

Factors influencing luteal angiogenesis and alternative splicing in the bovine ovary

Robert F. Thompson BVMedSci

Thesis submitted to the University of Nottingham for the degree of Master of Research

MAY 15, 2017

Declaration.

I declare that this research is a presentation of my own work and has not been submitted for any other degree. Wherever contributions from any other individual's work were presented, every effort was made to ensure this was clearly acknowledged and referenced. I have also acknowledged all support provided during this research.

Acknowledgements.

I would like to acknowledge and express my gratitude to my supervisors Dr. Bob Robinson and Dr. Katie Woad for their guidance, technical knowledge and expertise. I would not have been able to complete this research without their support and patience, for which I am very grateful.

I am very grateful for the assistance that Dr. Nigel Mongan provided, his technical expertise and advice were invaluable. I would also like to express my thanks to Professor David Bates and his team for their technical assistance. Equally, I would like to thank the technical team at the School of Veterinary Medicine and Science for their guidance. I would also like to acknowledge the contributions of Dr Jennifer Edwards and Emer Grant.

I am very grateful to the University of Nottingham, School of Veterinary Medicine and Science for funding this research.

I would also like to thank my family for their constant encouragement and support. Finally I would like to express my endless thanks to Annemieke Nicholls for her care, support and patience throughout the duration of this research.

Contents

List of Figures. 6
List of Tables 7
Abstract 8
Chapter 1. Review of the literature. 11
1.1 Introduction. 11
1.2 Corpus luteum formation and growth. 12
1.2.1 Luteinisation of the ovulated follicle. 12
1.2.2 Luteal growth and endocrine development. 14
1.3 The bovine oestrous cycle, hormonal regulation and synthesis. 14
1.3.1 The hypothalamic pituitary gonadal axis. 14
1.3.2 FSH, LH and oestradiol. 16
1.3.3 Progesterone. 17
1.3.3.1 Progesterone synthesis. 17
1.3.3.2 Interaction of progesterone with other reproductive hormones. 18
1.3.3.3 The effect of progesterone on the reproductive tract. 20
1.3.3.4 The importance of progesterone in the establishment of pregnancy. 21
1.4 Angiogenesis. 22
1.4.1 Mechanisms of angiogenesis in the CL. 22
1.4.2 Factors regulating angiogenesis in the bovine CL. 24
1.4.2.1 Pro-angiogenic factors in the bovine CL. 25
1.4.3 Vascular Endothelial Growth Factor A (VEGFA). 26
1.4.3.1 VEGFA structure and isoforms. 26
1.4.3.2 VEGF receptors. 28
1.4.3.3 VEGFA isoform expression in the bovine CL. 29
1.4.3.4 VEFGA effects on angiogenesis. 30
1.4.4 Fibroblast Growth Factor 2. 30
1.4.4.1 FGF2 structure and family. 30
1.4.4.2 FGF receptors (FGFR) 31
1.4.4.3 FGF2 expression in the bovine CL. 31
1.4.4.4 The effect of FGF2 on angiogenesis in the bovine CL. 32
1.4.4.5 The effect of FGF2 on progesterone production. 33
1.4.5 Platelet derived growth factor (PDGF) 34

1.4.5.1 Platelet derived growth factor structure and family. 34
1.4.5.2 PDGF receptors.....34
1.4.5.3 Expression of PDGF in the bovine CL.....34
1.4.5.4 The effect of PDGF on angiogenesis.....35
1.5 Anti-angiogenic factors in the bovine CL.....35
1.5.1 VEGFA.....37
1.5.1.1 Anti-VEGFA isoform formation. 37
1.5.1.2 The inhibitory role of soluble VEGF receptors and their expression in bovine CL. 38
1.5.1.3 Expression of $VEGFA_{xxxb}$ in the CL.....38
1.5.1.4 The anti-angiogenic properties of $VEGFA_{xxxb}$. 39
1.5.2 Pigment epithelium derived factor (PEDF) 40
1.5.2.1 PEDF structure.....40
1.5.2.2 PEDF expression and its activity.....40
1.5.2.3 The anti-angiogenic effects of PEDF.....41
1.5.3 Thrombospondins (THBS). 41
1.5.3.1 THBS family and structure.....41
1.5.3.2 THBS1 and THBS2 receptors.....42
1.5.3.3 THBS expression in the bovine ovary. 42
1.5.3.4 The effect of THBS on luteal angiogenesis.....43
1.5.4 Transforming growth factor-B. 44
1.5.4.1 TGFB family and structure.....44
1.5.4.2 TGFB receptors.....44
1.5.4.3 TGFB expression and effect in the bovine CL.....45
1.5.5 Vasohibin.....46
1.5.5.1 Vasohibin structure. 46
1.5.5.2 Vasohibin expression and effect on angiogenesis. 46
1.5.6 2-methoxyoestradiol (2-ME).....48
1.5.6.1 2-ME structure. 48
1.5.6.2 Production of 2-ME and its effect on angiogenesis. 48
1.6 Alternative splicing of pre-mRNA. 49
1.6.1 Mechanism of splicing. 50
1.6.2 Types of alternative splicing. 51
1.6.3 Regulation of alternative splicing. 53

1.6.4 Alternative splicing in the bovine ovary. 53
1.7 Conclusion.....55
Chapter 2. Anti-angiogenic factors. 56
2.1 Introduction. 56
2.1.2 Hypothesis and aims. 57
2.2 Materials and methods. 58
2.2.1 PEDF, VEGFA and $VEGFA_{165b}$ immunohistochemistry on bovine CL sections. 58
2.2.1.1 Tissue collection and processing. 58
2.2.1.2 Immunohistochemistry procedure for PEDF, VEGFA164b and VEGFA. 59
2.2.1.3 Image acquisition. 61
2.2.2 Western blotting. 61
2.2.2.1 Sample collection. 61
2.2.2.2 Sample preparation. 62
2.2.2.3 Protein determination, Bradford assay. 62
2.2.2.4 SDS-PAGE (protein separation). 63
2.2.2.5 Blotting/transfer of protein. 63
2.2.2.6 Immunoblotting. 64
2.2.2.7 Stripping blots. 64
2.2.3 In vitro luteal angiogenesis co-culture system. 65
2.2.3.1 Ovary collection and CL selection. 65
2.2.3.2 Fibronectin coating. 65
2.2.3.3. Culture procedure. 66
2.2.4. Immunohistochemistry for von Willebrand factor (VWF). 67
2.2.4.1 Image analysis of Von Willebrand Factor immunohistochemistry. 69
2.2.4.2 Statistical analysis. 69
2.2.5. Progesterone ELISA. 70
2.2.5.1. Preparation of progesterone standards. 70
2.2.5.2. Preparation of progesterone HRP conjugate. 70
2.2.5.3 Preparation of progesterone antiserum. 70
2.2.5.4. Coating plates. 71
2.2.5.5. Assay procedure. 71

2.2.5.6. Curve fitting. 71
2.2.5.7. Statistical analysis. 72
2.3 Results. 72
2.3.1 Localisation of VEGFA and $VEGFA_{164b}$ in the bovine CL. 72
2.3.1.1 Spatial-temporal expression of VEGFA in the bovine CL. 72
2.3.1.2 Spatial-temporal expression of $VEGFA_{164b}$ in the bovine CL. 73
2.3.1.3 Identification of VEGFA and VEGFA164b protein by Western blot. 75
2.3.2 PEDF expression in the bovine CL and its effect on luteal angiogenesis and function in vitro. 76
2.3.2.1 Spatial-temporal expression of PEDF in the bovine CL. 76
2.3.2.2 Western blot analysis of PEDF protein in the bovine CL. 79
2.3.2.3 The effect of PEDF on EC network morphology. 80
2.3.2.4 The effect of PEDF on key EC network formation parameters. 81
2.3.2.5 The effect of PEDF on progesterone production in vitro. 84
2.3.3 The role of THBS1 in early luteal angiogenesis and steroidogenesis. 84
2.3.3.1 The effect of THBS1 on luteal angiogenesis (initial experiment) 84
2.3.3.2 Effect of THBS1 on EC network parameters 88
2.3.3.3 Effect of THBS1 on early luteal angiogenesis and steroidogenesis (main experiment). 90
2.3.3.4 Morphological appearance of EC networks following THBS1 treatment. 90
2.3.3.5 Effect of THBS1 on key EC network parameters. 92
2.3.3.6 The effect of THBS1 on progesterone production in vitro. 95
2.4 Discussion. 96
Chapter 3. Alternative splicing in the bovine ovary. 106
3.1 Introduction. 106
3.1.1 Next generation sequencing. 106
3.1.2 Alternative splicing in the bovine ovary. 106
3.1.3 The effect of dietary protein on reproductive performance. 107
3.1.3.1 The effect of dietary protein on gene expression in bovine granulosa cells. 108
3.1.4 Hypothesis and aims. 108
3.2 Materials and Methods: Splicing. 109
3.2.1 Animals and diet 109

3.2.2 Tissue collection. 110
3.2.3 Sample processing. 110
3.2.4 RNA extraction. 111
3.2.5 Quality control and processing. 111
3.2.6 Alignment of transcript and alternative splicing analysis. 112
3.2.7 Gene ontology and identification of genes of interest. 112
3.2.8 Functional analysis and identification of resultant genes. 113
3.2.9 Sashimi plots and amino acid sequence analysis. 114
3.3 Results. 115
3.3.1 Alternatively spliced genes in bovine granulosa cells. 115
3.3.2 Analysis of known spliced genes in the bovine ovary. 116
3.3.3 Gene ontology. 118
3.3.4 Functional analysis and identification of resultant genes. 120
3.3.5 Functional analysis and identification of resultant genes [selected GO terms]. 122
3.3.6 Further analysis into genes of interest. 126
3.3.7 GO analysis for the genes of interest geneMANIA network. 127
3.3.8 Sashimi plots and amino acid sequences for genes of interest. 128
3.3.8.1 BCAS3. 128
3.3.8.2 IRF1. 129
3.3.8.3 PGF. 130
3.3.8.4 HPSE. 131
3.4 Discussion. 132
Chapter 4. Future directions. 145
5. Bibliography. 148
6. Appendix. 171

List of Figures.

Fig 1.1 Antral follicle structure. 13
Fig 1.2 The bovine oestrous cycle.....15
Fig 1.3 Progesterone synthesis.....20
Fig 1.4 Mechanism of sprouting angiogenesis. 23
Fig 1.5 The mechanism of splicing.....50
Fig 1.6 Alternative splicing events.....52
Fig 2.1 Plate layout for pigment epithelium-derived factor (PEDF) and thrombospondin-1 (THBS1) treatments.....67
Fig 2.2 Spatial-temporal expression of VEGFA throughout luteal development. 73
Fig 2.3 Spatial-temporal expression of VEGFA164b throughout luteal development.....75
Fig 2.4 Temporal expression of VEGFA protein throughout the CL lifespan....76
Fig 2.5 Spatial-temporal expression of PEDF throughout luteal development. 78
Fig 2.6 Temporal expression of PEDF protein throughout the CL lifespan. 79
Fig 2.7 Representative images of the effect of PEDF on luteal EC network development.....80
Fig 2.8 The effect of PEDF on luteal EC network development. 81
Fig 2.9 The effect of PEDF on luteal endothelial cell network parameters.....83
Fig 2.10 The effect of PEDF on luteal progesterone production.....84
Fig 2.11 Effect of THBS1 on luteal EC network development (Day 9).....86
Fig 2.12 Extracted EC networks following THBS1 treatment (Day 9).....87
Fig 2.13 Effect of THBS1 on luteal EC network parameters (Day 9).....89
Fig 2.14 Effect of THBS1 on luteal EC network development (Day 5).....91
Fig 2.15 Extracted EC networks following THBS1 treatment (Day 5).....92
Fig 2.16 Effect of THBS1 on luteal EC network parameters (Day 5).....94
Fig 2.17 Effect of THBS1 on luteal progesterone production.....95
Fig 3.1 GeneMANIA network analysis for all the differentially spliced genes121
Fig 3.2 GeneMANIA network analysis for differentially spliced genes in the (A) RNA binding and (B) regulation of cell cycle GO terms 123
Fig 3.3 GeneMANIA network analysis for differentially spliced genes in the (A) protein phosphatase and (B) immune system development GO terms. 125
Fig 3.4 GeneMANIA network analysis for four genes of interest: IRF1, HPSE, BCAS3 and PGF 126
Fig 3.5 Sashimi plot and mRNA sequence showing A5SS splicing of BCAS3. .128
Fig.3.6 Sashimi plot and amino acid sequence showing a skipped exon in the IRF1 gene 129
Fig 3.7 Sashimi plot and amino acid sequence showing a retained intron in the PGF gene. 130
Fig 3.8 Sashimi plot and amino acid sequence showing a skipped exon in the HPSE gene. 131

List of Tables

Table 1.1 Pro-angiogenic factors, their receptors and functions in the bovine CL. 26
Table 1.2 Anti-angiogenic factors, their receptors and functions. 36
Table 2.1 Primary and secondary antibodies utilised in the immunohistochemistry on CL sections.....61
Table 2.2 Antibodies and their dilutions utilised in Western blotting. 64
Table 3.1 Composition of the different dietary rations for the control and high protein dietary groups.....110
Table 3.2 Differentially spliced genes and their Ensembl IDs that were common to both 11CP vs 13HP and 12 CP vs. 13HP analyses (Novel genes are denoted by the Ensembl ID only).....117
Table 3.3 Gene ontology (GO) analysis of the common gene lists for each type of alternative splicing. 119
Table 3.4 The top 10 GO terms from the geneMANIA network analysis of the genes of interest.....127

Abstract.

The fertility of the cow is influenced by a variety of factors. Two factors of particular importance are the growth and development of corpus luteum (CL) and the impact of diet. Angiogenesis is integral to luteal development, and is critically regulated by the balance of pro- and anti-angiogenic factors. However, the role of many anti-angiogenic factors in the bovine CL has not been investigated.

Therefore, the first aim of this study was to identify the spatial and temporal expression of VEGFA, $VEGFA_{164b}$ and PEDF in the bovine CL, and to determine the effect of PEDF and THBS1 on early luteal angiogenesis and steroidogenesis *in vitro*.

Immunohistochemistry and Western blotting demonstrated that PEDF and VEGFA were expressed at all stages of luteal development, with PEDF concentration greatest in the early CL. In contrast, evidence of luteal $VEGFA_{164b}$ expression was limited. Luteal angiogenesis cultures were subsequently treated with PEDF (0, 10 and 100ng/ml; n=3) or THBS1 (0, 100, 1000ng/ml; n=3) for 5 days. Image analysis of von Willebrand factor immunohistochemistry was performed to quantify the effect on endothelial cell (EC) growth parameters. PEDF (10ng/ml) markedly reduced total and average EC area ($P<0.05$), total branch length and number of branch points, as well as the degree of branching within each EC cluster ($P<0.05$). A preliminary study showed that 1000ng/ml THBS1 reduced the degree of branching per EC cluster ($P=0.03$), but not other parameters. In further studies, THBS1 treatment had no effect on any EC

network parameter. In addition, neither treatment had any effect on progesterone production.

Adequate dietary protein is essential for growth, however the effect of a high level of dietary protein on the bovine ovary has not been established. The second aim of this study was to determine the effect of a high protein diet on alternative splicing of the granulosa cell transcriptome, and to predict the effects of alternative splicing on the genes and gene ontologies controlling granulosa cell function *in silico.*

Beef heifers were fed either control (11%) or high (14%) crude protein diets for at least 60 days. Granulosa cells were collected from multiple medium-sized follicles at slaughter and pooled by diet. Alternative splicing events were identified following rMATs analysis of RNAseq transcription data obtained from the granulosa cells of medium sized follicles from beef heifers fed a control (n=2) or high protein diet (n=1). 127 genes were found to be commonly spliced across analyses of the two controls vs. one high protein comparisons, with 5 genes undergoing two types of splicing simultaneously. Multiple patterns of splicing were observed and the most common type was skipped exon splicing. Gene ontology analysis showed that the high protein diet altered the splicing of genes involved in the regulation of the cell cycle and mitosis. Functional analysis with GeneMANIA showed that angiogenic factors including FLT1, VEGFA and NRP1 were linked to several of the spliced genes by shared domains, and physical or genetic interactions. BCAS3, PGF, IRF1 and HPSE were

identified as genes of interest, splicing caused the insertion of a premature stop codon in PGF, HPSE and IRF1 sequences and thus is predicted to alter function.

In conclusion, this study has shown that anti-angiogenic factors are expressed by the early corpus luteum and have the potential to dramatically inhibit luteal angiogenesis. In addition, ovarian function may also be regulated by dietary protein, whereby the degree and pattern of alternative splicing is predicted to influence critical processes including angiogenesis, cell division and mitosis.

Chapter 1. Review of the literature.

1.1 Introduction.

Over the past 4-5 decades dairy cow fertility has undergone a steady decline, with conception rates currently estimated to be around 35-45% globally (Lucy 2001, Dobson *et al.* 2007). Factors such as the past genetic drive towards higher milk yields at the expense of fertility, altered energy metabolism during times of stress (e.g. during periods of disease and peak milk yield) as well as influences such as the exposure to environmental endocrine disrupting chemicals have all been implicated in the reduction of fertility (Haile-Mariam *et al.* 2003, Ahmadzadeh *et al.* 2009, Roche *et al.* 2009, Sheldon *et al.* 2009, Rhind *et al.* 2010).

Central to the establishment and maintenance of a successful pregnancy is the development of a corpus luteum (CL) from the ovulated dominant follicle under the influence of luteinising hormone (LH), in a process termed luteinisation. The CL is the main steroidogenic tissue responsible for progesterone production, which has several essential roles in the maintenance of pregnancy. For pregnancy to be established, the CL must begin producing large quantities of progesterone within a short period of time (<5 days) of ovulation (Spencer *et al.* 2004). This requires a period of rapid luteal growth such that the rate of growth is only matched by the fastest growing of tumours (Robinson *et al.* 2008).

Integral to luteal growth is the development of a new vasculature network via the process of angiogenesis, and this is of critical importance during early CL

development (Schams & Berisha 2004). Angiogenesis is a highly regulated process that is controlled by a plethora of pro and anti-angiogenic factors (Galvão *et al.* 2013). It is highly likely that the balance of the pro and anti-angiogenic factors is particularly significant. Previous studies have largely focused upon pro-angiogenic factors, the importance of inhibitory factors is widely becoming recognised (Robinson *et al.* 2009).

It is plausible that when high-producing dairy cows are exposed to stresses such as disease and lactational negative energy balance that the ratio of angiogenic factors may become unbalanced (Carmeliet 2003, Woolf *et al.* 2015). The excess of certain inhibitory factors, or inadequate quantity of pro-angiogenic factors, may compromise the dynamic structural changes in the ovary that occur during the cycle. Therefore investigations into the role of anti-angiogenic factors in CL development is of utmost importance.

1.2 Corpus luteum formation and growth.

The CL develops from the remnants of the ovulated follicle in a process termed luteinisation. The follicular-luteal transition begins just prior to ovulation coinciding with the LH surge, and involves a period of structural reorganisation, cell differentiation and biochemical alteration (Reynolds & Redmer 1999, Niswender *et al.* 2000).

1.2.1 Luteinisation of the ovulated follicle.

The pre-ovulatory follicle consists of an oocyte located within a fluid-filled antrum surrounded by a layer of granulosa cells which are separated from the theca interna and externa cell layers by a basement membrane (Fig 1.1). The

theca layer is highly vascularised with vascular density particularly high just beneath the basement membrane, however the granulosa layer remains avascular as the basement membrane provides a physical barrier (Fraser 2006). After ovulation, in the cow the follicle collapses in on itself leading to the intermingling of granulosa and theca cells, alongside this the integrity of the basement membrane is disrupted allowing the in-growth of vessels from the thecal vasculature into the granulosa layer (Fraser & Lunn 2001). This allows an intense period of blood vessel growth to begin, whereby the developing CL becomes fully vascularised.

Simultaneously, a process of cellular differentiation takes place whereby the granulosa and theca cells differentiate into large and small luteal cells, respectively (Niswender *et al.* 2000). Progesterone becomes the primary steroid hormone produced by these cells, instead of oestradiol.

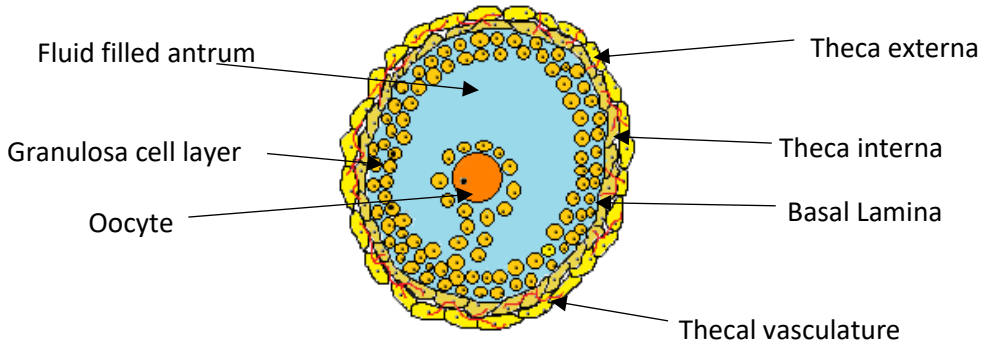


Fig 1.1 *Antral follicle structure.*
The structure of the dominant pre-ovulatory follicle. The follicle consists of an outer thecal cell layer, which is subdivided into theca interna and externa layers. These layers are separated from the inner granulosa cell layer, which surrounds an oocyte and a fluid filled antrum. A basal lamina separates the granulosa and thecal layers. The thecal layer is vascularised, whilst the granulosa layer remains avascular until luteinisation of the follicle following ovulation.

1.2.2 Luteal growth and endocrine development.

The newly formed CL undergoes near exponential growth, during which the number of endothelial cells (ECs) and pericytes, that are associated with new blood vessel formation, vastly increases (Robinson *et al.* 2009). The continuous rapid growth of the bovine CL takes place for 9 days following ovulation, by which time the CL has reached its maximal weight and circumference (Taylor & Rajamahendran 1991, Mann 2009) However, circulating progesterone concentrations continue to rise until around day 16, suggesting that there is continued endocrine development taking place. By the time the CL has finished developing every steroidogenic cell lies adjacent to an EC, giving the CL one of the highest blood flows of any tissue per given mass (Reynolds & Redmer 1999, Fraser & Lunn 2001). Any disruptions to this growth process would reduce the ability of the CL to develop rapidly and initiate the production of progesterone, potentially leading to a decline in fertility.

1.3 The bovine oestrous cycle, hormonal regulation and synthesis.

1.3.1 The hypothalamic pituitary gonadal axis.

The hypothalamic-pituitary-gonadal axis governs the phases and timing of the bovine oestrous cycle. It consists of several key hormones whose production and action are controlled by complex interlinked negative and positive feedback systems (Senger 2003).

In the cow, the development and recruitment of follicle cohorts occurs in waves (Fig 1.2), with at least two waves each oestrous cycle (Boer *et al.* 2011). The first wave takes place just after oestrus (Day 0), with a cohort of antral follicles

developing due to an increase in circulating follicle-stimulating hormone (FSH) (Fig 1.2). Due to the increasing levels of progesterone this follicular wave will not ovulate and the dominant follicle regresses (Richter *et al.* 2002). The second follicular wave develops due to a second spike in FSH production around the time the dominant follicle starts to regress. In the absence of progesterone's inhibitory effects, the dominant follicle from this wave is capable of ovulating under the influence of increasing LH (Ginther *et al.* 2002, Boer *et al.* 2011). The hormones that regulate the bovine oestrous cycle, their interactions and their important role in governing fertility, are discussed in detail below.

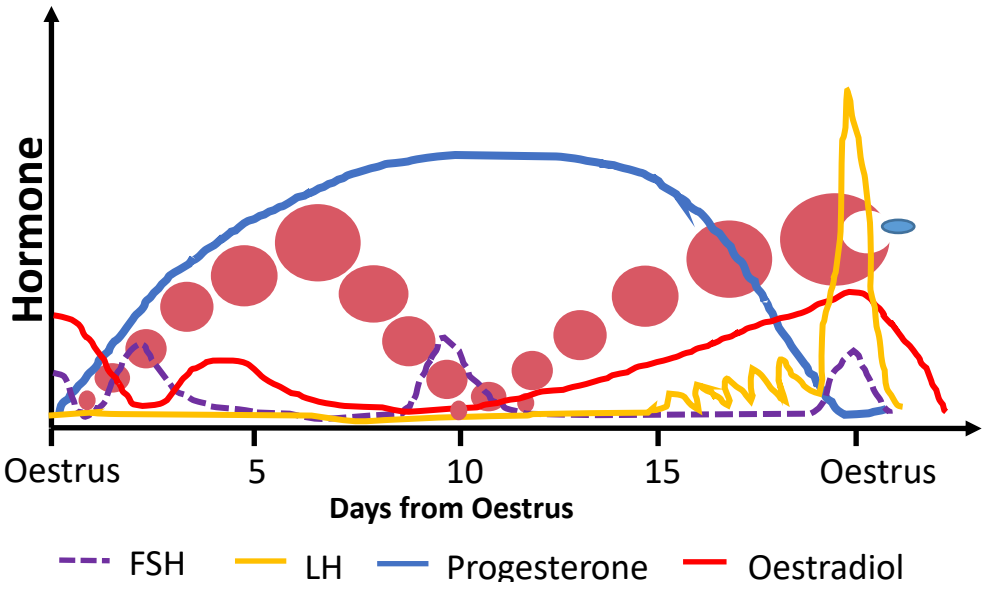


Fig 1.2 *The bovine oestrous cycle.*
Follicle stimulating hormone (FSH), luteinising hormone (LH), progesterone and oestradiol concentrations fluctuate throughout the cycle. A wave like pattern of follicular development occurs alongside these hormonal changes. The corpus luteum secretes progesterone until day 18, after which it regresses. Towards the end of the cycle elevated oestradiol levels prevent FSH production whilst stimulating the pulsatile release of LH. Once sufficient oestradiol is produced by the dominant follicle an LH surge is triggered causing ovulation. The ovulated follicle forms a corpus luteum which produces progesterone. (Adapted from https://research.vet.upenn.edu).

1.3.2 FSH, LH and oestradiol.

FSH and LH are gonadotrophic hormones produced by the anterior pituitary gland under the stimulation of gonadotrophin releasing hormone (GnRH). Low levels of GnRH are produced throughout the follicular phase by the tonic centre in the hypothalamus until sufficient circulating levels of oestradiol are reached to stimulate the surge centre, which ultimately brings about ovulation (Scaramuzzi *et al.* 2011).

FSH and LH act upon their respective receptors on the ovaries to influence oestradiol production, progesterone synthesis, follicle development and ovulation. The production of androgens by theca cells is regulated by LH stimulation, whilst the granulosa cells express aromatase under the influence of FSH (Kumar & Sait 2011). The close proximity of the cell layers means that theca-derived androgens are readily converted to oestradiol by aromatase in the granulosa cells (Bao & Garverick 1998). FSH also acts to increase the number of follicles recruited in each cohort, which further increases the amount of oestradiol produced, with the dominant follicle producing more oestradiol than the small antral follicles (Kumar & Sait 2011).

Elevated concentrations of oestradiol and inhibin produced by developing follicles exert a potent negative feedback effect upon the hypothalamus, leading to a fall in FSH production (Senger 2003). When sufficient LH receptors (LHCGR) are expressed on granulosa cells, LH replaces the role of FSH and supports further follicle development (Zeleznik & Hillier 1984). The decline in FSH coincides with the selection of the dominant follicle, which takes place due

to acquisition of LHCGR in granulosa cells. At this time, follicles are no longer dependent upon FSH (Fortune *et al.* 2001).

When circulating oestradiol levels reach a threshold, positive feedback is initiated leading to the release of large amounts of GnRH, triggering a surge in LH and ovulation (Lucy *et al.* 1992). The LH surge suppresses the growth of non-dominant follicles and granulosa cells, allowing the final stages of oocyte development to occur alongside the beginning of luteinisation (Kumar & Sait 2011).

1.3.3 Progesterone.

1.3.3.1 Progesterone synthesis.

The outline of progesterone synthesis in a generic luteal cell is shown in Fig 1.3. Whilst the underlying process is similar in both large and small luteal cells, there are key differences in the rates of synthesis and factors affecting production in the two cell types (Niswender *et al.* 2000). Large luteal cells are responsible for the majority of basal progesterone secretion, producing 2-40 times the quantity produced by unstimulated small luteal cells (Niswender *et al.* 2000). Small luteal cells are able to increase their progesterone output 5-20 fold following LH stimulation (Wiltbank *et al.* 1993). The reason for the cellular difference in LH responsiveness has yet be elucidated, although it has been suggested that it is related to phosphorylation of steroidogenic acute regulatory protein (StAR) along with involvement of the peripheral benzodiazepine receptor system that also transports steroids within the cell (Niswender *et al.* 2000).

The main substrate for luteal progesterone production is cholesterol. In ruminants this is predominantly acquired from high density lipoproteins (HDLs), however low density lipoproteins (LDLs) and cholesterol esters can be utilised (Wiltbank *et al.* 1990, Niswender 2002). Whilst it remains unclear how cholesterol is transported into cells, HDLs are up taken following binding to HDL receptors and LDLs by receptor mediated endocytosis, with the receptors then being recycled (Fig 1.3) (Grummer & Carroll 1988, Niswender *et al.* 2000).

Free cholesterol is required for steroidogenesis, however surplus cholesterol is stored as cholesterol esters within luteal cells, with conversion controlled by acyl coenzyme A: cholesterol acyltransferase. Cholesterol esterase permits free cholesterol to be reformed (Fig 1.3) (Pittman *et al.* 1975, Johnson *et al.* 1997, Niswender *et al.* 2000).

The movement of cholesterol across the inner mitochondrial membrane is the rate limiting step in progesterone synthesis, with steroid acute regulatory protein (StAR) playing a vital role in this process (Stevens *et al.* 1993, Clark *et al.* 1994). Following transportation into the mitochondria, cholesterol is converted to pregnenolone by cytochrome P450 side-chain cleavage (Tuckey 2005). Pregnenolone is converted to progesterone by 3β-hydroxysteroid dehydrogenase (HSD3B) within the smooth endoplasmic reticulum (Niswender *et al.* 2000). Progesterone then freely diffuses into surrounding tissues and vasculature.

1.3.3.2 Interaction of progesterone with other reproductive hormones.

Progesterone has several important effects upon the hypothalamic-pituitary

ovarian axis, in particular it plays an important role in governing the length of each oestrous cycle (Niswender *et al.* 2000). Progesterone has a negative feedback effect on both the pituitary and hypothalamus. In the hypothalamus, progesterone inhibits surges of GnRH, thus limiting the potential for an LH surge (Senger 2003). Whilst progesterone reduces the number of GnRH receptors in the pituitary, leading to a reduction in LH release, as well as decreasing LH and FSH gene expression (Laws *et al.* 1990, Brann *et al.* 1993, Di Gregorio & Nett 1995).

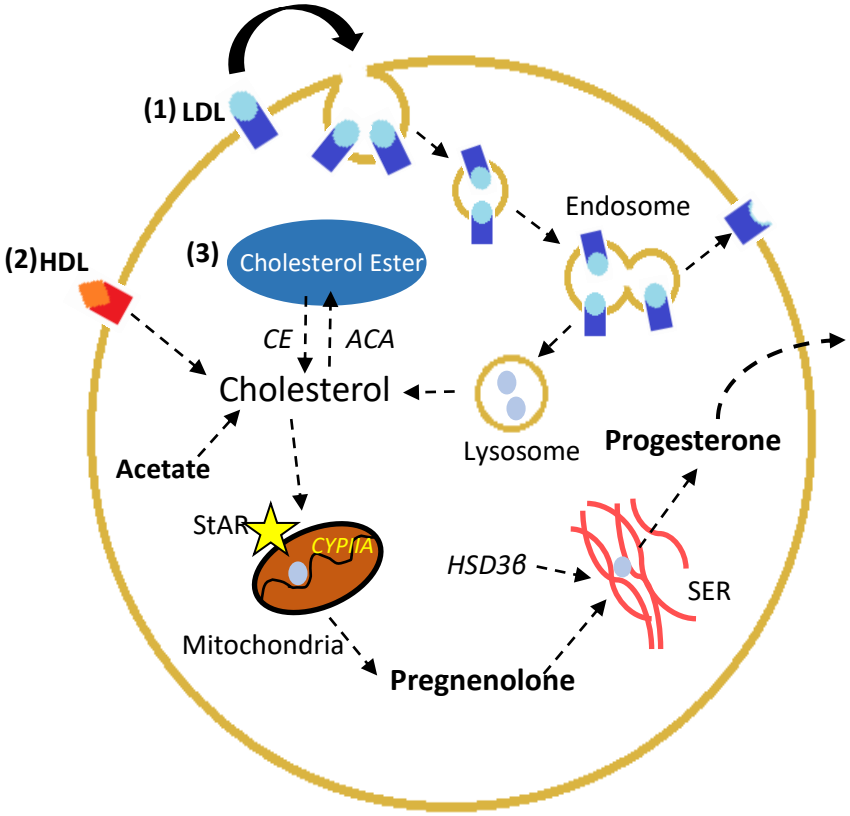


Fig 1.3 *Progesterone synthesis.*
A simplified outline of progesterone synthesis in a luteal cell. The three main sources of the cholesterol are (1) low density lipoproteins (LDLs), (2) high density lipoproteins (HDLs) and (3) cholesterol esters, with HDLs the most common source in cows. Cholesterol esters are hydrolysed by cholesterol esterase (CE) or reformed from cholesterol by acyl coenzyme A:cholesterol acyltransferase (ACAT). Free cholesterol is transported to the mitochondria, then transferred from the outer membrane to the inner membrane by steroidogenic acute regulatory protein (StAR). Inside the mitochondria, cytochrome P450 side chain cleavage enzyme (CYPIIA) converts cholesterol to pregnenolone. Pregnenolone is converted to progesterone by 3β-hydroxysteroid dehydrogenase (HSD3B) in the smooth endoplasmic reticulum (SER). Progesterone then freely diffuses from the cell. (Adapted from Niswender, 2002).

1.3.3.3 The effect of progesterone on the reproductive tract.

During the follicular phase, oestradiol acts on the uterus to promote the growth of endometrial stromal and epithelial cells (Padykula *et al.* 1989). In the luteal phase, progesterone downregulates oestradiol receptors, reducing endometrial growth and stimulating differentiation of the stroma, increasing

the volume of uterine secretions (Maslar *et al.* 1986, Padykula *et al.* 1989). This provides an ideal environment for embryo development, providing an abundance of nutritional support during the early stages of growth.

Progesterone prepares the reproductive tract for the establishment of pregnancy, by binding to the progesterone receptor (PGR) which regulates gene expression by binding to progesterone-responsive elements (Moutsatsou & Sekeris 1997). Progesterone also has a marked effect on the myometrium by inhibiting contractility through reducing calcium uptake, increasing the cellular resting potential, preventing cell to cell stimulation and blocking the actions of oestradiol (Bottari *et al.* 1983, Batra 1986). This aids in attachment of the conceptus to the endometrium, and prevents any myometrial contractions that would result in early expulsion of the fetus.

Any factors that impact upon the production of progesterone will reduce the potential fertility of an animal. Therefore, the processes governing progesterone synthesis are critically important for CL growth and angiogenesis. Any factor(s) that impact upon these mechanisms would decrease progesterone synthesis, delaying the post-ovulatory rise in progesterone and decreasing the final circulating progesterone concentration, both of which will impair reproductive success (Mann & Lamming 1995, Larson *et al.* 1997, Robinson *et al.* 2006).

1.3.3.4 The importance of progesterone in the establishment of pregnancy.

In order for a pregnancy to be established in the cow, the embryo must have developed sufficiently to produce enough interferon-tau to prevent luteolysis

(Mann *et al.* 1999). A low concentration of progesterone has been shown to reduce embryo development and interferon-tau production by the embryo (Mann & Lamming 2001). Therefore, inadequate progesterone production by the CL will reduce embryo growth, preventing the establishment of pregnancy (Mann *et al.* 1999).

1.4 Angiogenesis.

Angiogenesis is the formation of new blood vessels from pre-existing vasculature (Schams & Berisha 2004). The development of an expansive microvascular network is critically important during CL formation to support its rapid growth (Niswender et al. 2000, Woad et al. 2012). In the mature CL, the dense vasculature aids nutrient supply and the dissemination of progesterone to the circulation. In a physiological setting, angiogenesis is highly regulated by a plethora of pro and inhibitory factors (Galvão et al. 2013). An imbalance of these factors can lead to unchecked blood vessel growth (as in neoplasia) or inhibition of vasculature development, which would impair tissue remodelling (e.g. CL development) (Fidler 2000, Robinson et al. 2007). Indeed, it is highly plausible that during disease outbreaks or intense metabolic stress, there may be a shift in the equilibrium of these angiogenic factors leading to negative effects upon luteal growth and progesterone production.

1.4.1 Mechanisms of angiogenesis in the CL.

There are two distinct types of angiogenesis, sprouting and intussusceptive (Adair & Montani 2010). With the predominant form in the CL being sprouting angiogenesis (Amselgruber *et al.* 1999). Sprouting angiogenesis (Fig 1.4)

involves, 1) EC tip differentiation and sprouting, 2) EC stalk proliferation, 3) EC tip migration, junction, lumen and network formation and 4) remodelling and differentiation (Clapp *et al.* 2009, Adair & Montani 2010). Alongside this, recruitment of mural cells (e.g. pericytes) takes place to support the newly formed vascular networks (Bergers & Song 2005).

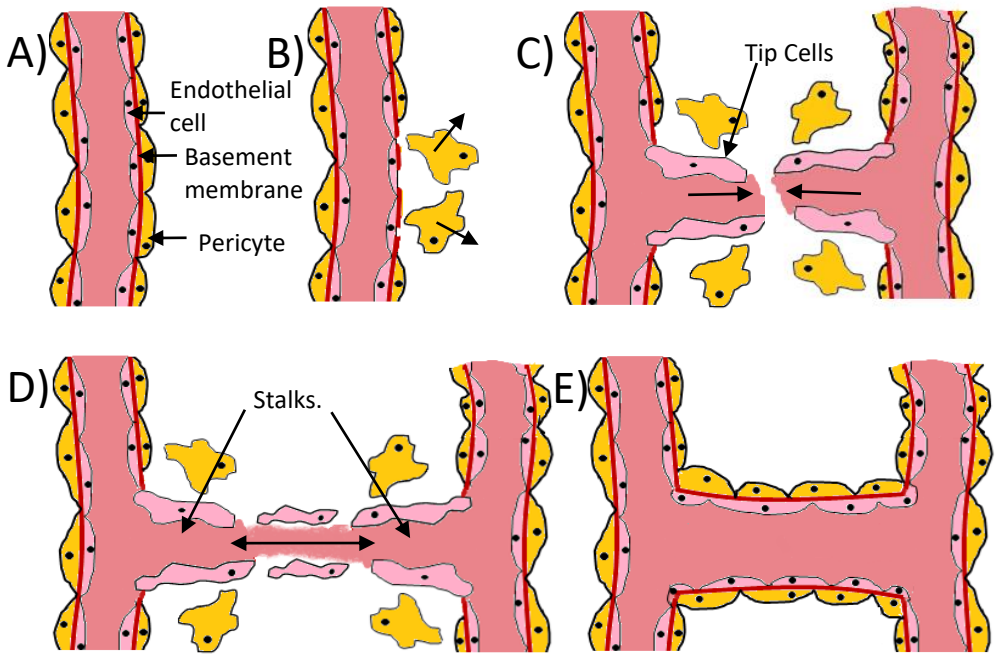


Fig 1.4 *Mechanism of sprouting angiogenesis.*
The steps involved in Sprouting angiogenesis. A) Shows a generic unbranched blood vessel prior to the onset of angiogenesis, B) Destabilization of the vessel wall through protease action increases vessel permeability, C) Sprouting of vessels takes place as endothelial cells (ECs) differentiate into tip cells and migrate, meanwhile further extracellular matrix degradation takes place, D) ECs migrate and proliferate, starting to form tubules. Networks form as capillary sprouts come in contact with each other, E) Vessel stabilisation occurs as pericytes are recruited and a new basement membrane is formed. *(Adapted from Clapp et al., 2009).*

Classically, sprouting angiogenesis is stimulated by hypoxia, which induces the upregulation of several pro-angiogenic factor genes such as vascular endothelial growth factor A (VEGFA) and nitric oxide (NO) which in turn cause an increase in vascular permeability and vasodilation, respectively. Through the Notch signalling pathway, a sub-population of ECs differentiate into tip cells and migrate along a temporary protein matrix towards angiogenic stimuli using filopodia. Whilst another population of ECs just behind the tip cells form a stalk, which is responsible for elongation of the sprouting vessel and is where the majority of EC proliferation takes place (Gerhardt *et al.* 2003, Clapp *et al.* 2009). The proliferating ECs release proteases such as matrix metalloproteases (MMPs) that breakdown and change the structural composition of the extracellular matrix (ECM), providing support and guidance for the migrating ECs (Serini *et al.* 2006, Clapp *et al.* 2009). The proliferating sprouts join together to form vascular networks and undergo lumen formation through cellular vacuole fusion (Kamei *et al.* 2006). Finally, pericytes are recruited and a basement membrane is established leading to stability of the newly formed vessels. Following this, an influx of blood flow raises the oxygen concentration in the surrounding tissues, reducing VEGFA secretion leading to a cessation of angiogenic mechanisms (Clapp *et al.* 2009).

