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Regulation of thecal endothelial cell function by growth factors in ruminants

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RÉSUMÉ

Cette étude a été réalisée afin d'étudier les effets de BMP4 et de TGFβ1 sur l'expression de gènes et de protéine des cellules endothéliales de la thèque chez l'ovin. Les ovaires ovins ont été obtenus à partir de brebis adultes, quel que soit le stade du cycle œstral, et les cellules endothéliales de la thèque ont été isolées à l'aide de la technologie pluriSelect et cultivées. Après exposition à BMP4 et à TGFβ1 pendant 24 heures, les acides ribonucléiques totaux (ARN) ont été isolés pour une analyse quantitative de la réaction en chaîne de la polymérase en temps réel (qRT-PCR) de gènes de rôles bien connus dans la prolifération des cellules endothéliales, la formation et/ou la perméabilité vasculaire. Une analyse protéomique a également été réalisée à l'aide de la spectrométrie de masse après exposition de cellules endothéliales de la thèque à BMP4 pendant 48h. Les résultats montrent que BMP4 et TGFβ1 régulent les gènes liés à la fonction endothéliale, incluant le facteur de croissance des fibroblastes 18 et l'endothéline-1. L'analyse par spectrométrie de masse a identifié 1488 protéines totales dont 28 protéines sont significativement régulées positivement (> 2 fois) et 29 protéines régulées négativement (<0,5 fois). L'analyse de l'interactome, réalisée à partir de deux bases de données biochimiques et biologiques distinctes, a mis en évidence un degré élevé d'interactions génétiques et physiques entre les voies de signalisation TIMP1, JUN, STAT3 et CD63. Ces observations ont été validées par une analyse par STRING. En conclusion, ces résultats montrent que l'administration exogène de BMP4 ou de TGFβ1 induit des modifications du protéome de la cellule endothéliale de la thèque chez l'ovin. Cependant, la présente étude ne portait que sur des changements d'expression des gènes liés à la fonction endothéliale. Des travaux additionnels seront nécessaires afin d'évaluer le transcriptome, grâce au séquençage d'ARN des cellules endothéliales primaires de

la thèque ovine pour identifier d'autres gènes susceptibles d'être régulés par la signalisation du TGF.

Mots-clés: facteur de croissance transformant β, ovaire ovin, cellules endothéliales, facteur de croissance fibroblastique de type 18, protéine osseuse morphogénétique.

ABSTRACT

This study was performed to investigate the effects of BMP4 and TGFβ1 on ovine thecal endothelial cell gene and protein expression. Ovine ovaries were obtained from adult ewes irrespective of stage of estrous cycle, and thecal endothelial cells were isolated using pluriSelect technology, and cultured. Following exposition to BMP4 and TGFβ1 for 24h, total ribonucleic acid (RNA) was isolated for quantitative real-time polymerase chain reaction (qRT-PCR) analysis of genes with well-validated roles in endothelial cell proliferation, vascular formation and/or permeability. Proteomic analysis was also undertaken using mass spectrometry following exposure of ovine thecal endothelial cells to BMP4 for 48h. The results show that BMP4 and TGFβ1 regulated genes related to endothelial function, including fibroblast growth factor 18 and endothelin-1. Mass spectrometry analysis identified 1488 proteins in total, with significant up-regulation (>2- fold) of 28 proteins and down-regulation (<0.5-fold) of 29 proteins. Interactome analysis, by two distinct biochemical and biological databases, identified a high degree of genetic and physical interactions between TIMP1 and JUN, STAT3, CD63 signaling pathways. These observations were validated by a STRING analysis. In conclusion, the results presented herein show that exogenous administration of BMP4 or TGFβ1 induces changes in the ovine thecal endothelial cell proteome. However, the current study only involved investigation of expression changes in genes related to endothelial function. Future work assessing the transcriptome using RNA sequencing in primary ovine theca ECs is required to identify other genes that may be regulated by TGF signaling.

Key words: Transforming growth factor β, ovine ovary, endothelial cells, fibroblast growth factor 18, bone morphogenetic protein.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Actc1	Actin, Alpha, Cardiac Muscle 1
ActR-1A	Type 1A activin receptor
ActR2	Type 2 activin receptor
ActR2B	Type 2B activin receptor
AKAP3	A-kinase anchoring protein 3
ATF	Activating transcription factor
BCL2L13	B cell lymphoma 2 like 13
BMPR-1A or ALK3	Type 1A BMP receptor
BMPR-1B or ALK6	Type 1B BMP receptor
BMPR2	Type 2 BMP receptor
BMPs	Bone morphogenetic proteins
BRAM1	Bone morphogenetic protein receptor associated molecule 1
BSA	Bovine serum albumin
CBP	CREB-binding protein
CGCs	Cumulus granulosa cells
DAG	Diacylglycerol
DBN1	Drebrin 1
DNA	Deoxyribonucleic acid
E1A	Early region 1A
ECs	Endothelial cells
ED	Embryonic day
ERK	Extracellular signal-regulated kinase

ESI	Electrospray ionization
FBLN1	Fibulin-1
FGF	Fibroblast growth factors
FGFRs	FGF receptors
FRS2α	FGFR substrate 2α
FSH	Follicle-stimulating hormone
FWHM	Full width at half maximum
GAB1	GRB2-associated binding protein 1
GCs	Granulosa cells
GDF	Growth differentiation factors
GDF-9	Growth differentiation factor-9
GDNFs	Glial derived neurotrophic factors
GNB4	G protein subunit beta 4
GPX7	Glutathione peroxidase
GRB2	Guanine nucleotide exchange factor 2
GREL	Gonadal ridge epithelial-like
GS	Glycine-serine
hESCs	human embryonic stem cells
Hoxc	Homeobox gene c
HPLC	High performance liquid chromatography
HRAM	High-resolution and accurate-mass
HSD3B	3β-hydroxysteroid dehydrogenase
IGF-1	Insulin-like growth factor I

IGFBP6	**Insulin like growth factor binding protein 6**
IGFBP7	**Insulin-like growth factor-binding protein-7**
IP3	**Inositol triphosphate**
iPSCs	**induced pluoripotent stem cells**
JNK	**C-jun N-terminal kinase**
KGF	**Keratinocyte-growth factor**
LAP3	**Leucine aminopeptidase 3**
LH	**Luteinizing hormone**
LHCGR	**luteinizing hormone/choriogonadotropin receptor**
LIF	**leukemia inhibitory factor**
LMNB@	**Lamin B2**
MAPK	**Mitogen activated protein kinase**
MARCKSL1	**MARCKS like 1**
MGCs	**Mural granulosa cells**
MKP1	**MAP kinase phosphatase**
MS	**Mass spectrometry**
MSG	**Melanocyte-specific gene or mad-supporting gene**
NF-κB	**Nuclear factor kappa beta**
NGF	**Nerve growth factors**
OAZ	**Olf1/EBF associated zinc finger**
PBS	**Phosphate-buffered saline**
PCSK	**Proprotein convertase subtilisin/kexin**
PEBP	**Polyomavirus-enhancer-binding protein**

PGCs	Primordial germ cells
PHB2	Prohibitin 2
PI3	Phosphoinositide 3
PIP2	Phosphatidylinositol (4,5)-bisphosphate
PKC	Protein kinase C
PLCγ	Phospholipase C gamma
PTB	Phosphotyrosine binding
RNA	Ribonucleic acid
R-SMAD	Receptor-regulated SMADs
SCF	Stem cell factor
SH2	Src homology-2
SHP2	Tyrosine phosphatase
SIP	Smad-interacting protein
SNIP	Smad nuclear interacting protein
SOS	Son of sevenless
SSXS	C-terminal Ser-Ser-X-Ser
STAT3	Signal transducers and activators of transcription
TAB1	TAK1 binding protein
TAK1	TGF-β activated kinase 1
TBCB	Tubulin folding cofactor B
TCs	Theca cells
TFA	Trifluoroacetic acid
TFE	Transcription factor µ E3

TGFBI	**Transforming growth factor beta induced**
TGF- β	**transforming growth factor-β**
TGM2	**Transglutaminase 2**
TIMP1	**Metalloproteinase inhibitor 1**
TMOD	**Tropomodulin**
VEZF1	**Vascular endothelial zinc finger 1**
XIAP	**X-linked inhibitor of apoptosis protein**

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INTRODUCTION

The development of the ovarian follicle is a highly complex process that involves physiological actions of multiple endocrine and paracrine signaling pathways (Price 2016). The role of the pituitary gonadotrophins, the main endocrine drivers, in various stages of follicular development is well-established (Price 2016). Indeed, the current methods available to regulate ovarian function in order to suppress or enhance fertility ultimately influence the hypothalamic–pituitary–ovarian axis (Apter 1997). In recent years, considerable attention has been focused on the paracrine signaling pathways between the oocyte and its adjacent somatic cells, namely the cumulus, granulosa and thecal cells. In specific, it has become increasingly evident that intraovarian growth factors, such as the members of transforming growth factor (TGF)-β super family, have potential roles as local regulators of ovarian function and fertility (Juengel and McNatty 2005).

The TGF-β superfamily is a structurally conserved, but functionally diverse, group of proteins with at least 35 members in vertebrates, and regulates a variety of biological processes in mammals by influencing cell proliferation, growth, differentiation, and apoptosis (Pangas 2007). The members of this TGF-β superfamily have been further classified into several subfamilies, including the TGF-β, bone morphogenetic protein (BMP), the growth and differentiation factor (GDF), the activin/inhibin, the glial cell-derived neurotrophic factor (GDNF), as well as the anti-Mullerian hormone (AMH) and nodal (Knight and Glister 2006). Research over the past two decades has revealed extremely important oocyte-derived paracrine (and possibly autocrine) roles for GDF9, BMP15 and BMP6 in the regulation of

oocyte maturation and cumulus and granulosa cell function (Knight and Glister 2006).

Previous work in our laboratory has shown a pro-apoptotic role for fibroblast growth factor (FGF)-18 in bovine granulosa cells (Portela et al. 2010, Portela et al. 2015). Furthermore, recent immunohistochemical studies have identified FGF18 protein in the small blood vessels of theca layer and the medulla of sheep ovary, and FGF18 messenger RNA (mRNA) and protein in ovine thecal endothelial cells following bone morphogenetic protein (BMP)-4 treatment in vitro (unpublished data). These data suggest a plausible crosstalk signaling between the endothelial cells and the theca and/or granulosa cells. This study was performed to investigate the effects of BMP4 and TGFβ1 on ovine thecal endothelial cell gene and protein expression. Recognition of the central role of intraovarian growth factors in controlling the follicular development and ovulation rate may lead to a paradigm shift in our understanding of the ovarian function. In addition, this information may form the basis for the development of new therapeutics for regulating fertility in mammals.

CHAPTER 1: LITERATURE REVIEW

1. Literature Review

1.1. Sheep Ovary

The ovaries are the female gonads that are located in the pelvic area of the ewe's reproductive system (Bartlewski et al. 2011). The ovarian development begins with the differentiation of gonadal ridge epithelial-like (GREL) cells from mesonephric epithelial cells (Smith et al. 2014). The primordial germ cells (PGCs) migrate from the endoderm of the yolk sac, through the developing hindgut and dorsal mesentery, to the developing gonads. This migration of the PGCs has been established to occur between embryonic day (ED) 17 to 21 in the ewe (Ledda et al. 2010). The ovary is apparent as a thickening of the coelomic epithelium on the medial aspect of the mesonephros around ED 22 in sheep (Smith et al. 2014). The gonadal primordia form a paired thickening of the coelomic epithelium and arise within the intermediate mesoderm between the pronephros (initial kidney) and metanephros (the definitive kidney) between ED 34– 38 in sheep (Oktem and Oktay 2008a, Smith et al. 2014).

Follicles are the functional units of the ovary and each follicle consists of an oocyte (germ cells) surrounded by one or more layers of somatic cells, viz. granulosa, theca and stromal cells. The complex cascade of interactions between these cells regulate the formation of follicles and development of oocytes/somatic cells (Richards and Pangas 2010). The two primary reproductive functions of the ovary include gametogenesis and endocrine function (Barnett et al. 2006). Gametogenesis is the process responsible for the differentiation and release of a mature oocyte for fertilization (Barnett et al. 2006). The endocrine role involves ovarian hormonal and growth factors production which is essential for follicle development, and maintenance of estrous cycle and the normal function of the reproductive tract (Barnett et al. 2006).

