

intracellular cAMP concentrations and activating protein kinase A (PKA) signalling (Jiang et al., 2011a, Yalu et al., 2015, Kowalewski et al., 2015). The results did not fully agree with the hypothesis that PKA inhibition (with H89) would decrease the formation of EC networks by down-regulating HIF1A protein expression. Firstly, H89 treatment had no effect on the abundantly expressed BNIP3 protein. In this study, though, BNIP3 protein expression was increased by low oxygen. Secondly, there was no overall effect of H89 treatment on the total area of EC networks but there was a H89 by oxygen interaction on this endpoint only. This interaction indicated that H89 reduced the total EC network area but only when cells were cultured in 20% oxygen. The negative effect of low oxygen was reversed when the cells were treated with H89. This suggests that the suppressive effect of low oxygen was mediated at least in part by PKA signalling. The limited evidence available, supports that inhibition of PKA signalling has a negative effect on angiogenesis. Namely, H89 decreased VEGFA expression in rat brain endothelial cells (Yang et al., 2013) and human cardiac myocytes (Weiss et al., 2004). Moreover, prostaglandin E2-induced tubule formation by rat luteal endothelial cells was suppressed by H89 treatment (Sakurai et al., 2011). Thus, PKA signalling might be important for EC network formation in 20% oxygen but was not so in 3% oxygen. It is tempting to speculate that PKA signalling is involved in HIF1A up-regulation in 20% oxygen.

Alternatively, H89 treatment could be acting by suppressing the production of stanniocalcin 1 (STC1), a potent stimulator of angiogenesis (Basini et al., 2009, Law and Wong, 2013). STC1 was more abundantly expressed in the ovary than any other tissue (Varghese et al., 1998). Furthermore, STC1 expression in rat thecal cells was promoted by cAMP and inhibited by H89 (Paciga et al., 2002), while in swine granulosa cells hypoxia induced STC1 expression (Basini et al., 2010). Intriguingly, H89 treatment decreased the number of branch points and degree of branching. Similarly, STC1 particularly increased the sprouting of HUVECs (Law and Wong, 2013). The determination of STC1 expression in the different oxygen concentrations alongside the effects of PKA inhibition or stimulation certainly warrants further investigation.

Progesterone production was also influenced by H89 and oxygen concentration. Namely, that while there was no effect on day 3 of culture, H89 reduced progesterone production in luteinising follicular cells cultured

in 20% oxygen but not in 3% oxygen. This effect was independent of altered STAR, HSD3B1 and CYP11A1 expression. This is in agreement with Jiang et al. (2011a) who reported that hypoxia downregulated steroidogenesis through PKA signalling.

Limitation of the present Chapter was that HIFIA measurements using western blots were not totally consistent. It is presumed that this is related to the instability of HIF1A and its rapid degradation. This meant that it was not possible to confirm the activation of HIF1A under low oxygen. This meant that a proxy / HIF1A-responsive proteins were employed instead. While BNIP3 produced an intense band, the bands for GLUT1 and PGK1 were faint and not at their predicted molecular weight.

A second limitation is the level of oxygen selected for the study. The exact concentration of oxygen in follicular fluid are unclear with reports ranging from 1–7% (Van Blerkom et al., 1997, e Paula et al., 2008, Redding et al., 2008). This means that the 20% oxygen that was used as a "control" was clearly supra-physiological and thus not a true biological control. However, the vast majority of culture studies are conducted at 20% oxygen and thus it was decided to use this concentration as it would provide reference to previous studies including those conducted within the group (Woad et al., 2012, Laird et al., 2013). Thus, the study would have benefitted from incorporating a range of oxygen concentrations such as 1%, 3% and 6%. In the present study, 3% oxygen was selected because at the lower end of predicted range. At the same time, it would be more realistic if short windows such as 24 hours of low oxygen were investigated as these would represent the transient hypoxia after ovulation.

In conclusion, this study has refuted the hypothesis that low oxygen concentrations would increase EC network formation in luteinising follicular cells. Instead, follicular angiogenesis was suppressed in low oxygen. While cell proliferation was unaffected by low oxygen concentrations, progesterone production was decreased. It is also likely that HIF1A expression by luteinising follicular cells is upregulated in a non-hypoxic manner. This study has though demonstrated that the activation of HIFIA increases VEGFA production and HIFIA is important for the formation of EC networks. In addition, HIF1A activation promoted progesterone production, partly through the regulation of HSD3B1. In the final experiment, the decreased total area of EC networks in cells cultured in low oxygen conditions was

blocked by inhibition of PKA signalling with H89. A similar H89 by oxygen interaction was observed on progesterone production, although surprisingly PKA inhibition had no effect on the expression of key steroidogenic proteins. Collectively, these studies have provided a unique insight into the complex regulation of EC network formation and progesterone production by low oxygen concentrations and HIF1A in bovine luteinising follicular cells.

# Chapter 6: General Discussion

Reduced fertility and increased prevalence of reproductive pathologies adversely affect the productive efficiency of dairy farms and are a major source of economic and financial losses to the industry. Furthermore, inadequate nutrition has been linked to reproductive failure in dairy cows (Roche, 2006, Dupont et al., 2012). For example, negative energy balance (NEB) occurs when energy intake is insufficient to meet the requirement of maintenance and production in ruminants and commonly occurs in dairy cows (McClure, 1994). In dairy cows, this period of energy deficiency will typically happen for 1-2 months after calving and in some cases even longer. However, during this time, the cow might become pregnant meaning that the survival and growth of embryo/fetus is dependent on balanced nutrition and adequate metabolic supply from the dam. Progesterone is critical for the establishment and continuation of pregnancy. In turn, intensive angiogenesis is required for the development of ovarian follicles and subsequent formation of the corpus luteum (CL) after ovulation. Indeed, luteal inadequacy is associated with a reduced conception rate in cows, which is characterised by inadequate vascularisation and reduced capability to synthesise progesterone.

The vasculature to and within the ovary enables a supply of balanced nutrients, adequate hormone and oxygen supply for all physiological activities of the ovarian cycle. Thus, the central theme of this thesis investigated the role and regulation of angiogenesis during key stages of ovarian development and function. Specifically, the intention of this research was to provide mechanistic insights into the link between adequate ovarian function and maternal protein restriction, the insulin-like growth factor system and low oxygen concentration. The key endpoint investigated in all chapters was the degree to which endothelial cell networks were formed *ex vivo* and *in vitro*.

The first study investigated the effect of maternal dietary protein restriction during the first 65 days of gestation on the development of the fetal sheep ovary. In this study, ewes were fed a 50% decreased crude protein (LP; n=8; 8.7 g versus 17.0 g.kg$^{1}$MJ) or control diet (CP; n=7) from conception to day 65 of gestation. The characteristics of the germ cells and vasculature in the fetal sheep ovary were generated from histological and immunohistochemical analysis. The mean fetal weight was unaffected by

protein restriction on day 65 of gestation whilst kidney function and its vasculature was compromised (Dunford et al., 2014). The effects of maternal protein restriction on the fetal ovary were more limited, but the estimated total germ cell weight and fetal ovary weight were reduced in the LP group. This suggested that the fetal ovary in the LP group might have been slightly developmentally delayed and the full effects, such as delayed primordial follicular development, might not be observed until later in mid-late gestation as previously reported (Rae et al., 2001). The percentage area of cortex and density of germ cells within it were unaffected by maternal diet. The germ cells were OCT4, DAZL and VASA-positive indicating that the germ cells were at various stages of oogenesis. OCT4 was the most abundantly expressed suggesting they were principally in the primordial germ cell/early oogonia stage. More importantly, the proportion of the OCT4, DAZL and VASA-positive cells was similar in the two diets. There was extensive proliferation, with a relatively low proportion of Caspase 3-positive cells. These characteristics are similar to previous reports around gestation day 65 (Gaytan et al., 1999, Sawyer et al., 2002, Lea et al., 2006, Anderson et al., 2007, Hummitzsch et al., 2013). In contrast to that observed in the fetal kidney of the same sheep, the number, area and perimeter of the larger medullary blood vessels and degree of microvascularisation in the cortex were unaffected by dietary protein restriction.

Collectively, the relatively few differences in the fetal ovary structure and development between the dietary groups suggest that the fetal ovary might be under some kind of "protection" from the influence of maternal protein restriction. This would enable the fetal ovary to be less sensitive to maternal protein insults. However, other body systems including the heart, liver, and kidney were affected by maternal diet in these animals (Dunford et al., 2014) revealing that detrimental effect of undernutrition can affect different organs differently. This "protection" might be the result of different mechanisms regulating angiogenesis in the fetal ovary as opposed to that in other organs. Consequently, ovarian endothelial cells are less sensitive to metabolic insults. This would enable the developing fetal ovary to receive continual moderate nutrient supply, hormones, and oxygen for growth and appropriate development of the germ cells and ultimately their associated granulosa cells. The "protection" of the ovary would enable reproductive capability to be maintained which from an evolutionary perspective is essential.

Further work should be conducted on the blood samples from the sheep fetuses to analyse them for insulin, IGF1, beta-hydroxybutyrate (BHB), non-esterified fatty acids (NEFAs), ghrelin and leptin to check the effect of maternal dietary restriction on the metabolic signals in these sheep. More importantly, future studies are required to confirm whether the fetal ovary remains "protected" from the maternal dietary insults at later time points in gestation.

In the adult cow, altered metabolism and endocrine function are associated with nutrient shortage. This can then result in delayed resumption of normal reproductive cycle's post-partum, ovulation failure and reduced conception rates. The key signalling molecules between the metabolic and reproductive axes include insulin, insulin-like growth factor 1 (IGF1) and leptin (Zhao et al., 2002, Shimizu et al., 2008). IGF1 (and IGF2) decrease plasma glucose and increase glucose uptake while low plasma insulin and IGF1 has been assiociated with poor fertility that is linked to the NEB. However, the effect of the IGF system on vascularisation during the follicular-luteal transition is unclear.

The second study utilised an *in vitro* bovine luteinising follicular angiogenesis culture system that mimicked the follicular-luteal transition. Contrary to expectations, $LR^3$-IGF1 and IGF2 treatment at physiologically relevant doses (10 & 100 ng/ml) had limited or no effect on endothelial cell (EC) network development (e.g. total EC area, perimeter and number of branch points). This suggested that the effects of IGF1 or IGF2 treatment on EC development might have been masked by the significant production of endogenous IGF1 and/or IGF2 in our culture system. An important observation was that LH, at similar doses to that observed during the LH surge, increased IGF1 production in luteinising follicular cells. Collectively, this could be a mechanism by which gonadotropins and growth factors synergise to stimulate EC development during the follicular-luteal transition. It should be though noted that the endogenous IGF1 production (100 pg/ml) and low level of endogenous IGF2 production are significantly below the levels found in follicular fluid (100-200 ng/ml) and plasma (10-100 ng/ml) (Ortega et al., 2008, Rodríguez et al., 2017).

The addition of IGF1 and IGF2 marginally stimulated the overall cell proliferation, most likely of the non-endothelial cells. The surprising lack of effect of IGF1 or IGF2 on progesterone production did not corroborate with

other studies (Armstrong et al., 2000, Schams et al., 2002, Spicer et al., 2002, Spicer and Aad, 2007). This would suggest the IGFs did not alter the proliferation (and hypertrophy) of steroidogenic luteal cells. Thus, it is feasible that IGF1/IGF2 might preferentially stimulate the proliferation and/or differentiation of myofibroblasts and pericytes (vascular smooth muscle cells). This might have shifted the distribution of cell type (i.e. decreased space for endothelial cell growth) and stimulated the proliferation of SMA-positive myofibroblasts/mural cells.

IGF1 receptor inhibition (PPP) reduced EC development (number, total area, perimeter, mean area, and number of branch points) and progesterone concentration by 60-70%. This confirmed that IGF1 and/or IGF2 acting through IGF1R promote EC network formation and progesterone production in bovine luteinising follicular cells. These findings demonstrated that IG1R activation and signalling was vital for EC proliferation and development as well as progesterone production in bovine luteinising follicular cells. This also suggests that the endogenous production of IGF1 and/or IGF2 was sufficient to stimulate IGF1R activation. IGF1R expression was present in steroidogenic cells and possibly endothelial cells, which would suggest a direct action, which is supported by previous findings (Lucy, 2008, Silva et al., 2009, Rodríguez et al., 2017).

In the final experiment, the aim was to sequester endogenously produced IGFs with IGFBP4 but this had no effect on EC network formation or progesterone synthesis. This might have been the result of IGFBP4 degradation by PAPP-A (Spicer and Echternkamp, 1995, Stewart et al., 1995, Meyerholz et al., 2015). Thus, a future experiment would be to include an IGFBP protease inhibitor or use an alternative approach such siRNA to knockdown IGF synthesis. The study has clearly demonstrated however that the activation of IGF1R will promote both steroidogenesis and, for the first time, angiogenesis in the developing CL. Thus, extending this to the whole cow, it can be hypothesised that a cow in moderate NEB with low IGF1 levels (Fenwick et al., 2008, Velazquez et al., 2008) would have reduced IGF1R activation and thus luteal angiogenesis and function.

Physiological oxygen concentration within the body varies depending on the distance of each tissue from its blood vessels (Bates, 2012). The supply of blood and degree of vascularisation can be affected during ovarian follicular and CL development and thus the oxygen supply to these structures can

vary (Fischer et al., 1992). Low oxygen concentration as a consequence of decreased blood supply will influence steroid production and angiogenesis during follicle development, ovulation and CL formation (Nishimura and Okuda, 2010). This last study investigated the effect of low oxygen concentration on EC network formation and progesterone production in bovine luteinising follicular cells. This study revealed that low oxygen conditions reduced most EC network parameters and progesterone production by 35% and 45% respectively. These effects were broadly consistently observed across 3 different studies. The implication of this result for the whole animal is that if the developing CL was maintained for several days in a reduced oxygen environment then blood supply would be restricted due to inadequate blood vessel development. This reduced blood flow and the action of low oxygen on progesterone synthesis would result in a delayed rise in post-ovulatory plasma progesterone concentrations. There were four notable observations from chapter 5:

*1) HIF1A activity appeared to be up-regulated in both low and high oxygen conditions*

This present study provided further evidence that HIF1A expression in luteinising follicular cells is regulated through non-hypoxic mechanisms. The granulosa layer is avascular and thus it is possible that these cells are adapted to functioning in a low oxygen environment. The most plausible explanation for the apparent up-regulation of HIF1A in 20% oxygen is the presence of an abundance of growth factors or the LH-activation of PKA signalling. In the present study, there was no evidence to support the role for PKA signalling increasing HIF1A activity. It is feasible though that LH-induction of intracellular cAMP stimulated HIF1A activity in a non-PKA dependent manner. This has been observed for progesterone production in human luteinising granulosa cells (Chin and Abayasekara, 2004). Indeed, treating mouse granulosa cells with forskolin and the PKC-activator, phorbol myristate acetate resulted in HIF1A upregulation to a similar extent to that observed in chemical-induction of hypoxia. *In vivo*, the pre-ovulatory follicular cells are exposed to the LH surge resulting in steroidogenic cells undergoing luteinisation. There is also an abundance of growth factors (e.g. IGFs and VEGFA amongst others) in the bovine early CL (Berisha et al., 2006, Robinson et al., 2007). This would suggest that HIF1A protein would be present *in vivo* irrespective of whether there was hypoxia or normoxia.

One aspect of the effects of hypoxia on follicular cells that was not investigated due to time constraints was the relatively recent observation that granulosa cells synthesise haemoglobin (Thompson et al., 2015, Brown et al., 2015). Moreover, the LH surge up-regulated the production of haemoglobin in the pre-ovulatory follicle. While, the role of haemoglobin in the follicular-luteal transition is unclear, it is hypothesised that this causes a short, transient decrease in oxygen concentrations. This in turn would promote HIF1A expression and hence angiogenesis. Further work should be conducted to evaluate the role of haemoglobin in the luteinising follicular angiogenesis culture system and whether it causes a local hypoxia in these cells.

*2) Exposure of luteinising follicular cells to low oxygen reduced* endothelial cell *network formation and progesterone production.*

The most surprising observation was that EC network formation was inhibited in low oxygen, which is in stark contrast to the established literature. It is unlikely to be because there was a lack of cellular ATP, as the energy consuming process of proliferation was similar in 20% and 3% oxygen. An important point to note is that 20% oxygen is not a physiological concentration of oxygen, while it was used as the reference concentration (based on the vast majority of culture systems using 20% oxygen) it should not really be considered as a control. That said, the *in vivo* implications of these results are that prolonged low oxygen concentrations would, particularly, inhibit the growth of endothelial cells (and steroidogenic cells), with the other cell types in the developing CL less affected. This would ultimately lead to a dysfunctional CL. A more elaborate experiment would be to culture luteinising follicular cells in 1, 3 or 5% oxygen and observe the impacts on EC network formation. Indeed a more transient exposure to lower oxygen concentration might be particularly informative of the *in vivo* situation. Equally, the results from the present study also challenge the rationale for using 20% oxygen as a standard physiological condition for culturing follicular or luteal cells in general.

The inhibitory effect of low oxygen concentration on progesterone production by steroidogenic cells was more predicted and was most likely achieved by downregulating STAR protein expression. The STAR protein is responsible for the rate-limiting step of cholesterol transportation from outer to inner mitochondria during synthesis of progesterone production. However, the

action of low oxygen on STAR appeared to be HIF1A-independent. This might be part of the cell's adaption to low oxygen by minimising cellular translation.

*3) HIF1A is central to the up-regulation angiogenesis even in the absence of hypoxia*.

It is widely recognised that VEGFA is the most potent proangiogenic growth factor regulated by hypoxia. VEGFA mediates physiological angiogenesis and increases the permeability of blood vessels (Ferrara et al., 2003). Our findings clearly support a HIF-dependent mechanism for VEGFA production and this inhibition could be responsible for the reduction in EC development (angiogenesis) in bovine luteinising follicular cells. Surprisingly, the process occurred in both 20% and 3% oxygen, from which it can be hypothesised that the HIF1A-VEGFA-angiogenesis axis is a central mechanism that is activated when cells require vascularisation. Importantly and potentially uniquely to female reproductive tissues, this axis is activated even when oxygen concentrations are high.

There has been relatively little attention paid to the interplay between hypoxia, HIF1A and FGF2 production. In the bovine follicular-luteal transition, FGF2 plays a more important role than VEGFA in stimulating angiogenesis, in particular EC sprouting. Intriguingly, in HUVECs, the induction of HIF1A increased FGF2 production, which then acted in an autocrine manner to further increase HIF1A production (Calvani et al., 2006). Given, the very high FGF2 concentrations that are induced by the LH surge in the collapsed follicle (Robinson et al., 2007), this could easily trigger the expression of HIF1A (Fig. 6.1 for proposed mechanism). The induction of HIF1A by FGF2 is further supported by observations in other cells (Yao et al., 2013, Fábián et al., 2014, Gouazé-Andersson et al., 2016). Equally, there is recent evidence to support the regulation of the potent pro-angiogenic factor, stanniocalcin by HIF1A in luteinising follicular cells (Basini et al., 2009, Law and Wong, 2013).

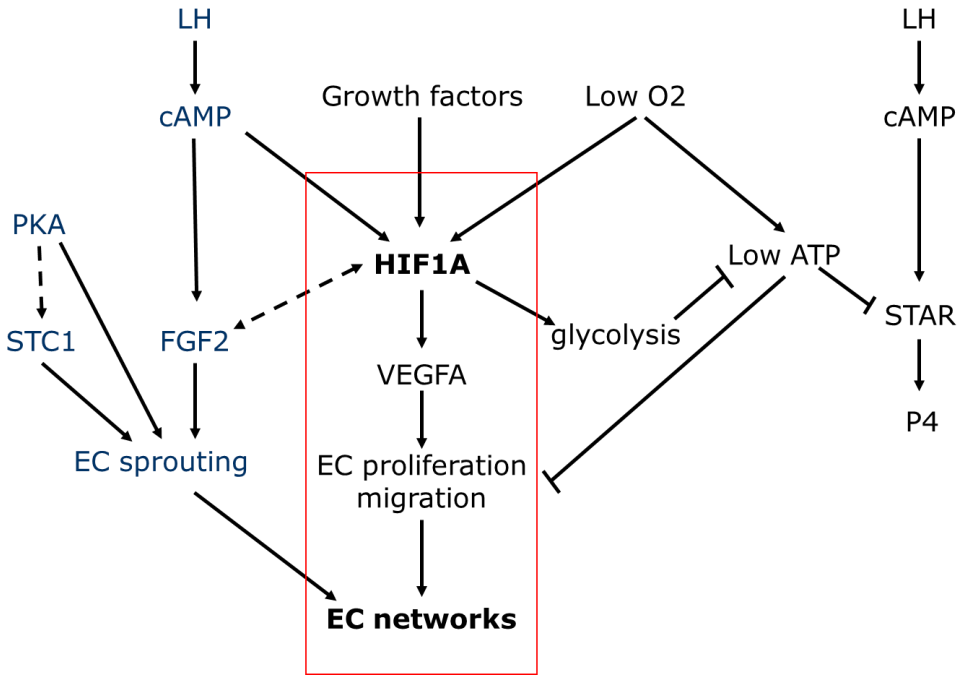



Fig. 6.1: The central HIF1A axis in the promotion of angiogenesis (highlighted in the red box) and its proposed regulation in luteinising follicular cells. The factors written in black are those investigated in this study, while associated factors, integrated from the literature (Woad et al., 2012, Meidan et al., 2013, Laird et al., 2013) are shown in blue. The dashed lines indicate proposed interactions.

*4) The regulation of energy production in luteinising follicular cells is complex*

Increased lactate production is a major indicator of the activation of glycolysis to maintain ATP production. The consumption of glucose and subsequent lactate production increased as the follicle developed *in vitro* (Boland et al., 1993, Harris et al., 2007). Lactate concentrations were decreased by echinomycin, but were unaffected by oxygen conditions. The decreased lactate production is likely to indicate decreased conversion of pyruvate to lactate by lactate dehydrogenase (LDH). It is recognised that LDH is a HIF1A-responsive protein in other tissues (Firth et al., 1995) and the evidence presented here supports that this is also the case in luteinising follicular cells. Extending this to the whole animal, then the increased LDH expression (via HIF1A action) would enable the luteinising follicular cells to accommodate increased glycolysis and maintenance of ATP production. This would be necessary until the new and extensive blood supply was established to maximise oxygen delivery to the luteal cells for their high metabolic demands. The reason for the lack of an effect of low oxygen on

lactate concentrations is unclear. However, given the abundance of nutrients in the media (i.e. glucose, amino and fatty acids), it is feasible that the luteinising follicular cells had adapted to maintain ATP production without increasing glycolysis. This would explain why low oxygen did not increase PGK1 expression or affect GLUT1 expression. It would be useful to repeat this experiment in nutrient-restricted MCDB 131 media to determine whether in this scenario, low oxygen influenced lactate production.

In summary, this thesis has clearly demonstrated that maternal protein restriction had limited effects on the number of germ cells and the degree of vascularisation in the fetal sheep ovary. Next, it was shown that bovine follicular luteinising follicular cells appeared to produce IGF1 and/or IGF2 endogenously that stimulated endothelial cell network formation and angiogenesis. Finally, low oxygen reduced progesterone production by bovine luteinising follicular cells as expected, but contrary to our hypothesis, low oxygen did not stimulate EC growth. In fact, EC network formation was decreased in low oxygen. Furthermore, many of the effects of inhibiting HIF1A actions were observed in both 20% and 3% oxygen. This indicated that HIF1A plays a critical role in angiogenesis and steroidogenesis that is independent of oxygen environment. The negative effects of low oxygen on EC networks are possibly mediated in part by PKA signalling. In conclusion, this thesis further highlights the complex regulation of bovine follicular angiogenesis during the follicular-luteal transition.