1.4.2 Factors regulating angiogenesis in the bovine CL.

The regulation of luteal angiogenesis has long been a topic of profound interest, not only due to its importance in fertility but also as a model for the intense angiogenesis observed in cancer. It has been extensively reviewed (Schams & Berisha 2004, Clapp *et al.* 2009, Robinson *et al.* 2009, Woad &

Robinson 2016). The majority of the research has focused upon promoting angiogenesis. However, the ratio of pro and inhibitory factors is likely to be important and has been overlooked to date.

1.4.2.1 Pro-angiogenic factors in the bovine CL.

In the bovine CL, VEGFA and fibroblast growth factor 2 (FGF2) are the two pro-angiogenic factors of greatest importance. VEGFA induces EC tip migration and stalk proliferation(Gerhardt *et al.* 2003), whilst FGF2 plays a role in network sprouting and tubule formation (Woad *et al.* 2012). However, there are a variety of other pro-angiogenic factors that act in the bovine CL, such as platelet derived growth factor (PDGF) and angiopoietins (ANGPT), these are outlined in Table 1.1.

Table 1.1 *Pro-angiogenic factors, their receptors and functions in the bovine CL.*

Ligand	Isoforms	Receptors	Function	References
VEGFA	$VEGFA_{120}$ $VEGFA_{144}$ $VEGFA_{164}$ $VEGFA_{188}$ $VEGFA_{205}$	FLT1, KDR, sFLT1, sKDR.	Vascular permeability, EC growth, migration and lumen formation.	(Ferrara *et al.* 2003, Woad *et al.* 2012)
FGF2	18-kDa (cytoplasmic). 22.5, 23.1 and 24.2-kDa (intranuclear).	FGFR1-4 & their isoforms.	EC island growth, tubule growth and vascular sprouting. Mural cell growth.	(Ornitz *et al.* 1996, Powers *et al.* 2000, Laird *et al.* 2013)
PDGF	PDGFA PDGFB PDGFC PDGFD	PDGFR-AA, PDGFR-AB & PDGFR-BB.	Pericyte recruitment following PDGFR-ββ binding by PDGF-BB.	(Fredriksson *et al.* 2004, Woad *et al.* 2009)
ANGPT	ANGPT1 ANGPT2	Tie2	ANGPT1: vessel maturation. ANGPT2: Disrupts EC-pericyte connections.	(Augustin *et al.* 2009)

1.4.3 Vascular Endothelial Growth Factor A (VEGFA).

VEGFA is present in a variety of tissues and is implicated in both physiological and pathological angiogenesis (Ferrara & Davis-Smyth 1997, Ferrara *et al.* 2003).

1.4.3.1 VEGFA structure and isoforms.

VEGFA exists in several isoforms, all of which are formed from the alternative splicing of a single gene (Ferrara *et al.* 2003). In humans, the VEGFA gene is

composed of 8 exons divided by 7 introns (Houck *et al.* 1991, Tischer *et al.* 1991, Ferrara *et al.* 2003). The isoforms are denoted using the formula $VEGFA_{xxx}$ where $_{XXX}$ signifies the number of amino acids present in that particular isoform. In humans, the main isoform expressed is $VEGFA_{165}$ which is a glycoprotein dimer with a molecular weight of 45kDa. In this isoform, the peptide sequence encoded by exon 6 is missing, whilst $VEGFA_{121}$ lacks the components expressed by exon 6 and 7 (Houck *et al.* 1992, Ferrara *et al.* 2003). In the bovine, each isoform is 1 amino acid shorter, therefore $VEGFA_{120}$ and $VEGFA_{164}$ are the predominant isoforms expressed in the bovine CL (Schams & Berisha 2004). Alternative splicing of the VEGA isoforms is further complicated since $VEGFA_{xxx}$ can undergo splicing to form $VEGFA_{xxxb}$. For example, $VEGFA_{165}$ undergoes splicing to give rise to $VEGFA_{165b,}$ which has anti-angiogenic properties (Rennel *et al.* 2008) and will be discussed in detail later.

The different isoforms possess slightly different properties in terms of heparin binding and extracellular matrix (ECM) interaction. $VEGFA_{165}$, $VEGFA_{189}$ and $VEGAF_{206}$ all bind heparin whilst $VEGFA_{121}$ is acidic and does not (Houck *et al.* 1992). $VEGFA_{189}$ and $VEGFA_{206}$ are highly bound to the ECM whilst $VEGFA_{121}$ has minimal interaction with the ECM (Houck *et al.* 1992). Interestingly, $VEGFA_{165}$ has properties somewhere between these different isoforms, with a fraction binding to the ECM or remaining in contact with the cell, whilst another fraction remains diffuse (Park *et al.* 1993). The properties of $VEGFA_{165}$ mean that it has high bioavailability, and readily interacts with its receptors and other factors (Ferrara *et al.* 2003).

1.4.3.2 VEGF receptors.

VEGFA binds to both vascular endothelial growth factor receptor 1 (FLT1) and vascular endothelial growth factor receptor 2 (KDR), which are closely related tyrosine kinase receptors (Ferrara *et al.* 2003). The receptors consist of a single trans-membrane domain, an extra-cellular region of 7 immunoglobulin-like domains and an intracellular tyrosine kinase domain (Shibuya 2001a, Ferrara *et al.* 2003). Both VEGFA receptors (VEGFRs) have been detected in ECs from the bovine CL using RT-PCR while KDR has been localised using immunohistochemistry (Gabler *et al.* 2004, Hünigen *et al.* 2008). Both receptors also exist as soluble isoforms in the bovine ovary (Macias *et al.* 2012). The soluble isoforms have an inhibitory role in angiogenesis, binding to VEGFA but not possessing the necessary trans-membrane or intra-cellular domains for further signalling (Ortega Serrano *et al.* 2016).

The role of FLT1 is an area of contention, with pro and inhibitory roles for this receptor reported (Shibuya & Claesson-Welsh 2006, Roskoski 2007). FLT1 may act as a "decoy" receptor for VEGFA, competing with KDR for VEGFA binding, however its binding does not result in activation and leads to a decrease in VEGFA signalling (Park *et al.* 1994). This idea is supported by the finding that FLT1 has a higher affinity than KDR for VEGFA, and binding does not result in FLT1 phosphorylation. This suggests an inhibitory role (Waltenberger *et al.* 1994). Guzmán et al. (2015) found that *FLT1* mRNA expression was highest during the late luteal stage, at a time when the inhibitory role of FLT1 would coincide with luteal regression. Conversely, placental growth factor (PGF) binds FLT1 to weakly induce vascular permeability, and *FLT1* knockout mice have

disorganised vessel development suggesting an important vascular role (Fong *et al.* 1995, Shibuya & Claesson-Welsh 2006). This suggests that the role of FLT1 is likely to be ligand and situation-dependent.

KDR is the receptor principally implicated in promoting the actions of VEGFA in angiogenesis and is mostly highly expressed in the early to mid CL (Berisha *et al.* 2000b, Ferrara *et al.* 2003, Hünigen *et al.* 2008). Activation of KDR leads to EC growth, migration and vessel formation (Harper & Bates 2008, Guzmán *et al.* 2015).

Neuropillin (NRP) 1 and NRP2 are receptors expressed on the surface of certain ECs and are involved in VEGFA signalling (Soker *et al.* 1996, Ferrara *et al.* 2003). Presence of NRP1 alongside KDR increased $VEGFA_{165}$ binding to KDR (Soker *et al.* 1998). However, there is no evidence that NRP1 or NRP2 alone are capable of signalling after binding VEGFA (Neufeld *et al.* 2002, Ferrara *et al.* 2003).

1.4.3.3 VEGFA isoform expression in the bovine CL.

VEGFA protein expression increases slowly throughout early CL development, reaching maximal levels around day 12 of the oestrus cycle, before decreasing prior to luteolysis (Robinson *et al.* 2007). Similarly, the mRNA expression of the most common VEGFA isoforms increased from day 12 of the luteal phase onwards with all isoforms reaching a maximal level by day 15. $VEGFA_{120a}$ expression remained high on day 18 whilst $VEGFA_{164a}$ and $VEGF_{120b}$ expression decreased (Guzmán *et al.* 2015).

1.4.3.4 VEFGA effects on angiogenesis.

VEGFA has numerous effects on the vasculature, however one of its key roles is induction of vascular permeability, an important early step in angiogenesis (Connolly 1991). Furthermore, VEGFA is an endothelial cell mitogen that also induces bovine EC migration *in vitro* (Leung *et al.* 1989, Bernatchez *et al.* 1999). In the bovine ovary, immunoneutralisation of VEGFA reduced CL growth and progesterone production *in vivo* (Yamashita *et al.* 2008). Whilst in a physiologically relevant luteal angiogenesis culture system, inhibition of KDR resulted in a 50-60% reduction in EC island number, total EC network area and EC network length (Woad *et al.* 2009). Furthermore, KDR inhibition reduced progesterone production *in vitro (Laird et al. 2013)*. These results suggest that VEGFA acting through KDR is involved in EC network formation and proliferation in the bovine CL.

1.4.4 Fibroblast Growth Factor 2.

1.4.4.1 FGF2 structure and family.

FGF2 is a small heparin binding protein which belongs to a family of 22 structurally similar proteins that are involved in a variety of cellular processes (Imamura 2014). Despite sharing a similar structure, the action of the individual members of the FGF family differs greatly and thus the family is further divided into subfamilies based upon their function. FGF2 belongs to the FGF1 sub-family (Imamura 2014). The *FGF2* gene is capable of transcribing four FGF2 isoforms of different molecular weights; an 18-kDa cytoplasmic molecule and

3 intra-nuclear molecules of weights 22.5, 23.1 and 24.2-kDa (Gospodarowicz *et al.* 1987, Powers *et al.* 2000)

1.4.4.2 FGF receptors (FGFR)

The FGF family act through fibroblast growth factor receptors 1–4 (FGFR1–FGFR4) (Powers *et al.* 2000). The receptors have an intracellular tyrosine kinase domain and an extracellular domain consisting of 2-3 immunoglobulin-like regions (Berisha *et al.* 2006). Alternative splicing of FGFR1, 2 and 3 leads to formation of receptor variants IIIb and IIIc, which differ in their extracellular domains so have altered binding affinities and tissue specific expression (Powers *et al.* 2000, Berisha *et al.* 2004). The FGFRs are highly ligand specific, with FGF2 predominately binding to FGFR1IIIc and FGFR2IIIc isoforms (Ornitz *et al.* 1996). Both FGFR1IIIc and FGFR2IIIc have been identified in the bovine follicle at various stages of development and in the newly formed CL, with FGFR2IIIc levels similar at all stages of the cycle. Conversely, FGFR1IIIc levels were highest during early follicle development and lowest in the early CL (Berisha *et al.* 2006). This suggests FGF2 has an important role throughout follicular growth and early luteal development, but potentially by acting through different receptors.

1.4.4.3 FGF2 expression in the bovine CL.

FGF2 mRNA expression was highest during early antral follicle development, then decreased throughout the rest of follicular development before increasing again in the early CL (Berisha *et al.* 2006). Similarly, FGF2 protein levels are

highest in the very early CL (1-2 days), before a marked reduction as the CL ages (Robinson *et al.* 2007).

Alongside the temporal change in FGF2 expression, there is also a spatial change in FGF2 expression around the time of ovulation. Namely, prior to the LH surge FGF2 is located in the pericytes and ECs of the thecal layer, however after the LH surge FGF2 expression shifted to the luteinising granulosa cells before being again detected in the ECs (Berisha *et al.* 2006). FGF2 expression is LH responsive, with LH increasing FGF2 production and vascular sprouting (Laird *et al.* 2013), consistent with the raised FGF2 levels around the time of follicle-luteal transition.

When viewed alongside VEGFA expression, FGF2 clearly has an important function at specific time points (e.g. early CL) whilst VEGFA appears to be important throughout CL growth (Robinson *et al.* 2007). This suggests that the two factors act differently, but are closely linked, in regulating luteal angiogenesis with FGF2 having a particular important function for vascular sprouting around ovulation, whilst VEGFA is responsible for vessel development and maturation (Robinson *et al.* 2008, Laird *et al.* 2013).

1.4.4.4 The effect of FGF2 on angiogenesis in the bovine CL.

Treatment of bovine luteal cells with FGF2 *in vitro* stimulated EC network formation (Robinson *et al.* 2008). Inhibition of FGF2 signalling via the specific FGFR1 inhibitor, SU5402, had a maximal effect when added from days 3-6 of culture, and resulted in an 81% reduction in total EC network area (Woad *et al.* 2012). Furthermore, FGFR1 inhibition on days 3-6 of culture resulted in a large

decrease in the extent of vascular sprouting. This suggested that FGF2 influenced angiogenesis by promoting EC island growth and vascular sprouting in the bovine CL. A study utilising a similar culture technique demonstrated that addition of FGF2 increased precocious EC sprouting in luteinising follicular cells. Additionally FGF2 had an important effect upon mural cell growth, by altering their morphology and increasing the extent to which they formed clusters (Laird *et al.* 2013). This demonstrates that FGF2 may also play an important part in vessel maturation, during the stabilisation of vessels in the final stages of angiogenesis.

1.4.4.5 The effect of FGF2 on progesterone production.

Intriguingly, inhibition of the FGFR receptor increased progesterone production *in vitro*, whilst addition of FGF2 typically had limited effect on progesterone production (Laird *et al.* 2013). Interestingly, the addition of both VEGFA and FGF2 increased progesterone production *in vitro*, given their seemingly opposing effects on progesterone production, it is likely that progesterone production was increased due to VEGFA overcoming the inhibitory effect of FGF2 (Robinson *et al.* 2008).

1.4.5 Platelet derived growth factor (PDGF)

1.4.5.1 Platelet derived growth factor structure and family.

The PDGF family consists of four different polypeptides; PDGFA, PDGFB, PDGFC and PDGFB with a different gene encoding each one (Fredriksson *et al.* 2004). The four polypeptides give rise to five dimeric isoforms; PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC and PDGF-DD. Each member of the PDGF family shares the same basic structure, with a growth factor domain responsible for dimerisation and receptor interaction. The polypeptides vary in length from 196 to 370 amino acids, with a common domain in all chains is around (~100 amino acids) while extension from this common region yields different biological activities (Fredriksson *et al.* 2004).

1.4.5.2 PDGF receptors.

PDGFs exert their action through different tyrosine kinase receptors; PDGFRA, PDGFRB and the heterodimer PDGF receptor PDGFRAB. PDGF-AA binds specifically to PDGFRA, PDGF-AB can bind PDGFRA and PDGFRAB. PDGF-BB is unique in that it is capable of binding to all 3 receptors (Donovan *et al.* 2013).

1.4.5.3 Expression of PDGF in the bovine CL.

To date, there have been no studies investigating the expression and localisation of PDGF in the bovine ovary. PDGF expression has been localised to the rat ovary and promotes early follicle development (Nilsson *et al.* 2006, Sleer & Taylor 2007a). Following hCG administration, PDGFA, PDGFB and PDGFC were localised in steroidogenic cells of the rat CL, with PDGFRB localised to the luteal vasculature (Sleer & Taylor 2007b).

1.4.5.4 The effect of PDGF on angiogenesis.

Pericytes closely attach themselves to smaller capillaries, such as those seen in the CL, and these cells play an important role in angiogenesis by promoting vessel maturation and patency (Armulik *et al.* 2011). Endothelial tip cells produce PDGFB which leads to recruitment of pericytes that express PDGFRB, this means that pericytes lag only slightly behind the migrating endothelial tip cells (Gerhardt & Betsholtz 2003, Armulik *et al.* 2011). Inhibition of PDGF in rat ovaries reduced the number of CL alongside evidence of widespread ovarian haemorrhage in some treated individuals, suggesting that PDGF plays an important role in CL formation and vascular stability (Sleer & Taylor 2007b).

Inhibition of PDGFR signalling reduced the number of EC networks formed and the total area of EC networks, demonstrating that PDGF plays a key part in the stimulation of luteal angiogenesis (Woad *et al.* 2009). Interestingly, despite affecting luteal angiogenesis, PDGF inhibition had no effect on luteal progesterone production, suggesting that PDGF has a less important role in influencing steroidogenic function (Spicer & Stewart 1996, Laird *et al.* 2013).

1.5 Anti-angiogenic factors in the bovine CL.

The ratio of pro-angiogenic factors to inhibitory factors is crucial in the control of vascular growth (Woad & Robinson 2016). Factors capable of inhibiting angiogenesis are particularly interesting in pathological conditions where they are often dysregulated and are a focus of many investigations (Díaz *et al.* 2008, Sharma *et al.* 2013). The importance of inhibitory factors has not gone unnoticed in normal physiology, and is an area of interest in the ovary due to

their potential impact upon mechanisms such as follicular and luteal development (Qiu *et al.* 2012, Zalman *et al.* 2012, Chuderland *et al.* 2013, Guzmán *et al.* 2015). Inhibitory factors of potential importance in luteal angiogenesis are displayed in Table 1.2.

Table 1.2 *Anti-angiogenic factors, their receptors and functions.*

Ligand	Receptors/Interaction	Function	References
$VEGFA_{165b}$	FLT1, KDR.	Inhibits the pro-angiogenic effects of $VEGFA_{165}$	(Woolard et al. 2004, Woolard et al. 2008)
PEDF	PEDFR, ATP-synthase, phospholipase A2, various others.	Inhibits VEGFA, FGF2 and PDGF. Induces apoptosis of ECs.	(Dawson et al. 1999, Kanda et al. 2005, Manalo et al. 2011)
THBS1	CD36, CD47.	Activates TGFB, induces apoptosis and alters nitric oxide production.	(Dawson et al. 1997, Isenberg et al. 2009, Lawler and Lawler 2012)
TGFB	ACVRL1, TGFBR1.	Inhibits EC growth, proliferation and network formation. Induces fibroblast migration.	(Goumans et al. 2003, Maroni and Davis 2011)
VASH	---	Inhibits VEGFA.	(Watanabe et al. 2004, Shirasuna et al. 2012)
2-ME	Numerous different receptors.	Inhibits VEGFA.	(Rogerson and Eagon 1986, Kohen et al. 2013)

1.5.1 VEGFA.

1.5.1.1 Anti-VEGFA isoform formation.

Alternative splicing of exon 8 leads to the formation of two different $VEGFA_{xxx}$ isoforms (Perrin *et al.* 2005). The family denoted $VEGFA_{xxxa,}$ which previously was referred to as $VEGFA_{xxx}$ until the different splice isoforms were discovered, has pro-angiogenic properties as already discussed. The alternate splice isoform family formed is denoted $VEGFA_{xxxb}$ and has anti-angiogenic properties (Harper & Bates 2008). These two isoform families with seemingly opposing properties are formed depending upon the splice site in the terminal exon 8. The exon 8a acts as the proximal splice site and yields the $VEGFA_{xxxa}$ family, whilst the distal splice location exon 8b leads to formation of the $VEGFA_{xxxb}$ family (Nowak *et al.* 2008). The members of the $VEGFA_{xxxb}$ family identified as having anti-angiogenic properties are $VEGFA_{121b}$, $VEGFA_{145b}$, $VEGFA_{165b}$ and $VEGFA_{189b}$ (Harper & Bates 2008). $VEGFA_{165b}$ is the most common isoform, it has been the best characterised to date (Peiris-Pagès *et al.* 2010).

The receptor binding domains are retained in the inhibitory isoforms with $VEGFA_{165b}$ capable of binding to FLT1 and KDR. Although binding results in limited receptor phosphorylation, this blocks the interaction of the pro-angiogenic form with KDR (Woolard *et al.* 2004, Cébe Suarez *et al.* 2006). The other $VEGFA_{xxxb}$ isoforms are believed to inhibit angiogenesis through a similar mechanism (Perrin *et al.* 2005, Nowak *et al.* 2008).

1.5.1.2 The inhibitory role of soluble VEGF receptors and their expression in bovine CL.

The function of the soluble form of FLT1 (sFLT1) is clearer than for the membrane bound FLT1, inhibiting angiogenesis in a number of tissues where it is expressed (Barleon *et al.* 1996, Fong *et al.* 1999, Guzmán *et al.* 2015). However, the expression of sFLT1 mRNA in the bovine CL did not change throughout the luteal phase, suggesting that this receptor does not play a major regulatory role in *de novo* vascular development in the CL (Guzmán *et al.* 2015). Conversely, the ratio of soluble KDR (sKDR) to membrane bound KDR expression changed throughout the lifespan of the CL (Guzmán *et al.* 2015). Indeed the KDR:sKDR ratio decreased around the time of luteal regression due to an increase in sKDR expression. It can be hypothesised that since sKDR has an anti-angiogenic role, it would limit vascular growth around the time of luteal regression. This suggests that the often-overlooked ratio of the soluble to membrane bound receptors is of particular importance in controlling the development and regression of the luteal vasculature.

1.5.1.3 Expression of $VEGFA_{xxxb}$ in the CL.

Luteal $VEGFA_{164b}$ mRNA expression was detected at low levels throughout the luteal phase in the pig CL. Relative expression was highest on day 1 post ovulation before falling and remaining constant until day 17, when expression decreased further (Ribeiro *et al.* 2007). In contrast to the pig, $VEGFA_{165b}$ accounted for almost two thirds of the entire VEGFA content of marmoset ovaries with CLs (Qiu *et al.* 2012).

To date, only one study has investigated $VEGFA_{164b}$ mRNA expression in the cow, in which no expression was detected at any time point during the luteal phase. In the same study, $VEGFA_{120b}$ expression was detected and increased drastically on day 15 before declining on day 18. Despite these changes, the ratio of $VEGFA_{120a}$:$VEGFA_{120b}$ did not change throughout the cycle (Guzmán *et al.* 2015). However, this study was performed on *Bos indicus* x *Bos taurus* cows therefore the characterisation of $VEGFA_{164b}$ and other splice variants in *Bos taurus* may warrant further investigation.

1.5.1.4 The anti-angiogenic properties of $VEGFA_{xxxb}$.

Overexpression of $VEGFA_{165b}$ in mice resulted in a reduction in embryo number, CL size and number, as well as decreased follicular development that was associated with reduced vascular density (Qiu *et al.* 2012). The neutralisation of $VEGFA_{xxxb}$ isoforms in perinatal rats increased follicular vascular density and increased follicular development when compared to both controls and ovaries treated with pro-angiogenic VEGF isoforms, highlighting the key role that anti-angiogenic factors play in vascular function and ovarian development (Artac *et al.* 2009).

In pathological situations, such as cancer, $VEGFA_{165b}$ inhibited the pro-angiogenic effects of certain tumour secreted factors (Rennel *et al.* 2008). $VEGFA_{165b}$ directly inhibited the pro-angiogenic effects of $VEGFA_{165}$ on branch point density, sprouting, EC proliferation, vessel density and mean vessel length (Woolard *et al.* 2004). However, the effects of $VEGFA_{164b}$ in the bovine

CL have yet to be elucidated, therefore the characterisation of this factors effect on luteal ECs and steroidogenesis is an area that warrants further study.

1.5.2 Pigment epithelium derived factor (PEDF)

1.5.2.1 PEDF structure.

Pigment epithelium derived factor (PEDF, originally named early population doubling level cDNA-1) is a member of the Serpin superfamily, however it does not possess the ability to inhibit proteases (Pignolo *et al.* 1993, Becerra *et al.* 1995). It is encoded by the SERPINF1 gene and forms a 50-kDa glycoprotein that is 418 amino acids long (Becerra *et al.* 1995, Becker *et al.* 2011, Chuderland *et al.* 2013). Although first discovered for its neurotrophic properties, extracellular phosphorylation converts PEDF to an anti-angiogenic factor (Maik-Rachline *et al.* 2005).

1.5.2.2 PEDF expression and its activity.

The PEDF gene is expressed in a multitude of tissues, including the ovary and PEDF is secreted by both mouse and human granulosa cells (Tombran-Tink *et al.* 1996, Chuderland *et al.* 2013). *SERPINF1* expression has also been detected in bovine follicular cells by microarray (Hayashi *et al.* 2011). However, the presence of PEDF in the bovine CL has yet to be investigated.

PEDF has been found to interact with numerous different proteins/receptors such as; membrane-bound ATP synthase and phospholipase A2, as well as binding to collagen and heparin (Meyer *et al.* 2002, Notari *et al.* 2010, Hirsch *et al.* 2011, Johnston *et al.* 2015). Of particular relevance to angiogenesis, PEDF

binds to a 60-kDa PEDF receptor (PEDFR) found on the surface of ECs and reduces angiogenesis, as well as triggering EC apoptosis (Manalo *et al.* 2011). *In vitro* PEDF blocked the interaction of VEGFA with KDR in retinal capillary ECs and stimulated the intracellular proteolysis of both VEGFRs (Cai *et al.* 2006, Zhang *et al.* 2006, Ablonczy *et al.* 2009).

1.5.2.3 The anti-angiogenic effects of PEDF.

The anti-angiogenic effects of PEDF are mediated via inhibitory interactions with several pro-angiogenic factors. In addition to interacting with VEGFA signalling, PEDF inhibited FGF2 induced capillary morphogenesis in ECs (Kanda *et al.* 2005, Cai *et al.* 2006). This finding is particularly interesting given the importance of FGF2 in angiogenesis in the early CL. Similarly, PDGF is also affected by PEDF with EC migration towards this factor inhibited by PEDF (Dawson *et al.* 1999). PEDF is also known to induce EC apoptosis, which is another possible mechanism through which it could act to downregulate blood vessel growth (Chen *et al.* 2006, Ho *et al.* 2009).

1.5.3 Thrombospondins (THBS).

1.5.3.1 THBS family and structure.

The thrombospondins are a family of five secreted multi-domain glycoproteins that are part of a group of matricellular proteins. They have a varied set of roles integrating cells and the ECM (Adams & Lawler 2004, Stenina-Adognravi 2014). The thrombospondin (THBS) family is further divided into sub groups A and B determined by the presence or absence of type 1 repeats, respectively (Stenina-Adognravi 2014). THBS1 and THBS2 share the same structural

domains, with both having regions related to pro-collagen and type 1 repeats, whereas THBS3, THBS4 and THBS5 lack these domains but contain an additional type 2 repeat (Adams & Lawler 1993). Whilst THBS share a high level of similarity in their protein structure, they exhibit little homology in their DNA sequences, with the exception of their protein-coding regions (Adolph *et al.* 1997). THBS1 has been studied extensively and is involved in the negative regulation of angiogenesis. Several of these properties are shared by THBS2 due to their similar structures (Helkin *et al.* 2015).

1.5.3.2 THBS1 and THBS2 receptors.

THBS1 has been shown bind to heparin sulphate proteoglycan, fibronectin, fibrinogen and several vascular integrins (Chen *et al.* 2000, Feitsma *et al.* 2000, Gutierrez 2008). The presence of type 1 repeats enables THBS1 and THBS2 to bind to the receptor CD36, which triggers events such as induction of EC apoptosis (Dawson *et al.* 1997). CD36 is an 88-kDa glycoprotein transmembrane receptor that has a large extracellular region, and is expressed on certain EC subtypes (Greenwalt *et al.* 1992, Swerlick *et al.* 1992). Both THBS1 and THBS2 are also capable of binding to CD47, although THBS1 has a greater affinity. This interaction along with the binding of CD36, is responsible for an alteration in nitric oxide production that can also inhibit angiogenesis (Isenberg *et al.* 2009, Lawler & Lawler 2012).

1.5.3.3 THBS expression in the bovine ovary.

THBS1 and *THBS2* mRNA is expressed throughout bovine follicular development with levels highest in the early antral follicle before declining to

low levels in large antral follicles. Immunohistochemistry has localised THBS and CD36 to granulosa cells, with staining intensity decreasing as follicle size increases. Moreover, there was no evidence of THBS expression in large follicles and only low levels of CD36 evident (Greenaway *et al.* 2005). Expression of *THBS1*, *THBS2* and *CD36* mRNA in the bovine CL has been demonstrated, with expression evident in early CL and prostaglandin-F2α (PGF2α) significantly upregulating their expression in the CL 11 days after ovulation (Zalman *et al.* 2012).

1.5.3.4 The effect of THBS on luteal angiogenesis.

THBS1 inhibited bovine luteal angiogenesis in both the presence and absence of FGF2 (Zalman *et al.* 2012). Furthermore, THBS1 activated latent transforming growth factor-β (TGFB) which itself is capable of disrupting angiogenesis in bovine luteal ECs (Maroni & Davis 2011, Zalman *et al.* 2012). Additionally, THBS1 inhibited angiogenesis by inducing apoptosis in ECs (Dawson *et al.* 1997). These studies demonstrate that THBS1 is a potent anti-angiogenic factor in the bovine CL that is capable of inhibiting angiogenesis by several different mechanisms. Given the variety of mechanisms through which this factor can act its actions in the bovine CL warrant further investigation, in particular its effect on luteal function.

1.5.4 Transforming growth factor-B.

1.5.4.1 TGFB family and structure.

TGFB is a member of the TGFB superfamily, a group of nearly 100 proteins that share at least one region of homologous amino acid sequence. The superfamily consists of members such as the bone morphogenic proteins (BMPs) and is involved in a large number of cellular functions from apoptosis to cell growth and differentiation (Massague 1990, Burt & Law 1994).

TGFB exists as three isoforms; TGFB1, TGFB2 and TGFB3. Each protein is encoded by a different gene, but they share a high degree of homology (Elliott & Blobe 2005, Kubiczkova *et al.* 2012). The first protein to be discovered was TGFB1, consequently it is the most studied isoform and when referring to TGFB most literature is referring to this protein unless otherwise stated (Chin *et al.* 2004).

The biologically active form of TGFB1 is a dimer composed of two identical chains of 112 amino acids (Massague 1990). TGFB is secreted from cells alongside a glycoprotein called latent TGFB binding protein (LTBP) which is responsible for the association of TGFB with the ECM. It also means that TGFB requires further processing before it is biologically active (Miyazono *et al.* 1988, Taipale *et al.* 1994).

1.5.4.2 TGFB receptors.

The TGFB receptors are divided into two subfamilies; type 1 and type 2 receptors depending on their structure and function. Receptors were given the

general nomenclature ALK (activin receptor-like kinase) until their ligand was discovered, they were then given a more descriptive name (Massague 1998).

Of particular interest to angiogenesis are receptors ACVRL1 (ALK1) and TGFBR1 (ALK5), which are expressed on endothelial cells and activated by TGFB binding (Attisano *et al.* 1993, Franzén *et al.* 1993, Goumans *et al.* 2003). Of the two receptors, TGFB binds to TGFBR1 with greatest affinity (Ten Dijke *et al.* 1994). Binding to these receptors activates a SMAD signalling pathway whereby phosphorylation of specific receptor regulated SMADs (R-SMADs) allows them to bind with SMAD4, which leads to the transcription of specific target genes (Itoh *et al.* 2000, Goumans *et al.* 2003). Binding of TGFB to TGFBR1 leads to activation of SMAD2 and SMAD3, whilst activation of ACVRL1 leads to phosphorylation of SMAD1 and SMAD5 (Goumans *et al.* 2003).

1.5.4.3 TGFB expression and effect in the bovine CL.

Luteal *TGFB1* mRNA production was induced with luteolytic doses of PGF2α *in vivo* (Hou *et al.* 2008). Bovine luteal cells produce large amounts of TGFB, with TGFB1 accounting for almost 90% of the TGFB produced by these cells, which was further increased by prostaglandin treatment *in vitro* (Gangrade *et al.* 1993, Hou *et al.* 2008). The effect of TGFB is often highly tissue and situation-dependent, with reviews noting seemingly pro- and inhibitory angiogenic roles depending upon the biological setting (Goumans *et al.* 2003, Kelly & Morris 2010). However, several studies have begun to elucidate the role of TGFB in the bovine CL.

TGFB inhibited the proliferation of luteal fibroblasts whilst simultaneously promoting their migration and ability to contract collagen matrices. This suggested that TGFB plays an important role in luteal remodelling that would be particularly important in the regressing CL (Maroni & Davis 2012). Furthermore, TGFB inhibited luteal progesterone production (Hou *et al.* 2008). TGFB had an inhibitory effect on bovine luteal endothelial cells, reduced EC migration, disrupted capillary formation, altered permeability and under certain conditions induced cell death (Maroni & Davis 2011). Whilst these studies point towards a role in luteal regression the effect of TGFB in the early bovine CL has not yet been established, it would be prudent to investigate this.

1.5.5 Vasohibin.

1.5.5.1 Vasohibin structure.

Vasohibin-1 (VASH1) and Vasohibin-1 (VASH2) are angiogenic factors encoded by the homologous genes *VASH1* and *VASH2,* respectively (Watanabe *et al.* 2004, Shibuya *et al.* 2006). VASH1 exists as two isoforms; VASH1A and the shorter splice variant VASH1B (Sato 2013). In the human, VASH1A is 365 amino acids long whilst VASH1B is 204 amino acids long. Despite being significantly shorter VASH1B retains the anti-angiogenic properties of VASH1A (Shimizu *et al.* 2005, Kern *et al.* 2008).

1.5.5.2 Vasohibin expression and effect on angiogenesis.

Integral to the function of vasohibin is a 66 amino acid protein called small vasohibin-binding protein (SVBP) (Suzuki *et al.* 2010). SVBP is responsible for

the secretion of vasohibin from cells by forming a heterodimer with vasohibin (Sato 2013).

VASH1 expression is very low in ECs that are undergoing angiogenic sprouting, with expression much higher in surrounding non-proliferating ECs. Whilst VASH2 expression is highest in mononuclear cells surrounding angiogenic sprouts but low in non-proliferating regions. This suggests that VASH1 and VASH2 have opposing roles with VASH1 possessing anti-angiogenic properties whilst VASH2 has a role in promoting angiogenesis. Thus, the balance of these two closely related factors will be important in their regulation of angiogenesis (Kimura *et al.* 2009, Maeshima & Makino 2010). VASH expression is induced by VEGFA and FGF2, the fact that VASH1 is capable of blocking the pro-angiogenic effects of VEGFA, which would suggest VASH1 is involved in inhibiting angiogenesis through negative feedback (Watanabe *et al.* 2004, Shimizu *et al.* 2005, Heishi *et al.* 2010).

In the bovine CL, VASH1 mRNA expression increased from the early to mid-stage CL before declining in the regressing and late CL. VASH1 protein levels remained constant from the early to late CL, with VASH1 predominately localised to luteal ECs. Furthermore VEGFA was shown to induce VASH1 in these cells, whilst VASH1 inhibited VEGFA induced formation of capillary-like structures (Shirasuna *et al.* 2012).

1.5.6 2-methoxyoestradiol (2-ME).

1.5.6.1 2-ME structure.

2-methoxyoestradiol (2-ME) is a metabolite of 17β-oestradiol that has no oestrogenic activity, the enzymes CYP1A1 and catechol-O-methyltransferase are critical in the formation of this metabolite (Zhu & Conney 1998, Mueck & Seeger 2010). 2-ME binds to sex hormone-binding globulin with very high affinity and is known to bind to a variety of tissue receptors (Dunn *et al.* 1980, Rogerson & Eagon 1986).

1.5.6.2 Production of 2-ME and its effect on angiogenesis.

In the human CL, 2-ME production was highest in the late CL with the lowest levels observed in the mid-stage CL (Kohen *et al.* 2013). In the same study, 2-ME inhibited VEGFA production by large luteal cells and reduced basal angiogenic activity, but did not affect progesterone production. Whilst the effect of 2-ME in the bovine CL has not been directly investigated, 2-ME inhibited proliferation of bovine ECs, even in the presence of FGF2 (Fotsis *et al.* 1994, Klauber *et al.* 1997). Furthermore, 2-ME has been shown to reduce the proliferation of bovine granulosa cells (Fotsis *et al.* 1994).

1.6 Alternative splicing of pre-mRNA.

Gene transcription produces a pre-mRNA molecule that requires further processing to form mature mRNA that can then undergo translation (Bentley 2014). Splicing is the process whereby any introns are removed from the pre-mRNA to leave the exons which then make up the strand of mature mRNA (Pandya-Jones 2011). There are splice sites located at the 5' and 3' ends of introns that enable the specific cleavage of the intron. There are specific consensus sequences, which consist of the dinucleotides GU at the 5' end and AG at the 3' end. A branch site is located a short distance upstream of the 3' splice site and always contains an adenine and is critical for splicing (Clancy 2008). Alternative splicing is the mechanism by which a gene is differentially processed to yield multiple different transcripts through alterations in the introns and/or exons that are included or excluded in the mature mRNA (Tazi *et al.* 2009). Thus, alternative splicing can yield different isoforms of the same gene/protein that have different biological properties. Equally, it may lead to non-sense mediated decay of RNA where any RNA containing premature stop codons are degraded (Clark & Thanaraj 2002, Lewis *et al.* 2003).

1.6.1 Mechanism of splicing.

Five small nuclear ribonucleoproteins (snRNPs); U1, U2, U4, U5 and U6 along with numerous other proteins/RNA form a large and highly dynamic complex called the spliceosome. This is responsible for the coordination of splicing by catalysing the removal of introns. The structures and mechanisms involved in splicing have been extensively reviewed (Chen & Manley 2009, Will & Lührmann 2011, Lee & Rio 2015). Fig. 1.5 illustrates the key mechanism by which splicing occurs.

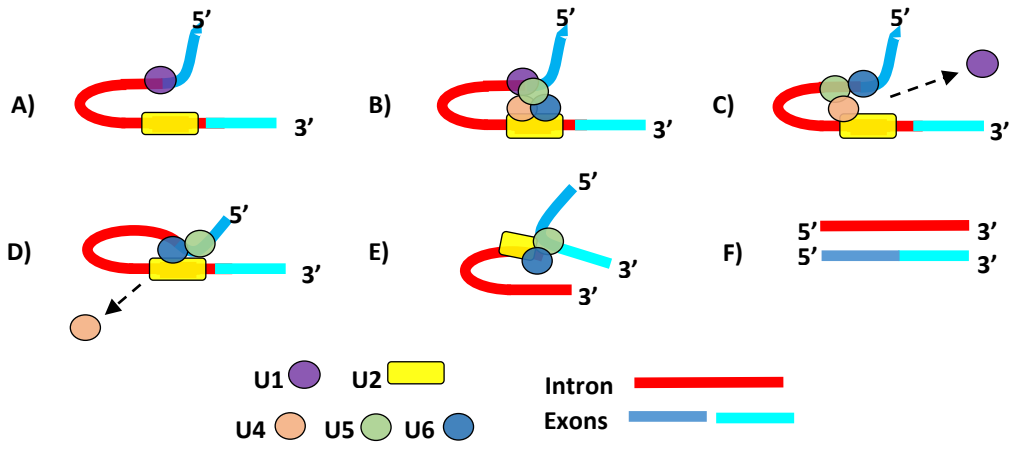


Fig 1.5 *The mechanism of splicing.*
Five small nuclear ribonucleoproteins (snRNPs); U1, U2, U4, U5 and U6 along with several other proteins form the spliceosome, the unit responsible for catalysing splicing. A) U1 binds to the 5' end of the intron, U2 binds to the branch site. B) The trimer consisting of U4, U5 and U6 then forms with U5 binding to the exon whilst U6 binds U2. C) This releases U1 and U5 moves to the intron whilst U6 binds to the intron 5' splice site. D) U4 is then released, enabling U6 and U2 to catalyse a transesterification reaction and U5 binds to the exon 3' splice site. This results in the cleavage of the intron 5' end and the formation of a lariat. E) The 3' intron site is cleaved and the exons covalently bound together. U2, U5 and U6 remain bound to the lariat. F) The spliced RNA is released and the lariat debranched. Adapted from Lewin, 2008.

Briefly, splicing begins with binding of U1 to its complimentary sequence at the 5' end of the intron (Clancy 2008). U2 binds to the branch site, after which the U4, U5 and U6 trimer attaches, with U5 binding to the exon initially and U6 binding to U2 (Lewin 2008). U1 is then released and U5 moves to the intron whilst U6 binds to the 5' splice site. Next, U4 is released and the U6/U2 complex catalyses the cleavage of the 5' intron site and formation of a lariat through a transesterification reaction between the guanine at the end of the ligated intron and the adenine located at the branching site (Clancy 2008). Meanwhile, U5 binds to the exon at the 3' splice site. U2, U5 and U6 remain bound to the lariat and through interaction with U5 the 3' end of the intron is cleaved, resulting in release of the lariat and the joining of the two exons through covalent bonding (Lewin 2008).

