**Oocyte-derived forms of ruminant BMP15 and GDF9
and a theoretical model to explain their synergistic
response**

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*To the memory of the Wallaceville Animal Research Centre:
where the journey began.*

ABSTRACT

Bone morphogenetic factor 15 (BMP15) and growth differentiation factor 9 (GDF9) are two oocyte-secreted factors with well documented effects on ovarian follicular development and ovulation-rate. The aims of these studies were to: (i) identify the molecular forms of BMP15 and GDF9 that are produced and secreted by both the ovine and bovine oocyte using highly specific monoclonal antibodies; (ii) assess the biological activity of some recombinant molecular forms of BMP15 and GDF9; (iii) visualise the various molecular forms using protein modelling techniques and; (iv) provide a hypothetical model of how oocyte-secreted form(s) of BMP15, GDF9 and their cell surface receptors may interact.

Using genetic modifications and transformations of HEK293 cells, recombinant forms of ovine (o) BMP15, including a BMP15 (S356C) mutant capable of forming covalent dimers, and oGDF9 were produced. The bioactivity of these proteins was established using a rat granulosa cell proliferation bioassay. The specificity of the monoclonal antibodies MN2-61A (anti-BMP15) and 37A (anti-GDF9) used in these studies, and determination of the forms they recognise, was examined by Western blotting. The recombinant forms of oBMP15 were further interrogated by purification using both immobilised metal affinity chromatography (IMAC) and reverse phase HPLC. The BMP15 and GDF9 proteins produced and/or secreted by ovine and bovine oocytes, before and after *in vitro* incubation, were identified and compared with the molecular forms(s) of recombinant oBMP15 or oGDF9 using Western blotting under non-reducing, reducing and cross-linking conditions.

The molecular forms of recombinant oBMP15 and oGDF9 comprise mainly mature monomers with a lesser amount of the uncleaved pro-mature form. Mature domains, in the dimeric mature form, were detected for oGDF9 and oBMP15 (S356C), but not oBMP15. These mature domains were almost entirely located within high molecular weight multimeric complexes, which likely also contain the pro-region. In contrast, BMP15 and GDF9 secreted from ruminant oocytes under *in vitro* conditions were found mainly in an unprocessed promature form, along with some fully processed mature domains that did not interact to form detectable mature homodimers or heterodimers. Throughout ovarian follicular development, BMP15 and GDF9 are co-expressed and it has been established that these two factors have synergistic effects on granulosa cell proliferation both *in vitro* and *in vivo* and also on follicular maturation and ovulation-rate *in vivo*. Moreover, the recombinant proteins oBMP15 and oGDF9 generated for this study, when added together,

also demonstrated a synergistic effect in the granulosa cell proliferation assay but this was not observed for oBMP15 (S356C) and oGDF9.

Currently, no adequate model has been proposed to explain how interactions between the cell membrane and forms of oocyte-derived BMP15 and GDF9 achieve their synergistic effects. To investigate this, two homology models of the promature BMP15 and GDF9 proteins were generated using promature porcine TGFB1 and human BMP9 as templates. These models, together with the previously determined forms of GDF9 and BMP15 produced by the ruminant oocyte, were used to visualise their potential interactions, both with each other and with their receptors. This report describes a model showing the possible interactions involved in a synergistic response. In this model, the mature domain is presented to the type II receptor by the proregion and heterodimers form at the level of the receptor. Differences, following heterodimerisation in the conformation and orientation between GDF9 and its type I receptor, as well as between type I and type II receptors, relative to that in homodimers, could explain how heterodimerisation leads to increased Smad3 phosphorylation and subsequent down-stream somatic cell responses.

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ABBREVIATIONS

17 α OH	17 α hydroxylase
3 β HSD	3 β -Hydroxy steroid dehydrogenase
Å	Angstrom
aa	Amino acid
ACN	Acetonitrile
Act	Activin
ACVR1	Activin A receptor, type 1 (a.k.a ALK2)
ACVR1B	Activin A receptor, type 1B (a.k.a ALK4)
ACVR1C	Activin A receptor, type 1C (a.k.a ALK7)
ACVRL1	Activin A receptor, type II like I (a.k.a ALK1)
ACVR2A	Activin A receptor, type IIA
ACVR2B	Activin A receptor, type IIB
ALK	Activin receptor like kinase
ALK1	Activin receptor like kinase 1 (a.k.a ACVRL1)
ALK2	Activin receptor like kinase 2 (a.k.a ACVR1)
ALK3	Activin receptor like kinase 3 (a.k.a BMPR1A)
ALK4	Activin receptor like kinase 4 (a.k.a ACVR1B)
ALK5	Activin receptor like kinase 5 (a.k.a TGF β R1)
ALK6	Activin receptor like kinase 6 (a.k.a BMPR1B)
ALK7	Activin receptor like kinase 7 (a.k.a ACVR1C)
AMH	Anti Mullerian hormone (a.k.a. MIS)
AMHRII	Type II anti mullerian hormone receptor
AMSH	<u>A</u> ssociated <u>m</u> olecule with the <u>S</u> H3 domain of STAM
BAMBI	BMP and activin membrane-bound inhibitor
BMP	Bone morphogenetic protein
BMP15	Bone Morphogenetic Protein 15
BMP15 (S356C)	Bone Morphogenetic Protein 15, where the serine at position 356 of the promature domain has been replaced with a cysteine
BMPR1A	Bone morphogenetic protein receptor like kinase 1 (a.k.a ALK3)
BMPR1B	Bone morphogenetic protein receptor like kinase 1 (a.k.a ALK6)
BMPR2	Type II bone morphogenetic protein receptor
BS ³	Bis(sulfosuccinimidyl)suberate (cross-linker)
CDMP1	Cartilage-derived morphogenetic protein 1 (a.k.a GDF5)
CDMP2	Cartilage-derived morphogenetic protein 2 (a.k.a GDF6)

Co-Smad	Common-smad
cpm	Counts per minute
DAN	Deadenylation nuclease (a.k.a PARN, poly(A)-specific ribonuclease)
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
dTTP	Deoxythymidine triphosphate
ECD	Extracellular domain
<i>E. coli</i>	Escherichia coli
EM	Expression medium (medium in which transfected HEK293 cells have grown and expressed recombinant proteins)
ERK	extracellular signal-regulated kinases
EST	Expressed sequence tag
FecX ^I	Fecundity gene Inverdale on X chromosome
FecG ^H	Fecundity gene (G, GDF9; H, high fertility)
FSH-R	Follicle stimulating hormone receptor
GDF	Growth differentiation factor
GDF9	Growth differentiation factor 9
GDNF	Glial cell-derived neurotrophic factor
HEK	Human embryonic kidney
h/o	Human prodomain / ovine mature domain
HPLC	High-performance liquid chromatography
h	Hour
I+	Heterozygous Inverdale gene carrier
IgG	Immunoglobulin, sub type G.
II	Homozygous Inverdale gene carrier
IMAC	Immobilised metal ion affinity chromatography
INH α	Inhibin α
I-Smad	Inhibitory-smad
IVF	<i>In vitro</i> fertilization
JNK	c-Jun N-terminal kinases
KL	Kit ligand (a.k.a. Steel factor)
LAP	Latency associated protein
LH	Luteinising hormone
LHR	Luteinising hormone receptor

LNCaP	Human prostate adenocarcinoma cell line
MAPK	Mitogen activated protein kinase
MH60	Murine B cell hybridoma cell line
MIC-1	Macrophage inhibitory cytokine 1 (a.k.a GDF15)
min	Minute
MIS	Mullerian inhibiting substance (a.k.a. AMH)
Ned4-2	Neural precursor cell expressed, developmentally down-regulated 4-like
NFκB	Nuclear factor kappa B
ODG2	Ovarian dysgenesis 2
o/o	Ovine prodomain / ovine mature domain
Op2	Osteogenic protein 2 (a.k.a BMP7)
Op3	Osteogenic protein 3 (a.k.a BMP8a)
P450 _{scc}	Cytochrome P450, cholesterol side chain cleavage
P450 _{arom}	<i>Aromatase Cytochrome P450</i> , (a.k.a aromatase, estrogen synthetase or estrogen synthase)
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
POF	Premature ovarian failure
POF4	Premature ovarian failure 4
PRDC	Protein related to DAN and cerberus
RGM	Repulsive guidance molecule
RGMa	Repulsive guidance molecule A
RIA	Radioimmunoassay
R-Smad	Receptor regulated-Smad
s	Second
(S356C)	Mutated BMP15 (Serine to Cysteine replacement at position 356 of mature domain).
SDS	Sodium dodecyl sulphate (a.k.a sodium lauryl sulphate)
SF-1	Steroidogenic factor 1
SFM	Serum free medium
Smad	Sma and Mad related family, derived from a fusion of the gene names <i>sma</i> (in <i>Caenorhabditis elegans</i>) and <i>Mad</i> (in <i>Drosophila</i>).
Smurf	Smad ubiquitination regulatory factor

SOSTDC1	Sclerostin domain containing 1
StAR	Steroidogenic acute regulatory protein
STRAP	Serine/threonine kinase receptor-associated protein
TAK1	TGF-beta-activated kinase 1
TFA	Trifluoroacetic acid
TGFB	Transforming growth factor Beta
TGFB1	Transforming growth factor Beta 1
TGFB2	Transforming growth factor Beta 2
TGFB3	Transforming growth factor Beta 3
TGFBR1	Transforming growth factor beta receptor like kinase 1 (a.k.a ALK5)
TGFBR2	Type II transforming growth factor beta receptor
TGFBRIIB	Type II transforming growth factor beta receptor B (<i>an alternatively spliced TGFβ type II receptor</i>)
Tween20	Polyethylene glycol sorbitan monolaurate, CAS # 9005-64-5
U	Unit
Vgr1	Vegetal-1 related protein (a.k.a BMP6)
Vgr2	VG-1-related protein 2 (a.k.a GDF3)
WWP1	WW domain containing E3 ubiquitin protein ligase 1
X-Gal	5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside

CHAPTER 1: INTRODUCTION

The subject matter in this thesis is concerned with the biological functions of two protein members of the transforming growth factor beta (TGFB) superfamily, namely bone morphogenetic protein 15 (BMP15) and growth differentiating factor 9 (GDF9). Over the past 15-20 years, BMP15 and GDF9, either alone or together, have emerged as being essential for: ovarian follicular development; determining the number of eggs (oocytes) released at each ovulation (ovulation rate) and; most likely, the quality of the oocyte at the time of fertilization.

In this introductory chapter, the general principles of ovarian and follicular development together with oocyte characteristics will be reviewed, followed by our current understanding of the biological actions of the TGFB superfamily, including that of BMP15 and GDF9.

1.1 The ovary

In mammals, ovaries are two paired organs/glands each of which is attached to the uterus by a uterine ligament and in close proximity to the end of the fallopian tube. One of the main roles served by the ovary is the production of oocytes, each of which is enclosed within a follicular structure consisting of two main somatic cell-types. The cells immediately adjacent to the oocyte are known as the granulosa cells and those immediately adjacent, but outside the basement membrane of the follicle, as the theca cells. These somatic cells together with the oocyte all need to develop in a highly synchronized manner to ensure oocytes are capable of being fertilised following ovulation, and developing into an embryo. Moreover, following ovulation, the follicular somatic cells are transformed into luteal cells and these are needed to support the early maintenance of pregnancy (Peters *et al.* 1975). Another key role for the ovary is to produce steroid and protein hormones which act as endocrine, paracrine and autocrine factors. Hormones produced by the ovary play diverse roles, such as in the development of secondary sex characteristics (Juul 2001), development of follicles and the timing of ovulation (Swerdlow *et al.* 1972; Richards *et al.* 1976), tissue remodelling of the uterus ready for implantation (Thibault *et al.* 1993), maintenance of early term pregnancy (Cumming *et al.* 1974), suppression of oestrus and ovulation during pregnancy (Meites *et al.* 1951) as well as effects on bone growth (Juul 2001).

1.2 Ovarian follicular development

1.2.1 The ontogeny of follicular formation

Sawyer *et al.* (2002) published a comprehensive study of follicular formation in the ewe. The evidence in humans and cattle indicates that the sequence of events leading to follicular formation is similar although the timing of events in fetal life is different but consistent with the duration of pregnancy in these species (van Wageningen and Simpson 1965; Garverick *et al.* 2010). In the mouse, follicular formation differs as it extends into early neonatal life but the origins of the somatic cell-types appears to be the same as in the aforementioned species (Mork *et al.* 2012). For the purposes herein, the sequence of events leading to follicular formation will be that described for sheep. In ewes, follicular formation begins during early fetal life. Briefly, by Day 38 after conception, dividing germ cells (oogonia) have migrated into the newly forming ovaries, continue to proliferate and also, to establish contacts with mesenchymal (pregranulosa) cells. These oogonia continue to migrate towards the cortical regions (outer periphery) of the ovary, where they are most numerous. Some are even found within the surface epithelium, previously described, albeit incorrectly, as the germinal epithelium. The formation of ovigerous cords, as the result of extensive contacts of oogonia with the pregranulosa cells and the formation of a basal lamina, is evident by Day 38 and this process continues until Day 75 of gestation. These cords are “sock like” in appearance and remain open to the surface epithelium whereby proliferating oogonia can recruit only epithelial (pregranulosa) cells within the cord. By Day 75, the ovary contains the maximum number of germ cells that it will ever have. However, between Day 75 and Day 90 the vast majority of germ cells die, although there is no corresponding cell death or proliferation observed in the pregranulosa cells. This prompted Sawyer *et al.* (2002) to propose that the surviving pregranulosa cells then form new associations with the remaining germ cells so that the process of germ cell death is a mechanism by which oocytes can acquire additional numbers of pregranulosa cells. Beginning on Day 55, some of these germ cells enter the prophase of the first meiotic division (meiosis I) and consequently to become oocytes. These were found mainly at the base of the ovigerous cords (the toe of the sock) located at the presumptive cortical-medulla interface. This process continues progressively up the cord, in an outwards direction, towards the surface of the ovary and by Day 75, large numbers of oogonia have entered meiosis I and become oocytes. Between Days 75 and 100, fully formed primordial follicles consisting of an oocyte surrounded by a single layer of granulosa cells, all encompassed in a basement membrane, begin to bud off from the “toe” of the ovigerous cord. This process continues progressively up the cord with the first primordial follicles to

form being located at the deepest region of the cortex and the last follicles to be formed being found adjacent to the surface epithelium. By Day 100, the process of follicular formation is complete. The earliest forming follicles are likely to have granulosa cells of mesenchymal origin (~5-10% of follicles in sheep) whereas the remainder contain granulosa cell of epithelial origin.

These primordial follicles remain in this state until growth is initiated. This begins around the time the first follicles are formed during fetal life and thereafter continues in a sequential manner throughout the life of the animal. However, while follicles remain at the primordial state, they are not completely quiescent as they actively transcribe genes. For example, in sheep, the mRNA for over 2300 genes have been isolated from primordial follicles (Kezele *et al.* 2005). Perhaps the term “developmentally arrested” would be a more accurate portrayal of the state of a primordial follicle. For example, proliferating cell nuclear antigen (PCNA) has been located in both the oocyte and the granulosa cells of primordial follicles in the sheep. (Lundy *et al.* 1999). PCNA plays a role not only in cell proliferation but also in cell repair (Essers *et al.* 2005). The ultimate role of the oocyte is to pass on an accurate copy of the genetic code to the next generation and as such it is not unreasonable to find proteins present which are involved in DNA repair.

1.2.2 Follicular development

Follicular development starts with the initiation of growth of a primordial follicle. Both the process by which an individual follicle is recruited for growth, and the mechanism responsible for initiating growth, are unknown. However, once this process has begun it is irreversible and unceasing with primordial follicles being recruited sequentially for further development (Peters *et al.* 1975). Moreover, a primordial follicle, once recruited, will continue to grow, without pause, until the follicle either reaches a stage where the oocyte is ovulated or undergoes atresia (Peters *et al.* 1975). This process of follicular development continues unabated throughout life even during the periods of anoestrus and pregnancy (Peters *et al.* 1975).

Once growth has been initiated, the diameter of the oocyte enlarges and the number of granulosa cells continue to increase and both occur in a coordinated manner (Lundy *et al.* 1999). As mentioned earlier, a primordial follicle consists of an oocyte surrounded by a single layer of granulosa cells, containing at least one flattened granulosa cell.