# References

AAD, P., ECHTERNKAMP, S. & SPICER, L. 2013. Possible role of IGF2 receptors in regulating selection of 2 dominant follicles in cattle selected for twin ovulations and births. *Domestic Animal Endocrinology,* 45**,** 187-195.

ABRAMOVICH, D., CELIN, A. R., HERNANDEZ, F., TESONE, M. & PARBORELL, F. 2009. Spatiotemporal analysis of the protein expression of angiogenic factors and their related receptors during folliculogenesis in rats with and without hormonal treatment. *Reproduction,* 137**,** 309-320.

ABU-SAAD, K. & FRASER, D. 2010. Maternal nutrition and birth outcomes. *Epidemiologic Reviews,* 32**,** 5-25.

ACOSTA, T. J., GASTAL, E., GASTAL, M., BEG, M. & GINTHER, O. 2004. Differential blood flow changes between the future dominant and subordinate follicles precede diameter changes during follicle selection in mares. *Biology of Reproduction,* 71**,** 502-507.

ACOSTA, T. J., HAYASHI, K., OHTANI, M. & MIYAMOTO, A. 2003. Local changes in blood flow within the preovulatory follicle wall and early corpus luteum in cows. *Reproduction,* 125**,** 759-767.

ALIG, S. K., STAMPNIK, Y., PIRCHER, J., ROTTER, R., GAITZSCH, E., RIBEIRO, A., WORNLE, M., KROTZ, F. & MANNELL, H. 2015. The tyrosine phosphatase SHP-1 regulates hypoxia inducible factor-1alpha (HIF-1alpha) protein levels in endothelial cells under hypoxia. *PlOS One,* 10**,** 121-113.

AMSELGRUBER, W., SCHÄFER, M. & SINOWATZ, F. 1999. Angiogenesis in the bovine corpus luteum: an immunocytochemical and ultrastructural study. *Anatomia, Histologia, Embryologia,* 28**,** 157-166.

AMSELGRUBER, W., SINOWATZ, F., SCHAMS, D. & SKOTTNER, A. 1994. Immunohistochemical aspects of insulin-like growth factors I and II in the bovine corpus luteum. *Journal of Reproduction and Fertility,* 101**,** 445-451.

ANDERSON, R. A., FULTON, N., COWAN, G., COUTTS, S. & SAUNDERS, P. T. 2007. Conserved and divergent patterns of expression of DAZL, VASA and OCT4 in the germ cells of the human fetal ovary and testis. *BMC Developmental Biology,* 7**,** 136.

ANDRADE, L. P., RHIND, S. M., RAE, M. T., KYLE, C. E., JOWETT, J. & LEA, R. G. 2013. Maternal undernutrition does not alter Sertoli cell numbers or the expression of key developmental markers in the mid-gestation ovine fetal testis. *Journal of Negative Results in Biomedicine,* 12**,** 1.

ANNETT, R. & CARSON, A. 2006. Effects of plane of nutrition during the first month of pregnancy on conception rate, foetal development and lamb output of mature and adolescent ewes. *Animal Science,* 82**,** 947-954.

ANTHONY, R., BELLOWS, R., SHORT, R., STAIGMILLER, R., KALTENBACH, C. & DUNN, T. 1986. Fetal GRowth of Beef Calves. II. Effect of Sire on Prenatal Development of the Calf and Related Placental Characteristics 1. *Journal of Animal Science,* 62**,** 1375-1387.

ARAGONÉS, J., FRAISL, P., BAES, M. & CARMELIET, P. 2009. Oxygen sensors at the crossroad of metabolism. *Cell Metabolism,* 9**,** 11-22.

ARENDS, M., MORRIS, R. & WYLLIE, A. 1990. Apoptosis. The role of the endonuclease. *The American Journal of Pathology,* 136**,** 593.

ARMSTRONG, D., BAXTER, G., GUTIERREZ, C., HOGG, C., GLAZYRIN, A., CAMPBELL, B., BRAMLEY, T. & WEBB, R. 1998. Insulin-like growth factor binding protein-2 and-4 messenger ribonucleic acid expression in bovine ovarian follicles: effect of gonadotropins and developmental status. *Endocrinology,* 139**,** 2146-2154.

ARMSTRONG, D., GUTIERREZ, C., BAXTER, G., GLAZYRIN, A., MANN, G., WOAD, K., HOGG, C. & WEBB, R. 2000. Expression of mRNA encoding IGF-I, IGF-II and type 1 IGF receptor in bovine ovarian follicles. *Journal of Endocrinology,* 165**,** 101-113.

ARMSTRONG, L. C. & BORNSTEIN, P. 2003. Thrombospondins 1 and 2 function as inhibitors of angiogenesis. *Matrix Biology,* 22**,** 63-71.

ARMULIK, A., ABRAMSSON, A. & BETSHOLTZ, C. 2005. Endothelial/pericyte interactions. *Circulation Research,* 97**,** 512-523.

ARNQVIST, H. 2008. The role of IGF-system in vascular insulin resistance. *Hormone and Metabolic Research,* 40**,** 588-592.

ARRAZTOA, J. A., MONGET, P., BONDY, C. & ZHOU, J. 2002. Expression patterns of insulin-like growth factor-binding proteins 1, 2, 3, 5, and 6 in the mid-cycle monkey ovary. *The Journal of Clinical Endocrinology & Metabolism,* 87**,** 5220-5228.

ASHWORTH, C. J., TOMA, L. M. & HUNTER, M. G. 2009. Nutritional effects on oocyte and embryo development in mammals: implications for reproductive efficiency and environmental sustainability. *Philosophical Transactions of the Royal Society of Biological Sciences,* 364**,** 3351-3361.

AUGUSTIN, H. G. 2000. Vascular morphogenesis in the ovary. *Best Practice of Research Clinical Obstetrics and Gynaecology,* 14**,** 867-882.

AUGUSTIN, H. G., KOH, G. Y., THURSTON, G. & ALITALO, K. 2009. Control of vascular morphogenesis and homeostasis through the angiopoietin–Tie system. *Nature Reviews Molecular Cell Biology,* 10**,** 165.

AZAR, W. J., AZAR, S. H., HIGGINS, S., HU, J.-F., HOFFMAN, A. R., NEWGREEN, D. F., WERTHER, G. A. & RUSSO, V. C. 2011. IGFBP-2 enhances VEGF gene promoter activity and consequent promotion of angiogenesis by neuroblastoma cells. *Endocrinology,* 152**,** 3332-3342.

BACH, L. A. 1999. Insulin-like growth factor binding protein-6: the "forgotten" binding protein? *Hormone and Metabolic Research,* 31**,** 226-234.

BACH, L. A. 2015. Endothelial cells and the IGF system. *Journal of Molecular Endocrinology,* 54**,** 1-13.

BACH, L. A., HEADEY, S. J. & NORTON, R. S. 2005. IGF-binding proteins–the pieces are falling into place. *Trends in Endocrinology & Metabolism,* 16**,** 228-234.

BADDELA, V. S., SHARMA, A., VIERGUTZ, T., KOCZAN, D. & VANSELOW, J. 2018. Low oxygen levels induce early luteinization associated changes in bovine granulosa cells. *Frontiers in Physiology,* 9.

BALASA, A., SANCHEZ-VALLE, A., SADIKOVIC, B., SANGI-HAGHPEYKAR, H., BRAVO, J., CHEN, L., LIU, W., WEN, S., FIOROTTO, M. L. & VAN DEN VEYVER, I. B. 2011. Chronic Maternal Protein Deprivation in Mice Is Associated with Overexpression of the Cohesin-Mediator Complex in Liver of Their Offspring–. *The Journal of Nutrition,* 141**,** 2106-2112.

BAO, B. & GARVERICK, H. A. 1998. Expression of steroidogenic enzyme and gonadotropin receptor genes in bovine follicles during ovarian follicular waves: a review. *Journal of Animal Science,* 76**,** 1903-1921.

BAR, R. S. & BOES, M. 1984. Distinct receptors for IGF-I, IGF-II, and insulin are present on bovine capillary endothelial cells and large vessel endothelial cells. *Biochemical and Biophysical Research Communications,* 124**,** 203-209.

BAR, R. S., CLEMMONS, D. R., BOES, M., BUSBY, W. H., BOOTH, B. A., DAKE, B. L. & SANDRA, A. 1990. Transcapillary permeability and subendothelial distribution of endothelial and amniotic fluid insulin-like growth factor binding proteins in the rat heart. *Endocrinology,* 127**,** 1078-1086.

BARKER, D. & CLARK, P. M. 1997. Fetal undernutrition and disease in later life. *Reviews of Reproduction,* 2**,** 105-112.

BARKER, D. J., GODFREY, K. M., GLUCKMAN, P. D., HARDING, J. E., OWENS, J. A. & ROBINSON, J. S. 1993. Fetal nutrition and cardiovascular disease in adult life. *The Lancet,* 341**,** 938-941.

BASINI, G., BAIONI, L., BUSSOLATI, S., GROLLI, S., KRAMER, L. H., WAGNER, G. F. & GRASSELLI, F. 2010. Expression and localization of stanniocalcin 1 in swine ovary. *General and Comparative Endocrinology,* 166**,** 404-8.

BASINI, G., BIANCO, F., GRASSELLI, F., TIRELLI, M., BUSSOLATI, S. & TAMANINI, C. 2004. The effects of reduced oxygen tension on swine granulosa cell. *Regulatory Peptides,* 120**,** 69-75.

BASINI, G., BUSSOLATI, S., SANTINI, S. E. & GRASSELLI, F. 2009. Stanniocalcin, a potential ovarian angiogenesis regulator, does not affect endothelial cell apoptosis. *Annals of the New York Academy of Sciences,* 1171**,** 94-9.

BATES, M. K. 2012. Culturing cells under hypoxic conditions for biologically relevant results. *American Laboratory*.

BAUFELD, A. & VANSELOW, J. 2018. Lactate promotes specific differentiation in bovine granulosa cells depending on lactate uptake thus mimicking an early post-LH stage. *Reproductive Biology and Endocrinology,* 16**,** 15.

BAUMGARTEN, S. C., CONVISSAR, S. M., FIERRO, M. A., WINSTON, N. J., SCOCCIA, B. & STOCCO, C. 2014. IGF1R signaling is necessary for FSH-induced activation of AKT and differentiation of human Cumulus granulosa cells. *The Journal of Clinical Endocrinology and Metabolism,* 99**,** 2995-3004.

BAUMGARTEN, S. C., CONVISSAR, S. M., ZAMAH, A. M., FIERRO, M. A., WINSTON, N. J., SCOCCIA, B. & STOCCO, C. 2015. FSH regulates IGF-2 expression in human granulosa cells in an AKT-dependent manner. *The Journal of Clinical Endocrinology and Metabolism,* 100**,** 1046-1055.

BAXTER, R. C. 2000. Insulin-like growth factor (IGF)-binding proteins: interactions with IGFs and intrinsic bioactivities. *American Journal of Physiology-Endocrinology and Metabolism,* 278**,** E967-E976.

BAXTER, R. C. 2014. IGF binding proteins in cancer: mechanistic and clinical insights. *Nature Reviews Cancer,* 14**,** 3720.

BEATTIE, J., ALLAN, G. J., LOCHRIE, J. D. & FLINT, D. J. 2006. Insulin-like growth factor-binding protein-5 (IGFBP-5): a critical member of the IGF axis. *Biochemical Journal,* 395**,** 1-19.

BECKMAN, J. D., GRAZUL-BILSKA, A. T., JOHNSON, M. L., REYNOLDS, L. P. & REDMEL, D. A. 2006. Isolation and characterization of ovine luteal pericytes and effects of nitric oxide on pericyte expression of angiogenic factors. *Endocrine,* 29**,** 467-476.

BEHROOZ, A. & ISMAIL-BEIGI, F. 1997. Dual control of glut1 glucose transporter gene expression by hypoxia and by inhibition of oxidative phosphorylation. *Journal of Biological Chemistry,* 272**,** 5555-5562.

BEINDORFF, N., HONNENS, A., PENNO, Y., PAUL, V. & BOLLWEIN, H. 2009. Effects of human chorionic gonadotropin on luteal blood flow and progesterone secretion in cows and in vitro–microdialyzed corpora lutea. *Theriogenology,* 72**,** 528-534.

BELFIORE, C. J., HAWKINS, D. E., WILTBANK, M. C. & NISWENDERS, G. D. 1994. Regulation of cytochrome P450scc synthesis and activity in the ovine corpus luteum. *The Journal of Steroid Biochemistry and Molecular Biology,* 51**,** 283-290.

BENNETT, A., WILSON, D. M., LIU, F., NAGASHIMA, R., ROSENFELD, R. G. & HINTZ, R. L. 1983. Levels of insulin-like growth factors I and II in human cord blood. *The Journal of Clinical Endocrinology & Metabolism,* 57**,** 609-612.

BEREKET, A., LANG, C. H., BLETHEN, S. L., FAN, J., FROST, R. A. & WILSON, T. A. 1995. Insulin-like growth factor binding protein-3 proteolysis in children with insulin-dependent diabetes mellitus: a possible role for insulin in the regulation of IGFBP-3 protease activity. *The Journal of Clinical Endocrinology & Metabolism,* 80**,** 2282-2288.

BERGERS, G. & SONG, S. 2005. The role of pericytes in blood-vessel formation and maintenance. *Neuro-Oncology,* 7**,** 452-64.

BERISHA, B., SCHAMS, D., KOSMANN, M., AMSELGRUBER, W. & EINSPANIER, R. 2000. Expression and localisation of vascular endothelial growth factor and basic fibroblast growth factor during the final growth of bovine ovarian follicles. *Journal of Endocrinology,* 167**,** 371-382.

BERISHA, B., SINOWATZ, F. & SCHAMS, D. 2004. Expression and localization of fibroblast growth factor (FGF) family members during the final growth of bovine ovarian follicles. *Molecular Reproduction and Development: Incorporating Gamete Research,* 67**,** 162-171.

BERISHA, B., STEFFL, M., AMSELGRUBER, W. & SCHAMS, D. 2006. Changes in fibroblast growth factor 2 and its receptors in bovine follicles before and after GnRH application and after ovulation. *Reproduction,* 131**,** 319-329.

BERRA, E., ROUX, D., RICHARD, D. E. & POUYSSÉGUR, J. 2001. Hypoxia-inducible factor-1α (HIF-1α) escapes O2-driven proteasomal degradation irrespective of its subcellular localization: nucleus or cytoplasm. *EMBO Reports,* 2**,** 615-620.

BERRIDGE, M. V., HERST, P. M. & TAN, A. S. 2005. Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction. *Biotechnology Annual Review,* 11**,** 127-152.

BESNARD, N., PISSELET, C., MONNIAUX, D., LOCATELLI, A., BENNE, F., GASSER, F., HATEY, F. & MONGET, P. 1996. Expression of messenger ribonucleic acids of insulin-like growth factor binding proteins-2,-4, and-5 in the ovine ovary: localization and changes during growth and atresia of antral follicles. *Biology of Reproduction,* 55**,** 1356-1367.

BID, H. K., ZHAN, J., PHELPS, D. A., KURMASHEVA, R. T. & HOUGHTON, P. J. 2012. Potent inhibition of angiogenesis by the IGF-1 receptor-targeting antibody SCH717454 is reversed by IGF-2. *Molecular Cancer Therapeutics,* 11**,** 649-659.

BIEGHS, L., LUB, S., FOSTIER, K., MAES, K., VAN VALCKENBORGH, E., MENU, E., JOHNSEN, H. E., OVERGAARD, M. T., LARSSON, O. & AXELSON, M. 2014. The IGF-1 receptor inhibitor picropodophyllin potentiates the anti-myeloma activity of a BH3-mimetic. *Oncotarget,* 5**,** 11193.

BLAIS, J. D., ADDISON, C. L., EDGE, R., FALLS, T., ZHAO, H., WARY, K., KOUMENIS, C., HARDING, H. P., RON, D., HOLCIK, M. & BELL, J. C. 2006. Perk-dependent translational regulation promotes tumor cell

adaptation and angiogenesis in response to hypoxic stress. *Molecular Cell Biology,* 26**,** 9517-32.

BLOCK, E. 1952. Quantitative morphological investigations of the follicular system in women. *Cells Tissues Organs,* 14**,** 108-123.

BLOOMFIELD, F., OLIVER, M. & HARDING, J. 2006. The late effects of fetal growth patterns. *Archives of Disease in Childhood-Fetal and Neonatal Edition,* 91**,** 299-304.

BLOOMFIELD, F. H., OLIVER, M. H., HAWKINS, P., CAMPBELL, M., PHILLIPS, D. J., GLUCKMAN, P. D., CHALLIS, J. R. & HARDING, J. E. 2003. A periconceptional nutritional origin for noninfectious preterm birth. *Science,* 300**,** 606-606.

BLUM, W. F., ALBERTSSON-WIKLAND, K., ROSBERG, S. & RANKE, M. B. 1993. Serum levels of insulin-like growth factor I (IGF-I) and IGF binding protein 3 reflect spontaneous growth hormone secretion. *The Journal of Clinical Endocrinology and Metabolism,* 76**,** 1610-1616.

BLUM, W. F. & SCHWEIZER, R. 2003. Insulin-like growth factors and their binding proteins. *Diagnostics of endocrine function in children and adolescents.*: Karger Publishers. Pages 166-199.

BLUNDELL, T., BEDARKAR, S., RINDERKNECHT, E. & HUMBEL, R. 1978. Insulin-like growth factor: a model for tertiary structure accounting for immunoreactivity and receptor binding. *Proceedings of the National Academy of Sciences,* 75**,** 180-184.

BOEKSTEGERS, P., RIESSEN, R. & SEYDE, W. 1990. Oxygen partial pressure distribution within skeletal muscle: indicator of whole body oxygen delivery in patients? *Oxygen Transport to Tissue XII.* Springer. Pages 507-514.

BOHNERT, D., SCHAUER, C. & DELCURTO, T. 2002. Influence of rumen protein degradability and supplementation frequency on performance and nitrogen use in ruminants consuming low-quality forage: cow performance and efficiency of nitrogen use in wethers. *Journal of Animal Science,* 80**,** 1629-1637.

BOLAND, M. P., LONERGAN, P. & O'CALLAGHAN, D. 2001. Effect of nutrition on endocrine parameters, ovarian physiology, and oocyte and embryo development. *Theriogenology,* 55**,** 1323-1340.

BOLAND, N., HUMPHERSON, P., LEESE, H. & GOSDEN, R. 1993. Pattern of lactate production and steroidogenesis during growth and maturation of mouse ovarian follicles in vitro. *Biology of Reproduction,* 48**,** 798-806.

BOONYAPRAKOB, U., GADSBY, J. E., HEDGPETH, V., ROUTH, P. A. & ALMOND, G. W. 2005. Expression and localization of hypoxia inducible factor-1α mRNA in the porcine ovary. *Canadian Journal of Veterinary Research,* 69**,** 215.

BORWICK, S., RHIND, S., MCMILLEN, S. & RACEY, P. 1996. Effect of undernutrition of ewes from the time of mating on fetal ovarian development in mid gestation. *Reproduction, Fertility, and Development,* 9**,** 711-715.

BRADLEY, T., HODGSON, G. & ROSENDAAL, M. 1978. The effect of oxygen tension on haemopoietic and fibroblast cell proliferation in vitro. *Journal of Cellular Physiology,* 97**,** 517-522.

BRAUN, R. D., LANZEN, J. L., SNYDER, S. A. & DEWHIRST, M. W. 2001. Comparison of tumor and normal tissue oxygen tension measurements using OxyLite or microelectrodes in rodents. *American Journal of Physiology-Heart and Circulatory Physiology,* 280**,** 2533-2544.

BREZIS, M. & ROSEN, S. 1995. Hypoxia of the renal medulla—its implications for disease. *New England Journal of Medicine,* 332**,** 647-655.

BROOKS, A., EASTWOOD, J., BECKINGHAM, I. & GIRLING, K. 2004. Liver tissue partial pressure of oxygen and carbon dioxide during partial hepatectomy. *British Journal of Anaesthesia,* 92**,** 735-737.

BROWN, H. M., ANASTASI, M. R., FRANK, L. A., KIND, K. L., RICHANI, D., ROBKER, R. L., RUSSELL, D. L., GILCHRIST, R. B. & THOMPSON, J. G. 2015. Hemoglobin: a gas transport molecule that is hormonally

regulated in the ovarian follicle in mice and humans. *Biology of Reproduction,* 92**,** 26, 1-10.

BROWN, J., JONES, E. Y. & FORBES, B. E. 2009. Keeping IGF-II under control: Lessons from the IGF-II–IGF2R crystal structure. *Trends in Biochemical Sciences,* 34**,** 612-619.

BROXMEYER, H., COOPER, S., RUBIN, B. Y. & TAYLOR, M. W. 1985. The synergistic influence of human interferon-gamma and interferon-alpha on suppression of hematopoietic progenitor cells is additive with the enhanced sensitivity of these cells to inhibition by interferons at low oxygen tension in vitro. *The Journal of Immunology,* 135**,** 2502-2506.

BRUICK, R. K. 2000. Expression of the gene encoding the proapoptotic Nip3 protein is induced by hypoxia. *Proceedings of the National Academy of Sciences,* 97**,** 9082-9087.

BRUICK, R. K. 2003. Oxygen sensing in the hypoxic response pathway: regulation of the hypoxia-inducible transcription factor. *Genes & Development,* 17**,** 2614-2623.

BURKHART, M., JUENGEL, J., SMITH, P., HEATH, D., PERRY, G., SMITH, M. & GARVERICK, H. 2010. Morphological development and characterization of aromatase and estrogen receptors alpha and beta in fetal ovaries of cattle from days 110 to 250. *Animal Reproduction Science,* 117**,** 43-54.

BURRI, P. H. & DJONOV, V. 2002. Intussusceptive angiogenesis––the alternative to capillary sprouting. *Molecular Aspects of Medicine,* 23**,** 1-27.

BURROUGHS, W., NELSON, D. & MERTENS, D. 1975. Evaluation of Protein Nutrition by Metabolizable Protein and Urea Fermentation Potential1, 2. *Journal of Dairy Science,* 58**,** 611-619.

BUSBY, W. H., SNYDER, D. K. & CLEMMONS, D. R. 1988. Radioimmunoassay of a 26,000-dalton plasma insulin-like growth factor-binding protein: control by nutritional variables. *The Journal of Clinical Endocrinology and Metabolism,* 67**,** 1225-1230.

BUSSOLINO, F., MANTOVANI, A. & PERSICO, G. 1997. Molecular mechanisms of blood vessel formation. *Trends in Biochemical Sciences,* 22**,** 251-256.

BUTLER, W. R. 1998. Review: effect of protein nutrition on ovarian and uterine physiology in dairy cattle. *Journal of Dairy Science,* 81**,** 2533-2539.

BUTLER, W. R. 2003. Energy balance relationships with follicular development, ovulation and fertility in postpartum dairy cows. *Livestock Production Science,* 83**,** 211-218.

BYSKOV, A.-G. & LINTERN-MOORE, S. 1973. Follicle formation in the immature mouse ovary: the role of the rete ovarii. *Journal of Anatomy,* 116**,** 207.

BYSKOV, A. G. 1986. Differentiation of mammalian embryonic gonad. *Physiological Reviews,* 66**,** 71-117.