1.6.2 Types of alternative splicing.

The five most commonly occurring types of alternative splicing are shown in Fig.1.6. Skipped exon (SE) splice events occur when an exon and its adjacent introns are removed from the transcript, this accounts for almost 40% of the known alternative splicing events observed in both humans and mice (Sugnet *et al.* 2004). When there are multiple possible splice sites available at the beginning or end of an exon alternative 5' splice selection (A5SS) or alternative 3' splice selection (A3SS) can take place and account for 18% and 8% of alternative splicing, respectively (Sugnet *et al.* 2004). Mutually exclusive exons (MXEs) are a fourth possible type of alternative splicing event in which one of two possible exons is removed with the other exon remaining. This means the size of the coded protein can remain unchanged if the two exons involved in

MXE splicing are of equal length. However, the other alternative splicing events will result in alterations to the length of the proteins (Pohl *et al.* 2013). The final type of alternative splicing event is retained intron where an intron can be removed or retained, this is subtly different to other the other forms of alternative splicing as there is no process of selection between multiple splice locations and requires the breaching of a nuclear checkpoint (Black 2003, Ner-Gaon *et al.* 2004). In addition to these common types, alternative promotor and alternative polyadenylation splicing can take place, however these events are less frequently recognised so are not discussed here.

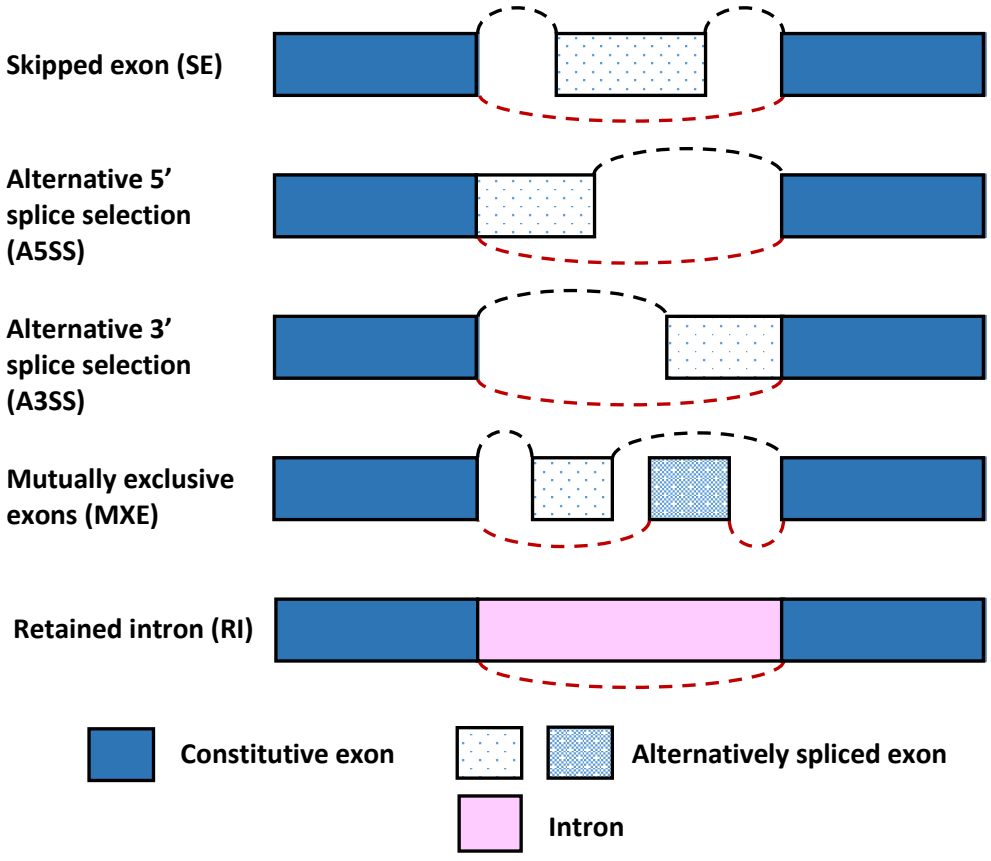


Fig 1.6 *Alternative splicing events.*
The five most commonly occurring alternative splicing events. The alternatively spliced exons have a spotted pattern, differently coloured dashed lines indicate how the different splice variants can be formed. Adapted from Park et al., 2013.

1.6.3 Regulation of alternative splicing.

Splicing is regulated in a variety of ways, but one of the main regulatory mechanisms is the presence of *cis*-regulatory elements in the RNA sequence. These sequences and their associated protein regulators can determine where splicing takes place (Chen & Manley 2009). There are 4 types of *cis*-regulatory elements; exonic splicing enhancers (ESE), exonic splicing silencers (ESS), intronic splicing enhancers (ISE) and intronic splicing silencers (ISS) (Chen & Manley 2009). The serine-arginine-rich splicing factor (SRSF) protein family typically binds to ESE, whilst heterogeneous nuclear ribonucleoprotein (HNRNP) usually bind to ISS and ESS sites, numerous factors bind to ISE, many of which also bind to ESS (Smith & Valcárcel 2000, Dreyfuss *et al.* 2002, Long & Caceres 2008, Wang *et al.* 2012).

Further regulation takes place through environmental influences and the interaction of transcription factors and RNA polymerase II with splicing factors, as well as through numerous other mechanisms (Pleiss *et al.* 2007, Clancy 2008, Chen & Manley 2009). The role that key environmental factors, such as diet may play in the regulation of splicing requires further investigation.

1.6.4 Alternative splicing in the bovine ovary.

In the human ovary, over 20% of genes undergo alternative splicing, which is unsurprising given the cyclic changes that this structure undergoes (Yeo *et al.* 2004). In the bovine, most studies investigating splicing have focused on gene products in granulosa cells. The *SRSF9* gene is associated with RNA splicing and was upregulated in bovine granulosa cells of dominant follicles. This suggests

that splicing of other genes would be taking place at this stage of follicular development (Mihm *et al.* 2008). Alternative splicing of the *LHCGR* gene in bovine granulosa cells leads to the formation of multiple LH receptor (LHCGR) isoforms. However, this was not associated with functional changes to LHCGR function in granulosa cells *in vitro* (Nogueira *et al.* 2007). Similarly, alternative splicing yielded 4 different LHCGR receptor isoforms in the bovine CL, with the expression of each isoform increasing during luteal development (Kawate & Okuda 1998). Furthermore, during the luteinisation of bovine granulosa cells, there was a change in *FSH-receptor* mRNA splicing that resulted in the production of shortened transcripts that only coded for the extra-cellular region of the FSH-receptor. This suggested that alternative splicing could be responsible for the loss of sensitivity to FSH that occurs during CL formation (Rajapaksha *et al.* 1996). As previously discussed, $VEGFA_{xxx}$ undergoes alternative splicing to give rise to the inhibitory $VEGFA_{xxxb}$ isoforms which are present in the bovine ovary (Guzmán *et al.* 2015), and inhibit angiogenesis in other tissues (Woolard *et al.* 2004). These findings highlight that alternative splicing could play a key role in the regulation of a variety of ovarian mechanisms. Therefore determining which genes are alternatively spliced and the factors that affect splicing is an area that warrants further investigation.

1.7 Conclusion.

In conclusion, angiogenesis in the bovine ovary and alternative spicing are both highly regulated biological processes that play a key role in ovarian function. Angiogenesis is controlled by a plethora of pro- and anti-angiogenic factors, with pro-angiogenic factors being the subject of many studies whilst the role of the anti-angiogenic factors on CL function is an area that warrants further investigation (Galvão *et al.* 2013). Similarly, alternative splicing plays an important role in ovarian function, including in the regulation of angiogenesis through the generation of alternative splicing of the pro- and anti-angiogenic isoforms of VEGFA (Guzmán *et al.* 2015).

Whilst alternative splicing is regulated by a variety of factors, the effect of environmental factors such as diet on the generation of alternative splice variants in the bovine ovary remains largely unexplored. Further investigation is warranted given the impact that alternative splicing can have on protein function. The effect of diet upon this process is particularly worthy of investigation given that diet plays an important role in the productivity and reproductive success of animal (Pushpakumara *et al.* 2003).

Chapter 2. Anti-angiogenic factors.

2.1 Introduction.

The rapidly developing CL produces large quantities of progesterone shortly after ovulation and is vital for the establishment of pregnancy (Niswender *et al.* 2000). The CL is formed from the remnants of the ovulated follicle, with the ingress of blood vessels from the theca layer into the avascular granulosa cell layer occurring (Fraser & Lunn 2001, Fraser 2006). Simultaneously, granulosa cells differentiate into large luteal cells, whilst theca cells form small luteal cells (Niswender *et al.* 2000).

Vital to early luteal development is angiogenesis, with the development of a new and expansive vascular network crucial to support cellular differentiation and structural remodelling (Reynolds & Redmer 1999). Numerous pro and anti-angiogenic factors play an essential role in controlling angiogenesis, with the ratio of these factors likely being central to the regulation of this process (Woad & Robinson 2016). An excess of an inhibitory factor or an inadequate production of pro-angiogenic factors is likely to reduce new vessel development, and limit the growth and function of the new CL. To date, the majority of studies have focused upon the factors that are key to promoting angiogenesis in in the bovine CL, namely VEGFA and FGF2 (Berisha *et al.* 2000b, Robinson *et al.* 2007, Woad *et al.* 2012). There has been very little research investigating the role of factors that could potentially inhibit angiogenesis in the early CL. Based on the literature review, the inhibitory factors of particular interest are $VEGFA_{xxxb}$ isoforms, PEDF and THBS1.

The $VEGFA_{xxxb}$ isoform, $VEGFA_{165b}$ has anti-angiogenic activity in a variety of species and tissues (Woolard *et al.* 2004, Perrin *et al.* 2005, Rennel *et al.* 2008, Qiu *et al.* 2012). However, to date relatively little research has been performed to determine the role of $VEGFA_{164b}$ in the CL. Guzmán et al. 2015 were unable to detect *$VEGFA_{164b}$* mRNA in CL from *B. indicus* x *B. taurus* cattle. However they did detect the expression of *$VEGFA_{120b}$,* suggesting that anti-angiogenic factors could have a role in luteal development.

PEDF is expressed in the ovaries of mice, cows and humans (Tombran-Tink *et al.* 1996, Hayashi *et al.* 2011, Chuderland *et al.* 2013). However, its expression in the bovine CL has not yet been characterised. PEDF inhibited the action of VEGFA and FGF2 directly, as well as stimulating EC apoptosis by binding to PEDFR (Kanda *et al.* 2005, Cai *et al.* 2006, Zhang *et al.* 2006, Manalo *et al.* 2011). Given its potential interaction with VEGFA and FGF2 in the bovine CL, PEDF if present could play a role in regulating luteal angiogenesis.

THBS1 and its receptor CD36 are expressed in the early bovine CL, with their expression upregulated during luteal regression and by PGF2α (Zalman *et al.* 2012). Furthermore, THBS1 inhibited angiogenesis in the presence of FGF2 (Zalman *et al.* 2012). This suggests that THBS1 is likely involved in the luteolytic mechanism, when the cessation of angiogenesis is important. However, the role of THBS1 in early luteal development has not yet been investigated.

2.1.2 Hypothesis and aims.

It is likely that anti-angiogenic factors play an important role in "balancing" angiogenesis and thus luteal function throughout the lifespan of the CL. Thus

the hypothesis was that anti-angiogenic factors would be expressed in the bovine CL and their expression would differ at key time points in the lifespan of the CL, such as early growth and luteal regression. Additionally, these anti-angiogenic factors would suppress luteal angiogenesis but not steroidogenic function *in vitro*. Therefore, the aims of this study were:

1. To identify the spatial and temporal expression of $VEGFA_{164b}$ and PEDF in the bovine CL.
2. To determine the effect of PEDF and THBS1 on early luteal angiogenesis and steroidogenesis *in vitro*.

2.2 Materials and methods.

2.2.1 PEDF, VEGFA and $VEGFA_{165b}$ immunohistochemistry on bovine CL sections.

The methods in this chapter were approved by the ethical review panel at the School of Veterinary Medicine and Science, University of Nottingham and all tissue collection was performed with the informed consent of the abattoir. Immunohistochemistry was performed on CL sections at the following estimated time points: days 1-2 (n=4), 3-4 (n=4), 5-7 (n=4), 8-12 (n=4) and 12-16 (n=4) to determine the spatial and temporal expression of PEDF, VEGFA and $VEGFA_{164b}$ during luteal development.

2.2.1.1 Tissue collection and processing.

Ovaries from Angus beef cross heifers were collected at the time of slaughter, and CL morphologically assigned (Ireland *et al.* 1980) to the following categories: luteal day 1-2, 3-4, 5-7, 8-12 or 12-16. The CL were then fixed in

Bouin's for 6h and mounted in paraffin blocks. Sections (5µm) were cut from each CL using the microtome (Leica) and mounted onto polysine slides.

2.2.1.2 Immunohistochemistry procedure for PEDF, VEGFA164b and VEGFA.

Sections were dewaxed and rehydrated by immersing slides in the following solutions for the allotted times:

- Xylene: 2 x 5 min
- 100% ethanol: 5 min
- 95% ethanol: 5 min
- 70% ethanol: 5 min
- PBS: 2 x 5 min

After dewaxing, citrate antigen retrieval was performed by microwaving slides in 10mM citrate buffer (pH 6; appendix 6.1). After cooling, slides were washed in PBS for 2 x 5 min. Endogenous peroxidase activity was blocked using 3% H_2O_2 in methanol, before another washing step. Antigen blocking was then performed using the appropriate 4% (v/v) normal serum (Table 2.1). Anti-$VEGFA_{165b}$, anti-VEGFA and anti-PEDF primary antibodies were diluted in PBS, then added to slides. Slides were incubated at 4°C overnight in a humidified chamber, alongside control slides treated with control IgG of equivalent concentration (Table 2.1). Following incubation, a washing step was performed and the AB complex (Vector) prepared 30 min before use. Next, biotinylated horse anti-mouse secondary antibody (for anti-$VEGFA_{165b}$) or biotinylated goat anti-rabbit secondary antibody (for anti-PEDF and anti-VEGFA) was added (Table 2.1). The slides were then incubated at room temperature (RT) for 30

min then washed thoroughly with PBS. Next, AB complex was added and incubated at RT for 30 min, the slides were then washed again. DAB substrate (Vector) was prepared according to the manufacturer's instructions and added for 5 min with colour development closely monitored. Slides were rinsed in running tap water for 10 min before counterstaining was performed by immersing slides in the following:

- Harris's haematoxylin: 20 seconds.
- Running tap water: 2 minutes.
- 1% ammoniated IMS: 8 dips.
- 70% ethanol: 2 minutes.
- 95% ethanol: 2 minutes.
- 100% ethanol: 2 minutes.
- Xylene: 2 x 5 minutes.

Slides were then mounted in DPX mountant, coverslips added and allowed to air dry.

Table 2.1 *Primary and secondary antibodies utilised in the immunohistochemistry on CL sections.*

Primary Antibody	**Final Conc.**	**Cat number; Company:**	**Blocking solution**	**Control**
Anti-$VEGFA_{165b}$ *(Mouse anti-human)*	10µg/ml	MABC595 clone 56/1; Sigma.	4% NHS for 30 min	Mouse IgG
Anti-VEGFA *(Rabbit anti-human)*	2µg/ml	ab52917 (EP1176Y; Abcam)	4% NGS for 30 min	Rabbit IgG
Anti-PEDF *(Rabbit anti-human)*	10µg/ml	Ab180711; Abcam	4% NGS for 30 min	Rabbit IgG
Secondary Antibody	**Dilution**	**Serum concentration**	**Used with**	**Company:**
Biotinylated horse anti-mouse	1/200.	2% NHS	$VEGFA_{165b}$	PK-4002, Vectastain
Biotinylated goat anti-rabbit	1/200.	2% NGS	VEGFA and PEDF	PK-4001, Vectastain

2.2.1.3 Image acquisition.

The extent of staining was determined under x5 lens on a Leica microscope and representative images acquired to allow comparison of the staining obtained across the different time points.

2.2.2 Western blotting.

Western blotting was performed to confirm primary antibody specificity and to identify the expression of VEGFA, $VEGFA_{165b}$ and PEDF protein during luteal development.

2.2.2.1 Sample collection.

Bovine ovaries were collected from a local abattoir and CL were morphologically assigned (Ireland *et al.* 1980) to the following categories: luteal

day 1-2, 3-4, 5-7, 8-12, 12-16 and regressing. The CL was dissected away from the ovarian stroma using sharp and blunt dissection. The weight, diameter, colour and surface description of each CL was then recorded. CL tissue was cut into smaller sections (~600mg) and stored at -80°C.

2.2.2.2 Sample preparation.

Samples were defrosted on ice and 200mg of luteal tissue placed in a MACS M tube (Miltenyi Biotec) along with 1ml of cell extraction buffer (Fisher) containing a cocktail of protease inhibitors (Sigma). Samples were homogenised (easyMACS machine) using either a slow speed, short duration program repeatedly (early CL) or using a high speed program for a longer time (late CL). This was repeated if necessary, with samples placed on ice between homogenisations. Homogenised samples were centrifuged at 2000**g** for 15 min and the supernatant aliquoted and stored at -80°C.

2.2.2.3 Protein determination, Bradford assay.

Bovine serum albumin (BSA) stock solution (2000µg/ml) was serially diluted to 7.8µg/ml in PBS. Each standard or sample (diluted 1/100; 10µl) was added in duplicate to the appropriate well of a 96-well plate (Nunc). PBS (10µl) was added to the blank wells. Bradford solution (200µl; B6916, Sigma) was added to all wells and incubated on a plate shaker at RT for 30 min. Absorbance was determined at 595nm using an OPTIMA plate reader. A standard curve was plotted using the BSA standards and used to determine the sample protein concentration.

2.2.2.4 SDS-PAGE (protein separation).

Twelve well pre-cast gels (NuPAGE 12% Bis-Tris 1.0mm) were used for all blots. The required sample volume was diluted in 2x Laemmli sample buffer (Appendix 6.2), then heated at 100°C for 10 min before being pulse centrifuged. Gels were allowed to warm to RT before being placed into the SureLock XCell electrophoresis chamber. All wells were washed using MOPS running buffer (Thermo Fisher) and the inner chamber filled with running buffer. Molecular weight markers (NEB, Colour PreStained broad range) were loaded into the first well, samples (20-50µg protein) were added to subsequent wells along with a positive control. A range of samples were run on each gel so that the entire life span of the CL was covered. The outer chamber was filled with MOPS running buffer and the gel was run at 200V (~100mA) for 50 min.

2.2.2.5 Blotting/transfer of protein.

NuPAGE transfer buffer (Thermo Fisher) was prepared whilst the PVDF membrane (BioRad) was activated in methanol and then transfer buffer. Blotting pads were pre-soaked in transfer buffer alongside two pieces of filter paper. Once the electrophoresis was completed, the plates were separated, the gel removed and PVDF membrane placed onto the gel. The membrane and gel were then sandwiched between filter paper and blotting pads to form the blotting module. The module was placed within the tank and the inner chamber filled with transfer buffer. The outer chamber was filled with cold distilled water. The tank was run at 30V (~160mA) for 1h at 4°C. Once transfer was completed the PVDF membrane was removed, stained with Ponceau S to confirm transfer, and then rinsed in distilled water.

2.2.2.6 Immunoblotting.

The PVDF membrane was blocked with 5% milk in PBS-Tween (PBST) for 1h at RT on a shaking plate. The membrane was incubated with primary antibodies against PEDF, VEGFA or $VEGFA_{165b}$ (Table 2.2) in blocking solution (5% milk in PBST) overnight at 4°C on a shaking plate. Following incubation the membrane was washed in PBST for 5 x 10 min. Membranes were incubated with secondary antibody diluted in blocking solution for 1h at RT. For rabbit primary antibodies, this was goat anti-rabbit HRP (Sigma; diluted 1/80000), while for $VEGFA_{165b}$, sheep anti-mouse HRP secondary antibody (GE Healthcare; diluted 1/50000) was used. Membranes were washed in PBST for 5 x 10 min and rinsed in distilled water. Membranes were covered with ECL prime (GE Healthcare) solution for 5 minutes. Then wrapped in clingfilm, transferred on to X-ray film and developed using a SRX-101A processor (Konica Minolta).

Table 2.2 *Antibodies and their dilutions utilised in Western blotting.*

Antibody	Dilution	Company
Mouse anti-human $VEGFA_{165b}$	1/1000	MABC595, Sigma.
Rabbit anti-human VEGFA	1/100	sc-152, Santa Cruz Biotechnology.
Rabbit anti-human PEDF	1/1000	ab180711, Abcam.

2.2.2.7 Stripping blots.

PVDF membranes were stored at 4°C in PBS until ready to strip. Membranes were then incubated in a mild stripping buffer (Appendix 6.3) at RT for 10 min, this step was repeated using fresh buffer. Membranes were washed in PBS (2

x 10 min), followed by a wash in PBST (2 x 5 min). ECL Prime was added and a hyperfilm was developed to check that stripping had occurred. Membranes were washed in PBST for 10 minutes and then reprobed for the housekeeping protein Histone H3 (rabbit anti-human ab-1791, diluted 1/18000) to confirm equal protein loading.

2.2.3 In vitro luteal angiogenesis co-culture system.

A physiologically relevant culture system (Robinson *et al.* 2008) that mimics luteal angiogenesis and progesterone production was utilised to determine the effect of PEDF and THBS1 treatment *in vitro* upon EC development and steroidogenesis.

2.2.3.1 Ovary collection and CL selection.

Bovine ovaries were collected from a local abattoir and CL were selected based on established criteria (Ireland *et al.* 1980). Namely, a very early CL (1-3 days post ovulation) that was <12mm in diameter with a soft texture and a red ovulatory point.

2.2.3.2 Fibronectin coating.

Coverslips (19mm) were soaked in 70% IMS for >1h. Individual coverslips were submerged in 70% IMS, allowed to dry for 1h then placed in 12 well plates. Next, 0.5ml of 20μg/ml bovine fibronectin (Sigma) was added to each well and incubated at 39°C for 4h. The fibronectin was removed and the plate dried overnight at 39°C. The following day, wells were washed with sterile water (1ml), then equilibrated with endothelial cell media (ECM; Appendix 6.4) in a 39°C incubator until required.

2.2.3.3. Culture procedure.

Ovaries were sprayed with 70% IMS and rinsed in sterile PBS, from this point onwards they were handled aseptically. The CL was dissected out with any connective tissue trimmed away and then cut up into small sections. Then 10 ml of dissociation solution (2mg/ml collagenase I and 25µg/ml DNase I) was added and incubated for 30 min at 37°C. After dissociation, the solution was passed through a 70µm filter into a new falcon tube. The remaining tissue was re-suspended in fresh 10ml of dissociation solution and incubated for a further 30 minutes at 37°C. Whilst the second dissociation was taking place, the first dissociation was centrifuged at 300**g** for 10 min, with the supernatant removed and cells re-suspended in 10 ml of ECM. After the second dissociation, the tissue solution was filtered through 70µm gauze and combined with the first cell dissociation. This was centrifuged for 5 min at 300**g**, the resulting cell pellet was then washed in 10ml of ECM. The process of centrifugation and washing was performed twice more with the final cell pellet being re-suspended in 2-5ml of ECM depending upon its size.

Cell viability and number were determined using a haemocytometer, typical total cell yields were 1-2 x 10^8 cells. The cells were diluted to 10^5 cells/ml in ECM supplemented with FGF2 and VEGFA (both 1ng/ml). To each well, 2 ml was added and cells were incubated at 39°C in humidified 5% CO_2 / 95% air. After 18 h, the cell media was changed and treatments with PEDF (Abcam; n=3 cultures) and THBS1 (R&D systems; n=3 cultures) commenced (Fig 2.1).

Spent media was collected, stored at -20°C for progesterone analysis and replaced every 48h thereafter. Cells were fixed on day 5 of culture in acetone:methanol (1:1) solution at 4°C for 10 min.

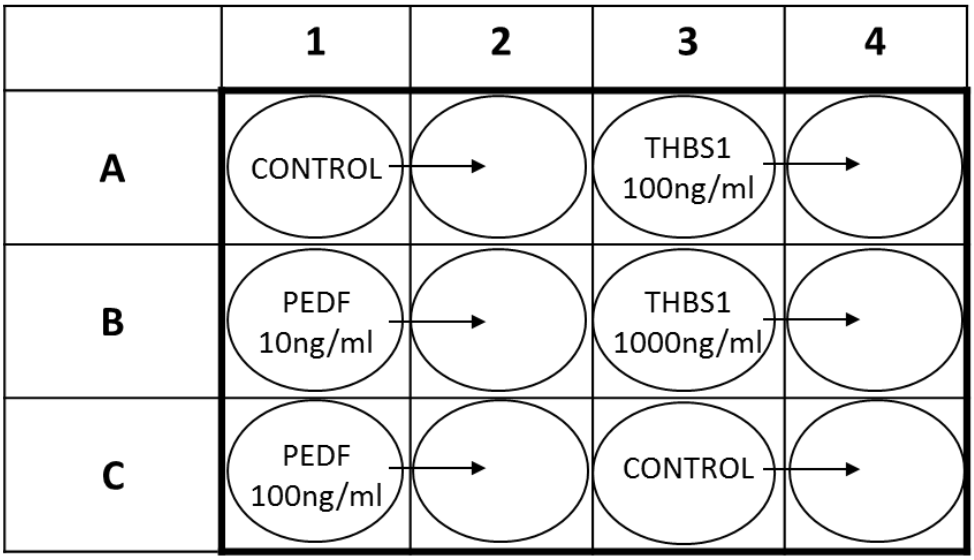


Fig 2.1 *Plate layout for pigment epithelium-derived factor (PEDF) and thrombospondin-1 (THBS1) treatments*. Factors were administered at two different doses in duplicate. All wells received ECM supplemented with FGF2 and VEGFA.

2.2.4. Immunohistochemistry for von Willebrand factor (VWF).

VWF immunohistochemistry was performed to determine the degree of endothelial cell (EC) network development. Coverslips were washed in PBS (2 x 5 min), then endogenous peroxidase activity was blocked in 3% (v/v) hydrogen peroxide in methanol for 10 min. Coverslips were then washed in PBS (2 x 5 min). Followed by blocking of non-specific binding with 20% (v/v) normal goat serum (NGS) for 10 min. Rabbit anti-human VWF (Dako) antibody at 5μg/ml in 2% NGS was added to coverslips and incubated in a humidified chamber at 4°C overnight. Due to the repeated validation of staining in numerous past experiments and the distinct characteristics of EC staining a negative control

was not utilised at this point. Following incubation, all wells were washed as previously described. Avidin-biotin (AB) complex (Vector Laboratories) was prepared in PBS, 30 minutes prior to use. Meanwhile, coverslips were incubated with biotinylated goat anti-rabbit secondary antibody (Vector Laboratories) diluted 1:150 in 2% NGS for 30 min at RT before washing. The AB complex was added to all wells and incubated at RT for 30 min. DAB substrate (Vector) was prepared in distilled water according to manufacturer recommendations and added to coverslips for 2-5 minutes depending on colour development. All sections were then rinsed in running tap water for 5 min.

Counterstaining and mounting

Following solutions were added to coverslips as follows

- Harris's haematoxylin: 20 sec
- Running tap water: 2 min
- 1% ammoniated IMS: 8 dips.
- 70% ethanol: 2 min
- 95% ethanol: 2 min
- 100% ethanol: 2 min
- Xylene: 2 x 20 sec

Coverslips were mounted on slides using DPX mountant. Slides were then allowed to dry overnight in a fume hood.

2.2.4.1 Image analysis of Von Willebrand Factor immunohistochemistry.

In an initial experiment, separate cultures and staining were performed by Emer Grant (University of Nottingham, BSc thesis). In this experiment, the cells were treated with the 0-1000ng/ml THBS1 (R&D systems) for a total of 9 days before fixation and staining for VWF (n=4 cultures). This experiment was conducted in the same manner as section 2.2.3 except that 24 well plates with 12mm coverslips were used. The image analysis was performed by myself, as described below.

Twelve randomly selected field of view images were obtained per coverslip under a 5x lens on a Leica microscope (Leica Microsystems). The extent of VWF staining was analysed using Image-Pro Premier 9.2 (MediaCybernetics®). Images were tiled into a 2x2 square, brown areas of staining representing EC networks were drawn around by hand and an appropriate colour palette selected to ensure all networks were equally highlighted. A minimum network (500μm^2) threshold was determined such that only networks and visible islands were selected and smaller areas of cell staining ignored. The total number of EC networks per tile was recorded. The EC networks were then extracted onto a grey background and sorted by size. The area, perimeter, branch number and branch length of the sorted EC networks was analysed using Image Premier. The number of branches per EC Island was then calculated.

2.2.4.2 Statistical analysis.

All EC network endpoints (total EC area, number of EC networks, number of branch points, branch points per EC island and branch length) were expressed

as percentage of their respective culture controls and then analysed by randomised block one-way ANOVA using the culture number as the block. The effects of PEDF and THBS1 treatment were analysed in separate analyses. Tukeys multiple comparisons were performed to determine where the differences lay.

2.2.5. Progesterone ELISA.

The progesterone content of spent media was analysed using an in-house ELISA to determine the effect of PEDF and THBS1 on luteal progesterone production.

2.2.5.1. Preparation of progesterone standards.

A progesterone stock solution (100µg/ml) was prepared in ethanol. This was diluted 1:1000 to give 100ng/ml progesterone, which was serially diluted 1 in 2 to give a range of standards from 0.195-50ng/ml.

2.2.5.2. Preparation of progesterone HRP conjugate.

Ultrapure water (250µl) was added to progesterone-HRP conjugate (Coralie Munro, University of California-Davis), vortexed and allowed to reconstitute for 30 min. Aliquots (10µl) were stored at -20°C. Prior to use, an aliquot of progesterone-HRP conjugate was defrosted and 0.99ml of ELISA buffer (appendix 6.5) added yielding a 1:100 conjugate. Immediately prior to use, this conjugate was diluted to a working concentration of 1:12500.

2.2.5.3 Preparation of progesterone antiserum.

Ultrapure water (400µl) was added to the progesterone antiserum (CL425, Coralie Munro), vortexed and allowed to reconstitute for 30 min. This solution

was then diluted 1:50 in coating buffer (Appendix 6.6), aliquoted into 80µl and stored at -20°C. Immediately prior to use one aliquot was defrosted and 62.5µl added to 15ml of coating buffer to give a 1:12,000 antiserum dilution.

2.2.5.4. Coating plates.

Progesterone antiserum (50µl) was added to all wells of a 96-well plate (Nunc Maxisorp), except for non-specific binding (NSB) wells. The plates were gently shaken, sealed and then incubated for two nights at 4°C.

2.2.5.5. Assay procedure.

The progesterone-antibody coated plate was washed 5 times with 0.15M NaCl + 0.05% Tween20 solution. ELISA blocking buffer (Appendix 6.7) was then added to each well and incubated for 1h at RT followed by 5 washes with wash solution. ELISA buffer (50µl) was added to NSB and total binding (TB) wells in triplicate. Then standards and diluted samples (50µl) were added in triplicate to the appropriate wells. To this, progesterone-HRP conjugate (50µl; 1:12500) was added and incubated on a plate shaker at RT. After 2h, the plate was washed again 5 times with wash solution then thoroughly blotted dry. TMB substrate (50µl) was added to each well and incubated at RT on a plate shaker for 30 min. Sulphuric acid (2M) was added to stop colour development. Optical density (OD) was then measure on an OPTIMA plate reader at a wavelength of 405nm.

2.2.5.6. Curve fitting.

The mean NSB was subtracted from each OD reading and the resulting OD expressed as a percentage of the TB. The progesterone standards were logged

and plotted using a 4-parameter logistic fit in GraphPad Prism. The sample progesterone concentration was calculated and the mean of the triplicate values calculated. The mean intra-assay coefficient of variation was 13.9% with samples from each culture analysed in a single assay.

2.2.5.7. Statistical analysis.

Progesterone concentrations were analysed by randomised block one-way ANOVA using the culture number as the block. The effects of PEDF and THBS1 treatment on progesterone concentration were analysed in separate analyses. Tukeys multiple comparisons were performed to determine where the differences lay.

2.3 Results.

2.3.1 Localisation of VEGFA and VEGFA$_{164b}$ in the bovine CL.

2.3.1.1 Spatial-temporal expression of VEGFA in the bovine CL.

Immunohistochemistry was utilised to characterise the spatial-temporal expression of VEGFA at different stages of luteal development. There was abundant staining for VEGFA at all stages of luteal development (Fig 2.2A-E). Tissue architecture was poorly conserved in the very early CL (Fig 2.2A), however VEGFA staining was still evident. An increase in cell number and size was evident as the CL developed alongside a greater degree of structural organisation (Fig 2.2D-E). VEGFA staining was largely confined to the cytoplasm of small and large luteal cells, with no evidence of staining in endothelial cells.

The intensity of staining was visibly similar at all time points. There was low level background staining evident in the controls (Fig 2.2F).

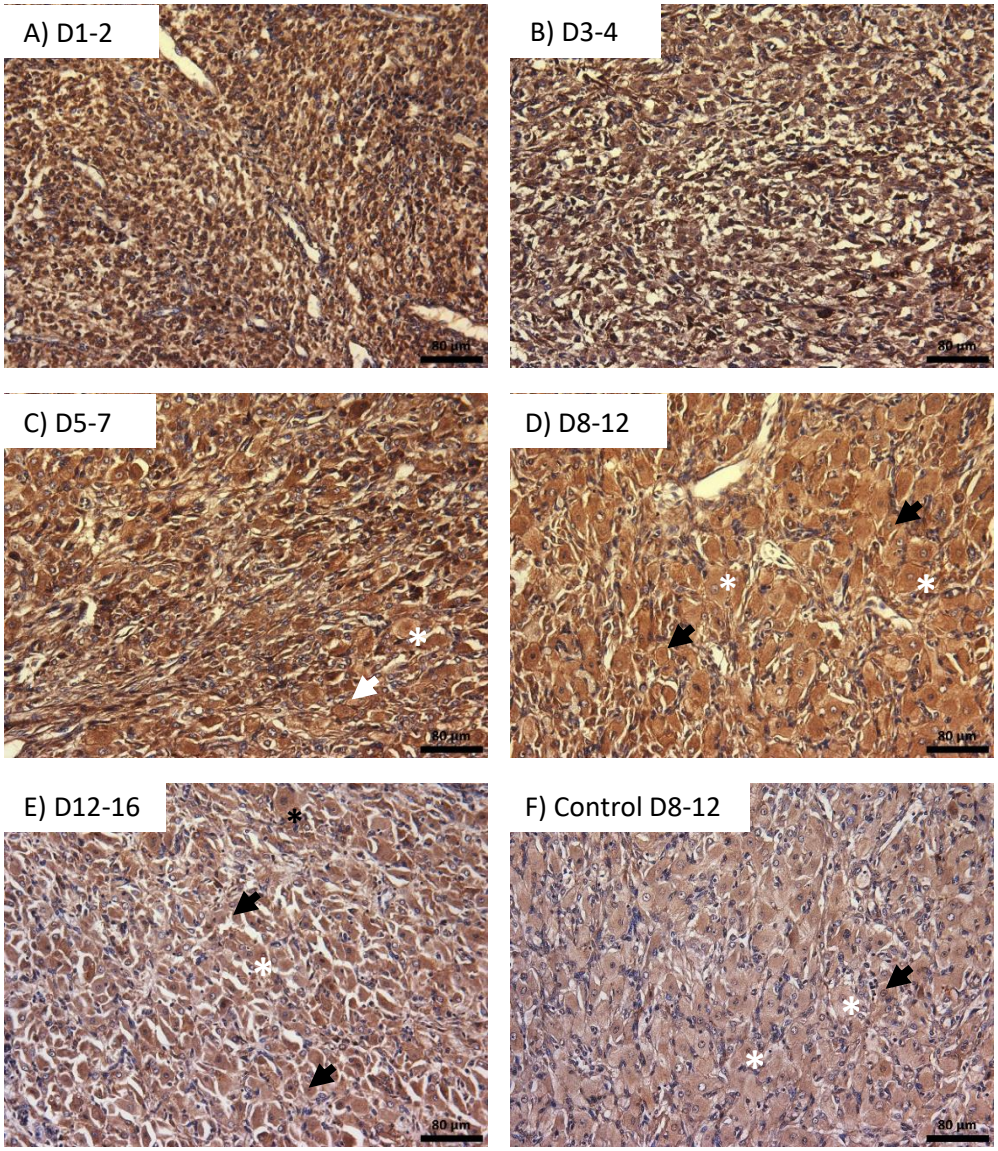


Fig 2.2 *Spatial-temporal expression of VEGFA throughout luteal development.* Immunohistochemistry for VEGFA was performed on sections from different CL stages with representative images obtained at ×20 magnification. In comparison to the control (F), there was a high degree of staining for VEGFA at all time points which was predominately confined to large (asterisk) and small (arrow) luteal cells, with no evidence of endothelial cell staining. Scale bar = 80µm

2.3.1.2 Spatial-temporal expression of $VEGFA_{164b}$ in the bovine CL.

$VEGFA_{164b}$ immunohistochemistry was performed on sections from the same tissue blocks as VEGFA. There was low level staining in all sections (Fig 2.3A-E), including the control sections (Fig 2.3F). This made interpretation of the extent of $VEGFA_{164b}$ staining difficult. It did appear that there was increased staining in the more mature luteal sections (Fig 2.3D-E). This suggested that any $VEGFA_{164b}$ expression in the bovine CL was seemingly limited to the mature CL and that expression levels are low even at these time points. $VEGFA_{164b}$ expression appeared to be localised to steroidogenic cells.

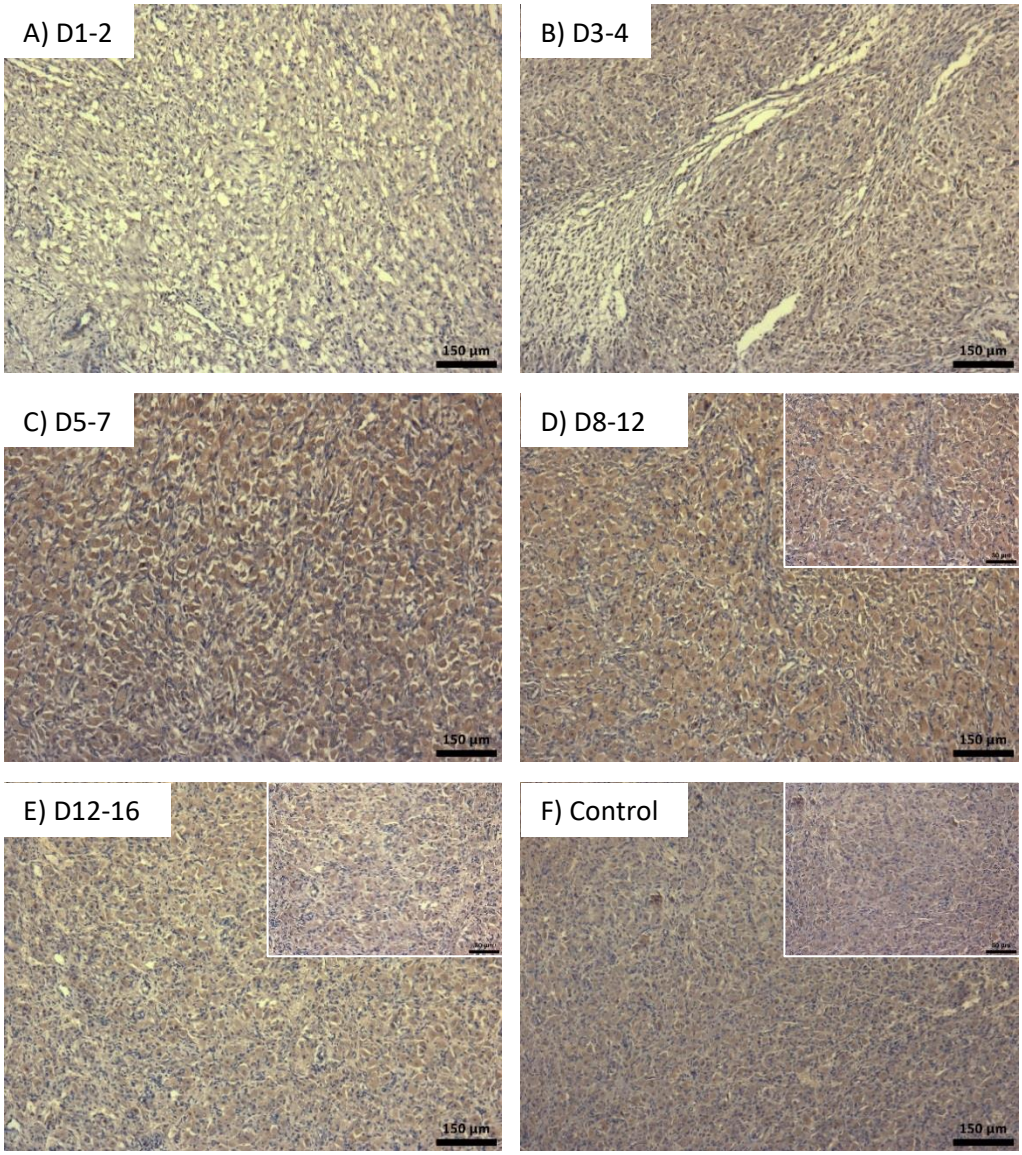


Fig 2.3 *Spatial-temporal expression of VEGFA164b throughout luteal development*. Luteal tissue was collected at different estimated days after oestrus and immunohistochemistry for $VEGFA_{164b}$ performed. Low levels of background staining were evident in the control (F) and at all time points. However, there appeared to be a visible increase in staining intensity in the more mature luteal sections (D) and (E). Images were obtained at x10 and x20 magnification (inset).