This follicle will be approximately 40 μ m in diameter and contain an oocyte with a diameter of about 35 μ m (Figure 1.1A; Sawyer *et al.* 2002). The first morphologically distinct sign that a follicle has been selected to grow is when the single layer of granulosa

cells has become composed solely of cuboidal shaped cells (Figure 1.1B). Using sheep as an example of the events occurring during follicular development, Lundy *et al.* (1999) has termed follicles at this stage of development as type 2 or primary follicles. In addition, the diameter of the oocyte increases by about 50% during the transition from a primordial to a primary follicle (Lundy *et al.* 1999). Follicles at this phase of growth continue to develop as pre-antral follicles. The largest pre-antral follicles consist of 4-6 layers of granulosa cells surrounding an oocyte which has now obtained a diameter of ~90 μ m, some 2.5 fold greater than when it was enclosed in a primordial follicle (Figure 1.1C). By this stage of follicular development, all oocytes have formed a completed zona pellucida and theca cells have been recruited from the surrounding stromal cells to enclose the follicle (Peters and McNatty 1980; Lundy *et al.* 1999). Small, fluid-filled, intracellular spaces also start to appear between the granulosa cells (Peters and McNatty 1980). The production and accumulation of this fluid continues in a progressive manner until the spaces coalesce and a fluid-filled antrum is formed. At this stage, the follicle has reached, what is referred to, as the small antral follicle stage (Figure 1.1D): these follicles will now contain an average of 11,000 granulosa cells. This represents more than a 700-fold increase in granulosa cell number from that of a primordial follicle, and has taken 9-10 cell doublings to achieve this state.

Figure 1.1 Schematic of ovarian follicles at the (A) primordial, (B) primary, (C), pre-antral, (D) small antral and (E) large antral stages of development. The cell types comprising the follicle are marked; oocyte (o), granulosa cells (g) and theca (t).

The oocyte now will have reached 120µm, almost its maximum diameter and the follicle will have a diameter of approximately 350µm (Lundy *et al.* 1999). As the follicle continues to develop and the antrum becomes larger, the granulosa cells segregate into two distinct phenotypes. The first, referred to as the mural granulosa, are located around the periphery of the follicle near to the basement membrane. The second, referred to as the cumulus cells, are those cells surrounding the oocyte (Figure 1.1E). It is uncertain as to how this segregation of phenotypes occurs. One view is that the oocyte actively induces the cumulus cell phenotype by the production and secretion of specific factors. The other view is that up to this stage all granulosa cells are “cumulus cells” and those (i.e mural granulosa cells) that become more distant from the oocyte as the follicle grows, default to a different phenotype as they escape the action or influence of the oocyte-secreted factors. Whichever view is correct, either a mural granulosa cell actively changing to a cumulus cell, or a cumulus cell passively defaulting to a mural granulosa cell, the one point they have in common is the involvement of the oocyte. The theca cells have also segregated into a highly vascularised, well-defined theca interna and a relatively avascular, and less well-defined, theca externa.

Antrum formation is a perilous time for a follicle. While very few follicles undergo atresia prior to this stage, many follicles fail to survive during the transition to antrum formation or during early antral development and those that fail, enter an apoptotic pathway. In the sheep, follicles are able to grow up to 3 mm in diameter without the requirement for gonadotrophins. Although granulosa cells can respond to gonadotrophins prior to reaching 3 mm in diameter, they become gonadotrophin-dependent for continued growth beyond this stage (Figure 1.2; McNatty *et al.* 1990). In order to develop into a preovulatory follicle, granulosa cells must develop a functional receptor for luteinising hormone (LH) (Webb and England, 1981). Should an ovine follicle avoid atresia, it will ultimately develop into a preovulatory follicle with a diameter of 5-7mm, and contain approximately 5×10^6 granulosa cells. The number of granulosa cells present in preovulatory follicles varies between species. For example in humans, the preovulatory follicle is >15 mm diameter and contains $>50 \times 10^6$ granulosa cells (Gougeon 2010)

The oestrous or menstrual cycle in mammals includes a series of endocrine events coordinated through hormonal signals between the hypothalamus, pituitary and ovary. In sheep and cattle, the oestrous cycle lasts 17 and 21 days respectively.

B

Developmental stage	First appearance of mRNA or protein
Developmentally arrested	GDF9 ^(o) , BMP6 ^(o) , BMPR1A ^(o, g) , TGFBR1 ^(o) , BMPR1B ^(o) , BMPR2 ^(g) , Betaglycan ^(o) , Follicle stimulating hormone receptor (FSHR) ^(g) , Steroidogenic factor-1 (SF-1) ^(g) , Steroidogenic acute regulatory protein (StAR) ^(o) , 3 β -hydroxy steroid dehydrogenase (3 β -HSD) ^(g)
Non-responsive to gonadotrophins	BMP15 ^(o) , BMPR1B ^(g) , ACVR2B ^(o, g) , Inhibin beta B ^(g) , Inhibin alpha ^(g)
Gonadotrophin-responsive	TGFB1 ^(t) , TGFB2 ^(t) , BMPR1A ^(t) , TGFBR1 ^(g, t) , BMPR1B ^(t) , ACVR2B ^(t) , BMPR2 ^(t) , TGFBR2 ^(t) , Betaglycan ^(g, t) , Follistatin ^(g, t) , Follistatin-related protein (FSRP) ^(g, t) , SF-1 ^(t) , StAR ^(t) , Cholesterol side-chain cleavage enzyme (P450scc) ^(g, t) , 17 α -Hydroxylase (17 α OH) ^(t) , 3 β -HSD ^(g) , LHR ^(t) , FSHR ^(g) , Inhibin beta A ^(g)
Gonadotrophin-dependent	BMP6 ^(g, t) ? inconsistent expression, StAR ^(g) , P450arom ^(g)
LH-dependent	LHR ^(g)

Figure 1.2 Schematic diagram depicting the recruitment and growth of follicles throughout an oestrous cycle, illustrating how a single follicle may continue its growth towards ovulation. (A) Primordial follicles are continually and sequentially recruited to enter the growing pool. These may either progress to ovulation or undergo atresia at some point along the way (as shown by upward then downward pointing arrows). As follicles reach the large antral stage and become gonadotrophin dependent, they secrete more estradiol which at the later stages of the oestrous cycle, feeds back on the pituitary to suppress FSH, and increase LH secretion. This

ultimately culminates in the LH surge and ovulation. Adapated from (Peters and McNatty 1980). (B) The ontogeny of expression of selected genes during follicular development. Members of the TGFB superfamily family (Juengel and McNatty 2005), steroidogenic pathway (Logan *et al.* 2002), LHR (Logan *et al.* 2002), FSHR (Tisdall *et al.* 1995) and Inhibins (Montgomery *et al.* 2001) genes/proteins within the oocyte (o), granulosa cells (g) and theca (t) compartments of the ovine follicle).

The cycle is regulated by the hypothalamus secreting episodic pulses of gonadotrophin-releasing hormone (GnRH) that act on the anterior pituitary, which in turn, secretes follicle-stimulating hormone (FSH) in a wave-like manner and LH in pulses synchronised to those of GnRH.

The circulating levels of gonadotrophic hormones exert their actions on the ovary. By acting on granulosa cells, FSH promotes follicular maturation, while LH stimulates androgen synthesis in thecal cells. At the same time, oocyte-secreted factors (such as BMP15; see Section 1.4.3) regulate the actions of FSH and LH on the granulosa cells. The secreted androgen, mainly in the form of androstenedione, is secreted into the blood stream and also metabolized to oestradiol by the granulosa cells under the influence of FSH. Likewise, antral follicles and specifically the granulosa cells also produce inhibin. Follicular development to the preovulatory stage leads to increasing plasma concentrations of both oestradiol and inhibin, which, in turn, initiates feedback effects on the hypothalamus and/or pituitary. The increasing oestradiol levels, through positive feedback results in an increase in the pulse frequency of LH secretion, and together with inhibin, by negative feedback, a decline in FSH levels. The levels of circulating inhibin are highly correlated with the number of granulosa cells present in the large antral follicles (McNatty *et al.* 1993). At this stage, the dominant oestrogen-secreting follicle(s) develop a functional LH receptor on their granulosa cells. This enables the oestrogen-secreting follicle(s) to continue development, by maintaining aromatase activity, in the face of declining FSH levels. In contrast, any remaining follicles (at the gonadotrophin-dependent stage of development), but without LH-responsive granulosa cells, are unable to sustain growth and undergo atresia (Figure 1.2). Ultimately, the increasing levels of oestradiol secretion from the preovulatory follicle(s) reach a threshold that triggers a large preovulatory surge of both LH and FSH (Baird and McNeilly 1981). This gonadotrophin surge induces ovulation (i.e. the rupture of the dominant follicle(s) and release of the oocyte-cumulus cell complex) together with luteinisation of both the granulosa and theca cells leading to formation of a corpus luteum (CL). During the luteal phase, the secretion of CL-derived plasma

progesterone limits the ability of follicular-derived oestradiol to stimulate gonadotrophin secretion. However, if pregnancy does not eventuate, the CL regresses leading to a rapid decline in progesterone levels.

1.2.3 Oocyte maturation

As mentioned earlier, follicular somatic cell development occurs concurrently with the closely-associated process of oocyte maturation. Somatic cell development results in the formation of a mature follicle capable of ovulating. Moreover, oocyte maturation involves the oocyte gaining the ability to resume meiosis and upon fertilization, to develop into a viable embryo (developmental competency). These processes require a highly coordinated degree of communication between the oocyte and the somatic cells. This ‘intra-follicular’ communication is carried out, not only by molecules acting in a paracrine fashion between oocytes and cumulus/granulosa cells, but also by molecules which are transported through gap junctions between the cell-types. Cumulus/granulosa cell processes occur between the granulosa cells, granulosa and cumulus cells and also extend through the zona pellucida to form attachments with the oocyte (Thibault *et al.* 1993). The zona pellucida plays important roles during fertilization and preimplantation development (Wassarman and Litscher 2012).

Oocyte maturation culminates in the oocyte gaining the competence to undergo meiotic maturation. During the growth phase of the follicle, the ovine oocyte increases 3.5 fold in diameter from 35µm to >120µm. This equates to an increase in volume of over 40-fold. Generally, the oocyte needs to reach 80% of its maximum size before it is capable of meiotic maturation (Thibault *et al.* 1993). However, the oocyte remains in meiotic arrest awaiting the signal to resume meiosis, that is, the preovulatory LH surge. Following the surge, gap junctional connectivity between the oocyte and its granulosa/cumulus cell syncytium is broken, resulting in a rapid decline of cAMP concentrations within the oocyte (Sela-Abramovich *et al.* 2006). This allows the oocyte to resume meiosis by moving from diakinesis, where it has been arrested during the growth phase of the follicle and into metaphase of meiosis I (Thibault *et al.* 1993).

1.3 Transforming Growth Factor-Beta (TGFB) super family

1.3.1 General characteristics

The transforming growth factor-beta (TGFB) super family comprised of structurally similar proteins, with diverse functions, and consists of over 35 members. The members

can be classified further, into the TGFB, activin, bone morphogenetic protein (BMP) and growth differentiation factor (GDF) sub-families, as well as more distant members such as Mullerian inhibiting substance (also known as anti-Mullerian hormone, AMH), and inhibin α (Massague 1998). Members of the TGFB family are produced as pre-proproteins consisting of a signal peptide, a prodomain and a mature domain (Massague 1990).

Figure 1.3 Monomer and dimer ribbon structures. (A) The monomer of ovine BMP15 (oBMP15) has been likened to the shape of a left hand which consists of two “fingers” composed of anti-parallel beta strands (blue), a “wrist” region constructed from an alpha helix (green). Two disulphide bridges (red) complete a ring of covalent bonds that is bisected by a third disulphide bond (purple) to form the cysteine knot. This forms a rigid “palm” (Daopin *et al.* 1992). (B) The structure of a dimer of BMP2. The covalent bond between the seventh conserved cysteine (yellow) linking the two monomers is shown. The BMP2 dimer was obtained from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (www.pdb.org; Accession number 1ES7; Kirsch *et al.* 2000b). The oBMP15 was created using the Swiss model comparative protein modelling server (Schwede *et al.* 2003; Arnold *et al.* 2006; Kopp and Schwede 2006; Kiefer *et al.* 2009) using BMP2 (1ES7) as a template.

One of the defining characteristics of this family is the presence of a “cysteine knot” within the mature region, formed from disulphide bonds among six conserved cysteines. (Figure 1.3A; Daopin *et al.* 1992). It is thought that the fully mature and biologically active form of most family members consists of dimers of the mature region. Most exist as homodimers, however some such as activin AB, inhibin A and inhibin B are heterodimers. It has also been shown that coexpressed recombinant BMP2, with BMP5, 6 or 7 are able to form heterodimers that are more biologically active than their homodimer counterparts (Israel *et al.* 1996). BMP15 has also been shown to act in a synergistic manner with GDF9, possibly as heterodimers (McNatty *et al.* 2005b). These dimers are usually bound covalently through a seventh conserved cysteine (Figure 1.3B; McDonald and Hendrickson 1993). However in some family members such as BMP15, GDF9, GDF3, lefty 1, and lefty 2, this seventh cysteine has been mutated to a serine, therefore any dimers that exist between these forms must be due to a non-covalent association.

1.3.2 Role of the proregion

In general, the role of the proregion of the TGFB family members has not been well defined. However, for some members of the family, interactions between the proregion and the mature domains have been demonstrated (Young and Murphy-Ullrich 2004; McIntosh *et al.* 2008; Walton *et al.* 2009). Moreover, mutations within the proregion of the TGFB superfamily members have been linked to disease states. For example, mutations in: AMH, result in persistent Mullerian duct syndrome (Belville *et al.* 2004); GDF1, results in heart defects (Karkera *et al.* 2007); GDF5, leads to a brachydactyly condition (Schwabe *et al.* 2004) and; BMP15 to hypergonadotropic ovarian failure (Di Pasquale *et al.* 2004). With respect to normal physiological functions, it is thought that the pro-region folds around and binds non-covalently to the mature region, thereby causing or assisting the mature domain to fold correctly (Walton *et al.* 2009). The mature region is cleaved from the pro-region by furin-like proprotein convertases (Dubois *et al.* 1995; Thomas 2002; Jin *et al.* 2004). It has also been suggested that the pro-region, while still attached non-covalently to the mature region, plays a role in dimer formation and in export from the cell (Walton *et al.* 2009) with two proregions and a mature dimer being secreted as a complex. Exceptions to this do occur as uncleaved GDF8 has been found bound to the extracellular matrix surrounding skeletal muscle cells (Anderson *et al.* 2008). The proregion is also thought to be involved in the presentation of these dimers to receptors on target cells or to anchor them to the extracellular matrix (Brown *et al.* 2005; Anderson *et al.* 2008; Sengle *et al.* 2008a). The proregion of some family members, such as TGFB and myostatin (Brown *et al.* 2005) bind strongly to and thus inhibit the action of the mature dimer, thereby acting as a latency protein. Moreover, the proregion of TGFB1 is also known as latency-associated protein (LAP).

1.3.3 Receptors

Receptors for the TGFB family members are composed of two types of serine-threonine kinases. Type I receptors (also known as activin receptor like kinases: ALK) are comprised of seven members, ALK1 (ACVRL1; activin A receptor, type II-like I), ALK2 (ACVR1; activin A receptor, type I), ALK3 (BMPR1A; bone morphogenetic protein receptor, type IA), ALK4 (ACVR1B; activin A receptor, type IB), ALK5 (TGFB1; transforming growth factor beta receptor 1), ALK6 (BMPR1B; bone morphogenetic protein receptor, type IB) and ALK7 (ACVR1C; activin A receptor, type IC). The five type II receptors are ACVR2A (activin A receptor, type IIA), ACVR2B (activin A receptor, type IIB), BMPR2 (bone morphogenetic protein receptor, type II (serine/threonine kinase)), TGFB2

(transforming growth factor, beta receptor II (70/80 kDa)), and AMHR2 (anti-Mullerian hormone receptor, type II) (de Caestecker 2004). The onset of expression of some of these receptors in the ewe are shown in Figure 1.2.

Figure 1.4 A BMP2 homodimer: type II receptor: type I receptor complex. Adapted from (McNatty *et al.* 2004) and (Lin *et al.* 2006). The BMP2 homodimer was obtained from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (1ES7; Kirsch *et al.* 2000b).

A receptor ligand complex consists of a dimer of mature proteins bound to two type I receptors and two type II receptors (Figure 1.4). Upon formation of the complex, the constitutively-active type II receptor phosphorylates and activates a highly conserved glycine- and serine-rich region of the type I receptor, termed the GS domain (Wrana *et al.* 1994). In turn the type I receptor recruits and phosphorylates intracellular signalling molecules called Smads. (Miyazono 1998; Fujii *et al.* 1999).