19

1.2 Oocyte

The oocyte is the female germ cell that is produced in the ovary by the process of female gametogenesis (Sigel and Minier 2005). Prior to fertilization, the number of oocytes in the mammalian ovary is fixed early in life. The development of an oocyte begins with the transformation of the PGCs to oogonia (Voronina and Wessel 2003). The oogonia subsequently differentiate into primary oocytes, through meiosis by replicating their deoxyribonucleic acid (DNA), and arrests at the diplotene stage of the first prophase with homologous chromosomes (Matova and Cooley 2001). Thus, at birth all oocytes are arrested in the stage of prophase I. The prophase oocyte then may spend various periods of time in the arrested state until puberty and the onset of follicle development (Voronina and Wessel 2003). In this protracted period, the oocyte accumulates an extensive collection of ribonucleic acid (RNAs), proteins, and organelles such as cortical granules, ribosomes, and mitochondria (Liu 2011, Conner et al. 1997).

Meiosis resumes in preovulatory follicles in response to a surge of luteinizing hormone (LH) from the pituitary gland, which ultimately induces rupture of the follicle, release of the oocyte and oocyte maturation (Mehlmann 2005). Oocyte maturation is a dynamic and highly coordinated process, in which the oocytes finish the first meiotic division, undergoes cytoplasmic alteration and progress to metaphase II (Mehlmann 2005). The first meiotic division ends with the emergence of two haploid cells, the oocyte and the first polar body (Mehlmann 2005). Subsequently, the oocytes become arrested at metaphase II and await the process of fertilization. The development and maturation of oocytes are tightly regulated by actions of several growth hormones (reviewed in detail elsewhere (Voronina and Wessel 2003, Smith et al. 2014, Webb et al. 2016)).

1.3 Granulosa cells

The granulosa cells (GCs) are vital for oocyte maturation as they provide nutrients that support further development (Albertini et al. 2001). During folliculogenesis, the GC layer separates into two anatomically and phenotypically different subtypes, the cumulus granulosa cells (CGCs) and the mural granulosa cells (MGCs) (Mcnatty et al. 1979). The CGCs are in direct contact with the oocyte, have a high rate of proliferation, low steroidogenic capacity, low or absent luteinizing hormone (LH) receptor (LHCGR) expression and express high levels of insulin-like growth factor I (IGF-1) (Makabe et al. 2006, Gilchrist et al. 2004). In contrast, the MGCs have a primary endocrine function and support follicular growth, and undergo terminal differentiation to luteal cells following ovulation (Makabe et al. 2006, Gilchrist et al. 2004).

The interaction between oocytes and CGCs, via gap junctions containing connexins 32, 43 and 45 (Gilchrist et al. 2004), is essential for normal growth and development of both the oocyte and the follicles, and plays a key role in disseminating local and endocrine signals (Gilchrist et al. 2004). Small molecules, such as ions, metabolites, and amino acids, are transported via the gap junctions, whereas larger molecules are transported by receptor-mediated endocytosis (Gilchrist et al. 2004, Senbon et al. 2003). Specifically, connexin 43 has been demonstrated to be essential for continued follicular growth and GC proliferation (Ackert et al. 2001). Indeed, mutations in the gene encoding connexin 43 leads to retarded oocyte growth and poor development of the zona pellucida (Ackert et al. 2001). The GCs produce sex steroids and numerous growth factors that interact with the oocyte during its development. The sex steroid production consists of the conversion of androgens produced by theca cells to estradiol by the aromatase enzyme (CYP19A1) under the

control of follicle- stimulating hormone (FSH) (Gilchrist et al. 2004). However, after ovulation the GCs differentiate into luteinized cells that produce progesterone (Erickson et al. 1985).

1.4   Theca cells

The theca consists of two layers–the outer layer called the theca externa and the inner layer known as the theca interna–both of which originate from mesenchymal cells (Gougeon 1996). Theca cells (TCs) are highly differentiated with structural features characteristic of steroid-secreting cells, with abundant mitochondria, agranular endoplasmic reticulum and lipid vesicles (Magoffin 2005).

The TCs are endocrine cells that play essential roles within the ovary by producing androgens under the control of LH (Adams et al. 2008). The hyperactivity of theca cells causes infertility due to hyperandrogenism and lack of estrogen (Magoffin 2005). Androgens are required for ovarian estrogen biosynthesis, and provide structural support for the growing follicle as it progresses through various developmental stages (Hillier et al. 1994). Unlike the GC layers, the TC layers are highly vascularized and this vascularization provides the rest of the follicle with essential nutrients and endocrine hormones from the pituitary axis (Young and McNeilly 2010).

The TCs are not associated with the primordial follicle, but only appear in the early secondary stage of follicular development (Magoffin 2005). Hence, it was hypothesized that the differentiation of TCs was stimulated by chemogenic signals originating from the growing follicles. Early work proposed the involvement of small-molecular-weight proteins secreted by the GCs to stimulate the differentiation of the TCs (Magoffin and Magarelli 1995). It is now believed that intraovarian growth factors such as insulin-like growth factor (IGF)-I and/or -II, growth

differentiation factor-9 (GDF-9), stem cell factor (SCF), and follistatin may contribute to thecal recruitment and differentiation (Magoffin 2005, Webb et al. 2016, Smith et al. 2014).

1.5 Stromal Cells

Ovarian stroma is primarily composed of peculiar spindle-shaped stromal cells which are similar to fibroblasts and are arranged into a characteristic whorled texture or storiform pattern (Furuya, 2012). The ovarian stroma is divided into cortex and medulla. In addition to the spindle shaped cells, they also contain other cell types including the polygonal-shaped luteinized stromal cells, decidualized cells in pregnant women, endometrial stromal-type cells, smooth muscle cells, fat cells, and stromal Leydig cells (Weidner et al., 2009). The ovarian stroma surrounding the follicle differentiates into theca interna and theca externa cells. Unlike any other connective tissue, ovarian stroma is highly cellular and vascular, while the supporting fibers (both reticular fibers and ordinary collagen) are inconspicuous (Furuya, 2012). The interstitial cells of the ovarian stroma possess steroidogenic enzymes, and secrete androgens and progesterone (Weidner et al., 2009). Therefore, stromal cells of adult ovary actively contribute to the synthesis and remodeling of extracellular matrix and blood vessels (Weidner et al., 2009). In adults, the ovarian stroma demonstrates active tissue remodeling during and after ovulation.

1.6 The ovarian follicle: classification, structure and folliculogenesis

The ovarian follicles are the basic structural and functional unit of the ovary which ensures proper oocyte development and maturation (Webb et al. 2016). Folliculogenesis, the growth and development of ovarian follicles from primordial to pre-ovulatory, is a complex cascade of events dependent on interactions between the oocyte and the somatic cells (Barnett et al. 2006). The structure of mammalian ovarian follicles changes during development and is classified as primary,

secondary, pre-antral, antral and pre-ovulatory follicles (Figure 1) according to their size and structure (Webb et al. 2016).
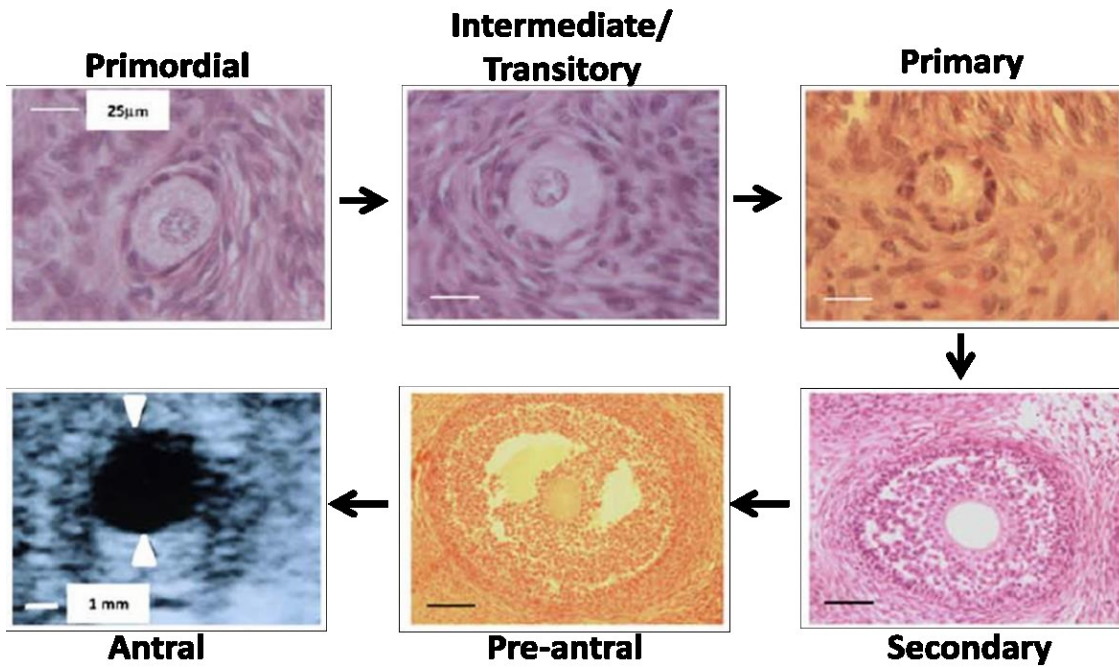
Figure 1. Stages of ovine ovarian follicular development. Adapted and redrawn from Bartlewski et al (2011). Primordial follicle is an oocyte surrounded by partial or complete layer of squamous follicular cells. In intermediate/transitory state, the follicles contain both squamous and cuboidal follicular cells. Primary follicle has a single layer of cuboidal granulosa cells. Secondary follicle has two or more layers of cuboidal granulosa cells and a small number of theca cells. In pre-antral phase, the follicles have antral cavity, filled with follicular fluid, and distinctive layers of theca cells separated from granulosa cells. Antral or Graafian follicle is the last stage of follicle development. Antral follicles are larger, have more antral fluid and may contain a secondary oocyte.

The process of folliculogenesis is systematically controlled not only by the actions of the pituitary gonadotropins, FSH and LH, but also by other hormones and growth factors (Barnett et al. 2006). Thus, the stages of follicular development are also classified depending upon their response to gonadotropins. These include, early gonadotropin- independent (primordial to early pre-antral), gonadotropin-responsive (pre-antral to small antral) and gonadotropin-dependent (antral to large antral) stages (Webb et al. 2016).

The process of folliculogenesis begins with the recruitment of the dormant primordial follicles. However, the mechanism underlying the activation of the resting primordial follicles remains to be elucidated (Oktem and Oktay 2008b). The resting primordial follicles are formed during fetal development in ruminants, with the first set of follicles formed around 70 days of gestation in sheep (Evans 2003). It has been estimated that the lamb ovary contains ~100,000 – 200,000 follicles at birth, with 50 antral follicles present in the ovaries of an adult sheep (Land 1970, Driancourt 1991). Hence, the proportion of primordial follicles that undergo folliculogenesis and reach the antral stage is very low as most of the follicles undergo regression and atresia (Webb et al. 2016).

The transition of the quiescent primordial follicles into primary and secondary follicles involves orchestrated communication between the oocytes and the somatic cells, as well as a multitude of extra-cellular components and growth factors (Oktem and Urman 2010). These include anti-Müllerian hormone, bone morphogenetic proteins (BMPs), growth differentiation factors

(GDF), leukemia inhibitory factor (LIF), nerve growth factors (NGF), fibroblast growth factors (FGF) and keratinocyte-growth factor (KGF) among others (Oktem and Urman 2010, Oktem and Oktay 2008b). The primary follicles are characterized by a single layer of cuboidal granulosa cells (GCs) surrounding the oocyte, whereas secondary follicles have two or three layers of cuboidal GCs (Bartlewski et al. 2011). Importantly, it is established that FSH is not required for transition of the primordial follicles, since they do not express FSH receptors (Oktay et al. 1997, Rannikki et al. 1995).