2.3.1.3 Identification of VEGFA and VEGFA164b protein by Western blot.

Western blots were performed to further characterise the temporal expression of VEGFA and $VEGFA_{164b}$ expression in the bovine CL. Following immunoblotting for VEGFA, a strong band was identifiable at approximately

50kDa across luteal samples from a range of CL stages (Fig 2.4). This band was different to the expected molecular weight of 21kDa (or 42KDa for the dimer) and it is unclear whether this band corresponded to VEGFA. The band intensity appeared to be similar throughout the lifespan of the bovine CL. Another VEGFA antibody (VG76e; Abcam) was tested but no bands were detected. Probing for $VEGFA_{164b}$ did not yield a band at any molecular weight but the housekeeping protein histone H3 was detected confirming protein transfer. Based on these observations in conjunction with the immunohistochemistry results, no further investigations into the role of $VEGFA_{164b}$ in the early bovine CL were conducted.

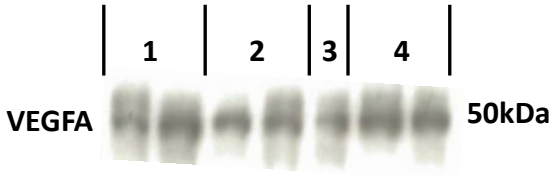


Fig 2.4 *Temporal expression of VEGFA protein throughout the CL lifespan.* Identification of VEGFA protein was performed by Western blot analysis of luteal samples of different ages; (1) 1-2 day, (2) 3-4 days, (3) 8-12 days and (4) regressing CL. VEGFA specific antibody was used. A single band at 50kDa was observed, but it was unclear if this was VEGFA protein. The expected molecular weight was 21 or 42 kDa (dimer).

2.3.2 PEDF expression in the bovine CL and its effect on luteal angiogenesis and function in vitro.

2.3.2.1 Spatial-temporal expression of PEDF in the bovine CL.

Immunohistochemistry for PEDF was optimised using different antibody dilutions and then performed across a range of different luteal stages. Staining intensity for PEDF was highest in the very early CL (Fig 2.5A) with the intensity of staining appearing to decrease in the later CL stages, but it was still clearly

evident in the mature CL (Fig 2.5E). Staining for PEDF was more prevalent in small than large luteal cells (Fig 2.5C) but was also present in some ECs, albeit at a lower intensity.

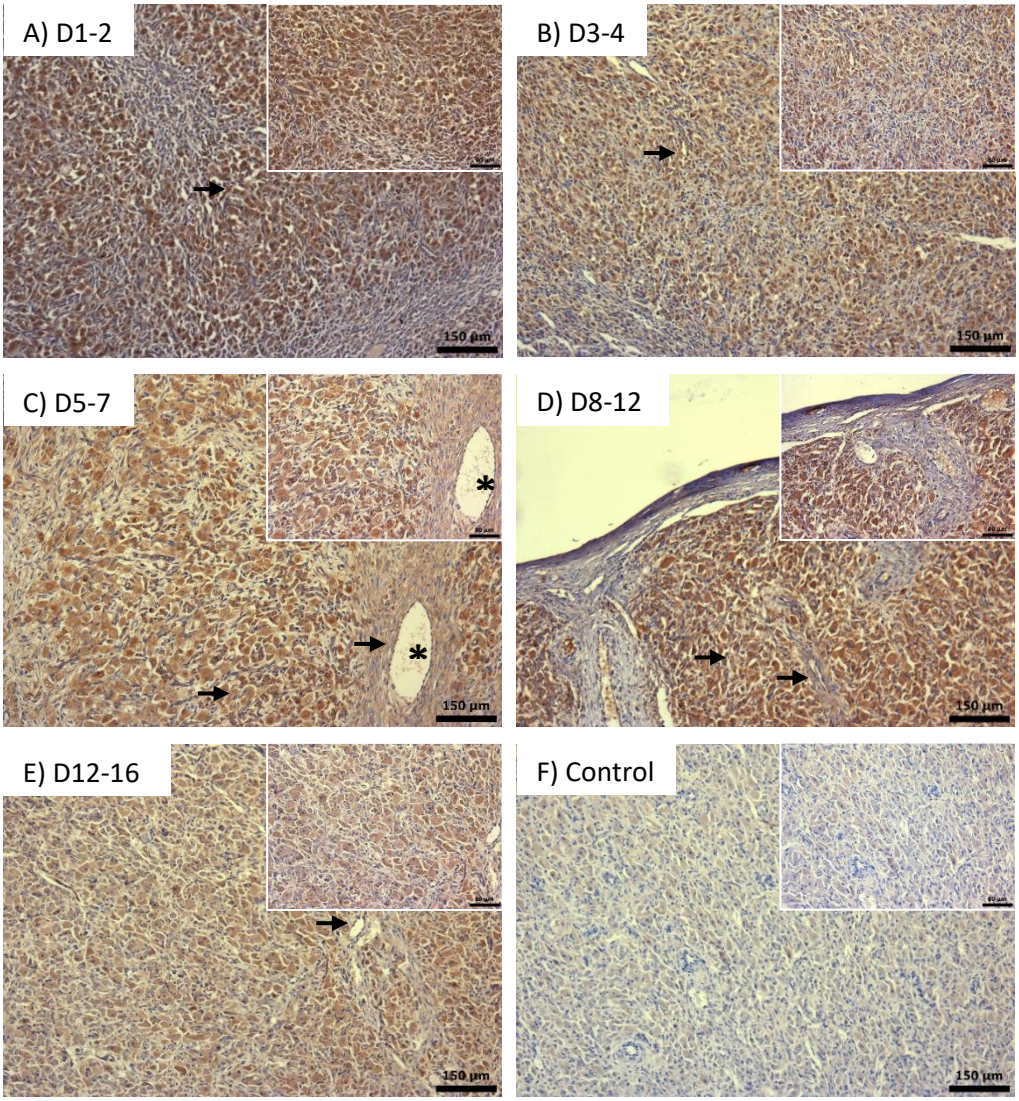


Fig 2.5 *Spatial-temporal expression of PEDF throughout luteal development.* Immunohistochemistry for PEDF was performed on luteal sections from bovine CL at different estimated days after oestrus. The most abundant staining was evident in the early CL samples (A) and (B), with the degree of staining decreasing as CL age increased (C-E). The intensity of staining was greater in small than large luteal cells, with some staining of endothelial cells evident (arrows). A large blood vessel (*) is visible in (C). Images were acquired at x10 and x20 (inset) magnifications.

2.3.2.2 Western blot analysis of PEDF protein in the bovine CL.

A Western blot was performed to characterise the specificity of the PEDF antibody. A band was observed at 50kDa which corresponds to the expected molecular weight of PEDF. Expression of PEDF was greatest in the early CL, with reduced expression in the mid-late CL samples.

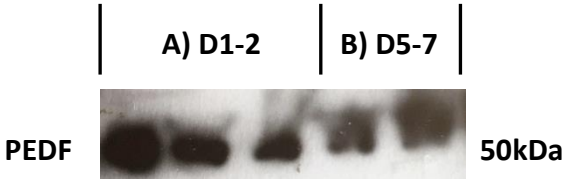


Fig 2.6 *Temporal expression of PEDF protein throughout the CL lifespan.* Identification of PEDF protein was performed by Western blot analysis of luteal samples of different ages; (A) 1-2 day, (B) 5-7 days (12-16 days and regressing CL are not shown due to the presence of artefacts on the blot). PEDF specific antibody was used. A single band at 50kDa was observed.

2.3.2.3 The effect of PEDF on EC network morphology.

In control, there were extensive and elaborate EC networks dispersed between other luteal-derived cells (Fig 2.7A). Treatment with 10ng/ml PEDF changed the morphological appearance of EC networks (Fig 2.7B) with the networks appearing smaller, with an apparent reduction in the number and length of EC network branches in comparison to the control and 100ng/ml PEDF-treated cells. This effect was particularly evident in (Fig 2.8) when the EC networks were extracted and then sorted by area during image analysis.

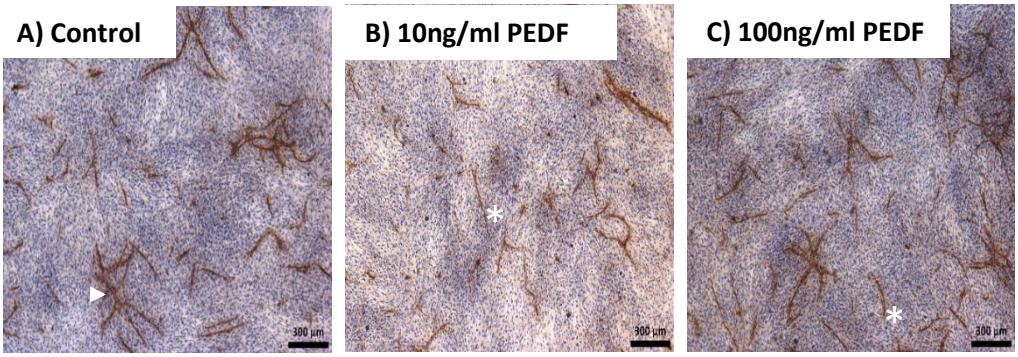
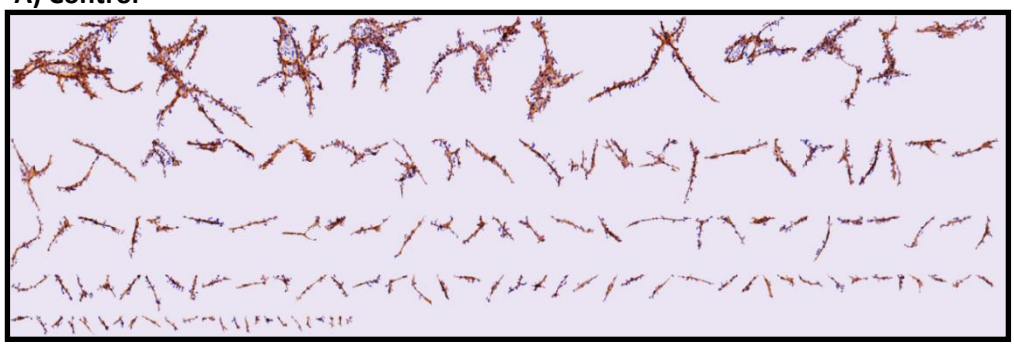


Fig 2.7 *Representative images of the effect of PEDF on luteal EC network development*. Cells were treated either 0, 10 or 100 ng/ml PEDF for 5 days. Areas of complex endothelial cell (EC) network formation were evident (arrow) alongside small areas of unbranched EC development (asterisk). The morphological appearance of EC networks was altered by 10ng/ml PEDF, with a visually reduction in the area of networks alongside a reduction in the number and length of EC network branches compared to control and 100ng/ml PEDF. Images were obtained at ×5 magnification; scale bar = 300µm.

A) Control



B) 10ng/ml PEDF



C) 100ng/ml PEDF

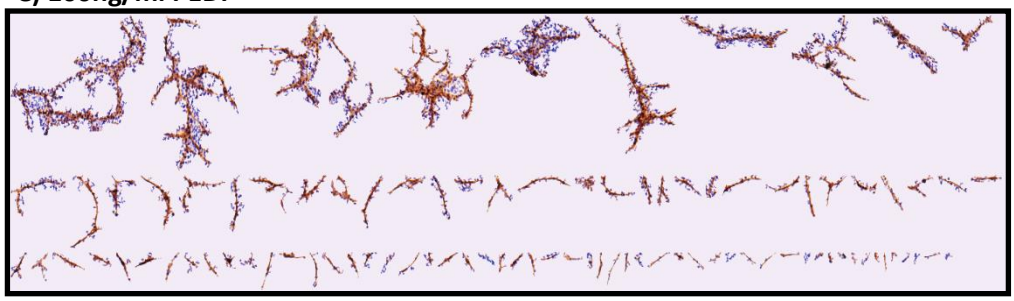


Fig 2.8 *The effect of PEDF on luteal EC network development.*
Bovine luteal cells were treated either 0, 10 or 100ng/ml PEDF. On day 5, cells were fixed and immunohistochemistry for von Willebrand factor performed, followed by image analysis to extract and sort the different EC networks present on the coverslip. Representative images were obtained for the following treatments (A) control, (B) 10ng/ml PEDF and (C) 100ng/ml PEDF. These were then used for quantification of the number of branch points.

2.3.2.4 The effect of PEDF on key EC network formation parameters.

PEDF, in a dose-dependent manner reduced total EC area ($P<0.05$) by almost 50% in comparison to the untreated control (Fig 2.9A). Similarly, PEDF at 10ng/ml reduced the average area of each EC network compared to both control and 100ng/ml PEDF treatments ($P<0.05$; Fig 2.9B). Furthermore,

10ng/ml PEDF reduced both total branch length (by 50%; $P<0.05$; Fig 2.9D) and number of branch points (by 3-fold; $P<0.01$; Fig 2.9E) in comparison to the controls ($P<0.05$). The lowest dose of PEDF also decreased the degree of branching in each EC cluster ($P<0.05$; Fig 2.9F), but had no effect on the number of EC islands ($P>0.05$; Fig 2.9C). For all EC network parameters, PEDF treatment at 100ng/ml was similar to the controls.

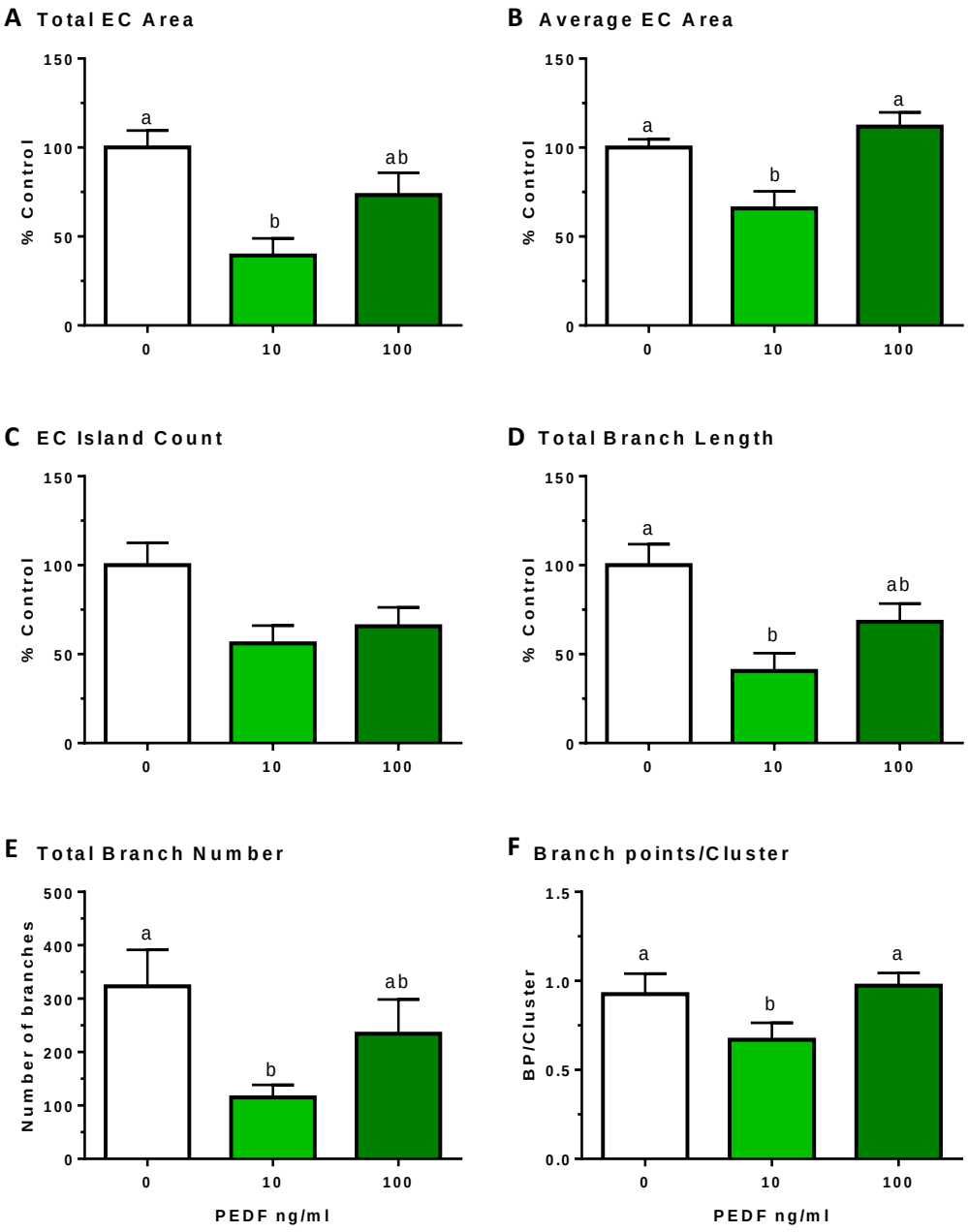


Fig 2.9 *The effect of PEDF on luteal endothelial cell network parameters.* Image analysis of von Willebrand factor staining to quantify the effect of PEDF treatment on the following endothelial cell (EC) network parameters; (A) total EC area, (B) average EC area, (C) EC island count, (D) total branch length, (E) total branch number and (F) branch points per cluster. Significant differences ($P<0.05$) between treatments are illustrated with different letters where a>b. Values are mean + S.E.M from n=3 cultures.

2.3.2.5 The effect of PEDF on progesterone production in vitro.

Spent media was collected on day 3 and 5. Despite inhibiting luteal angiogenesis, PEDF treatment had no effect on luteal progesterone production at any time point (Fig 2.10). However, progesterone production increased by 50% across all treatments from day 3 to day 5 (P<0.05).

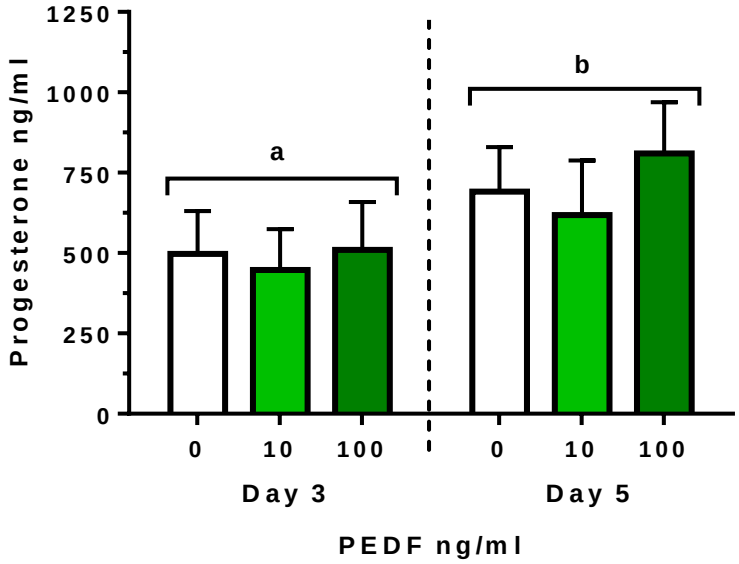


Fig 2.10 *The effect of PEDF on luteal progesterone production.*
Luteal cells were cultured for 5 days with either control, 10ng/ml or 100ng/ml PEDF. Spent cell media was collected on days 3 and 5 and progesterone content analysed by ELISA. Progesterone production was greater on day 5 compared with day 3 (P<0.05; a<b) but PEDF treatment had no effect progesterone production. Values are mean+SEM from n=3 cultures.

2.3.3 The role of THBS1 in early luteal angiogenesis and steroidogenesis.

2.3.3.1 The effect of THBS1 on luteal angiogenesis (initial experiment)

An initial experiment was conducted by Emer Grant, University of Nottingham to investigate the effect of different THBS1 doses on EC growth in the presence of FGF2 and VEGFA. The cultures were performed for 9 days with all images

captured and analysed by myself. Representative images of the effect of THBS1 on EC network growth in comparison to controls were obtained (Fig 2.11). On day 9 of culture, small branched EC networks were visible in all treatments. The degree of VWF staining appeared similar across all treatments and the control, with no visually appreciable differences in EC growth and network morphology being noted. There was also similar level of cell confluency across all treatments.

The images of extracted EC networks (Fig 2.12) clearly shows the number of networks, as well as the number and length of branches stemming from each network, these were broadly similar between each treatment. The higher doses of THBS1 (Fig 2.12C and D) tended to produce more EC networks with plaque-like centres, however the presence of these structures was not consistent between cultures.

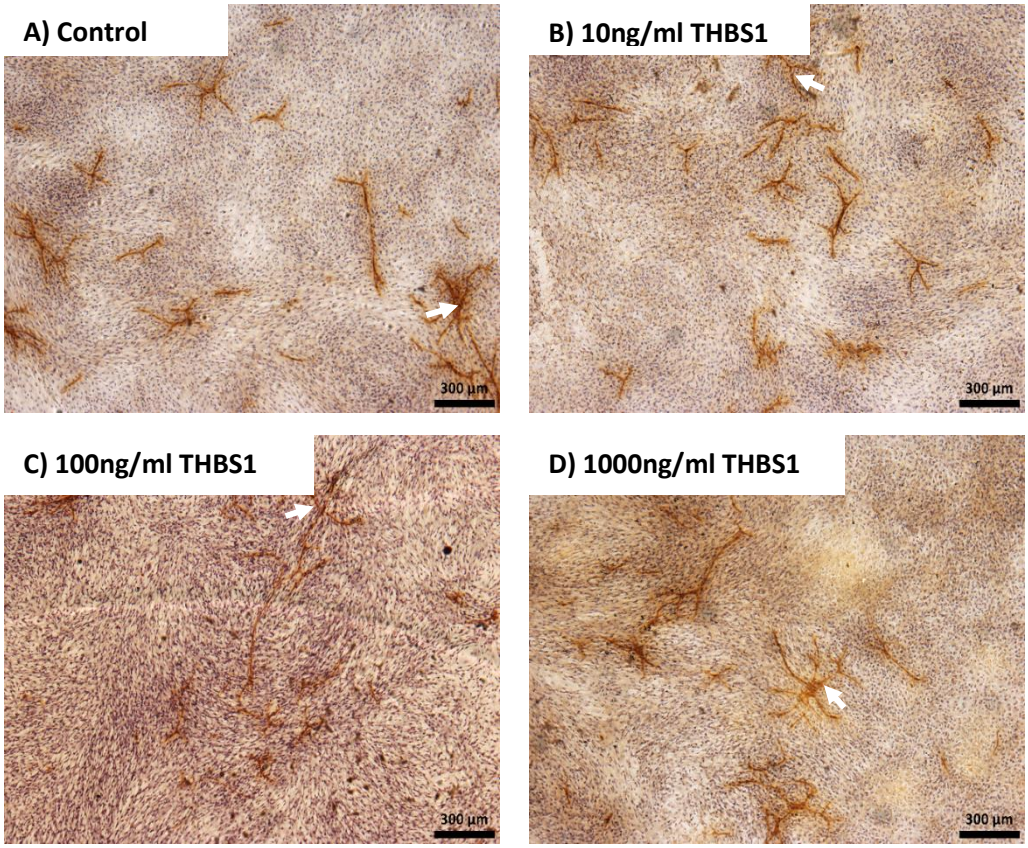
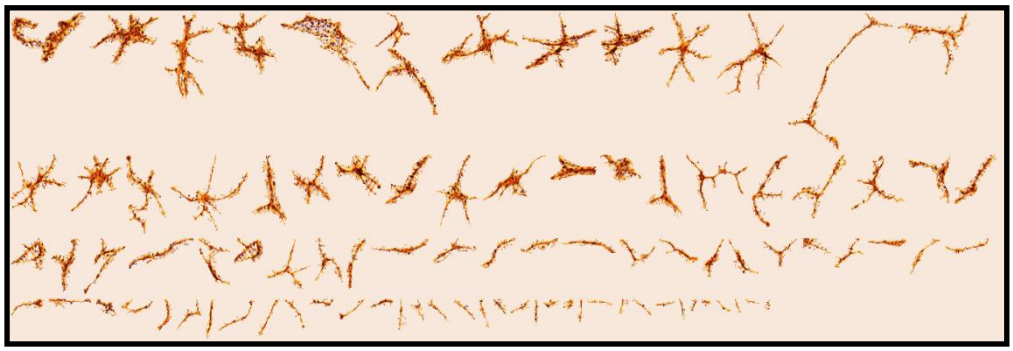


Fig 2.11 *Effect of THBS1 on luteal EC network development (Day 9).*
Representative images obtained of endothelial cell (EC) network growth following continuous treatment with either (A) 0, (B) 10ng/ml, (C) 100ng/ml or (D) 1000ng/ml THBS1. Cells were fixed on day 9 and immunohistochemistry for von Willebrand factor was performed to identify ECs. Large and complex EC networks had formed (arrows). Representative images obtained at ×5 magnification; scale bar = 300µm.

A) Control



B) 10ng/ml THBS1



C) 100ng/ml THBS1



D) 1000ng/ml THBS1



Fig 2.12 *Extracted EC networks following THBS1 treatment (Day 9).*
Early bovine luteal cells were treated with either 0, 10, 100 or 1000ng/ml THBS1, for 9 days, then fixed and immunostained for von Willebrand factor to identify endothelial cell (EC) development. Representative images of EC networks (sorted by size) were obtained following treatment with (A) control, (B) 10ng/ml THBS1, (C) 100ng/ml THBS1 and (D) 1000ng/ml THBS1. Evidence of networks with plaque-like centres are visible at higher THBS1 doses.

2.3.3.2 Effect of THBS1 on EC network parameters

The total EC network and average size of each EC network size was numerically higher with 1000ng/ml THBS1 but there was no significant treatment effect on the total area (P=0.39) or average EC area (P=0.13) of the networks formed at any dose in comparison to the untreated control (Fig 2.13A-B). Also THBS1 had no effect on the number of EC islands formed (P=0.78; Fig 2.13C). There was a tendency for THBS1 treatment to reduce the number of EC branch points (P=0.06), although THBS1 had no effect on the total branch length (P=0.12). However, 1000ng/mlTHBS1 significantly reduced the number of branch points per EC cluster from 2.6 to 1.4 (P=0.03).

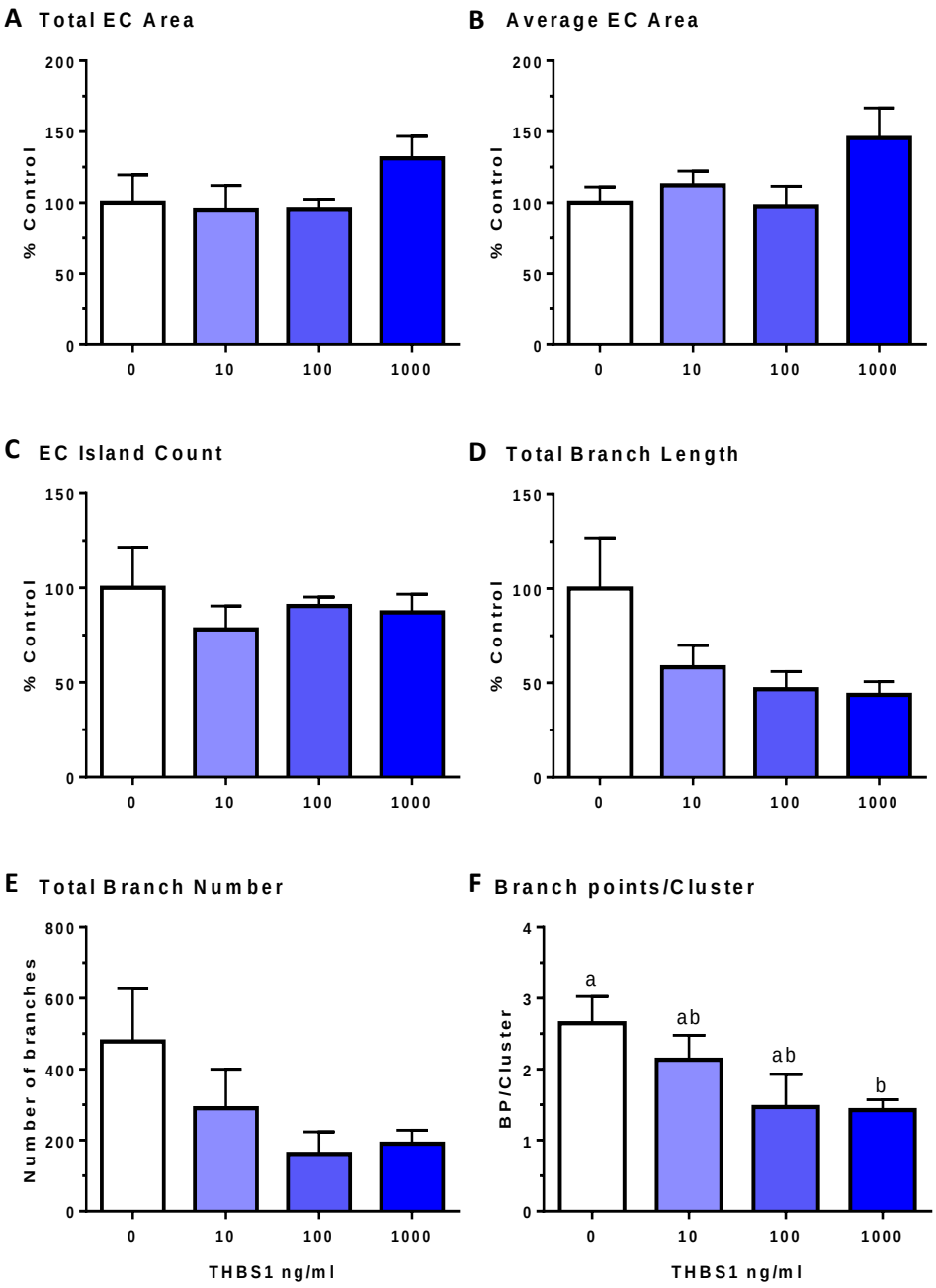


Fig 2.13 *Effect of THBS1 on luteal EC network parameters (Day 9).*
Following treatment with either 0, 10, 100 or 1000ng/ml THBS1, fixed cells were immunostained for von Willebrand factor and image analysis performed to determine endothelial cell (EC) network formation. The parameters shown are; (A) total EC area, (B) average EC area, (C) number of EC clusters/islands, (D) total branch length, (E) total branch number and (F) branch points per cluster. Significant differences ($P<0.05$) between treatments are illustrated with different letters where a>b. Values are mean+SEM from n=3 cultures.

2.3.3.3 Effect of THBS1 on early luteal angiogenesis and steroidogenesis (main experiment).

The results from this initial experiment warranted further investigation, to confirm the subtle effect of higher THBS1 doses (100 and 1000ng/ml). Therefore, cultures were established from early bovine CL and cells were treated with either 0, 100 or 1000ng/ml THBS1 in the presence of FGF2 and VEGFA but this time cells were fixed on day 5 of culture due to an increased rate of growth of the luteal tissue utilised in the culture system.

2.3.3.4 Morphological appearance of EC networks following THBS1 treatment.

On day 5 of culture, large and branched EC networks had formed across all treatments, however there were still smaller areas of unbranched EC islands evident alongside the larger networks formed. There was no observable difference in the visual appearance of the EC networks between the control (Fig 2.14A) and THBS1 treatments (Fig. 2.14B-C). In comparison to the initial experiment, there were no large plaque-like networks observed in the 1000ng/ml THBS1 treatment wells. The similarity between the controls and THBS1 treated EC networks can be further appreciated in Fig 2.15 which highlights the size and degree of branching in the larger networks across the treatment groups. It also emphasized that on day 5 of culture, there was still a large number of unbranched small EC islands. Furthermore, the number of networks observed across all treatments appeared to be very similar (Fig 2.15).

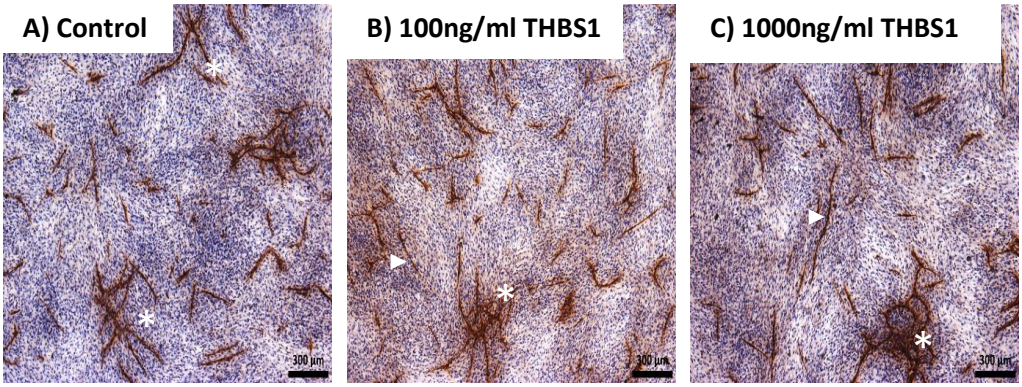
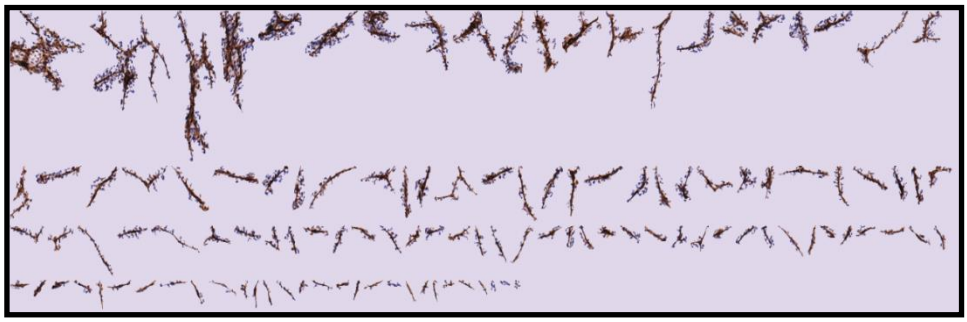


Fig 2.14 *Effect of THBS1 on luteal EC network development (Day 5).*
Representative images obtained of endothelial cell (EC) network formation following culture for 5 days with either (A) control, (B) 100ng/ml THBS1 or (C) 1000ng/ml THBS1. Cells were fixed on day 5 and immunohistochemistry for von Willebrand factor was performed to identify EC. Large and complex EC networks had formed (asterisk) across all treatments, however there were still some smaller regions of unbranched EC growth (arrows). Images were acquired at x5 magnification; scale = 300µm.

A) Control



B) 100ng/ml THBS1



C) 1000ng/ml THBS1

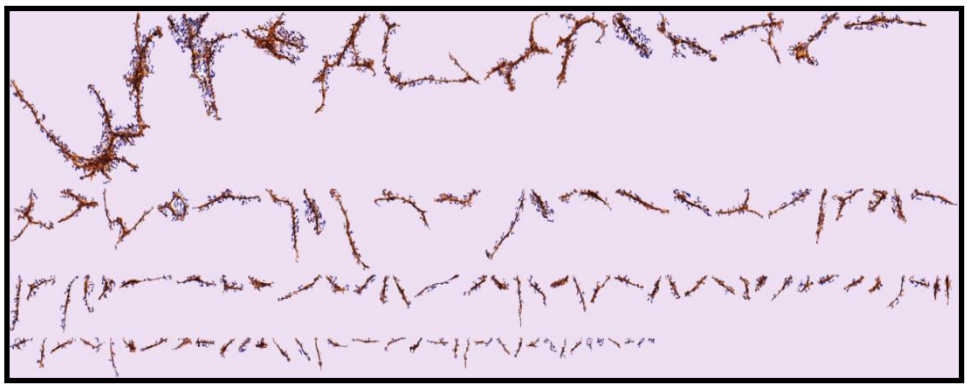


Fig 2.15 *Extracted EC networks following THBS1 treatment (Day 5).*
Early bovine luteal cells were treated with either 0, 100 or 1000ng/ml THBS1 in culture. On day 5, cells well fixed and immunostained for von Willebrand factor to identify endothelial cell (EC), image analysis was performed. Representative images were obtained following treatment with (A) control, (B) 100ng/ml THBS1 and (C) 1000ng/ml THBS1, with EC networks sorted by total area.

2.3.3.5 Effect of THBS1 on key EC network parameters.

THBS1 treatment had no significant effect on the total EC area (P=0.43) or average EC area (P=0.57; Fig 2.16A-B). Equally, the number of EC islands were also unaffected by THBS1 treatment (P=0.47; Fig 2.16C). While the total branch

length and total branch number appeared lower in the 100ng/ml THBS1 in relation to the control, they were not significantly altered by THBS1 treatment (P=0.16 and P=0.15, respectively). In contrast to the initial experiment, THBS1 did not significantly reduce the number of branch points per EC cluster (P=0.06). Although there was a tendency for the 1000ng/ml THBS1 to increase the number of branch points per cluster in comparison to the 100ng/ml dose, but this was not significant.

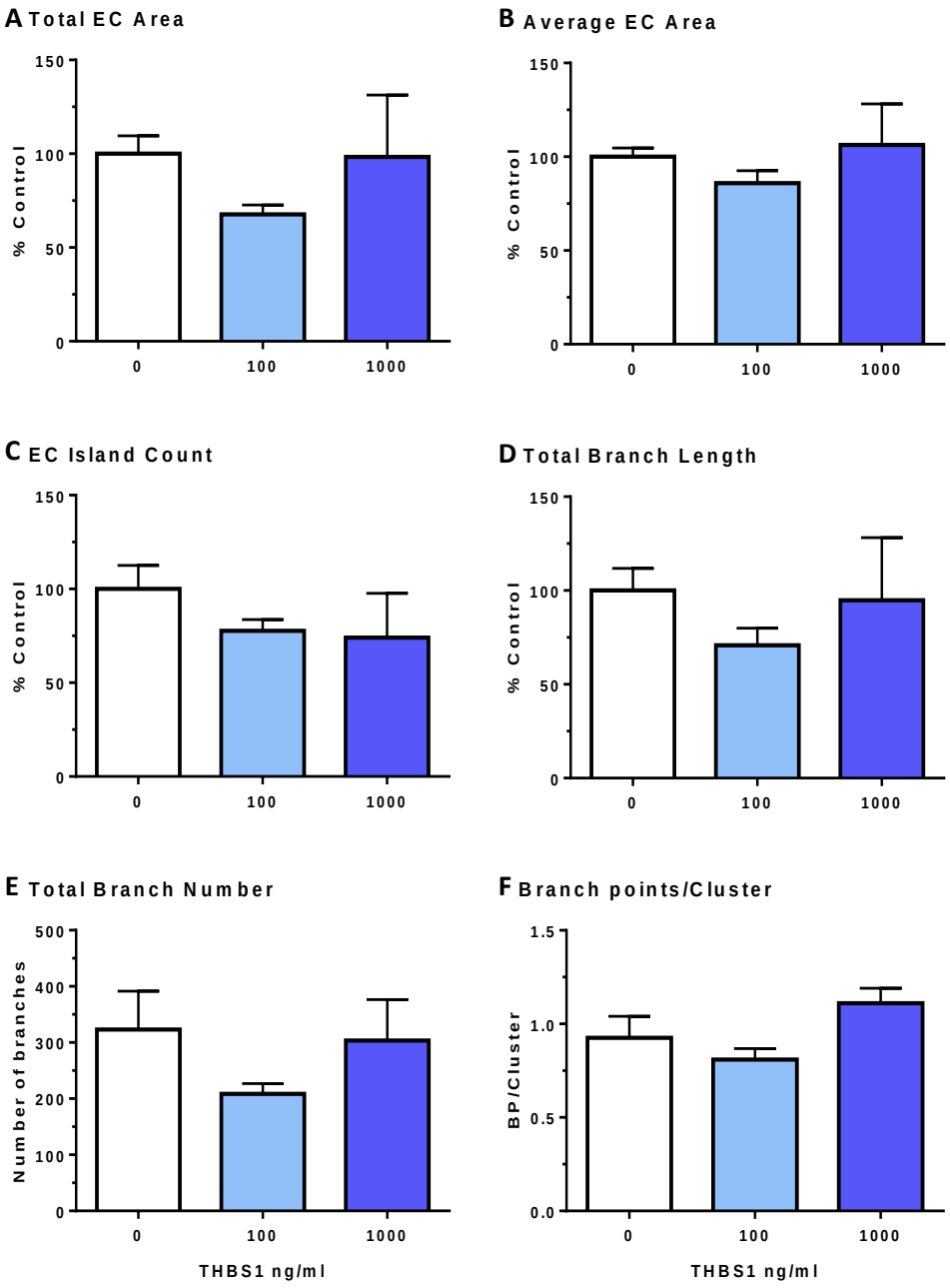


Fig 2.16 *Effect of THBS1 on luteal EC network parameters (Day 5).*
Image analysis of von Willebrand factor staining was performed to quantify the effect of THBS1 treatment on the following EC network parameters; (A) total EC area, (B) average EC area, (C) number of EC islands, (D) total branch length, (E) total branch number and (F) branch points per cluster. No significant differences were detected between treatments. Values are mean + S.E.M.