CALVANI, M., RAPISARDA, A., URANCHIMEG, B., SHOEMAKER, R. H. & MELILLO, G. 2006. Hypoxic induction of an HIF-1alpha-dependent bFGF autocrine loop drives angiogenesis in human endothelial cells. *Blood,* 107**,** 2705-12.

CAMPBELL, B., BAIRD, D. & WEBB, R. 1998. Effects of dose of LH on androgen production and luteinization of ovine theca cells cultured in a serum-free system. *Journal of Reproduction and Fertility,* 112**,** 69-77.

CAMPBELL, P., SKAAR, T., VEGA, J. & BAUMRUCKER, C. 1991. Secretion of insulin-like growth factor-I (IGF-I) and IGF-binding proteins from bovine mammary tissue in vitro. *Journal of Endocrinology,* 128**,** 219-228.

CARMELIET, P. 2005. Angiogenesis in life, disease and medicine. *Nature,* 438**,** 932.

CARMELIET, P., COLLEN, D., DE FALCO, S. & MENOTTI, R. 2009. Inhibitors of placental growth factor for the treatment of pathological

angiogenesis, pathological arteriogenesis, inflammation, tumor formation and/or vascular leakage.: Google Patents. Pages 9 - 11.

CARMELIET, P., DOR, Y., HERBERT, J.-M., FUKUMURA, D., BRUSSELMANS, K., DEWERCHIN, M., NEEMAN, M., BONO, F., ABRAMOVITCH, R. & MAXWELL, P. 1998. Role of HIF-1α in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. *Nature,* 394**,** 485.

CARREAU, A., HAFNY-RAHBI, B. E., MATEJUK, A., GRILLON, C. & KIEDA, C. 2011. Why is the partial oxygen pressure of human tissues a crucial parameter? Small molecules and hypoxia. *Journal of Cellular and Molecular Medicine,* 15**,** 1239-1253.

CASTRILLON, D. H., QUADE, B. J., WANG, T., QUIGLEY, C. & CRUM, C. P. 2000. The human VASA gene is specifically expressed in the germ cell lineage. *Proceedings of the National Academy of Sciences,* 97**,** 9585-9590.

CATON, J., VONNAHME, K., REED, J., NEVILLE, T., EFFERTZ, C., HAMMER, C., LUTHER, J., TAYLOR, J., REDMER, D. & REYNOLDS, L. 2007. Effects of maternal nutrition on birth weight and postnatal nutrient metabolism. *European Association for Animal Production,* 124**,** 101.

CELIK-OZENCI, C., AKKOYUNLU, G., KAYISLI, U. A., ARICI, A. & DEMIR, R. 2003. Localization of vascular endothelial growth factor in the zona pellucida of developing ovarian follicles in the rat: a possible role in destiny of follicles. *Histochemistry and Cell Biology,* 120**,** 383-390.

CHASE, C., KIRBY, C., HAMMOND, A., OLSON, T. & LUCY, M. 1998. Patterns of ovarian growth and development in cattle with a growth hormone receptor deficiency. *Journal of Animal Science,* 76**,** 212-219.

CHASSEIGNEAUX, S., MORACA, Y., COCHOIS-GUEGAN, V., BOULAY, A. C., GILBERT, A., LE CROM, S., BLUGEON, C., FIRMO, C., CISTERNINO, S., LAPLANCHE, J. L., CURIS, E., DECLEVES, X. & SAUBAMEA, B. 2018. Isolation and differential transcriptome of vascular smooth muscle cells and mid-capillary pericytes from the rat brain. *Scientific Reports,* 8**,** 12272.

CHEN, E. Y., MAZURE, N. M., COOPER, J. A. & GIACCIA, A. J. 2001. Hypoxia activates a platelet-derived growth factor receptor/phosphatidylinositol 3-kinase/Akt pathway that results in glycogen synthase kinase-3 inactivation. *Cancer Research,* 61**,** 2429-2433.

CHEN, L., ENDLER, A. & SHIBASAKI, F. 2009. Hypoxia and angiogenesis: regulation of hypoxia-inducible factors via novel binding factors. *Experimental and Molecular Medicine,* 41**,** 849.

CHIN, E. & ABAYASEKARA, D. 2004. Progesterone secretion by luteinizing human granulosa cells: a possible cAMP-dependent but PKA-independent mechanism involved in its regulation. *Journal of Endocrinology,* 183**,** 51-60.

CHRISTENSON, L. K. & STOUFFER, R. L. 1997. Follicle-stimulating hormone and luteinizing hormone/chorionic gonadotropin stimulation of vascular endothelial growth factor production by macaque granulosa cells from pre-and periovulatory follicles. *The Journal of Clinical Endocrinology and Metabolism,* 82**,** 2135-2142.

CLAPP, C., THEBAULT, S., JEZIORSKI, M. C. & MARTINEZ DE LA ESCALERA, G. 2009. Peptide hormone regulation of angiogenesis. *Physiological Reviews,* 89**,** 1177-1215.

CLARK, B. J., RANGANATHAN, V. & COMBS, R. 2000. Post-translational regulation of steroidogenic acute regulatory protein by cAMP-dependent protein kinase A. *Endocrine Research,* 26**,** 681-689.

CLARK, B. J., WELLS, J., KING, S. R. & STOCCO, D. M. 1994. The purification, cloning, and expression of a novel luteinizing hormone-induced mitochondrial protein in MA-10 mouse Leydig tumor cells. Characterization of the steroidogenic acute regulatory protein (StAR). *Journal of Biological Chemistry,* 269**,** 28314-28322.

CLAYCOMB, R. W., DELWICHE, M. J., MUNRO, C. J. & BONDURANT, R. H. 1998. Rapid enzyme immunoassay for measurement of bovine progesterone. *Biosensors and Bioelectronics,* 13**,** 1165-1171.

CLEMMONS, D. R. 2006. Involvement of insulin-like growth factor-I in the control of glucose homeostasis. *Current Opinion in Pharmacology,* 6**,** 620-625.

CLEMMONS, D. R. 2007. Modifying IGF1 activity: an approach to treat endocrine disorders, atherosclerosis and cancer. *Nature Reviews Drug Discovery,* 6**,** 821.

COCKMAN, M. E., MASSON, N., MOLE, D. R., JAAKKOLA, P., CHANG, G.-W., CLIFFORD, S. C., MAHER, E. R., PUGH, C. W., RATCLIFFE, P. J. & MAXWELL, P. H. 2000. Hypoxia inducible factor-α binding and ubiquitylation by the von Hippel-Lindau tumor suppressor protein. *Journal of Biological Chemistry,* 275**,** 25733-25741.

CONTOIS, L. W., NUGENT, D. P., CARON, J. M., CRETU, A., TWEEDIE, E., AKALU, A., LIEBES, L., FRIESEL, R., ROSEN, C. & VARY, C. 2012. Insulin-like growth factor binding protein-4 differentially inhibits growth factor-induced angiogenesis. *Journal of Biological Chemistry,* 287**,** 1779-1789.

CORDEIRO, M. B., PERES, M. S., DE SOUZA, J. M., GASPAR, P., BARBIERE, F., SÁ FILHO, M. F., MATURANA FILHO, M., DINARDI, R. N., NOGUEIRA, G. P. & MESQUITA, F. S. 2015. Supplementation with sunflower seed increases circulating cholesterol concentrations and potentially impacts on the pregnancy rates in Bos indicus beef cattle. *Theriogenology,* 83**,** 1461-1468.

CORDIN, O., BANROQUES, J., TANNER, N. K. & LINDER, P. 2006. The DEAD-box protein family of RNA helicases. *Gene,* 367**,** 17-37.

CORY, A. H., OWEN, T. C., BARLTROP, J. A. & CORY, J. G. 1991. Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture. *Cancer Communications,* 3**,** 207-212.

COUTU, D. L. & GALIPEAU, J. 2011. Roles of FGF signaling in stem cell self-renewal, senescence and aging. *Aging (Albany NY),* 3**,** 920.

CURSIEFEN, C., KÜCHLE, M. & NAUMANN, G. 1998. Angiogenesis in corneal diseases: histopathologic evaluation of 254 human corneal buttons with neovascularization. *Cornea,* 17**,** 611-613.

CUSHMAN, R., MCNEEL, A. K. & FREETLY, H. C. 2014. The impact of cow nutrient status during the second and third trimesters on age at puberty, antral follicle count, and fertility of daughters. *Livestock Science,* 162**,** 252-258.

DAVIS, J. S., RUEDA, B. R. & SPANEL-BOROWSKI, K. 2003. Microvascular endothelial cells of the corpus luteum. *Reproductive Biology and Endocrinology,* 1**,** 89.

DAVIS, S., HODGKINSON, S., PROSSER, C., GLUCKMAN, P., BUONOMO, F. & COLLIER, R. 1992. Effect of growth hormone treatment on the distribution of insulin-like growth factor-I between plasma and lymph of lactating sheep. *Journal of Endocrinology,* 132**,** 339-344.

DE MEYTS, P., CHRISTOFFERSEN, C. T., URSØ, B., WALLACH, B., GRØNSKOV, K., YAKUSHIJI, F. & SHYMKO, R. M. 1995. Role of the time factor in signaling specificity: application to mitogenic and metabolic signaling by the insulin and insulin-like growth factor-I receptor tyrosine kinases. *Metabolism,* 44**,** 2-11.

DELAFONTAINE, P., SONG, Y.-H. & LI, Y. 2004. Expression, regulation, and function of IGF-1, IGF-1R, and IGF-1 binding proteins in blood vessels. *Arteriosclerosis, Thrombosis, and Vascular Biology,* 24**,** 435-444.

DEPALO, R., NAPPI, L., LOVERRO, G., BETTOCCHI, S., CARUSO, M. L., VALENTINI, A. M. & SELVAGGI, L. 2003. Evidence of apoptosis in human primordial and primary follicles. *Human Reproduction,* 18**,** 2678-2682.

DEVOTO, L., K. CHRISTENSON, L., M. MCALLISTER, J., MAKRIGIANNAKIS, A. & F. STRAUSS III, J. 1999. Insulin and insulin-like growth factor-I and-II modulate human granulosa–lutein cell steroidogenesis: enhancement of steroidogenic acute regulatory protein (StAR) expression. *Molecular Human Reproduction,* 5**,** 1003-1010.

DI PIETRO, M., PARBORELL, F., IRUSTA, G., PASCUALI, N., BAS, D., BIANCHI, M. S., TESONE, M. & ABRAMOVICH, D. 2015. Metformin

regulates ovarian angiogenesis and follicular development in a female polycystic ovary syndrome rat model. *Endocrinology,* 156**,** 1453-63.

DISKIN, M., MACKEY, D., ROCHE, J. & SREENAN, J. 2003. Effects of nutrition and metabolic status on circulating hormones and ovarian follicle development in cattle. *Animal Reproduction Science,* 78**,** 345-370.

DONOVAN, P. J. & DE MIGUEL, M. P. 2003. Turning germ cells into stem cells. *Current Opinion in Genetics & Development,* 13**,** 463-471.

DOR, J., BEN-SHLOMO, I., LUNENFELD, B., PARIENTE, C., LEVRAN, D., KARASIK, A., SEPPÄLÄ, M. & MASHIACH, S. 1992. Insulin-like growth factor-I (IGF-I) may not be essential for ovarian follicular development: evidence from IGF-I deficiency. *The Journal of Clinical Endocrinology and Metabolism,* 74**,** 539-542.

DRAKE, C. J., LARUE, A., FERRARA, N. & LITTLE, C. D. 2000. VEGF regulates cell behavior during vasculogenesis. *Developmental Biology,* 224**,** 178-188.

DUAN, C. & XU, Q. 2005. Roles of insulin-like growth factor (IGF) binding proteins in regulating IGF actions. *General and Comparative Endocrinology,* 142**,** 44-52.

DUNCAN, W. C., VAN DEN DRIESCHE, S. & FRASER, H. M. 2008. Inhibition of vascular endothelial growth factor in the primate ovary up-regulates hypoxia-inducible factor-1α in the follicle and corpus luteum. *Endocrinology,* 149**,** 3313-3320.

DUNFORD, L. J., SINCLAIR, K. D., KWONG, W. Y., STURROCK, C., CLIFFORD, B. L., GILES, T. C. & GARDNER, D. S. 2014. Maternal protein-energy malnutrition during early pregnancy in sheep impacts the fetal ornithine cycle to reduce fetal kidney microvascular development. *The FASEB Journal,* 28**,** 4880-4892.

DUNWOODIE, S. L. 2009. The role of hypoxia in development of the Mammalian embryo. *Developmental Cell,* 17**,** 755-773.

DUPONT, C., CORDIER, A., JUNIEN, C., MANDON-PÉPIN, B., LEVY, R. & CHAVATTE-PALMER, P. 2012. Maternal environment and the

reproductive function of the offspring. *Theriogenology,* 78**,** 1405-1414.

DUPONT, J. & HOLZENBERGER, M. 2003. Biology of insulin-like growth factors in development. *Birth Defects Research Part C: Embryo Today: Reviews,* 69**,** 257-271.

DURLINGER, A., VISSER, J. A. & THEMMEN, A. 2002. Regulation of ovarian function: the role of anti-Mullerian hormone. *Reproduction,* 124**,** 601-609.

E PAULA, L. D. C., ANDRZEJEWSKI, J., JULIAN, D., SPICER, L. & HANSEN, P. 2008. Oxygen and steroid concentrations in preovulatory follicles of lactating dairy cows exposed to acute heat stress. *Theriogenology,* 69**,** 805-813.

ERICKSON, B. 1966. Development and radio-response of the prenatal bovine ovary. *Journal of Reproduction and Fertility,* 11**,** 97-105.

ESFAHANIAN, N., SHAKIBA, Y., NIKBIN, B., SORAYA, H., MALEKI-DIZAJI, N., GHAZI-KHANSARI, M. & GARJANI, A. 2012. Effect of metformin on the proliferation, migration, and MMP-2 and -9 expression of human umbilical vein endothelial cells. *Molecular Medicine Reports,* 5**,** 1068-74.

EVANS, A., MOSSA, F., WALSH, S., SCHEETZ, D., JIMENEZ-KRASSEL, F., IRELAND, J., SMITH, G. & IRELAND, J. 2012. Effects of maternal environment during gestation on ovarian folliculogenesis and consequences for fertility in bovine offspring. *Reproduction in Domestic Animals,* 47**,** 31-37.

EVANS, A. C. O. & WALSH, S. W. 2011. The physiology of multifactorial problems limiting the establishment of pregnancy in dairy cattle. *Reproduction, Fertility and Development,* 24**,** 233-237.

FÁBIÁN, Z., RAMADURAI, S., SHAW, G., NASHEUER, H.-P., KOLCH, W., TAYLOR, C. & BARRY, F. 2014. Basic fibroblast growth factor modifies the hypoxic response of human bone marrow stromal cells by ERK-mediated enhancement of HIF-1α activity. *Stem Cell Research,* 12**,** 646-658.

FADHILLAH, YOSHIOKA, S., NISHIMURA, R. & OKUDA, K. 2014. Hypoxia Promotes Progesterone Synthesis During Luteinization in Bovine Granulosa Cells. *Journal of Reproduction and Development,* 60**,** 194-201.

FADHILLAH, YOSHIOKA, S., NISHIMURA, R., YAMAMOTO, Y., KIMURA, K. & OKUDA, K. 2017. Hypoxia-inducible factor 1 mediates hypoxia-enhanced synthesis of progesterone during luteinization of granulosa cells. *Journal of Reproduction and Development,* 63**,** 75-85.

FALOON, P., ARENTSON, E., KAZAROV, A., DENG, C. X., PORCHER, C., ORKIN, S. & CHOI, K. 2000. Basic fibroblast growth factor positively regulates hematopoietic development. *Development,* 127**,** 1931-1941.

FEDERICI, M., PORZIO, O., ZUCARO, L., FUSCO, A., BORBONI, P., LAURO, D. & SESTI, G. 1997. Distribution of insulin/insulin-like growth factor-I hybrid receptors in human tissues. *Molecular and Cellular Endocrinology,* 129**,** 121-126.

FENG, Y., CUI, P., LU, X., HSUEH, B., BILLIG, F. M., YANEZ, L. Z., TOMER, R., BOERBOOM, D., CARMELIET, P. & DEISSEROTH, K. 2017. CLARITY reveals dynamics of ovarian follicular architecture and vasculature in three-dimensions. *Scientific Reports,* 7**,** 44810.

FENWICK, M. A., FITZPATRICK, R., KENNY, D. A., DISKIN, M. G., PATTON, J., MURPHY, J. J. & WATHES, D. C. 2008. Interrelationships between negative energy balance (NEB) and IGF regulation in liver of lactating dairy cows. *Domestic Animal Endocrinology,* 34**,** 31-44.

FERRARA, N. 2005. The role of VEGF in the regulation of physiological and pathological angiogenesis. *Mechanisms of angiogenesis.* Springer. Pages 209 - 231.

FERRARA, N., CHEN, H., DAVIS-SMYTH, T., GERBER, H.-P., NGUYEN, T.-N., PEERS, D., CHISHOLM, V., HILLAN, K. J. & SCHWALL, R. H. 1998. Vascular endothelial growth factor is essential for corpus luteum angiogenesis. *Nature Medicine,* 4**,** 336.

FERRARA, N. & DAVIS-SMYTH, T. 1997. The biology of vascular endothelial growth factor. *Endocrine Reviews,* 18**,** 4-25.

FERRARA, N., GERBER, H.-P. & LECOUTER, J. 2003. The biology of VEGF and its receptors. *Nature Medicine,* 9**,** 669-676.

FIELD, S. L., DASGUPTA, T., CUMMINGS, M. & ORSI, N. M. 2014. Cytokines in ovarian folliculogenesis, oocyte maturation and luteinisation. *Molecular Reproduction and Development,* 81**,** 284-314.

FINDLAY, J. 1995. Molecular Biology of the Female Reproductive System. *General and Comparative Endocrinology,* 98**,** 332.

FINDLAY, J. K. & DRUMMOND, A. E. 1999. Regulation of the FSH receptor in the ovary. *Trends in Endocrinology and Metabolism,* 10**,** 183-188.

FINDLAY, J. K., HUTT, K. J., HICKEY, M. & ANDERSON, R. A. 2015. How is the number of primordial follicles in the ovarian reserve established? *Biology of Reproduction,* 93**,** 111, 1-7.

FIRTH, J., EBERT, B., PUGH, C. & RATCLIFFE, P. 1994. Oxygen-regulated control elements in the phosphoglycerate kinase 1 and lactate dehydrogenase A genes: similarities with the erythropoietin 3'enhancer. *Proceedings of the National Academy of Sciences,* 91**,** 6496-6500.

FIRTH, J. D., EBERT, B. L. & RATCLIFFE, P. J. 1995. Hypoxic regulation of lactate dehydrogenase A Interaction between hypoxia-inducible factor 1 and cAMP response elements. *Journal of Biological Chemistry,* 270**,** 21021-21027.

FIRTH, S. M. & BAXTER, R. C. 2002. Cellular actions of the insulin-like growth factor binding proteins. *Endocrine Reviews,* 23**,** 824-854.

FISCHER, B. & BAVISTER, B. 1993. Oxygen tension in the oviduct and uterus of rhesus monkeys, hamsters and rabbits. *Journal of Reproduction and Fertility,* 99**,** 673-679.

FISCHER, B., KÜNZEL, W., KLEINSTEIN, J. & GIPS, H. 1992. Oxygen tension in follicular fluid falls with follicle maturation. *European Journal of Obstetrics and Gynecology,* 43**,** 39-43.

FISKE, R. & ADAMS, L. 1985. Immune responsiveness and lymphoreticular morphology in cattle fed hypo-and hyperalimentative diets. *Veterinary Immunology and Immunopathology,* 8**,** 225-244.

FITZ, T., MAYAN, M., SAWYER, H. & NISWENDER, G. 1982. Characterization of two steroidogenic cell types in the ovine corpus luteum. *Biology of Reproduction,* 27**,** 703-711.

FLAMME, I. & RISAU, W. 1992. Induction of vasculogenesis and hematopoiesis in vitro. *Development,* 116**,** 435-439.

FORTUNE, J., CUSHMAN, R., WAHL, C. & KITO, S. 2000. The primordial to primary follicle transition. *Molecular and Cellular Endocrinology,* 163**,** 53-60.

FORTUNE, J. E. 1988. Ovarian follicular dynamics during the estrous cycle in heifers monitored by real-time ultrasonography. *Biology of Reproduction,* 39**,** 308-317.

FRASER, H. M., BELL, J., WILSON, H., TAYLOR, P. D., MORGAN, K., ANDERSON, R. A. & DUNCAN, W. C. 2005. Localization and quantification of cyclic changes in the expression of endocrine gland vascular endothelial growth factor in the human corpus luteum. *The Journal of Clinical Endocrinology and Metabolism,* 90**,** 427-434.

FRASER, H. M. & LUNN, S. F. 2001. Regulation and manipulation of angiogenesis in the primate corpus luteum. *Reproduction,* 121**,** 355-362.

FRASER, H. M. & WULFF, C. 2001. Angiogenesis in the primate ovary. *Reproduction, Fertility and Development,* 13**,** 557-566.

FREDRIKSSON, L., LI, H. & ERIKSSON, U. 2004. The PDGF family: four gene products form five dimeric isoforms. *Cytokine & Growth Factor Reviews,* 15**,** 197-204.

FRIEDRICH, N., WOLTHERS, O. D., ARAFAT, A. M., EMENY, R. T., SPRANGER, J., ROSWALL, J., KRATZSCH, J., GRABE, H. J., HÜBENER, C. & PFEIFFER, A. F. 2014. Age-and sex-specific reference intervals across life span for insulin-like growth factor binding protein 3 (IGFBP-3) and the IGF-I to IGFBP-3 ratio measured by new automated chemiluminescence assays. *The Journal of Clinical Endocrinology and Metabolism,* 99**,** 1675-1686.

GAO, J., YE, J., YING, Y., LIN, H. & LUO, Z. 2018. Negative regulation of TGF-β by AMPK and implications in the treatment of associated disorders. *Acta Biochimica et Biophysica Sinica,* 50**,** 523-531.

GARDNER, B. A., DOLEZAL, H. G., OWENS, F. N., BRYANT, L. K., NELSON, J. L., SCHUTTE, B. R. & SMITH, R. A. 1998. Impact of health on profitability of feedlot steers. *Oklaham Agriculture Experiment Station, Animal Science Research Report. Pages 102 - 108*.

GARDNER, D., BUTTERY, P., DANIEL, Z. & SYMONDS, M. 2007. Factors affecting birth weight in sheep: maternal environment. *Reproduction,* 133**,** 297-307.

GARDNER, D. S., OZANNE, S. E. & SINCLAIR, K. D. 2009. Effect of the early-life nutritional environment on fecundity and fertility of mammals. *Philosophical Transactions of the Royal Society of London Biological Sciences,* 364**,** 3419-3427.

GARMEY, J. C., DAY, R. N., DAY, K. H. & VELDHUIS, J. D. 1993. Mechanisms of regulation of ovarian sterol metabolism by insulin-like growth factor type II: in vitro studies with swine granulosa cells. *Endocrinology,* 133**,** 800-808.

GARVERICK, H., JUENGEL, J., SMITH, P., HEATH, D., BURKHART, M., PERRY, G., SMITH, M. & MCNATTY, K. 2010. Development of the ovary and ontongeny of mRNA and protein for P450 aromatase (arom) and estrogen receptors (ER) α and β during early fetal life in cattle. *Animal Reproduction Science,* 117**,** 24-33.