Table 1.1 Relationships between ligands, receptors and the Smad signalling pathways of TGF β superfamily members

Ligand	aka	Type II receptor	Type I receptor	Co-Receptor	R-Smad	References
Inhibin(s)		ACVR2A ACVR2B BMPR2		Betaglycan		Lewis <i>et al.</i> 2000; Bernard <i>et al.</i> 2001; Wiater and Vale 2003
Activin(s)		ACVR2A ACVR2B	ACVR1B		Smad2/3	Attisano <i>et al.</i> 1996; Macias-Silva <i>et al.</i> 1998; Bernard <i>et al.</i> 2001
Activin B		ACVR2A ACVR2B	ACVR1C		Smad2/3	Bernard <i>et al.</i> 2006; Bertolino <i>et al.</i> 2008
TGFβ1		TGFBR2	ACVRL1 TGFBR1	Endoglin Betaglycan	Smad2/3	Boyd <i>et al.</i> 1990; Yamashita <i>et al.</i> 1994; Altomonte <i>et al.</i> 1996; Nakao <i>et al.</i> 1997b; Lux <i>et al.</i> 1999
TGFβ2		TGFBR2B	TGFBR1	Betaglycan	Smad3	Boyd <i>et al.</i> 1990; Rotzer <i>et al.</i> 2001; Zode <i>et al.</i> 2009)
TGFβ3		TGFBR2	ACVRL1	Endoglin	Smad2 /3	Altomonte <i>et al.</i> 1996; Lux <i>et al.</i> 1999; Dudas <i>et al.</i> 2004
BMP2	BMP2a	BMPR2	BMPR1A BMPR1B	Betaglycan, Repulsive Guidance Molecule (RGM)	Smad1/5/8	Sieber <i>et al.</i> 2009; Babitt <i>et al.</i> 2005; Kirkbride <i>et al.</i> 2008
BMP3	Osteogenin	ACVR2B	ACVR1B		No signal transduction	Gamer <i>et al.</i> 2005; Allendorph <i>et al.</i> 2007
BMP4		BMPR2	BMPR1A BMPR1B	Betaglycan, RGMa	Smad1/5/8	Baade Ro <i>et al.</i> 2004; ten Dijke <i>et al.</i> 1994; Nohno <i>et al.</i> 1995; Babitt <i>et al.</i> 2005; Gromova <i>et al.</i> 2007; Kirkbride <i>et al.</i> 2008)
BMP5		?	?		Smad1/8	Zuzarte-Luís <i>et al.</i> 2004
BMP6	Vgr1	ACVR2A BMPR2	ACVR1	(EGF-CFC factor)	Smad1/5	Baade Ro <i>et al.</i> 2004; Ebisawa <i>et al.</i> 1999; Cheng <i>et al.</i> 2003
BMP7	OP1	ACVR2A ACVR2B	ACVR1	Betaglycan	Smad1/5	Sieber <i>et al.</i> 2009; ten Dijke <i>et al.</i> 1994; Macias-Silva <i>et al.</i> 1998; Kirkbride <i>et al.</i> 2008 Baade Ro <i>et al.</i> 2004; Motazed <i>et al.</i> 2008
BMP8a	OP2	similar to BMP7			?	Zhao <i>et al.</i> 2001
BMP8b	BMP8/ OP2/Op3	?	?		Smad1/5	Jorgez <i>et al.</i> 2005
BMP10		ACVR2A BMPR2	BMPR1A BMPR1B		Smad1/5/8	Mazerbourg <i>et al.</i> 2005
BMP15	GDF9b	BMPR2	BMPR1B		Smad1/5/8	Moore <i>et al.</i> 2003
Nodal		ACVR2B	ACVR1B ACVR1C	Cripto (EGF-CFC factor)	Smad2	Reissmann <i>et al.</i> 2001; Yeo and Whitman 2001

Table 1.1 continued. Relationships between ligands, receptors and the Smad signalling pathways of TGFB superfamily members.

Ligand	aka	Type II receptor	Type I receptor	Co-Receptor	R-Smad	References
GDF1		ACVR2B	ACVR1B	(EGF-CFC factor)	Smad2	Cheng <i>et al.</i> 2003
GDF2	BMP9	BMPR2	ACVRL1	Endoglin	Smad1/5	Scharpfenecker <i>et al.</i> 2007
GDF3	Vgr2	ACVR2A ACVR2B	ACVR1C	Cripto		Andersson <i>et al.</i> 2007; Andersson <i>et al.</i> 2008
GDF5	CDMP1	BMPR2 ACVR2A	BMPR1A BMPR1B	Betaglycan	Smad1/5/8	Nishitoh <i>et al.</i> 1996; Aoki <i>et al.</i> 2001; Kirkbride <i>et al.</i> 2008; Upton <i>et al.</i> 2008
GDF6	BMP13/ CDMP2	ACVR2A BMPR2	BMPR1A BMPR1B		Smad1/5/8	Mazerbourg <i>et al.</i> 2005
GDF7	BMP12	ACVR2A BMPR2	BMPR1A BMPR1B		Smad1/5/8	Mazerbourg <i>et al.</i> 2005
GDF8	Myostatin	ACVR2B	ACVR1B TGFBRI		Smad2/3	Rebbapragada <i>et al.</i> 2003
GDF9		BMPR2	TGFBRI		Smad2/3	Vitt <i>et al.</i> 2002; Kaivo-Oja <i>et al.</i> 2003; Mazerbourg <i>et al.</i> 2004; Wang <i>et al.</i> 2009
GDF10	BMP3b	?	?		?	
GDF11	BMP11	ACVR2A ACVR2B	TGFBRI		Smad2	Oh <i>et al.</i> 2002; Andersson <i>et al.</i> 2006
GDF15	MIC-1	?	?		Smad2/3	Xu <i>et al.</i> 2006
AMH	MIS	AMHR2	ACVRL1 BMPR1A BMPR1B		Smad1	di Clemente <i>et al.</i> 1994; Gouedard <i>et al.</i> 2000; Clarke <i>et al.</i> 2001; Jamin <i>et al.</i> 2002

? Not described in the literature

Generally, ligands bind first to the type II receptor which then recruits and trans-phosphorylates the type I receptor. However, exceptions to this occur and both BMP2 and BMP4 have been shown to bind to a type I receptor first, and to then recruit the type II receptor to the complex (de Caestecker 2004). It has also been reported that type I and type II BMP receptors can form complexes in the absence of ligand (Gilboa *et al.* 2000). Several studies using BMP2 have shown that it can bind either to the type I receptor and recruit the type II receptor or that it can bind to pre-formed receptor complexes of type I and II receptors (Gilboa *et al.* 2000; Hassel *et al.* 2003; Sieber *et al.* 2009). Moreover, the intracellular signalling pathway which is activated by BMP2 is dependent upon the method by which BMP2 associates with its receptors. BMP2 binding to preformed receptor complexes initiates the canonical Smad pathway. In contrast, when binding first to the type I receptor and recruiting the type II receptor, the mitogen-activated protein kinase (MAPK) pathway is activated (Sieber *et al.* 2009).

Numerous combinations between type I and II receptors are possible, and each of these combinations may also bind more than one member of the TGFB superfamily. In addition to the type I & II receptors, there are also co-receptors which interact with TGFB superfamily members and these may be required for correct signalling (Table 1.1). One such molecule is betaglycan, and has been named as a TGFB type III receptor (de Caestecker 2004). Betaglycan plays a role in presenting ligands to their receptor. This may be a positive role when presenting TGFB2 to its receptor but it may play a more negative role when presenting inhibin to ACVR1A, where it acts as an antagonist to the action of activin and possibly other BMPs (de Caestecker 2004; Juengel and McNatty 2005).

Conversely, receptors can also act as inhibitors of signalling by sequestering ligands in a non-signalling receptor complex. For example ACVR1 can block the action of activin by binding to its type II receptor and prevent activin from signalling. Moreover, this action can itself be blocked by overexpression by AMHR1, which is postulated to sequester ACVR1. This would allow the activin type II receptor to recruit the correct type I receptor and activate the activin signalling pathway (Renlund *et al.* 2007).

1.3.4 Signalling pathways

The canonical signalling pathway for members of the TGFB superfamily is through the Smad pathway. There are eight Smads in total with Smads 1, 2, 3, 5 and 8 being activated upon phosphorylation by a type I receptor. These are known as receptor-regulated Smads (R-Smads). The R-Smads fall into two categories namely Smads2/3 which are activated by TGFB/Activin/Nodal via ACVR1, ACVR1B, TGFBR1 or ACVR1C and Smads1/5/8 which are activated by BMPs via ACVR1, BMPR1A or BMPR1B (Table 1.1). Once phosphorylated, these R-Smads, in the form of homodimers, interact with Smad4. Smad4 is a binding partner to all of the R-Smads and is known as the common Smad (Co-Smad). Upon complex formation with the Co-Smad, R-Smads translocate to the nucleus where they bind to Smad response elements as part of a larger transcriptional complex. For example, Smad3 in complex with Smad2 and Smad4 binds to the palindromic Smad binding element GTCTAGAC (von Gersdorff *et al.* 2000). Smad2 has been shown (in complex with Smad4) to recruit histone acetylases as well as ATP dependent chromatin remodelling complexes to the DNA and to orchestrate chromatin remodelling prior to recruitment of the RNA pol II transcription machinery to promoter regions. Not only does Smad2 cause remodelling of chromatin but it also shows an absolute requirement for chromatin and is unable to activate transcription from naked DNA (Ross *et al.* 2006).

Smad-independent pathways are also activated by members of the TGFB superfamily. The mitogen-activated protein kinase (MAPK) pathway(s) including c-Jun N-terminal kinases (JNKs), extracellular signal-regulated kinases (ERKs) and p38 MAPK are all activated by TGF-beta-activated kinase 1 (TAK1) (Attisano and Wrana 2002). The activation of TAK1 is downstream of receptors for both BMP and TGFB (Moustakas and Heldin 2005). Mullerian-inhibiting substance has been shown to work through nuclear factor- κ B (NF κ B) to inhibit androgen-stimulated growth of LNCaP cells (Tran *et al.* 2006). Moreover BMP15 and GDF9 together have also been shown to activate non-Smad signalling pathways in a species-specific manner (Reader *et al.* 2011; Mottershead *et al.* 2012; Reader *et al.* 2014).

Signalling is mediated by a wide range of receptor combinations (with and without co-receptor modification). Although there is the appearance of a potential bottleneck in the signalling pathway with a limited number of Smads signalling through one of two main groupings (Smads1/5/8 or Smads2/3), the interactions and crosstalk between Smad and non-Smad mediated pathways enables a wide range of responses to be mediated by members of the TGFB superfamily. In addition, signalling may be modified by Smad binding proteins. These binding proteins consist of proteins with extremely diverse functions. They include membrane receptors, cytoskeletal components, nuclear transport proteins, cytoplasmic proteins, transcriptional coactivators and repressors, and transcription factors (Itoh *et al.* 2000). Given the numerous ligand/receptor complexes which are capable of being formed, often with antagonising actions, it may no longer be enough to simply ask what action a particular ligand mediates. "...à la John F. Kennedy: ask not what the signal does with the cell, but what the cell does with the signal." (Joan Massagué, 2006)

1.3.5 Intra- and extracellular regulators of TGFB family members

The actions of TGFB superfamily members are regulated at most points of the signalling pathway(s). In some cases, such as for TGFB and GDF8, the mature dimer is secreted in a latent form with the pro-region bound strongly to the mature dimer thus preventing access to receptors. This is achieved either by binding to and covering the receptor binding area of the mature domain or by sequestering the mature dimer to the extracellular matrix by binding to both the mature domain and extracellular matrix proteins, creating an extracellular store. However, many family members do not bind strongly to their pro-region and their actions can be regulated by other inhibitors. Numerous extracellular

inhibitors have been identified, such as follistatin (Shimonaka *et al.* 1991), noggin (Zimmerman *et al.* 1996), decorin (Yamaguchi *et al.* 1990), the cerberus/Dan family of secreted BMP inhibitors consisting of DAN, cerberus, gremlin, protein related to DAN and cerberus (PRDC) (Balemans and Van Hul 2002), sclerostin (Kusu *et al.* 2003), sclerostin domain containing 1 (SOSTDC1, also known as ectodin) (Laurikkala *et al.* 2003) and coco (Bell *et al.* 2003) and the chordin family, chordin, chordin-like (Balemans and Van Hul 2002), brorin (Koike *et al.* 2007) and brorin-like (Miwa *et al.* 2009). Even members of the TGFB superfamily itself can act as extracellular inhibitors to other members. GDF3 can act as a BMP inhibitor by forming inactive heterodimers (Levine and Brivanlou 2006). Inhibin on the other hand acts to sequester type II activin receptors in a complex with betaglycan, thus blocking the action of activin (Shimasaki *et al.* 2004).

To add another level of complexity, pseudoreceptors such as BAMBI (BMP and activin membrane-bound inhibitor) exist. BAMBI is structurally similar to the extracellular domain of type I receptors but lacks an intracellular kinase domain. BAMBI can form stable complexes with type II receptors and thus prevent signal transduction (Onichtchouk *et al.* 1999). Although all of the above act to inhibit the action of TGFB superfamily members, it should be remembered that members of this family exert their effect by means of concentration gradients. The role of the inhibitors is probably to help create these gradients and ensure that the correct signal strength is delivered to the target cell and not simply to block their action.

Intracellular regulation of the signalling pathway also occurs. Smads 6 and 7 are inhibitory Smads (I-Smads) and exert their inhibitory effects in several distinct ways. Smad7 is a general inhibitor of TGFB superfamily signal transduction, while Smad6 appears to be an inhibitor of BMP signalling (Itoh *et al.* 2000). I-Smads interact with activated type I receptors and thereby prevent phosphorylation of the R-Smads (Imamura *et al.* 1997; Nakao *et al.* 1997a). In addition, Smad7 forms a complex with Smurf (Smad ubiquitination regulatory factor) 2, and upon binding to the type I receptor, both Smad7 and the receptor are ubiquitinated (Itoh and ten Dijke 2007). Moreover, Smad7 can also recruit a complex containing protein phosphatase (PP) 1c which dephosphorylates and inactivates the TGFBR1 receptor. In a similar manner, ACVRL1 is also dephosphorylated by PP1 α in a Smad7-dependent manner (Itoh and ten Dijke 2007). An accessory molecule, STRAP (serine/threonine kinase receptor-associated protein), interacts with and recruits Smad7 to the receptor (Datta and Moses 2000) although no protein performing a similar role for

Smad6 has yet been discovered. However, AMSH (associated molecule with the SH3 domain of STAM) has the reverse effect and is a negative regulator of both I-Smads (Itoh *et al.* 2001). Smad6 competes with the Co-Smad (Smad4) for activated Smad1 and thereby specifically inhibits BMP activated Smad1 (Hata *et al.* 1998). Smad7 acts as an adaptor molecule for Smurfs 1 and 2, Nedd4-2 and WWP1/Tiul1 and thereby promotes the degradation of both R-Smads and the Co-Smad (Itoh and ten Dijke 2007). Smad6 also acts as a transcriptional co-repressor by forming a complex with the homeobox Hoxc-8 thereby inhibiting the formation of a Smad-1:Hoxc-8 complex and Smad1 induced transcription (Bai *et al.* 2000). In addition, Smad6 has also been reported to bind directly to TAK1 (TGFB-activated kinase 1) and interfere with BMP induced non-Smad mediated apoptosis in MH60 cells (Kimura *et al.* 2000).

1.3.6 The role of TGFB family members in follicular development and ovulation rate

In recent years, members of the TGFB superfamily have become increasingly recognised as key regulators of ovarian follicular development as well as influencing ovulation-rate and thus the number of offspring. Their importance in this role has come from studying naturally-occurring mutations in sheep, mouse knockout models as well as *in vivo* and *in vitro* studies (Chang *et al.* 2002; Findlay *et al.* 2002; Juengel and McNatty 2005; Knight and Glister 2006)

The first TGFB superfamily member that was identified affecting fecundity in sheep was BMP15. Originally, two independent germ-line point mutations were reported. The first resulted in a non-conserved amino acid substitution in the mature domain and was termed the Inverdale gene while the second resulted in a premature stop codon and was termed the Hanna gene (Galloway *et al.* 2000). Subsequently, four other BMP15 mutations that influence follicular development and ovulation-rate have been identified in other highly prolific sheep breeds (Hanrahan *et al.* 2004; Bodin *et al.* 2007; Monteagudo *et al.* 2009). Two mutations in GDF9 have also been found to influence ovarian follicular development and ovulation-rate in sheep in a similar manner to that for BMP15. For both the BMP15 and GDF9 heterozygous mutant ewes, higher ovulation rates occur whereas the homozygous animals are sterile (Galloway *et al.* 2000; Hanrahan *et al.* 2004; Nicol *et al.* 2009). Another mutation affecting fecundity was originally identified in Booroola sheep: this was present in the TGFB, type I receptor, BMPR1B, (otherwise known as ALK6) (Mulsant *et al.* 2001; Souza *et al.* 2001; Wilson *et al.* 2001). The Booroola mutation in BMPR1B causes an increase in ovulation-rate in a dose-dependent manner (i.e. wild-type <

heterozygote < homozygote). Heterozygous carriers of any of the above mutations, all have increased ovulation-rates, showing that they participate in a pathway(s) involved in the recruitment of follicles for ovulation.