2.3.3.6 The effect of THBS1 on progesterone production in vitro.

There was a significant increase in progesterone production from day 3 to day 5 ($P<0.05$), however THBS1 treatment did not significantly impact upon luteal progesterone production at either dose or time point.

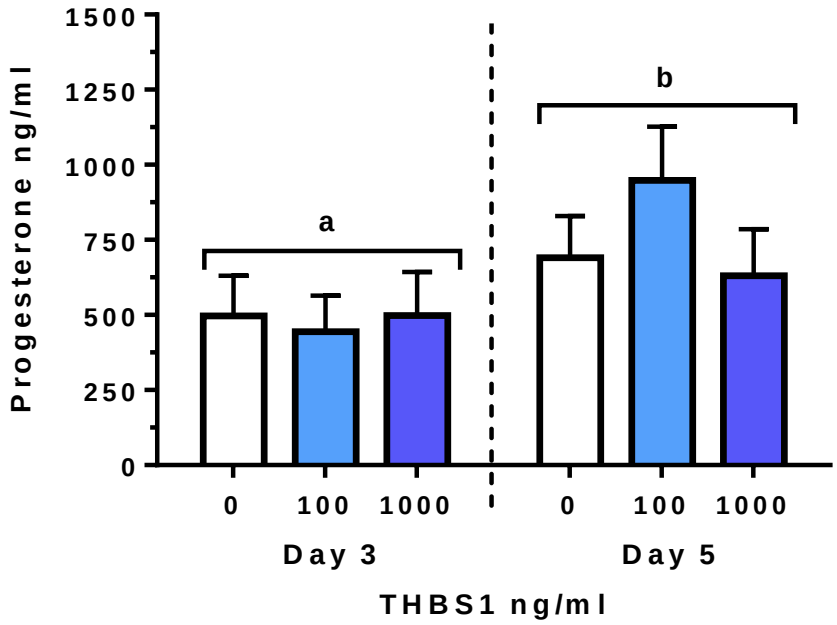


Fig 2.17 *Effect of THBS1 on luteal progesterone production.*
Luteal cells were treated with either 0, 100 or 1000 ng/ml THBS1. Spent cell media was analysed for progesterone content by ELISA on day 3 and 5 of culture progesterone concentrations were greater on day 5 compared with day 3 ($P<0.05$; a<b). However, THBS1 treatment had no effect on progesterone levels at either dose. Values are mean + S.E.M.

2.4 Discussion.

The key findings of chapter 2 were as follows; that there is limited evidence for $VEGFA_{164b}$ expression in the bovine CL, and if present, expression is likely to be minimal and confined to the mature CL. This is in contrast to VEGFA, which is expressed throughout the entire lifespan of the CL at apparently high levels. It was also demonstrated that THBS1 had a varied effect on luteal angiogenesis and seemingly no effect on luteal progesterone production *in vitro*. The principal novel finding demonstrated that PEDF was expressed at all stages of luteal development, with levels highest in the early CL before expression declined with luteal development. Alongside this, PEDF inhibited early luteal angiogenesis in the presence of FGF2 and VEGFA, but had no effect on luteal progesterone production.

VEGFA and $VEGFA_{165b}$ expression in the bovine CL.

VEGFA mRNA and protein expression has been characterized in the bovine CL previously. Expression of the most abundant *VEGFA* mRNA isoforms reached maximal levels by day 15 of the luteal phase, whilst VEGFA protein levels were found to increase slowly throughout CL development, reaching a maximal level on day 12 of the luteal phase (Robinson *et al.* 2007, Guzmán *et al.* 2015). The current VEGFA immunohistochemistry and Western blot analysis found VEGFA expression throughout the CL lifespan. However, the temporal changes in VEGFA expression seen in other studies (Redmer *et al.* 1996, Berisha *et al.* 2000a, Robinson *et al.* 2007, Guzmán *et al.* 2015), were not observed here.

In the present study, VEGFA was predominantly expressed in the small and large luteal cells, with no expression in ECs. Similarly in CLs from primates and horses, VEGFA is predominantly located in steroidogenic cells (Kamat *et al.* 1995, Al-zi'abi *et al.* 2003). Interestingly, in sheep, there is minimal VEGFA expression in the steroidogenic luteal cells, with VEGFA mainly localised to vascular pericytes (Redmer *et al.* 2001). The reason for the species differences in the cellular localisation of VEGFA is not known, but both locations are in close proximity to ECs where FLT1 and KDR are localised in the bovine CL (Gabler *et al.* 2004).

The present study did not show a change in VEGFA expression at the different stages of luteal development. This could be attributed to the presence of background staining that was evident in the control immunoglobulin sections that might have obscured subtle changes in VEGFA staining. Similarly, the results of the Western blot did not show any obvious changes in the levels of VEGFA, although this was not quantified. A single band was detected following immunoblotting for VEGFA at 50kDA, this was higher than expected (21kDa for bovine VEGFA monomer). The dimeric form which should not be present yields a band at 42kDa. This discrepancy was not investigated further since the focus of the project was the role of anti-angiogenic factors in early CL development.

$VEGFA_{164b}$ expression in the bovine CL.

There were high levels of background staining with the $VEGFA_{164b}$ immunohistochemistry, which made interpretation of possible $VEGFA_{164b}$ expression difficult to determine. The principal reason for the high staining in

the control section was the high concentration of IgG that was required to obtain a signal. If $VEGFA_{164b}$ is expressed at all, then expression levels are minimal and almost entirely confined to the mature CL. When the immunohistochemistry results were interpreted alongside the Western blotting results it became evident that $VEGF_{164b}$ expression was apparently absent during the early stage of luteal development. Whilst we cannot definitively conclude that this factor is absent in bovine luteal tissue, its expression is minimal. Similarly, Guzmán et al. (2015) did not detect *$VEGFA_{164b}$* mRNA at any stage of luteal development. Collectively, it appears that $VEGFA_{164b}$ does not play an important role in regulating luteal angiogenesis. In contrast, $VEGFA_{165b}$ was expressed at low levels throughout the luteal phase in the porcine CL (Ribeiro *et al.* 2007). Equally, $VEGFA_{165b}$ expression in the marmoset ovary was highly dynamic, with expression of this isoform accounting for almost 100% of the VEGFA protein in ovaries without CL and 65% of the VEGFA protein in ovaries with CL. It was hypothesised that this reduction in expression could trigger the promotion of luteal angiogenesis in this species (Qiu *et al.* 2012). In mice, $VEGFA_{165b}$ overexpression reduced the number of follicles transitioning from the primary to secondary stages of development, and consequently the number of mature follicles and CL observed. Additionally, the size of those CL was reduced suggesting that this factor plays an important role in both follicle and CL development (Qiu *et al.* 2012).

An aim of future investigations would be to quantify the expression of $VEGFA_{164b}$ in bovine ovaries without CL. This would determine if this factor is

highly expressed in the follicular phase but is then downregulated around ovulation to enable luteal angiogenesis to take place. Alterations to the expression of $VEGFA_{165b}$ expression in response to such an event would support the concept that there is a necessity to balance pro- and inhibitory angiogenic factors. Indeed, a change in the ratio of the pro- and inhibitory isoforms of VEGFA was important in pre-eclampsia in humans, although this is a pathological rather than physiological setting (Bates *et al.* 2006).

The VEGFA antibody utilised in this study revealed that VEGFA was highly expressed in the bovine CL. However, it binds non-selectively to multiple VEGFA isoforms so it is not possible to determine which specific isoforms are being expressed. The current findings would suggest that $VEGFA_{164b}$ can be excluded as it was absent in the bovine CL. Guzmán et al. (2015) observed that $VEGFA_{165a}$ and $VEGFA_{120a}$ mRNA expression increased throughout CL development, reaching maximal levels on day 15. Similarly $VEGFA_{120b}$ was also expressed in the CL, reaching maximum expression levels on day 15 and has been implicated in inhibition of angiogenesis during luteal regression by counteracting pro-angiogenic VEGFA isoforms. However, the role of larger VEGFA isoforms such as $VEGFA_{189}$ is yet to be explored and more work is required in order to identify these in the bovine CL. This would give us a greater understanding of the complex interplay of the pro- and anti-angiogenic isoforms of VEGFA in the regulation of angiogenesis in the bovine ovary.

PEDF expression and effect on angiogenesis and steroidogenesis in the bovine CL.

The Western blot and immunohistochemistry results have demonstrated for the first time, not only the presence of PEDF protein, but also the temporal and spatial expression of PEDF in the bovine CL. Expression of the *SERPINF1 (PEDF)* gene has previously been identified in the bovine follicle, however the expression of this anti-angiogenic factor has not been studied in the bovine CL (Hayashi *et al.* 2011). PEDF staining intensity was greatest in the early CL and declined throughout the CL lifespan, suggesting that PEDF plays an important role in the regulation of early luteal angiogenesis. PEDF expression was predominantly localised to the large and small luteal cells, with little apparent difference in staining between these two cell types. Previous studies have localised PEDF expression in the granulosa cells of mice and humans, and in luteinised human granulosa cells (Chuderland *et al.* 2013, Kampfer *et al.* 2014). Furthermore, low levels of PEDF expression were evident in ECs, which is important given that the PEDF interacts with KDR and PEDFR that are located on these cells (Cai *et al.* 2006, Zhang *et al.* 2006, Ablonczy *et al.* 2009, Manalo *et al.* 2011). If it were possible to separate the different luteal cells then future investigations into the expression of PEDF in specific luteal cell types, utilising qPCR would allow for further confirmation and quantification of this factor in the CL. This would give an insight into which luteal cells are responsible for the majority of PEDF expression. Similarly, it would be interesting to determine if PEDF expression was upregulated by the LH surge.

The effect of PEDF on luteal angiogenesis.

PEDF acted as a potent inhibitor of *in vitro* luteal angiogenesis by reducing total and average EC area, branch number and length as well as reducing the degree of branching. However, this effect was only detected at the lower PEDF dose. The only parameter seemingly unaffected by PEDF was the total EC island number. These findings are supported by numerous studies that have shown PEDF inhibits angiogenesis in a variety of systems (Dawson *et al.* 1999, Stellmach *et al.* 2001, Loegl *et al.* 2016). PEDF can inhibit angiogenesis by blocking the interaction of VEGFA with KDR and inducing proteolysis of both VEGFRs (Cai *et al.* 2006, Zhang *et al.* 2006). Furthermore, PEDF inhibits the effects of FGF2 on ECs as well as inducing EC apoptosis (Kanda *et al.* 2005, Manalo *et al.* 2011).

Inhibition of FGFR signalling at a similar time point to when PEDF was administered also reduced the total area of EC networks, number of branch points and degree of branching per EC island, whilst VEGFR inhibition alone had more limited effects on EC network development (Woad *et al.* 2012). It is therefore feasible that PEDF predominantly elicits its anti-angiogenic effects in the bovine CL by inhibition of FGF2. However, it is likely that PEDF inhibited luteal angiogenesis by several different mechanisms. Future work to identify the mechanism by which PEDF acts in the bovine CL would be beneficial and could be achieved through quantification of the degree of EC apoptosis induced by PEDF treatment, and by analysing the effect of PEDF treatment on VEGFR and FGFR1 function and signalling capabilities.

Interestingly, the inhibition of luteal angiogenesis by PEDF was seemingly dose dependent with only the lower dose of PEDF having an effect. This type of dose-dependent effect of PEDF has not been reported in other studies, however it has been reported that the balance of VEGFA and PEDF determines whether cells survive or undergo apoptosis, with the expression of these factors varying depending upon whether cells are actively dividing or quiescent (Pollina *et al.* 2008). Similarly, TGFB inhibits angiogenesis at high doses but stimulates it at lower doses (Carmeliet 2003, Ferrari *et al.* 2009). There are reports of several other anti-angiogenic factors such as endostatin and interferon-α eliciting a "U" shaped dose-response curve, whereby low concentrations inhibit angiogenesis, but at higher concentrations this effect is lost (Slaton *et al.* 1999, Celik *et al.* 2005, Tjin Tham Sjin *et al.* 2006, Reynolds 2010).

It is therefore possible that at low doses of PEDF stimulates EC apoptosis, whilst at high doses the ratio shifts to favour EC survival and that this mechanism is closely linked to *VEGFA* expression. Evidently further investigations are required to determine the reason for the contrasting effects of low and high PEDF doses. Given these findings, it would be beneficial to conduct future experiments using more PEDF doses, including a dose lower than 10ng/ml to give a greater insight into the mechanism by which it reduces angiogenesis.

The effect of PEDF on luteal progesterone production.

Despite drastically reducing angiogenesis, neither PEDF dose had an effect on luteal progesterone production. This would suggest that despite being

predominantly expressed in the steroidogenic cells of the bovine CL, PEDF has no direct effect upon the function of these cells. In the bovine CL, almost every steroidogenic cell is located adjacent to an EC, suggesting that these progesterone producing cells are reliant upon a good vascular supply (Reynolds *et al.* 2000). Therefore, the dramatic inhibition of angiogenesis by PEDF observed *in vitro* would likely impact upon the growth and function of luteal steroidogenic cells *in vivo*. Further experiments are required in order to establish this. A previous study has shown TGFB inhibited angiogenesis to a similar extent as PEDF, but TGFB profoundly reduced the concentration of progesterone in the spent media (Thompson 2014). This finding nicely illustrates that despite the numerous anti-angiogenic factors having a similar effect upon EC growth and differentiation, the mechanism by which they act and the other cell types with which they interact are often profoundly different.

The effect of THBS1 on luteal angiogenesis and steroidogenesis.

THBS1 reduced the number of branches per EC cluster by 50% in the initial experiments (principally at 1000ng/ml), however this effect was not observed in the subsequent experiment conducted under similar conditions. Other than this difference, the results of both experiments demonstrated that THBS1 had no effect upon the total area of luteal EC networks in the presence of FGF2 and VEGFA. These inconsistent results indicate that luteal-derived ECs in this co-culture system are less sensitive to THBS1 than others previously described, where THBS1 lowered the number of luteal ECs in the presence of FGF2 and was capable of inducing EC apoptosis (Dawson *et al.* 1997, Zalman *et al.* 2012).

One mechanism through which THBS1 can inhibit angiogenesis is by sequestering FGF2 and preventing the release of this pro-angiogenic factor (Colombo *et al.* 2010). The presence of VEGFA in the culture media may account for differences observed in the current study and that conducted by Zalman et al. (2012), since VEGFA is capable of stimulating angiogenesis to such an extent that the sequestration of FGF2 by THBS1 had no impact upon angiogenesis. However, THBS1 binds to VEGFA causing the cellular internalization of VEGFA in the murine ovary. This would then reduce VEGFA-mediated angiogenesis (Greenaway *et al.* 2007). The addition of VEGFA and FGF2 together to the culture system produces a response greater than the addition of either factor alone, so it is possible that in combination these factors overcame the inhibitory effects of THBS1 (Robinson *et al.* 2008). A possible way of determining if FGF2 and VEGFA were compensating for the inhibitory effects of THBS1 would be to treat ECs with THBS1 in the absence of FGF2 and VEGFA.

The other mechanism through which THBS1 inhibits angiogenesis is by inducing EC apoptosis via the CD36 receptor (Dawson *et al.* 1997, Jimenez *et al.* 2000, Zalman *et al.* 2012). Given that the only reduction in EC network formation observed in the current study was a decline in the number of branches per cluster, it is unlikely that the EC cells were undergoing a significant amount of apoptosis. However, ECs undergoing branching following their conversion to tip cells may be more sensitive to THBS1 and/or induction of apoptosis. Therefore it is not possible to determine whether THBS1 induced apoptosis in the luteal angiogenesis culture system. Nevertheless, determining if THBS1 is

inducing EC apoptosis would be a useful direction of future work, which could be achieved by performing dual staining for caspase 3 alongside an EC marker.

In the current study, THBS1 had no effect on progesterone production, suggesting that this factor does not act upon the steroidogenic cells in the bovine CL. Previous studies have not investigated the effect of THBS1 on luteal steroidogenesis, so it is not possible to draw comparisons. However, further confidence in the reliability of the result that THBS1 did not impact progesterone production, was provided by the observation that progesterone concentrations increased from day 3 to 5 in culture. This suggests that the luteal cells were functioning normally and mimicking the physiological rise in progesterone that is observed *in vivo* (Gilbert *et al.* 1989).

In conclusion, the results of this chapter show that VEGFA was highly expressed at all stages of luteal development, however $VEGFA_{165b}$ expression was minimal if at all present. THBS1 has an inconsistent effect on luteal angiogenesis *in vitro* and did not affect luteal progesterone production. Whilst PEDF was highly expressed in the early CL and inhibited early luteal angiogenesis in a biphasic manner, but had no effect on luteal progesterone production.

These results suggest that there is a need for balance even during periods of intense luteal angiogenesis, and that the interplay between the pro and anti-angiogenic factors is highly complex.

Chapter 3. Alternative splicing in the bovine ovary.

3.1 Introduction.

3.1.1 Next generation sequencing.

Next generation sequencing (e.g. RNAseq) allows millions of short DNA strands to be sequenced in parallel, these short strands can then be mapped to the genome of interest, enabling entire genomes to be sequenced with greater sensitivity than more traditional methods (Behjati & Tarpey 2013). These technologies have greatly improved our understanding of a variety of physiological and pathological processes, from the determination of sex to the identification of neoplastic mutations (Kandoth *et al.* 2013, Haussmann *et al.* 2016). Since techniques such as RNAseq measure global gene expression, this allows for the identification of splice variants, alterations in splicing and detection of novel transcripts (Wang *et al.* 2009, Zhao *et al.* 2014). These techniques rely heavily upon bioinformatics to analyse the large quantities of data obtained, with processes such as mapping the short reads obtained from RNAseq to the genome of interest and identification of alternative splicing being particularly challenging (Wang *et al.* 2009).

3.1.2 Alternative splicing in the bovine ovary.

Alternative splicing of candidate genes has been identified in the bovine ovary, with many of these splicing events likely to have an impact upon ovarian function. These have included the splicing of LH and FSH receptors in granulosa cells (Rajapaksha *et al.* 1996, Nogueira *et al.* 2007). In the CL, alternative

splicing of VEGFA isoforms has been observed and is likely to play an important role in the regulation of luteal angiogenesis (Guzmán *et al.* 2015). Furthermore SRSF9, a constituent of the spliceosome, is upregulated in granulosa cells of dominant follicles (Mihm *et al.* 2008), suggesting an important role in follicular development.

3.1.3 The effect of dietary protein on reproductive performance.

The effect of diet on reproductive performance has been extensively studied in several species (Pushpakumara *et al.* 2003, Garnsworthy *et al.* 2008, Lazarin *et al.* 2012, Eborn *et al.* 2013). This is particularly important in cattle where the production of the calf is central for both dairy and beef units. Of particular interest, is the effect of feeding high levels of dietary protein to promote growth and development, which has had varying effects on reproductive performance (Ferguson & Chalupa 1989). High dietary protein levels decreased oocyte quality and first service conception rates, most likely as a consequence of increased circulatory urea levels (Canfield *et al.* 1990, Armstrong *et al.* 2001). Additionally, feeding a high protein diet that was urea-generating reduced the metabolic activity of ovine granulosa cells and increased the apoptotic rate (Nandi *et al.* 2016). Conversely, Barton et al., (1996) observed that a high protein intake only had negative implications when there were concurrent health issues. It is evident from these studies, that the effect of dietary protein on reproductive performance requires further investigation.

3.1.3.1 The effect of dietary protein on gene expression in bovine granulosa cells.

Recently, it was demonstrated using RNAseq that feeding a high protein diet to beef heifers altered gene expression in granulosa cells, with several key genes such as *FN1* and STAR upregulated (Edwards 2016). *FN1* has a role in granulosa cell proliferation and cell adhesion, whilst STAR is responsible for the transport of cholesterol in the mitochondria, the rate limiting step in steroid synthesis (Colman-Lerner *et al.* 1999, Miller 2007, Kantola *et al.* 2008, Yamashita *et al.* 2011, Edwards 2016). Whilst others, such as *CH25H* and *KLF4* were downregulated in granulosa cells in the high dietary protein animals (Edwards 2016). CH25H is important in cholesterol synthesis whilst KLF4 regulates follicular development (Wang & Tuohimaa 2006, Batista *et al.* 2007, Natesampillai *et al.* 2008). It is interesting to note that while both STAR and CH25H are involved in steroidogenesis, a high level of dietary protein had contrasting effects on their expression. Further investigation is required to determine the extent of these dietary effects on alternative mRNA splicing in the ovary so that a deeper knowledge of gene expression is understood.

3.1.4 Hypothesis and aims.

The hypothesis was that feeding a high-protein diet to beef heifers would induce changes in the alternative splicing of genes in the granulosa cells. Therefore, the aims of this study were:

1. To determine the effect of feeding a high-protein diet on the alternative spicing of the granulosa cell transcriptome.

2. To investigate the potential effects *in silico* of the alternative splicing of particular genes / gene ontologies on biological processes controlling granulosa cell function.

3.2 Materials and Methods: Splicing.

This study utilised samples collected and processed by Dr Jennifer Edwards (University of Nottingham, PhD 2017). This experiment was conducted under Project Licence number PPL 40/3560 in accordance with the Animals (Scientific Procedures) Act 1986. Sample collection, processing and RNA extraction were performed by Dr Nigel Mongan (University of Nottingham), who also performed the quality control, transcript alignment and alternative splicing analysis steps.

3.2.1 Animals and diet.

Non-pregnant 20-30 months old Angus cross dairy heifers (n=320) were housed on the same commercial farm unit (Manor Farm, Fritwell, Bicester, Oxfordshire). The different diets, formulated by David Hendy, Blackbrook Farm were fed to groups of heifers from February-September 2014. The control (CP) diet contained 10.4% crude protein while the high protein (HP) diet contained 14.5% crude protein. The nutritional composition of the two dietary rations is shown in Table 3.1. After being fed the diet for at least 60 days, the heifers were sent for slaughter at a commercial abattoir (Dovecote Ltd., Skellingthorpe, Lincoln, UK).

Table 3.1 *Composition of the different dietary rations for the control and high protein dietary groups.*

	Dietary group	
Diet composition	**Control (CP)**	**High (HP)**
Dry Matter (DM) (g/kg)	436	431
Metabolisable Energy (MJ/kg DM)	11.8	11.2
Crude protein (g/kg DM)	**104**	**145**
Oil (g/kg DM)	41.4	40
Non Digestible Fibre (g/kg DM)	320	395
Starch (g/kg DM)	302	145
Sugar (g/kg DM)	59	113
Long roughage (%)	49.2	49.3

3.2.2 Tissue collection.

Samples were collected in batches of animals (n=7-37), with a total of 9 CP and 8 HP diet batches. Following slaughter, the reproductive tract was immediately collected and examined to ensure no gross abnormalities or signs of disease were present. Both ovaries were removed and stored in pairs in PBS at 36°C for transport back to the University of Nottingham.

3.2.3 Sample processing.

Medium sized follicles (4-9mm diameter) were aspirated using a 25ga needle and syringe to remove follicular fluid and harvest granulosa cells (GCs). Follicular fluid from 10-12 ovaries was pooled together and allowed to settle for 5 minutes before the fluid was removed. Cells from the pooled fluid were placed in a petri dish and all oocytes removed, the GCs were collected and washed in 1% BSA in PBS. The GCs were placed in 5-10 volumes of RNALater (Invitrogen, Life Technologies) and stored at -80°C.

3.2.4 RNA extraction.

Total RNA was isolated from GCs using the QIAGEN RNAEasy® Mini kit (QIAGEN, Crawley West Sussex, UK) with minor alterations to the manufacturer's guidelines, the extracted RNA was stored at -80°C. A NanoDrop N-8000 Spectrophotometer (Labtech International Ltd., East Sussex, UK) was used to measure the quantity and quality of the RNA. Samples selected for sequencing had a total RNA concentration >100ng/µL and an RNA integrity number (RIN) greater than 7.0, as measured by an Agilent 2100 Bioanalyzer (Agilent Technologies UK Ltd., Stockport, UK). In total, 4 samples (CP [n=2]; HP [n=2]) were sent for sequencing. cDNA Library preparation and transcriptome sequencing were performed in the Genome Centre (Barts, London) whilst quality control and alignment was carried out by Dr Nigel Mongan. RNA transcription was measured using next generation sequencing (NGS) using the Illumina NextSeq platform.

3.2.5 Quality control and processing.

The following steps were performed by Dr Mongan. Adapter sequences added during library preparation were removed from the mRNA using Cutadapt (Martin 2011). Quality control checks were performed using FastQC, a phred score of 30 was used as the quality threshold and any poor quality reads were removed. The Trimgalore wrapper was used for Cutadapt and FastQC processing steps. All reads were then trimmed to 100 base pairs using the rMATs trimfastq python script.

3.2.6 Alignment of transcript and alternative splicing analysis.

rMATs software detects and analyses five major types of alternative splicing events (see Fig 1.6); skipped exons (SE), alternative 5' splice site (A5SS), alternative 3' splice site (A3SS), mutually exclusive exons (MXE) and retained introns (RI) (Park *et al.* 2013). rMATs was utilised to align (Shen *et al.* 2014) the processed reads to the bovine reference genome UMB3.1 using STAR software (Dobin *et al.* 2013) and to identify differences in alternative splicing events between the control and high protein diet samples. rMATs was used to analyse the difference in the inclusion levels of an exon/intron between the diets, calculating when the P-value and false discovery rate (FDR) exceeded a threshold value with a FDR of <0.01 used as the cut off.

Due to an unexpected high degree of variation, one of the HP samples did not match or overlap with any other sample, this sample was excluded from the analysis. Therefore, the 2 controls (Sample numbers 11CP and 12CP) and 1 HP diet sample (Sample number 13HP) were analysed, with each control sample separately compared to the 1 HP diet sample. The results of these 2 separate analyses were compared to generate a list of genes that were alternatively spliced in both separate analyses. The list of "common" genes were organised by the type of alternative splicing observed.

3.2.7 Gene ontology and identification of genes of interest.

Common gene lists for each type of splicing event were analysed using WebGestalt 2013 (http://www.webgestalt.org/webgestalt_2013/option.php; Wang *et al.* (2013)) to identify the pathways that the commonly spliced genes

were involved in. A gene ontology (GO) enrichment analysis was run using the BH multiple-test adjustment as described by Benjamini & Hochberg, (1995) with the organism set as human, as bovine ontologies are not as robust. Orthologous genes were examined and $p<0.05$ set as the cut-off. This was organised by the following sub-roots; biological process (BP), molecular function (MF) or cellular component (CC). GO category name and GO ID were listed with the following values; number of genes in the reference category, expected number of genes in the category, ratio of enrichment, p-value and the p-value adjusted by the multiple test adjustment.

Additionally, using gene terms and Ensembl ID, genes in each splice variant list were manually searched for using Ensembl and NCBI gene using both the human and bovine genomes as reference. The function of each gene was manually recorded and genes of interest identified if involved in angiogenesis directly, mechanisms linked to angiogenesis or involved in steroidogenesis.

3.2.8 Functional analysis and identification of resultant genes.

The whole list of differentially spliced genes (n=127) was analysed for potential interactions with other proteins using GeneMANIA (http://genemania.org/; Warde-Farley *et al.* (2010)). GeneMANIA searches a gene list to establish the pathways, physical interactions, shared protein domains and genetic interactions that are shared. The analysis was performed with *Homo sapiens* selected as the reference database. GeneMANIA also suggests "resultant" genes that are involved in the same functions and pathways as those on the input list. The analysis generates a network with the input gene nodes centrally

located and surrounded by the resultant genes. Coloured-code lines between the nodes show the type of interaction between the genes with its thickness indicating the weight of the interaction. The co-expression, co-localisation and predicted data was omitted from the networks generated to allow greater network clarity. Based on gene ontology outputs from Section 3.2.7, additional geneMANIA analyses were performed to provide greater insight for the following GO terms:

- RNA binding (n=11 genes)
- Regulation of the cell cycle (n=14 genes)
- Protein phosphatase activity (n=5 genes)
- Immune system development (n=3 genes)
- Genes of interests that have been associated with angiogenesis (n=4 genes)

3.2.9 Sashimi plots and amino acid sequence analysis.

Sashimi plots were generated using the Integrative Genomics Viewer (IGV) version 2.3 (Robinson *et al.* 2011) to highlight the position of the key alternative splicing event. Alignment data from samples 11CP, 12CP and 13HP was uploaded and compared to the reference bovine genome UMD3.1. Genes of interest were then searched for and alignment data compared between samples. Depending upon the gene size, Sashimi plots for all 3 samples were generated, focusing upon the region of the major splicing event.

The information from the Sashimi plots was then utilised to predict the effect of splicing on the amino acid sequence encoded by the spliced region. The DNA sequence for each gene was searched for using Ensembl, using the UMD3.1

cow genome as reference. The exonic DNA sequence was used to generate the amino acid sequence encoded using EMBOSS Sixpack (Rice *et al.* 2000). The reference DNA sequence was then altered to reflect the observed splicing event and run through EMBOSS Sixpack. The original and spliced sequences for each gene were then compared to determine the possible effects of splicing on protein function.

3.3 Results.

3.3.1 Alternatively spliced genes in bovine granulosa cells.

Individual analysis of 11CP *vs.* 13HP and 12CP *vs.* 13HP showed that numerous genes underwent more than one type of alternative splicing, although this was not always significant. However, in the list of common genes that were spliced in both 11CP *vs.* 13HP and 12CP *vs.* 13HP, only 5 genes appeared in more than one alternative splicing group, with no gene undergoing more than 2 types of splicing. The 5 genes that underwent 2 different types of alternative splicing were PHF3 (SE; A3SS), IFT20 (MXE; SE), ENSBTAG00000025485 (A5SS; SE), TAF1A (SE; A3SS) and GEMIN6 (SE; A5SS). PHF3 contains motifs often observed in transcription factors (Fischer *et al.* 2001). IFT20 codes for a protein that is essential to the formation of cilia, is localized to the Golgi complex and several splice variants have been identified (Follit *et al.* 2006). ENSBTAG00000025485 is a novel gene, whilst TAF1A and GEMIN6 proteins form part of RNA polymerase transcription factors and small nuclear ribonucleoprotein assembly, respectively (Comai *et al.* 1994, Pellizzoni *et al.* 2002).

A list of genes that were commonly spliced in both 11CP *vs* 13HP and 12CP *vs* 13HP lists was generated (Table 3.2). The most common splicing event was SE splicing with 96 genes observed, 17 genes underwent A5SS splicing, 11 MXE splicing, whilst 3 genes were found in the A3SS and RI groups.

3.3.2 Analysis of known spliced genes in the bovine ovary.

To further interrogate the splicing analysis, genes associated with follicular function (e.g. LHCGR, FSHR and VEGFA) and known to undergo alternative splicing were manually searched for excluding any dietary influence. FSHR was shown to undergo SE, MXE, A5SS and A3SS splicing but was unaffected by diet. No LHCGR or VEGFA splicing events were observed.

Table 3.2 *Differentially spliced genes and their Ensembl IDs that were common to both 11CP vs 13HP and 12 CP vs. 13HP analyses (Novel genes are denoted by the Ensembl ID only).*

Splice type	Ensembl ID and Gene Symbol					
A5SS	ENSBTAG00000006529	*PDSS1*	ENSBTAG00000008065	*CDPF1*	ENSBTAG00000006245	*MORG1*
	ENSBTAG00000019219	*SH2B1*	ENSBTAG00000019971	*CDC16*	ENSBTAG00000004268	*TRAPPC5*
	ENSBTAG00000007779	*GTF3C3*	ENSBTAG00000027453	*RNF4*	ENSBTAG00000018067	*S100PBP*
	ENSBTAG00000007566	*CEP170*	ENSBTAG00000002663	*BCAS3*	ENSBTAG00000011571	*ACIN1*
	ENSBTAG00000006638	*BCL2L12*	ENSBTAG00000025485	---	ENSBTAG00000001506	*HTT*
	ENSBTAG00000015521	*GEMIN6*	ENSBTAG00000005213	*ZWILCH*		
MXE	ENSBTAG00000038190	*SMAP1*	ENSBTAG00000008755	*MARCH7*	ENSBTAG00000009547	*ZDHHC4*
	ENSBTAG00000032902	*SLC11A2*	ENSBTAG00000000808	*COX14*	ENSBTAG00000008695	*TCF3*
	ENSBTAG00000021943	*NFYC*	ENSBTAG00000015798	*UCKL1*	ENSBTAG00000004145	*ANO4*
	ENSBTAG00000008110	*IFT20*	ENSBTAG00000027321	*CCDC18*		
RI	ENSBTAG00000009694	*PUS10*	ENSBTAG00000013688	*PGF*	ENSBTAG00000004999	*KIAA1551*
SE	ENSBTAG00000047434	*TSNAX*	ENSBTAG00000012658	*TMA16*	ENSBTAG00000004873	*CCNL2*
	ENSBTAG00000005726	*HNRNPA2B1*	ENSBTAG00000009275	*MTRF1L*	ENSBTAG00000014598	*BPTF*
	ENSBTAG00000018000	---	ENSBTAG00000013483	*TAF1A*	ENSBTAG00000005753	*PARP6*
	ENSBTAG00000003788	*RCC1*	ENSBTAG00000019182	*TIA1*	ENSBTAG00000002515	*DNAAF3*
	ENSBTAG00000006025	*AHSA2*	ENSBTAG00000003340	*HDDC2*	ENSBTAG00000006070	*RANBP3*
	ENSBTAG00000035226	*TOR1AIP1*	ENSBTAG00000017550	*CSNK1G3*	ENSBTAG00000011425	*PTPRA*
	ENSBTAG00000008362	*CTDSP1*	ENSBTAG00000002279	*LUC7L3*	ENSBTAG00000008771	*MYEF2*
	ENSBTAG00000006071	*CTTN*	ENSBTAG00000000687	*WDR51B*	ENSBTAG00000007817	*BRCC3*
	ENSBTAG00000005092	*ROR2*	ENSBTAG00000003516	*TM2D2*	ENSBTAG00000002735	*C25H16ORF13*
	ENSBTAG00000003017	*TTC38*	ENSBTAG00000001352	*SDCCAG3*	ENSBTAG00000017123	*ZNF354B*
	ENSBTAG00000004722	*THUMPD2*	ENSBTAG00000014786	*PBRM1*	ENSBTAG00000008110	*IFT20*
	ENSBTAG00000019193	*SETD5*	ENSBTAG00000001164	*ACAD10*	ENSBTAG00000014458	*MROH1*
	ENSBTAG00000025485	---	ENSBTAG00000031439	*CDC14A*	ENSBTAG00000015611	*TBC1D9B*
	ENSBTAG00000015310	*SLC25A36*	ENSBTAG00000000869	*RNF219*	ENSBTAG00000011471	*CARS2*
	ENSBTAG00000003087	*DCUN1D5*	ENSBTAG00000019436	*SLC39A13*	ENSBTAG00000012077	*C26H10ORF6*
	ENSBTAG00000011017	*ABI2*	ENSBTAG00000014451	*YEATS2*	ENSBTAG00000016924	*CAP2*
	ENSBTAG00000015521	*GEMIN6*	ENSBTAG00000046514	*ASMTL*	ENSBTAG00000017543	*XRCC6BP1*
	ENSBTAG00000005745	*HPSE*	ENSBTAG00000002625	*MAP3K7*	ENSBTAG00000009109	*CASD1*
	ENSBTAG00000010210	*SUV39H2*	ENSBTAG00000008321	*RNF8*	ENSBTAG00000010743	*FASTKD1*
	ENSBTAG00000004495	*UBA5*	ENSBTAG00000019373	*CAPRIN2*	ENSBTAG00000019139	*PHF3*
	ENSBTAG00000010613	---	ENSBTAG00000030174	*ACOX1*	ENSBTAG00000015312	*LTBR*
	ENSBTAG00000038434	*ATRX*	ENSBTAG00000004517	*TARBP1*	ENSBTAG00000012501	*FTO*
	ENSBTAG00000032493	*FAM134C*	ENSBTAG00000012387	*PAM*	ENSBTAG00000020066	*ZW10*
	ENSBTAG00000020164	*BAZ1A*	ENSBTAG00000012955	*TRUB1*	ENSBTAG00000025788	*MPV17L2*
	ENSBTAG00000010105	*TMEM20*	ENSBTAG00000039117	*FAM179B*	ENSBTAG00000019496	*CDKN3*
	ENSBTAG00000010533	*HMGXB4*	ENSBTAG00000017750	*RHBDD3*	ENSBTAG00000019954	*ABHD2*
	ENSBTAG00000008510	*FBXO42*	ENSBTAG00000021164	*SLMAP*	ENSBTAG00000011401	*PDZD8*
	ENSBTAG00000011589	*CDC14B*	ENSBTAG00000013277	*CCDC22*	ENSBTAG00000010450	*WIZ*
	ENSBTAG00000016297	*CCNYL1*	ENSBTAG00000018671	*DCUN1D2*	ENSBTAG00000017999	*TNRC6A*
	ENSBTAG00000005221	*WNK1*	ENSBTAG00000019771	*PALM*	ENSBTAG00000031231	*IRF1*
	ENSBTAG00000020059	*GEN1*	ENSBTAG00000007476	*BTRC*	ENSBTAG00000030962	*EEFSEC*
	ENSBTAG00000014680	*ZBTB49*	ENSBTAG00000016895	*NIPA2*	ENSBTAG00000032521	*PLEKHH2*
A3SS	ENSBTAG00000017755	*EWSR1*	ENSBTAG00000019139	*PHF3*	ENSBTAG00000013483	*TAF1A*

3.3.3 Gene ontology.

The most signficant number of GO terms and those with the highest number of associated genes came from the A5SS and SE types, whilst no significant GO terms were observed following analysis of the RI and A3SS common gene lists (Table 3.3). The GO terms with the highest number of input genes per category were the transcription regulating ISWI complex (33.3%), and the related NURF complex (33.3%), acyl-CoA dehydrogenase activity (13.3%) and protein K11-linked ubiquitination (7.7%). The most highly significant GO terms were protein K11-linked ubiquitination (p=0.0155) and regulation of cell cycle process (p=0.0155). Interestingly, a large number of GO terms linked to the cell cycle and mitosis, and organelle fission were observed. These findings could indicate that the HP diet effected the splicing (and hence function) of genes involved in the regulation of the cell cycle and mitosis of granulosa cells. A surprising finding was that in the MXE splicing category, GO terms related to immune system development, haemopoiesis, haematopoietic or lymphoid organ development and 2 categories relating to erythrocytes were observed.

Table 3.3 *Gene ontology (GO) analysis of the common gene lists for each type of alternative splicing.*

Splice type	Gene ontology term	Category	Set size	Input genes in set (%)	Adjusted P-value
A5SS	GO:0070979 protein K11-linked ubiquitination	BP	26	2 (7.69%)	0.0155
	GO:0010564 regulation of cell cycle process	BP	430	4 (0.93%)	0.0155
	GO:0022402 cell cycle process	BP	1062	5 (0.47%)	0.0232
	GO:0000279 M phase	BP	539	4 (0.74%)	0.0232
	GO:0007049 cell cycle	BP	1393	5 (0.36%)	0.0255
	GO:0051726 regulation of cell cycle	BP	734	4 (0.54%)	0.0255
	GO:0000278 mitotic cell cycle	BP	780	4 (0.51%)	0.0255
	GO:0048285 organelle fission	BP	392	3 (0.77%)	0.0255
	GO:0007067 mitosis	BP	368	3 (0.82%)	0.0255
	GO:0006996 organelle organization	BP	2113	6 (0.28%)	0.0255
	GO:0043226 organelle	CC	10651	13 (0.12%)	0.0256
	GO:0016604 nuclear body	CC	274	3 (1.09%)	0.0256
	GO:0043229 intracellular organelle	CC	10636	13 (0.12%)	0.0256
	GO:0044451 nucleoplasm part	CC	820	4 (0.49%)	0.0256
	GO:0005654 nucleoplasm	CC	1478	5 (0.34%)	0.0266
	GO:0005829 cytosol	CC	2372	6 (0.25%)	0.0323
	GO:0032991 macromolecular complex	CC	3864	7 (0.18%)	0.0482
	GO:0043233 organelle lumen	CC	3340	6 (0.18%)	0.0482
	GO:0044424 intracellular part	CC	12237	13 (0.11%)	0.0482
	GO:0005813 centrosome	CC	376	2 (0.53%)	0.0482
MXE	GO:0030097 hemopoiesis	BP	498	3 (0.60%)	0.0464
	GO:0002520 immune system development	BP	564	3 (0.53%)	0.0464
	GO:0034101 erythrocyte homeostasis	BP	94	2 (2.13%)	0.0464
	GO:0030218 erythrocyte differentiation	BP	87	2 (2.30%)	0.0464
	GO:0048534 hematopoietic or lymphoid organ development	BP	532	3 (0.56%)	0.0464
SE	GO:0006338 chromatin remodelling	BP	97	5 (5.15%)	0.0389
	GO:0004722 protein serine/threonine phosphatase activity	MF	48	3 (6.24%)	0.0438
	GO:0008138 protein tyrosine/serine/threonine phosphatase activity	MF	45	3 (6.67%)	0.0438
	GO:0004721 phosphoprotein phosphatase activity	MF	173	5 (2.89%)	0.0438
	GO:0004725 protein tyrosine phosphatase activity	MF	103	4 (3.88%)	0.0438
	GO:0003995 acyl-CoA dehydrogenase activity	MF	15	2 (13.33%)	0.0494
	GO:0003723 RNA binding	MF	854	11 (1.29%)	0.0498
	GO:0031010 ISWI complex	CC	6	2 (33.33%)	0.0138
	GO:0016589 NURF complex	CC	6	2 (33.33%)	0.0138

BP=Biological process CC=Cellular component MF=Molecular function

3.3.4 Functional analysis and identification of resultant genes.

The geneMANIA network for all the genes that were differentially alternatively spliced are presented in Fig 3.1. The majority of the interactions were physical (83%) but there were also genetic (7.8%), shared protein domains (6.2%) and pathway (3.4%) interactions. The gene nodes with numerous interactions were HTT, ZW10 and MAP3K7. The five most highly weighted resultant genes were KNTC1, TSN, CANX, TRPT1 and GEMIN7.