The progression of the primary follicle to the pre-antral stage is a continuous process. The pre- antral phase is characterized by zona pellucida formation, GC proliferation, recruitment of thecal cells to the follicular basal lamina and a dramatic increase in oocyte volume (Duranthon and Renard 2001). Like the primordial follicle transition phase, the pre-antral phase is also believed to be a gonadotropin- independent process (Oktay et al. 1998). Further progression of the developing pre-antral follicle to antral phase is characterized by the formation of the antrum (central liquid-filled cavity) as well as increased vascularization, oocyte growth, and the formation of a fibrous layer around the theca interna (Duranthon and Renard 2001). In contrast to during the pre-antral phase, FSH becomes a critical determinant during antral follicle growth whereas subsequent follicular development is independent of FSH concentrations (Duggavathi et al. 2005). Finally, the pre-ovulatory follicle (Figure 2) consists of an oocyte surrounded by the zona pellucida, several layers of cumulus cells, an antrum, and a basal lamina that separates mural GCs from the theca cells (Smith et al. 2014).
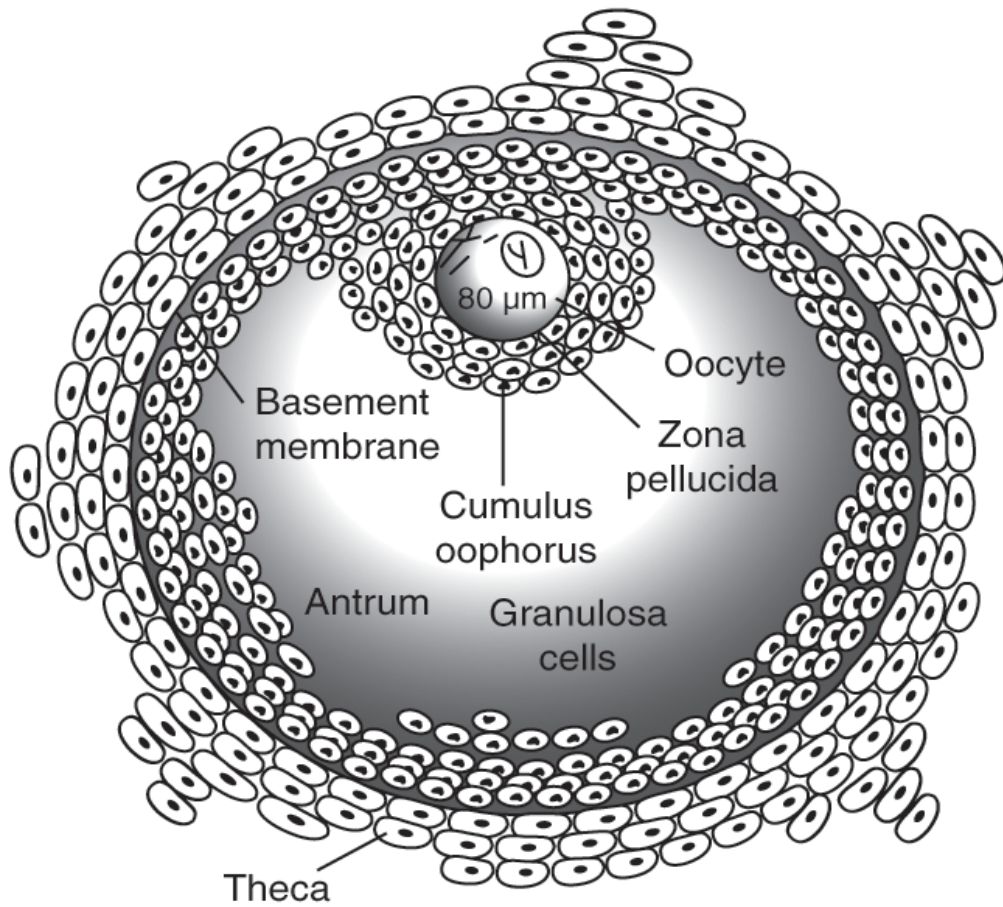
Figure 2: Schematic representation of a pre-ovulatory mammalian follicle. Shown in the diagram are the various cell types of a pre-ovulatory follicle (Kase 2013).

The growth of ovarian follicles exhibits a distinct wave-like pattern at all stages of the breeding season and throughout seasonal anoestrus in ewes (Bartlewski et al. 2011) as well as in non- seasonal breeders including cattle and humans. There are typically 3 or 4 waves of follicle emergence per inter-ovulatory interval (Evans 2003). This characteristic pattern of follicular development has been associated with temporal fluctuations in the levels of FSH and oestradiol (Cahill and Mauleon 1980a, Webb et al. 2016). It has been estimated that the period of follicular growth from the primordial to the pre-ovulatory stage in ewes exceeds 6 months (Cahill and Mauleon 1980a). Specifically, the growth from the primordial to the early preantral stage takes about 130 days, and it takes a further 35 – 55 days to reach the pre-ovulatory stage (Cahill and Mauleon 1980b). Thus, the ovine primordial follicles undergo sequential growth and development, under the control of endocrine and paracrine factors, forming pre-ovulatory follicle containing a mature oocyte.

1.7. Follicular selection, dominance and atresia

The selection process during a follicular wave is characterized by the continued growth of a developing 'dominant' follicle and reduced growth of the subordinate follicles (Aerts and Bols 2010b, a). Although the exact mechanisms of the dominant follicular selection remain to be elucidated, it has been suggested that the selected dominant follicle exhibits increased expression of FSH receptors, luteinizing hormone/choriogonadotropin receptor (LHCGR) and 3β- hydroxysteroid dehydrogenase (HSD3B) in the GCs (Scaramuzzi et al. 2011). This in turn has been proposed to facilitate the follicle's responsiveness to LH and survival in the face of lower FSH concentrations (Scaramuzzi et al. 2011).

A functionally dominant follicle has the ability to inhibit the development of other competing follicles within both ovaries while continuing to thrive itself (Fortune 1994). The dominant follicles express high oestradiol, low progesterone, and LH and FSH receptors on the GCs (Driancourt 1991). Thus, there is a marked hierarchy between the cohort of activated follicles in terms of diameter and steroid production, resulting in dominance (Driancourt 1991). Furthermore, the emergence of a follicular wave occurs only after the dominant follicle diminishes its inhibitory effect on other follicles, subsequent to an increase in FSH concentrations (Adams et al. 1992). Removal of the dominant follicle has been shown to delay the regression of the largest subordinate follicle and/or induce early emergence of the next follicle wave (Ko et al. 1991, Adams et al. 1993).

More recent work has also attributed a role for insulin growth factor (IGF) signalling system as a 'switch' for follicular dominance (Webb and Campbell 2007, Scaramuzzi et al. 2011). It has been hypothesized that follicular dominance arises because one follicle first acquires proteolytic activity against the IGF binding proteins-4 and -5 before its subordinate follicles, resulting an increase in free IGF and oestradiol (Scaramuzzi et al. 2011). The resultant free IGF may then amplify the effects of FSH, facilitating an increased oestradiol production and decreased circulatory FSH (Webb and Campbell 2007, Scaramuzzi et al. 2011).

The above description describes best follicle dynamics in mono-ovulatory species such as cattle and humans. In the di- and tri-ovulatory sheep, there is broad agreement that the ovulatory follicles exerts dominance over other follicles during the follicular phase (Evans et al. 2000), however, contradictory reports exists for this phenomena during the luteal phase (Evans et al.

2000). In sheep, follicles in a subsequent wave has been demonstrated to emerge after the largest follicle in the previous wave had stopped growing (Bartlewski et al. 1999, Evans et al. 2000).

The process of folliculogenesis is also tightly regulated by crosstalk between cell death and survival signals (Webb et al. 2016). Throughout life, there is continous depletion of the ovarian follicular reserve and only a small proportion of the primordial follicles reach the ovulatory stage, whilst the rest undergo a degenerative process called atresia (Smith et al. 2014). Generally, atresia can occur at any stage of follicular development and is accompanied by a series of changes in steroidogenesis and morphology (Smith et al. 2014). It is believed that the process of atresia is initiated by loss of growth factors such as tumour growth factor (TGF)-α or FSH, and/or expression of death factors such as Fas ligand (Barnett et al. 2006).

1.8 Bone Morphogenetic Proteins

The bone morphogenetic proteins (BMPs) are part of the transforming growth factor-β (TGF-β) superfamily of proteins, which includes TGF-βs, activins, inhibins, GDFs, glial derived neurotrophic factors (GDNFs) and anti-Müllerian hormone (Wang et al. 2014). The TGF-β superfamily members have a wide array of functions in the body including regulation of cell proliferation/apoptosis, differentiation, migration, invasion and extracellular matrix production (Wang et al. 2014).

The physiological relevance of the BMPs was first identified in the 1960s when its role in the induction of ectopic bone formation was discovered (Urist 1965). Since then, BMP-dependent signalling has been shown to play a significant role in cell growth, apoptosis and differentiation, adult tissue homeostasis (i.e., fracture repair, vascular remodeling etc.), and bone and cartilage formation (Bragdon et al. 2011). To date, over 20 members of phylogenetically conserved BMPs

have been identified (Lochab and Extavour 2017a). These include BMP 2/4, BMP 5/6/7/8a/8b, BMP 9/10, and BMP 12/13/14 subgroups based on phylogenetic analysis (Ali and Brazil 2014).

1.8.1 Biological functions of BMP in reproductive system

The expression of BMP system in the ruminant ovary is well-documented. The mRNA for *BMP2*, *BMP4*, *BMP6*, *BMP7*, *BMP15* have been detected in the ovaries of sheep and cattle (Souza et al. 2003, Pierre et al. 2004, Glister et al. 2005). The expression of BMP receptors, BMPR-1A and BMPR-1B, and that of downstream signaling proteins, Smad1/2, has been reported in ovine oocytes, GCs and TCs (Souza et al. 2002, Pierre et al. 2004). The importance of BMPs in the reproductive physiology of sheep is further attested by the phenotypes of mutant ewes (*FecX, FecG and FecB*) which involve mutations in BMPR genes (Souza et al. 2002, Pierre et al. 2004).

The physiological functions in the reproductive system are exerted by tissue-specific expression of members of the BMP family (Shimasaki et al. 2004). A significant role for BMP signaling in the establishment of the germ line has been well-established (McLaren 1999). Specifically, the role of BMP4 in PGC specification has been demonstrated in mice, axolotl and crickets (Donoughe et al. 2014, Johnson et al. 2003, Ying and Zhao 2001). BMP4, but not BMP7/8, has been shown to induce germ cell differentiation from human embryonic stem cells (hESCs), and promote *in vitro* differentiation of cultured pluripotent stem cells into PGC-like cells from human and ruminant induced pluripotent stem cells (iPSCs) (Malaver-Ortega et al. 2016, Shah et al. 2015, Kee et al. 2006).

BMP signaling pathways have been shown to promote PGC proliferation and/or migration in chickens (Whyte et al. 2015), mice (Dudley et al. 2010, Dudley et al. 2007, Ross et al. 2007, Ying

et al. 2001, Lawson et al. 1999, Ying et al. 2000, Lopes et al. 2004, Tremblay et al. 2001, Chang and Matzuk 2001) and fruit flies (Sato et al. 2010, Gilboa and Lehmann 2004, Deshpande et al. 2014). Several studies have shown the vital role for BMP ligands in regulation of gametogenesis and folliculogenesis in chordates, nematodes and arthropods (Lochab and Extavour 2017a). These include BMP2, BMP4, BMP6, BMP7, BMP8, and BMP15, with specialized sex-specific reproductive roles for BMP6, BMP8 and BMP15 (Sugiura et al. 2010, Ying et al. 2000, Ying et al. 2001, Yan et al. 2001). Taken together, research over the last three decades have elucidated the evolutionarily conserved role for BMPs in PGC specification, PGC proliferation, PGC migration, and gametogenesis across protostome and deuterostome lineages (reviewed in detail elsewhere (Lochab and Extavour 2017b)), indicating the vital role of BMPs in the physiology of reproductive system.

1.8.2 Structure

TGF-β superfamily members are synthesized as precursor proteins comprising an N-terminal signal peptide, a prodomain for folding and secretion, and a C-terminal mature domain (Ali and Brazil 2014). The precursors are formed in the cytoplasm as dimeric pro-protein complexes and are then cleaved by pro-protein convertases to generate the N- and C-terminal fragments (Bragdon et al. 2011, Wang et al. 2014). The proprotein convertase family is composed of nine members called proprotein convertase subtilisin/kexin (PCSK1-PCSK9). A mature BMP protein contains seven cysteine residues, six of which form intramolecular disulfide bonds, also known as cysteine knots. A seventh cysteine amino acid facilitates dimerization with another BMP monomer via a disulfide bond (Ali and Brazil 2014).

1.8.3 Signaling pathways of BMPs

1.8.3.1 SMAD-dependent pathways

The physiological effects of BMPs are mediated via both canonical and non-canonical pathways. In the canonical signaling pathway, the BMPs initiate the signal transduction cascade by binding to cell surface receptors and forming a heterotetrameric complex (Heldin et al. 1997). The BMPs bind to two cell-surface serine/threonine kinase receptors, TGF-β type I and type II receptors. Amongst the 7 type-I and 5 type II TGF-β receptors, the BMPs are known to bind to type 1A activin receptor (ActR-1A), type 1A BMP receptor (BMPR-1A or ALK3), type 1B BMP receptor (BMPR-1B or ALK6), type 2 activin receptor (ActR2), type 2B activin receptor (ActR2B) and type 2 BMP receptor (BMPR2) (Heldin et al. 1997, Heldin 1997).