While some members of this super family play a role in the recruitment of follicles for ovulation, others appear to influence follicular development, if not ovulation-rate. By comparing mice null for anti-Mullerian hormone (AMH) with wild-type, it was found that AMH null mice have an increased rate of recruitment of primordial follicles into the growing pool. This suggests that AMH plays an important role in regulating the number of growing follicles (Durlinger *et al.* 1999; Visser and Themmen 2005). Likewise, a similar approach identified inhibin α as a negative regulator of stromal cell proliferation in gonads (Matzuk *et al.* 1992). Mice null for inhibin α , develop tumours of mixed granulosa and thecal cell origins. Like sheep (Hanrahan *et al.* 2004), mice lacking GDF9 are infertile (Dong *et al.* 1996; Yan *et al.* 2001) as follicular growth is unable to proceed from the primary stage of growth. Moreover, there are possibly indirect roles for GDF9 for both the formation of the theca and for oocyte meiotic competence (Yan *et al.* 2001). Activins, BMP6, GDF9 and BMP15 have all been implicated in modulating the actions of FSH and promoting proliferation of granulosa cells (Knight and Glister 2006). Likewise, the various TGFB isoforms have been shown to have roles in granulosa cell proliferation and to also modulate some FSH actions. It is important to note at least some of these roles are species-dependent (Juengel and McNatty 2005). TGFB also inhibits androgen production by thecal cells by down regulation of 17 α -hydroxylase and steroidogenic acute regulatory protein (Juengel and McNatty 2005). Members of the TGFB family, their receptors and binding proteins are all produced by cell types within the mammalian ovary, and have been the subject of several articles (Kristensen *et al.* 2014) and reviews (Findlay *et al.* 2002; Erickson and Shimasaki 2003; Knight and Glister 2003; Shimasaki *et al.* 2004; Juengel and McNatty 2005; Knight and Glister 2006).

1.4 Bone Morphogenetic Protein 15 (BMP15)

1.4.1 General characteristics

Bone morphogenetic protein 15 (BMP15), also known as growth differentiation factor 9B (GDF9B), was identified by two independent groups, in 1998. The first group used polymerase chain reaction (PCR), utilising degenerate oligonucleotides, based upon consensus sequences from known BMP's, to identify both the mouse *Bmp15* and human *BMP15* genes (Dube *et al.* 1998). The second group searched the GenBank Expressed

Sequence Tag (EST) database with the mouse GDF9 sequence. From this search, they identified a putative member of the TGFB superfamily from a mouse 2-cell embryo library, which they named GDF9B (Laitinen *et al.* 1998). BMP15 has also been reported as ovarian dysgenesis 2 (ODG2) (Di Pasquale *et al.* 2004) and as premature ovarian failure-4 (POF4) (Dixit *et al.* 2006).

Human BMP15 (hBMP15) is encoded by a gene on the X chromosome at location Xp11.2 (Dube *et al.* 1998). The gene consists of two exons. Exon 1 codes for part of the pro-region while exon 2 codes for the remainder of the proregion and the entire mature region. The gene product of hBMP15 is a 391 amino acid (aa) preproprotein consisting of a 17 aa signal peptide, a 251aa proregion and a 124aa mature region. Like other members of the TGFB superfamily, BMP15 is synthesized as a prepropeptide that requires cleavage, by a furin-like proprotein convertase, at an arginine rich cleavage site (RRXR), to produce a mature protein. However, the specific proprotein convertase that functions in this capacity is presently unknown.

1.4.2 Role in fertility

The effect of BMP15 on fertility has now been documented in human (Di Pasquale *et al.* 2004), mouse (Yan *et al.* 2001), sheep (Galloway *et al.* 2000), cow (Juengel *et al.* 2009), goat (Chu *et al.* 2007) and chicken (Elis *et al.* 2007). Previously, follicular development and ovulation-rate was thought to be controlled mainly by gonadotrophins. However, in more recent times, it has been recognised that oocyte-secreted factors and therefore the oocyte itself, plays a significant role in follicular development and ovulation-rate (Eppig *et al.* 2002) Juengel and McNatty 2005). The discovery of the oocyte-secreted factor, BMP15, along with the phenotypes expressed in sheep, either by naturally-occurring inactivating mutations (Galloway *et al.* 2000) or from immunisations against BMP15 (McNatty *et al.* 2004), have shown that the oocyte regulates both follicular development and ovulation-rate. Ewes heterozygous for inactivating mutations in BMP15, whereby BMP15 levels are reduced, have increased fecundity due to an increase in ovulation rate while homozygous ewes, with no biologically-active BMP15, are sterile. The absence of biologically-active BMP15 has been shown to block follicular development from the primary stage of development in the sheep (Galloway *et al.* 2000; Juengel *et al.* 2002; Hanrahan *et al.* 2004). However, the number of primordial follicles remaining in the non-growing pool of follicles is the same as in wild-type animals at both two and five years of

age, suggesting that BMP15 does not play a crucial role in the initiation of follicular growth (McNatty *et al.* 2001).

Heterozygous mutations in the BMP15 gene have been linked to premature ovarian failure (POF) in woman (Di Pasquale *et al.* 2004; Di Pasquale *et al.* 2006). Compared to sheep homozygous for inactivating BMP15 mutations, BMP15 knockout mice are fertile with little effect on follicular development (Yan *et al.* 2001), although reduced litter size and an altered interaction with GDF9 was reported (Yan *et al.* 2001). The reason(s) for this difference is unknown. However, it demonstrates that there are species differences in the role(s) of BMP15 and other TGFB family members in mono- versus polyovulatory species (McNatty *et al.* 2001; Shimasaki *et al.* 2004).

1.4.3 Actions of BMP15

Several actions of this growth factor have been revealed through *in vitro* experiments using recombinant BMP15. Both recombinant murine and human BMP15 stimulate rat granulosa cell (GC) proliferation as determined by ³H-thymidine incorporation (Otsuka *et al.* 2000; Moore *et al.* 2003; McNatty *et al.* 2005a). However, ovine BMP15 which had a minimal effect on its own, had a synergistic effect when added with either murine or ovine GDF9 (McNatty *et al.* 2005a). In contrast, ovine BMP15 alone stimulated proliferation of both ovine and bovine GC. From co-culturing experiments with rat GC, incubated with rat oocytes, the effect of neutralising antibodies showed that only GDF9 caused GC proliferation, and blocking BMP15 had no effect (Lin *et al.* 2012), probably due to little, if any, BMP15 being expressed and secreted (Crawford and McNatty 2012). However, from rat GC incubated with sheep oocytes, neutralising antibodies showed that both GDF9 and BMP15 were able to cause GC proliferation. Thus, the ability of BMP15 to increase GC proliferation is dependent upon the species of both BMP15 and of the target GC. In sheep, the lack of BMP15 blocks folliculogenesis *in vivo* while addition of recombinant BMP15 promotes *in vitro* granulosa cell proliferation.

The second biological effect of BMP15 is its ability to modulate the way granulosa cells respond to gonadotrophins. Human Flag-tagged BMP15 mature domains have been shown to inhibit follicle-stimulating hormone receptor (FSHR) expression in rat granulosa cells (Otsuka *et al.* 2001). This study also showed that this subsequently inhibited the expression of mRNA for StAR, P450_{scc}, 3 β HSD, LH receptor and the inhibin α and activin β A & β B subunits (Otsuka *et al.* 2001; Figure 1.5). In the absence of FSH, oBMP15 alone had no

effect on inhibin production by rat granulosa cells. However, co-culture with oGDF9 and oBMP15, containing a mixture of promature and mature forms, synergistically increased production of inhibin (McNatty *et al.* 2005a). BMP15 was also recognised as a luteinization inhibitor based on its ability to inhibit FSH-induced progesterone synthesis (Otsuka *et al.* 2001; Shimasaki *et al.* 2004). The extent of LH contamination in the human FSH preparation in the above studies was not indicated. In contrast, no effect of BMP15 on granulosa cell responsiveness to an LH-free, FSH preparation was observed in sheep. Inverdale ewes heterozygous (I+) for the inactivating BMP15 mutation (FecX^I) showed no significant differences from wild-type ewes in FSH-responsiveness at any stage of follicular development or stage of the oestrus cycle (McNatty *et al.* 2009). Although there was no difference in the response to FSH in I+ ewes, there was an increased proportion of follicles that developed a functional luteinizing hormone receptor (LHR) earlier than in wild-type ewes (McNatty *et al.* 2009). This is consistent with the notion that BMP15 can act to suppress LHR formation in granulosa cells.

Figure 1.5 Role of BMP15 in cross-talk between oocytes and rat granulosa cells. Adapted from Otsuka and Shimasaki (2002). In the rat, BMP15 binds to its receptor on granulosa cells and inhibits FSH-R expression, the consequence of this is that mRNA for StAR, P450_{SCC}, 3βHSD, LH receptor and the inhibin-α and activin βA & βB subunits are all subsequently inhibited in response to FSH. BMP15 also stimulates mitosis both directly and possibly via a kit ligand (KL)/c-kit mediated pathway. In turn KL acting via c-kit inhibits the expression of BMP15 in a negative feedback loop. It is not known what signal(s) stimulate BMP15 expression.

The third action of BMP15 is the stimulation of kit ligand (KL) expression in rat granulosa cells (Otsuka and Shimasaki 2002). Kit ligand, also known as stem cell factor or steel

factor, is produced by granulosa cells and is the ligand for the tyrosine receptor kinase, c-kit, which is located on the oocyte (Zsebo *et al.* 1990). BMP15 secreted by the oocyte binds to its receptors on granulosa cells to stimulate KL expression. KL subsequently acts through its receptor on the oocyte to inhibit BMP15 expression, thus creating a negative feedback loop (Otsuka and Shimasaki 2002). Due to the need to have both granulosa cells and oocytes present in the culture (only oocytes express c-kit), it is possible that BMP15 is not the only mitogenic factor secreted by the oocyte that is influenced by kit ligand (Figure 1.5; Otsuka and Shimasaki 2002). The KL-c-kit pair is also present in ovine granulosa cells and oocytes respectively (Tisdall *et al.* 1999; Juengel *et al.* 2000). A reduction in BMP15, as found in I+ ewes, had no effect on the ontogeny of expression for both c-kit and KL compared to wild-type ewes. (Juengel *et al.* 2000). However, the levels of protein expression were not investigated in this study.

1.5 Growth Differentiation Factor (GDF9)

Growth differentiation 9 (GDF9) was first identified by (McPherron and Lee 1993) using degenerate oligonucleotides, based upon conserved regions of known members of the TGFB family, in order to probe a range of tissues. From this study, they found that GDF9 was located specifically to ovarian tissue. In later studies, GDF9 mRNA was also located in the hypothalamus and testis in mice, rodents and humans (Fitzpatrick *et al.* 1998). Both ovine and human GDF9 are encoded by a gene on chromosome 5, with the human gene being located at 5q31.1, whereas the bovine gene is located on chromosome 7. The human, bovine and ovine genes consist of six, three and two exons, respectively. Both the ovine and bovine genes encode a preproprotein consisting of a 453 amino acid (aa), of which 135aa comprises the mature domain. GDF9 is thought to signal through the type II receptor BMPR2 (Vitt *et al.* 2002) and the type I receptor TGFB1 (Mazerbourg *et al.* 2004) to activate the Smad2/3 pathway (Mottershead *et al.* 2008).

As mentioned, in part earlier, GDF9 has important effects on fertility in mice (Dong *et al.* 1996), sheep (Juengel *et al.* 2002; Hanrahan *et al.* 2004; Nicol *et al.* 2009) and chicken (Huang *et al.* 2015) and to be associated with premature ovarian failure in woman (Dixit *et al.* 2005; Laissue *et al.* 2006; Kovanci *et al.* 2007; Zhao *et al.* 2007). Ovarian GDF9 is located specifically within oocytes (Crawford and McNatty 2012) and is found at all stages of follicular development beginning during follicular formation and from the earliest primordial follicle stage onwards (Bodensteiner *et al.* 2000). GDF9 has been found to be essential for ovarian folliculogenesis in all species studied so far. A lack of biological GDF9

activity, as a result of homozygous inactivating mutations (sheep: Thoka, Nicol *et al.* 2009; Belclare, Hanrahan *et al.* 2004); gene knockout mice (Dong *et al.* 1996) or immunisations against GDF9 in sheep; (Juengel *et al.* 2002), all share a similar phenotype. A lack of GDF9 results in a disruption of normal follicular development from the primary stage causing sterility. In contrast, ewes heterozygous for inactivating mutations in GDF9 express a phenotype, similar to that with heterozygous BMP15 mutations, whereby ovulation rate is increased.

GDF9 has also been shown to play a role in a range of regulatory functions such as: inhibition of 3'5'-adenosine monophosphate-stimulated steroidogenesis in human granulosa and theca cells (Yamamoto *et al.* 2002), enhanced cell transition from G(0)/G(1) to S and G(2)/M phases (Huang *et al.* 2009), suppression of the effect of activin A on StAR expression and progesterone production by upregulating expression of inhibin B (Shi *et al.* 2010), suppression of follistatin and follistatin-like 3 production (Shi *et al.* 2011a) and in conjunction with BMP15, promoted follicular development in humans (Kedem *et al.* 2011) and granulosa cell proliferation *in vitro* (McNatty *et al.* 2005a;b).

1.6 Aims of this study

The aims of this study were to: (i) identify the molecular forms of BMP15 and GDF9 that are produced and secreted by both the ovine and bovine oocyte; (ii) assess the biological activity of these molecular forms; (iii) visualise the various molecular forms using protein modelling techniques and; (iv) provide a hypothetical model of how oocyte-secreted form(s) of BMP15, GDF9 and their cell surface receptors may interact.

CHAPTER 2: MATERIALS AND METHODS

Unless stated below, additional details including sources of equipment and reagents used are described in Appendix 5. The methodological details described below are relevant for Chapters 3 and 4. Additional methodology used for the modelling studies are described in Chapter 5.

2.1 Sequence alignments

Protein sequences (accession number) used in this study were obtained from the National Center for Biotechnology Information biosystems database (www.ncbi.nlm.nih.gov): mouse Gdf9 (NP_032136); rat Gdf9 (NP_067704); porcine GDF9 (NP_001001909); cat GDF9 (NP_001159372); ovine GDF9 (AAC28089); caprine GDF9 (AAU09020); bovine GDF9 (NP_777106); water buffalo GDF9 (ACL68182); human GDF9 (NP_005251); shrew GDF9 (ACE77690); *Callicebus moloch* GDF9 (ACB21290); bat GDF9 (ACC68858); *Otolemur garnettii* GDF9 (ACH53052); mouse Bmp15 (NP_033887); rat Bmp15 (NP_067702); porcine BMP15 (NP_001005155); cat BMP15 (NP_001159370); ovine BMP15 (NP_001108239); caprine BMP15 (ACJ61256); bovine BMP15 (NP_001026922); water buffalo BMP15 (ABN05299); human BMP15 (NP_005439); chimpanzee BMP15 (XP_529247); rhesus BMP15 (XP_001083980); human INH α (NP_002182); human ActA (NP_002183); human ActB (NP_002184); human ActC (NP_005529); human ActE (NP_113667); human TGFB1 (NP_000651); human TGFB2 (AAA50405); human TGFB3 (NP_003230); human BMP2 (NP_001191); human BMP3 (NP_001192); human BMP4 (NP_570912); human BMP5 (NP_066551); human BMP6 (NP_001709); human BMP7 (NP_001710); human BMP8a (NP_861525); human BMP8b (NP_001711); human BMP10 (NP_055297); human BMP15 (NP_005439); human NODAL (NP_060525); human GDF1 (NP_001483); human GDF2 (NP_057288); human GDF3 (NP_065685); human GDF5 (CAB89416); human GDF6 (NP_001001557); human GDF7 (NP_878248); human GDF8 (NP_005250); human GDF10 (NP_004953); human GDF11 (NP_005802); human GDF15 (NP_004855); human MIS (NP_000470); human LEFTY1 (NP_066277) and human LEFTY2 (NP_003231). The protein sequences for Inverdale (V299S; (Galloway *et al.* 2000); Belclare (S367I; (Hanrahan *et al.* 2004) and Laucune (C321Y; (Bodin *et al.* 2007) BMP15 were generated by modifying ovine BMP15 (NP_001108239)

and the protein sequence for Thoka (S427R; (Nicol *et al.* 2009) GDF9 by modification of ovine GDF9 (NP_001136360). Sequence alignments were generated using Clustal W (Thompson *et al.* 1997).