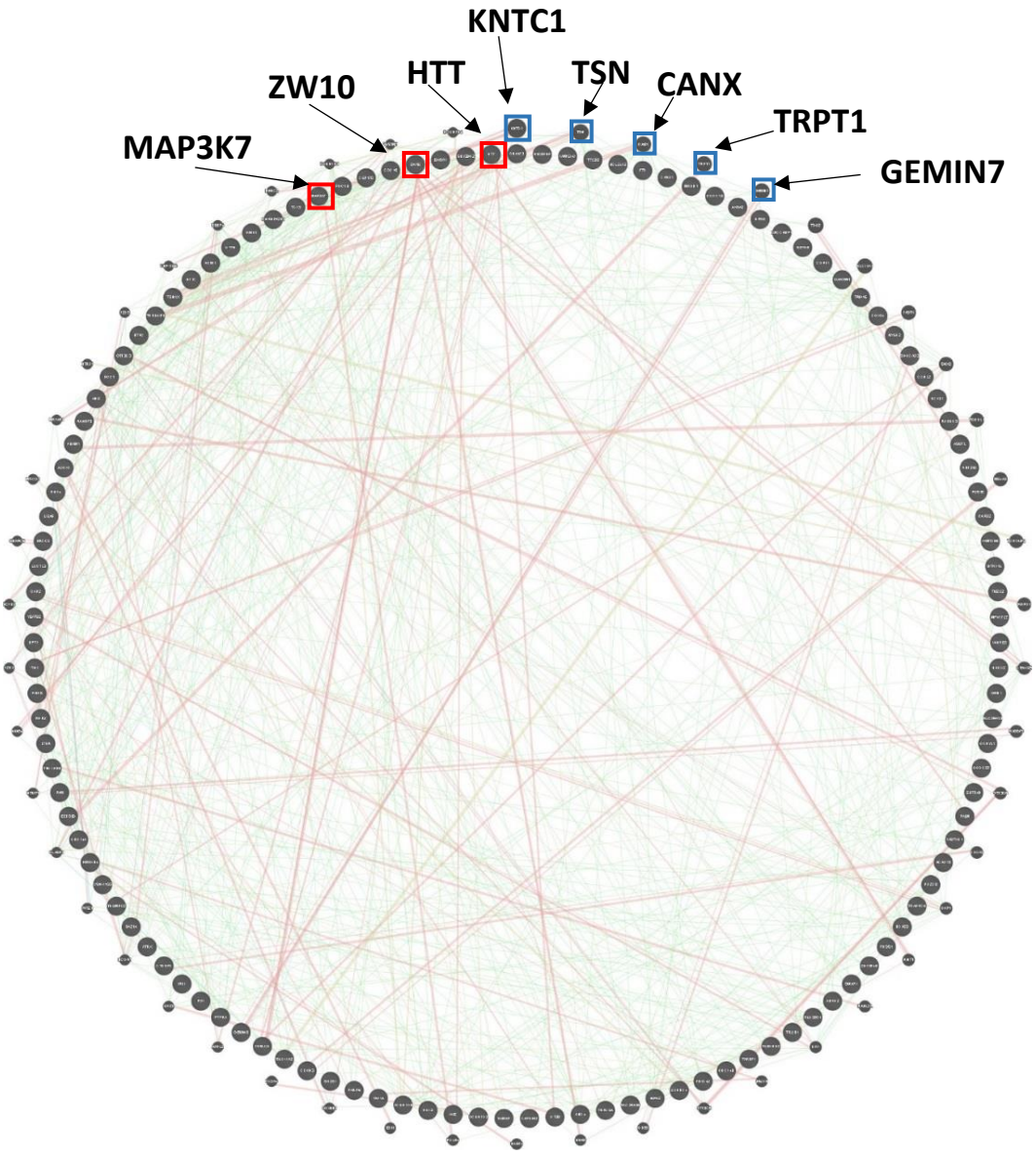


Fig 3.1 *GeneMANIA network analysis for all the differentially spliced genes*. Input genes are located centrally with resultant genes forming the outer circle (limited to 50). Different interactions are shown as follows: physical interactions (red line), pathways (blue line), genetic interactions (light green line), shared protein domains (dark green line). The line thickness relates to its weighting. Red boxes=key nodes. Blue boxes=most heavily weighted resultant genes.

3.3.5 Functional analysis and identification of resultant genes [selected GO terms].

In the RNA binding gene list (n=11), the majority of the interactions were shared protein domains (79%), with the remaining consisting of genetic interactions (21%) (Fig 3.2A). The majority of these interactions were between the input genes and their resultant genes. However, there was a genetic interaction between the input genes TARBP1, HNRNPA2B1 and EEFSEC. In the resultant genes, two genes encoded proteins with methyltransferase activity, namely RNMTL1 and MRM1. In the regulation of cell cycle gene list (n=14), the majority of the interactions were physical (90%), with shared protein domains (9%) and pathway (1.6%) interactions (Fig 3.2B) also detected. The genes ZW10, CDC16 and CDKN3 had multiple interactions with other input and resultant genes.

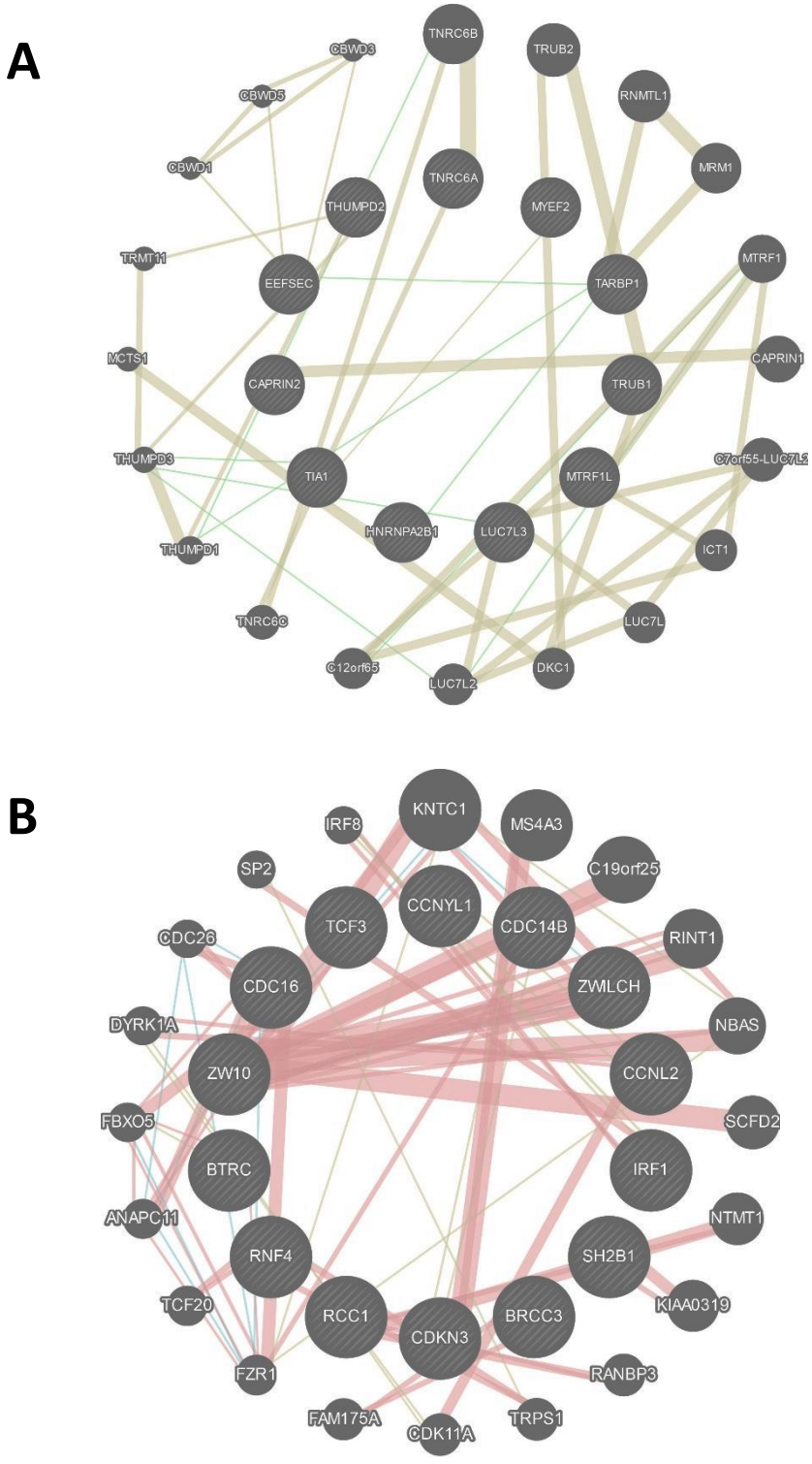


Fig 3.2 *GeneMANIA network analysis for differentially spliced genes in the (A) RNA binding and (B) regulation of cell cycle GO terms*. Input genes are located centrally (dashed grey circles) with resultant genes forming the outer circle (limited to 20). Different interactions are shown as follows: physical interactions (red line), pathways (blue line), genetic interactions (light green line), shared protein domains (dark green line). The line thickness relates to its weighting.

In the protein phosphatase activity category gene list (n=5), the vast majority of the interactions were physical (92%), with the remaining consisting of pathway (6.9%), genetic (2.4%) and shared protein domains (1.1%) interactions (Fig 3.3A). There appeared to be two principal networks, one surrounding CTD small phosphatase 1 (CTDSP1) and the other incorporating protein tyrosine phosphatase receptor type A (PTPRA), cyclin-dependent kinase inhibitor 3 (CDKN3), cell division cyclin 14A (CDC14A) and CDC14B genes. In the resultant genes, there were a number that are known regulators of granulosa cell function such as insulin receptor (INSR), members of SMAD family, cyclin-dependent kinase (CDK) members and androgen receptor (AR). In the network for the immune system development (n=3) gene list, most of the interactions were physical (90%) with transcription factor 3 (TCF3) forming a central node (Fig 3.3B). Interestingly, inhibitor of DNA binding 1 (ID1) and ID2 physically interact with TCF3 and are regulated by members of the TGFB superfmaily (Hogg *et al.* 2010).

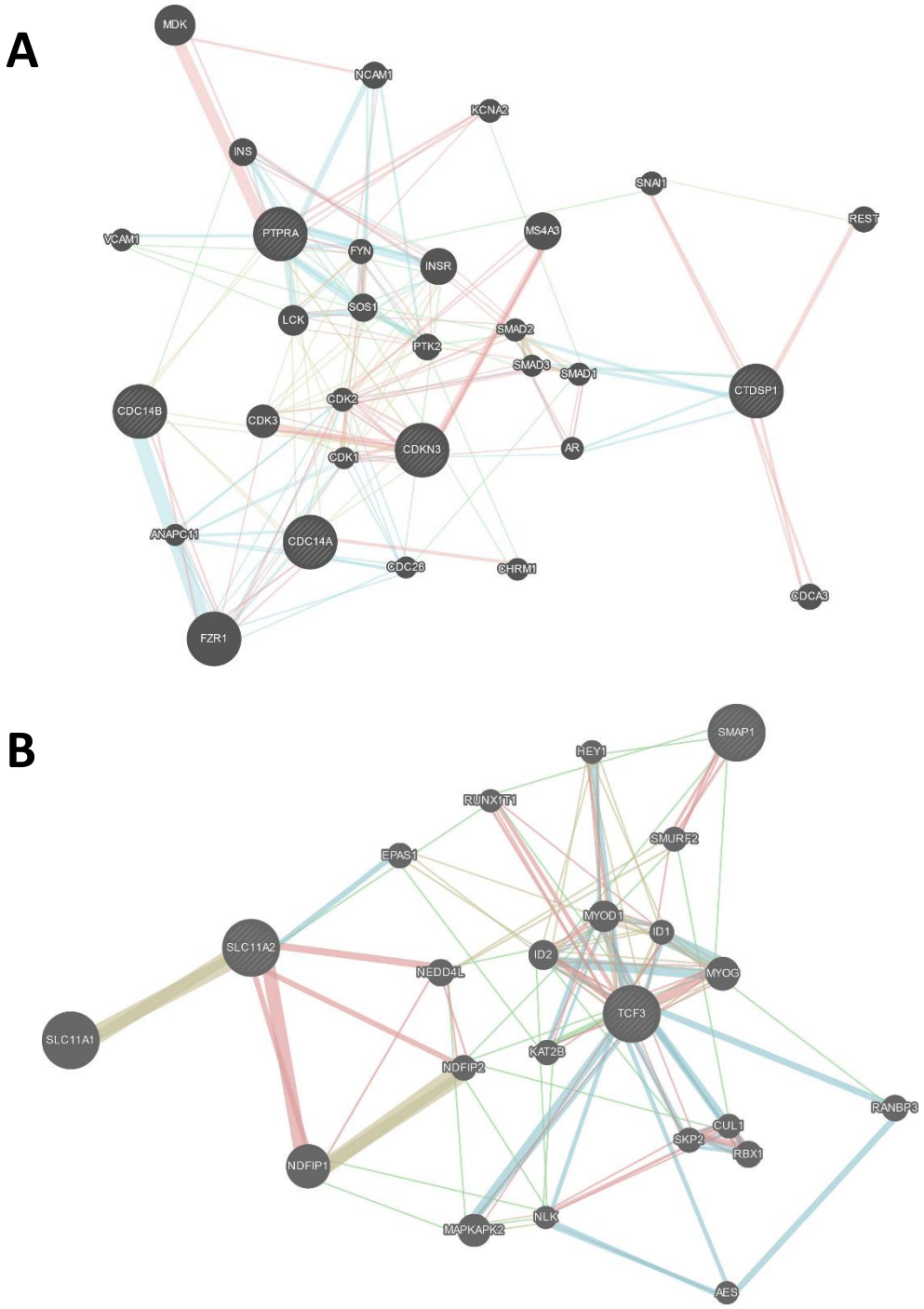


Fig 3.3 *GeneMANIA network analysis for differentially spliced genes in the (A) protein phosphatase and (B) immune system development GO terms.* Input genes are located within nodes (dashed grey circles) with resultant genes (solid grey circles) surrounding them. Different interactions are shown as follows: physical interactions (red line), pathways (blue line), genetic interactions (light green line), shared protein domains (dark green line). The majority of the interactions were "shared protein domains" (bright green) in (A), while physical interactions were predominant in (B). The line thickness relates to its weighting.

3.3.6 Further analysis into genes of interest.

Following these investigations, four genes (BCAS3, HPSE, IRF1, and PGF) were identified as of particular interest due to their association with angiogenesis. GeneMANIA network analysis revealed numerous physical interactions (90%) but that the four gene nodes were relatively independent. It was evident that PGF can physically interact with several other key angiogenic proteins including FLT1, NRP1, NRP2 and VEGFA.

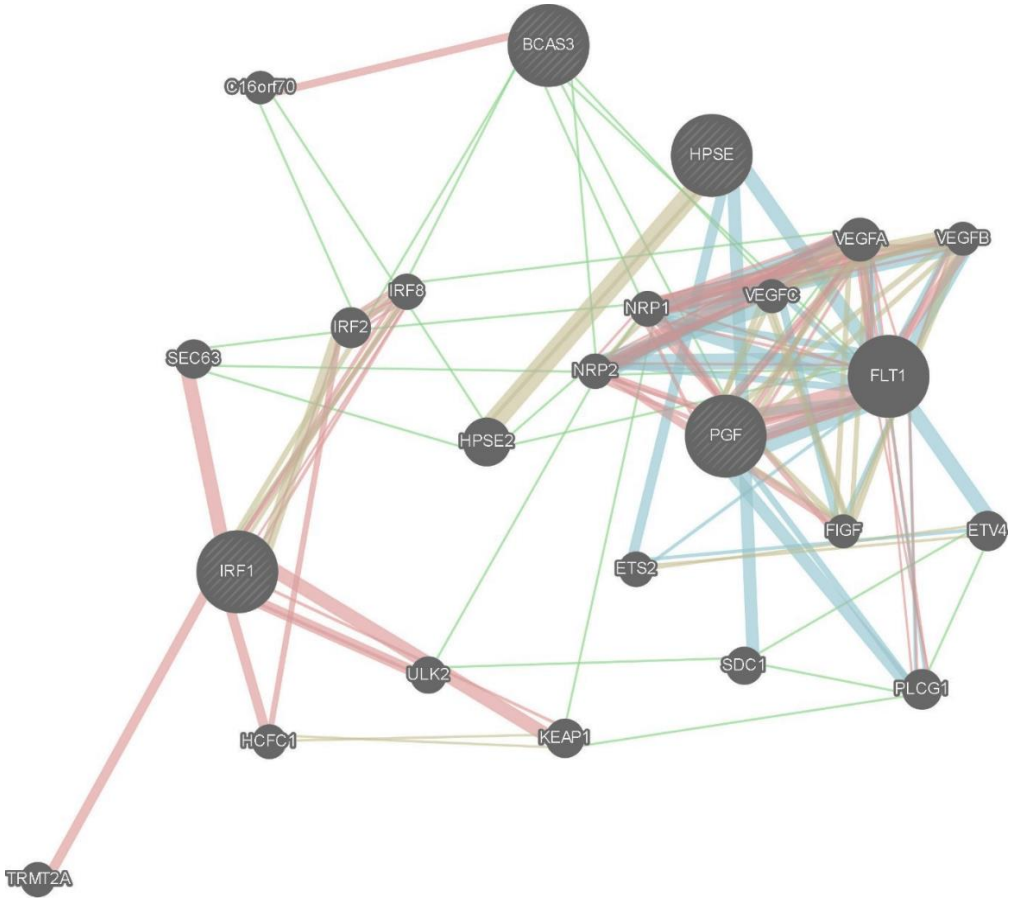


Fig 3.4 *GeneMANIA network analysis for four genes of interest: IRF1, HPSE, BCAS3 and PGF*. Input genes are located within nodes (dashed grey circles) with resultant genes (solid grey circles) surrounding them. Different interactions are shown as follows: physical interactions (red line), pathways (blue line), genetic interactions (light green line), shared protein domains (dark green line). The line thickness relates to its weighting.

3.3.7 GO analysis for the genes of interest geneMANIA network.

GO analysis revealed that the GO term with the smallest adjusted P-value was VEGFR signalling. There were a number of GO terms relating to the stimulation of endothelial cell function. Additional GO terms affected included PDGFR signalling pathway and leukocyte chemotaxis (Table 3.4)

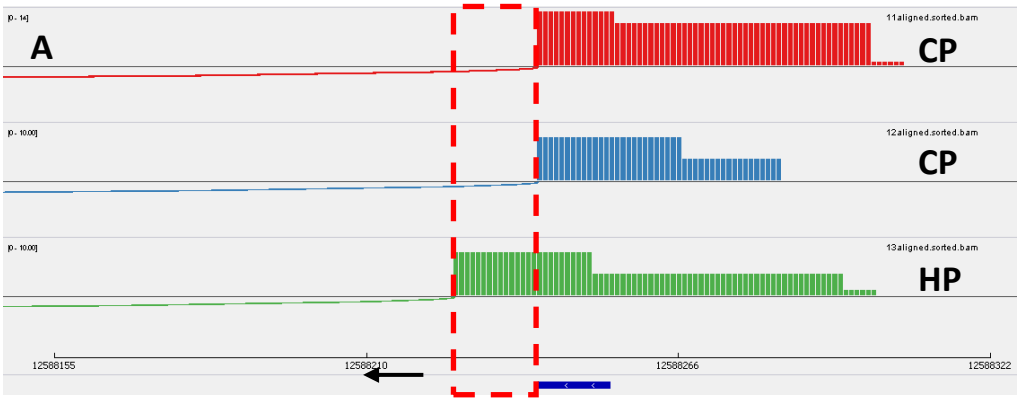
Table 3.4 *The top 10 GO terms from the geneMANIA network analysis of the genes of interest.*

Gene ontology term	Category	Set size	Genes in network (%)	Adjusted P-value
GO:0048010 vascular endothelial growth factor receptor signalling pathway	BP	37	8 (21.6)	<0.0001
GO:0030335 positive regulation of cell migration	BP	198	10 (5.1)	<0.0001
GO:0005161 platelet-derived growth factor receptor signalling pathway	BP	12	5 (41.7)	<0.0001
GO:0031093 platelet alpha granule lumen	CC	48	6 (12.5)	<0.0001
GO:0001938 positive regulation of endothelial cell proliferation	BP	45	5 (11.1)	<0.0001
GO:0030595 leukocyte chemotaxis	BP	106	6 (5.7)	<0.0001
GO:0010595 positive regulation of endothelial cell migration	BP	47	5 (10.6)	<0.0001
GO:0032103 positive regulation of response to external stimulus	BP	130	6 (4.6)	<0.0001
GO:0010634 positive regulation of epithelial cell migration	BP	63	5 (7.9)	<0.0001
GO:0060333 interferon gamma mediated signalling pathway	BP	73	5 (6.8)	<0.0001

3.3.8 Sashimi plots and amino acid sequences for genes of interest.

3.3.8.1 BCAS3.

The high protein diet altered the A5SS splicing of the BCAS3 gene. The two control samples showed identical splicing locations for exon 1 of the BCAS3 gene whilst the high protein sample underwent splicing at an alternate 5' location, downstream of the control samples splice site (Fig 3.5A). A5SS splicing inserted a short 18 nucleotide sequence after the end of exon 1, this was located in the 5' UTR (Fig 3.5B).



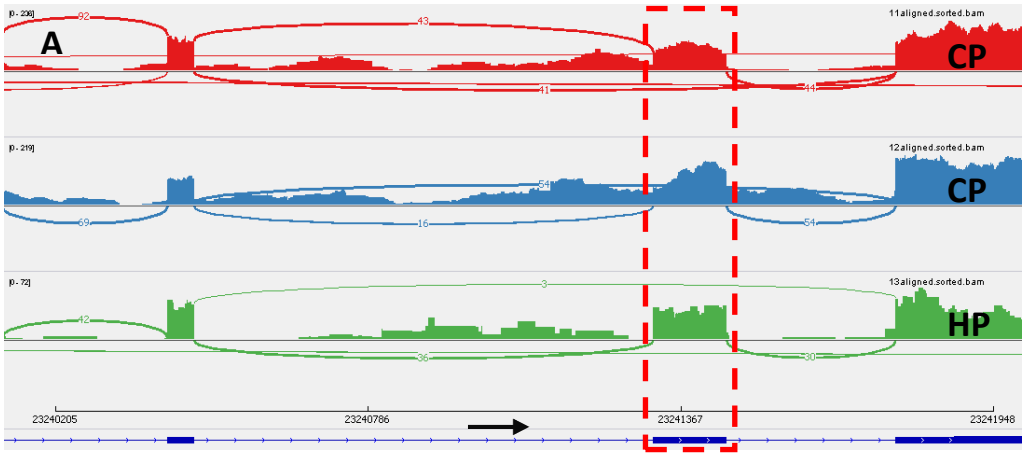
B

Effect on BCAS3 mRNA sequence:	
Original (CP)	GGCGGGGCGGCCG
Spliced (HP)	GGCGGGGCGGCCGGTGAGAGGGCTGACGGTG

Fig 3.5 *Sashimi plot and mRNA sequence showing A5SS splicing of BCAS3.* Exon alignment read densities are shown as vertical coloured bars, with each sample colour coded (Red = 11CP, Blue = 12CP and green = 13HP). Exons are shown as horizontal blue bars along the bottom of the plot, which correspond to the exon location in the bovine reference genome, arrow shows exon direction. Splice junction reads are drawn as arcs between exons, the arc width and number displayed show the number of reads that align to the splice site. (A) A5SS splicing of exon 1 in the BCAS3 gene is evident in the high protein sample. Red box=splicing event location. (B) A short 18 nucleotide sequence was inserted following splicing.

3.3.8.2 IRF1.

The HP diet effected the splicing of IRF1, the exon skipped was exon 9, which was frequently skipped in the control samples, whilst exon 9 was included more often, with very few splice junction reads in the HP sample (Fig 3.6A). The HP sample showed significantly fewer skipped exon splicing events (3 events) compared to the controls (41 and 54 events). Skipping of exon 9 resulted in formation of a premature stop codon in control mRNA, this formed a truncated amino acid (aa) sequence (Fig 3.6B), which was considerably shorter (76aa). The N-terminal is associated with IRF transcription factor and DNA binding activity, but the function of the C-terminal is unknown.



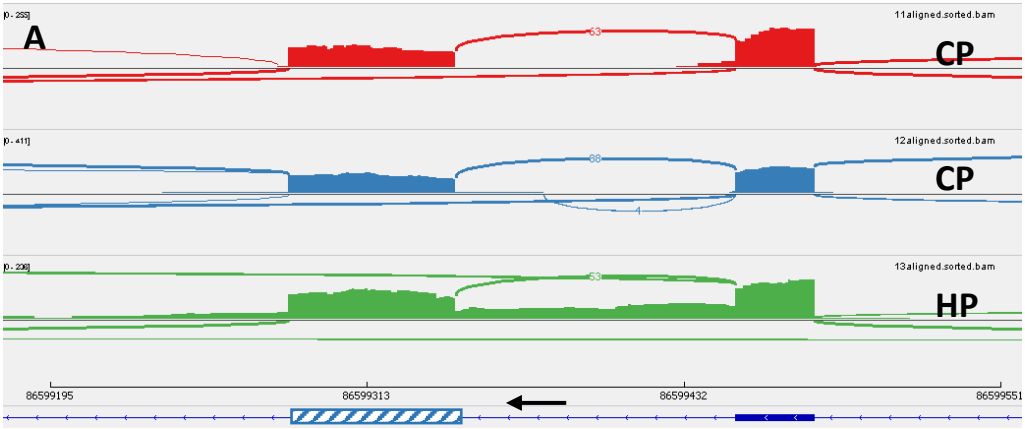
B

Effect on IRF1 amino acid sequence:	
Original (HP)	...EEGKLTEDIMKLLEQTG... (322aa)
Spliced (CP)	...EEGKLTEDIMKVILA*STOP* (244aa)

Fig.3.6 *Sashimi plot and amino acid sequence showing a skipped exon in the IRF1 gene*. Exon alignment read densities are shown as vertical coloured bars, with each sample colour coded (Red = 11CP, Blue = 12CP and green = 13HP). Exons are shown as horizontal blue bars along the bottom of the plot, which correspond to the exon location in the bovine reference genome, arrow shows exon direction. Splice junction reads are drawn as arcs between exons, the arc width and number displayed show the number of reads that align to the splice site. (A) Exon 9 was skipped in controls, but not the high protein sample. Red box=skipped exon. (B) Formation of a premature stop codon in controls.

3.3.8.3 PGF.

The HP diet resulted in the retention of a portion of an intron, which is removed in control samples. The intronic portion retained is located between exons 5 and 6 according to the reference bovine genome (Fig 3.7A). The retained intron results in the formation of a premature stop codon shortly after exon 5, causing a truncated amino acid sequence by 8aa (Fig 3.7B).



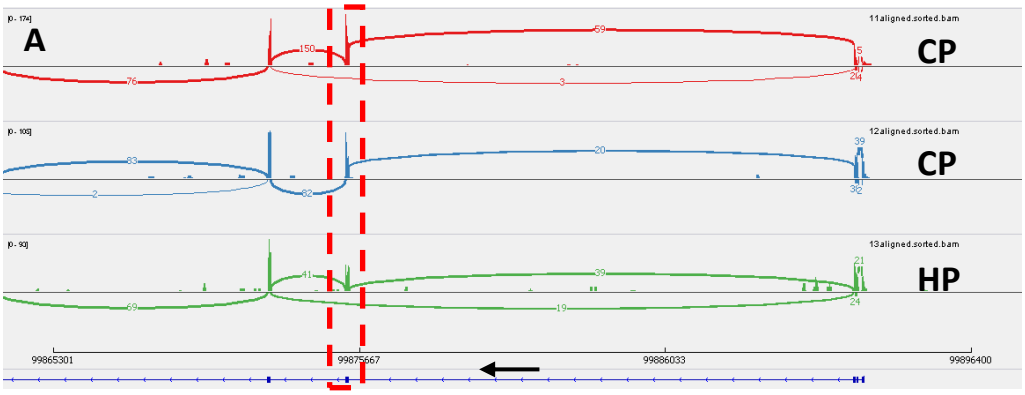
B

Effect on PGF amino acid sequence:	
Original (CP)	...WEKMKQTRCGDTISQR... (149aa)
Spliced (HP)	...WEKMKQTR*STOP* (141aa)

Fig 3.7 *Sashimi plot and amino acid sequence showing a retained intron in the PGF gene.* Exon alignment read densities are shown as vertical coloured bars, with each sample colour coded (Red = 11CP, Blue = 12CP and green = 13HP). Exons are shown as horizontal blue bars along the bottom of the plot, which correspond to the exon location in the bovine reference genome, arrow shows exon direction. In this instance the reference genome is seemingly incorrect so a blue bar denoting an exon is missing. Splice junction reads are drawn as arcs between exons, the arc width and number displayed show the number of reads that align to the splice site. (A) A portion of the intron between exons 5 and 6 is retained in the HP sample. (B) RI splicing results in insertion of a premature stop codon in the HP sample. Striped blue bar=location of putative exon 6.

3.3.8.4 HPSE.

In the HP diet sample, exon 4 of the HPSE gene was frequently skipped, in comparison to the controls where this exon was usually read. This is evident in the Sashimi plot in (Fig 3.8A) where a thick arc is present that runs between exons 3 and 5 in the HP sample, whilst in the controls this arc is much thinner or absent. Skipping of exon 4 results in the formation of a premature stop codon in the high protein sample, causing a truncated amino acid sequence by 458aa (Fig 3.8B). This would result in the exclusion of the glycoside hydrolase catalytic domain (161-372aa).



B

Effect on HPSE amino acid sequence:	
Original (CP)	...ATDPRFFTFLGSSKLRTLARG... (545aa)
Spliced (HP)	...ATDPRFFTFLGYLQIRIHPF*STOP* (87aa)

Fig 3.8 *Sashimi plot and amino acid sequence showing a skipped exon in the HPSE gene.* Exon alignment read densities are shown as vertical coloured bars, with each sample colour coded (Red = 11CP, Blue = 12CP and green = 13HP). Exons are shown as horizontal blue bars along the bottom of the plot, which correspond to the exon location in the bovine reference genome, arrow shows exon direction. Splice junction reads are drawn as arcs between exons, the arc width and number displayed show the number of reads that align to the splice site. (A) Exon 4 was skipped in the HP diet sample, but not the controls, red box=skipped exon. (B) Formation of a premature stop codon due to the skipped exon.

3.4 Discussion.

Alternatively spliced genes in bovine granulosa cells.

The discovery that a high protein diet influences the alternative splicing of genes expressed by bovine granulosa cells builds upon the findings of Edwards (2016) who observed from a different bioinformatic approach on the same data set that a high protein diet altered the expression of 26 genes including STAR and fibronectin.

The finding that SE splicing was the most common splicing event in bovine granulosa cells is in agreement with Sugnet et al. (2004) who observed that this splicing type accounts for 40% of alternative splicing events in mice and humans. Whilst this splicing produces a shorter amino acid chain, the overall effect of skipping an exon on a proteins' function depends upon the function of the missing exon or impact on overall tertiary structure. However, this shows that a high protein diet affected SE splicing in a large number of genes in bovine granulosa cells by either increasing or reducing the number of SE events. The finding that 5 genes in the common gene list underwent 2 types of alternative splicing and that numerous genes under went multiple types of splicing was unsurprising since several alternative splicing events can occur simulatenously in the same gene. Furthermore, this accounts for the fact that many proteins exist as multiple isoforms (Human Genome Sequencing 2004, Chen *et al.* 2012, Roy *et al.* 2013).

Analysis of genes known to be spliced in the bovine ovary.

The current study showed that the FSHR gene underwent SE, MXE, A5SS and A3SS splicing in the granulosa cells of medium follicles, although this was not influenced by diet. It is supported by Rajapaksha et al. (1996) who demonstrated that FSHR undergoes alternative splicing in luteinising bovine granulosa cells. Whilst FSHR splicing has been suggested to play a role in the loss of FSH sensivity during luteal formation, it is unclear what role of FSHR splicing has in medium sized follicles (Rajapaksha *et al.* 1996). In rats a reduction in FSH binding to granulosa cells resulted in follicle atresia (Peluso & Steger 1978). Based on this, the FSHR splicing observed in bovine granulosa cells could be a mechanism to fine-regulate granulosa cell growth. Alternatively, it might be involved in regulating follicle atresia as a consequence of reduced FSH binding or signalling. For example, if FSHR splicing resulted in the generation of non-functional FSHR isoforms then FSH signalling would be reduced and could result in follicle atresia. Further analysis of the FSHR splice variants is required to determine the possible effect of each type of alternative splicing on FSHR function, as well as exact quantification of the splice variant expression by qPCR in the granulosa cells of follicles of different stages and sizes.

The non-detection of LHCGR splicing was expected since expression of LHCGR mRNA is absent from granulosa cells until they achieve dominance (Xu *et al.* 1995). However, the non-detection of VEGFA splicing was surpising as VEGFA isoform expression is documented in bovine granulosa and luteal cells (Shimizu & Miyamoto 2007, Rosales-Torres *et al.* 2010, Guzmán *et al.* 2015). It is

essential that qPCR using isoform-specific primers is performed to confirm this. A possible explanation for the absence of VEGFA in this study is that the granulosa cell layer is avascular, therefore VEGFA expression is likely to be minimal in this region. Furthermore, the absence of VEGFA may be important in providing a gradient for EC migration in later stages of follicular development.

Gene ontology.

Intepretation of the GO analysis shows that the most statistically significant terms and the best represented terms were associated with cell division. Protein K11-linked ubiquitination and regulation of cell cycle process were the terms with the lowest P-values. Suggesting that a high protein diet affects the A5SS splicing of genes involved in regulating processes within the cell cycle, with protein ubiquitination identified as an essential cell cycle regulatory process (Wickliffe *et al.* 2011). Other significant GO terms of particular note in the A5SS and SE groups were mitosis, organelle fission, organelle organization and chromatin remodelling, all of which are processes that are key to the cell cycle and cellular division. The GO terms with the greatest number of input genes were ISWI complex, NURF complex and acyl-CoA dehydrogenase activity, all of which were in the SE group and linked to the cell cycle. NURF is a protein complex that contains ISWI which is involved chromatin transcription (Badenhorst *et al.* 2002). Whilst the term acyl-CoA dehydrogenase activity is associated with metabolism, where acyl-CoA dehydrogenase has a key role in the β-oxidation of fatty acids, which is a significant source of energy (Sherratt 1988, Stankovich *et al.* 1999). Together these observations suggest that a high

protein diet effects the splicing of genes involved in the energy intensive process of cell division. Further investigations to determine the functional effect of the splicing of genes involved in these processes is crucial as granulosa cells in medium follicles undergo rapid growth, therefore any alternative splicing events that effect genes involved in this process could limit follicle development.

The GO terms of the MXE group show that the genes are involved in an altogether different set of processes with GO terms relating to erythrocytes, immune system development and hematopoiesis observed. These findings were related to the same 3 genes and were unexpected. The importance of immune regulation in granulosa cells is an area that has limited researched. Interestingly, haemoglobin gene and protein expression has been documented in both mouse and human granulosa cells, with expression in mice influenced by hormone administration (Kind *et al.* 2013, Brown *et al.* 2015). Whilst the role of haemoglobin in granulosa cells requires further investigation, the findings of Brown et al. (2015) may explain why GO terms relating to hemopoiesis and erythrocyte homeostasis were observed.

Functional analysis and identification of resultant genes.

The network shows that the majority of the spliced and resultant genes are involved in similar processes, with most of the proteins encoded by the genes physically interacting with several other proteins. However, given the low percentage of pathway interactions the genes are likely involved in similar or closely related processes, rather then being part of the same pathway.

Of the nodes with the greatest number of interactions, ZW10 and HTT are involved in mitotic spindle orientation and the mitotic spindle-assembly checkpoint, respectively (Basto *et al.* 2000, Godin *et al.* 2010). MAP3K7 is a protein kinsase involved in regulating the effect of TGFB and is important in the response of cells to environmental stress (Shirakabe *et al.* 1997, Edlund *et al.* 2003). The finding that MAP3K7 splicing is influenced by a high protein diet is intriguing and would suggest that granulosa cells are responding to the diet as an environmental stress. Furthermore, TGFB inhibited ovarian angiogenesis and steroidogenesis, therefore splicing of MAP3K7 could affect ovarian function (Thompson 2014). The highest weighted resultant genes are involved in cell division, DNA binding, protein folding and splicing. This demonstrates that the high protein diet could have widespread effects on granulosa cell function.

RNA binding

The interactions in the RNA binding gene list were predominantly shared protein domains. This shows that the high protein diet influences the splicing of genes that share a similar structure and function to several resulant genes involved in RNA binding. Demonstrating that the spliced and resultant genes encode proteins with similar RNA binding capabilities.

Furthemore, the presence of genetic interactions between three input genes shows that the high protein diet was influencing the splicing of several closely related genes, with splicing of one of these genes having the potential to effect the function of the others. TARBP1, HNRNPA2B1 and EEFSEC are involved in RNA methylation, RNA splicing and selenoprotein translation, respectively

(Fagegaltier *et al.* 2000, Wu *et al.* 2008, Alarcon *et al.* 2015). Splicing of these genetically linked genes as a result of the high protein diet could have widespread effects on protein production in granulosa cells. Similarly, the two closely related resultant genes RNMTL1 and MRM1 play a role in the modification of the mitochondrial ribosome, therefore their interaction with spliced genes could effect mitochondrial function in granulosa cells (Lee & Bogenhagen 2014, Rorbach *et al.* 2014).

Regulation of cell cycle.

The most prevalent type of interaction between the regulation of cell cycle genes was physical, suggesting that they are involved in similar or closely linked processes. Of the genes with multiple interactions, ZW10 encodes a mitotic checkpoint protein (Chan *et al.* 2000). CDC16 encodes a ubiquitin ligase which forms part of the anaphase promoting complex/cyclosome which governs the transition from metaphase to anaphase (Tugendreich *et al.* 1995). While, CDKN3 is a tumour suppressor which regulates the G1 to S phase transition (Nalepa *et al.* 2013). Many of the spliced genes encode proteins which physically interact with numerous other resultant genes linked to the cell cycle, therefore the diet-induced changes in splicing of a few genes has the potential to affect numerous cell cycle processes. This in turn could adversely impact upon granulosa cell growth and differentiation, and ultimately impair follicular development.

Protein phosphatase activity.

CTDSP1 forms one of the two principal networks observed in the protein phosphatase activity GO term and encodes a protein that downregulates RNA polymerase II transcription (Yeo *et al.* 2003). Furthermore, CTDSP1 is involved in the regulation of SMAD2/3 signalling, and is shown to physically interact with several of the SMAD family resultant genes in the network. The SMAD2/3 pathway is activated by TGFB and plays a key role in numerous ovarian processes which are vital to fertility (Sapkota *et al.* 2006, Li *et al.* 2008). Therefore splicing of CTDSP1 may severely impact upon granulosa cell function and subsequent fertility.

The other principal network is composed of input genes mainly associated with the cell cycle, further demonstrating that the high protein diet may affect granulosa cell growth by splicing genes involved in cell division. Interestingly, several of the resultant genes are important regulators of granulosa cell function, which further supports the notion that the high protein diet may have a major effect on granulosa cell growth, function and hence fertility by causing alternative splicing of key regulatory genes.

Immune system development.