GAYTAN, F., MORALES, C., GARCIA-PARDO, L., REYMUNDO, C., BELLIDO, C. & SANCHEZ-CRIADO, J. E. 1999. A quantitative study of changes

in the human corpus luteum microvasculature during the menstrual cycle. *Biology of Reproduction,* 60**,** 914-919.

GEALEKMAN, O., GURAV, K., STRAUBHAAR, J., THOMPSON, M., MALKANI, S., HARTIGAN, C. & CORVERA, S. 2014. Control of adipose tissue expandability in response to high fat diet by the insulin-like growth factor-binding protein-4. *Journal of Biological Chemistry,* 289**,** 18327-18338.

GERHARDT, H. & BETSHOLTZ, C. 2003. Endothelial-pericyte interactions in angiogenesis. *Cell and Tissue Research,* 314**,** 15-23.

GINTHER, O., BERGFELT, D., BEG, M. & KOT, K. 2001. Follicle selection in cattle: role of luteinizing hormone. *Biology of Reproduction,* 64**,** 197-205.

GLASS, C., HARPER, S. & BATES, D. 2006. The anti-angiogenic VEGF isoform VEGF165b transiently increases hydraulic conductivity, probably through VEGF receptor 1 in vivo. *The Journal of Physiology,* 572**,** 243-257.

GLUCKMAN, P. & AMBLER, G. 1993. What is the function of circulating insulin-like growth factor-2 in postnatal life? *Molecular and Cellular Endocrinology,* 92**,** C1-C3.

GONG, J. G., BRAMLEY, T. & WEBB, R. 1991. The effect of recombinant bovine somatotropin on ovarian function in heifers: follicular populations and peripheral hormones. *Biology of Reproduction,* 45**,** 941-949.

GONZALEZ, J., CAMACHO, L., EBARB, S., SWANSON, K., VONNAHME, K., STELZLENI, A. & JOHNSON, S. 2013. Realimentation of nutrient restricted pregnant beef cows supports compensatory fetal muscle growth. *Journal of Animal Science,* 91**,** 4797-4806.

GOOTWINE, E., SPENCER, T. E. & BAZER, F. W. 2007. Litter-size-dependent intrauterine growth restriction in sheep. *The Animal Consortium. Pages 547–564*.

GOSPODAROWICZ, D., CHENG, J., LUI, G., BAIRD, A., ESCH, F. & BOHLEN, P. 1985. Corpus luteum angiogenic factor is related to fibroblast growth factor. *Endocrinology,* 117**,** 2383-2391.

GOUAZÉ-ANDERSSON, V., DELMAS, C., TAURAND, M., MARTINEZ-GALA, J., EVRARD, S., MAZOYER, S., TOULAS, C. & MOYAL, E. C.-J. 2016. FGFR1 induces glioblastoma radioresistance through the PLCγ/Hif1α pathway. *Cancer Research***,** 2015 -2058.

GRANATA, R., TROVATO, L., GARBARINO, G., TALIANO, M., PONTI, R., SALA, G., GHIDONI, R. & GHIGO, E. 2004. Dual effects of IGFBP-3 on endothelial cell apoptosis and survival: involvement of the sphingolipid signaling pathways. *The FASEB Journal,* 18**,** 1456-1458.

GRANATA, R., TROVATO, L., LUPIA, E., SALA, G., SETTANNI, F., CAMUSSI, G., GHIDONI, R. & GHIGO, E. 2007. Insulin-like growth factor binding protein-3 induces angiogenesis through IGF-I-and SphK1-dependent mechanisms. *Journal of Thrombosis and Haemostasis,* 5**,** 835-845.

GRANT, M., MAMES, R., FITZGERALD, C., ELLIS, E., ABOUFRIEKHA, M. & GUY, J. 1993. Insulin-like growth factor I acts as an angiogenic agent in rabbit cornea and retina: comparative studies with basic fibroblast growth factor. *Diabetologia,* 36**,** 282-291.

GRASSELLI, F., BASINI, G., TIRELLI, M., CAVALLI, V., BUSSOLATI, S. & TAMANINI, C. 2003. Angiogenic activity of porcine granulosa cells co-cultured with endothelial cells in a microcarrier-based three-demensional fibrin gel. *Journal of Physiology and Pharmacology,* 54**,** 361-370.

GREENAWAY, J., GENTRY, P. A., FEIGE, J.-J., LAMARRE, J. & PETRIK, J. J. 2005. Thrombospondin and vascular endothelial growth factor are cyclically expressed in an inverse pattern during bovine ovarian follicle development. *Biology of Reproduction,* 72**,** 1071-1078.

GREENWOOD, P. & CAFE, L. 2007. Prenatal and pre-weaning growth and nutrition of cattle: long-term consequences for beef production. *Animal,* 1**,** 1283-1296.

GREENWOOD, P. L., BELL, A. W., VERCOE, P. E. & VILJOEN, G. J. 2010. *Managing the prenatal environment to enhance livestock productivity*, Springer. Pages 3 - 37.

GRIMES, R. W. & HAMMOND, J. M. 1992. Insulin and insulin-like growth factors (IGFs) stimulate production of IGF-binding proteins by ovarian granulosa cells. *Endocrinology,* 131**,** 553-558.

GRUNERT, E., SCHULZ, L. C. & ESSER, J. 1973. The effect of intrauterine injections of iodine solution on the duration of the oestrous cycle in cows. *Journal of Reproduction and Fertility,* 33**,** 497-499.

GULL, I., GEVA, E., LERNER-GEVA, L., LESSING, J. B., WOLMAN, I. & AMIT, A. 1999. Anaerobic glycolysis: the metabolism of the preovulatory human oocyte. *European Journal of Obstetrics & Gynecology and Reproductive Biology,* 85**,** 225-228.

GUNN, R., SIM, D. & HUNTER, E. 1995. Effects of nutrition in utero and in early life on the subsequent lifetime reproductive performance of Scottish Blackface ewes in two management systems. *Animal Science,* 60**,** 223-230.

GUO, C.-L., LIU, L., JIA, Y.-D., ZHAO, X.-Y., ZHOU, Q. & WANG, L. 2012. A novel variant of Oct3/4 gene in mouse embryonic stem cells. *Stem Cell Research,* 9**,** 69-76.

HACKETT, J. A., REDDINGTON, J. P., NESTOR, C. E., DUNICAN, D. S., BRANCO, M. R., REICHMANN, J., REIK, W., SURANI, M. A., ADAMS, I. R. & MEEHAN, R. R. 2012. Promoter DNA methylation couples genome-defence mechanisms to epigenetic reprogramming in the mouse germline. *Development,* 139**,** 3623-3632.

HAFEZ, E. S. E. & HAFEZ, B. 2000. *Reproduction in farm animals*, 7th Edition,Baltimore, Maryland, USA, Blackwell Publishing. Pages 13 - 30.

HALES, C., DESAI, M., OZANNE, S. & CROWTHER, N. 1996. Fishing in the stream of diabetes: from measuring insulin to the control of fetal organogenesis. Biochemical Society Transactions, 24, 341 -350

HANKINS, G. R., DE, A. S., BENTLEY, R. C., PATEL, M. R., MARKS, J. R., IGLEHART, J. D. & JIRTLE, R. L. 1996. M6P/IGF2 receptor: a candidate breast tumor suppressor gene. *Oncogene,* 12**,** 2003-2009.

HANSSON, H.-A., BRANDSTEN, C., LOSSING, C. & PETRUSON, K. 1989. Transient expression of insulin-like growth factor I immunoreactivity by vascular cells during angiogenesis. *Experimental and Molecular Pathology,* 50**,** 125-138.

HARDING, J. & JOHNSTON, B. 1995. Nutrition and fetal growth. *Reproduction, Fertility and Development,* 7**,** 539-547.

HARRIS, S. E., ADRIAENS, I., LEESE, H. J., GOSDEN, R. G. & PICTON, H. M. 2007. Carbohydrate metabolism by murine ovarian follicles and oocytes grown in vitro. *Reproduction,* 134**,** 415-424.

HASTON, K. M., TUNG, J. Y. & PERA, R. A. R. 2009. Dazl functions in maintenance of pluripotency and genetic and epigenetic programs of differentiation in mouse primordial germ cells in vivo and in vitro. *PLOS One,* 4**,** 5654.

HATZIRODOS, N., HUMMITZSCH, K., IRVING-RODGERS, H. F., HARLAND, M. L., MORRIS, S. E. & RODGERS, R. J. 2014. Transcriptome profiling of granulosa cells from bovine ovarian follicles during atresia. *BMC Genomics,* 15**,** 40.

HAZZARD, T. M. & STOUFFER, R. L. 2000. Angiogenesis in ovarian follicular and luteal development. *Best Practice and Research Clinical Obstetrics and Gynaecology,* 14**,** 883-900.

HE, J., STEWART, K., KINNELL, H. L., ANDERSON, R. A. & CHILDS, A. J. 2013. A developmental stage-specific switch from DAZL to BOLL occurs during fetal oogenesis in humans, but not mice. *PLOS One,* 8**,** 73996.

HEASMAN, L., CLARKE, L., STEPHENSON, T. & SYMONDS, M. 1999. The influence of maternal nutrient restriction in early to mid-pregnancy on placental and fetal development in sheep. *Proceedings of the Nutrition Society,* 58**,** 283-288.

HELDIN, C.-H. & ÖSTMAN, A. 1996. Ligand-induced dimerization of growth factor receptors: variations on the theme. *Cytokine & Growth Factor Reviews,* 7**,** 3-10.

HERMANN, B. P., SUKHWANI, M., LIN, C. C., SHENG, Y., TOMKO, J., RODRIGUEZ, M., SHUTTLEWORTH, J. J., MCFARLAND, D., HOBBS, R. M. & PANDOLFI, P. P. 2007. Characterization, cryopreservation, and ablation of spermatogonial stem cells in adult rhesus macaques. *Stem Cells,* 25**,** 2330-2338.

HERZOG, K., BROCKHAN-LÜDEMANN, M., KASKE, M., BEINDORFF, N., PAUL, V., NIEMANN, H. & BOLLWEIN, H. 2010. Luteal blood flow is a more appropriate indicator for luteal function during the bovine estrous cycle than luteal size. *Theriogenology,* 73**,** 691-697.

HERZOG, K., VOSS, C., KASTELIC, J., BEINDORFF, N., PAUL, V., NIEMANN, H. & BOLLWEIN, H. 2011. Luteal blood flow increases during the first three weeks of pregnancy in lactating dairy cows. *Theriogenology,* 75**,** 549-554.

HILLENSJÖ, T. 1976. Oocyte maturation and glycolysis in isolated pre-ovulatory follicles of PMS-injected immature rats. *Acta Endocrinologica,* 82**,** 809-830.

HILLIER, S., PUROHIT, A. & REICHERT JR, L. 1985. Control of granulosa cell lactate production by follicle-stimulating hormone and androgen. *Endocrinology,* 116**,** 1163-1167.

HOEBEN, A., LANDUYT, B., HIGHLEY, M. S., WILDIERS, H., VAN OOSTEROM, A. T. & DE BRUIJN, E. A. 2004. Vascular endothelial growth factor and angiogenesis. *Pharmacological Reviews,* 56**,** 549-580.

HUANG, L. E., WILLMORE, W. G., GU, J., GOLDBERG, M. A. & BUNN, H. F. 1999. Inhibition of hypoxia-inducible factor 1 activation by carbon monoxide and nitric oxide Implications for oxygen sensing and signaling. *Journal of Biological Chemistry,* 274**,** 9038-9044.

HUBER, T. L., KOUSKOFF, V., FEHLING, H. J., PALIS, J. & KELLER, G. 2004. Haemangioblast commitment is initiated in the primitive streak of the mouse embryo. *Nature,* 432**,** 625.

HUDSON, C. D. & GREEN, M. J. 2018. Associations between routinely collected Dairy Herd Improvement data and insemination outcome in UK dairy herds. *Journal of dairy science,* 101**,** 11262-11274.

HUEY, S., ABUHAMAD, A., BARROSO, G., HSU, M.-I., KOLM, P., MAYER, J. & OEHNINGER, S. 1999. Perifollicular blood flow Doppler indices, but not follicular pO2, pCO2, or pH, predict oocyte developmental competence in in vitro fertilization. *Fertility and Sterility,* 72**,** 707-712.

HUMMITZSCH, K., ANDERSON, R. A., WILHELM, D., WU, J., TELFER, E. E., RUSSELL, D. L., ROBERTSON, S. A. & RODGERS, R. J. 2014. Stem Cells, Progenitor Cells, and Lineage Decisions in the Ovary. *Endocrine Reviews,* 36**,** 65-91.

HUMMITZSCH, K., ANDERSON, R. A., WILHELM, D., WU, J., TELFER, E. E., RUSSELL, D. L., ROBERTSON, S. A. & RODGERS, R. J. 2015. Stem cells, progenitor cells, and lineage decisions in the ovary. *Endocrine Reviews,* 36**,** 65-91.

HUMMITZSCH, K., IRVING-RODGERS, H. F., HATZIRODOS, N., BONNER, W., SABATIER, L., REINHARDT, D. P., SADO, Y., NINOMIYA, Y., WILHELM, D. & RODGERS, R. J. 2013. A new model of development of the mammalian ovary and follicles. *PLOS One,* 8**,** e55578.

HÜNIGEN, H., BISPLINGHOFF, P., PLENDL, J. & BAHRAMSOLTANI, M. 2008. Vascular dynamics in relation to immunolocalisation of VEGF-A, VEGFR-2 and Ang-2 in the bovine corpus luteum. *Acta Histochemica,* 110**,** 462-472.

ILIEV, D., KANNENBERG, K., WEBER, K. & BINDER, G. 2014. IGF-I sensitivity in Silver–Russell syndrome with IGF2/H19 hypomethylation. *Growth Hormone & IGF Research,* 24**,** 187-191.

IVANOVIC, Z. 2009. Hypoxia or in situ normoxia: The stem cell paradigm. *Journal of Cellular Physiology,* 219**,** 271-275.

JAAKKOLA, P., MOLE, D. R., TIAN, Y.-M., WILSON, M. I., GIELBERT, J., GASKELL, S. J., VON KRIEGSHEIM, A., HEBESTREIT, H. F., MUKHERJI, M. & SCHOFIELD, C. J. 2001. Targeting of HIF-α to the

von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. *Science,* 292**,** 468-472.

JABLONKA-SHARIFF, A., GRAZUL-BILSKA, A., REDMER, D. & REYNOLDS, L. 1993. Growth and cellular proliferation of ovine corpora lutea throughout the estrous cycle. *Endocrinology,* 133**,** 1871-1879.

JACOBS, S., KULL, F., EARP, H. S., SVOBODA, M., VAN WYK, J. & CUATRECASAS, P. 1983. Somatomedin-C stimulates the phosphorylation of the beta-subunit of its own receptor. *Journal of Biological Chemistry,* 258**,** 9581-9584.

JIANG, B.-H., SEMENZA, G. L., BAUER, C. & MARTI, H. H. 1996. Hypoxia-inducible factor 1 levels vary exponentially over a physiologically relevant range of O2 tension. *American Journal of Physiology-Cell Physiology,* 271**,** C1172-C1180.

JIANG, J., MACCHIARELLI, G., TSANG, B. & SATO, E. 2003. Capillary angiogenesis and degeneration in bovine ovarian antral follicles. *Reproduction,* 125**,** 211-223.

JIANG, Y. F., TSUI, K. H., WANG, P. H., LIN, C. W., WANG, J. Y., HSU, M. C., CHEN, Y. C. & CHIU, C. H. 2011a. Hypoxia regulates cell proliferation and steroidogenesis through protein kinase A signaling in bovine corpus luteum. *Animal Reproduction Science,* 129**,** 152-161.

JIANG, Z. L., RIPAMONTE, P., BURATINI, J., PORTELA, V. & PRICE, C. 2011b. Fibroblast growth factor-2 regulation of sprouty and NR4A genes in bovine ovarian granulosa cells. *Journal of Cellular Physiology,* 226**,** 1820-1827.

JIMENEZ-KRASSEL, F., FOLGER, J., IRELAND, J., SMITH, G., HOU, X., DAVIS, J., LONERGAN, P., EVANS, A. & IRELAND, J. 2009. Evidence that high variation in ovarian reserves of healthy young adults has a negative impact on the corpus luteum and endometrium during estrous cycles in cattle. *Biology of Reproduction,* 80**,** 1272-1281.

JOHNSON, S., NGUYEN, V. & CODER, D. 2013. Assessment of cell viability. *Current Protocols in Cytometry***,** 9.2. 1-9.2. 26.

JONES, J. I. & CLEMMONS, D. R. 1995. Insulin-like growth factors and their binding proteins: biological actions. *Endocrine Reviews,* 16**,** 3-34.

JUENGEL, J. L., GARVERICK, H. A., JOHNSON, A. L., YOUNGQUIST, R. S. & SMITH, M. F. 1993. Apoptosis during luteal regression in cattle. *Endocrinology,* 132**,** 249-254.

JUENGEL, J. L., LARRICK, T. L., MEBERG, B. M. & NISWENDER, G. D. 1998. Luteal expression of steroidogenic factor-1 mRNA during the estrous cycle and in response to luteotropic and luteolytic stimuli in ewes. *Endocrine,* 9**,** 227-232.

JUENGEL, J. L., SAWYER, H. R., SMITH, P. R., QUIRKE, L. D., HEATH, D. A., LUN, S. & MCNATTY, K. P. 2002. Origins of follicular cells and ontogeny of steroidogenesis in ovine fetal ovaries. *Molecular and Cellular Endocrinology,* 191**,** 1-10.

JUENGEL, J. L. & SMITH, P. 2014. Formation of Ovarian follicles in ruminants. *In:* JL JUENGEL, A. M., C PRICE, LP REYNOLDS, MF SMITH AND R WEBB (ed.) *Ninth International Symposium on Reproduction in Domestic Ruminants.* Obhiro Hokkaido, Japan. Pages 277 - 294.

JUNGERMANN, K. & KIETZMANN, T. 1997. Role of oxygen in the zonation of carbohydrate metabolism and gene expression in liver. *Kidney International,* 51**,** 402-412.

JURGENS, M. H. 2002. *Animal feeding and nutrition*, Kendall/Hunt. Pages 588.

JUUL, A., DALGAARD, P., BLUM, W. F., BANG, P., HALL, K., MICHAELSEN, K., MÜLLER, J. & SKAKKEBAEK, N. 1995. Serum levels of insulin-like growth factor (IGF)-binding protein-3 (IGFBP-3) in healthy infants, children, and adolescents: the relation to IGF-I, IGF-II, IGFBP-1, IGFBP-2, age, sex, body mass index, and pubertal maturation. *The Journal of Clinical Endocrinology & Metabolism,* 80**,** 2534-2542.

KAJIMURA, S., AIDA, K. & DUAN, C. 2006. Understanding hypoxia-induced gene expression in early development: in vitro and in vivo analysis of hypoxia-inducible factor 1-regulated zebra fish insulin-like growth

factor binding protein 1 gene expression. *Molecular and Cellular Biology,* 26**,** 1142-1155.

KALLEN, C. B., BILLHEIMER, J. T., SUMMERS, S. A., STAYROOK, S. E., LEWIS, M. & STRAUSS, J. F. 1998. Steroidogenic acute regulatory protein (StAR) is a sterol transfer protein. *Journal of Biological Chemistry,* 273**,** 26285-26288.

KAMURA, T., SATO, S., IWAI, K., CZYZYK-KRZESKA, M., CONAWAY, R. C. & CONAWAY, J. W. 2000. Activation of HIF1α ubiquitination by a reconstituted von Hippel-Lindau (VHL) tumor suppressor complex. *Proceedings of the National Academy of Sciences,* 97**,** 10430-10435.

KANE, K., CREIGHTON, K., PETERSEN, M., HALLFORD, D., REMMENGA, M. & HAWKINS, D. 2002. Effects of varying levels of undegradable intake protein on endocrine and metabolic function of young post-partum beef cows. *Theriogenology,* 57**,** 2179-2191.

KANG, D.-H., JOLY, A. H., OH, S.-W., HUGO, C., KERJASCHKI, D., GORDON, K. L., MAZZALI, M., JEFFERSON, J. A., HUGHES, J. & MADSEN, K. M. 2001. Impaired angiogenesis in the remnant kidney model: I. Potential role of vascular endothelial growth factor and thrombospondin-1. *Journal of the American Society of Nephrology,* 12**,** 1434-1447.

KAPLAN, S. A. & COHEN, P. 2007. The somatomedin hypothesis 2007: 50 years later. *The Journal of Clinical Endocrinology & Metabolism,* 92**,** 4529-4535.

KEHLER, J., TOLKUNOVA, E., KOSCHORZ, B., PESCE, M., GENTILE, L., BOIANI, M., LOMELÍ, H., NAGY, A., MCLAUGHLIN, K. J. & SCHÖLER, H. R. 2004. Oct4 is required for primordial germ cell survival. *EMBO Reports,* 5**,** 1078-1083.

KELLEMS, R. O. & CHURCH, D. C. 2002. *Livestock feeds and feeding*, 5th Edition, Prentice Hall Upper saddle River, New Jersery. Pages 86 - 116.

KENYON, P., STAFFORD, K., JENKINSON, C., MORRIS, S. & WEST, D. 2007. The body composition and metabolic status of twin-and triplet-

bearing ewes and their fetuses in late pregnancy. *Livestock Science,* 107**,** 103-112.

KENYON, P. & WEBBY, R. 2007. Pastures and supplements in sheep production systems. In pasture and supplements for grazing animals (Eds PV Rattray, IM Brookes, AM Nicol) *New Zealand Society of Animal Production: Hamilton, New Zealand. Pages 255–274*.

KENYON, P. R. 2008. *A review of in-utero environmental effects on sheep production*, New Zealand Society of Animal Production. 68: 142-155.

KETY, S. S. & SCHMIDT, C. F. 1948. The effects of altered arterial tensions of carbon dioxide and oxygen on cerebral blood flow and cerebral oxygen consumption of normal young men. *The Journal of Clinical Investigation,* 27**,** 484-492.

KIAER, T. & KRISTENSEN, K. 1988. Intracompartmental pressure, PO 2, PCO 2 and blood flow in the human skeletal muscle. *Archives of Orthopaedic and Traumatic Surgery,* 107**,** 114-116.

KIKUTA, A., MACCHIARELLI, G. & MURAKAMI, T. 1991. Microvasculature of the ovary. *Ultrastructure of the Ovary***,** 239-254.

KIM, H.-S., INGERMANN, A. R., TSUBAKI, J., TWIGG, S. M., WALKER, G. E. & OH, Y. 2004. Insulin-like growth factor-binding protein 3 induces caspase-dependent apoptosis through a death receptor-mediated pathway in MCF-7 human breast cancer cells. *Cancer Research,* 64**,** 2229-2237.

KIM, J., BAGCHI, I. C. & BAGCHI, M. K. 2009. Signaling by hypoxia-inducible factors is critical for ovulation in mice. *Endocrinology,* 150**,** 3392-3400.

KIM, J. H., PARK, S. W., YU, Y. S., KIM, K.-W. & KIM, J. H. 2012. Hypoxia-induced insulin-like growth factor II contributes to retinal vascularization in ocular development. *Biochimie,* 94**,** 734-740.

KIM, Y.-H., KIM, B.-J., KIM, B.-G., LEE, Y.-A., KIM, K.-J., CHUNG, H.-J., HWANG, S., WOO, J.-S., PARK, J.-K. & SCHMIDT, J. 2013. Stage-specific embryonic antigen-1 expression by undifferentiated

spermatogonia in the prepubertal boar testis. *Journal of Animal Science,* 91**,** 3143-3154.