Upon formation of heterotetrameric complex, the constitutively active type-II receptor transphosphorylates the type-I receptor at glycine-serine rich motif (Heldin 1997). This in turn allows phosphorylation of downstream substrate proteins known as the receptor- regulated SMADs (R-SMADs) at a C-terminal Ser-Ser-X-Ser (SSXS) motif (Horbelt et al. 2012). The R-SMAD family includes SMAD-1/-5/-8, the co-mediator SMAD (SMAD-4) and the inhibitory SMADs (SMAD-6 and -7) (de Caestecker 2004). The BMP type-I receptors activate the canonical SMAD-1/-5/-8 group, which then associates with SMAD4. This complex translocates into the nucleus to exert functions that regulate gene expression. The inhibitory SMAD-6 and -7 regulate the feedback inhibition of the BMP signaling pathway (Heldin 1997, Heldin et al. 1997, Wang et al. 2014) (Figure 3).

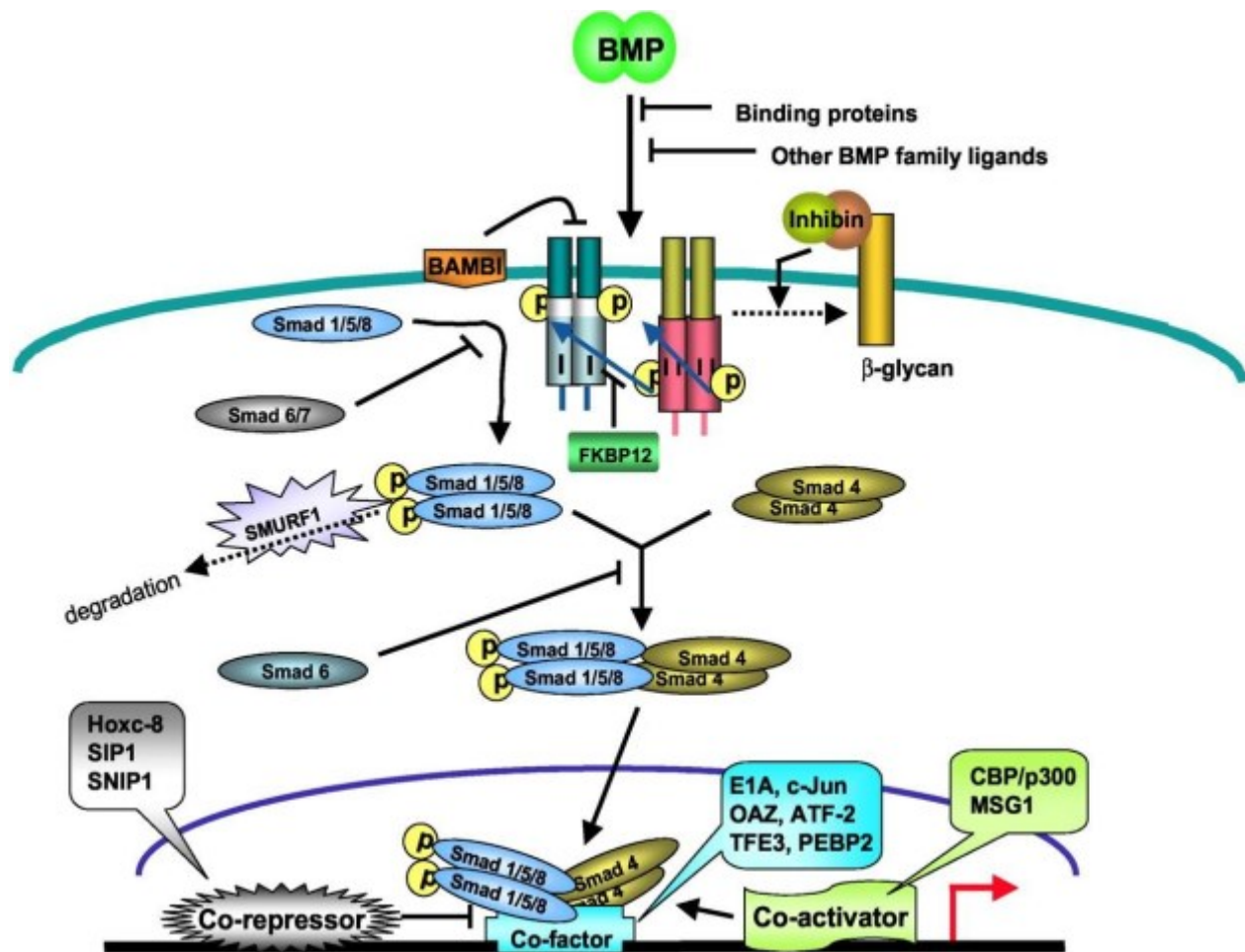**Figure 3. BMP signalling pathways.** ATF, activating transcription factor; CBP, CREB-binding protein; E1A, early region 1A; Hoxc, homeobox gene c; MSG, melanocyte-specific gene or mad-supporting gene; OAZ, Olf1/EBF associated zinc finger; PEBP, polyomavirus-enhancer-binding protein; SIP, Smad-interacting protein; SNIP, Smad nuclear interacting protein; TFE, transcription factor μ E3 (Shimasaki et al. 2004).

1.8.3.2 SMAD-independent signaling

The activation of the type-I BMP receptors may also activate SMAD-independent pathways such as extracellular signal-regulated kinase (ERK), mitogen activated protein kinase (MAPK), C-jun N-terminal kinase (JNK) and nuclear factor kappa beta (NF-κB) (Lochab and Extavour 2017a, Wang et al. 2014). The activation of SMAD-independent pathways is believed to be achieved though protein–protein interactions of bone morphogenetic protein receptor associated molecule 1 (BRAM1), X-linked inhibitor of apoptosis protein (XIAP), TGF-β activated kinase 1 (TAK1) or TAK1 binding protein (TAB1) with the BMP type-I receptors (Oktay et al. 1997, Ornitz and Itoh 2001). BRAM1 directly associates with the cytoplasmic tail of type-I BMP receptors to link it to TAB1 (Chung et al. 2002). On the other hand, XIAP is recruited by type-I BMP receptors to link it with TAB1–TAK1 complex (Lu et al. 2007). It has been suggested that the activation of TAK-1 leads to downstream activation of the p38 MAPK, JNK and NF-κB pathways (Lu et al. 2007).

1.9 Fibroblast Growth Factors

The FGF family consists of structurally related polypeptides involved in several physiologic processes. These include embryonic development, organogenesis, tissue maintenance, repair, regeneration and metabolism (Ornitz and Itoh 2015). At the cellular level, FGF regulates cell proliferation, survival, migration, differentiation and metabolism (Ornitz and Itoh 2015). Genetic studies in mice have shown that mutations leading to disruption of FGF signaling cause a variety of developmental disorders including dominant skeletal diseases, infertility, and cancer (Turner and Grose 2010).

The FGFs exert their physiological roles through binding to one of four FGF receptors (FGFRs; FGFR 1 – 4), which are highly conserved transmembrane tyrosine kinase receptors (Teven et al. 2014). Although a fifth FGF receptor (FGF receptor-like 1; FGFRL1) lacking intracellular tyrosine kinase domain was discovered, its function remains controversial and poorly understood (Steinberg et al. 2010). The FGFRs contain 3 domains–an extracellular ligand-binding domain, a transmembrane domain and an intracellular tyrosine kinase domain (Turner and Grose 2010). The extracellular region contains two or three immunoglobulin (Ig)-like domains, and a heparin- binding domain (Yan et al. 2001). Activation of the FGFRs induces dimerization and phosphorylation of specific cytoplasmic residues, resulting in activation of cytoplasmic signal transduction pathways (Teven et al. 2014).

According to current consensus, the FGF family comprises 22 genes encoding 18 secreted FGF ligands (i.e., FGF1-10 and FGF16-23) (Yun et al. 2010). Four FGF family members, FGF11, FGF12, FGF13, and FGF14, are intracellular proteins that do not bind to FGFR, and are referred to as FGF homologous factors (Yun et al. 2010). Based on phylogenetic analysis and sequence homology, the FGF family can be divided into seven subfamilies, viz. FGF1, FGF4, FGF7, FGF8, FGF9, FGF11, and FGF19, with each containing two to four members (Itoh 2007).

1.9.1 Biological role of FGF Signaling Pathways in Reproduction

The fundamental physiological roles of FGF in mitogenesis, cellular migration and differentiation, angiogenesis and wound healing have been long been recognized (Itoh et al. 2015, Krejci et al. 2016). The expression of specific members of the FGF family within the reproductive system of humans and ruminants is documented (Chaves et al. 2012). These, taken together with the

critical role of FGF family ligands in the formation of gonadotropin-releasing hormone system in humans, rodents and ruminants, have suggested a plausible role for FGF in reproductive physiology (Chaves et al. 2012, Miraoui et al. 2011). In further support of this notion, humans with loss-of-function mutations in FGF receptor 1 (*FGFR1*) and *FGF8* genes develop hypogonadotropic hypogonadism, delayed puberty and hypothalamic amenorrhea (Falardeau et al. 2008).

The expression of some FGF family members in the ovary is tissue specific and others are widely expressed. In the ovary, FGFs are predominantly expressed in theca cells, and granulosa cells mostly express FGF receptors (Chaves et al. 2012). Specifically, theca cells of rodents, ruminants and humans are known to express a number FGFs, including FGF1, FGF2, FGF7, FGF10 and FGF18 (Hawkins and Matzuk 2010, Price 2016). Within the female reproductive tract, the effects of FGFs have been focused on the ovary, and the most studied one is FGF2. Functional theca-to-granulosa signaling has been suggested by the ability of FGF2 to inhibit steroidogenesis, promote GC proliferation and prevent apoptosis in GCs (Buratini and Price 2011). More recently, several studies have implicated a role for FGF8 and FGF18 in folliculogenesis, oocyte and follicle survival, steroidogenesis and corpus luteum formation (discussed in detail in (Chaves et al. 2012, Price 2016)). FGF8 at first was detected and expressed in oocytes of small and large antral follicles of adult mice ovaries (Falardeau et al. 2008). In cattle FGF8 is detected in the oocyte, TCs and GCs, and both FGF8 receptors, FGFR3c and FGFR4, are expressed within the follicle (Buratini, Teixeira, et al. 2005, Buratini, Glapinski, et al. 2005). Likewise, FGF18 has been detected in oocytes in mice, and in GCs and TCs of cattle (Buratini, Teixeira, et al. 2005, Buratini, Glapinski, et al. 2005). Both FGF8 and FGF18 have emerged as key players of mesenchymal-epithelial signaling in a variety of

tissues, especially during organogenesis, and in modulation of ovarian function (Falardeau et al. 2008).

Not all FGFs appear to be mitotic, as previous work in our laboratory has shown that FGF18 increases the rate of granulosa cell death by apoptosis in cattle, through a caspase-3 mediated mechanism (Portela et al. 2010, Portela et al. 2015). Further, abundance of *FGF18* in the theca of atretic follicles is higher than that in healthy follicles in cattle (Portela et al. 2010, Portela et al. 2015). More recently, immunohistochemical studies localized FGF18 protein to the small blood vessels in the theca layer and in the medulla of the sheep ovary, which suggest a plausible crosstalk signaling between the endothelial cells and the TCs and/or GCs.

1.9.2 FGF Signaling Pathways

The activation of FGFR results in recruitment of specific molecules that bind to phosphorylated tyrosines of the intracellular receptor domain (Ornitz and Itoh 2001). These subsequently serve as docking sites for the recruitment of Src homology-2 (SH2) or phosphotyrosine binding (PTB) domains of adaptor or docking proteins (Yun et al. 2010). Currently, the well-understood signaling pathways of FGF include the RAS/MAPK, PI3 kinase/AKT and PLCγ pathways (Figure 4; (Yun et al. 2010)).

The RAS/MAPK pathway is a common mechanism of signal transduction in eukaryotic cells. It is composed of multiple serine/threonine kinases that react to extracellular stimuli and regulate developmental changes in organisms (Teven et al. 2014, Ornitz and Itoh 2015). As already noted, a key event in the FGF signaling pathway is phosphorylation of the tyrosine residues. This leads to recruitment of FGFR substrate 2α (FRS2α), guanine nucleotide exchange factor 2 (GRB2),

the son of sevenless (SOS), the tyrosine phosphatase (SHP2) and the GRB2-associated binding protein 1 (GAB1), resulting in activation of the RAS/MAPK and the PI3 kinase/AKT pathways (Ornitz and Itoh 2015). On the other hand, FGF-FGFR mediated signaling that induces a MAPK-mediated negative feedback loop, causing threonine phosphorylation of FRS2α and reduction of tyrosine phosphorylation and recruitment of GRB2, has also been documented (Lax et al. 2002).