TCF3 was a central node, connected to resultant genes by predominantly physical interactions. TCF3 undergoes alternative splicing and encodes a member of the E protein transcription factor family that is fundamental for lymphopoesis (e.g. it regulates B and T cell development) (Bain *et al.* 1994). The physical interaction of TCF3 with ID1, ID2 and indirectly with TGFB superfamily

members is particularly interesting. Thus there are a large number of cellular processes that splicing of TCF3 could effect through it's link to TGFB. However, the importance of TCF3 splicing and its influence on lymphopoesis in relation to granulosa cell function remains unclear.

Genes of interest GeneMANIA network analysis.

The majority of the resultant genes were observed to be indirectly involved in angiogenesis. This is likely due to the low number of genes in this group and the presence of PGF, which had numerous interactions. PGF is a pro-angiogenic factor that functions predominantly through interaction with VEGFA and VEGFRs (Carmeliet *et al.* 2001). Given the low number of genes in the network, it is important that the findings of this analysis are not over interpreted.

Notably, variants of VEGFA and NRP were present as resultant genes, both of which are involved in the regulation of angiogenesis as a factor and receptor, respectively (Ferrara *et al.* 2003, Herzog *et al.* 2011). FLT1 had the largest node of any of the suggested new genes, it encodes a VEGFR and is fundamental in the regulation of angiogenesis (Shibuya 2001b, Herzog *et al.* 2011). Consequently, it is linked to multiple genes in the network. Given the pro-angiogenic effects and the large number of resultant genes it interacts with, splicing of PGF as a result of the high protein diet could have potentially widespread effects on ovarian angiogenesis and function.

GO analysis of genes of interest.

The most notable results observed in the GO analysis were the terms VEGFR signalling pathway, PDGFR signalling pathway, positive regulation of

endothelial cell proliferation, positive regulation of EC migration and response to external stimulus. These findings suggest that the splicing of these genes of interest due to the high protein diet is likely to have a consequence upon subsequent follicular angiogenesis. However, given that VEGFA and VEGFR splicing were not observed in this investigation it is difficult to infer the exact effect that splicing is having. This would require further characterisation of the effect of splicing on each gene of interest, determining the most prevalent isoforms of each gene through qPCR and further investigation into the importance of each gene in ovarian angiogenesis.

Genes of interest Sashimi plot and amino acid sequence interpretation.

BCAS3.

Microtubule associated cell migration factor, BCAS3, underwent A5SS alternative splicing. This resulted in an alteration in the length of the first exon, with the high protein diet sample having a longer exon in comparison to the control samples. In this instance A5SS splicing occurred in an exon that forms part of the 5' UTR.

The function of BCAS3 in the cow has not been investigated, however in humans it has a role in angiogenesis, specifically EC migration (Jain *et al.* 2012). Additionally, BCAS3 is activated by oestrogen, as well as acting as a co-activator of oestrogen receptor alpha (Gururaj *et al.* 2007). Further investigations are required to determine if changes to the 5' UTR result in alterations in the BCAS3 protein formation and function.

Interferon regulatory factor 1 (IRF1).

Interestingly, IRF1 was the only gene that was in the commonly spliced gene list and also present in the list of genes significantly altered by the high protein diet (Edwards 2016). In this case, IRF1 expression was reduced by the high protein diet. Alternative splicing analysis of IRF1 gene showed that exon 9 was frequently skipped in control samples, while in the high protein diet sample exon 9 was often included. Further analysis showed that skipping exon 9 inserted a premature stop codon. A premature stop codon would result in the formation of a truncated protein. Interestingly this occurred in the control samples. While in the HP sample, exon 9 was commonly retained producing a longer peptide sequence. These findings are supported by Lee et al. (2006), who observed in cancerous human cervical tissue that skipping of exon 9 in the IRF1 gene resulted in the formation of a truncated IRF1 protein. This study also showed that SE splice variants were more highly expressed in cancerous than normal tissue, with the variants capable of reducing the transcriptional activity of full length IRF1. Full length IRF1 inhibited VEGFA mediated angiogenesis through interaction with KDR and downregulation of VEGFA. However, the IRF1 variant missing exon 9 did not downregulate VEGFA (Lee *et al.* 2008). These results suggest the skipping of exon 9 could reduce the effect of IRF1, hence promoting angiogenesis. Whilst the high protein diet encoding the full length IRF1 would potentially increase the activity of IRF1 and inhibit angiogenesis. However, quantification of which isoform is most prevalent in the control and high protein samples by qPCR is required. Alongside investigations into the

effect of the IRF1 isoforms in the ovary to determine the exact effect of this alternative splicing.

Placental growth factor (PGF).

Analysis of RI splicing in the PGF gene showed the presence of a partially RI between exons 5 and 6 in the high protein sample. This alternative splicing event leads to the generation of a slightly premature stop codon, resulting in the formation of a truncated protein. This alternative splicing event has been discovered in the human PGF gene, where an additional exon that corresponds to the location of the RI in the high protein sample is evident. It is therefore likely that the annotation of the bovine reference genome is incorrect at this location and there is in fact another exon present at this location. In the human isoforms of PGF this exon has been linked to heparin binding (Cao *et al.* 1997). The formation of a stop codon as a result of alternative splicing would result in changes to the heparin binding properties of the PGF protein. PGF is a pro-angiogenic factor that binds exclusively to FLT1, therefore splicing in this region could affect the PGFs ability to promote angiogenesis (De Falco 2012).

Heparanase (HPSE).

The high protein diet induced skipping of exon 4 in the HPSE gene, with further analysis showing this inserted a premature stop codon in exon 5 and markedly shortened the peptide sequence. Skipping of exon 4 in the HPSE gene has been reported in another species, with the shortened product of the alternatively spliced HPSE lacking enzymatic activity (Bertolesi *et al.* 2008).

HPSE encodes an endoglycosidase, which breaks down heparan sulphate resulting in the formation of biologically active fragments (Pikas *et al.* 1998, Parish *et al.* 2001, Vlodavsky & Friedmann 2001). The breakdown of heparan sulphate is a vitally important process in the remodelling of the ECM (Zetser *et al.* 2006). Heparanase upregulated VEGFA expression and increased the adhesion of ECs to the ECM. The latter process occurred independently of heparanase enzyme activity, demonstrating that heparanase likely has an important role in angiogenesis (Goldshmidt *et al.* 2003, Zetser *et al.* 2006). The skipping of exon 4 may result in the formation of an inactive heparanase isoform that affects VEGFA expression.

Of particular relevance to the bovine ovary, LH upregulated HPSE in granulosa cells during the pre-ovulatory stage, suggesting that HPSE could contribute to the ECM degradation required for the rupturing of the follicle during ovulation (Klipper *et al.* 2009). Therefore, if the spliced HPSE lacks enzyme activity, it is possible that the high protein diet may impair ovulation of the follicle, as well as reducing subsequent angiogenesis. Further work would involve determining the effect that skipping exon 4 has on the activity of heparanase, by isolating the spliced protein and determing it's enzymatic activity.

Conclusion.

In conclusion, this chapter shows for the first time that a high protein diet causes the alternative splicing of numerous genes in granulosa cells. Many of the spliced genes are involved in processes relating to cell growth such as the regulation of the cell cycle and mitosis. This demonstrates that a high protein

diet could alter granulosa cell growth through alternative splicing, showing that dietary changes have the potential to impact upon bovine fertility. Furthermore, several of the spliced genes were linked to angiogenesis. Analysis of these genes showed that alternative splicing resulted in the formation of truncated proteins encoded by these genes. This could change the function of the proteins or cause them to be non-functional, suggesting that a high protein diet may alter angiogenesis in the ovary. This could have negative repercussions for the establishment and maintenance of pregnancy.

Further analysis is required to determine the effects of alternative splicing caused by dietary intervention on the function of many of the proteins encoded by spliced genes. However, the results of the current study provide a mechanistic insight into how a high protein diet causes the alternative splicing of numerous granulosa cell genes, that may alter granulosa cell growth, angiogenesis and ovarian function. Furthermore, it is plausible that a high protein diet will induce the splicing of genes in other cell types, which could alter not only reproductive success but also other key processes, such as the growth and immunity of the cows on this diet. However, analysis of alternative splicing in other cell types is required to investigate this. These findings demonstrate that the perception that feeding a high protein diet is beneficial to productivity may be incorrect, suggesting that further analysis of the impact of dietary alterations is required.

Chapter 4. Future directions.

The current study found that VEGFA and PEDF are expressed throughout luteal development, however $VEGFA_{164b}$ expression was minimal at all stages of luteal development. PEDF but not THBS1, inhibited early luteal angiogenesis, whilst luteal progesterone production was unaffected by both factors. A high protein diet induced the splicing of 127 common genes in granulosa cell samples, the most prevalent type of splicing event was SE splicing. The most significant and well represented genes were linked to mitosis and the regulation of the cell cycle. Spliced genes were shown to interact with multiple resultant proteins that were involved in a variety of cellular processes. Further analysis of genes of interest showed that the high protein diet induced splicing events that altered the amino acid sequence of the genes, with 3 out of the 4 genes developing a premature stop codon.

In the marmoset $VEGF_{165b}$ expression accounts for nearly 100% of VEGFA protein in ovaries without CL, before its expression is downregulated during CL development (Qiu *et al.* 2012). Therefore, determining the spatial and temporal expression of VEGFA and $VEGFA_{164b}$ in follicles at different stages of development would show if $VEGFA_{164b}$ is present in the bovine ovary prior to CL formation. Furthermore, this would establish if this inhibitory factor's expression is dynamic and if its downregulation is a pre-requisite to CL development. This could be achieved by using similar immunohistochemistry and Western blotting techniques to those used in the current study. Similarly, quantifying PEDF expression in the bovine follicle would also be beneficial.

In the current study the effect of PEDF was dose dependent, with the lower dose inhibiting angiogenesis, whilst the high dose had no effect. Performing further luteal cultures using a 1ng/ml PEDF treatment would show if the inhibitory effects are present at an even lower dose. It has been reported that the ratio of PEDF and VEGF is important in cell survival, therefore altering the concentration of VEGF added to the culture media would establish if the balance of these two factors influences luteal angiogenesis (Pollina *et al.* 2008). Performing dual immunohistochemistry for caspase 3 and an EC marker would reveal if PEDF is inhibiting angiogenesis by inducing EC apoptosis. Similarly, staining for FLT1 and FGFR2 would show which receptors PEDF was eliciting its effect through. Characterisation of anti-angiogenic factors in the bovine ovary could also be performed, with 2-ME and VASH1 both warranting further investigation.

A future aim of the alternative splicing investigation would be to determine the effect of alternative splicing on the amino acid sequence of a greater number of genes. Similarly, determining the effect that the altered amino acid sequences have on protein function would provide a valuable insight into the effect of the high protein diet on granulosa cell function. Furthermore, following the recognition of alternatively spliced genes it would be beneficial to establish the effect of the high protein diet on the expression of the spliced genes, as well as determining which splice variant is most prevalent, which could be performed using qPCR. The focus of another future study would be to determine how a high protein diet alters the expression and alternative splicing of granulosa cell genes, throughout follicular development. This could be

achieved by repeating the current study using granulosa cell samples acquired from follicles at different stages of development.

5. Bibliography.

Ablonczy Z, Prakasam A, Fant J, Fauq A, Crosson C & Sambamurti K 2009 Pigment epithelium-derived factor maintains retinal pigment epithelium function by inhibiting vascular endothelial growth factor-R2 signaling through γ-Secretase. *Journal of Biological Chemistry* **284** 30177-30186.

Adair T & Montani J 2010 Angiogenesis. In *Integrated Systems Physiology: From Molecule to Function*, pp. 1-84.

Adams J & Lawler J 1993 The thrombospondin family. *Current Biology* **3** 188-190.

Adams JC & Lawler J 2004 The thrombospondins. *The International Journal of Biochemistry & Cell Biology* **36** 961-968.

Adolph KW, Liska DJ & Bornstein P 1997 Analysis of the promoter and transcription start sites of the human thrombospondin 2 gene (THBS2). *Gene* **193** 5-11.

Ahmadzadeh A, Frago F, Shafii B, Dalton JC, Price WJ & McGuire MA 2009 Effect of clinical mastitis and other diseases on reproductive performance of Holstein cows. *Animal Reproduction Science* **112** 273-282.

Al-zi'abi M, Watson E & Fraser H 2003 Angiogenesis and vascular endothelial growth factor expression in the equine corpus luteum. *Reproduction* **125** 259-270.

Alarcon CR, Goodarzi H, Lee H, Liu X, Tavazoie S & Tavazoie SF 2015 HNRNPA2B1 is a mediator of m(6)A-dependent nuclear rna processing events. *Cell* **162** 1299-1308.

Amselgruber WM, Schäfer M & Sinowatz F 1999 Angiogenesis in the bovine corpus luteum: An immunocytochemical and ultrastructural study. *Anatomia, Histologia, Embryologia* **28** 157-166.

Armstrong DG, McEvoy TG, Baxter G, Robinson JJ, Hogg CO, Woad KJ, Webb R & Sinclair KD 2001 Effect of dietary energy and protein on bovine follicular dynamics and embryo production in vitro: associations with the ovarian insulin-like growth factor system. *Biology of Reproduction* **64** 1624-1632.

Armulik A, Genové G & Betsholtz C 2011 Pericytes: Developmental, physiological, and pathological perspectives, problems, and promises. *Developmental Cell* **21** 193-215.

Artac RA, McFee RM, Longfellow Smith RA, Baltes-Breitwisch MM, Clopton DT & Cupp AS 2009 Neutralization of vascular endothelial growth factor antiangiogenic isoforms is more effective than treatment with proangiogenic isoforms in stimulating vascular development and follicle progression in the perinatal rat ovary. *Biology of Reproduction* **81** 978-988.

Attisano L, Cárcamo J, Ventura F, Weis FMB, Massagué J & Wrana JL 1993 Identification of human activin and TGFβ type I receptors that form heteromeric kinase complexes with type II receptors. *Cell* **75** 671-680.

Augustin HG, Young Koh G, Thurston G & Alitalo K 2009 Control of vascular morphogenesis and homeostasis through the angiopoietin-Tie system. *Nature Reviews Molecular Cell Biology* **10** 165-177.

Badenhorst P, Voas M, Rebay I & Wu C 2002 Biological functions of the ISWI chromatin remodeling complex NURF. *Genes & Development* **16** 3186-3198.

Bain G, Maandag EC, Izon DJ, Amsen D, Kruisbeek AM, Weintraub BC, Krop I, Schlissel MS, Feeney AJ, van Roon M & et al. 1994 E2A proteins are required for proper B cell development and initiation of immunoglobulin gene rearrangements. *Cell* **79** 885-892.

Bao B & Garverick HA 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.

Barleon B, Sozzani S, Zhou D, Weich H, Mantovani A & Marme D 1996 Migration of human monocytes in response to vascular endothelial growth factor (VEGF) is mediated via the VEGF receptor flt-1. *Blood* **87** 3336-3343.

Barton BA, Rosario HA, Anderson GW, Grindle BP & Carroll DJ 1996 Effects of dietary crude protein, breed, parity, and health status on the fertility of dairy cows. *Journal of Dairy Science* **79** 2225-2236.

Basto R, Gomes R & Karess RE 2000 Rough deal and Zw10 are required for the metaphase checkpoint in Drosophila. *Nature Cell Biology* **2** 939-943.

Bates DO, MacMillan P, Manjaly J, Qiu Y, Hudson S, Bevan H, Hunter A, Soothill P, Read M, Donaldson LF & Harper S 2006 The endogenous anti-angiogenic family of splice variants of VEGF, VEGFxxxb, are down-regulated in pre-eclamptic placentae at term. *Clinical Science* **110** 575.

Batista F, Vaiman D, Dausset J, Fellous M & Veitia RA 2007 Potential targets of FOXL2, a transcription factor involved in craniofacial and follicular development, identified by transcriptomics. *Proceedings of the National Academy of Sciences of the United States of America* **104** 3330-3335.

Batra S 1986 Effect of estrogen and progesterone treatment on calcium uptake by the myometrium and smooth muscle of the lower urinary tract. *European Journal of Pharmacology* **127** 37-42.

Becerra SP, Sagasti A, Spinella P & Notario V 1995 Pigment epithelium-derived factor behaves like a noninhibitory serpin: Neutrotrophic activity does not require the serpin reactive loop. *Journal of Biological Chemistry* **270** 25992-25999.

Becker J, Semler O, Gilissen C, Li Y, Bolz Hanno J, Giunta C, Bergmann C, Rohrbach M, Koerber F, Zimmermann K, de Vries P, Wirth B, Schoenau E, Wollnik B, Veltman Joris A, Hoischen A & Netzer C 2011 Exome sequencing identifies truncating mutations in human SERPINF1 in autosomal-recessive osteogenesis imperfecta. *American Journal of Human Genetics* **88** 362-371.

Behjati S & Tarpey PS 2013 What is next generation sequencing? *Archives of Disease in Childhood. Education and Practice Edition* **98** 236-238.

Benjamini Y & Hochberg Y 1995 Controlling the false discovery rate: A practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society. Series B (Methodological)* **57** 289-300.

Bentley DL 2014 Coupling mRNA processing with transcription in time and space. *Nature Reviews Genetics* **15** 163-175.

Bergers G & Song S 2005 The role of pericytes in blood-vessel formation and maintenance. *Neuro-Oncology* **7** 452-464.

Berisha B, Schams D, Kosmann M, Amselgruber W & Einspanier R 2000a 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, Schams D, Kosmann M, Amselgruber W & Einspanier R 2000b Expression and tissue concentration of vascular endothelial growth factor, its receptors, and localization in the bovine corpus luteum during estrous cycle and pregnancy. *Biology of Reproduction* **63** 1106-1114.

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* **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.

Bernatchez PN, Soker S & Sirois MG 1999 Vascular endothelial growth factor effect on endothelial cell proliferation, migration, and platelet-activating factor synthesis is Flk-1-dependent. *Journal of Biological Chemistry* **274** 31047-31054.

Bertolesi GE, Michaiel G & McFarlane S 2008 Two heparanase splicing variants with distinct properties are necessary in early Xenopus development. *The Journal of Biological Chemistry* **283** 16004-16016.

Black DL 2003 Mechanisms of alternative pre-messenger rna splicing. *Annual Review of Biochemistry* **72** 291-336.

Boer HMT, Röblitz S, Stötzel C, Veerkamp RF, Kemp B & Woelders H 2011 Mechanisms regulating follicle wave patterns in the bovine estrous cycle investigated with a mathematical model. *Journal of Dairy Science* **94** 5987-6000.

Bottari SP, Vokaer A, Kaivez E, Lescrainier J & Vauquelin GP 1983 Differential regulation of α-adrenergic receptor subclasses by gonadal steroids in human myometrium. *The Journal of Clinical Endocrinology & Metabolism* **57** 937-941.

Brann DW, O'Conner JL, Wade MF, Zamorano PL & Mahesh VB 1993 Regulation of anterior pituitary gonadotropin subunit mRNA levels during the preovulatory gonadotropin surge: A physiological role of progesterone in regulating LH-β and FSH-β mRNA levels. *The Journal of Steroid Biochemistry and Molecular Biology* **46** 427-437.

Brown HM, Anastasi MR, Frank LA, Kind KL, Richani D, Robker RL, Russell DL, Gilchrist RB & Thompson JG 2015 Hemoglobin: A gas transport

molecule that is hormonally regulated in the ovarian follicle in mice and humans. *Biology of Reproduction* **92** 26, 21-10-26, 21-10.

Burt DW & Law AS 1994 Evolution of the transforming growth factor-beta superfamily. *Progress in Growth Factor Research* **5** 99-118.

Cai J, Jiang WG, Grant MB & Boulton M 2006 Pigment epithelium-derived factor inhibits angiogenesis via regulated intracellular proteolysis of vascular endothelial growth factor receptor 1. *Journal of Biological Chemistry* **281** 3604-3613.

Canfield RW, Sniffen CJ & Butler WR 1990 Effects of excess degradable protein on postpartum reproduction and energy balance in dairy cattle. *Journal of Dairy Science* **73** 2342-2349.

Cao Y, Ji W-R, Qi P, Rosin Å & Cao Y 1997 Placenta growth factor: Identification and characterization of a novel isoform generated by rna alternative splicing. *Biochemical and Biophysical Research Communications* **235** 493-498.

Carmeliet P 2003 Angiogenesis in health and disease. *Nature Medicine* **9** 653-660.

Carmeliet P, Moons L, Luttun A, Vincenti V, Compernolle V, De Mol M, Wu Y, Bono F, Devy L, Beck H, Scholz D, Acker T, DiPalma T, Dewerchin M, Noel A, Stalmans I, Barra A, Blacher S, Vandendriessche T, Ponten A, Eriksson U, Plate KH, Foidart J-M, Schaper W, Charnock-Jones DS, Hicklin DJ, Herbert J-M, Collen D & Persico MG 2001 Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. *Nature Medicine* **7** 575-583.

Cébe Suarez S, Pieren M, Cariolato L, Arn S, Hoffmann U, Bogucki A, Manlius C, Wood J & Ballmer-Hofer K 2006 A VEGF-A splice variant defective for heparan sulfate and neuropilin-1 binding shows attenuated signaling through VEGFR-2. *Cellular and Molecular Life Sciences* **63** 2067-2077.

Celik I, Surucu O, Dietz C, Heymach JV, Force J, Hoschele I, Becker CM, Folkman J & Kisker O 2005 Therapeutic efficacy of endostatin exhibits a biphasic dose-response curve. *Cancer Research* **65** 11044-11050.

Chan GK, Jablonski SA, Starr DA, Goldberg ML & Yen TJ 2000 Human Zw10 and ROD are mitotic checkpoint proteins that bind to kinetochores. *Nature Cell Biology* **2** 944-947.

Chen H, Herndon ME & Lawler J 2000 The cell biology of thrombospondin-1. *Matrix Biology* **19** 597-614.

Chen L, Tovar-Corona JM & Urrutia AO 2012 Alternative splicing: A potential source of functional innovation in the eukaryotic genome. *International Journal of Evolutionary Biology* **2012** 10.

Chen L, Zhang SS-M, Barnstable CJ & Tombran-Tink J 2006 PEDF induces apoptosis in human endothelial cells by activating p38 MAP kinase dependent cleavage of multiple caspases. *Biochemical and Biophysical Research Communications* **348** 1288-1295.

Chen M & Manley JL 2009 Mechanisms of alternative splicing regulation: insights from molecular and genomics approaches. *Nature Reviews Molecular Cell Biology* **10** 741-754.

Chin D, Boyle GM, Parsons PG & Coman WB 2004 What is transforming growth factor-beta (TGF-β)? *British Journal of Plastic Surgery* **57** 215-221.

Chuderland D, Ben-Ami I, Kaplan-Kraicer R, Grossman H, Komsky A, Satchi-Fainaro R, Eldar-Boock A, Ron-El R & Shalgi R 2013 Hormonal regulation of pigment epithelium-derived factor (PEDF) in granulosa cells. *Molecular Human Reproduction* **19** 72-81.

Clancy S 2008 RNA splicing: introns, exons and spliceosome. *Nature Education* **1** 31.

Clapp C, Thebault S, Jeziorski MC & Martínez De La Escalera G 2009 Peptide hormone regulation of angiogenesis. *Physiological Reviews* **89** 1177-1215.

Clark BJ, Wells J, King SR & Stocco DM 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.

Clark F & Thanaraj TA 2002 Categorization and characterization of transcript-confirmed constitutively and alternatively spliced introns and exons from human. *Human Molecular Genetics* **11** 451-464.

Colman-Lerner A, Fischman ML, Lanuza GM, Bissell DM, Kornblihtt AR & Baranao JL 1999 Evidence for a role of the alternatively spliced ED-I sequence of fibronectin during ovarian follicular development. *Endocrinology* **140** 2541-2548.

Colombo G, Margosio B, Ragona L, Neves M, Bonifacio S, Annis DS, Stravalaci M, Tomaselli S, Giavazzi R, Rusnati M, Presta M, Zetta L, Mosher DF, Ribatti D, Gobbi M & Taraboletti G 2010 Non-peptidic thrombospondin-1 mimics as fibroblast growth factor-2 inhibitors: An integrated strategy for the development of new antiangiogenic compounds. *The Journal of Biological Chemistry* **285** 8733-8742.

Comai L, Zomerdijk JC, Beckmann H, Zhou S, Admon A & Tjian R 1994 Reconstitution of transcription factor SL1: exclusive binding of TBP by SL1 or TFIID subunits. *Science* **266** 1966-1972.

Connolly DT 1991 Vascular permeability factor: A unique regulator of blood vessel function. *Journal of Cellular Biochemistry* **47** 219-223.

Dawson DW, Pearce SFA, Zhong R, Silverstein RL, Frazier WA & Bouck NP 1997 CD36 mediates the in vitro inhibitory effects of thrombospondin-1 on endothelial cells. *The Journal of Cell Biology* **138** 707-717.

Dawson DW, Volpert OV, Gillis P, Crawford SE, Xu HJ, Benedict W & Bouck NP 1999 Pigment epithelium-derived factor: A potent inhibitor of angiogenesis. *Science* **285** 245.

De Falco S 2012 The discovery of placenta growth factor and its biological activity. *Experimental & Molecular Medicine* **44** 1-9.

Di Gregorio GB & Nett TM 1995 Estradiol and progesterone influence the synthesis of gonadotropins in the absence of gonadotropin-releasing hormone in the ewe. *Biology of Reproduction* **53** 166-172.

Díaz R, Peña C, Silva J, Lorenzo Y, García V, García JM, Sánchez A, Espinosa P, Yuste R, Bonilla F & Domínguez G 2008 p73 isoforms affect VEGF,

VEGF165b and PEDF expression in human colorectal tumors: VEGF165b downregulation as a marker of poor prognosis. *International Journal of Cancer* **123** 1060-1067.

Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M & Gingeras TR 2013 STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* **29** 15-21.

Dobson H, Smith RF, Royal MD, Knight CH & Sheldon IM 2007 The high producing dairy cow and its reproductive performance. *Reproduction in Domestic Animals* **42** 17-23.

Donovan J, Shiwen X, Norman J & Abraham D 2013 Platelet-derived growth factor alpha and beta receptors have overlapping functional activities towards fibroblasts. *Fibrogenesis & Tissue Repair* **6** 10.

Dreyfuss G, Kim VN & Kataoka N 2002 Messenger-RNA-binding proteins and the messages they carry. *Nature Reviews Molecular Cell Biology* **3** 195-205.

Dunn JF, Merriam GR, Eil C, Kono S, Loriaux DL & Nisula BC 1980 Testosterone-estradiol binding globulin binds to 2-methoxyestradiol with greater affinity than to testosterone. *Journal of Clinical Endocrinology & Metabolism* **51** 404-406.

Eborn DR, Cushman RA & Echternkamp SE 2013 Effect of postweaning diet on ovarian development and fertility in replacement beef heifers. *Journal of Animal Science* **91** 4168-4179.

Edlund S, Bu S, Schuster N, Aspenstrom P, Heuchel R, Heldin NE, ten Dijke P, Heldin CH & Landstrom M 2003 Transforming growth factor-beta1 (TGF-beta)-induced apoptosis of prostate cancer cells involves Smad7-dependent activation of p38 by TGF-beta-activated kinase 1 and mitogen-activated protein kinase kinase 3. *Molecular Biology of the Cell* **14** 529-544.

Edwards JL. 2016 The influences of dietary protein on bovine placental development and ovarian function. In *Veterinary Medicine and Science*, pp. 285 University of Nottingham.

Elliott RL & Blobe GC 2005 Role of transforming growth factor beta in human cancer. *Journal of Clinical Oncology* **23** 2078-2093.

Fagegaltier D, Hubert N, Yamada K, Mizutani T, Carbon P & Krol A 2000 Characterization of mSelB, a novel mammalian elongation factor for selenoprotein translation. *The EMBO Journal* **19** 4796-4805.

Feitsma K, Hausser H, Robenek H, Kresse H & Vischer P 2000 Interaction of thrombospondin-1 and heparan sulfate from endothelial cells: Structural requirements of heparan sulfate. *Journal of Biological Chemistry* **275** 9396-9402.

Ferguson JD & Chalupa W 1989 Impact of protein nutrition on reproduction in dairy cows. *Journal of Dairy Science* **72** 746-766.

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.

Ferrari G, Cook BD, Terushkin V, Pintucci G & Mignatti P 2009 Transforming growth factor-beta 1 (tgf-β1) induces angiogenesis through vascular endothelial growth factor (vegf)-mediated apoptosis. *Journal of Cellular Physiology* **219** 449-458.

Fidler IJ 2000 Regulation of neoplastic angiogenesis. *Journal of the National Cancer Institute Monographs* **2000** 10-14.

Fischer U, Struss AK, Hemmer D, Michel A, Henn W, Steudel WI & Meese E 2001 PHF3 expression is frequently reduced in glioma. *Cytogenetics and Cell Genetics* **94** 131-136.

Follit JA, Tuft RA, Fogarty KE & Pazour GJ 2006 The intraflagellar transport protein IFT20 is associated with the Golgi complex and is required for cilia assembly. *Molecular Biology of the Cell* **17** 3781-3792.

Fong GH, Rossant J, Gertsenstein M & Breitman ML 1995 Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature* **376** 66-70.

Fong GH, Zhang L, Bryce DM & Peng J 1999 Increased hemangioblast commitment, not vascular disorganization, is the primary defect in flt-1 knock-out mice. *Development* **126** 3015-3025.

Fortune JE, Rivera GM, Evans ACO & Turzillo AM 2001 Differentiation of dominant versus subordinate follicles in cattle. *Biology of Reproduction* **65** 648-654.

Fotsis T, Zhang Y, Pepper MS, Adlercreutz H, Montesano R, Nawroth PP & Schweigerer L 1994 The endogenous oestrogen metabolite 2-methoxyoestradiol inhibits angiogenesis and suppresses tumour growth. *Nature* **368** 237-239.

Franzén P, ten Dijke P, Ichijo H, Yamashita H, Schulz P, Heldin C-H & Miyazono K 1993 Cloning of a TGFβ type I receptor that forms a heteromeric complex with the TGFβ type II receptor. *Cell* **75** 681-692.

Fraser H & Lunn S 2001 Regulation and manipulation of angiogenesis in the primate corpus luteum. *Reproduction* **121** 355-362.

Fraser HM 2006 Regulation of the ovarian follicular vasculature. *Reproductive Biology and Endocrinology* **4** 18.

Fredriksson L, Li H & Eriksson U 2004 The PDGF family: four gene products form five dimeric isoforms. *Cytokine & Growth Factor Reviews* **15** 197-204.

Gabler C, Plath-Gabler A, Killian GJ, Berisha B & Schams D 2004 Expression pattern of fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) system members in bovine corpus luteum endothelial cells during treatment with FGF-2, VEGF or oestradiol. *Reproduction in Domestic Animals* **39** 321-327.

Galvão AM, Ferreira-Dias G & Skarzynski DJ 2013 Cytokines and angiogenesis in the corpus luteum. *Mediators of Inflammation* **2013** 420186.

Gangrade BK, Gotcher ED, Davis JS & May JV 1993 The secretion of transforming growth factor-beta by bovine luteal cells in vitro. *Molecular and Cellular Endocrinology* **93** 117-123.

Garnsworthy PC, Gong JG, Armstrong DG, Newbold JR, Marsden M, Richards SE, Mann GE, Sinclair KD & Webb R 2008 Nutrition, metabolism, and

fertility in dairy cows: 3. Amino acids and ovarian function. *Journal of Dairy Science* **91** 4190-4197.

Gerhardt H & Betsholtz C 2003 Endothelial-pericyte interactions in angiogenesis. *Cell and Tissue Research* **314** 15-23.

Gerhardt H, Golding M, Fruttiger M, Ruhrberg C, Lundkvist A, Abramsson A, Jeltsch M, Mitchell C, Alitalo K, Shima D & Betsholtz C 2003 VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. *The Journal of Cell Biology* **161** 1163-1177.

Gilbert CL, Lamming GE, Parkinson TJ, Flint APF & Wathes DC 1989 Oxytocin infusion from Day 10 after oestrus extends the luteal phase in non-pregnant cattle. *Journal of Reproduction and Fertility* **86** 203-210.

Ginther O, Bergfelt D, Beg M & Kot K 2002 Role of low circulating FSH concentrations in controlling the interval to emergence of the subsequent follicular wave in cattle. *Reproduction* **124** 475-482.

Godin JD, Colombo K, Molina-Calavita M, Keryer G, Zala D, Charrin BC, Dietrich P, Volvert ML, Guillemot F, Dragatsis I, Bellaiche Y, Saudou F, Nguyen L & Humbert S 2010 Huntingtin is required for mitotic spindle orientation and mammalian neurogenesis. *Neuron* **67** 392-406.

Goldshmidt O, Zcharia E, Cohen M, Aingorn H, Cohen I, Nadav L, Katz B-z, Geiger B & Vlodavsky I 2003 Heparanase mediates cell adhesion independent of its enzymatic activity. *The FASEB Journal* **17** 1015-1025.

Gospodarowicz D, Ferrara N, Schweigerer L & Neufeld G 1987 Structural characterization and biological functions of fibroblast growth factor. *Endocrine Reviews* **8** 95-114.

Goumans M-J, Lebrin F & Valdimarsdottir G 2003 Controlling the angiogenic switch: A balance between two distinct TGF-β receptor signaling pathways. *Trends in Cardiovascular Medicine* **13** 301-307.

Greenaway J, Gentry PA, Feige J-J, LaMarre J & Petrik JJ 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.

Greenaway J, Lawler J, Moorehead R, Bornstein P, Lamarre J & Petrik J 2007 Thrombospondin-1 inhibits vegf levels in the ovary directly by binding and internalization via the low density lipoprotein receptor-related protein-1 (lrp-1). *Journal of cellular physiology* **210** 807-818.

Greenwalt DE, Lipsky RH, Ockenhouse CF, Ikeda H, Tandon NN & Jamieson GA 1992 Membrane glycoprotein CD36: a review of its roles in adherence, signal transduction, and transfusion medicine. *Blood* **80** 1105.

Grummer R & Carroll D 1988 A review of lipoprotein cholesterol metabolism: importance to ovarian function. *Journal of Animal Science* **66** 3160-3173.

Gururaj AE, Peng S, Vadlamudi RK & Kumar R 2007 Estrogen induces expression of BCAS3, a novel estrogen receptor-α coactivator, through proline-, glutamic acid-, and leucine-rich protein-1 (PELP1). *Molecular Endocrinology* **21** 1847-1860.

Gutierrez LS 2008 The role of thrombospondin 1 on intestinal inflammation and carcinogenesis. *Biomarker Insights* **3** 171-178.

Guzmán A, Macías-Valencia R, Fierro-Fierro F, Gutiérrez CG & Rosales-Torres AM 2015 The corpora lutea proangiogenic state of VEGF system components is turned to antiangiogenic at the later phase of the oestrous cycle in cows. *Animal* **9** 301-307.

Haile-Mariam M, Bowman PJ & Goddard ME 2003 Genetic and environmental relationship among calving interval, survival, persistency of milk yield and somatic cell count in dairy cattle. *Livestock Production Science* **80** 189-200.

Harper SJ & Bates DO 2008 VEGF-A splicing: the key to anti-angiogenic therapeutics? *Nature Reviews Cancer* **8** 880-887.

Haussmann IU, Bodi Z, Sanchez-Moran E, Mongan NP, Archer N, Fray RG & Soller M 2016 m6A potentiates Sxl alternative pre-mRNA splicing for robust Drosophila sex determination. *Nature* **540** 301-304.

Hayashi K-G, Ushizawa K, Hosoe M & Takahashi T 2011 Differential gene expression of serine protease inhibitors in bovine ovarian follicle: possible involvement in follicular growth and atresia. *Reproductive Biology and Endocrinology* **9** 72-72.

Heishi T, Hosaka T, Suzuki Y, Miyashita H, Oike Y, Takahashi T, Nakamura T, Arioka S, Mitsuda Y, Takakura T, Hojo K, Matsumoto M, Yamauchi C, Ohta H, Sonoda H & Sato Y 2010 Endogenous angiogenesis inhibitor vasohibin1 exhibits broad-spectrum antilymphangiogenic activity and suppresses lymph node metastasis. *American Journal of Pathology* **176** 1950-1958.

Helkin A, Maier KG & Gahtan V 2015 Thrombospondin-1, -2 and -5 have differential effects on vascular smooth muscle cell physiology. *Biochemical and biophysical research communications* **464** 1022-1027.

Herzog B, Pellet-Many C, Britton G, Hartzoulakis B & Zachary IC 2011 VEGF binding to NRP1 is essential for VEGF stimulation of endothelial cell migration, complex formation between NRP1 and VEGFR2, and signaling via FAK Tyr407 phosphorylation. *Molecular Biology of the Cell* **22** 2766-2776.

Hirsch J, Johnson CL, Nelius T, Kennedy R, Riese Wd & Filleur S 2011 PEDF inhibits IL8 production in prostate cancer cells through PEDF receptor/phospholipase A2 and regulation of NFκB and PPARγ. *Cytokine* **55** 202-210.

Ho T-C, Chen S-L, Yang Y-C, Lo T-H, Hsieh J-W, Cheng H-C & Tsao Y-P 2009 Cytosolic phospholipase A2-α is an early apoptotic activator in PEDF-induced endothelial cell apoptosis. *American Journal of Physiology - Cell Physiology* **296** 273-284.

Hogg K, Etherington SL, Young JM, McNeilly AS & Duncan WC 2010 Inhibitor of differentiation (Id) genes are expressed in the steroidogenic cells of the ovine ovary and are differentially regulated by members of the transforming growth factor-beta family. *Endocrinology* **151** 1247-1256.

Hou X, Arvisais EW, Jiang C, Chen D-b, Roy SK, Pate JL, Hansen TR, Rueda BR & Davis JS 2008 Prostaglandin F2α stimulates the expression and secretion of transforming growth factor B1 via induction of the early

growth response 1 gene (EGR1) in the bovine corpus luteum. *Molecular Endocrinology* **22** 403-414.

Houck KA, Ferrara N, Winer J, Cachianes G, Li B & Leung DW 1991 The Vascular endothelial growth factor family: Identification of a fourth molecular species and characterization of alternative splicing of RNA. *Molecular Endocrinology* **5** 1806-1814.

Houck KA, Leung DW, Rowland AM, Winer J & Ferrara N 1992 Dual regulation of vascular endothelial growth factor bioavailability by genetic and proteolytic mechanisms. *Journal of Biological Chemistry* **267** 26031-26037.

Human Genome Sequencing 2004 Finishing the euchromatic sequence of the human genome. *Nature* **431** 931-945.

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.

Imamura T 2014 Physiological functions and underlying mechanisms of fibroblast growth factor (FGF) family members: Recent findings and implications for their pharmacological application. *Biological and Pharmaceutical Bulletin* **37** 1081-1089.

Ireland JJ, Murphee RL & Coulson PB 1980 Accuracy of predicting stages of bovine estrous cycle by gross appearance of the corpus luteum. *Journal of Dairy Science* **63** 155-160.

Isenberg JS, Annis DS, Pendrak ML, Ptaszynska M, Frazier WA, Mosher DF & Roberts DD 2009 Differential interactions of thrombospondin-1, -2, and -4 with CD47 and effects on cGMP signaling and ischemic injury responses. *The Journal of Biological Chemistry* **284** 1116-1125.

Itoh S, Itoh F, Goumans M-J & ten Dijke P 2000 Signaling of transforming growth factor-β family members through Smad proteins. *European Journal of Biochemistry* **267** 6954-6967.

Jain M, Bhat GP, Vijayraghavan K & Inamdar MS 2012 Rudhira/BCAS3 is a cytoskeletal protein that controls Cdc42 activation and directional cell migration during angiogenesis. *Experimental Cell Research* **318** 753-767.

Jimenez B, Volpert OV, Crawford SE, Febbraio M, Silverstein RL & Bouck N 2000 Signals leading to apoptosis-dependent inhibition of neovascularization by thrombospondin-1. *Nature Medicine* **6** 41-48.

Johnson WJ, Phillips MC & Rothblat GH 1997 Lipoproteins and cellular cholesterol homeostasis. *Cholesterol*235-276.

Johnston EK, Francis MK & Knepper JE 2015 Recombinant pigment epithelium-derived factor PEDF binds vascular endothelial growth factor receptors 1 and 2. *In Vitro Cellular & Developmental Biology* **51** 730-738.

Kamat BR, Brown LF, Manseau EJ, Senger DR & Dvorak HF 1995 Expression of vascular permeability factor/vascular endothelial growth factor by human granulosa and theca lutein cells. Role in corpus luteum development. *The American Journal of Pathology* **146** 157-165.

Kamei M, Brian Saunders W, Bayless KJ, Dye L, Davis GE & Weinstein BM 2006 Endothelial tubes assemble from intracellular vacuoles in vivo. *Nature* **442** 453-456.

Kampfer C, Saller S, Windschuttl S, Berg D, Berg U & Mayerhofer A 2014 Pigment-Epithelium Derived Factor (PEDF) and the human ovary: a role in the generation of ROS in granulosa cells. *Life Sciences* **97** 129-136.