KIMURA, H., WEISZ, A., OGURA, T., HITOMI, Y., KURASHIMA, Y., HASHIMOTO, K., D'ACQUISTO, F., MAKUUCHI, M. & ESUMI, H. 2001. Identification of hypoxia-inducible factor 1 ancillary sequence and its function in vascular endothelial growth factor gene induction by hypoxia and nitric oxide. *Journal of Biological Chemistry,* 276**,** 2292-2298.

KING, G. L., GOODMAN, A., BUZNEY, S., MOSES, A. & KAHN, C. 1985. Receptors and growth-promoting effects of insulin and insulinlike growth factors on cells from bovine retinal capillaries and aorta. *The Journal of Clinical Investigation,* 75**,** 1028-1036.

KLAGSBRUN, M. & D'AMORE, P. A. 1996. Vascular endothelial growth factor and its receptors. *Cytokine & Growth Factor Reviews,* 7**,** 259-270.

KLIPPER, E., LEVIT, A., MASTICH, Y., BERISHA, B., SCHAMS, D. & MEIDAN, R. 2010. Induction of endothelin-2 expression by luteinizing hormone and hypoxia: possible role in bovine corpus luteum formation. *Endocrinology,* 151**,** 1914-1922.

KOBAYASHI, Y., BOYD, C., BRACKEN, C., LAMBERSON, W., KEISLER, D. & LUCY, M. 1999. Reduced growth hormone receptor (GHR) messenger ribonucleic acid in liver of periparturient cattle is caused by a specific down-regulation of GHR 1A that is associated with decreased insulin-like growth factor I. *Endocrinology,* 140**,** 3947-3954.

KONG, D., PARK, E. J., STEPHEN, A. G., CALVANI, M., CARDELLINA, J. H., MONKS, A., FISHER, R. J., SHOEMAKER, R. H. & MELILLO, G. 2005. Echinomycin, a small-molecule inhibitor of hypoxia-inducible factor-1 DNA-binding activity. *Cancer Research,* 65**,** 9047-9055.

KÖNIG, H., AMSELGRUBER, W. & RÜSSE, I. 1988. Zur Mikrozirkulation in Follikeln und Corpora lutea des Rinderovars-eine korrosionsanatomische Studie. *Tierärztl. Prax,* 16**,** 25-31.

KOOS, R. D. & FEIERTAG, M. A. 1989. The effect of reduced oxygen tension on progesterone accumulation in rat granulosa cell cultures. *Steroids,* 54**,** 553-562.

KOOS, R. D. & OLSON, C. E. 1989. Expression of basic fibroblast growth factor in the rat ovary: detection of mRNA using reverse transcription-polymerase chain reaction amplification. *Molecular Endocrinology,* 3**,** 2041-2048.

KOWALEWSKI, M. P., GRAM, A. & BOOS, A. 2015. The role of hypoxia and HIF1α in the regulation of STAR-mediated steroidogenesis in granulosa cells. *Molecular and Cellular Endocrinology,* 401**,** 35-44.

KWON, H., CHOI, D.-H., BAE, J.-H., KIM, J.-H. & KIM, Y.-S. 2010. mRNA expression pattern of insulin-like growth factor components of granulosa cells and cumulus cells in women with and without polycystic ovary syndrome according to oocyte maturity. *Fertility and Sterility,* 94**,** 2417-2420.

LAEMMLI, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature,* 227**,** 680.

LAIRD, M., WOAD, K. J., HUNTER, M. G., MANN, G. E. & ROBINSON, R. S. 2013. Fibroblast growth factor 2 induces the precocious development of endothelial cell networks in bovine luteinising follicular cells. *Reproduction, Fertility and Development,* 25**,** 372-386.

LANGLEY-EVANS, S. C. & SCULLEY, D. V. 2006. The association between birthweight and longevity in the rat is complex and modulated by maternal protein intake during fetal life. *FEBS Letters,* 580**,** 4150-4153.

LANGLEY, S. C., BROWNE, R. F. & JACKSON, A. A. 1994. Altered glucose tolerance in rats exposed to maternal low protein diets in utero. *Comparative Biochemistry and Physiology Part A: Physiology,* 109**,** 223-229.

LANGLEY, S. C. & JACKSON, A. A. 1994. Increased systolic blood pressure in adult rats induced by fetal exposure to maternal low protein diets. *Clinical Science,* 86**,** 217-222.

LARSSON, O. & AXELSON, M. 2007. Cyclolignans as inhibitors of the insulin-like growth factor-I receptor. *Cancer Research,* 67**,** 2899-2899.

LAUGHNER, E., TAGHAVI, P., CHILES, K., MAHON, P. C. & SEMENZA, G. L. 2001. HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1α (HIF-1α) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression. *Molecular and Cellular Biology,* 21**,** 3995-4004.

LAVOIE, H. A., SINGH, D. & HUI, Y. Y. 2004. Concerted regulation of the porcine steroidogenic acute regulatory protein gene promoter activity by follicle-stimulating hormone and insulin-like growth factor I in granulosa cells involves GATA-4 and CCAAT/enhancer binding protein β. *Endocrinology,* 145**,** 3122-3134.

LAW, A. Y. & WONG, C. K. 2013. Stanniocalcin-1 and -2 promote angiogenic sprouting in HUVECs via VEGF/VEGFR2 and angiopoietin signaling pathways. *Molecular Cell Endocrinology,* 374**,** 73-81.

LE, Q.-T., CHEN, E., SALIM, A., CAO, H., KONG, C. S., WHYTE, R., DONINGTON, J., CANNON, W., WAKELEE, H. & TIBSHIRANI, R. 2006. An Evaluation of tumor oxygenation and gene expression in patients with early stage non–small cell lung cancers. *Clinical Cancer Research,* 12**,** 1507-1514.

LE ROITH, D., BONDY, C., YAKAR, S., LIU, J.-L. & BUTLER, A. 2001. The somatomedin hypothesis: 2001. *Endocrine Reviews,* 22**,** 53-74.

LEA, R. G., ANDRADE, L., RAE, M., HANNAH, L. T., KYLE, C., MURRAY, J. F., RHIND, S. & MILLER, D. 2006. Effects of maternal undernutrition during early pregnancy on apoptosis regulators in the ovine fetal ovary. *Reproduction,* 131**,** 113-124.

LEDDA, S., BOGLIOLO, L., BEBBERE, D., ARIU, F. & PIRINO, S. 2010. Characterization, isolation and culture of primordial germ cells in domestic animals: recent progress and insights from the ovine species. *Theriogenology,* 74**,** 534-543.

LEE, D. C., SOHN, H. A., PARK, Z. Y., OH, S., KANG, Y. K., LEE, K. M., KANG, M., JANG, Y. J., YANG, S. J., HONG, Y. K., NOH, H., KIM, J. A., KIM,

D. J., BAE, K. H., KIM, D. M., CHUNG, S. J., YOO, H. S., YU, D. Y., PARK, K. C. & YEOM, Y. I. 2015. A lactate-induced response to hypoxia. *Cell,* 161**,** 595-609.

LEE, J.-W., BAE, S.-H., JEONG, J.-W., KIM, S.-H. & KIM, K.-W. 2004. Hypoxia-inducible factor (HIF-1) α: its protein stability and biological functions. *Experimental & Molecular Medicine,* 36**,** 1.

LEROITH, D. & YAKAR, S. 2007. Mechanisms of disease: metabolic effects of growth hormone and insulin-like growth factor 1. *Nature Reviews Endocrinology,* 3**,** 302.

LEROITH, D. L. 2003. The insulin-like growth factor system. *Journal of Diabetes Research,* 4**,** 205-212.

LI, G., BARRETT, E. J., WANG, H., CHAI, W. & LIU, Z. 2005. Insulin at physiological concentrations selectively activates insulin but not insulin-like growth factor I (IGF-I) or insulin/IGF-I hybrid receptors in endothelial cells. *Endocrinology,* 146**,** 4690-4696.

LIN, Y. & PAGE, D. C. 2005. Dazl deficiency leads to embryonic arrest of germ cell development in XY C57BL/6 mice. *Developmental Biology,* 288**,** 309-316.

LIU, J., KOENIGSFELD, A. T., CANTLEY, T. C., BOYD, C. K., KOBAYASHI, Y. & LUCY, M. C. 2000. Growth and the initiation of steroidogenesis in porcine follicles are associated with unique patterns of gene expression for individual componentsof the ovarian insulin-like growth factor system. *Biology of Reproduction,* 63**,** 942-952.

LIU, L., CASH, T. P., JONES, R. G., KEITH, B., THOMPSON, C. B. & SIMON, M. C. 2006. Hypoxia-induced energy stress regulates mRNA translation and cell growth. *Mol Cell,* 21**,** 521-31.

LIU, X., MALKOWSKI, M., GUO, Y., ERICKSON, G. F., SHIMASAKI, S. & LING, N. 1993. Development of specific antibodies to rat insulin-like growth factor-binding proteins (IGFBP-2 to-6): analysis of IGFBP production by rat granulosa cells. *Endocrinology,* 132**,** 1176-1183.

LOGSDON, E. A., FINLEY, S. D., POPEL, A. S. & GABHANN, F. M. 2014. A systems biology view of blood vessel growth and remodelling. *Journal of Cellular and Molecular Medicine,* 18**,** 1491-1508.

LONG, N., PRADO-COOPER, M., KREHBIEL, C., DESILVA, U. & WETTEMANN, R. 2010. Effects of nutrient restriction of bovine dams during early gestation on postnatal growth, carcass and organ characteristics, and gene expression in adipose tissue and muscle 1. *Journal of Animal Science,* 88**,** 3251-3261.

LU, X., GUO, S., CHENG, Y., KIM, J. H., FENG, Y. & FENG, Y. 2017. Stimulation of ovarian follicle growth after AMPK inhibition. *Reproduction,* 153**,** 683-694.

LUCAS, A. 1991. Programming by early nutrition in man. *The Childhood Environment and Adult Disease,* 1991**,** 38-55.

LUCY, M. 2001. Reproductive loss in high-producing dairy cattle: where will it end? *Journal of Dairy Science,* 84**,** 1277-1293.

LUCY, M. 2008. Functional differences in the growth hormone and insulin-like growth factor axis in cattle and pigs: implications for post-partum nutrition and reproduction. *Reproduction in Domestic Animals,* 43**,** 31-39.

LUCY, M., JIANG, H. & KOBAYASHI, Y. 2001. Changes in the somatotrophic axis associated with the initiation of lactation. *Journal of Dairy Science,* 84**,** 113-119.

LUCY, M. C. 2011. Growth hormone regulation of follicular growth. *Reproduction, Fertility and Development,* 24**,** 19-28.

LUNDY, T., SMITH, P., O'CONNELL, A., HUDSON, N. & MCNATTY, K. 1999. Populations of granulosa cells in small follicles of the sheep ovary. *Journal of Reproduction and Fertility,* 115**,** 251-262.

LÜTTGENAU, J., BEINDORFF, N., ULBRICH, S. E., KASTELIC, J. & BOLLWEIN, H. 2011. Low plasma progesterone concentrations are accompanied by reduced luteal blood flow and increased size of the dominant follicle in dairy cows. *Theriogenology,* 76**,** 12-22.

LYMAN, C. M., KUIKEN, K. & HALE, F. 1956. Amino acids in feedstuffs, essential amino acid content of farm feeds. *Journal of Agricultural and Food Chemistry,* 4**,** 1008-1013.

MACCHIARELLI, G., NOTTOLA, S. A., VIZZA, E., FAMILIARI, G., KIKUTA, A., MURAKAMI, T. & MOTTA, P. M. 1993. Microvasculature of growing and atretic follicles in the rabbit ovary: a SEM study of corrosion casts. *Archives of Histology and Cytology,* 56**,** 1-12.

MACMILLAN, K., LEAN, I. & WESTWOOD, C. 1996. The effects of lactation on the fertility of dairy cows. *Australian Veterinary Journal,* 73**,** 141-147.

MAGOFFIN, D. A. 2005. Ovarian theca cell. *The International Journal of Biochemistry & Cell Biology,* 37**,** 1344-1349.

MAGOFFIN, D. A. & WEITSMAN, S. R. 1993. Insulin-like growth factor-I stimulates the expression of 3β-hydroxysteroid dehydrogenase messenger ribonucleic acid in ovarian theca-interstitial cells. *Biology of Reproduction,* 48**,** 1166-1173.

MAGOFFIN, D. A. & WEITSMAN, S. R. 1994. Insulin-like growth factor-I regulation of luteinizing hormone (LH) receptor messenger ribonucleic acid expression and LH-stimulated signal transduction in rat ovarian theca-interstitial cells. *Biology of Reproduction,* 51**,** 766-775.

MAHESHWARI, A. & FOWLER, P. 2008. Primordial follicular assembly in humans–revisited. *Zygote,* 16**,** 285-296.

MAHON, P. C., HIROTA, K. & SEMENZA, G. L. 2001. FIH-1: a novel protein that interacts with HIF-1α and VHL to mediate repression of HIF-1 transcriptional activity. *Genes & Development,* 15**,** 2675-2686.

MAISONPIERRE, P. C., SURI, C., JONES, P. F., BARTUNKOVA, S., WIEGAND, S. J., RADZIEJEWSKI, C., COMPTON, D., MCCLAIN, J., ALDRICH, T. H. & PAPADOPOULOS, N. 1997. Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. *Science,* 277**,** 55-60.

MANNA, P. R., WANG, X.-J. & STOCCO, D. M. 2003. Involvement of multiple transcription factors in the regulation of steroidogenic acute regulatory protein gene expression. *Steroids,* 68**,** 1125-1134.

MARTELLI, A., BERNABÒ, N., BERARDINELLI, P., RUSSO, V., RINALDI, C., DI GIACINTO, O., MAURO, A. & BARBONI, B. 2009. Vascular supply as a discriminating factor for pig preantral follicle selection. *Reproduction,* 137**,** 45-58.

MARTIN, J., VONNAHME, K., ADAMS, D., LARDY, G. & FUNSTON, R. 2007. Effects of dam nutrition on growth and reproductive performance of heifer calves1. *Journal of Animal Science,* 85**,** 841-847.

MARTIN, J. L., COVERLEY, J. A. & BAXTER, R. C. 1994. Regulation of immunoreactive insulin-like growth factor binding protein-6 in normal and transformed human fibroblasts. *Journal of Biological Chemistry,* 269**,** 11470-11477.

MARTINEZ-CHEQUER, J., STOUFFER, R., HAZZARD, T., PATTON, P. & MOLSKNESS, T. 2003. Insulin-like growth factors-1 and-2, but not hypoxia, synergize with gonadotropin hormone to promote vascular endothelial growth factor-A secretion by monkey granulosa cells from preovulatory follicles. *Biology of Reproduction,* 68**,** 1112-1118.

MASON, H. D., CWYFAN-HUGHES, S., HOLLY, J. M. & FRANKS, S. 1998. Potent inhibition of human ovarian steroidogenesis by insulin-like growth factor binding protein-4 (IGFBP-4). *The Journal of Clinical Endocrinology & Metabolism,* 83**,** 284-287.

MASSAGUE, J. & CZECH, M. 1982. The subunit structures of two distinct receptors for insulin-like growth factors I and II and their relationship to the insulin receptor. *Journal of Biological Chemistry,* 257**,** 5038-5045.

MATSUI, M. & MIYAMOTO, A. 2009. Evaluation of ovarian blood flow by colour Doppler ultrasound: practical use for reproductive management in the cow. *The Veterinary Journal,* 181**,** 232-240.

MAURES, T. J. & DUAN, C. 2002. Structure, developmental expression, and physiological regulation of zebrafish IGF binding protein-1. *Endocrinology,* 143**,** 2722-2731.

MAXWELL, P. H., WIESENER, M. S., CHANG, G.-W., CLIFFORD, S. C., VAUX, E. C., COCKMAN, M. E., WYKOFF, C. C., PUGH, C. W., MAHER, E. R. & RATCLIFFE, P. J. 1999. The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. *Nature,* 399**,** 271.

MAZERBOURG, S., BONDY, C., ZHOU, J. & MONGET, P. 2003. The insulin-like growth factor system: a key determinant role in the growth and selection of ovarian follicles? A comparative species study. *Reproduction in Domestic Animals,* 38**,** 247-258.

MCCLURE, T. 1994. *Nutritional and metabolic infertility in the cow*, Wallingford : CAB International. Pages 128.

MCCRABB, G., HOSKING, B. & EGAN, A. 1992. Changes in the maternal body and feto-placental growth following various lengths of feed restriction during mid-pregnancy in sheep. *Australian Journal of Agricultural Research,* 43**,** 1429-1440.

MCFEE, R. M. & CUPP, A. S. 2013. Vascular contributions to early ovarian development: potential roles of VEGFA isoforms. *Reproduction, Fertility and Development,* 25**,** 333-342.

MCKNIGHT, J. R., SATTERFIELD, M. C., LI, X., GAO, H., WANG, J., LI, D. & WU, G. 2011. Obesity in pregnancy: problems and potential solutions. *Front Bioscience (Elite Edition),* 3**,** 442-452.

MCMILLEN, I. C. & ROBINSON, J. S. 2005. Developmental origins of the metabolic syndrome: prediction, plasticity, and programming. *Physiological Reviews,* 85**,** 571-633.

MCNATTY, K., FIDLER, A., JUENGEL, J., QUIRKE, L., SMITH, P., HEATH, D., LUNDY, T., O'CONNELL, A. & TISDALL, D. 2000. Growth and paracrine factors regulating follicular formation and cellular function. *Molecular and Cellular Endocrinology,* 163**,** 11-20.

MCNATTY, K., SMITH, P., HUDSON, N. L., HEATH, D., TISDALL, D. & BRAW-TAL, R. 1994. Development of the sheep ovary during fetal and early neonatal life and the effect of fecundity genes. *Journal of Reproduction and Fertility. Supplement,* 49**,** 123-135.

MEIDAN, R., KLIPPER, E., ZALMAN, Y. & YALU, R. 2013. The role of hypoxia-induced genes in ovarian angiogenesis. *Reproduction, Fertility and Development,* 25**,** 343-350.

MEIXENSBERGER, J., DINGS, J., KUHNIGK, H. & ROOSEN, K. 1993. Studies of tissue PO2 in normal and pathological human brain cortex. *Monitoring of Cerebral Blood Flow and Metabolism in Intensive Care.* Acta Neurochirurgica book series (NEUROCHIRURGICA, volume 59). Springer. Pages 58 -63.

MELLIN, T. & BUSCH, R. 1976. Corpus luteum function in the ewe: Effect of PGF 2α and prostaglandin synthetase inhibitors. *Prostaglandins,* 12**,** 303-319.

MEYERHOLZ, M. M., MENSE, K., LIETZAU, M., KASSENS, A., LINDEN, M., KNAACK, H., WIRTHGEN, E., HOEFLICH, A., RALIOU, M. & RICHARD, C. 2015. Serum IGFBP4 concentration decreased in dairy heifers towards day 18 of pregnancy. *Journal of Veterinary Science,* 16**,** 413-421.

MICKE, G., SULLIVAN, T., MAGALHAES, R. S., ROLLS, P., NORMAN, S. & PERRY, V. 2010. Heifer nutrition during early-and mid-pregnancy alters fetal growth trajectory and birth weight. *Animal Reproduction Science,* 117**,** 1-10.

MICKE, G. C., SULLIVAN, T. M., MCMILLEN, I. C., GENTILI, S. & PERRY, V. E. 2011. Heifer nutrient intake during early-and mid-gestation programs adult offspring adiposity and mRNA expression of growth-related genes in adipose depots. *Reproduction,* 141**,** 697-706.

MIHM, M. & AUSTIN, E. 2002. The final stages of dominant follicle selection in cattle. *Domestic Animal Endocrinology,* 23**,** 155-166.

MIHM, M., AUSTIN, E., GOOD, T., IRELAND, J., KNIGHT, P., ROCHE, J., IRELAND & JJ 2000. Identification of potential intrafollicular factors

involved in selection of dominant follicles in heifers. *Biology of Reproduction,* 63**,** 811-819.

MIYAMOTO, A., OKUDA, K., SCHWEIGERT, F. & SCHAMS, D. 1992. Effects of basic fibroblast growth factor, transforming growth factor-β and nerve growth factor on the secretory function of the bovine corpus luteum in vitro. *Journal of Endocrinology,* 135**,** 103-114.

MOHAN, S., NAKAO, Y., HONDA, Y., LANDALE, E., LESER, U., DONY, C., LANG, K. & BAYLINK, D. J. 1995. Studies on the mechanisms by which insulin-like growth factor (IGF) binding protein-4 (IGFBP-4) and IGFBP-5 modulate IGF actions in bone cells. *Journal of Biological Chemistry,* 270**,** 20424-20431.

MOHR, S., STRYKER, J. M. & LAMBOWITZ, A. M. 2002. A DEAD-box protein functions as an ATP-dependent RNA chaperone in group I intron splicing. *Cell,* 109**,** 769-779.

MOLINA, P. E. & MOLINA, P. E. 2006. *Endocrine physiology*, 4th Edition. New York. Lange Medical Books/McGraw-Hill. Pages 216 -217.

MONGET, P. & MONNIAUX, D. 1995. Growth factors and the control of folliculogenesis. *Journal of Reproduction and Fertility. Supplement,* 49**,** 321-333.

MORENO, M. J., BALL, M., ANDRADE, M. F., MCDERMID, A. & STANIMIROVIC, D. B. 2006. Insulin-like growth factor binding protein-4 (IGFBP-4) is a novel anti-angiogenic and anti-tumorigenic mediator secreted by dibutyryl cyclic AMP (dB-cAMP)-differentiated glioblastoma cells. *Glia,* 53**,** 845-857.

MORRIS, S. & KENYON, P. 2004. The effect of litter size and sward height on ewe and lamb performance. *New Zealand Journal of Agricultural Research,* 47**,** 275-286.

MORRISON, J. L. 2008. Sheep models of intrauterine growth restriction: fetal adaptations and consequences. *Clinical and Experimental Pharmacology and Physiology,* 35**,** 730-743.

MOSSA, F., CARTER, F., WALSH, S. W., KENNY, D. A., SMITH, G. W., IRELAND, J. L., HILDEBRANDT, T. B., LONERGAN, P., IRELAND, J. J. & EVANS, A. C. 2013. Maternal undernutrition in cows impairs ovarian and cardiovascular systems in their offspring. *Biology of Reproduction,* 88**,** 92.

MOSSA, F., WALSH, S., BUTLER, S., BERRY, D., CARTER, F., LONERGAN, P., SMITH, G., IRELAND, J. & EVANS, A. 2012. Low numbers of ovarian follicles≥ 3mm in diameter are associated with low fertility in dairy cows. *Journal of Dairy Science,* 95**,** 2355-2361.

NAKAO-HAYASHI, J., ITO, H., KANAYASU, T., MORITA, I. & MUROTA, S.-I. 1992. Stimulatory effects of insulin and insulin-like growth factor I on migration and tube formation by vascular endothelial cells. *Atherosclerosis,* 92**,** 141-149.

NASU, K., NISHIDA, M., MATSUMOTO, H., BING, S., INOUE, C., KAWANO, Y. & MIYAKAWA, I. 2005. Regulation of proliferation, motility, and contractivity of cultured human endometrial stromal cells by transforming growth factor-beta isoforms. *Fertility Sterility,* 84 Supplement 2**,** 1114-23.