2.2 Preparation of recombinant proteins.

2.2.1 Preparation of cloning vectors.

The DNA sequence encoding the human proregion/ovine mature BMP15 (S356C) mutant (h/oBMP15 (S356C); Appendix 1) inserted into the cloning vector pGEM-T Easy was kindly gifted from Dr D.G. Mottershead (Robinson Institute, The University of Adelaide, Adelaide, Australia). The DNA sequence encoding the human BMP15 pro-region (hBMP15pro, Appendix 3), was amplified from h/oBMP15 (S356C) using the primers, 5' primer; [5'-ACTCGAGGCCACCATGAAGTG-3'] and 3' primer; [5'-GAATTCAGGAGAAGAGATTCCCTTTC-3'] prepared by Invitrogen custom primers, (Invitrogen, Auckland, New Zealand). Polymerase chain reaction (PCR) was carried out using the GC-RICH PCR system (Roche Applied Science, Auckland New Zealand) as per manufacturers instructions. Each PCR reaction mixture contained 0.2 mM deoxynucleotide triphosphates (dNTPs; 0.05 mM each of dATP, dTTP, dGTP and dCTP), 0.5 M GC-RICH resolution solution, 1x GC-RICH PCR reaction buffer (includes 1.5 mM MgCl₂), 2 U GC-RICH enzyme mix, 0.3 µM of each primer and <0.3 µg template DNA. Two additional reaction mixtures were prepared and run concurrently, the first of which included ovine cDNA as the template and primers for β-actin [5' primer; (5'-GCATGGGCCAGAAGGACTCC-3') and 3' primer; (5'-CGTAGATGGGCACCGTGTGG-3')] (Invitrogen custom primers). The second reaction mixture combined all four of the above primers in the absence of a template. The PCR conditions were 1 cycle of 3 minutes (min) at 94°C, followed by 10 cycles of 20 seconds (s) at 94°C, 20 s at 60°C, 20 s at 72°C; followed by 25 cycles of 20 s at 94°C, 20 s at 60°C, 20 s (extended by 5 s after each cycle) at 72°C and finished with a 7 min incubation at 72°C. Both the presence and the expected size of the PCR product(s) was confirmed by agarose gel electrophoresis. The PCR product(s) was pre-incubated with SYBR® Green (Invitrogen), following the manufacturers instructions, for 5 min at room temperature before being run on a 1% (w/v) agarose gel at 85 volts for 40-60 min. Thereafter, the bands were visualised with ultra violet (UV) light. Images of the gel were obtained using a UVIPRO gel doc system (UVItec Limited, Cambridge, United Kingdom). Human BMP15pro DNA was extracted from the gel using a QIAprep gel extraction kit (QIAGEN Pty. Ltd) as per the manufacturers instructions. DNA was stored at -20°C until it was

ligated into the cloning vector pGEM-T Easy using the LigaFast™ Rapid DNA Ligation System (PROMEGA, Sydney, Australia) as per the manufacturers instructions. Briefly, vector (pGEM-T Easy) DNA and insert (hBMP15pro) DNA were combined in a 1:3 ratio in the presence of T4 DNA ligase in 1x rapid ligation buffer and incubated for 2 h at room temperature. The sequence of the insert (Appendix 3) was confirmed by the Waikato DNA Sequencing Facility, University of Waikato, Hamilton, New Zealand.

2.2.2 Generation of expression plasmids

Inserts (h/oBMP15 (S356C) or hBMP15pro) contained within pGEM-T Easy were subcloned into the expression vector pEFIREs-P. Competent *E. coli* (DH5α strain) cells, provided by Mr A. Bibby, (Victoria University of Wellington, Wellington, New Zealand), were transformed by incubating with the insert containing the pGEM-T Easy plasmid on ice for 30 min, followed by a 2 min heat shock at 37°C, after which the cells were placed back on ice for an additional 2 min. The cells were made up to 1 mL with lysogeny broth (LB broth) and incubated for 1 hr at 37°C, with shaking at 250 rpm, after which they were centrifuged at 1800 g for 5 min, then re-suspended in 0.1 mL LB broth and spread out on LB agar plates coated with 1mg X-Gal. Blue-white selection was used to determine those colonies derived from transformed clones and a single white colony was selected and grown in LB broth containing 50 µg/mL ampicillin. Plasmid DNA was subsequently purified using a QIAprep spin miniprep kit (QIAGEN), as per the manufacturers instructions. The h/oBMP15 (S356C) insert was released from the plasmid DNA by the use of XhoI and XbaI restriction enzymes, and the hBMP15pro insert was released with the restriction enzymes XhoI and EcoRI. A total of 2.2 µg of plasmid DNA was incubated with both restriction enzymes concurrently for 2.5 h at 37°C. Both the presence and the expected size of the released insert was confirmed by running the enzyme cut plasmid, pre-incubated with SYBR® Green, on a 1% (w/v) agarose gel. Thereafter, the DNA band was visualised with UV light. The DNA was extracted from the band using a QIAprep gel extraction kit, as per the manufacturers instructions, and the sequence was confirmed by sequencing (Waikato DNA Sequencing Facility). The gel-purified DNA was subsequently ligated into linearised pEFIREs-P using T4 DNA ligase as described above. The expression vector, pEFIREs-P, used to ligate the h/oBMP15 (S356C) mutant, was previously linearised using the restriction enzymes XhoI and XbaI by Mr Adrian Bibby. The DNA sequence for the ovine prodomain/ovine mature BMP15 (o/oBMP15; Appendix 2) that was inserted into pEFIREs-P plasmid was also provided by Mr Bibby. The empty

pEFIRES-P plasmid was provided by Dr S.M. Hobbs at the CRC Centre for Cancer Therapeutics, Institute of Cancer Research, Surrey, UK. (Hobbs *et al.* 1998).

2.2.3 Transfection of HEK293 cells.

The human embryonic kidney cells [HEK293; American Type Culture Collection, Manassas, VA, USA, (CRL-1573TM)] were grown in 3 mL growth medium, namely Dulbecco's Modified Eagle Medium (D-MEM), supplemented with 10% _(v/v) fetal calf serum (FCS), penicillin (50 IU/mL), streptomycin (50 µg/mL) [PenStrep] and Glutamax at 1x strength in a 6-well culture plate at 37°C in 5% CO₂ in air until the cell monolayer was approximately 90% confluent. A total of 2 µg of pEFIRES-P-h/oBMP15 (S356C), pEFIRES-P-o/oBMP15, or empty pEFIRES-P plasmid DNA was incubated with 8 µL Eugene HD (Roche) transfection reagent for 15min at room temperature in 100 µL serum free medium (SFM; D-MEM plus Glutamax) prior to being added dropwise to the HEK293 monolayers. Negative controls comprising of SFM alone and SFM plus Eugene HD were also included. The cells were incubated at 37°C in 5% CO₂ in air for an additional 24 h. Cells were detached from the surface of the well using TrypLETM Express (a recombinant trypsin alternative), then split 1:2 and 1:6 and each replated in the presence of 1 µg/mL puromycin dihydrochloride. Puromycin selection was continued on the transfected (surviving) cells, such that cells were allowed to reach 90% confluence before being again split 1:3 into three new wells. The puromycin concentration was increased 1-5 fold at each split until 50 µg/mL was reached; thereafter the puromycin concentrations were increased by 10-50% until a final concentration of 100 µg/mL was achieved.

2.2.4 Production and harvesting of recombinant proteins secreted by HEK293 cells.

HEK293 cells transfected with the pEFIRES-P-h/oBMP15 (S356C) mutant, pEFIRES-P-o/oBMP15 or empty pEFIRES-P plasmid were cultured in growth media plus 100 µg/mL puromycin to 80-90% confluency in a total of twenty culture flasks, each with a growth area of 150 cm². The growth medium was then removed and the cells washed gently, twice with sterile 0.15 M phosphate buffered saline, pH 7.4 that had been pre-warmed to 37°C. To each flask, 25 mL expression medium (EM) was added (D-MEM:Hams F12 (1:1) supplemented with penicillin (50 IU/mL), streptomycin (50 µg/mL), Glutamax (1x), bovine serum albumin (BSA; 0.01%, _{w/v}) and heparin (100 µg/mL), and the flasks incubated at 37°C in 5% CO₂ in air. The EM containing the recombinant (oBMP15-EM, oBMP15 (S356C)-EM or Null-EM) proteins was harvested 96.0 ± 2.4 h (mean ± S.E.M.) later. Whole cells present in the EM were removed by centrifugation for 5 min at 200g and

the resultant supernatant was subsequently cleared by centrifugation for 10 min at 3200g. The protease inhibitors (i.e. complete, EDTA-free protease inhibitor cocktail, Roche) were added to the cleared media at the dosage recommended by the manufacturer and the media stored at -20°C.

2.3 Protein analysis.

2.3.1 Preparation of samples for SDS-Polyacrylamide gel electrophoresis (PAGE).

SDS-PAGE was run under non-reducing, reducing or cross-linking/reducing conditions. Preparation of samples for cross-linking using the non-cleavable cross-linker bis(sulfosuccinimidyl)suberate (BS³) followed the protocol described in (McIntosh *et al.* 2008). EM containing either o/oGDF9 (supplied by Dr Kenneth P. McNatty, Victoria University of Wellington), o/oBMP15, h/oBMP15 (S356C), o/oGDF9 + o/oBMP15 (1:1) or o/oGDF9 + h/oBMP15 (S356C) (1:1) were incubated overnight at room temperature. Subsequently the EM, lysates of oocytes and oocyte incubation medium were cross-linked by the addition of BS³ to a final concentration of 2 mM and incubated for 2h at room temperature. Cross-linked samples were then mixed with 5x reducing sample buffer. Non cross-linked samples were mixed with either 5x reducing sample buffer or 5x non-reducing sample buffer, (Appendix 5). Under reducing, but not non-reducing, conditions, the sample plus sample buffer was heated to 100°C for 5 min. This was followed by a short incubation on ice in order to condense water vapour, and then centrifugation at 6000 g for 5 s to collect all liquid in the bottom of the tube, prior to loading the solution onto the gel.

2.3.2 Separation of proteins by SDS-PAGE.

Utilising solutions listed in Appendix 5, 13.5% (w/v) polyacrylamide gels were prepared as described in Table 2.1. The gels were prepared, as per the manufacturer's instructions utilising the Mini-PROTEAN Tetra Electrophoresis System (Bio-Rad). The separating gel solution was poured between two glass plates held apart by a 1mm spacer and allowed to polymerise under a layer of distilled water. After polymerisation was complete, the water overlay was tipped off and residual moisture removed by use of blotting paper inserted between the glass plates. Subsequently, a stacking gel solution was added and a comb was inserted to form sample wells within the gel. Usually, the gels were prepared on the day of use. However, on some occasions, the gels (with comb still in place) were wrapped in wet paper towels and stored at 4°C within a sealed plastic bag but were always used within 2 days of preparation. The samples were loaded onto gels (typically the two outside lanes

were occupied by molecular size markers) and electrophoresis carried out in Tris-glycine buffer, pH 8.5 at 180 V for 60min at room temperature.

Table 2.1 Table of reagents and recipe for 13.5% polyacrylamide gels

	13.5% Separating gel	4% stacking gel
Distilled water	2.85 mL	3.0 mL
1.5 M TRIS-HCl (pH 8.8)	2.50 mL	-
0.5 M TRIS-HCl (pH 6.8)	-	1.25 mL
10% (w/v) SDS	100 µL	50 µL
30% Acrylamide/Bis solution (37.5:1)	4.50 mL	0.65 mL
10% (w/v) Ammonium persulphate	50 µL	25 µL
	5 µL	5 µL

2.3.3 Western blotting

The Western blotting procedure involved the transfer of proteins from a PAGE gel to a nitrocellulose membrane, using the Mini-PROTEAN Tetra Electrophoresis System. Briefly, 1x blotting paper, 1x PAGE gel (with staking gel removed), 1x nitrocellulose membrane (pore size 0.45 µm) and 1x blotting paper were sandwiched between 2x Scotch pads (Bio-Rad) and placed into the transblot sandwich holder (orientated so that the nitrocellulose membrane was positioned between the gel and the positively charged electrode). Transfer was carried out at 100 volts for 1 h on ice, in 192 mM Tris-glycine buffer containing 20% (v/v) methanol at 4°C. Following removal of the membrane from the transblot sandwich holder, the location of molecular size markers on the membrane were marked by pencil. Thereafter, the membranes were washed twice for 10 minutes in low salt buffer (20 mM Tris-HCl containing 0.876 mg/L NaCl and 0.1% (v/v) Tween 20, pH 7.5; Appendix 5) at room temperature.. They were then blocked with 5% blocking buffer (low salt buffer containing 5% (w/v) low fat milk powder) for 1 h and finally, incubated with the selected primary antibody diluted in 5% blocking buffer overnight. The primary antibodies were monoclonal antibodies to either BMP15 (clone MN2-61A; 1 µg/mL; McNatty *et al.* 2005a) or GDF9 (clone 37A; 0.5 µg/mL; Juengel *et al.* 2002). The membranes were then washed three times for 10 minutes each in low salt buffer, incubated with horseradish peroxidase conjugated rabbit anti mouse IgG (1:6000), for 2 h and then washed again three times for 10 minutes each in low salt buffer. Chemiluminescent detection of proteins was performed using the Western Blotting detection system (Western Bright™ ECL spray) as per the manufacturer's instructions. The images were captured using an Omega Lum G

(Aplegen, Pleasanton CA) gel documentation system. The images were routinely captured at the following exposure times, 0.75, 1.5, 2.25, 3.0, 3.75, 4.5, 5.25, 6.0, 6.75, 7.5 and 10 min.

On some occasions, membranes were incubated in 0.1 M Tris-HCl buffer containing 1.25 mM Luminol, 0.2 mM p-coumaric acid and 0.01% (v/v) hydrogen peroxide, pH 8.5 for 60 s, blotted dry, encased a plastic (saran) wrap (Gladwrap) and then exposed to X-ray film for 10-300 s within a X-ray cassette. The film was developed for 4 min in both developer and fixer (KODAK). The locations of the molecular size markers were copied directly from the membrane onto the film. All incubations were performed at room temperature.

2.3.4 Densitometry of Western blots

The freeware ImageJ (<http://rsb.info.nih.gov/ij/index.html>) was used to determine the density and area of bands within Western blots. The method employed is fully described in (Miller 2010)

Figure 2.1 Density plot of a Western blot. Showing peaks (1-3) without (A) and with (B) artificial baselines (—). (C) Relative peak area and percent area for peaks 1, 2 and 3.

Briefly, all images were first converted to 8-bit grey scale and identically-sized rectangular slices were taken of each lane within a Western blot. From these, a density plot was generated showing the relative density along the length of each lane; with peaks within this plot corresponding to each band in the lane. Where the peaks did not reach down to the baseline (perfect white) due to background noise, an artificial baseline was created to close off the peak. (Figure 2.1A and B). The area under the curve for each peak was then

determined. Thereafter, the percent area for each peak was calculated relative to the total area under the curve (Figure 2.1C).

2.4 Statistical analysis

Results from ^3H -thymidine incorporation bioassay were expressed as the mean $\pm$ S.E.M. (fold difference relative to control) of at least 6 separate experiments, with triplicate determinations for each treatment. Differences between treatment groups were analyzed for statistical significance by using ANOVA or paired t tests (SPSS: IBM Corp. Released 2013. IBM SPSS, Statistics for Windows, Version 22.0. Armonk, NY, USA). P values <0.05 were accepted as statistically significant.

CHAPTER 3: PRODUCTION AND CHARACTERISATION OF RECOMBINANT FORMS OF BONE MORPHOGENETIC PROTEIN 15 (BMP15)

3.1. Introduction

Members of the TGFB superfamily, such as BMP15, are produced as preproteins and cleaved at an arginine rich cleavage site (RRXR) by a furin-like proprotein convertase to release the mature domain. Consequently, several molecular forms are possible: (i) an uncleaved promature form (covalently bound promature; mature domain yet to be cleaved by a proprotein convertase); (ii) a cleaved promature form (mature domain cleaved from the prodomain by a proprotein convertase, yet still attached to the prodomain in a non-covalent complex) and; (iii) a free mature domain (mature domain not attached to the prodomain). While members of the TGFB superfamily exert their biological activity in the form of homo- or heterodimers of mature domains, some members, such as BMP15, lack the conserved cysteine integral to the maintenance of these dimers. Although GDF9 also lacks this cysteine, recombinant GDF9 has been shown to form homodimeric mature complexes (Edwards *et al.* 2008). Recombinantly-derived mature hBMP15, with either a N-terminal FLAG tag (Liao *et al.* 2004; Hashimoto *et al.* 2005), a N-terminal MycHis tag (Rossetti *et al.* 2009) or from re-introduction of the missing cysteine (Pulkki *et al.* 2012), have all been shown to include a mature dimeric form. Moreover, cross-linked purified recombinant hBMP15 has also been reported to form homodimers (Pulkki *et al.* 2012). Therefore, should unmodified oBMP15 mature domains also form non-covalent homodimers, various dimeric combinations of those mentioned above may result.