Similar to the RAS/MAPK pathway, the phosphoinositide 3 (PI3) kinase/AKT pathway is initiated by forming an FRS2α complex and activation of FGFR by linked GAB1 protein, resulting in a complex between the p110 catalytic unit of PI3 kinase and p85 adapter protein (Lamothe et al. 2004). The phospholipase C gamma (PLCγ) binds to the phosphorylated Tyr-766 of the FGFR and results in activation of the PLCγ pathway. The activated PLCγ hydrolyzes phosphatidylinositol, generating inositol triphosphate (IP3) and diacylglycerol (DAG) (Mohammadi et al. 1991).
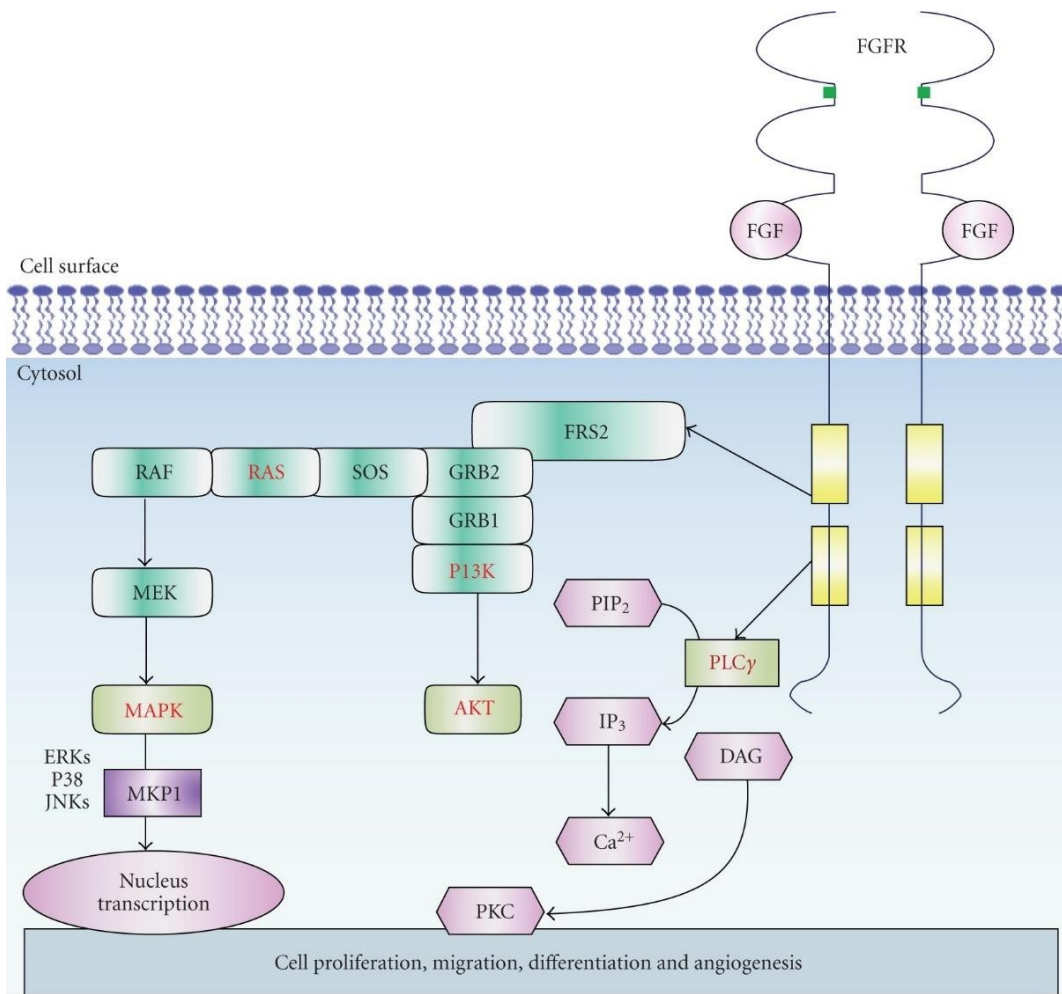
Figure 4. The fibroblast growth factor (FGF) signaling pathway (Yun et al. 2010). FGFs stimulate tyrosine phosphorylation of the docking protein FRS. This then forms the GRB2- SHP2-GAB-1 complex, resulting in activation of RAS-MAP kinase pathway and PI3 kinase/AKT pathway. In PLCγ pathway, activated PLCγ hydrolyzes phosphatidylinositol, generating IP3 and DAG. This in turn activates PKC. FRS2: fibroblast growth factor receptor substrate 2, GRB: guanine nucleotide exchange factor, SOS: son of sevenless, RAS: monomeric G-protein, RAF: kinase, MEK: kinase, MKP1: MAP kinase phosphatase, PIP2: phosphatidylinositol (4,5)-bisphosphate, IP3: inositol triphosphate, DAG: diacylglycerol, PKC: protein kinase C.

2. HYPOTHESIS AND OBJECTIVES

2.1 Hypothesis

As this laboratory has preliminary data to suggest that FGF18 is expressed in follicular endothelial cells, and that TGFβ family members regulate endothelial cell migration and proliferation, we hypothesize that TGFβ family members alter function of endothelial cells derived from the theca of the sheep.

2.2 Objectives

The specific objectives of this work were:

1. To determine the effect of BMP4 and TGFβ1 on ovine endothelial cell gene expression.
2. To determine the effect of BMP4 on the ovine endothelial cell proteome.

CHAPTER 2: MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1 **Cell Culture**

Ovine ovaries were obtained from adult ewes, irrespective of their estrous cycle phase, from an abattoir. The ovaries were the transported to the laboratory at 30˚C in phosphate-buffered saline (PBS) solution containing penicillin (100 μg/mL) and streptomycin (100 μg/mL). Endothelial cells were isolated from the theca layer using S-pluriBead® Maxi Reagent Kit (pluriSelect Life Science, Germany). Briefly, the surface of the follicle was cut with a scalpel blade. The theca layer was removed using forceps and collected into 10 ml of DMEM/F12 (Thermo Fisher Scientific, Burlington, Canada). The theca layer was then incubated in a solution containing collagenase (1 mg/ml; 500μl;), trypsin inhibitor (100 μl/ml; 500μl) and 4 ml of DMEM/F12 at 37°C for 45min. Subsequently, the cell suspension was centrifuged at 800g for 10 min and the resultant cell pellet was resuspended in 1 ml of DPBS. Following osmotic lysis of the red blood cells using milliQ water, the cell suspension was filtered through a 30 μm pluristrainer. The cell viability was assessed using trypan blue dye and the cells were then seeded onto 24-well tissue culture plates (Sarstedt Inc., Montreal, Canada) at a density of 0.5 million in 1 mL DMEM/F12 containing bovine serum albumin (BSA) (0.1%; Sigma-Aldrich, St. Louis, Missouri, United States). The cells were grown to ~70 – 90% confluency prior to treatments. The cell cultures were maintained at 37°C in 5% CO_2, 95% O_2 for 5 days, with medium changes on days 2 and 4.

2.1.1 Experimental treatments

To determine the effects of TGFβ1 and BMP4 on endothelial cells gene expression, and the effect of BMP4 on endothelial cells gene expression and secretory activity, cells were serum-starved for 8 h and treated from day 5 with different doses of human recombinant BMP4

(PeproTech, Rocky Hill, US) and different doses of human recombinant TGFβ1 acquaired from the same company . While some cells were recovered 24 h post- TGFβ1 and BMP4 treatment for RNA extraction, other cells and respective media were recovered after 48 h post-BMP4 treatment for total protein extraction using RIPA buffer for mass spectrometry analysis. All experiments were performed with three different pools of cells each collected on a different occasion.

2.2 Mass spectrometry

Proteins were extracted from cell samples and bottom-up proteomic analysis was performed. The total amount of protein in each sample was determined using Bradford assay (Thermofisher, Rockford US). Briefly, a volume corresponding to 50 μg of proteins was used for each sample. Proteins were isolated using a precipitation procedure with a ratio 1:3 (v:v) of acetone. The samples were centrifuged at 9,000 g for 10 min and the acetone was discarded. The resultant protein pellet was dried for 20 min in a vacuum centrifuge set at 60˚C. The protein pellet was then dissolved in 100 μL of 50 mM ammonium bicarbonate (pH 8.0) and the solution was sonicated for 60 min at maximum intensity to improve protein dissolution. Reduction and alkylation were performed as previously described [Ruiz et al. 2015], and then 2 μg of proteomic-grade trypsin was added. The reaction was performed at 40˚C for 24 h. The protein digestion was quenched by adding 10 μL of a 2% trifluoroacetic acid (TFA) solution. Samples were centrifuged at 12 000 g for 10 min and the supernatants were transferred into injection vials for analysis.

The HPLC system was a Thermo Scientific™ UltiMate™ 3000 Rapid Separation UHPLC system (San Jose, CA, USA). The chromatography was achieved using a gradient mobile phase in a microbore C8 column (Thermo Biobasic) with a particle size of 5μm. The initial mobile phase consisted of acetonitrile and water (both fortified with 0.1% of formic acid) at a ratio of 5:95.

From 0 to 1 min, the ratio was maintained at 5:95. From 1 to 61 min, a linear gradient was applied up to a ratio of 50:50 and maintained for 2 min. The mobile phase composition ratio was reverted to the initial conditions and the column was allowed to re-equilibrate for 14 min for a total run time of 77 min. The flow rate was fixed at 75 µL/min and 2 µL of sample was injected.

The Q Exactive™ Orbitrap™ Mass Spectrometer (San Jose, CA, USA) was interfaced with the UHPLC system using a pneumatic assisted heated electrospray ion source. MS detection was performed in positive ion mode and operating in scan mode at high-resolution, and accurate- mass (HRAM). Nitrogen was used for sheath and auxiliary gases set at 10 and 5 arbitrary units. The Electrospray ionization (ESI) voltage was set at 4000V and the ion transfer tube temperature was set at 300℃. The default scan range was set at m/z 400 -1500. Data was acquired at a resolving power of 140,000 (full width at half maximum; FWHM) using automatic gain control targets of 3.0 x 10^6 and maximum ion injection time of 200 ms. Additionally, MS data was acquired using a data-dependent top-10 method to dynamically choose the most abundant precursor ions from the survey scans (400–1500 Da) and generate MS/MS spectra. Instrument calibration was performed prior to all analysis and mass accuracy was notably below 1 ppm using Thermo Pierce calibration solution and automated instrument protocol.

Database surveys were performed using Proteome Discoverer software (v2.1) with Uniprot ovine protein database (extracted FASTA file). Mass tolerance of precursor and fragment (i.e. typically b and y) were set at 5 ppm and 10 ppm, respectively. Phosphorylation at Y and T amino acids was set as a variable post translational modification. Quantification was based on MS1 ion intensity and peptide identification was based on precursor ion (MS1) and at least three characteristic (MS2). Label-free MS1 quantification of peptide/protein via peak intensity was

performed using SIEVE (v2.1), a label-free differential expression software that aligns the MS spectra over time from different data sets and then determines structures in the data (m/z and retention time pairs) that differ. These differences were examined using Uniprot ovine protein database and then sorted based on biological function obtained from the data of each biological replicate. STRING interfaces were used to perform the interactomic analysis. In addition to STRING, GeneMANIA (http://genemania.org) was used to evidence putative interactive functional association network illustrating the relationships among genes.

2.3   Total RNA extraction and RT-PCR

Total RNA was isolated from theca cells using RNA Mini Kit (Invitrogen) according to the manufacturer's instructions. Real-time PCR was performed in a 15μl reaction volume with 2× Power SYBR Green PCR Master Mix on CFX-96 Real-Time PCR Detection System (Bio-Rad Laboratories Ltd. CA, USA). The ovine-specific primer sequences were designed as shown in Table 1. The thermal cycling conditions were 3 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. Melting curve analyses were performed to verify product identity. Samples were run in duplicate and the results are expressed relative to the geometric mean of three housekeeping genes (*RPL19*, *YWHAZ* and *SDHA*). Data were normalized to a calibrator sample using the ΔΔCt method with correction for amplification efficiency.

2.4   Statistical Analysis

Data were tested for homogeneity of variance with Bartlett test, and log transformations were performed as appropriate. One-way ANOVA were performed with Tukey's multiple comparisons tests, or Dunnett's compare with control, as appropriate. Culture data included culture replicate as a random variable. Data are presented as means ± SEM with $p < 0.05$ considered significant.