NEEMAN, M., GILAD, A. A., DAFNI, H. & COHEN, B. 2007. Molecular imaging of angiogenesis. *Journal of Magnetic Resonance Imaging: An Official Journal of the International Society for Magnetic Resonance in Medicine,* 25**,** 1-12.

NICOSIA, R. F., NICOSIA, S. V. & SMITH, M. 1994. Vascular endothelial growth factor, platelet-derived growth factor, and insulin-like growth factor-1 promote rat aortic angiogenesis in vitro. *The American Journal of Pathology,* 145**,** 1023.

NILSSON, I. 2006. Hypoxia, PDGF and VEGF in Vascular Development. Acta Universitatis Upsaliensis. Digital Comprehensive Summaries of Uppsala Disertations from the Faculty of Medicine. Page 11. .

NISHI, S., HOSHI, N., KASAHARA, M., ISHIBASHI, T. & FUJIMOTO, S. 1999. Existence of human DAZLA protein in the cytoplasm of human oocytes. *Molecular Human Reproduction,* 5**,** 495-497.

NISHIMURA, R., KOMIYAMA, J., TASAKI, Y., ACOSTA, T. J. & OKUDA, K. 2008. Hypoxia promotes luteal cell death in bovine corpus luteum. *Biology of Reproduction,* 78**,** 529-536.

NISHIMURA, R. & OKUDA, K. 2010. Hypoxia is important for establishing vascularization during corpus luteum formation in cattle. *Journal of Reproduction and Development,* 56**,** 110-116.

NISHIMURA, R., OKUDA, K., GUNJI, Y., KHALID, A. M., YAMANO, Y., YAMASHITA, Y. & HISHINIMA, M. 2018. BNIP3 expression in bovine follicle and corpus luteum. *Journal of Veterinary Medical Science,* 80**,** 368-374.

NISHIMURA, R., SAKUMOTO, R., TATSUKAWA, Y., ACOSTA, T. J. & OKUDA, K. 2006. Oxygen concentration is an important factor for modulating progesterone synthesis in bovine corpus luteum. *Endocrinology,* 147**,** 4273-4280.

NISHINA, H., GREEN, L. R., MCGARRIGLE, H. H., NOAKES, D. E., POSTON, L. & HANSON, M. A. 2003. Effect of nutritional restriction in early pregnancy on isolated femoral artery function in mid-gestation fetal sheep. *The Journal of Physiology,* 553**,** 637-647.

NISWENDER, G., SCHWALL, R. H., FITZ, T., FARIN, C. & SAWYER, H. 1985. Regulation of luteal function in domestic ruminants: new concepts. Proceedings of the 1984 Laurentian Hormone Conference. Elsevier, 101-151.

NISWENDER, G. D. 2002. Molecular control of luteal secretion of progesterone. *Reproduction,* 123**,** 333-339.

NISWENDER, G. D., JUENGEL, J. L., SILVA, P. J., ROLLYSON, M. K. & MCINTUSH, E. W. 2000. Mechanisms controlling the function and life span of the corpus luteum. *Physiological Reviews,* 80**,** 1-29.

NORMAN, H., WRIGHT, J., KUHN, M., HUBBARD, S., COLE, J. & VANRADEN, P. 2009. Genetic and environmental factors that affect gestation length in dairy cattle. *Journal of Dairy Science,* 92**,** 2259-2269.

O'SHEA, J., RODGERS, R. & D'OCCHIO, M. 1989. Cellular composition of the cyclic corpus luteum of the cow. *Journal of Reproduction and Fertility,* 85**,** 483-487.

O'GORMAN, D. B., WEISS, J., HETTIARATCHI, A., FIRTH, S. M. & SCOTT, C. D. 2002. Insulin-like growth factor-II/mannose 6-phosphate receptor overexpression reduces growth of choriocarcinoma cells in vitro and in vivo. *Endocrinology,* 143**,** 4287-4294.

ORTEGA, H. H., PALOMAR, M. M., ACOSTA, J. C., SALVETTI, N. R., DALLARD, B. E., LORENTE, J. A., BARBEITO, C. G. & GIMENO, E. J. 2008. Insulin-like growth factor I in sera, ovarian follicles and follicular fluid of cows with spontaneous or induced cystic ovarian disease. *Research in Veterinary Science,* 84**,** 419-427.

OSGERBY, J., WATHES, D., HOWARD, D. & GADD, T. 2002. The effect of maternal undernutrition on ovine fetal growth. *Journal of Endocrinology,* 173**,** 131-141.

OSMOND, C., BARKER, D., WINTER, P., FALL, C. & SIMMONDS, S. 1993. Early growth and death from cardiovascular disease in women. *British Medical Journal,* 307**,** 1519-1524.

OZANNE, S. E. & HALES, C. N. 2004. Lifespan: catch-up growth and obesity in male mice. *Nature,* 427**,** 411.

PACIGA, M., WATSON, A. J., DIMATTIA, G. E. & WAGNER, G. F. 2002. Ovarian stanniocalcin is structurally unique in mammals and its production and release are regulated through the luteinizing hormone receptor. *Endocrinology,* 143**,** 3925-34.

PAN, H.-A., TSAI, S.-J., CHEN, C.-W., LEE, Y., LIN, Y.-M. & KUO, P.-L. 2002. Expression of DAZL protein in the human corpus luteum. *Molecular Human Reproduction: Basic Science of Reproductive Medicine,* 8**,** 540-545.

PAN, X. Y., ZHANG, Z. H., WU, L. X. & WANG, Z. C. 2015. Effect of HIF-1a/VEGF signaling pathway on plasma progesterone and ovarian prostaglandin F(2)a secretion during luteal development of pseudopregnant rats. *Genetics Molecular Resources,* 14**,** 8796-809.

PAPADOPOULOS, V., AMRI, H., BOUJRAD, N., CASCIO, C., CULTY, M., GARNIER, M., HARDWICK, M., LI, H., VIDIC, B. & BROWN, A. 1997. Peripheral benzodiazepine receptor in cholesterol transport and steroidogenesis. *Steroids,* 62**,** 21-28.

PAPAPETROPOULOS, A., GARCÍA-CARDEÑA, G., MADRI, J. A. & SESSA, W. C. 1997. Nitric oxide production contributes to the angiogenic properties of vascular endothelial growth factor in human endothelial cells. *The Journal of Clinical Investigation,* 100**,** 3131-3139.

PARK, J. Y., CHANG, J. H., BAE, K. S., LEE, K. H., CHOI, S. J., PARK, J. Y., RYANG, Y. S. & KIM, S. K. 2008. NF-κB-dependency and consequent regulation of IL-8 in echinomycin-induced apoptosis of HT-29 colon cancer cells. *Cell Biology International,* 32**,** 1207-1214.

PARMAR, K., MAUCH, P., VERGILIO, J.-A., SACKSTEIN, R. & DOWN, J. D. 2007. Distribution of hematopoietic stem cells in the bone marrow according to regional hypoxia. *Proceedings of the National Academy of Sciences,* 104**,** 5431-5436.

PASANEN, A., HEIKKILÄ, M., RAUTAVUOMA, K., HIRSILÄ, M., KIVIRIKKO, K. I. & MYLLYHARJU, J. 2010. Hypoxia-inducible factor (HIF)-3α is subject to extensive alternative splicing in human tissues and cancer cells and is regulated by HIF-1 but not HIF-2. *The International Journal of Biochemistry & Cell Biology,* 42**,** 1189-1200.

PATE, J. L. 1996. Intercellular communication in the bovine corpus luteum. *Theriogenology,* 45**,** 1381-1397.

PAULA, L. D. C., ANDRZEJEWSKI, J., JULIAN, D., SPICER, L. & HANSEN, P. 2008. Oxygen and steroid concentrations in preovulatory follicles of lactating dairy cows exposed to acute heat stress. *Theriogenology,* 69**,** 805-813.

PERKS, C., NEWCOMB, P., NORMAN, M. & HOLLY, J. 1999. Effect of insulin-like growth factor binding protein-1 on integrin signalling and the induction of apoptosis in human breast cancer cells. *Journal of Molecular Endocrinology,* 22**,** 141-150.

PERKS, C. & WATHES, D. 1996. Expression of mRNAs for insulin-like growth factor binding proteins-2,-3 and-4 in the ovine ovary throughout the oestrous cycle. *Journal of Endocrinology,* 151**,** 241-249.

PESCE, M., WANG, X., WOLGEMUTH, D. J. & SCHÖLER, H. R. 1998. Differential expression of the Oct-4 transcription factor during mouse germ cell differentiation. *Mechanisms of Development,* 71**,** 89-98.

PIECEWICZ, S. M., PANDEY, A., ROY, B., XIANG, S. H., ZETTER, B. R. & SENGUPTA, S. 2012. Insulin-like growth factors promote vasculogenesis in embryonic stem cells. *PlOS One,* 7**,** e32191.

PLENDL, J. 2000. Angiogenesis and Vascular Regression in the Ovary. *Anatomia, Histologia, Embryologia,* 29**,** 257-266.

PNG, K. J., HALBERG, N., YOSHIDA, M. & TAVAZOIE, S. F. 2012. A microRNA regulon that mediates endothelial recruitment and metastasis by cancer cells. *Nature,* 481**,** 190.

POLLAK, M. 2008. Insulin and insulin-like growth factor signalling in neoplasia. *Nature Reviews Cancer,* 8**,** 915.

POOLE, T. J., FINKELSTEIN, E. B. & COX, C. M. 2001. The role of FGF and VEGF in angioblast induction and migration during vascular development. *Developmental Dynamics,* 220**,** 1-17.

PRESTA, M., DELL'ERA, P., MITOLA, S., MORONI, E., RONCA, R. & RUSNATI, M. 2005. Fibroblast growth factor/fibroblast growth factor receptor system in angiogenesis. *Cytokine Growth Factor Reviews,* 16**,** 159-78.

PUTOWSKI, L., ROHAN, R. M., CHOI, D. S., SCHERZER, W. J., RICCIARELLI, E., MORDACQ, J., MAYO, K. E. & ADASHI, E. Y. 1997. Rat ovarian insulin-like growth factor binding protein-4: a hormone-dependent granulosa cell-derived antigonadotropin. *Journal of the Society for Gynecologic Investigation,* 4**,** 144-151.

QUIGLEY, S., KLEEMANN, D., WALKER, S., SPECK, P., RUDIGER, S., NATTRASS, G., DEBLASIO, M. & OWENS, J. 2008. Effect of variable

long-term maternal feed allowance on the development of the ovine placenta and fetus. *Placenta,* 29**,** 539-548.

RAE, M., KYLE, C., MILLER, D., HAMMOND, A., BROOKS, A. & RHIND, S. 2002. The effects of undernutrition, in utero, on reproductive function in adult male and female sheep. *Animal Reproduction Science,* 72**,** 63-71.

RAE, M., PALASSIO, S., KYLE, C., BROOKS, A., LEA, R., MILLER, D. & RHIND, S. 2001. Effect of maternal undernutrition during pregnancy on early ovarian development and subsequent follicular development in sheep fetuses. *Reproduction,* 122**,** 915-922.

RANKIN, E. & GIACCIA, A. 2008. The role of hypoxia-inducible factors in tumorigenesis. *Cell Death and Differentiation,* 15**,** 678.

RAPPOLEE, D. A., STURM, K. S., BEHRENDTSEN, O., SCHULTZ, G. A., PEDERSEN, R. A. & WERB, Z. 1992. Insulin-like growth factor II acts through an endogenous growth pathway regulated by imprinting in early mouse embryos. *Genes & Development,* 6**,** 939-952.

RAZ, E. 2000. The function and regulation of vasa-like genes in germ-cell development. *Genome Biology,* 1**,** Reviews. 1017 - 1018.

REDDING, G. P., BRONLUND, J. E. & HART, A. L. 2008. Theoretical investigation into the dissolved oxygen levels in follicular fluid of the developing human follicle using mathematical modelling. *Reproduction, Fertility and Development,* 20**,** 408-417.

REDING, S. C., STEPNOSKI, A. L., CLONINGER, E. W. & OATLEY, J. M. 2010. THY1 is a conserved marker of undifferentiated spermatogonia in the pre-pubertal bull testis. *Reproduction,* 139**,** 893-903.

REDMER, D. A., DORAISWAMY, V., BORTNEM, B. J., FISHER, K., JABLONKA-SHARIFF, A., GRAZUL-BILSKA, A. T. & REYNOLDS, L. P. 2001. Evidence for a role of capillary pericytes in vascular growth of the developing ovine corpus luteum. *Biology of Reproduction,* 65**,** 879-889.

REDMER, D. A. & REYNOLDS, L. P. 1996. Angiogenesis in the ovary. *Reviews of Reproduction,* 1**,** 182-192.

REDMER, D. A., WALLACE, J. M. & REYNOLDS, L. P. 2004. Effect of nutrient intake during pregnancy on fetal and placental growth and vascular development. *Domestic Animal Endocrinology,* 27**,** 199-217.

REINHART, A. J., WILLIAMS, S. C. & STOCCO, D. M. 1999. Transcriptional regulation of the StAR gene. *Molecular and Cellular Endocrinology,* 151**,** 161-169.

REKAWIECKI, R., NOWIK, M. & KOTWICA, J. 2005. Stimulatory effect of LH, PGE2 and progesterone on StAR protein, cytochrome P450 cholesterol side chain cleavage and 3β hydroxysteroid dehydrogenase gene expression in bovine luteal cells. *Prostaglandins and Other Lipid Mediators,* 78**,** 169-184.

REYNAUD, K. & DRIANCOURT, M. 2000. Oocyte attrition. *Molecular and Cellular Endocrinology,* 163**,** 101-108.

REYNOLDS, L., BOROWICZ, P., CATON, J., VONNAHME, K., LUTHER, J., HAMMER, C., MADDOCK CARLIN, K., GRAZUL-BILSKA, A. & REDMER, D. 2010. Developmental programming: The concept, large animal models, and the key role of uteroplacental vascular development 1, 2. *Journal of Animal Science,* 88**,** E61-E72.

REYNOLDS, L. & REDMER, D. 1999. Growth and development of the corpus luteum. *Journal of Reproduction and Fertility. Supplement,* 54**,** 181-191.

REYNOLDS, L. P., GRAZUL-BILSKA, A. T., KILLILEA, S. D. & REDMER, D. A. 1994. Mitogenic factors of corpora lutea. *Progress in Growth Factor Research,* 5**,** 159-175.

REYNOLDS, L. P., GRAZUL-BILSKA, A. T. & REDMER, D. A. 2000. Angiogenesis in the corpus luteum. *Endocrine,* 12**,** 1-9.

REYNOLDS, L. P. & REDMER, D. A. 1992. Growth and microvascular development of the uterus during early pregnancy in ewes. *Biology of Reproduction,* 47**,** 698-708.

REYNOLDS, N. & COOKE, H. J. 2005. Role of the DAZ genes in male fertility. *Reproductive Biomedicine Online,* 10**,** 72-80.

RHIND, S., MCKELVEY, W., MCMILLEN, S., GUNN, R. & EISTON, D. 1989. Effect of restricted food intake, before and/or after mating, on the reproductive performance of Greyface ewes. *Animal Production,* 48**,** 149-155.

RHIND, S. M. 2004. Effects of maternal nutrition on fetal and neonatal reproductive development and function. *Animal Reproduction Science,* 82**,** 169-181.

RHO, S. B., DONG, S. M., KANG, S., SEO, S.-S., YOO, C. W., LEE, D. O., WOO, J. S. & PARK, S.-Y. 2008. Insulin-like growth factor-binding protein-5 (IGFBP-5) acts as a tumor suppressor by inhibiting angiogenesis. *Carcinogenesis,* 29**,** 2106-2111.

RICO, C., DODELET-DEVILLERS, A., PAQUET, M., TSOI, M., LAPOINTE, E., CARMELIET, P. & BOERBOOM, D. 2014. HIF1 activity in granulosa cells is required for FSH-regulated Vegfa expression and follicle survival in mice. *Biology of Reproduction,* 90.

RINDERKNECHT, E. & HUMBEL, R. E. 1978a. The amino acid sequence of human insulin-like growth factor I and its structural homology with proinsulin. *Journal of Biological Chemistry,* 253**,** 2769-2776.

RINDERKNECHT, E. & HUMBEL, R. E. 1978b. Primary structure of human insulin-like growth factor II. *FEBS letters,* 89**,** 283-286.

RINEHART, L. 2008. *Ruminant nutrition for graziers.*, National Sustainable Agriculture Information Service. Pages 1 - 20.

RISAU, W. 1997. Mechanisms of angiogenesis. *Nature,* 386**,** 671-674.

RISAU, W. & FLAMME, I. 1995. Vasculogenesis. *Annual Review of Cell and Developmental Biology,* 11**,** 73-91.

ROBERTS, A. E., ARBOGAST, L. K., FRIEDMAN, C. I., COHN, D. E., KAUMAYA, P. T. & DANFORTH, D. R. 2007. Neutralization of endogenous

vascular endothelial growth factor depletes primordial follicles in the mouse ovary. *Biology of Reproduction,* 76**,** 218-223.

ROBINSON, J., ASHWORTH, C., ROOKE, J., MITCHELL, L. & MCEVOY, T. 2006. Nutrition and fertility in ruminant livestock. *Animal Feed Science and Technology,* 126**,** 259-276.

ROBINSON, J. J., SINCLAIR, K. D. & MCEVOY, T. G. 1999. Nutritional effects on foetal growth. *Animal Science,* 68**,** 315-331.

ROBINSON, R., HAMMOND, A., MANN, G. & HUNTER, M. 2008. A novel physiological culture system that mimics luteal angiogenesis. *Reproduction,* 135**,** 405-413.

ROBINSON, R., NICKLIN, L., HAMMOND, A., SCHAMS, D., HUNTER, M. & MANN, G. 2007. Fibroblast growth factor 2 is more dynamic than vascular endothelial growth factor A during the follicle-luteal transition in the cow. *Biology of Reproduction,* 77**,** 28-36.

ROBINSON, R. S., WOAD, K. J., HAMMOND, A. J., LAIRD, M., HUNTER, M. G. & MANN, G. E. 2009. Angiogenesis and vascular function in the ovary. *Reproduction,* 138**,** 869-81.

ROCHE, J., MACKEY, D. & DISKIN, M. 2000. Reproductive management of postpartum cows. *Animal Reproduction Science,* 60**,** 703-712.

ROCHE, J. F. 2006. The effect of nutritional management of the dairy cow on reproductive efficiency. *Animal Reproduction Science,* 96**,** 282-296.

RODRÍGUEZ, F., GAREIS, N., HEIN, G., SALVETTI, N., AMWEG, A., HUBER, E., STASSI, A., ORTEGA, H. & REY, F. 2017. Role of Components of the Insulin-like Growth Factor System in the Early Stages of Ovarian Follicular Persistence in Cattle. *Journal of Comparative Pathology,* 157**,** 201-214.

ROSNER, M. H., VIGANO, M. A., OZATO, K., TIMMONS, P. M., POIRIE, F., RIGBY, P. W. & STAUDT, L. M. 1990. A POU-domain transcription factor in early stem cells and germ cells of the mammalian embryo. *Nature,* 345**,** 686.

ROTWEIN, P. 2018. The complex genetics of human insulin-like growth factor 2 are not reflected in public databases. *Journal of Biological Chemistry***,** RA117. 001573.

ROTWEIN, P., POLLOCK, K., DIDIER, D. & KRIVI, G. 1986. Organization and sequence of the human insulin-like growth factor I gene. Alternative RNA processing produces two insulin-like growth factor I precursor peptides. *Journal of Biological Chemistry,* 261**,** 4828-4832.

ROY, S., KHANNA, S., WALLACE, W. A., LAPPALAINEN, J., RINK, C., CARDOUNEL, A. J., ZWEIER, J. L. & SEN, C. K. 2003. Characterization of perceived hyperoxia in isolated primary cardiac fibroblasts and in the reoxygenated heart. *Journal of Biological Chemistry,* 278**,** 47129-47135.

ROY, S. K. 1994. Regulation of ovarian follicular development: a review of microscopic studies. *Microscopy Research and Technique,* 27**,** 83-96.

ROYAL, M., DARWASH, A., FLINT, A., WEBB, R., WOOLLIAMS, J. & LAMMING, G. 2000. Declining fertility in dairy cattle: changes in traditional and endocrine parameters of fertility. *Animal Science,* 70**,** 487-501.

ROZARIO, K., LLOYD, C. & RYAN, F. 2000. GH and IGF-1 physiology in childhood.

RUBIN, J. B., SHIA, M. A. & PILCH, P. F. 1983. Stimulation of tyrosine-specific phosphorylation in vitro by insulin-like growth factor I. *Nature,* 305**,** 438.

RUGGIU, M. & COOKE, H. J. 2000. In vivo and in vitro analysis of homodimerisation activity of the mouse Dazl1 protein. *Gene,* 252**,** 119-126.

RUGGIU, M., SPEED, R., TAGGART, M., MCKAY, S. J., KILANOWSKI, F., SAUNDERS, P., DORIN, J. & COOKE, H. J. 1997. The mouse Dazla gene encodes a cytoplasmic protein essential for gametogenesis. *Nature,* 389**,** 73.

RUTLAND, C., LATUNDE-DADA, A., THORPE, A., PLANT, R., LANGLEY-EVANS, S. & LEACH, L. 2007. Effect of gestational nutrition on vascular integrity in the murine placenta. *Placenta,* 28**,** 734-742.

SAKUMOTO, R., MURAKAMI, S. & OKUDA, K. 2000. Tumor necrosis factor-α stimulates prostaglandin F2α secretion by bovine luteal cells via activation of mitogen-activated protein kinase and phospholipase A2 pathways. *Molecular Reproduction and Development: Incorporating Gamete Research,* 56**,** 387-391.

SAKURAI, T., SUZUKI, K., YOSHIE, M., HASHIMOTO, K., TACHIKAWA, E. & TAMURA, K. 2011. Stimulation of tube formation mediated through the prostaglandin EP2 receptor in rat luteal endothelial cells. *Journal of Endocrinology,* 209**,** 33-43.

SALCEDA, S., BECK, I., SRINIVAS, V. & CARO, J. 1997. Complex role of protein phosphorylation in gene activation by hypoxia. *Kidney International,* 51**,** 556-559.

SALCEDA, S. & CARO, J. 1997. Hypoxia-inducible factor 1α (HIF-1α) protein Is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions its stabilization by hypoxia depends on redox-induced changes. *Journal of Biological Chemistry,* 272**,** 22642-22647.

SÁNCHEZ, F. & SMITZ, J. 2012. Molecular control of oogenesis. *Biochimica et Biophysica Acta. Molecular Basis of Disease,* 1822**,** 1896-1912.

SANSON, D., CLANTON, D. & RUSH, I. G. 1990. Intake and digestion of low-quality meadow hay by steers and performance of cows on native range when fed protein supplements containing various levels of corn. *Journal of Animal Science,* 68**,** 595-603.

SASAKI, N., REES-JONES, R., ZICK, Y., NISSLEY, S. & RECHLER, M. 1985. Characterization of insulin-like growth factor I-stimulated tyrosine kinase activity associated with the beta-subunit of type I insulin-like growth factor receptors of rat liver cells. *Journal of Biological Chemistry,* 260**,** 9793-9804.

SAUERWEIN, H., MIYAMOTO, A., GÜNTHER, J., MEYER, H. & SCHAMS, D. 1992. Binding and action of insulin-like growth factors and insulin in

bovine luteal tissue during the oestrous cycle. *Journal of Reproduction and Fertility,* 96**,** 103-115.