Several different approaches have been taken to immuno-detect BMP15 by Western blotting techniques. Several reports have used recombinant mature BMP15 tagged with either N-terminal FLAG tag (Liao *et al.* 2004; Hashimoto *et al.* 2005), or a N-terminal MycHis tag (Rossetti *et al.* 2009). With antibodies to these tags, BMP15 has been detected indirectly by Western blotting. A commercially available polyclonal antibody, raised against the first 83 amino acids of mature hBMP15, has been used in Western blotting to detect BMP15 in the follicular fluid of both porcine (Paradis *et al.* 2009) and bovine (Behrouzi *et al.* 2016) pre-ovulatory follicles. The specificity of this polyclonal antibody was inferred from the lack of staining when either the polyclonal antibody was omitted or when the polyclonal antibody was replaced with preimmune rabbit IgG. The same bands were observed when using an alternative antibody, although the antibody used was not reported. Western blots using this polyclonal antibody showed a mature BMP15 band at 25

kDa. This was different than the expected 15-17 kDa molecular size that had previously been demonstrated (McNatty *et al.* 2005a; Pulkki *et al.* 2012). Pullki *et al.* (2012) reported the use of a monoclonal antibody (Mab28) which was raised against the hBMP15 sequence SAEVTASSSKHSGPENNNQC. This antibody was capable of detecting both monomeric and dimeric forms of hBMP15. The monomeric form was reported as corresponding to two bands at 16 and 17 kDa, with the dimeric form consisting of three bands corresponding to bands at 30 -34 kDa.

```

(Mab28)      hBMP15  268 QADGI SAEVTASSSKHSGPENNNQC SLHPFQISF 300
(MN2-61A)    oBMP15  269 QAGSIA SEVPGPSREHDGPES NQC SLHPFQVSF 301
                ** .*: : ** . * :*.***.*****.***

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Figure 3.1 Comparison of sequences recognised (yellow) by the antibodies Mab28 (Pulkki *et al.* 2011) and MN2-61A (McNatty *et al.* 2005a).

The monoclonal antibody (MN2-61A) recognises an epitope within the oBMP15 sequence SEVPGPSREHDGPES (Figure 3.1). This lies in a region homologous to the antigen that the Mab28 antibody was raised against. Moreover, MN2-61A recognises promature and mature forms in Western blotting (Edwards *et al.* 2008). Additionally, antibodies raised against the same sequence as the MN2-61A antibody are capable of inhibiting antral follicular growth in both sheep (McNatty *et al.* 2007) and cattle (Juengel *et al.* 2009). Therefore, since MN2-61A was proven to recognise biologically active forms of ovine and bovine BMP15, this monoclonal antibody was chosen for this present study.

Although the MN2-61A antibody has been shown previously to be specific for ovine BMP15 (McNatty *et al.* 2005a; Edwards *et al.* 2008) these studies did not detect any dimeric forms of BMP15. It was not determined whether this was due to a lack of dimeric forms or an inability of the clone MN2-61A to recognise them. Therefore, the major aim of this current study was to establish which molecular forms of BMP15 are recognised by the monoclonal antibody MN2-61A. To assist this aim, a mutant form of oBMP15 was created, whereby S356 was replaced with a cysteine [oBMP15 (S356C)], thereby permitting BMP15 to create covalently-bound homodimers. A human prodomain was used to increase the concentrations of BMP15 forms present in expression medium (Dr D.M. Mottershead, personal communication). From this cell line, it was anticipated that the expressed BMP15 (S356C) proteins could be used to confirm that the monoclonal antibody (MN2-61A) was capable of detecting such forms and as a positive control for detecting any dimeric forms in non-mutated oBMP15.

3.2. Methodology

Unless stated below, additional details including sources of equipment and reagents used are described in Appendix 5. The methodological details describing preparation of expression plasmids, transfection and culture of HEK293 cells and the production of recombinant proteins used in this study are described in Chapter 2.

3.2.1. Immobilised Metal ion Affinity Chromatography (IMAC)

An IMAC protocol supplied by Dr D.M. Mottershead (personal communication), employing a liquid chromatography approach, was modified to a batch process (Table 3.1). This modified protocol was used to further purify the expression medium from HEK293 cells transfected with either pEFIRES-P-h/oBMP15 (S356C), pEFIRES-P-o/oBMP15wt, or empty pEFIRES-P plasmid.

Table 3.1 Step wise IMAC protocol (All buffer recipes can be found in Appendix 5)

Step	Purpose	Resin volumes	Buffer
1	Ni-NTA-agarose preparation	50x	Distilled H ₂ O
2		25x	W5 buffer
3		25x	W5 buffer
4	Loading sample	250x	Expression medium containing: 50 mM TRIS-HCl.pH 7.4 + 300 mM NaCl + 5 mM Imidazole,
5			Rotating mixer (end over end) 8 rpm, for 2 hours @ 4°C
6	Wash	10x	W10 Buffer
7	Wash	10x	W20 Buffer
8-22	Elute 1: (mature domains)	5x	E1 Buffer 5 minute incubation, with intermittent mixing
23	Wash	10x	W20 Buffer
24	Elute 2: (Pro- & promature domains)	10x	E2 buffer
25		10x	E2 buffer
26	Wash	10x	W0 buffer
27	Wash	10x	W0 buffer
28	Ni-NTA-agarose storage (4°C)	10x	W0 buffer + 70% (v/v) Ethanol

Unless otherwise stated, all incubations with Ni-NTA-agarose were performed at room temperature for 30 s and the supernatants were recovered after centrifugation (200 g x 5min). Each batch of Ni-NTA-agarose was dedicated to purifying proteins from the

expression medium (EM) from cells producing BMP15, BMP15 (S356C) or cells with empty transfected vector (i.e. control) (Section 2.2.4).

3.2.2. High Performance Liquid Chromotography (HPLC)

IMAC purified fractions were further purified by HPLC using a method described by Pulkki *et al.* (2011) with minor modifications, namely an increased flow rate (1 mL/min) and more than one acetonitrile (ACN) gradient was used, (Table 3.2). Fractions 8-22 (Table 3.1) were pooled. Thereafter, trifluoroacetic acid (TFA) and ACN were added to a final concentration of 0.1% (v/v) and 6% (v/v), respectively and the resultant preparation(s) was passed through a 0.22 µm filter (Corning Inc., Corning, NY). Subsequently, 5 mL of this preparation was loaded onto a reverse phase HPLC column (Jupiter, 5 µm, C4, 300Å; Phenomenix) fitted with an upstream security guard cartridge (Widapore, C4, Phenomenix) which had been previously equilibrated with 0.1% (v/v) TFA and 6% (v/v) ACN in water (HPLC grade). Differing molecular forms of BMP15 were eluted from the column by employing a series of linear gradients of ACN while keeping the TFA concentration constant (Table 3.2) at a flow rate of 1 mL/min. One mL fractions were collected into microcentrifuge tubes and evaporated to dryness using an Eppendorf® centrifugal vacuum concentrator (SIGMA, model 5301) at room temperature.

Table 3.2 Composition of linear gradients of acetonitrile applied to HPLC column

Length of gradient (min)	Cumulative time (min)	Linear gradients	
		ACN percentage at start of time period	ACN percentage at end of time period
0	0	6	6
10	10	6	6
5	15	6	30
30	45	30	60
1	46	60	80
3	49	80	80
1	50	80	95
2	52	95	95
1	53	95	6
2	55	6	6
STOP	60	6	6

3.2.3. Bone morphogenetic protein 15 relative densitometry assay

An assay to determine the concentrations of BMP15 was developed based on the densitometry readings of the BMP15 immunoreactive bands on Western blots under reducing conditions (see Section 2.3).

Figure 3.2 Example of the BMP15 relative densitometry assay. Western immunoblots under reducing conditions showing the molecular forms of recombinant HEK293- or *E. coli*-derived oBMP15. Expression medium (EM) from HEK293 cells transfected with an empty pEFIREs-P (lane 1; 10 μ L), pEFIREs-o/oBMP15 (Lane 2; 10 μ L, Lane 3; 5 μ L, Lane 4; 2 μ L), pEFIREs-h/oBMP15 (S356C) (Lane 5; 5 μ L, Lane 6; 2 μ L), *E. coli*-derived oBMP15 mature domains (Lane 7; 0.78pg, Lane 8; 1.56pg, Lane 9; 3.13 pg, Lane 10; 6.25pg, Lane 11; 12.5pg and Lane 12; 25pg). Molecular size markers (kDa) are shown to the left of the image.

The Western blots included at least one lane with a preparation of *E. coli*-derived ovine BMP15 mature domain of known concentration (kindly gifted by Dr Jenny Juengel; Agresearch, Mosgiel, New Zealand). This was utilised as the standard against which preparations of unknown BMP15 concentration were measured. The area under the densitometry curve for the bands corresponding to the mature monomer for both the standard and sample preparations were determined and the percent area for each band was then calculated (General methods 2.4.4; Figure 2.C). From these, a relative density figure for each peak was produced using the formula:

$$\frac{\%(S)}{\%(Std)} = RD$$

where: $\%(S)$ = percent area for sample band; $\%(Std)$ = percent area for standard band and; RD = relative density.

A final sample concentration was determined using the formula:

$$\frac{RD \times [Std] \times (Vol_{Std})}{Vol_{(S)}} = [S]$$

where: RD = relative density; [Std] = concentration of standard (ng/mL); Vol_(Std) = volume of standard loaded on gel (μL); Vol_(S) = volume of sample loaded on gel (μL) and; [S] = concentration of sample (ng/mL).

As previously reported by McNatty *et al.* (2005b), *E. coli*-derived oBMP15 mature domains resolved at a different size to those produced by mammalian cells. This is most likely due to the *E. coli* construct containing a His tag and differences in the degree of linearisation of the polypeptide chains under the reducing conditions of the SDS-Page gel. The *E. coli*-derived proteins also formed aggregates which could not be fully dissociated.

3.2.4. Rat granulosa cell ³H-Thymidine incorporation bioassay

The rats used in this study were kindly supplied by the Department of Psychology, VUW and sacrificed in compliance with the VUW Animal Ethics Committee (VUW AEC 10695). Prepubertal female Sprague-Dawley rats (21-27 days of age) were euthanized by CO₂ asphyxiation, followed by cervical dislocation. A 70% ethanol solution was applied liberally to the abdominal region prior to dissection and collection of the ovaries. The ovaries along with a portion of oviductal tissue were collected into dissection medium (i.e. Medium A; DMA; see Appendix 5) at 37°C. Ovaries were kept at 37°C until dissection. Upon dissection, they were transferred into a 35x10 mm petri dish containing fresh Medium A and isolated from non-ovarian tissue under a dissection microscope at room temperature. The trimmed ovaries were transferred into a new petri dish containing fresh Medium A and visible follicles punctured (with additional gentle scraping) with a 20G needle fitted to a 1 mL syringe to encourage release of both granulosa cells and oocytes into the medium. Oocytes were removed by use of a 10 μL micro-pipette and the granulosa cells were kept at 37°C until dissections from all ovaries had been performed. Granulosa cells from all ovaries were harvested within 2 hours of ovary collection. Granulosa cells were then washed twice in incubation medium (i.e. Medium B; McCoys 5a containing glutamine, NaHCO₃, poly (vinyl alcohol) and antibiotics; see Appendix 5) by centrifugation at 453 g for 5 min. The supernatant was carefully decanted and the pelleted cells were re-suspended in 0.5 mL Medium B.

The number of clear (live) and blue (dead) cells were counted and the cell viability was determined by trypan blue exclusion. Briefly, 5 μ L trypan blue, 0.4% (w/v), was mixed with 50 μ L cell suspension and loaded onto a haemocytometer. The cell suspension was then diluted with Medium B to give a concentration of 0.5×10^6 live granulosa cells/mL. Aliquots of twenty thousand live cells (40 μ L) were pipetted into individual wells of a flat-bottomed 96 well plate (NUNC). Additional Medium B, containing the treatments described below were added to give a final volume of 55 μ L. For each bioassay, the test reagents in expression medium (EM) were investigated in triplicate, and included the following treatments: recombinant human activin A (10 ng/mL; R&D Systems), o/oBMP15-EM (19 ng/mL); h/oBMP15 (S356C)-EM (19 ng/mL); o/oGDF9-EM (50 ng/mL); o/oBMP15-EM and o/oGDF9-EM (19 ng/mL + 50 ng/mL, respectively); o/oBMP15-EM and o/oGDF9-EM (19 ng/mL + 50 ng/mL, respectively); and media control (EM from an untransfected HEK293 cell line). On some occasions, additional treatments of [h/oBMP15 (S356C)-EM, (90 ng/mL)] and [h/oBMP15 (S356C)-EM and o/oGDF9-EM (90 ng/mL + 50 ng/mL, respectively)] were also included. The volume of EM (5 μ L/well, 9.1%) was kept constant for all treatments. The granulosa cell preparations were incubated at 37°C in 5% CO₂ for 16 h. Thereafter to each well, 0.4 μ L ³H-thymidine (1 mCi/mL; Perkin-Elmer) in 10 μ L Medium B was added, giving a final concentration of 350 nM of thymidine, and incubated for a further 6 hours. The cells were then washed from the multi-well plates using a cell harvester (TOMTEC, model No. MACH 3-FM series) and the radiolabelled DNA captured on a filter mat (glass fibre filter mats, 90x120 mm, printed Filtermat A; Perkin Elmer). The filter mat together with 5 mL of scintillation fluid (Beta Scint; Perkin Elmer) was placed within a plastic sample bag (Perkin Elmer) and heat sealed. The level of ³H-thymidine incorporation was measured over a 2 minute interval, as counts per min (cpm) using a beta counter (Perkin Elmer; 1450-SLC MicroBeta Trilux). Mean values (cpm) for Medium B alone (negative control) were subtracted from test results. Results were then expressed as a fold-increase relative to the media control (i.e. EM from an untransfected HEK-293 cell line).

3.2.5. Surface Plasmon Resonance (SPR)

Surface Plasmon Resonance was used to investigate the ability and degree, of both promature and mature forms of o/oBMP15 and h/oBMP15 (S356C), to specifically interact with BMPR2. IMAC purified o/oBMP15 and h/oBMP15 (S356C) were further purified by HPLC (BIO-RAD, BioLogic DUOflow) using a reverse phase HPLC column (Jupiter,

5 μm , C4, 300Å; Phenomenix). The samples were eluted from the column over a 30 min period at a flow rate of 1 mL/minute using a linear gradient from 0-100% ACN, whilst keeping the TFA concentration constant at 0.1%. Aliquots of the resultant fractions were loaded onto a SDS page gel and electrophorised for 35 min at 280 volts. The gel was silver-stained and those fractions containing the promature and/or mature forms of BMP15 were freeze-dried, and reconstituted with 50 μL distilled water. The o/oGDF9-EM was dialysed 3x24 hours against 2 L Biocore running buffer (see Appendix 5). The o/oBMP15- and h/oBMP15 (S356C)-EM in 50 μL water were diluted further with the addition of 240 μL water and 290 μL of 2x Biocore running buffer. The diluted o/oBMP15-, h/oBMP15 (S356C)- and o/oGDF9-EM, samples were then serially diluted 1:2 with 1x Biocore running buffer to produce dilutions at 1:2, 1:4, 1:8, 1:16 and 1:32.

Surface Plasmon Resonance experiments were performed using Biacore 2000 system (Biacore, GE Healthcare, Chalfont, St. Giles, GB) (McDonnell 2001; Pattnaik 2005). The biosensor was prepared by attaching N-biotinylated, human BMPR2 extra-cellular domains to a Biacore sensor chip using equimolar concentrations of sulfo-NHS-LC-biotin (Pierce, Thermo Scientific, Rockford, IL, USA) using the method previously reported (Shen *et al.* 1996). The formation of ternary complexes and their subsequent dissociation from the biosensor was measured and recorded as described previously (Zhang *et al.* 2002).

3.3. Results

3.3.1. Production of pEFIRES-hBMP15 (S356C)

Utilizing the method outlined in Section 2.2.2, the h/oBMP15 (S356C) insert contained within pGEM-T Easy was sub-cloned into the expression vector pEFIRES-P and used to transform *E. coli*. The plasmid DNA was extracted from four replicate cultures of *E. coli* transformed with pGEM-T Easy-h/oBMP15 (S356C). The insert from one of these was excised from the plasmid, and pEFIRES-P was linearised, with two distinct pairs of restriction enzymes, EcoRI with XbaI and XhoI with XbaI (Figure 3.3A). The two bands corresponding to the pGEM-T Easy vector (upper band) and the h/oBMP15 (S356C) insert (lower band) are visualised in Figure 3.5A (Lanes 1 & 2). Using the regression equation (Figure 3.3B), the size of the released insert was calculated to be 1198 bp. This correlates well with the expected value of 1270bp.

Figure 3.3 Restriction enzyme digests of pGEM-T Easy-h/oBMP15 (S356C) and pEFIRES-P. (A) Restriction enzyme digests of pGEM-T Easy-h/oBMP15 (S356C) cut from pGEM-T Easy with EcoRI and XbaI (Lane 1), h/oBMP15 (S356C) cut from pGEM-T Easy with XhoI and XbaI (Lane 2), pEFIRES-P linearised with EcoRI and XbaI (Lane 3), pEFIRES-P linearised with XhoI and XbaI (Lane 4). The numbers on the side refer to number of base pairs. (B) Graphical depiction of the DNA ladder (diamonds). The values shown in blue diamonds were used to generate the regression line $Y = -16.4 \ln(x) + 158.3$, $R^2 = 0.995$