Table 1. Primer sequences used for real-time PCR

Gene symbol	Forward primer	Reverse primer
Target genes		
VEGFB	AACACAGCCAGTGCGAATGC	GTCACCTTCGCAGCTTCCG
VEGFC	GTTTACAGACAAGTCCATTCAAT	TGCAGACGTGGTTATTCC
VEGFD	GAAGAAGATCGCTGCTCCCA	ATGTGCTGACCACAGAGAGC
COX2	GATGACTGCCCAACACCCAT	AAAGGCGACGGTTATGCTGT
MMP9	GCTTCTGGAGGTTCGACGTG	ACGCCAGAAGAAGTGATCCTG
BMP4	TGGCTGTCAAGAATCATGGACT	ACGACCATCAGCATTCGGTT
IGFBP3	CTACACGCGTTGTTGGACG	GCTGTGGTCTTCTTCCGACT
ET1	AGGAAAAGAGCTCAAGGACCA	TTGATGCTGTTGCTGATGGC
Housekeeping genes		
RPL19	TATGGGTATAGGTAAGCGAAAG	TGGCGGTCAATCTTAG
YWHAZ	ACCAACACATCCTATCAGAC	CTCTCAGTAACTGCATTAGC
SDHA	GAATGGTCTGGAACACTGA	AGTAATCGTACTCGTCAACC

CHAPTER 3: RESULTS

3 RESULTS

3.1 Effect of BMP4 and TGFβ1 on ovine endothelial cell gene expression

Treatment of ovine theca endothelial cells (ECs) in culture with BMP4 or TGFβ1 altered the abundance of some but not all of the candidate target genes. Specifically, treatment of theca ECs with BMP4 caused a dose-dependent increase in the relative mRNA expressions of *ET1* (Figure 5A), *VEGFa* (Figure 5B) and *FGF18* (Figure 5C). However, treatment of the ECs with BMP4 did not significantly affect the relative mRNA expressions of *VEGFb* (Figure 5D), *VEGFc* (Figure 5E), *VEGFd* (Figure 5F), *MMP9* (Figure 5G), *IGFBP3* (Figure 5H) or *BMP4* (Figure 5I). In contrast, treatment of theca ECs with TGFβ1 only dose-dependently increased the mRNA expression of *ET1* (Figure 6A) but did not significantly affect the relative mRNA expressions of *VEGFa* (Figure 6B), *FGF18* (Figure 6C), *VEGFb* (Figure 6D), *VEGFc* (Figure 6E), *VEGFd* (Figure 6F), *MMP9* (Figure 6G), *IGFBP3* (Figure 6H) or *BMP4* (Figure 6I).

3.2 Effect of BMP4 on ovine endothelial cell proteome

Overall, 1488 proteins were identified by mass spectrometry in ovine theca ECs in both treated and non-treated group. Treatment of ECs with BMP4 resulted in an increased expression (>2-fold) of 28 proteins (Appendix A; Table 1) and decreased expression (<0.5-fold) of 29 proteins (Appendix A; Table 2). There were also 31 proteins that were slightly up-regulated (1.5 – 2-fold; Appendix A; Table 3) and 52 proteins that were slightly down-regulated (0.5 – 0.67-fold; Appendix A; Table 4). The three most significantly up-regulated and down-regulated proteins are given in Table 2 and Table 3, respectively. These proteins have well-characterized roles in endothelial cell proliferation and development (Marcelo et al. 2013).

Figure 5. Dose-dependent effects of BMP4 on (A) *ET1*, (B) *VEGFa*, (C) *FGF18*, (D) *VEGFb* (E) *VEGFc*, (F) *VEGFd*, (G) *MMP9*, (H) *IGFBP3* and (I) *BMP4* mRNA levels in ovine theca endothelial cells. *$p<0.05$ (One-way ANOVA; *posthoc*: Dunnett); *n.d. – not determined.
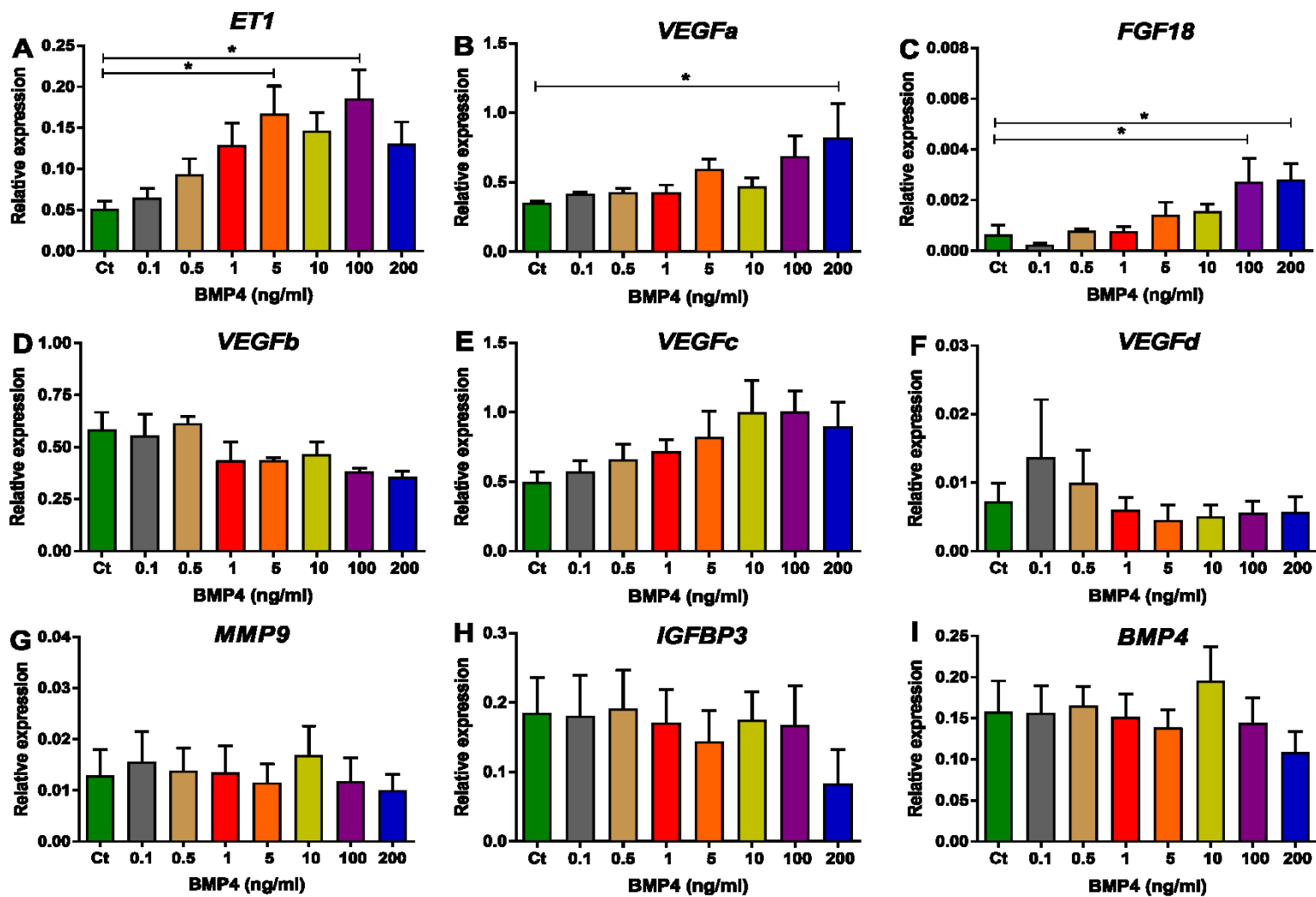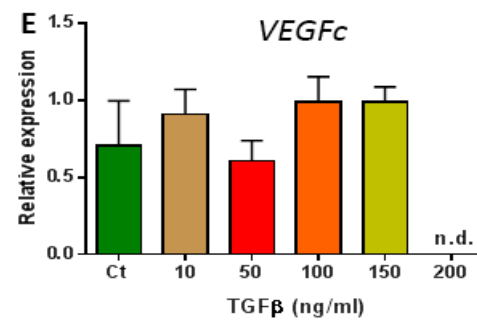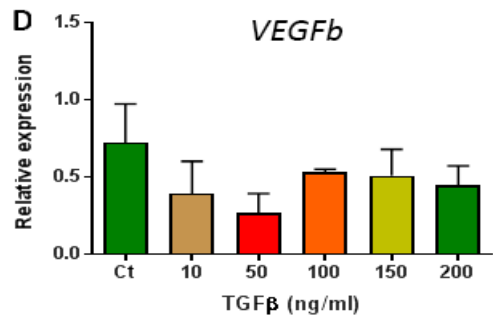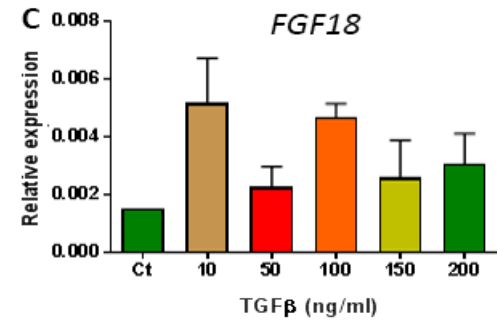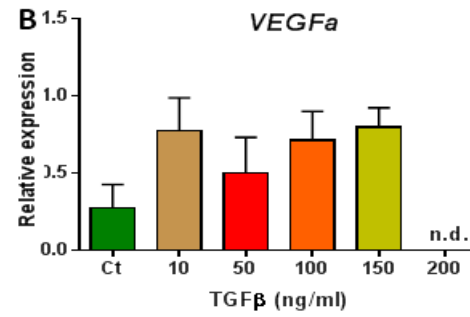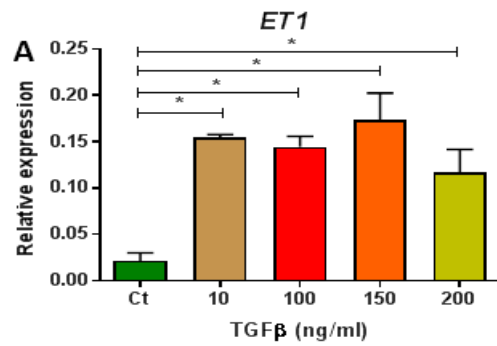
Figure 6. Dose-dependent effects of TGFβ1 on (A) *ET1*, (B) *VEGFa*, (C) *FGF18*, (D) *VEGFb* (E) *VEGFc*, (F) *VEGFd*, (G) *MMP9*, (H) *IGFBP3* and (I) *BMP4* mRNA levels in ovine theca endothelial cells. *p<0.05 (One-way ANOVA; *posthoc*: Dunnett); *n.d. – not determined.
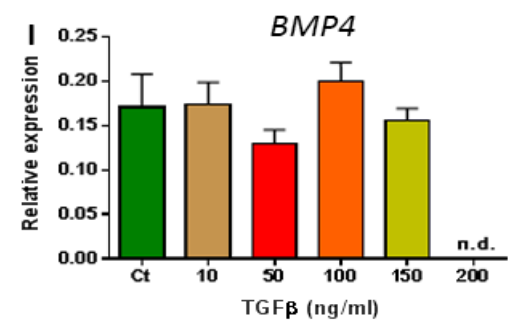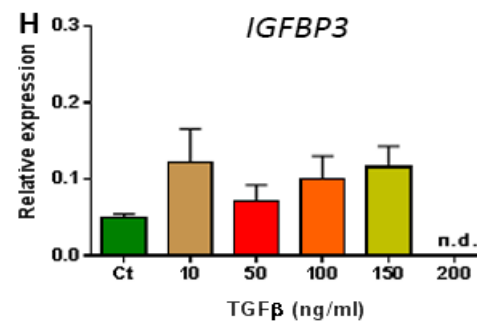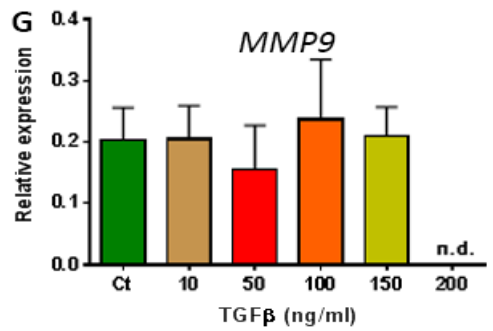
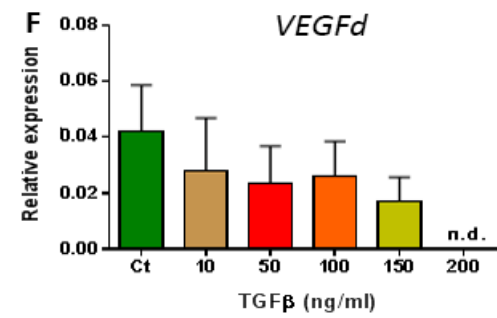**Table 2.** List of the top three up-regulated proteins in ovine theca endothelial cells following BMP4 treatment.

Protein	Accession	Abundance Ratio
Aldehyde dehydrogenase 6 family member A1 [OS=Ovis aries]	W5NS43	100
Bone morphogenetic protein 4 [OS=Ovis aries]	A8VTF8	100
NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 8, mitochondrial [OS=Ovis aries]	D5M8S1	100

Table 3. List of the top three down-regulated proteins in ovine theca endothelial cells following BMP4 treatment.