SAUNDERS, P., TURNER, J., RUGGIU, M., TAGGART, M., BURGOYNE, P., ELLIOTT, D. & COOKE, H. 2003. Absence of Dazl produces a final block on germ cell development at meiosis. *Reproduction,* 126**,** 589-597.

SAWYER, H., NISWENDER, K., BRADEN, T. & NISWENDER, G. 1990. Nuclear changes in ovine luteal cells in response to PGF2α. *Domestic Animal Endocrinology,* 7**,** 229-237.

SAWYER, H. R., SMITH, P., HEATH, D. A., JUENGEL, J. L. & MCNATTY, K. P. 2002. Formation of ovarian follicles during fetal development in sheep. *Biology of Reproduction,* 66**,** 1134-1150.

SCARAMUZZI, R., BAIRD, D., CAMPBELL, B., DRIANCOURT, M.-A., DUPONT, J., FORTUNE, J., GILCHRIST, R., MARTIN, G., MCNATTY, K. & MCNEILLY, A. 2011. Regulation of folliculogenesis and the determination of ovulation rate in ruminants. *Reproduction, Fertility and Development,* 23**,** 444-467.

SCARAMUZZI, R., MURRAY, J., DOWNING, J. & CAMPBELL, B. 1999. The effects of exogenous growth hormone on follicular steroid secretion and ovulation rate in sheep. *Domestic Animal Endocrinology,* 17**,** 269-277.

SCHAMS, D., AMSELGRUBER, W., EINSPANIER, R., SINOWATZ, F. & GOSPODAROWICZ, D. 1994. Localization and tissue concentration of basic fibroblast growth factor in the bovine corpus luteum. *Endocrine.*

SCHAMS, D. & BERISHA, B. 2004. Regulation of corpus luteum function in cattle–an overview. *Reproduction in Domestic Animals,* 39**,** 241-251.

SCHAMS, D., BERISHA, B., KOSMANN, M. & AMSELGRUBER, W. 2002. Expression and localization of IGF family members in bovine antral follicles during final growth and in luteal tissue during different stages of estrous cycle and pregnancy. *Domestic Animal Endocrinology,* 22**,** 51-72.

SCHAMS, D., BERISHA, B., KOSMANN, M., EINSPANIER, R. & AMSELGRUBER, W. 1999. Possible role of growth hormone, IGFs, and IGF-binding proteins in the regulation of ovarian function in large farm animals. *Domestic Animal Endocrinology,* 17**,** 279-285.

SCHAMS, D., KOSMANN, M., BERISHA, B., AMSELGRUBER, W. & MIYAMOTO, A. 2001. Stimulatory and synergistic effects of luteinising hormone and insulin like growth factor 1 on the secretion of vascular endothelial growth factor and progesterone of cultured bovine granulosa cells. *Experimental and Clinical Endocrinology & Diabetes,* 109**,** 155-162.

SCHNEIDER, G., BUBEL, M., POHLEMANN, T. & OBERRINGER, M. 2015. Response of endothelial cells and pericytes to hypoxia and erythropoietin in a co-culture assay dedicated to soft tissue repair. *Molecular Cell Biochemistry,* 407**,** 29-40.

SCHNEIDER, H. 1996. Ontogenic changes in the nutritive function of the placenta. *Placenta,* 17**,** 15-26.

SCHNEIDER, M. R. & YARDEN, Y. 2014. Structure and function of epigen, the last EGFR ligand. Seminars in Cell & Developmental Biology, 2014. Elsevier, 57-61.

SCHOENWOLF, G. C., BLEYL, S. B., BRAUER, P. R. & FRANCIS-WEST, P. H. 2014. *Larsen's Human Embryology: Study smart with Student Consult.*, 5th Edition. Philadelphia Elsevier Churchill Livingstone. Pages 304 - 312.

SCHRANS-STASSEN, B. H., SAUNDERS, P. T., COOKE, H. J. & DE ROOIJ, D. G. 2001. Nature of the spermatogenic arrest in Dazl−/− mice. *Biology of Reproduction,* 65**,** 771-776.

SEKAR, N., LAVOIE, H. A. & VELDHUIS, J. D. 2000. Concerted regulation of steroidogenic acute regulatory gene expression by luteinizing hormone and insulin (or insulin-like growth factor I) in primary cultures of porcine granulosa-luteal cells. *Endocrinology,* 141**,** 3983-3992.

SELIGMAN, J. & PAGE, D. C. 1998. The Dazl Gene Is Expressed in Male and Female Embryonic Gonads before Germ Cell Sex Differentiation. *Biochemical and Biophysical Research Communications,* 245**,** 878-882.

SEMENZA, G. L. 2007. Life with oxygen. *Science,* 318**,** 62-64.

SEMENZA, G. L., JIANG, B.-H., LEUNG, S. W., PASSANTINO, R., CONCORDET, J.-P., MAIRE, P. & GIALLONGO, A. 1996. Hypoxia response elements in the aldolase A, enolase 1, and lactate dehydrogenase A gene promoters contain essential binding sites for hypoxia-inducible factor 1. *Journal of Biological Chemistry,* 271**,** 32529-32537.

SENGER, P. 2003. Pathways to pregnancy and parturition. 2th edition, Pullman, Washington, Current Conceptions. . Inc. Pages 368.

SHIMIZU, T., MURAYAMA, C., SUDO, N., KAWASHIMA, C., TETSUKA, M. & MIYAMOTO, A. 2008. Involvement of insulin and growth hormone (GH) during follicular development in the bovine ovary. *Animal Reproduction Science,* 106**,** 143-152.

SHIRATSUKI, S., HARA, T., MUNAKATA, Y., SHIRASUNA, K., KUWAYAMA, T. & IWATA, H. 2016. Low oxygen level increases proliferation and metabolic changes in bovine granulosa cells. *Molecular and Cellular Endocrinology,* 437**,** 75-85.

SILVA, J., FIGUEIREDO, J. & VAN DEN HURK, R. 2009. Involvement of growth hormone (GH) and insulin-like growth factor (IGF) system in ovarian folliculogenesis. *Theriogenology,* 71**,** 1193-1208.

SIMMONS, R. A., TEMPLETON, L. J. & GERTZ, S. J. 2001. Intrauterine growth retardation leads to the development of type 2 diabetes in the rat. *Diabetes,* 50**,** 2279-2286.

SIMON, M. C., LIU, L., BARNHART, B. C. & YOUNG, R. M. 2008. Hypoxia-induced signaling in the cardiovascular system. *Annual Review in Physiology,* 70**,** 51-71.

SINCLAIR, K. D., RUTHERFORD, K., WALLACE, J., BRAMELD, J., STÖGER, R., ALBERIO, R., SWEETMAN, D., GARDNER, D. S., PERRY, V. & ADAM, C. 2016. Epigenetics and developmental programming of welfare and production traits in farm animals. *Reproduction, Fertility and Development,* 28**,** 1443-1478.

SINGH, P., DAI, B., DHRUVA, B. & WIDEN, S. G. 1994. Episomal expression of sense and antisense insulin-like growth factor (IGF)-binding protein-4 complementary DNA alters the mitogenic response of a human colon cancer cell line (HT-29) by mechanisms that are independent of and dependent upon IGF-I. *Cancer Research,* 54**,** 6563-6570.

SKALLI, O., PELTE, M., PECLET, M., GABBIANI, G., GUGLIOTTA, P., BUSSOLATI, G., RAVAZZOLA, M. & ORCI, L. 1989. Alpha-smooth muscle actin, a differentiation marker of smooth muscle cells, is present in microfilamentous bundles of pericytes. *Journal of Histochemistry & Cytochemistry,* 37**,** 315-321.

SKARZYNSKI, D., FERREIRA-DIAS, G. & OKUDA, K. 2008. Regulation of luteal function and corpus luteum regression in cows: hormonal control, immune mechanisms and intercellular communication. *Reproduction in Domestic Animals,* 43**,** 57-65.

SKARZYNSKI, D., PIOTROWSKA-TOMALA, K., LUKASIK, K., GALVAO, A., FARBEROV, S., ZALMAN, Y. & MEIDAN, R. 2013. Growth and regression in bovine corpora lutea: regulation by local survival and death pathways. *Reproduction in Domestic Animals,* 48**,** 25-37.

SMITH, O. & SOMADE, B. Interactions between nutrition and reproduction in farm animals. IFS (International Foundation for science) Proceedings of a Regional Seminar on Animal Reproduction, 1994. 17-21.

SMITH, P., HUDSON, N., SHAW, L., HEATH, D., CONDELL, L., PHILLIPS, D. & MCNATTY, K. 1993. Effects of the Booroola gene (FecB) on body weight, ovarian development and hormone concentrations during fetal life. *Journal of Reproduction and Fertility,* 98**,** 41-54.

SMITH, P., WILHELM, D. & RODGERS, R. J. 2014. Development of mammalian ovary. *Journal of Endocrinology,* 221**,** R145-R161.

SPICER, L., ALPIZAR, E. & ECHTERNKAMP, S. 1993. Effects of insulin, insulin-like growth factor I, and gonadotropins on bovine granulosa cell proliferation, progesterone production, estradiol production, and (or) insulin-like growth factor I production in vitro. *Journal of Animal Science,* 71**,** 1232-1241.

SPICER, L., ALVAREZ, P., PRADO, T., MORGAN, G. & HAMILTON, T. 2000. Effects of intraovarian infusion of insulin-like growth factor-I on ovarian follicular function in cattle☆. *Domestic Animal Endocrinology,* 18**,** 265-278.

SPICER, L. & ECHTERNKAMP, S. 1995. The ovarian insulin and insulin-like growth factor system with an emphasis on domestic animals. *Domestic Animal Endocrinology,* 12**,** 223-245.

SPICER, L., ECHTERNKAMP, S., CANNING, S. & HAMMOND, J. 1988. Relationship between concentrations of immunoreactive insulin-like growth factor-I in follicular fluid and various biochemical markers of differentiation in bovine antral follicles. *Biology of Reproduction,* 39**,** 573-580.

SPICER, L., VOGE, J. & ALLEN, D. 2004. Insulin-like growth factor-II stimulates steroidogenesis in cultured bovine thecal cells. *Molecular and Cellular Endocrinology,* 227**,** 1-7.

SPICER, L. J. & AAD, P. 2007. Insulin-like growth factor (IGF) 2 stimulates steroidogenesis and mitosis of bovine granulosa cells through the IGF1 receptor: role of follicle-stimulating hormone and IGF2 receptor. *Biology of Reproduction,* 77**,** 18-27.

SPICER, L. J. & CHAMBERLAIN, C. S. 1998. Influence of cortisol on insulin- and insulin-like growth factor 1 (IGF-1)-induced steroid production and on IGF-1 receptors in cultured bovine granulosa cells and thecal cells. *Endocrine,* 9**,** 153-161.

SPICER, L. J., CHAMBERLAIN, C. S. & MACIEL, S. M. 2002. Influence of gonadotropins on insulin-and insulin-like growth factor-I (IGF-I)-

induced steroid production by bovine granulosa cells. *Domestic Animal Endocrinology,* 22**,** 237-254.

STALLCUP, W. B. 2013. Bidirectional myoblast-pericyte plasticity. *Developmental Cell,* 24**,** 563-4.

STEWART, R., SPICER, L., HAMILTON, T. & KEEFER, B. 1995. Effects of insulin-like growth factor I and insulin on proliferation and on basal and luteinizing hormone-induced steroidogenesis of bovine thecal cells: involvement of glucose and receptors for insulin-like growth factor I and luteinizing hormone. *Journal of Animal Science,* 73**,** 3719-3731.

STIRLING, D., WATERMAN, M. & SIMPSON, E. 1991. Expression of mRNA encoding basic fibroblast growth factor (bFGF) in bovine corpora lutea and cultured luteal cells. *Journal of Reproduction and Fertility,* 91**,** 1-8.

STOCCO, D. M. 2001. StAR protein and the regulation of steroid hormone biosynthesis. *Annual Review of Physiology,* 63**,** 193-213.

STOCCO, D. M., CLARK, B. J., REINHART, A. J., WILLIAMS, S. C., DYSON, M., DASSI, B., WALSH, L. P., MANNA, P. R., WANG, X. & ZELEZNIK, A. J. 2001. Elements involved in the regulation of the StAR gene. *Molecular and Cellular Endocrinology,* 177**,** 55-59.

STOCKWELL, R. 1991. Morphometry of cytoplasmic components of mammalian articular chondrocytes and corneal keratocytes: species and zonal variations of mitochondria in relation to nutrition. *Journal of Anatomy,* 175**,** 251.

STOOP, H., HONECKER, F., COOLS, M., DE KRIJGER, R., BOKEMEYER, C. & LOOIJENGA, L. 2005. Differentiation and development of human female germ cells during prenatal gonadogenesis: an immunohistochemical study. *Human Reproduction,* 20**,** 1466-1476.

STOUFFER, R. L., MARTíNEZ-CHEQUER, J. C., MOLSKNESS, T. A., XU, F. & HAZZARD, T. M. 2001. Regulation and action of angiogenic factors in the primate ovary. *Archives of Medical Research,* 32**,** 567-575.

SUGAWARA, T., HOLT, J. A., DRISCOLL, D., STRAUSS, J. F., LIN, D., MILLER, W. L., PATTERSON, D., CLANCY, K. P., HART, I. M. & CLARK, B. J. 1995. Human steroidogenic acute regulatory protein: functional activity in COS-1 cells, tissue-specific expression, and mapping of the structural gene to 8p11. 2 and a pseudogene to chromosome 13. *Proceedings of the National Academy of Sciences,* 92**,** 4778-4782.

SULLIVAN, T., MICKE, G., GREER, R., IRVING-RODGERS, H., RODGERS, R. & PERRY, V. 2009. Dietary manipulation of Bos indicus× heifers during gestation affects the reproductive development of their heifer calves. *Reproduction, Fertility and Development,* 21**,** 773-784.

SUN, X., CHENG, L., DUAN, H. & LU, G. 2009. Effects of an endothelial cell-conditioned medium on the hematopoietic and endothelial differentiation of embryonic stem cells. *Cell Biology International,* 33**,** 1201-1205.

SUSSENBACH, J., STEENBERGH, P. & HOLTHUIZEN, P. 1992. Structure and expression of the human insulin-like growth factor genes. *Growth Regulation,* 2**,** 1-9.

SYMONDS, M., SEBERT, S. & BUDGE, H. 2010. Nutritional regulation of fetal growth and implications for productive life in ruminants. *Animal,* 4**,** 1075-1083.

SYMONDS, M. E., STEPHENSON, T., GARDNER, D. S. & BUDGE, H. 2006. Long-term effects of nutritional programming of the embryo and fetus: mechanisms and critical windows. *Reproduction, Fertility and Development,* 19**,** 53-63.

TAM, K. K., RUSSELL, D. L., PEET, D. J., BRACKEN, C. P., RODGERS, R. J., THOMPSON, J. G. & KIND, K. L. 2010. Hormonally regulated follicle differentiation and luteinization in the mouse is associated with hypoxia inducible factor activity. *Molecular and Cellular Endocrinology,* 327**,** 47-55.

TANAKA, S. S., TOYOOKA, Y., AKASU, R., KATOH-FUKUI, Y., NAKAHARA, Y., SUZUKI, R., YOKOYAMA, M. & NOCE, T. 2000. The mouse homolog

of Drosophila Vasa is required for the development of male germ cells. *Genes and Development,* 14**,** 841-853.

TANIMOTO, K., MAKINO, Y., PEREIRA, T. & POELLINGER, L. 2000. Mechanism of regulation of the hypoxia-inducible factor-1α by the von Hippel-Lindau tumor suppressor protein. *The EMBO Journal,* 19**,** 4298-4309.

TESONE, M., STOUFFER, R. L., BORMAN, S. M., HENNEBOLD, J. D. & MOLSKNESS, T. A. 2005. Vascular endothelial growth factor (VEGF) production by the monkey corpus luteum during the menstrual cycle: isoform-selective messenger RNA expression in vivo and hypoxia-regulated protein secretion in vitro. *Biology of Reproduction,* 73**,** 927-934.

THIBEAULT, S., RAUTUREAU, Y., OUBAHA, M., FAUBERT, D., WILKES, B. C., DELISLE, C. & GRATTON, J.-P. 2010. S-nitrosylation of β-catenin by eNOS-derived NO promotes VEGF-induced endothelial cell permeability. *Molecular Cell,* 39**,** 468-476.

THOMPSON, J. G., BROWN, H. M., KIND, K. L. & RUSSELL, D. L. 2015. The ovarian antral follicle: living on the edge of hypoxia or not? *Biology of Reproduction,* 92.

THORNTON, P. K. 2010. Livestock production: recent trends, future prospects. *Philosophical Transactions of the Royal Society of London B: Biological Sciences,* 365**,** 2853-2867.

TINGEN, C., KIM, A. & WOODRUFF, T. K. 2009. The primordial pool of follicles and nest breakdown in mammalian ovaries. *Molecular Human Reproduction,* 15**,** 795-803.

TÖNSHOFF, B., KIEPE, D. & CIARMATORI, S. 2005. Growth hormone/insulin-like growth factor system in children with chronic renal failure. *Pediatric Nephrology,* 20**,** 279-289.

TOYOOKA, Y., TSUNEKAWA, N., TAKAHASHI, Y., MATSUI, Y., SATOH, M. & NOCE, T. 2000. Expression and intracellular localization of mouse Vasa-homologue protein during germ cell development. *Mechanisms of Development,* 93**,** 139-149.

TROPEA, A., MICELI, F., MINICI, F., TIBERI, F., ORLANDO, M., GANGALE, M. F., ROMANI, F., CATINO, S., MANCUSO, S. & NAVARRA, P. 2006. Regulation of vascular endothelial growth factor synthesis and release by human luteal cells in vitro. *The Journal of Clinical Endocrinology & Metabolism,* 91**,** 2303-2309.

TSCHEUDSCHILSUREN, G., AUST, G., NIEBER, K., SCHILLING, N. & SPANEL-BOROWSKI, K. 2002. Microvascular endothelial cells differ in basal and hypoxia-regulated expression of angiogenic factors and their receptors. *Microvascular Research,* 63**,** 243-251.

TSUZUKI, T., OKADA, H., CHO, H., TSUJI, S., NISHIGAKI, A., YASUDA, K. & KANZAKI, H. 2012. Hypoxic stress simultaneously stimulates vascular endothelial growth factor via hypoxia-inducible factor-1alpha and inhibits stromal cell-derived factor-1 in human endometrial stromal cells. *Human Reproduction,* 27**,** 523-30.

TURGEON, J. L., CARR, M. C., MAKI, P. M., MENDELSOHN, M. E. & WISE, P. M. 2006. Complex actions of sex steroids in adipose tissue, the cardiovascular system, and brain: Insights from basic science and clinical studies. *Endocrine Reviews,* 27**,** 575-605.

ULLRICH, A., GRAY, A., TAM, A. W., YANG-FENG, T., TSUBOKAWA, M., COLLINS, C., HENZEL, W., LE BON, T., KATHURIA, S. & CHEN, E. 1986. Insulin-like growth factor I receptor primary structure: comparison with insulin receptor suggests structural determinants that define functional specificity. *The EMBO Journal,* 5**,** 2503-2512.

VAN BLERKOM, J., ANTCZAK, M. & SCHRADER, R. 1997. The developmental potential of the human oocyte is related to the dissolved oxygen content of follicular fluid: association with vascular endothelial growth factor levels and perifollicular blood flow characteristics. *Human Reproduction. Oxford, England.,* 12**,** 1047-1055.

VAN DEN DRIESCHE, S., MYERS, M., GAY, E., THONG, K. J. & DUNCAN, W. C. 2008. HCG up-regulates hypoxia inducible factor-1 alpha in luteinized granulosa cells: implications for the hormonal regulation of vascular endothelial growth factor A in the human corpus luteum. *MHR: Basic Science of Reproductive Medicine,* 14**,** 455-464.

VAN WEZEL, I. L. & RODGERS, R. J. 1996. Morphological characterization of bovine primordial follicles and their environment in vivo. *Biology of Reproduction,* 55**,** 1003-1011.

VARGHESE, R., WONG, C. K., DEOL, H., WAGNER, G. F. & DIMATTIA, G. E. 1998. Comparative analysis of mammalian stanniocalcin genes. *Endocrinology,* 139**,** 4714-25.

VELAZQUEZ, M., SPICER, L. & WATHES, D. 2008. The role of endocrine insulin-like growth factor-I (IGF-I) in female bovine reproduction. *Domestic Animal Endocrinology,* 35**,** 325-342.

VERBEEK, M. M., OTTE-HOLLER, I., WESSELING, P., RUITER, D. J. & DE WAAL, R. M. 1994. Induction of alpha-smooth muscle actin expression in cultured human brain pericytes by transforming growth factor-beta 1. *American Journal of Pathology,* 144**,** 372-82.

VONNAHME, K., HESS, B., NIJLAND, M., NATHANIELSZ, P. & FORD, S. 2006. Placentomal differentiation may compensate for maternal nutrient restriction in ewes adapted to harsh range conditions 1. *Journal of Animal Science,* 84**,** 3451-3459.

VONNAHME, K. A., HESS, B. W., HANSEN, T. R., MCCORMICK, R. J., RULE, D. C., MOSS, G. E., MURDOCH, W. J., NIJLAND, M. J., SKINNER, D. C. & NATHANIELSZ, P. W. 2003. Maternal undernutrition from early- to mid-gestation leads to growth retardation, cardiac ventricular hypertrophy, and increased liver weight in the fetal sheep. *Biology of Reproduction,* 69**,** 133-140.

WALLACE, J. M., AITKEN, R. P., MILNE, J. S. & HAY JR, W. W. 2004. Nutritionally mediated placental growth restriction in the growing adolescent: consequences for the fetus. *Biology of Reproduction,* 71**,** 1055-1062.

WALSH, S., MATTHEWS, D., BROWNE, J., FORDE, N., CROWE, M., MIHM, M., DISKIN, M. & EVANS, A. 2012a. Acute dietary restriction in heifers alters expression of genes regulating exposure and response to gonadotrophins and IGF in dominant follicles. *Animal Reproduction Science,* 133**,** 43-51.

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

WALSH, S. W., MEHTA, J. P., MCGETTIGAN, P. A., BROWNE, J. A., FORDE, N., ALIBRAHIM, R., MULLIGAN, F., LOFTUS, B., CROWE, M. A. & MATTHEWS, D. 2012b. Effect of the metabolic environment at key stages of follicle development in cattle: focus on steroid biosynthesis. *Physiological Genomics,* 44**,** 504-517.

WALTERS, T. D. & GRIFFITHS, A. M. 2009. Mechanisms of growth impairment in pediatric Crohn's disease. *Nature Reviews Gastroenterology & Hepatology,* 6**,** 513.

WANG, G. L., JIANG, B.-H. & SEMENZA, G. L. 1995. Effect of protein kinase and phosphatase inhibitors on expression of hypoxia inducible factor 1. *Biochemical and Biophysical Research Communications,* 216**,** 669-675.

WANG, G. L. & SEMENZA, G. L. 1995. Purification and characterization of hypoxia-inducible factor 1. *Journal of Biological Chemistry,* 270**,** 1230-1237.

WANG, L., XIONG, M., CHE, D., YUAN, Y. & LIU, S. 1999. Effect of hypoxia on proliferation and differentiation of the pulmonary vascular pericytes. *Zhonghua Jie He He Hu Xi Za Zhi,* 22**,** 176-8.