The insert and linearised pEFIRES-P (Figure 3.3A, Lanes 1 and 3, respectively), both cut with the restriction enzyme pair EcoRI and XbaI were extracted from the gel, ligated and used to transform *E. coli* bacteria. However no colonies were formed indicating that the linearisation, ligation or transformation failed. To test the linearization of pEFIRES-P, duplicate ligations were performed. This was undertaken by ligating the insert with either the linearised pEFIRES (Figure 3.3A; Lanes 2 and 4, respectively) or the pEFIRES-P that had been previously linearised with XhoI and XbaI (provided by Mr A. Bibby, Victoria University of Wellington). Successful transformation was only achieved with the ligation using the previously linearised pEFIRES-P. So it appears that for unknown reasons the linearisation of pEFIRES-P was unsuccessful. However from the successful transformation, the plasmid DNA from eight replicate cultures was extracted (Table 3.3).

The insert of pEFIRES-h/oBMP15 (S356C) from samples 1 and 3 were released by XhoI and XbaI and their sizes determined by agarose gel electrophoresis (Figure 3.4A). However, only the insert from sample 3 was released. From the regression equation (Figure 3.4B), the size of the released insert from sample 3 was calculated to be 1258 base pairs (bp), This is in agreement with the theoretical value of 1207bp. Sample 3 was sequenced to confirm the identity of the insert (Appendix 4) and subsequently, pEFIRES-h/oBMP15 (S356C) was used to transfect HEK293 cells.

Table 3.3 Quality of extracted pEFIREs-h/oBMP15 (S356C) plasmid DNA

	DNA	
	mg/mL	A ₂₆₀ /A ₂₈₀ ratio
Sample 1	0.35	1.87
Sample 2	0.65	1.81
Sample 3	0.62	1.80
Sample 4	0.63	1.79
Sample 5	0.61	1.79
Sample 6	0.65	1.82
Sample 7	0.55	1.77
Sample 8	0.66	1.82

Figure 3.4 Restriction enzyme digests of pEFIREs- h/oBMP15 (S356C).

(A) Enzyme digests, Sample 1 (Table 3.3) cut with XhoI and XbaI (Lane 1), Sample 3 (Table 3.3) cut with XhoI and XbaI (Lane 2). Numbers on side refer to number of base pairs. **(B)** Graphical depiction of DNA ladder (diamonds). The values shown in blue diamonds were used to generate the regression line

$$Y = -20.5 \ln(x) + 193.9, R^2=0.9924$$

3.3.2. Comparison of h/oBMP15 (S356C)-EM, o/oBMP15-EM and *E. coli* derived oBMP15 mature domains in the BMP15 relative densitometry assay

Experiments were performed to determine if the monoclonal antibody, MN2-61A, recognised the mature domains found in expression medium (EM) from HEK293 cells transfected with pEFIREs-o/oBMP15 or pEFIREs-h/oBMP15 (S356C) and *E. coli*-derived oBMP15 equally. Serial dilutions of h/oBMP15 (S356C)-EM, o/oBMP15-EM and *E. coli*-derived oBMP15 mature domains were analysed by Western blot under reducing conditions. Triplicate Western blots, each containing, a single replicate per dilution per test sample were performed. The density of the band corresponding to the monomeric mature

domain was determined for each sample and dilution, as described in Sections 3.2.3 and 3.2.4. The relative density (RD) of these bands underwent a logit transformation using the formula:

$$\text{logit} = \ln \frac{\left(\frac{RD}{8}\right)}{\left(1 - \left(\frac{RD}{8}\right)\right)}$$

Where the constant 8, is an arbitrary number used to generate a proportion, thus allowing the derived RD values to fit the equivalent equation:

$$\text{logit} = \ln \frac{(p)}{(1 - p)}$$

The slopes of the log-logit plots of h/oBMP15 (S356C)-EM (slope = 0.798x) and *E. coli*-derived oBMP15 (slope = 0.719x) were tested for parallelism by residual sum of squares and found to be not significantly different ($P > 0.05$, Figure 3.5).

Figure 3.5 Parallelism between h/oBMP15 (S356C)-EM and *E. Coli*-derived oBMP15. Log-logit plot of h/oBMP15 (S356C)-EM (◆) and *E. coli*-derived oBMP15 mature domains (◆)

It was not possible to analyse a similar dilution series of o/oBMP15, due to the low levels of expression by the pEFIREs-o/oBMP15-transfected HEK293 cells. Therefore, an alternative approach was used whereby Western blots were run under reducing conditions and the density of the reference and sample bands were then determined. Bands of three different concentrations of *E. coli*-derived BMP15 (625, 1250 and 2500 ng/mL) were each used to calculate the concentration in three different solutions of o/oBMP15-EM. This was performed using triplicate Western blots (Table 3.4). Having established that only a single reference standard was required, only one concentration of *E. coli*-derived oBMP15 (625 ng/mL) was used as a reference standard on all subsequent Western blots.

Table 3.4 Summary of calculated o/oBMP15 concentrations when using different reference standards

o/oBMP15-EM Sample number	BMP15 concentration (ng/mL): calculated using different concentrations of <i>E. coli</i> derived BMP15 as the reference standard			Difference between standards P=
	625 ng/mL	1250 ng/mL	2500 ng/mL	
1	213 ± 64	238 ± 75	244 ± 118	0.906
2	489 ± 60	529 ± 84	529 ± 159	0.878
3	402 ± 57	438 ± 86	441 ± 164	0.895

3.3.3. Production of o/oBMP15 and h/oBMP15 (S356C) proteins

Expression medium (EM) from HEK293 cells transfected with an empty pEFIREs-P, pEFIREs-o/oBMP15 or pEFIREs-h/oBMP15 (S356C) were harvested as described in Section 2.2.4. The o/oBMP15-EM and h/oBMP15 (S356C)-EM contained 414 ± 42 ng/mL ($n = 5$ replicate assays) and 1954 ± 171 ng/mL ($n = 6$ replicate assays) of *E. coli*-mature monomer equivalents.

Figure 3.6 Composite image showing western blot detection of molecular forms of o/oBMP15 and h/ oBMP15 (S356C).

Expression medium from HEK293 cells transfected with empty pEFIREs-P plasmid (Lane 1), pEFIREs-P-o/oBMP15 (Lane 2) or pEFIREs-P-h/oBMP15 (S356C) (Lane 3) under reducing or non-reducing conditions. Molecular size markers (kDa) are shown to the left of the image.

Western blot analyses of these EM showed bands corresponding to monomeric mature (~15 kDa) forms under reducing conditions in both o/oBMP15-EM and h/oBMP15 (S356C)-EM but not in pEFIREs-EM. Bands corresponding to uncleaved promature (~52 kDa ovine promature domain or ~56 kDa human/ovine promature domain) were also present. Under non-reducing conditions, the promature forms remained, however a band of

(~30 kDa) corresponding to the dimeric mature form was detected in h/oBMP15 (S356C)-EM but not o/oBMP15-EM. Higher molecular weight forms appeared under non-reducing conditions. Two bands in o/oBMP15-EM and one in h/oBMP15 (S356C)-EM corresponded to bands also seen in the empty pEFIRE5-EM. Interestingly, two additional bands appeared in h/oBMP15 (S356C)-EM: one of these corresponds in size to a dimeric promature form while the other corresponds to a complex between a promature and a mature monomeric form (Figure 3.6).

3.3.4. Purification of o/oBMP15 and h/oBMP15 (S356C) proteins

3.3.4.1. IMAC purification

Both h/oBMP15 (S356C) and o/oBMP15 bound to the Ni-NTA-agarose (Figures 3.7 and 3.8 respectively), via the N-terminal 8His tag on the prodomain and the mature domains, were eluted in the presence of 7 M urea. For increased clarity, the Figures below depict the use of a column, however the centrifugal batch methodology that was described in Section 3.2.1 was used in these experiments. Both the amount and composition of the eluted h/oBMP15 (S356C) material varied during sequential elution with 7 M urea. The eluted dimeric mature domains detected in Fractions 5-11 (Figure 3.7), also included very large amounts (>70%) of eluted monomeric mature domains (Table 3.5; page 50). Furthermore, the proportions of the mature BMP15 (S356C) domains relative to the uncleaved promature domains also varied. The Fractions 5-11, 12-14 and 15-19 contained 62.8 ± 1.3 , 85.0 ± 4.9 and 40.1 ± 1.0 (mean $\pm$ S.E.M.) percent mature domains, respectively. All the fractions eluted with 7 M urea included both uncleaved promature and mature domains (Figure 3.7). Overall, these fractions contained 1908 ± 220 ng/mL monomeric mature domain (mean $\pm$ S.E.M., $n = 5$). Residual materials bound to the column, containing promature, mature monomers and mature dimers were eluted in the presence of imidazol.

Similarly, o/oBMP15 was found predominantly as mature monomers and eluted mainly in Fractions 5-11 (Figure 3.8). Within these fractions, the uncleaved pro-mature domains comprised 7.8 ± 1.6 (mean $\pm$ S.E.M.) % of the immunoreactive BMP15 that was present. Fractions 12-14 and 17-19 contained no detectable BMP15, however Fractions 15 and 16 contained predominately uncleaved pro-mature domains, comprising 58% of all uncleaved promature forms that were eluted with 7 M urea.

Figure 3.7 Western blot of fractions collected from IMAC purification of h/oBMP15 (S356C)-EM, under reducing conditions. See Section 3.2.1 for detailed description of wash W10, wash W20, Elute 1 and Elute 2.

Figure 3.8. Western blot of fractions collected from IMAC purification of oBMP15-EM, under reducing conditions. See Section 3.2.1 for detailed description of wash W10, wash W20, Elute 1 and Elute 2.

Table 3.5. Concentration and percentage of BMP15 (S356C) and BMP15 mature domains in IMAC purified fractions (depicted in Figures 3.9 and 3.10 respectively). Monomer domains (M) and promature domains (PM)

Fraction	h/oBMP15 (S356C)			o/oBMP15		
	Monomer µg / mL	Cumulative % monomer (µg/fraction)	Percent free mature domains* (M x100) / (M+PM)	Monomer µg / mL	Cumulative % monomer (µg/fraction)	Percent free mature domains* (M x100) / (M+PM)
5	32.9	10.66	59.4	0.86	32.43	96.44
6	44.3	25.041	62.1	0.68	57.93	85.52
7	46.7	40.14	67.3	0.35	71.1	92.98
8	44.5	54.55	67.6	0.20	78.42	92.12
9	28.4	63.75	63.3	0.01	78.83	89.54
10	28.6	73.02	58.9	0.56	100.00	90.79
11	22.2	80.21	60.8	ND		
12	19.9	86.65	76.9	ND		
13	117	90.44	93.9	ND		
14	5.1	92.10	84.1	ND		
15	4.3	93.49	41.9	ND		
16	4.0	94.78	41.4	ND		
17	3.2	95.82	38.2	ND		
18	7.7	98.32	37.0	ND		
19	5.2	100.0	42.0	ND		

* may be present in unreduced form as mature monomers, dimers or cleaved pro-mature domains

3.3.4.2. HPLC purification

The pooled eluates of h/oBMP15 (S356C) in 7 M urea were further fractionated by HPLC, as described in Section 3.2.2. The BMP15 within these fractions proved difficult to recover when evaporated to complete dryness. When reconstituted with water, these fractions contained no soluble BMP15 when aliquots were removed and tested by Western blotting. In contrast, if the water was removed and replaced with reducing sample buffer (Appendix 5) and heated to 100°C (as per standard protocol, Section 2.3.1), immunoreactive BMP15 bands were detectable in the Western blots (results not shown). Likewise, if the freeze-drying process was halted when only 20-30 µL of the HPLC elution solvent was still present, then BMP15 was also detectable (Figure 3.9). A protocol similar to that used to purify human BMP15 (Pulkki *et al.* 2012) and human cumulin (Mottershead *et al.* 2015) was used, however it appeared that ovine BMP15 has a greater propensity to bind to the walls of the microcentrifuge tubes in which they were completely evaporated.

Aliquots (1 µL) of the residual HPLC elution solvent (after evaporation to near dryness) were diluted to 12 µL with Milli-Q H₂O and then analysed by Western blotting under both reducing and non-reducing conditions. Under non-reducing conditions, promature forms eluted at 44-45% ACN (Figure 3.11A, Lanes 2-3 and 9-10) whereas the mature domains (predominantly in dimeric form) were eluted with $\geq 45\%$ ACN (Figure 3.11A; Lanes 3-4 and 10-12). The loading material, previously purified by IMAC, also showed the presence of bands corresponding to both promature and dimeric mature domains (Figure 3.9A; Lane 7), along with two high molecular weight forms. Under reducing conditions, the loading material resolved fully into uncleaved promature and monomeric mature domains (Figure 3.9B, Lane 7). Similarly, uncleaved promature domains were still observed in Lanes 2-3 and 9-10 (Figure 3.9B) as they were under non-reducing conditions. Mature domains were present mainly as monomers and were identified in fractions eluted with $\geq 45\%$ ACN (Figure 3.9B; Lanes 2-4 and 9-12). Additional bands, intermediate to the dimeric mature and the promature domains also appeared under non-reducing conditions. These bands were not present in the loading material. The most likely explanation for this was the concentration difference between the IMAC- and HPLC-purified fractions. The identity of these bands was not determined.

Figure 3.9 Molecular forms of HPLC purified BMP15 (S356C).

Western blots of fractions from HPLC under (A) non-reducing and (B) reducing conditions. Fractions eluted from replicate runs at 43% ACN (Lanes 1 and 8), 44% ACN (Lanes 2 and 9), 45% ACN (Lanes 3 and 10), 46% ACN (Lanes 4 and 11), 47% ACN (Lanes 5 and 12) and 48% ACN (Lane 6). The pooled IMAC purified material is shown in Lane 7. The molecular size references (kDa) are shown to left of the images.

3.3.5. Bioactivity of o/oBMP15, h/oBMP15 (S356C) and o/oGDF9

A ^3H -thymidine incorporation bioassay using rat granulosa cells was used to determine the bioactivities of o/oBMP15-EM, h/oBMP15 (S356C)-EM alone and also in combination with o/oGDF9-EM.

In response to human activin A, ^3H -thymidine incorporation increased more than ten-fold (10.61 ± 2.08 ; mean $\pm$ S.E.M.; $p < 0.001$). This confirmed that the incubation conditions for the rat granulosa cells were conducive to proliferation and that the cells were able to respond to a purified TGFB family member. Factors produced and secreted into expression medium by un-transfected HEK-293 cells, at the volume used in the bioassay, had no significant impact on the amount of ^3H -thymidine incorporation by rat granulosa cells. Incubation media with and without addition of HEK-293 expression medium resulted in

mean cpm of 249 ± 23 (n=9) and 293 ± 16 cpm (n=9) respectively: these values were not significantly different from one another. The results presented as fold-differences relative to the media control, are shown in Figure 3.12.

Figure 3.10 Rat granulosa cell ^3H -thymidine incorporation bioassay. The effects of addition of activin A (10 ng/ml), oGDF9 (50 ng/mL), oBMP15 (19 ng/mL), BMP (S356C) (19 ng/mL), BMP15 + GDF9 (19 and 50 ng/mL respectively), oBMP (S356C) + oGDF9 (19 and 50 ng/mL respectively), oBMP15 (S356C) (high; 90 ng/mL) and oBMP15 (S356C) (high) + oGDF9 (90 and 50 ng/mL respectively) on ^3H -thymidine incorporation by rat granulosa cells. Values represent mean $\pm$ S.E.M. relative to control medium. Numbers above each histogram refer to number of independent rat granulosa cell pools tested. Different letters denote significant difference relative to control; a, $P<0.001$; b, $P<0.005$. The additive effect of oGDF9 and oBMP15 versus co-culture of oBMP15 + oGDF9 indicated a synergistic response; c, $P<0.02$.

The o/oGDF9-EM (0.97 ± 0.09 fold) had no effect on ^3H -thymidine incorporation, however o/oBMP15-EM (19 ng/mL) increased ^3H -thymidine incorporation both without (2.42 ± 0.28 fold; $p<0.005$) and with o/oGDF9-EM (3.76 ± 0.35 fold; $p<0.001$). Neither the low dose (19 ng/mL) of h/oBMP15 (S356C)-EM alone, or in combination with o/oGDF9-EM affected ^3H -thymidine incorporation (1.00 ± 0.08 and 1.15 ± 0.12 fold, respectively). Likewise, a high dose (90 ng/mL) of h/oBMP15 (S356C)-EM, neither alone, nor in combination with o/oGDF9-EM affected ^3H -thymidine incorporation (1.30 ± 0.17

and 1.32 ± 0.23 fold, respectively). Increasing the dose of h/oBMP15 (S356C) from 19 to 90 ng/mL increased the fold increase over control from 1.00 ± 0.08 to 1.30 ± 0.17 . Although this was not significantly different, this modest increase may be attributable to a small concentration of mature monomer present in this preparation (Figure 3.9A).

To test for interactions between oBMP15 and oGDF9, paired t-tests were performed comparing the ^3H -thymidine incorporation between the additive effect of oBMP15 and oGDF9 when added alone, compared to that when co-cultured. The additive effect of o/oBMP15-EM (19 ng/mL) and o/oGDF9-EM (2.39 ± 0.35 fold) was less than found when they were co-cultured (3.76 ± 0.35 fold; paired t-test, $p < 0.02$) demonstrating a synergistic response. However, the additive effect of o/oBMP15 (S356C)-EM (19 ng/mL) and o/oGDF9-EM when each was added alone (0.97 ± 0.15 fold), compared to that when co-cultured (1.15 ± 0.12) was not different. Thus, an interaction between o/oGDF9-EM and o/oBMP15-EM (19 ng/mL), but not with h/oBMP15 (S356C)-EM (19 ng/mL) was observed.

An original aim of this thesis was to assess the biological activity of differing BMP15 molecular forms. However, the HPLC purified mature and promature forms of oBMP15 and oBMP15 (S356C) were not tested. This was due to the failure to obtain soluble BMP15 from HPLC fractions, following evaporation to dryness (see Section 3.3.5.2). Moreover, when testing BMP15 activity in media with residual TFA in the fractions (i.e. those samples used for Figure 3.9), there was significant suppression of rat granulosa cell proliferation in the ^3H -thymidine incorporation bioassay. The residual fractions eluted from the HPLC contained 1.3×10^{-2} M TFA prior to drying. Tests showed rat granulosa cells were particularly sensitive to TFA. When exposed to 0, 4.5×10^{-5} M or 4.5×10^{-4} M TFA in the presence of 100 ng/mL activin A, the level of ^3H -thymidine incorporation by rat granulosa cells was 15710 ± 4163 [100%], 5269 ± 1609 [66.4%] or 905 ± 266 [94.3%] (mean $\pm$ S.E.M., [% suppression relative to no TFA control], $n=3$) cpm respectively. These values were significantly different, $P < 0.001$. Several future strategies that may alleviate this problem of insolubility are: (i) the addition of a carrier protein e.g. bovine serum albumin, to the HPLC purified BMP15 prior to freeze drying (ii) the inclusion of a carrier protein when the lyophilised BMP15 is reconstituted and (iii) altering the charge on the BMP15 by reconstituting with dilute acid i.e. 4mM HCl. These strategies could be trialled either singularly or in combination.