Protein	Accession	Abundance Ratio
Core histone macro-H2A [OS=Ovis aries]	W5P731	0.496
Gastrin [OS=Ovis aries]	W5Q7J9	0.484
amine oxidase [OS=Ovis aries]	W5NQS5	0.477

The proteins that showed significant fold changes were also classified according to their biological processes (Figure 7).
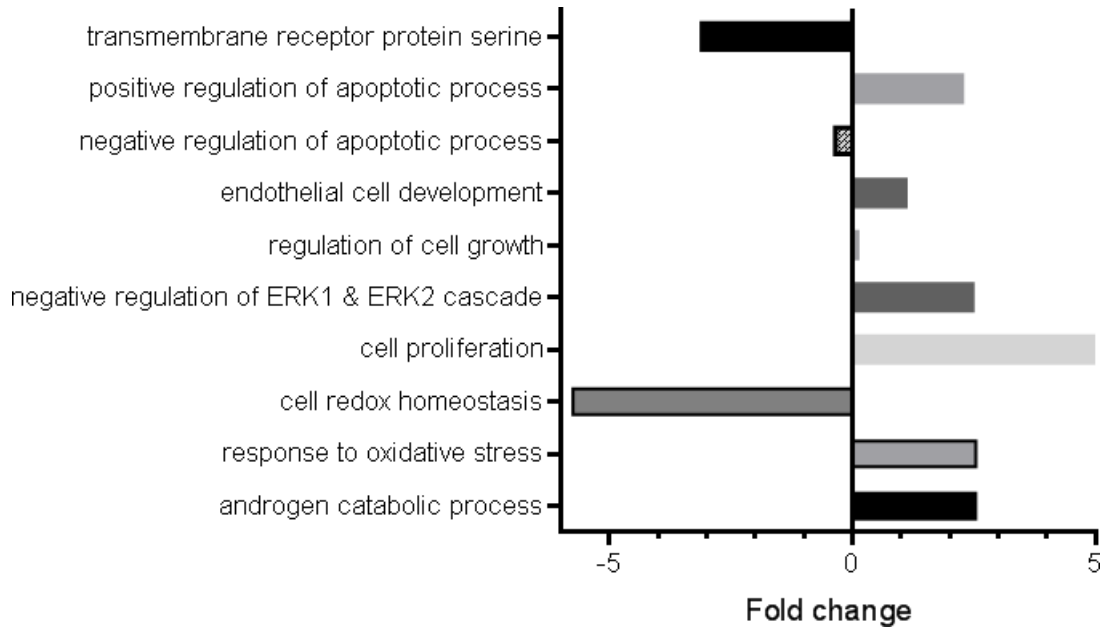
Figure 7. Biological function of proteins identified by mass spectrometry.

Although TIMP1 was not upregulated by more than 1.5-fold, interactome analysis of the differentially expressed proteins identified a high degree of genetic and physical interactions between TIMP1 and JUN, STAT3, CD63 signaling pathways following the exposition of endothelial cell to BMP4 for 48h (Figure 8A). This observation was also validated with a STRING analysis (i.e. predicted protein–protein interactions) in Figure 8B.
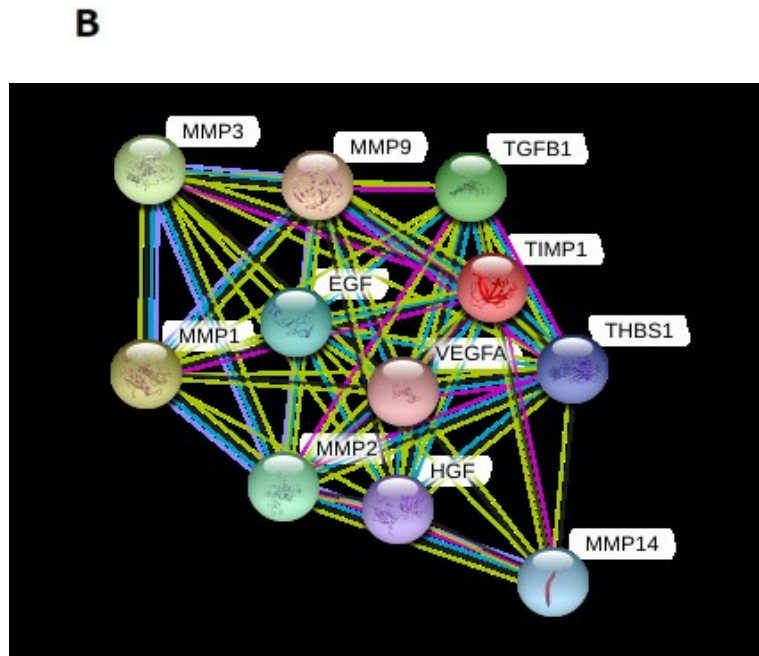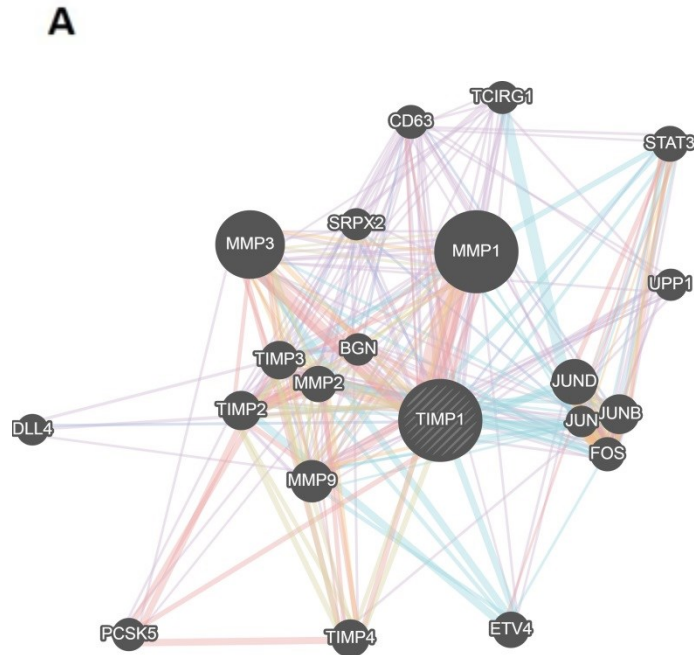
Figure 8. Interactome analysis network. (A) Complete mapping of the Interactome Networks. (B) protein-protein interaction network maps using a distinct database (STRING).

CHAPTER 4: DISCUSSION & CONCLUSION

4 DISCUSSION

The present study investigated the effects of TGFβ1 on ovine endothelial cell (EC) gene expression, and BMP4 on EC gene expression and protein secretion. The findings of the present study demonstrate that exogenous administration of TGFβ1 and BMP4 induced expression of endothelin-1 (ET-1) in ovine theca ECs. BMP4, but not TGFβ1, also significantly increased the expression of VEGF and FGF18. Proteomic analysis performed following 48h incubation of ovine theca ECs with BMP4 identified significant up-regulation (>2-fold) of 28 proteins and significant down-regulation (<0.5-fold) of 29 proteins. To the best of our knowledge, this is the first study that elucidates the effect of BMP4 and/or TGF-β1 on gene expression and proteome in primary ruminant theca ECs.

In this study, TGFβ1 stimulated the abundance of *ET1* mRNA, as has been previously demonstrated in human pulmonary artery endothelial cells (Park et al. 2012, Star et al. 2009), and we also demonstrate that BMP4 also stimulated *ET1* mRNA levels, which has not been previously reported. Other genes important for endothelial cell proliferation, vascular formation and/or permeability include VEGFa/b/c/d, MMP9, FGF18 and IGFBP3 (Datta et al. 2004, Rizov et al. 2017; Rossi et al. 2016), and these genes are regulated by TGFβ1 in human cancer cells and endothelial cells (Laulan and St-Pierre 2015, Genersch et al. 2000, Rossi et al. 2016), however in the present study only *VEGFa* and *FGF18* mRNA levels were altered by BMP4. This growth factor has been shown to increase *VEGFa* mRNA levels in ruminant theca/granulosa cells and neuroblastoma cells (Nichols et al. 2019, HaDuong et al. 2015). The inability of TGFβ1/BMP4 to upregulate angiogenic genes in this study may be owing to the use of primary EC from the follicle, which likely exhibit different properties compared with cell lines.

Exposition of ovine theca ECs to BMP4 for 48h resulted in significant changes in the proteome. Specifically, mass spectrometry analysis identified 1488 proteins in total, with significant up-regulation (>2-fold) of 28 proteins and down-regulation (<0.5-fold) of 29 proteins. Consistent with the well-documented role of BMP4 in apoptosis and cell proliferation in ECs (Kiyono and Shibuya 2006, 2003, Tian et al. 2012, David et al. 2009), the proteome analyses revealed significant up-regulation of proteins regulating these biological pathways. Interactome analysis, using two distinct biochemical and biological databases, identified a high degree of genetic and physical interactions between TIMP1 and JUN, STAT3, CD63 signaling pathways.

Of interest was the significant up-regulation of neutralizing tissue inhibitor of metalloproteinase 1 (TIMP1), which has been previously associated with apoptosis, cell growth, and angiogenesis (Chirco et al. 2006). Curiously, this protein was downregulated by TGFβ1 in mouse brain microcapillary endothelial cells (Siqueira et al. 2017), which may reflect a difference between species (mouse vs sheep) or cell origin (brain vs ovary). Interactomic analysis also identified a high degree of genetic and physical interactions between TIMP1 and signal transducers and activators of transcription 3 (STAT3). STAT3, a member of the STAT family identified as a DNA-binding factor, has been implicated in accelerating cell growth, inhibiting apoptosis and promoting tumorigenesis (Niu et al. 2002, Bromberg and Darnell 2000). STAT3 has also been known to be an upstream regulator of BMP signaling pathway (Fukuda et al. 2007). Furthermore, STAT3 has been widely described as an oncogene correlated with tumor progression and up-regulated in a wide variety of tumors, including hematological malignancies and solid tumors such as breast, lung, gastric, colorectal, ovarian and prostate cancers (Yu et al. 2009).

A role for TIMP1 is also documented in the regulation of ovarian extracellular matrix remodeling during follicular growth, ovulation, corpus luteum formation, and regression (Smith et al. 1999, Li and Curry 2009). It has been suggested that the induction of TIMP1 by TGFβ family members occurs through the activation of the protein kinase A pathway and downstream mediators such as extracellular-signal-regulated kinase (ERK) and mitogen activated protein kinases (MAPK) (Salvador et al. 2002). However, mass spectrometry analysis failed to identify up-regulation of these downstream proteins. Previous works by others have also observed similar discordance (Castagnino et al. 1998, Su et al. 1998, Nareyeck et al. 2005, Zhou et al. 2004), and have proposed differences in ubiquitination or posttranscriptional changes as a reason.

One puzzling feature of the present study was the lack of changes detected in protein levels of FGF18, VEGFa or ET1. This may be because (a) these factors are secreted and therefore differences were not detected in terms of intracellular levels, (b) the sheep protein database is insufficiently annotated to correctly detect and identify these proteins, and/or (c) concentrations of these proteins were too low to be accurately detected by mass spectrometry. It is also possible that culture conditions may have amplified changes in RNA and/or reduced protein production. Further studies with alternative techniques such as Western blotting or ELISA are required to resolve this issue.

Development and loss of the thecal capillary bed is critical for follicle development, and reduced vascularity of the theca has been associated with follicle atresia (Geva and Jaffe 2000 Wulff et al. 2001). Collectively, the present data suggest a role for TGFβ and BMP4 in regulating these cells. These ligands stimulate FGF18, ET1 and VEGFa expression, which are involved in promoting endothelial cell migration and proliferation (Antoine), which suggests that TGFβ signaling stimulates folliculogenesis at least in part through enhancing thecal vascularity.

4   CONCLUSION

In summary, our results demonstrate that exogenous administration of BMP4 or TGFβ1 induces differential changes in the expression of EC function-related genes and proteins in ovine thecal endothelial cells. A major limitation of this work was the use of a targeted gene approach (i.e. only genes involved in endothelial function), and future work assessing the genome-wide transcriptome changes using RNA sequencing in ovine theca ECs is required to further gain insights of the downstream signaling mediators of TGFβ family members. As already noted, this is the first study that has used ovine primary theca ECs to elucidate proteomic changes. Given the possibility of tissue- or species-specific role of BMPs, future work assessing proteomic changes in human umbilical vein endothelial cells (HUVEC) following BMP4 treatment and/or assessing BMP4-associated downstream signaling protein changes using molecular biology techniques is required to better understand the crosstalk between the endothelial cells and the TCs and/or GCs.

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69

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APPENDIX A

Table 1. List of proteins significantly up-regulated greater than 2-fold change by BMP4 treatment in ovine endothelial cells.