Kanda S, Mochizuki Y, Nakamura T, Miyata Y, Matsuyama T & Kanetake H 2005 Pigment epithelium-derived factor inhibits fibroblast-growth-factor-2-induced capillary morphogenesis of endothelial cells through Fyn. *Journal of Cell Science* **118** 961.

Kandoth C, McLellan MD, Vandin F, Ye K, Niu B, Lu C, Xie M, Zhang Q, McMichael JF, Wyczalkowski MA, Leiserson MDM, Miller CA, Welch JS, Walter MJ, Wendl MC, Ley TJ, Wilson RK, Raphael BJ & Ding L 2013 Mutational landscape and significance across 12 major cancer types. *Nature* **502** 333-339.

Kantola AK, Keski-Oja J & Koli K 2008 Fibronectin and heparin binding domains of latent TGF-beta binding protein (LTBP)-4 mediate matrix targeting and cell adhesion. *Experimental Cell Research* **314** 2488-2500.

Kawate N & Okuda K 1998 Coordinated expression of splice variants for luteinizing hormone receptor messenger RNA during the development of bovine corpora lutea. *Molecular Reproduction and Development* **51** 66-75.

Kelly RJ & Morris JC 2010 Transforming growth factor-beta: A target for cancer therapy. *Journal of Immunotoxicology* **7** 15-26.

Kern J, Bauer M, Rychli K, Wojta J, Ritsch A, Gastl G, Gunsilius E & Untergasser G 2008 Alternative splicing of vasohibin-1 generates an inhibitor of endothelial cell proliferation, migration, and capillary tube formation. *Arteriosclerosis, Thrombosis, and Vascular Biology* **28** 478.

Kimura H, Miyashita H, Suzuki Y, Kobayashi M, Watanabe K, Sonoda H, Ohta H, Fujiwara T, Shimosegawa T & Sato Y 2009 Distinctive localization and opposed roles of vasohibin-1 and vasohibin-2 in the regulation of angiogenesis. *Blood* **113** 4810.

Kind KL, Banwell KM, Gebhardt KM, Macpherson A, Gauld A, Russell DL & Thompson JG 2013 Microarray analysis of mRNA from cumulus cells following in vivo or in vitro maturation of mouse cumulus-oocyte complexes. *Reproduction, Fertility and Development* **25** 426-438.

Klauber N, Parangi S, Flynn E, Hamel E & Amato RJ 1997 Inhibition of angiogenesis and breast cancer in mice by the microtubule inhibitors 2-methoxyestradiol and taxol. *Cancer Research* **57** 81.

Klipper E, Tatz E, Kisliouk T, Vlodavsky I, Moallem U, Schams D, Lavon Y, Wolfenson D & Meidan R 2009 Induction of heparanase in bovine granulosa cells by luteinizing hormone: Possible role during the ovulatory process. *Endocrinology* **150** 413-421.

Kohen P, Henríquez S, Rojas C, Gerk PM, Palomino WA, Strauss Iii JF & Devoto L 2013 2-Methoxyestradiol in the human corpus luteum throughout the luteal phase and its influence on lutein cell steroidogenesis and angiogenic activity. *Fertility and Sterility* **100** 1397-1404.

Kubiczkova L, Sedlarikova L, Hajek R & Sevcikova S 2012 TGF-β – an excellent servant but a bad master. *Journal of Translational Medicine* **10** 183.

Kumar P & Sait SF 2011 Luteinizing hormone and its dilemma in ovulation induction. *Journal of Human Reproductive Sciences* **4** 2-7.

Laird M, Woad KJ, Hunter MG, Mann GE & Robinson RS 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.

Larson SF, Butler WR & Currie WB 1997 Reduced fertility associated with low progesterone postbreeding and increased milk urea nitrogen in lactating cows. *Journal of Dairy Science* **80** 1288-1295.

Lawler PR & Lawler J 2012 Molecular basis for the regulation of angiogenesis by thrombospondin-1 and -2. *Cold Spring Harbor Perspectives in Medicine* **2** a006627.

Laws SC, Beggs MJ, Webster JC & Miller WL 1990 Inhibin increases and progesterone decreases receptors for gonadotropin-releasing hormone in ovine pituitary culture. *Endocrinology* **127** 373-380.

Lazarin GB, Alves NG, Perez JRO, Lima RRd, Garcia IFF, José Neto A, Vale DNC & Saunders GA 2012 Plasma urea nitrogen and progesterone concentrations and follicular dynamics in ewes fed proteins of different degradability. *Revista Brasileira de Zootecnia* **41** 1638-1647.

Lee E-J, Jo M, Park J, Zhang W & Lee J-H 2006 Alternative splicing variants of IRF-1 lacking exons 7, 8, and 9 in cervical cancer. *Biochemical and Biophysical Research Communications* **347** 882-888.

Lee JH, Chun T, Park S-Y & Rho SB 2008 Interferon regulatory factor-1 (IRF-1) regulates VEGF-induced angiogenesis in HUVECs. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* **1783** 1654-1662.

Lee KW & Bogenhagen DF 2014 Assignment of 2'-O-methyltransferases to modification sites on the mammalian mitochondrial large subunit 16 S ribosomal RNA (rRNA). *Journal of Biological Chemistry* **289** 24936-24942.

Lee Y & Rio DC 2015 Mechanisms and regulation of alternative pre-mrna splicing. *Annual Review of Biochemistry* **84** 291-323.

Leung D, Cachianes G, Kuang W, Goeddel D & Ferrara N 1989 Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science* **246** 1306-1309.

Lewin B 2008 *Genes IX*: Jones & Bartlett Learning.

Lewis BP, Green RE & Brenner SE 2003 Evidence for the widespread coupling of alternative splicing and nonsense-mediated mRNA decay in humans. *Proceedings of the National Academy of Sciences* **100** 189-192.

Li Q, Pangas SA, Jorgez CJ, Graff JM, Weinstein M & Matzuk MM 2008 Redundant roles of SMAD2 and SMAD3 in ovarian granulosa cells in vivo. *Molecular Cell Biology* **28** 7001-7011.

Loegl J, Nussbaumer E, Hiden U, Majali-Martinez A, Ghaffari-Tabrizi-Wizy N, Cvitic S, Lang I, Desoye G & Huppertz B 2016 Pigment epithelium-derived factor (PEDF): A novel trophoblast-derived factor limiting feto-placental angiogenesis in late pregnancy. *Angiogenesis* **19** 373-388.

Long Jennifer C & Caceres Javier F 2008 The SR protein family of splicing factors: master regulators of gene expression. *Biochemical Journal* **417** 15.

Lucy MC 2001 Reproductive loss in high-producing dairy cattle: Where will it end? *Journal of Dairy Science* **84** 1277-1293.

Lucy MC, Savio JD, Badinga L, De La Sota RL & Thatcher WW 1992 Factors that affect ovarian follicular dynamics in cattle. *Journal of Animal Science* **70** 3615-3626.

Macias VR, Pinzón C, Fierro F, Vergara M, Martínez D, Rosado A, Gutiérrez CG & Rosales-Torres AM 2012 Identification of soluble forms of vascular endothelial growth factor receptors, sVEGFR-1 and sVEGFR-2, in bovine dominant follicles. *Reproduction in Domestic Animals* **47** e39-e42.

Maeshima Y & Makino H 2010 Angiogenesis and chronic kidney disease. *Fibrogenesis & Tissue Repair* **3** 13-13.

Maik-Rachline G, Shaltiel S & Seger R 2005 Extracellular phosphorylation converts pigment epithelium–derived factor from a neurotrophic to an antiangiogenic factor. *Blood* **105** 670.

Manalo KB, Choong PFM & Dass CR 2011 Pigment epithelium-derived factor as an impending therapeutic agent against vascular epithelial growth factor-driven tumor-angiogenesis. *Molecular Carcinogenesis* **50** 67-72.

Mann GE 2009 Corpus luteum size and plasma progesterone concentration in cows. *Animal Reproduction Science* **115** 296-299.

Mann GE & Lamming GE 1995 Progesterone inhibition of the development of the luteolytic signal in cows. *Journal of Reproduction and Fertility* **104** 1-5.

Mann GE & Lamming GE 2001 Relationship between maternal endocrine environment, early embryo development and inhibition of the luteolytic mechanism in cows. *Reproduction* **121** 175-180.

Mann GE, Lamming GE, Robinson RS & Wathes DC 1999 The regulation of interferon-tau production and uterine hormone receptors during early pregnancy. *Journal of Reproduction and Fertility Supplement* **54** 317-328.

Maroni D & Davis JS 2011 TGFB1 disrupts the angiogenic potential of microvascular endothelial cells of the corpus luteum. *Journal of Cell Science* **124** 2501.

Maroni D & Davis JS 2012 Transforming growth factor beta 1 stimulates profibrotic activities of luteal fibroblasts in cows. *Biology of Reproduction* **87** 127, 121-111.

Martin M 2011 Cutadapt removes adapter sequences from high-throughput sequencing reads. *Bioinformatics in action* **17**.

Maslar IA, Powers-Craddock P & Ansbacher R 1986 Decidual prolactin production by organ cultures of human endometrium: effects of continuous and intermittent progesterone treatment. *Biology of Reproduction* **34** 741-750.

Massague J 1990 The transforming growth factor-beta family. *Annual Review of Cell and Developmental Biology* **6** 597-641.

Massague J 1998 TGF-beta signal transduction. *Annual Review of Biochemistry* **67** 753-791.

Meyer C, Notari L & Becerra SP 2002 Mapping the type I collagen-binding site on pigment epithelium-derived factor: Implications for its antiangiogenic activity. *Journal of Biological Chemistry* **277** 45400-45407.

Mihm M, Baker PJ, Fleming LM, Monteiro AM & O'Shaughnessy PJ 2008 Differentiation of the bovine dominant follicle from the cohort upregulates mRNA expression for new tissue development genes. *Reproduction* **135** 253-265.

Miller WL 2007 Steroidogenic acute regulatory protein (StAR), a novel mitochondrial cholesterol transporter. *Biochimica et Biophysica Acta* **1771** 663-676.

Miyazono K, Hellman U, Wernstedt C & Heldin CH 1988 Latent high molecular weight complex of transforming growth factor beta 1. Purification from human platelets and structural characterization. *Journal of Biological Chemistry* **263** 6407-6415.

Moutsatsou P & Sekeris CE 1997 Estrogen and progesterone receptors in the endometrium. *Annals of the New York Academy of Sciences* **816** 99-115.

Mueck AO & Seeger H 2010 2-Methoxyestradiol—Biology and mechanism of action. *Steroids* **75** 625-631.

Nalepa G, Barnholtz-Sloan J, Enzor R, Dey D, He Y, Gehlhausen JR, Lehmann AS, Park SJ, Yang Y, Yang X, Chen S, Guan X, Chen Y, Renbarger J, Yang FC, Parada LF & Clapp W 2013 The tumor suppressor CDKN3 controls mitosis. *The Journal of Cell Biology* **201** 997-1012.

Nandi S, Mondal S, Pal DT & Gupta PSP 2016 Effect of ammonia-generating diet on ovine serum and follicular fluid ammonia and urea levels, serum oestrogen and progesterone concentrations and granulosa cell functions. *Journal of Animal Physiology and Animal Nutrition* **100** 309-315.

Natesampillai S, Kerkvliet J, Leung PC & Veldhuis JD 2008 Regulation of Kruppel-like factor 4, 9, and 13 genes and the steroidogenic genes LDLR, StAR, and CYP11A in ovarian granulosa cells. *The American Journal of Physiology - Endocrinology and Metabolism* **294** E385-391.

Ner-Gaon H, Halachmi R, Savaldi-Goldstein S, Rubin E, Ophir R & Fluhr R 2004 Intron retention is a major phenomenon in alternative splicing in Arabidopsis. *The Plant Journal* **39** 877-885.

Neufeld G, Cohen T, Shraga N, Lange T, Kessler O & Herzog Y 2002 The neuropilins: Multifunctional semaphorin and VEGF receptors that modulate axon guidance and angiogenesis. *Trends in Cardiovascular Medicine* **12** 13-19.

Nilsson EE, Detzel C & Skinner MK 2006 Platelet-derived growth factor modulates the primordial to primary follicle transition. *Reproduction* **131** 1007-1015.

Niswender G 2002 Molecular control of luteal secretion of progesterone. *Reproduction* **123** 333-339.

Niswender GD, Juengel JL, Silva PJ, Rollyson MK & McIntush EW 2000 Mechanisms controlling the function and life span of the corpus luteum. *Physiological Reviews* **80** 1-29.

Nogueira MFG, Buratini J, Price CA, Castilho ACS, Pinto MGL & Barros CM 2007 Expression of LH receptor mRNA splice variants in bovine granulosa cells: changes with follicle size and regulation by FSH in vitro. *Molecular Reproduction and Development* **74** 680-686.

Notari L, Arakaki N, Mueller D, Meier S, Amaral J & Becerra SP 2010 Pigment epithelium-derived factor (PEDF) binds to cell-surface F(1)-ATP synthase. *The Federation of European Biochemical Societies Journal* **277** 2192-2205.

Nowak DG, Woolard J, Amin EM, Konopatskaya O, Saleem MA, Churchill AJ, Ladomery MR, Harper SJ & Bates DO 2008 Expression of pro- and anti-angiogenic isoforms of VEGF is differentially regulated by splicing and growth factors. *Journal of Cell Science* **121** 3487-3495.

Ornitz DM, Xu J, Colvin JS, McEwen DG, MacArthur CA, Coulier F, Gao G & Goldfarb M 1996 Receptor specificity of the fibroblast growth factor family. *Journal of Biological Chemistry* **271** 15292-15297.

Ortega Serrano PV, Guzmán A, Hernández–Coronado CG, Castillo-Juárez H & Rosales-Torres AM 2016 Reduction in the mRNA expression of sVEGFR1 and sVEGFR2 is associated with the selection of dominant follicle in cows. *Reproduction in Domestic Animals* **51** 985-991.

Padykula HA, Coles LG, Okulicz WC, Rapaport SI, McCracken JA, King NW, Longcope C & Kaiserman-Abramof IR 1989 The basalis of the primate endometrium: a bifunctional germinal compartment. *Biology of Reproduction* **40** 681-690.

Pandya-Jones A 2011 Pre-mRNA splicing during transcription in the mammalian system. *Wiley Interdisciplinary Reviews.* **2** 700-717.

Parish CR, Freeman C & Hulett MD 2001 Heparanase: a key enzyme involved in cell invasion. *Biochimica et Biophysica Acta* **1471** M99-108.

Park JE, Chen HH, Winer J, Houck KA & Ferrara N 1994 Placenta growth factor. Potentiation of vascular endothelial growth factor bioactivity, in vitro and in vivo, and high affinity binding to Flt-1 but not to Flk-1/KDR. *Journal of Biological Chemistry* **269** 25646-25654.

Park JE, Keller GA & Ferrara N 1993 The vascular endothelial growth factor (VEGF) isoforms: differential deposition into the subepithelial extracellular matrix and bioactivity of extracellular matrix-bound VEGF. *Molecular Biology of the Cell* **4** 1317-1326.

Park JW, Tokheim C, Shen S & Xing Y 2013 Identifying differential alternative splicing events from rna sequencing data using RNAseq-mats. In *Deep Sequencing Data Analysis*, pp. 171-179.

Peiris-Pagès M, Harper SJ, Bates DO & Ramani P 2010 Balance of pro- versus anti-angiogenic splice isoforms of vascular endothelial growth factor as a regulator of neuroblastoma growth. *The Journal of Pathology* **222** 138-147.

Pellizzoni L, Baccon J, Rappsilber J, Mann M & Dreyfuss G 2002 Purification of native survival of motor neurons complexes and identification of

Gemin6 as a novel component. *The Journal of Biological Chemistry* **277** 7540-7545.

Peluso JJ & Steger RW 1978 Role of FSH in regulating granulosa cell division and follicular atresia in rats. *Journal of Reproduction and Fertility* **54** 275-278.

Perrin RM, Konopatskaya O, Qiu Y, Harper S, Bates DO & Churchill AJ 2005 Diabetic retinopathy is associated with a switch in splicing from anti- to pro-angiogenic isoforms of vascular endothelial growth factor. *Diabetologia* **48** 2422-2427.

Pignolo RJ, Cristofalo VJ & Rotenberg MO 1993 Senescent WI-38 cells fail to express EPC-1, a gene induced in young cells upon entry into the G0 state. *Journal of Biological Chemistry* **268** 8949-8957.

Pikas DS, Li J-p, Vlodavsky I & Lindahl U 1998 Substrate specificity of heparanases from human hepatoma and platelets. *Journal of Biological Chemistry* **273** 18770-18777.

Pittman R, Khoo JC & Steinberg D 1975 Cholesterol esterase in rat adipose tissue and its activation by cyclic adenosine 3': 5'-monophosphate-dependent protein kinase. *Journal of Biological Chemistry* **250** 4505-4511.

Pleiss JA, Whitworth GB, Bergkessel M & Guthrie C 2007 Rapid, transcript-specific changes in splicing in response to environmental stress. *Molecular Cell* **27** 928-937.

Pohl M, Bortfeldt RH, Grützmann K & Schuster S 2013 Alternative splicing of mutually exclusive exons—A review. *Biosystems* **114** 31-38.

Pollina EA, Legesse-Miller A, Haley E, Goodpaster T, Randolph-Habecker J & Coller HA 2008 Regulating the angiogenic balance in tissues: A potential role for the proliferative state of fibroblasts. *Cell Cycle* **7** 2056-2070.

Powers CJ, McLeskey SW & Wellstein A 2000 Fibroblast growth factors, their receptors and signaling. *Endocrine-Related Cancer* **7** 165-197.

Pushpakumara PGA, Gardner NH, Reynolds CK, Beever DE & Wathes DC 2003 Relationships between transition period diet, metabolic parameters and fertility in lactating dairy cows. *Theriogenology* **60** 1165-1185.

Qiu Y, Seager M, Osman A, Castle-Miller J, Bevan H, Tortonese DJ, Murphy D, Harper SJ, Fraser HM, Donaldson LF & Bates DO 2012 Ovarian VEGF(165)b expression regulates follicular development, corpus luteum function and fertility. *Reproduction* **143** 501-511.

Rajapaksha WS, Robertson L & O'Shaughnessy PJ 1996 Expression of follicle-stimulating hormone-receptor mRNA alternate transcripts in bovine granulosa cells during luteinization in vivo and in vitro. *Molecular and Cellular Endocrinology* **120** 25-30.

Redmer DA, Dai Y, Li J, Charnock-Jones DS, Smith SK, Reynolds LP & Moor RM 1996 Characterization and expression of vascular endothelial growth factor (VEGF) in the ovine corpus luteum. *Journal of Reproduction and Fertility* **108** 157-165.

Redmer DA, Doraiswamy V, Bortnem BJ, Fisher K, Jablonka-Shariff A, Grazul-Bilska AT & Reynolds LP 2001 Evidence for a role of capillary pericytes

in vascular growth of the developing ovine corpus luteum. *Biology of Reproduction* **65** 879-889.

Rennel ES, Waine E, Guan H, Schüler Y, Leenders W, Woolard J, Sugiono M, Gillatt D, Kleinerman ES, Bates DO & Harper SJ 2008 The endogenous anti-angiogenic VEGF isoform, VEGF(165)b inhibits human tumour growth in mice. *British Journal of Cancer* **98** 1250-1257.

Reynolds AR 2010 Potential relevance of bell-shaped and u-shaped dose-responses for the therapeutic targeting of angiogenesis in cancer. *Dose-Response* **8** 253-284.

Reynolds LP, Grazul-Bilska AT & Redmer DA 2000 Angiogenesis in the corpus luteum. *Endocrine* **12** 1-9.

Reynolds LP & Redmer DA 1999 Growth and development of the corpus luteum. *Journal of Reproduction and Fertility Supplement* **54** 181-191.

Rhind SM, Evans NP, Bellingham M, Sharpe RM, Cotinot C, Mandon-Pepin B, Loup B, Sinclair KD, Lea RG, Pocar P, Fischer B, van der Zalm E, Hart K, Schmidt JS, Amezaga MR & Fowler PA 2010 Effects of environmental pollutants on the reproduction and welfare of ruminants. *Animal* **4** 1227-1239.

Ribeiro LA, Bacci ML, Seren E, Tamanini C & Forni M 2007 Characterization and differential expression of vascular endothelial growth factor isoforms and receptors in swine corpus luteum throughout estrous cycle. *Molecular Reproduction and Development* **74** 163-171.

Rice P, Longden I & Bleasby A 2000 EMBOSS: the european molecular biology open software suite. *Trends in Genetics* **16** 276-277.

Richter TA, Robinson JE & Evans NP 2002 Progesterone blocks the estradiol-stimulated luteinizing hormone surge by disrupting activation in response to a stimulatory estradiol signal in the ewe. *Biology of Reproduction* **67** 119-125.

Robinson JT, Thorvaldsdottir H, Winckler W, Guttman M, Lander ES, Getz G & Mesirov JP 2011 Integrative genomics viewer. *Nature Biotechnology* **29** 24-26.

Robinson RS, Hammond AJ, Mann GE & Hunter MG 2008 A novel physiological culture system that mimics luteal angiogenesis. *Reproduction* **135** 405-413.

Robinson RS, Hammond AJ, Nicklin LT, Schams D, Mann GE & Hunter MG 2006 Endocrine and cellular characteristics of corpora lutea from cows with a delayed post-ovulatory progesterone rise. *Domestic Animal Endocrinology* **31** 154-172.

Robinson RS, Nicklin LT, Hammond AJ, Schams D, Hunter MG & Mann GE 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 RS, Woad KJ, Hammond AJ, Laird M, Hunter MG & Mann GE 2009 Angiogenesis and vascular function in the ovary. *Reproduction* **138** 869-881.

Roche JR, Friggens NC, Kay JK, Fisher MW, Stafford KJ & Berry DP 2009 Invited review: Body condition score and its association with dairy cow

productivity, health, and welfare. *Journal of Dairy Science* **92** 5769-5801.

Rogerson BJ & Eagon PK 1986 A male specific hepatic estrogen binding protein: characteristics and binding properties. *Archives of Biochemistry and Biophysics* **250** 70-85.

Rorbach J, Boesch P, Gammage PA, Nicholls TJ, Pearce SF, Patel D, Hauser A, Perocchi F & Minczuk M 2014 MRM2 and MRM3 are involved in biogenesis of the large subunit of the mitochondrial ribosome. *Molecular Biology of the Cell* **25** 2542-2555.

Rosales-Torres AM, Alonso I, Vergara M, Romano MC, Castillo-Juárez H, Ávalos A, Rosado A & Gutiérrez CG 2010 Vascular endothelial growth factor isoforms 120, 164 and 205 are reduced with atresia in ovarian follicles of sheep. *Animal Reproduction Science* **122** 111-117.

Roskoski R 2007 Vascular endothelial growth factor (VEGF) signaling in tumor progression. *Critical Reviews in Oncology/Hematology* **62** 179-213.

Roy B, Haupt LM & Griffiths LR 2013 Review: Alternative splicing (as) of genes as an approach for generating protein complexity. *Current Genomics* **14** 182-194.

Sapkota G, Knockaert M, Alarcon C, Montalvo E, Brivanlou AH & Massague J 2006 Dephosphorylation of the linker regions of Smad1 and Smad2/3 by small C-terminal domain phosphatases has distinct outcomes for bone morphogenetic protein and transforming growth factor-beta pathways. *Journal of Biological Chemistry* **281** 40412-40419.

Sato Y 2013 The vasohibin family: a novel family for angiogenesis regulation. *Journal of Biochemistry* **153** 5-11.

Scaramuzzi RJ, Baird DT, Campbell BK, Driancourt M-A, Dupont J, Fortune JE, Gilchrist RB, Martin GB, McNatty KP, McNeilly AS, Monget P, Monniaux D, Viñoles C & Webb R 2011 Regulation of folliculogenesis and the determination of ovulation rate in ruminants. *Reproduction, Fertility and Development* **23** 444-467.

Schams D & Berisha B 2004 Regulation of corpus luteum function in cattle – an overview. *Reproduction in Domestic Animals* **39** 241-251.

Senger PL 2003 *Pathways to Pregnancy and Parturition* Current Conceptions, Inc.

Serini G, Valdembri D & Bussolino F 2006 Integrins and angiogenesis: A sticky business. *Experimental Cell Research* **312** 651-658.

Sharma BK, Srinivasan R, Kapil S, Singla B, Saini N, Chawla YK, Chakraborti A, Duseja A, Kalra N & Dhiman RK 2013 Serum levels of angiogenic and anti-angiogenic factors: their prognostic relevance in locally advanced hepatocellular carcinoma. *Molecular and Cellular Biochemistry* **383** 103-112.

Sheldon IM, Cronin J, Goetze L, Donofrio G & Schuberth H-J 2009 Defining postpartum uterine disease and the mechanisms of infection and immunity in the female reproductive tract in cattle. *Biology of reproduction* **81** 1025-1032.

Shen S, Park JW, Lu Z, Lin L, Henry MD, Wu YN, Zhou Q & Xing Y 2014 rMATS: Robust and flexible detection of differential alternative splicing from

replicate RNA-Seq data. *Proceedings of the National Academy of Sciences of the United States of America* **111** E5593-E5601.

Sherratt HSA 1988 Introductory remarks. *Biochemical Society Transactions* **16** 409.

Shibuya M 2001a Structure and dual function of vascular endothelial growth factor receptor-1 (Flt-1). *The International Journal of Biochemistry & Cell Biology* **33** 409-420.

Shibuya M 2001b Structure and function of VEGF/VEGF-receptor system involved in angiogenesis. *Cell Structure and Function* **26** 25-35.

Shibuya M & Claesson-Welsh L 2006 Signal transduction by VEGF receptors in regulation of angiogenesis and lymphangiogenesis. *Experimental Cell Research* **312** 549-560.

Shibuya T, Watanabe K, Yamashita H, Shimizu K, Miyashita H, Abe M, Moriya T, Ohta H, Sonoda H, Shimosegawa T, Tabayashi K & Sato Y 2006 Isolation and characterization of vasohibin-2 as a homologue of VEGF-inducible endothelium-derived angiogenesis inhibitor vasohibin. *Arteriosclerosis, Thrombosis, and Vascular Biology* **26** 1051.

Shimizu K, Watanabe K, Yamashita H, Abe M, Yoshimatsu H, Ohta H, Sonoda H & Sato Y 2005 Gene regulation of a novel angiogenesis inhibitor, vasohibin, in endothelial cells. *Biochemical and Biophysical Research Communications* **327** 700-706.

Shimizu T & Miyamoto A 2007 Progesterone induces the expression of vascular endothelial growth factor (VEGF) 120 and Flk-1, its receptor, in bovine granulosa cells. *Animal Reproduction Science* **102** 228-237.

Shirakabe K, Yamaguchi K, Shibuya H, Irie K, Matsuda S, Moriguchi T, Gotoh Y, Matsumoto K & Nishida E 1997 TAK1 mediates the ceramide signaling to stress-activated protein kinase/c-Jun N-terminal kinase. *Journal of Biological Chemistry* **272** 8141-8144.

Shirasuna K, Kobayashi A, Nitta A, Nibuno S, Sasahara K, Shimizu T, Bollwein H & Miyamoto A 2012 Possible action of vasohibin-1 as an inhibitor in the regulation of vascularization of the bovine corpus luteum. *Reproduction* **143** 491-500.

Slaton JW, Perrotte P, Inoue K, Dinney CP & Fidler IJ 1999 Interferon-alpha-mediated down-regulation of angiogenesis-related genes and therapy of bladder cancer are dependent on optimization of biological dose and schedule. *Clinical Cancer Research* **5** 2726-2734.

Sleer LS & Taylor CC 2007a Cell-type localization of platelet-derived growth factors and receptors in the postnatal rat ovary and follicle. *Biology of Reproduction* **76** 379-390.

Sleer LS & Taylor CC 2007b Platelet-derived growth factors and receptors in the rat corpus luteum: Localization and identification of an effect on luteogenesis. *Biology of Reproduction* **76** 391-400.

Smith CWJ & Valcárcel J 2000 Alternative pre-mRNA splicing: The logic of combinatorial control. *Trends in Biochemical Sciences* **25** 381-388.

Soker S, Fidder H, Neufeld G & Klagsbrun M 1996 Characterization of novel vascular endothelial growth factor (VEGF) receptors on tumor cells that

bind VEGF via its exon 7-encoded domain. *Journal of Biological Chemistry* **271** 5761-5767.

Soker S, Takashima S, Miao HQ, Neufeld G & Klagsbrun M 1998 Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. *Cell* **92** 735-745.

Spencer TE, Burghardt RC, Johnson GA & Bazer FW 2004 Conceptus signals for establishment and maintenance of pregnancy. *Animal Reproduction Science* **82–83** 537-550.

Spicer LJ & Stewart RE 1996 Interactions among basic fibroblast growth factor, epidermal growth factor, insulin, and insulin-like growth factor-I (IGF-I) on cell numbers and steroidogenesis of bovine thecal cells: role of IGF-I receptors. *Biology of Reproduction* **54** 255-263.

Stankovich MT, Sabaj KM & Tonge PJ 1999 Structure/function of medium chain Acyl-CoA dehydrogenase: The importance of substrate polarization. *Archives of Biochemistry and Biophysics* **370** 16-21.

Stellmach V, Crawford SE, Zhou W & Bouck N 2001 Prevention of ischemia-induced retinopathy by the natural ocular antiangiogenic agent pigment epithelium-derived factor. *Proceedings of the National Academy of Sciences* **98** 2593-2597.

Stenina-Adognravi O 2014 Invoking the power of thrombospondins: Regulation of thrombospondins expression. *Matrix biology* 69-82.

Stevens VL, Xu T & Lambeth JD 1993 Cholesterol trafficking in steroidogenic cells. *European Journal of Biochemistry* **216** 557-563.

Sugnet CW, Kent WJ, Ares M, Jr. & Haussler D 2004 Transcriptome and genome conservation of alternative splicing events in humans and mice. *Pacific Symposium on Biocomputing*66-77.

Suzuki Y, Kobayashi M, Miyashita H, Ohta H, Sonoda H & Sato Y 2010 Isolation of a small vasohibin-binding protein (SVBP) and its role in vasohibin secretion. *Journal of Cell Science* **123** 3094.

Swerlick RA, Lee KH, Wick TM & Lawley TJ 1992 Human dermal microvascular endothelial but not human umbilical vein endothelial cells express CD36 in vivo and in vitro. *The Journal of Immunology* **148** 78-83.

Taipale J, Miyazono K, Heldin CH & Keski-Oja J 1994 Latent transforming growth factor-beta 1 associates to fibroblast extracellular matrix via latent TGF-beta binding protein. *Journal of Cell Biology* **124** 171-181.

Taylor C & Rajamahendran R 1991 Follicular dynamics and corpus luteum growth and function in pregnant versus nonpregnant dairy cows. *Journal of Dairy Science* **74** 115-123.

Tazi J, Bakkour N & Stamm S 2009 Alternative splicing and disease. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease* **1792** 14-26.

Ten Dijke P, Yamashita H, Ichijo H, Franzen P, Laiho M, Miyazono K & Heldin CH 1994 Characterization of type I receptors for transforming growth factor-beta and activin. *Science* **264** 101.

Thompson RF. 2014 The effect of transforming growth factor-β on luteal angiogenesis and function in vitro. In *School of Veterinary Medicine and Science*, pp. 61 University of Nottingham.

Tischer E, Mitchell R, Hartman T, Silva M, Gospodarowicz D, Fiddes JC & Abraham JA 1991 The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing. *Journal of Biological Chemistry* **266** 11947-11954.

Tjin Tham Sjin RM, Naspinski J, Birsner AE, Li C, Chan R, Lo KM, Gillies S, Zurakowski D, Folkman J, Samulski J & Javaherian K 2006 Endostatin therapy reveals a U-shaped curve for antitumor activity. *Cancer Gene Therapy* **13** 619-627.

Tombran-Tink J, Mazuruk K, Rodriguez IR, Chung D, Linker T, Englander E & Chader GJ 1996 Organization, evolutionary conservation, expression and unusual Alu density of the human gene for pigment epithelium-derived factor, a unique neurotrophic serpin. *Molecular Vision* **2** 11.

Tuckey RC 2005 Progesterone synthesis by the human placenta. *Placenta* **26** 273-281.

Tugendreich S, Tomkiel J, Earnshaw W & Hieter P 1995 CDC27Hs colocalizes with CDC16Hs to the centrosome and mitotic spindle and is essential for the metaphase to anaphase transition. *Cell* **81** 261-268.

Vlodavsky I & Friedmann Y 2001 Molecular properties and involvement of heparanase in cancer metastasis and angiogenesis. *Journal of Clinical Investigation* **108** 341-347.

Waltenberger J, Claesson-Welsh L, Siegbahn A, Shibuya M & Heldin CH 1994 Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. *Journal of Biological Chemistry* **269** 26988-26995.

Wang J, Duncan D, Shi Z & Zhang B 2013 WEB-based GEne SeT AnaLysis Toolkit (WebGestalt): update 2013. *Nucleic Acids Research* **41** W77-W83.

Wang JH & Tuohimaa P 2006 Regulation of cholesterol 25-hydroxylase expression by vitamin D3 metabolites in human prostate stromal cells. *Biochemical and Biophysical Research Communications* **345** 720-725.

Wang Y, Ma M, Xiao X & Wang Z 2012 Intronic splicing enhancers, cognate splicing factors and context-dependent regulation rules. *Nature Structural & Molecular Biology* **19** 1044-1052.

Wang Z, Gerstein M & Snyder M 2009 RNA-Seq: a revolutionary tool for transcriptomics. *Nature reviews. Genetics* **10** 57-63.

Warde-Farley D, Donaldson SL, Comes O, Zuberi K, Badrawi R, Chao P, Franz M, Grouios C, Kazi F, Lopes CT, Maitland A, Mostafavi S, Montojo J, Shao Q, Wright G, Bader GD & Morris Q 2010 The GeneMANIA prediction server: biological network integration for gene prioritization and predicting gene function. *Nucleic Acids Research* **38** W214-W220.

Watanabe K, Hasegawa Y, Yamashita H, Shimizu K, Ding Y, Abe M, Ohta H, Imagawa K, Hojo K, Maki H, Sonoda H & Sato Y 2004 Vasohibin as an endothelium-derived negative feedback regulator of angiogenesis. *Journal of Clinical Investigation* **114** 898-907.

Wickliffe KE, Williamson A, Meyer HJ, Kelly A & Rape M 2011 K11-linked ubiquitin chains as novel regulators of cell division. *Trends in Cell Biology* **21** 656-663.

Will CL & Lührmann R 2011 Spliceosome structure and function. *Cold Spring Harbor Perspectives in Biology* **3** a003707.

Wiltbank MC, Belfiore CJ & Niswender GD 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 MC, Diskin MG, Flores JA & Niswender GD 1990 Regulation of the corpus luteum by protein kinase C. II. Inhibition of lipoprotein-stimulated steroidogenesis by prostaglandin F2 alpha. *Biology of Reproduction* **42** 239-245.

Woad KJ, Hammond AJ, Hunter M, Mann GE, Hunter MG & Robinson RS 2009 FGF2 is crucial for the development of bovine luteal endothelial networks *in vitro*. *Reproduction* **138** 581-588.

Woad KJ, Hunter MG, Mann GE, Laird M, Hammond AJ & Robinson RS 2012 Fibroblast growth factor 2 is a key determinant of vascular sprouting during bovine luteal angiogenesis. *Reproduction* **143** 35-43.

Woad KJ & Robinson RS 2016 Luteal angiogenesis and its control. *Theriogenology* **86** 221-228.

Woolard J, Wang WY, Bevan HS, Qiu Y, Morbidelli L, Pritchard-Jones RO, Cui TG, Sugiono M, Waine E, Perrin R, Foster R, Digby-Bell J, Shields JD, Whittles CE, Mushens RE, Gillatt DA, Ziche M, Harper SJ & Bates DO 2004 VEGF165b, an inhibitory vascular endothelial growth factor splice variant: mechanism of action, in vivo effect on angiogenesis and endogenous protein expression. *Cancer Research* **64** 7822-7835.

Woolf EC, Curley KL, Liu Q, Turner GH, Charlton JA, Preul MC & Scheck AC 2015 The ketogenic diet alters the hypoxic response and affects expression of proteins associated with angiogenesis, invasive potential and vascular permeability in a mouse glioma model. *PLOS ONE* **10** e0130357.

Wu H, Min J, Zeng H & Plotnikov AN 2008 Crystal structure of the methyltransferase domain of human TARBP1. *Proteins: Structure, Function, and Bioinformatics* **72** 519-525.

Xu Z, Garverick HA, Smith GW, Smith MF, Hamilton SA & Youngquist RS 1995 Expression of follicle-stimulating hormone and luteinizing hormone receptor messenger ribonucleic acids in bovine follicles during the first follicular wave. *Biology of Reproduction* **53** 951-957.

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.

Yamashita H, Murayama C, Takasugi R, Miyamoto A & Shimizu T 2011 BMP-4 suppresses progesterone production by inhibiting histone H3 acetylation of StAR in bovine granulosa cells in vitro. *Molecular and Cellular Biochemistry* **348** 183-190.

Yeo G, Holste D, Kreiman G & Burge CB 2004 Variation in alternative splicing across human tissues. *Genome Biology* **5** R74.

Yeo M, Lin PS, Dahmus ME & Gill GN 2003 A novel RNA polymerase II C-terminal domain phosphatase that preferentially dephosphorylates serine 5. *The Journal of Biological Chemistry* **278** 26078-26085.

Zalman Y, Klipper E, Farberov S, Mondal M, Wee G, Folger JK, Smith GW & Meidan R 2012 Regulation of angiogenesis-related prostaglandin F2alpha-induced genes in the bovine corpus luteum. *Biology of Reproduction* **86** 92, 91-10.

Zeleznik AJ & Hillier SG 1984 The role of gonadotropins in the selection of the preovulatory follicle. *Clinical Obstetrics and Gynecology* **27** 927-940.

Zetser A, Bashenko Y, Edovitsky E, Levy-Adam F, Vlodavsky I & Ilan N 2006 Heparanase induces vascular endothelial growth factor expression: Correlation with p38 phosphorylation levels and src activation. *Cancer Research* **66** 1455.

Zhang SX, Wang JJ, Gao G, Parke K & Ma J-x 2006 Pigment epithelium-derived factor downregulates vascular endothelial growth factor (VEGF) expression and inhibits VEGF–VEGF receptor 2 binding in diabetic retinopathy. *Journal of Molecular Endocrinology* **37** 1-12.

Zhao S, Fung-Leung W-P, Bittner A, Ngo K & Liu X 2014 Comparison of RNA-Seq and Microarray in Transcriptome Profiling of Activated T Cells. *PLOS ONE* **9** e78644.

Zhu BT & Conney AH 1998 Functional role of estrogen metabolism in target cells: review and perspectives. *Carcinogenesis* **19** 1-27.

6. Appendix.

6.1. 10mM citrate buffer.

- Stock A citric acid buffer: 4.2g citric acid in 200ml dH_2O.
- Stock B citrate buffer: 14.7g sodium citrate in 500ml dH_2O.
- 10 mM citrate buffer: 16ml stock A, 84ml stock B and 900ml dH_2O. pH checked to pH 6.0

6.*2. Sample buffer.*

- 25ul of β-mecaptoethanol was added to 475ul of Laemmli buffer (Bio-Rad).

6.3. Stripping buffer.

- 7.5 g glycine
- 0.5 g SDS
- 5 ml Tween20
- 450ml RO water
- Adjust pH to 2.2
- Bring volume up to 500ml with RO water

6.4. Endothelial cell media.

Endothelial cell media (ECM-basic).
The following was added to 500ml EBM-2 bottle:

- 5ml of 500ng/ml LH
- 5ml of 100x ITS (Sigma)
- 5ml of 100x Penicillin and Streptomycin (Sigma)

Endothelial cell media complete (ECM-complete).
To 500ml of ECM basic added the following as per manufacturer's instructions:

- 500µl hEGF
- 500µl GA-1000
- 500µl heparin
- 500µl LR3-IGF-1
- 500µl ascorbic acid
- 200µl hydrocortisone
- 10% v/v FBS (Fetal bovine serum)

ECM-complete supplemented with FGF2 and VEGFA.
To ECM-complete add:

- 100µl (1µg/ml) hVEGFA per 100ml
- 400µl (250ng/ml) FGF2 per 100ml

6.5. ELISA buffer (0.1M Phosphate buffered saline containing 0.1% BSA, pH 7).

- Add 2 PBS tablets to 1L of dH_2O.
- Add 1g of bovine serum albumin.
- Check pH and alter as necessary.

6.6. Coating buffer (Sodium bicarbonate buffer 0.05M, pH 9.6).

- 1.59g Na_2CO_3
- 2.93g $NaHCO_3$

Make up to 1L with distilled water, check pH and alter if necessary.

6.7. ELISA block buffer (PBS + 1% BSA).

- Dissolve 1 PBS tablet in 500ml of dH_2O, add 5g BSA.