WANG, T.-H., HORNG, S.-G., CHANG, C.-L., WU, H.-M., TSAI, Y.-J., WANG, H.-S. & SOONG, Y.-K. 2002. Human chorionic gonadotropin-induced ovarian hyperstimulation syndrome is associated with up-regulation of vascular endothelial growth factor. *The Journal of Clinical Endocrinology and Metabolism,* 87**,** 3300-3308.

WANG, W., KUMAR, P., WANG, W., EPSTEIN, J., HELMAN, L., MOORE, J. V. & KUMAR, S. 1998. Insulin-like growth factor II and PAX3-FKHR cooperate in the oncogenesis of rhabdomyosarcoma. *Cancer Research,* 58**,** 4426-4433.

WATSON, E. & FRASER, H. 2003. Angiogenesis and vascular endothelial growth factor expression in the equine corpus luteum. *Reproduction,* 125**,** 259-270.

WEBB, R. & CAMPBELL, B. 2007. Development of the dominant follicle: mechanisms of selection and maintenance of oocyte quality. *Society of Reproduction and Fertility Supplement,* 64**,** 141.

WEISS, T. W., MEHRABI, M. R., KAUN, C., ZORN, G., KASTL, S. P., SPEIDL, W. S., PFAFFENBERGER, S., REGA, G., GLOGAR, H. D., MAURER, G., PACHER, R., HUBER, K. & WOJTA, J. 2004. Prostaglandin E1 induces vascular endothelial growth factor-1 in human adult cardiac myocytes but not in human adult cardiac fibroblasts via a cAMP-dependent mechanism. *Journal of Molecular and Cellular Cardiology,* 36**,** 539-46.

WELCH, W. J., BAUMGÄRTL, H., LÜBBERS, D. & WILCOX, C. S. 2001. Nephron pO2 and renal oxygen usage in the hypertensive rat kidney. *Kidney International,* 59**,** 230-237.

WENGER, R. H. 2002. Cellular adaptation to hypoxia: O2-sensing protein hydroxylases, hypoxia-inducible transcription factors, and O2-regulated gene expression. *The FASEB Journal,* 16**,** 1151-1162.

WENGER, R. H. & KATSCHINSKI, D. M. 2005. The hypoxic testis and post-meiotic expression of PAS domain proteins. Seminars in Cell and Developmental Biology, 2005. Elsevier, 547-553.

WHALEN, W. J., GANFIELD, R. & NAIR, P. 1970. Effects of breathing O2 or O2+ CO2 and of the injection of neurohumors on the PO2 of cat cerebral cortex. *Stroke,* 1**,** 194-200.

WILHELM, D., PALMER, S. & KOOPMAN, P. 2007. Sex determination and gonadal development in mammals. *Physiological Reviews,* 87**,** 1-28.

WILTBANK, M., BELFIORE, C. & NISWENDER, G. 1993. Steroidogenic enzyme activity after acute activation of protein kinase (PK) A and PKC in ovine small and large luteal cells. *Molecular and Cellular Endocrinology,* 97**,** 1-7.

WILTBANK, M., GALLAGHER, K., CHRISTENSEN, A., BRABEC, R. & KEYES, P. 1990. Physiological and immunocytochemical evidence for a new concept of blood flow regulation in the corpus luteum. *Biology of Reproduction,* 42**,** 139-149.

WOAD, K., BAXTER, G., HOGG, C., BRAMLEY, T., WEBB, R. & ARMSTRONG, D. 2000. Expression of mRNA encoding insulin-like growth factors I and II and the type 1 IGF receptor in the bovine corpus luteum at defined stages of the oestrous cycle. *Journal of Reproduction and Fertility,* 120**,** 293-302.

WOAD, K. J., HAMMOND, A. J., HUNTER, M., MANN, G. E., HUNTER, M. G. & ROBINSON, R. S. 2009. FGF2 is crucial for the development of bovine luteal endothelial networks in vitro. *Reproduction,* 138**,** 581-588.

WOAD, K. J., HUNTER, M. G., MANN, G. E., LAIRD, M., HAMMOND, A. J. & ROBINSON, R. S. 2012. Fibroblast growth factor 2 is a key determinant of vascular sprouting during bovine luteal angiogenesis. *Reproduction,* 143**,** 35-43.

WÖLFLE, D. & JUNGERMANN, K. 1985. Long-term effects of physiological oxygen concentrations on glycolysis and gluconeogenesis in hepatocyte cultures. *European Journal of Biochemistry,* 151**,** 299-303.

WOOD, A. W., SCHLUETER, P. J. & DUAN, C. 2005. Targeted knockdown of insulin-like growth factor binding protein-2 disrupts cardiovascular development in zebrafish embryos. *Molecular Endocrinology,* 19**,** 1024-1034.

WROBEL, K.-H. & SUSS, F. 1998. Identification and temporospatial distribution of bovine primordial germ cells prior to gonadal sexual differentiation. *Anatomy and Embryology,* 197**,** 451-467.

WU, G. 2009. Amino acids: metabolism, functions, and nutrition. *Amino Acids,* 37**,** 1-17.

WU, G., BAZER, F. W., CUDD, T. A., MEININGER, C. J. & SPENCER, T. E. 2004. Maternal nutrition and fetal development. *The Journal of Nutrition,* 134**,** 2169-2172.

WU, G., BAZER, F. W., WALLACE, J. M. & SPENCER, T. E. 2006. Board-invited review: intrauterine growth retardation: implications for the animal sciences. *Journal of Animal Science,* 84**,** 2316-2337.

WULFF, C., DICKSON, S. E., DUNCAN, W. C. & FRASER, H. M. 2001a. Angiogenesis in the human corpus luteum: simulated early pregnancy by HCG treatment is associated with both angiogenesis and vessel stabilization. *Human Reproduction,* 16**,** 2515-2524.

WULFF, C., WIEGAND, S. J., SAUNDERS, P. T., SCOBIE, G. A. & FRASER, H. M. 2001b. Angiogenesis during follicular development in the primate and its inhibition by treatment with truncated Flt-1-Fc (Vascular Endothelial Growth Factor TrapA40). *Endocrinology,* 142**,** 3244-3254.

WULFF, C., WILSON, H., WIEGAND, S. J., RUDGE, J. S. & FRASER, H. M. 2002. Prevention of thecal angiogenesis, antral follicular growth, and ovulation in the primate by treatment with vascular endothelial growth factor Trap R1R2. *Endocrinology,* 143**,** 2797-2807.

YAKAR, S., LIU, J.-L., STANNARD, B., BUTLER, A., ACCILI, D., SAUER, B. & LEROITH, D. 1999. Normal growth and development in the absence of hepatic insulin-like growth factor I. *Proceedings of the National Academy of Sciences,* 96**,** 7324-7329.

YALU, R., OYESIJI, A. E., EISENBERG, I., IMBAR, T. & MEIDAN, R. 2015. HIF1A-dependent increase in endothelin 2 levels in granulosa cells: role of hypoxia, LH/cAMP, and reactive oxygen species. *Reproduction,* 149**,** 11-20.

YAMASHITA, H., KAMADA, D., SHIRASUNA, K., MATSUI, M., SHIMIZU, T., KIDA, K., BERISHA, B., SCHAMS, D. & MIYAMOTO, A. 2008. Effect of local neutralization of basic fibroblast growth factor or vascular endothelial growth factor by a specific antibody on the development of the corpus luteum in the cow. *Molecular Reproduction and Development,* 75**,** 1449-1456.

YANG, J., SHI, Q. D., SONG, T. B., FENG, G. F., ZANG, W. J., ZONG, C. H. & CHANG, L. 2013. Vasoactive intestinal peptide increases VEGF expression to promote proliferation of brain vascular endothelial cells

via the cAMP/PKA pathway after ischemic insult in vitro. *Peptides,* 42**,** 105-11.

YAO, H.-C., LIU, T., MENG, X.-Y., HAN, Q.-F., ZHANG, M. & WANG, L.-X. 2013. Effect of basic fibroblast growth factor on the myocardial expression of hypoxia-inducible factor-1α and vascular endothelial growth factor following acute myocardial infarction. *Heart, Lung and Circulation,* 22**,** 946-951.

YATES, D. T., PETERSEN, J. L., SCHMIDT, T. B., CADARET, C. N., BARNES, T., POSONT, R. J. & BEEDE, K. A. 2018. Fetal origins of impaired muscle growth and metabolic dysfunction: lessons from the heat-stressed pregnant ewe. *Journal of Animal Science,* 96**,** 2987–3002.

YUNG, M., VANDEHAAR, M., FOGWELL, R. & SHARMA, B. 1996. Effect of energy balance and somatotropin on insulin-like growth factor I in serum and on weight and progesterone of corpus luteum in heifers. *Journal of Animal Science,* 74**,** 2239-2244.

ZAMBONI, L., BÉZARD, J. & MAULEON, P. 1979. The role of the mesonephros in the development of the sheep fetal ovary. Annales de Biologie Animale Biochimie Biophysique, 1979. EDP Sciences, 1153-1178.

ZEEBAREE, B. K., KWONG, W. Y., MANN, G. E., GUTIERREZ, C. G. & SINCLAIR, K. D. 2018. Physiological responses of cultured bovine granulosa cells to elevated temperatures under low and high oxygen in the presence of different concentrations of melatonin. *Theriogenology,* 105**,** 107-114.

ZHANG, Q., LI, Q., LI, J., LI, X., LIU, Z., SONG, D. & XIE, Z. 2008. b-DAZL: A novel gene in bovine spermatogenesis. *Progress in Natural Science,* 18**,** 1209-1218.

ZHAO, J., TAVERNE, M., VAN DER WEIJDEN, G., BEVERS, M. & VAN DEN HURK, R. 2002. Immunohistochemical localisation of growth hormone (GH), GH receptor (GHR), insulin-like growth factor I (IGF-I) and type I IGF-I receptor, and gene expression of GH and GHR in rat pre-antral follicles. *Zygote,* 10**,** 85-94.

ZHOU, J., KUMAR, T. R., MATZUK, M. M. & BONDY, C. 1997. Insulin-like growth factor I regulates gonadotropin responsiveness in the murine ovary. *Molecular Endocrinology,* 11**,** 1924-1933.

ZHOU, J., WANG, J., PENNY, D., MONGET, P., ARRAZTOA, J. A., FOGELSON, L. J. & BONDY, C. A. 2003. Insulin-like growth factor binding protein 4 expression parallels luteinizing hormone receptor expression and follicular luteinization in the primate ovary. *Biology of Reproduction,* 69**,** 22-29.

ZHOU, J., YAO, W., LI, C., WU, W., LI, Q. & LIU, H. 2017. Administration of follicle-stimulating hormone induces autophagy via upregulation of HIF-1α in mouse granulosa cells. *Cell Death & Disease,* 8**,** 3001.

ZIERATH, J., HE, L., GUMA, A., WAHLSTRÖM, E. O., KLIP, A. & WALLBERG-HENRIKSSON, H. 1996. Insulin action on glucose transport and plasma membrane GLUT4 content in skeletal muscle from patients with NIDDM. *Diabetologia,* 39**,** 1180.

ZIMMERMANN, R. C., XIAO, E., BOHLEN, P. & FERIN, M. 2002. Administration of antivascular endothelial growth factor receptor 2 antibody in the early follicular phase delays follicular selection and development in the rhesus monkey. *Endocrinology,* 143**,** 2496-2502.

ZIMMERMANN, R. C., XIAO, E., HUSAMI, N., SAUER, M. V., LOBO, R., KITAJEWSKI, J. & FERIN, M. 2001. Short-term administration of antivascular endothelial growth factor antibody in the late follicular phase delays follicular development in the rhesus monkey. *The Journal of Clinical Endocrinology and Metabolism,* 86**,** 768-772.

ZUCKERMAN, S. 1951. The number of oocytes in the mature ovary. *Recent Progress in Hormone Research,* 6**,** 63-109.

ZYGMUNT, M., HERR, F., MÜNSTEDT, K., LANG, U. & LIANG, O. D. 2003. Angiogenesis and vasculogenesis in pregnancy. *European Journal of Obstetrics & Gynecology and Reproductive Biology,* 110**,** S10-S18.

# Appendix

Abbreviations

| | |
|---|---|
| %B/TB | % Binding/Total Binding |
| ACTA2 | Alpha-smooth muscle actin |
| ACTB | Beta actin |
| AFC | Antral follicle counts |
| ALS | Acid labile subunit |
| AMH | Anti-Mullerian hormone |
| BHB | Beta-hydroxybutyrate |
| BMI | Body mass index |
| BMP4 | Bone morphogenetic protein 4 |
| BNIP3 | Apoptotic BCL-2 protein-interacting protein 3 |
| BSA | Bovine serum albumin |
| CD31 | Platelet endothelial cell adhesion molecule |
| CL | Corpus luteum |
| CP | Control protein |
| CYP11A1 | Cytochrome P450 cholesterol side-chain cleavage |
| CYP17A1 | Cytochrome P450 17A1 or 17α-hydroxylase |
| CYP19A1 | Aromatase |
| DAZL | Deleted azoospermia-like |
| DM | Dry matter |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| E2 | Oestradiol |
| EC | Endothelial cells |
| ECL | Electrochemiluminescence |
| ECM | Extracellular matrix |
| EGF | Epidermal growth factors |
| ELISA | Enzyme-linked immunosorbent assay |
| eNOS | Endothelial nitric oxide synthase |

| | |
|---|---|
| EPC | Endothelial progenitor cells |
| EPO | Erythropoietin |
| FGF2 | Fibroblast growth factor |
| FGFR1 | Fibroblast growth factor type 1 receptor |
| FGFR2 | Fibroblast growth factor type 2 receptor |
| FOXL2 | Forkhead box L2 |
| FSH | Follicle stimulating hormone |
| FSHR | Follicle stimulating hormone receptor |
| GC | Granulosa cells |
| GH | Growth hormone |
| GHR1A | Growth hormone receptor |
| GLUT1 | Glucose transporters |
| GnRH | Gonadotropin releasing hormone |
| GREL | Gonadal ridge epithelial-like |
| H&E | Haematoxylin and Eosin staining |
| HCG | Human chorionic gonadotrophin |
| HIF1A | Hypoxia-inducible factor 1A |
| HIST3H3 | Histone cluster 3 H3 |
| HPO | Hypothalamic-pituitary-ovarian |
| HRE | Hypoxia response elements |
| HRP | Horseradish peroxidase |
| HSD17B | 17β-hydroxysteroid dehydrogenase |
| HSD3B1 | 3β-hydroxysteroid dehydrogenase |
| HUVEC | Human umbilical vein endothelial cells |
| IGF1 | Insulin-like growth factor 1 |
| IGF1R | Insulin-like growth factor type 1 receptor |
| IGF2 | Insulin-like growth factor 2 |
| IGFBP | Insulin-like growth factor binding proteins |
| IMS | Industrial methylated spirits |
| IR | Insulin receptor |
| IRS | Insulin receptor substrate |

| | |
|---|---|
| ITS | Insulin, Transferrin and Selenium |
| IUGR | Intrauterine growth retardation |
| Ki67 | Cell proliferation marker |
| LDH | Lactate dehydrogenase |
| LH | Luteinising hormone |
| LHR | Luteinising hormone receptor |
| LP | Low protein |
| M6P/IGF2R | Mannose 6-phosphate/IGF type 2 receptor |
| MMP2 | Matrix metalloproteinase 2 |
| mRNA | Messenger RNA |
| NADH | Nicotinamide adenine dinucleotide |
| NEB | Negative energy balance |
| NEFAS | Non-esterified fatty acids |
| NGS | Normal goat serum |
| NHS | Normal horse serum |
| NO | Nitric oxide |
| OCT4 | Octamer binding transcription factor 4 |
| OD | Optical density |
| ODD | oxygen-dependent degradation domain |
| P4 | Progesterone; |
| PAS | Periodic-Acid Schiff staining |
| PBS | Phosphate-buffered saline |
| PDGF | Platelet-derived growth factor |
| PGC | Primordial germ cells |
| PGF2$\alpha$ | Prostaglandin F2$\alpha$ |
| PGK1 | Phosphoglycerate kinase 1 |
| PKA | Protein kinase A |
| PKC | Protein kinase C |
| pO2 | Physiological oxygen concentration |
| PPP | Picropodophyllin |
| PVDF | Polyvinylidene fluoride |

| | |
|---|---|
| RNA | Ribonucleic acid |
| RT | Room temperature |
| SDS | Sodium dodecyl sulphate |
| STAR | Steroidogenic acute regulatory protein |
| STC! | Stanniocalcin 1 |
| TBS | Tris-buffered saline |
| TC | Theca cells |
| TGF | Transforming growth factor |
| TMB | 3, 3', 5, 5'-tetramethylbenzidine |
| UK | United Kingdom |
| USA | United States of America |
| VASA | Dead Box: *DDX4/MVH* |
| VEGFA | Vascular endothelial growth factor |
| VEGFR1 | Vascular endothelial growth factor type 1 receptor |
| VEGFR2 | Vascular endothelial growth factor type 2 receptor |
| VWF | Von Willebrand factor |

The total number of ovarian tissue slides selection for the various histological and immunohistochemical analysis.

| Block | H&E | PAS | OCT4 | DAZL | CD31 | VASA | Ki67 | Caspase3 |
|---|---|---|---|---|---|---|---|---|
| **N110088** | 7 | 6 | 7 | 7 | 5 | 7 | 6 | 6 |
| **N110090** | 8 | 5 | 8 | 8 | 8 | 8 | 8 | 8 |
| **N110093** | 5 | 3 | 4 | 4 | 4 | 4 | 3 | 4 |
| **N110095** | 6 | 5 | 6 | 6 | 5 | 6 | 6 | 5 |
| **N110096** | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| **N110097** | 4 | 4 | 4 | 4 | 3 | 4 | 4 | 4 |
| **N110098** | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 4 |
| **N110102** | 7 | 4 | 7 | 7 | 7 | 7 | 7 | 7 |
| **N110104** | 5 | 3 | 4 | 4 | 4 | 4 | 4 | 4 |
| **N110108** | 6 | 6 | 6 | 5 | 5 | 5 | 5 | 5 |
| **N110109** | 4 | 2 | 4 | 4 | 4 | 5 | 4 | 4 |
| **N110110** | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| **N110111** | 5 | 2 | 5 | 5 | 4 | 5 | 5 | 4 |
| **N110134** | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| **N110135** | 7 | 6 | 7 | 7 | 7 | 6 | 6 | 6 |
| **Total Slides** | **83** | **65** | **81** | **80** | **75** | **80** | **77** | **75** |

**Paraffin embedded sections immunohistochemistry:**

10 mM citrate buffer:

- Stock A citric acid buffer: 4.2 g citric acid in 200 ml $dH_2O$.
- Stock B citrate buffer: 14.7 g sodium citrate in 500 ml $dH_2O$.
- 10 mM citrate buffer: 16ml stock A, 84 ml stock B and 900 ml $dH_2O$.
- pH checked to pH 6.0

***In vitro* cell culture dissociation solution:**

The following was added to 18.5 ml M199 media:

- 1 ml of 100mg/ml Collagenase 1A (Sigma-Aldrich Poole, UK)
- 400 µl of 50 mg/ml hyaluronidase type IS (Sigma-Aldrich, Poole, UK)
- 25 µl DNase I (Sigma-Aldrich, Poole, UK)

**Preparation of ELISA buffers:**

Coating buffer: (Sodium bicarbonate buffer 0.05 M, pH 9.6):

- 1.59 g $Na_2CO_3$
- 2.93 g $NaHCO_3$

Make up to 1 Litre with distilled water, check pH and alter if necessary.

ELISA buffer: (0.1 M phosphate buffered saline containing 0.1% BSA, pH 7).

- Add 2 PBS tablets to 1 litre of distilled water.
- Add 1 g of bovine serum albumin.
- Check pH and alter as necessary.

Wash solution: (0.15 M NaCl + 0.05% Tween 20).

- 8.76 g NaCl made up to 1 litre with distilled water.
- Add 0.5 ml Tween 20 and mix well.

ELISA block buffer: (PBS + 1% BSA).

- Dissolve 1 PBS tablet in 500 ml of distilled water, add 5 g BSA then aliquot into 50 ml aliquots and store in the freezer.

**Western blot buffers:**

Lysis buffer

- 155 mM ammonium chloride
- 10 mM potassium bicarbonate
- 0.01% of EDTA solution

Sample buffer:

25 µl of β-mecaptoethanol was added to 475 µl of Laemmli buffer (Bio-Rad).

Cell extraction buffer:

- 100 mM Tris, pH 7.4
- 2 mM $Na_3VO_4$
- 100 mM NaCL.
- 1% Triton X-100
- 1 mM EDTA
- 10% glycerol
- 1 mM EGTA
- 0.1% SDS
- 1 mM NaF
- 0.5% deoxycholate
- 20 mM $Na_4P_2O_7$

NuPAGE transfer buffer:

- 15 ml 20x TG buffer (ThermoFisher, USA).
- 255 ml $dH_2O$
- 30 ml methanol.

MOPS running buffer:

800ml of MOPS SDS running buffer was prepared by adding 40ml of 20x MOPS to 760 ml of distilled water.

Stripping buffer, 500 ml

- 7.5 g glycine
- 0.5 g SDS
- 5 ml Tween 20
- 450 ml RO water
- Adjust pH to 2.2

- Bring volume up to 500 ml with RO water

**IGF1 Assay**

PBS (R&D Systems, Catalog # DY006):

137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.2-7.4, 0.2 µm filtered.

Wash Buffer (R&D Systems, Catalog # WA126):

0.05% Tween 20 in PBS, pH 7.2-7.4.

Reagent Diluent (R&D Systems, Catalog # DY004):

5% Tween 20 in PBS, pH 7.2-7.4, 0.2 µm filtered.

Substrate Solution (R&D Systems, Catalog # DY999):

1:1 mixture of Color Reagent A ($H_2O_2$) and Color Reagent B (Tetramethylbenzidine).

Stop Solution (R&D Systems, Catalog # DY994):

2 N $H_2SO_4$

**VEGFA ASSAY**

Bovine VEGFA pre-coated plates (VS0286B-CP):

Polystyrene 96-well plates.

DPBS:

0.008M sodium phosphate, 0.002 M potassium phosphate, 0.14 M sodium chloride, 0.01 M potassium chloride, pH 7.4.

Wash Buffer:

0.05% Tween 20 in DPBS.

Reagent Diluent:

4% BSA in DPBS, 0.2 µm filtered. 2 g BSA in 50 ml DPBS sterile filtered.

Substrate Solution (Catalog # GWB-374D5C):

3,3',5,5'-tetramethylbenzidine (TMB) Substrate ELISA.

Stop Solution (Catalog # GWB-2009AD):

0.18 M Sulfuric Acid ELISA.

ITS added on an individual basis

12.5 µl of 4mg/ml human insulin (Gibco: 12585014),

500 µl of 10 mg/ml sodium selenite (Sigma-Aldrich: S5261)

275 µl of 10 mg/ml transferrin (Sigma-Aldrich: T1428).

The human $LR^3$-IGF1 (Sigma-Aldrich: I1271) was dissolved in 100 µl of 10 mM hydrochloric acid (HCl), 9.9 ml PBS and 0.1% BSA to get 10 µg/ml $LR^3$-IGF1. However, 2 µl or 20 µl of 10 µg/ml $LR^3$-IGF1 were further dissolved with 2 ml of MCDB 131 media for the final concentrations of 10 and 100 ng/ml $LR^3$-IGF1 per treatment for the experiment.

Insulin (Gibco: 12585014), transferrin (Sigma-Aldrich: T1428) and selenium (Sigma-Aldrich: S5261) was added individually at final concentrations of 100 ng/ml, 5 ng/ml and 5.5 µg/ml respectively.