Accession	Protein	Abundance Ratio
W5NS43	aldehyde dehydrogenase 6 family member A1 [OS=Ovis aries]	100
A8VTF8	Bone morphogenetic protein 4 [OS=Ovis aries]	100
D5M8S1	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 8, mitochondrial [OS=Ovis aries]	100
W5PNV2	Serine/threonine-protein phosphatase [OS=Ovis aries]	7.656
W5QIL2	Proteasome subunit beta [OS=Ovis aries]	7.475
W5Q0F3	transforming growth factor beta induced [OS=Ovis aries]	5.327
W5PXE7	copine 7 [OS=Ovis aries]	5.135
W5PWU4	Uncharacterized protein [OS=Ovis aries]	4.723
W5Q5R2	Neural cell adhesion molecule 1 [OS=Ovis aries]	4.379
W5P604	elongator acetyltransferase complex subunit 2 [OS=Ovis aries]	4.038
W5PQI9	microtubule associated protein 1 light chain 3 beta [OS=Ovis aries]	3.582
W5QHP5	G protein subunit beta 4 [OS=Ovis aries]	3.068
W5NSJ5	oxidative stress responsive 1 [OS=Ovis aries]	2.942
W5QHI4	cyclin dependent kinase like 1 [OS=Ovis aries]	2.814
W5NR86	transcription factor BTF3 [OS=Ovis aries]	2.709
W5PWZ3	MORC family CW-type zinc finger 3 [OS=Ovis aries]	2.633
W5P3W5	Hydroxysteroid 17-beta dehydrogenase 11 [OS=Ovis aries]	2.52
W5QDP8	Fibulin-1 [OS=Ovis aries]	2.458
W5QEQ3	DNA-(apurinic or apyrimidinic site) lyase [OS=Ovis aries]	2.392
W5QCX7	MYB binding protein 1a [OS=Ovis aries]	2.276
W5PTL7	ssemaphorin 4F [OS=Ovis aries]	2.23
W5P8E9	CD109 molecule [OS=Ovis aries]	2.214
W5P668	Glutathione peroxidase [OS=Ovis aries]	2.131
W5PLJ6	neudesin neurotrophic factor [OS=Ovis aries]	2.105
W5PVE5	peptidyl-prolyl cis-trans isomerase [OS=Ovis aries]	2.043
W5NPI6	Polyadenylate-binding protein [OS=Ovis aries]	2.025
W5QG21	Uncharacterized protein [OS=Ovis aries]	2.021
W5Q6J7	Tubulin-specific chaperone A [OS=Ovis aries]	2.001

Table 2. List of proteins that were significantly down-regulated (<0.5-fold) by BMP4 treatment in ovine endothelial cells.

Accession	Protein	Abundance Ratio
W5P731	Core histone macro-H2A [OS=Ovis aries]	0.496
W5Q7J9	Gastrin [OS=Ovis aries]	0.484
W5NQS5	amine oxidase [OS=Ovis aries]	0.477
W5PRA1	Uncharacterized protein [OS=Ovis aries]	0.466
W5PU70	Uncharacterized protein [OS=Ovis aries]	0.44
W5PZT1	Uncharacterized protein [OS=Ovis aries]	0.436
A0A0B4UE86	growth hormone [OS=Ovis aries]	0.413
B0FZM4	myosin light chain 6 [OS=Ovis aries]	0.41
W5PX18	BRO1 domain and CAAX motif containing [OS=Ovis aries]	0.405
W5PTM9	Uncharacterized protein [OS=Ovis aries]	0.387
W5QFH1	actin, alpha, cardiac muscle 1 [OS=Ovis aries]	0.383
W5PA52	Uncharacterized protein [OS=Ovis aries]	0.378
W5P5F6	Tubulin folding cofactor B [OS=Ovis aries]	0.378
W5NTP6	Uncharacterized protein [OS=Ovis aries]	0.376
W5Q3J3	collagen beta(1-O)galactosyltransferase 1 [OS=Ovis aries]	0.372
W5PZI0	Clusterin [OS=Ovis aries]	0.361
W5QB02	Transglutaminase 2 [OS=Ovis aries]	0.357
Q1A2D1	Beta-K globin chain [OS=Ovis aries]	0.346
W5PII6	A-kinase anchoring protein 3 [OS=Ovis aries]	0.322
W5Q5M3	Uncharacterized protein [OS=Ovis aries]	0.318
W5PGT5	Myosin heavy chain 11 [OS=Ovis aries]	0.297
W5Q009	X-prolyl aminopeptidase 2 [OS=Ovis aries]	0.266
W5PJI3	zinc finger CCHC-type containing 24 [OS=Ovis aries]	0.23
W5Q337	glutaredoxin 5 [OS=Ovis aries]	0.174
W5NU63	myosin heavy chain 10 [OS=Ovis aries]	0.163
W5QIH2	Tropomodulin 3 [OS=Ovis aries]	0.16
W5QBQ9	myosin heavy chain 9 [OS=Ovis aries]	0.157
W5NW47	Uncharacterized protein [OS=Ovis aries]	0.154
W5NWY3	Uncharacterized protein [OS=Ovis aries]	0.091
W5NWF1	syntaxin binding protein 5 [OS=Ovis aries]	0.082

Table 3. List of proteins that were slightly up-regulated (1.5 – 2-fold) by BMP4 treatment in ovine endothelial cells.

Accession	Protein	Abundance Ratio
W5Q6L8	Keratin 14 [OS=Ovis aries]	1.948
W5PE86	peptidylprolyl isomerase [OS=Ovis aries]	1.929
W5P4M1	Prohibitin 2 [OS=Ovis aries]	1.907
W5PER3	Transmembrane 9 superfamily member [OS=Ovis aries]	1.876
W5NRL8	Eukaryotic translation initiation factor 3 subunit A [OS=Ovis aries]	1.866
W5NQJ2	COP9 signalosome subunit 5 [OS=Ovis aries]	1.863
W5P1C2	nucleobindin 2 [OS=Ovis aries]	1.841
W5QFP1	Polyadenylate-binding protein [OS=Ovis aries]	1.829
W5Q2P6	Early endosome antigen 1 [OS=Ovis aries]	1.814
W5NPC7	acyl-CoA binding domain containing 3 [OS=Ovis aries]	1.807
W5PXC5	TRIO and F-actin binding protein [OS=Ovis aries]	1.761
W5QGI9	E3 ubiquitin-protein ligase RNF168 [OS=Ovis aries]	1.75
W5Q1Z8	Bromodomain PHD finger transcription factor [OS=Ovis aries]	1.716
W5Q952	Aly/REF export factor [OS=Ovis aries]	1.699
Q28552	fetal globin [OS=Ovis aries]	1.691
W5PVJ1	BCL2 like 13 [OS=Ovis aries]	1.69
W5P8C1	KIAA0319 [OS=Ovis aries]	1.682
W5PQR0	family with sequence similarity 129 member B [OS=Ovis aries]	1.679
W5NR48	Importin subunit alpha [OS=Ovis aries]	1.656
W5NXE7	peptidylprolyl isomerase like 4 [OS=Ovis aries]	1.641
W5PE07	NADH:ubiquinone oxidoreductase subunit S4 [OS=Ovis aries]	1.618
W5PUY7	PKHD1, fibrocystin/polyductin [OS=Ovis aries]	1.613
W5Q5T7	V-type proton ATPase subunit C [OS=Ovis aries]	1.566
W5PM07	solute carrier family 18 member A1 [OS=Ovis aries]	1.559
W5QFP0	Thrombospondin 1 [OS=Ovis aries]	1.559
W5P8B3	translocase of inner mitochondrial membrane 50 [OS=Ovis aries]	1.555
W5PBU7	Transmembrane protease, serine 13 [OS=Ovis aries]	1.542
W5PUX0	NADH dehydrogenase [ubiquinone] flavoprotein 1, mitochondrial [OS=Ovis aries]	1.539
W5P7W2	Stromal cell derived factor 4 [OS=Ovis aries]	1.538
W5QHP9	Guanine nucleotide-binding protein subunit gamma [OS=Ovis aries]	1.528
W5QBZ7	Uncharacterized protein [OS=Ovis aries]	1.516
B2MVW1	tetraspanin [OS=Ovis aries]	1.511

Table 4. List of proteins that were slightly down-regulated (0.5 – 0.67 fold) by BMP4 treatment in ovine endothelial cells.

Accession	Protein	Abundance Ratio
W5NS65	histone cluster 1 H1 family member e [OS=Ovis aries]	0.666
W5PIV7	Fragile X mental retardation 1 [OS=Ovis aries]	0.663
W5NTN0	Glutamine-fructose-6-phosphate transaminase 2 [OS=Ovis aries]	0.659
W5QIH4	MARCKS like 1 [OS=Ovis aries]	0.658
C8BKD6	superoxide dismutase [OS=Ovis aries]	0.654
A0A075B6D9	Caveolin [OS=Ovis aries]	0.653
W5Q1M0	Beta-galactosidase [OS=Ovis aries]	0.653
W5Q3E0	Thy-1 cell surface antigen [OS=Ovis aries]	0.652
W5PRP1	Na(+)/H(+) exchange regulatory cofactor NHE-RF [OS=Ovis aries]	0.651
W5NWK5	S100 calcium binding protein A13 [OS=Ovis aries]	0.649
W5Q039	tubulin beta chain [OS=Ovis aries]	0.648
W5NZQ2	leucine aminopeptidase 3 [OS=Ovis aries]	0.647
W5Q5W2	Uncharacterized protein [OS=Ovis aries]	0.646
W5PT12	40S ribosomal protein S30 [OS=Ovis aries]	0.643
W5PW39	Lamin B2 [OS=Ovis aries]	0.643
W5Q940	shootin 1 [OS=Ovis aries]	0.639
C6ZP47	I alpha globin [OS=Ovis aries musimon]	0.634
W5NUT0	Caveolae associated protein 1 [OS=Ovis aries]	0.629
W5P2E1	taste 1 receptor member 3 [OS=Ovis aries]	0.629
W5PHS7	solute carrier family 29 member 1 (Augustine blood group) [OS=Ovis aries]	0.628
W5PLK7	high mobility group AT-hook 1 [OS=Ovis aries]	0.626
W5PYU4	laminin subunit beta 2 [OS=Ovis aries]	0.622
W5Q687	Keratin 5 [OS=Ovis aries]	0.619
W5PHG0	Histone H3 [OS=Ovis aries]	0.614
W5P9M2	Prostaglandin reductase 1 [OS=Ovis aries]	0.613
W5PFT0	CDV3 homolog [OS=Ovis aries]	0.612
W5PIN0	Aldehyde dehydrogenase 1 family member A3 [OS=Ovis aries]	0.611
W5P6D1	Uncharacterized protein [OS=Ovis aries]	0.606
W5Q9T3	lamin B1 [OS=Ovis aries]	0.6
W5NZT7	NmrA like redox sensor 1 [OS=Ovis aries]	0.589
W5QBF8	Caveolae associated protein 3 [OS=Ovis aries]	0.587
W5NT34	barrier to autointegration factor 1 [OS=Ovis aries]	0.584

W5QAA1	14-3-3 protein sigma [OS=Ovis aries]	0.584
W5QGU0	OPA1, mitochondrial dynamin like GTPase [OS=Ovis aries]	0.583
W5Q160	Keratin 10 [OS=Ovis aries]	0.582
W5NZY8	Tubulin folding cofactor E [OS=Ovis aries]	0.571
W5PZW9	keratin 24 [OS=Ovis aries]	0.571
W5Q611	keratin 1 [OS=Ovis aries]	0.565
W5QIK8	Selenium binding protein 1 [OS=Ovis aries]	0.561
W5PNQ1	tropomodulin 1 [OS=Ovis aries]	0.561
W5NZJ5	Sulfotransferase [OS=Ovis aries]	0.553
W5PIF9	transforming growth factor beta 1 induced transcript 1 [OS=Ovis aries]	0.552
W5PKZ0	chromodomain helicase DNA binding protein 2 [OS=Ovis aries]	0.55
W5P3K9	SH3 and PX domains 2A [OS=Ovis aries]	0.547
D6QZ20	NADH dehydrogenase (ubiquinone) 1 beta [OS=Ovis aries]	0.545
W5P5J0	drebrin 1 [OS=Ovis aries]	0.543
W5Q5N9	Keratin 8 [OS=Ovis aries]	0.543
W5PIV2	diacylglycerol kinase [OS=Ovis aries]	0.541
W5QAX3	actin gamma 1 [OS=Ovis aries]	0.533
W5QGE5	serine peptidase inhibitor, Kunitz type 1 [OS=Ovis aries]	0.531
W5P2H4	Uncharacterized protein [OS=Ovis aries]	0.53
W5P374	ribosomal protein L15 [OS=Ovis aries]	0.521
W5NY08	atlastin GTPase 3 [OS=Ovis aries]	0.517