3.3.6. Surface Plasmon Resonance (SPR)

HPLC-purified h/oBMP15 (S356C) and o/oBMP15 failed to bind to a BIAcore sensor chip loaded with hBMPR2 extracellular domains. However, both a recombinant porcine (p) BMP15 (supplied by Dr Janet Pitman, Victoria University of Wellington) and an o/oGDF9 preparation were able to associate and disassociate from the sensor chip in a dose-dependent manner (Figure 3.11).

Figure 3.11 SPR sensograms of o/oBMP15, h/oBMP15 (S356C), o/oGDF9 and p/pBMP15 binding to human BMPR2. Binding profiles of (A) o/oBMP15, (B) h/oBMP15 (S356C), (C) o/oGDF9 and (D) p/pBMP15 binding to BMPR2 at 1:2 dilution (L4A2), 1:4 dilution (L4A3), 1:8 dilution (L4A4), 1:16 dilution (L4A5) and 1:32 dilution (L4A6). Biotinylated human BMPR2 captured on a BIAcore chip was equilibrated with running buffer (time <0 sec), injection of test ligands and complex formation with BMPR2 (time 0-120 sec) and disassociation of the complex (time 120 – 250 sec). RU, response units.

The reasons for these discrepancies were unclear. However, both oGDF9 and pBMP15 were prepared by simply dialysing the expression medium against the BIAcore running buffer. In contrast, both ovine BMP15 preparations, were purified by HPLC followed by freeze-drying to remove ACN and TFA, prior to SPR analysis. It is possible that by using this procedure, the oBMP15 was irretrievably bound to the tubes in which they were freeze-dried. This was thought to have occurred also when ovine BMP15 HPLC fractions were evaporated to dryness under vacuum (Section 3.3.5.2).

3.4 Discussion

HEK293 cells secrete oBMP15 largely as cleaved and uncleaved promature forms. Under non-reducing conditions, o/oBMP15-EM and h/oBM15 (S356C)-EM contained promature, but no monomeric mature, bands. However under reducing conditions both promature and monomeric mature domain were observed (Figure 3.6). It may be argued that the absence of observable monomeric mature bands under non-reducing conditions is due to a lack of sensitivity of clone MN2-61A (anti BMP15) and/or its inability to recognise this form under these conditions. The question of sensitivity, i.e. the level of detection of the different molecular forms, is one that cannot presently be answered. This will require the production of soluble purified forms of a known concentration. However MN2-61A was able to detect monomeric and dimeric forms of *E.coli* derived ovine mature BMP15 under both reducing and non-reducing conditions (Figure 3.12).

Figure 3.12 Western immunoblots showing the molecular forms of *E. coli* derived mature oBMP15 under (A) non-reducing and (B) reducing conditions. The blots depict bands from a dilution series where the total protein loaded onto the gel comprised 50ng (Lane 1); 25ng (Lane 2); 12.5ng (Lane 3); 6.25ng (Lane 4); 3.13ng (Lane 5) or 1.56ng (Lane 6). The molecular size references (kDa) are shown to the left of the images.

Moreover, HPLC-purified monomeric mature forms of oBMP15 (S356C) were also detected under non-reducing conditions (Figure 3.10). This same antibody was also able to immuno-neutralise thymidine incorporation when ovine oocytes were incubated with either rat or ovine granulosa cells (Lin *et al.* 2012). Assuming mature domains are indeed among the biologically active forms produced and secreted by ovine oocytes then MN2-61A is able to recognise this form under native, non-reducing conditions.

Although some of these mature domains in EM may be comprised within the high molecular weight complexes, the HPLC-purified forms showed much of the promature form was in a cleaved (non-covalently linked pro- and mature domains) state (Figure 3.9). This confirms that both cleaved and uncleaved promature forms were present. This is in agreement with published data for the mouse (McIntosh *et al.* 2008). However published data for recombinant ovine BMP15 have either: not commented on the promature forms (McNatty *et al.* 2005a), or not reported the presence of uncleaved promature forms (Edwards *et al.* 2008). Additional bands, not observed in o/oBMP15-EM, were present in h/oBMP15 (S356C)-EM (Figure 3.6). The size of these bands corresponded to forms that may represent dimeric mature protein, a complex between a promature and mature monomer and dimeric promature protein. Although the identities of these bands have not been confirmed, it is likely they are the result of covalent cysteine bonding between mature domains, which do not exist in o/oBMP15.

The IMAC procedure is based on the specific covalent bonding of amino acids, particularly histidine, to metals (Porath *et al.* 1975). The expected manner by which both o/oBMP15 and h/oBMP15 (S356C) bind to, and elute from, Ni-NTA-agarose, would have been via the 8His tagged pro- and promature domains binding directly to the Ni-NTA-agarose. Conversely, untagged proteins such as the mature domains would not bind. Thus cleaved promature domains should resolve into both pro- and mature domains in the presence of 7 M urea allowing for the mature domains to be eluted. While the remaining pro- and uncleaved promature domains would be expected to elute in the presence of imidazole which competes for the Ni²⁺ ions on the Ni-NTA-agarose. However this was not what was observed (Figures 3.7 and 3.8). Both h/oBMP (S356C) (Figure 3.9) and o/oBMP15 (Figure 3.8) eluted from the Ni-NTA-agarose in the presence of urea as promature and mature domains over an extended period. This pattern of elution may be explained by a complex. This complex could consist of various BMP15 molecular forms being bound together by both covalent and non-covalent bonds and then binding to the Ni-NTA-agarose via the

8His tag on a single pro- or promature domain. Indeed, high molecular weight complexes which resolve into promature and mature domains were observed in both the o/oBMP15-EM and h/oBMP15 (S356C)-EM preparations (Figure 3.6) Moreover, oBMP15 multimers have been reported previously (Edwards *et al.* 2008).

Table 3.6 Molecular forms of BMP15 recognised by clone MN2-61A in expression medium, IMAC-purified or HPLC-purified material.

Molecular forms recognised by clone MN2-61A		Molecular forms corresponding to Western blotting bands in:		
		Expression medium	IMAC purified	HPLC purified
Dimeric promature	✓	✓	✗
OR	Covalently bound mature monomer with promature. OR Dimeric mature:Prodomain complex	✓	✓	✓
Monomeric uncleaved promature	✓	✓	✓
Monomeric cleaved promature complex	✓	ND	✓
Dimeric mature domains	✓	✓	✓
Monomeric mature domains	✓	✓	✓

(✓) detected, (✗) not detected, (ND) not determined.

Notwithstanding the above, some degree of separation of promature and mature domains of h/oBMP15 (S356C) was achieved with the use of HPLC (Figure 3.9). The first fraction eluted contained both cleaved and uncleaved promature domains, the second fraction contained a mixture of promature and mature forms and the third fraction contained mostly the mature domains (Figure 3.9). The two high molecular weight bands observed in the IMAC purified material loaded onto the HPLC column (Figure 3.9, Lane7) were not present in fractions eluted from the HPLC column.

Interestingly, the promature bands of h/oBMP15 (S356C) found in both the IMAC- (Figure 3.9) and HPLC- (Figure 3.11) purified material appear to be composed of two bands located close together. The predominant form was found in the higher band under non-reducing conditions and in the lower band under reducing conditions. Not all associations between proteins were disrupted under reducing conditions as can be observed by the presence of some dimeric mature domains under reducing conditions (Figure 3.8 and Figure 3.9B). This raises the possibility that the lower of the two bands was composed largely of uncleaved promature domains along with some cleaved promature domains. Whereas the upper band was composed of mature dimers bound covalently or non-covalently to a single prodomain. It is also worth noting the presence of bands in Fractions 4-8 (Figure 3.8) corresponded in size to a dimeric promature form, similar to what was observed in h/oBMP15 (S356C)-EM (Figure 3.6). Western blotting using the monoclonal antibody MN2-61A identified many of the potential forms of BMP15 (S356C) (Figure 3.5). The most predominant forms were the promature and mature forms. Importantly, clone MN2-61A was able to detect the dimeric mature form of h/oBMP15 (S356C) but was unable to detect a corresponding band of o/oBMP15 (Table 3.5). This suggests that o/oBMP15 is different to hBMP15, in which mature dimers were detected (Pulkki *et al.* 2012).

Experiments using the ³H-thymidine incorporation bioassay and rat granulosa cells demonstrated o/oGDF9 and o/oBMP15 were biologically active. Moreover, co-incubating o/oBMP15-EM and o/oGDF9-EM confirmed the synergistic response that has been reported by others (Yan *et al.* 2001; Hanrahan *et al.* 2004; McNatty *et al.* 2005a; Edwards *et al.* 2008; McIntosh *et al.* 2008; Reader *et al.* 2011; Mottershead *et al.* 2012). The molecular form(s) responsible for this synergism were not able to be determined in the present study. However, for BMP15, it is likely to be the cleaved promature or monomeric mature forms, as these were the forms present in o/oBMP15-EM (Figure 3.6). Although

the uncleaved promature form was also present, this is known to be a biologically inactive form (Souza *et al.* 2014).

The response to human activin A was several fold greater than that observed with o/oBMP15 either alone or with o/oGDF9. Several factors may contribute to this difference. Different receptors are utilised by activin A compared to those used by oBMP15 or oGDF9. Furthermore, if cell proliferation is mediated by the Smad 2/3 pathway, then dimeric activin would signal through two type I receptors per receptor-ligand complex where as a BMP15-GDF9 receptor complex would contain just a single type I receptor capable of signalling via the Smad 2/3 pathway.

Interestingly, h/oBMP15 (S356C)-EM, unlike o/oBMP15, when cultured alone did not demonstrate biological activity. This is in agreement with Mottershead *et al.* (2015) who also reported the hBMP15 (S357C) variant, was unable to stimulate proliferation in mouse granulosa cells, either with or without co-culture with hGDF9. In contrast the hBMP15 and the hBMP15 (S357C) variant, were equally able to stimulate the Smad 1/5/8 activity and inhibin B production in COV434 cells (Pulkki *et al.* 2012). The reason(s) for these differences are unknown. However one reason may be the different end points examined. In the previous study, Smad1/5/8 activation and inhibin B production were investigated whilst this study investigated proliferation which is mediated via the Smad 2/3 pathway. Consequently, it will be important to determine in subsequent bioassays, that the biological activity of h/oBMP15 (S356C)-EM is dependent upon Smad 1/5/8 activity before its apparent inability to synergise with oGDF9 can be confirmed. Additionally, if the promature form is important for biological activity then the interaction between a human proregion and an ovine mature domain [h/oBMP15 (S356C)] may be sub-optimal in the thymidine incorporation bioassay. Indeed the synergistic response of BMP15 and GDF9 has been shown to involve, in addition to Smad 2/3, different non-smad signalling pathways. Which pathways are involved, appear to be dependent upon both the species of growth factors, and the species of the target granulosa cells (Reader *et al.* 2011; Mottershead *et al.* 2012; Reader *et al.* 2014).

CHAPTER4: DETERMINATION OF OOCYTE-DERIVED MOLECULAR FORMS OF BMP15 AND GDF9

4.1 Introduction

Oocyte-secreted factors and thus the oocyte itself are well-recognised to play a significant role in ovarian follicular development (Eppig *et al.* 2002). Two closely-related members of the TGFB superfamily, BMP15 and GDF9, are perhaps the best studied of these factors. In general, members of the TGFB superfamily are produced as proproteins, with both the pro- and mature regions being enzymatically cleaved from one another to form non-covalently linked pro-mature complexes (Massague 1998). A characteristic “cysteine knot” stabilising the structure of the mature growth factor domain is formed by six conserved cysteine residues and a seventh cysteine residue is involved in forming covalent bonds between mature dimers (McDonald and Hendrickson 1993). However, BMP15 and GDF9 belong to a small sub-family of TGFB proteins where this seventh cysteine has been substituted with a serine. Consequently, any mature homo- or hetero-dimers can only associate in a non-covalent manner.

Although the dimeric form of recombinant mature ovine and mouse GDF9 has been identified (Edwards *et al.* 2008), (McIntosh *et al.* 2008), the dimeric form of mature ovine BMP15 has not. However, the secreted forms of mature recombinant hBMP15, either without tags (Pulkki *et al.* 2012) or with either a N-terminal FLAG tag (Liao *et al.* 2004), (Hashimoto *et al.* 2005) or a N-terminal MycHis tag (Rossetti *et al.* 2009) have been reported to include mature dimers. It has been suggested that the synergistic effect from the combined action of BMP15 and GDF9 on the proliferation of granulosa cells may result from the formation of heterodimers (McNatty *et al.* 2004). Indeed, a covalently linked, biologically-active recombinant heterodimer (termed cumulin) was produced to test this hypothesis (Mottershead *et al.* 2015). However it should be noted that to date, neither homodimers of BMP15 nor heterodimers with GDF9 have been identified as being produced or secreted by oocytes of any species. Thus, a major aim of these studies was to determine the molecular forms of BMP15 and GDF9 that are being produced and/or secreted by ovine and bovine oocytes. Recombinant o/oBMP15, h/oBMP15 (S356C) and o/oGDF9 are useful tools to aid identification of these molecular forms.

Monoclonal antibodies, clone MN2-61A (anti-BMP15) and clone 37A (anti-GDF9) have previously been shown to be specific for ovine growth factors (McNatty *et al.* 2005a) but

this has not previously been determined for bovine (b)BMP15 and bGDF9. Both MN2-61A and 37A were raised against the *E. coli*-derived mature domains of ovine BMP15 (McNatty *et al.* 2005a) and ovine GDF9 (Lin *et al.* 2012), respectively. Epitope mapping revealed that MN2-61A recognised a region within the sequence SEVPGPSREHDGPES (McNatty *et al.* 2005a), which shares 100% homology between ovine and bovine. Whilst epitope mapping has not been performed with MN2-37A, the mature domains of ovine and bovine GDF9 share 131 of the 135 amino acid sequence and thus >97% homology. Therefore, based on these degrees of homology, each antibody should recognise bovine BMP15 or GDF9 with a high degree of specificity.

4.2 Methodology

Unless stated below, additional details including sources of equipment and reagents used are described in Appendix 5. The methodological details describing preparation of samples for SDS-PAGE and Western blotting are described in Chapter 2.

4.2.1 Ovary collection

In brief, ovine and bovine ovaries (from animals of unknown age) were collected at a local abattoir (Taylor Preston, Ngauranga, Wellington, New Zealand) and were transported to the laboratory at room temperature. Subsequently, ovine ovaries were dissected free of extraneous oviductal tissue and rinsed twice in sterile PBS (0.15M, pH7.4; SIGMA). Bovine ovaries were rinsed twice in PBS but did not have non-ovarian tissue removed prior to oocyte collection. The ovaries remained immersed in PBS at room temperature until oocyte collection.

4.2.2 Oocyte collection and incubation

Upon removal from PBS, ovine ovaries were dissected free of the medullary blood supply to aid follicular visualisation under a dissecting microscope. Cumulus cell-oocyte-complexes (COC) were recovered by flushing all the visible antral follicles on the ovarian surface with the dissection medium (i.e. Medium C; M199 containing Hepes, Glutamax (L-alanyl-L-glutamine dipeptide) and PenStrep, pH 7.4) using a 20G needle fitted to a 5 mL syringe.

Bovine ovaries were removed from the PBS and COC were collected by aspiration of the follicular contents directly into dissection medium using a 20G needle connected to a Venturi vacuum pump attached to a low pressure water supply.

A mixture of “naked oocytes” (oocytes devoid of a complete layer of cumulus cells) and COC were collected from the resultant media/follicular fluid, which will hereafter be referred to collectively as oocytes. Oocytes were extracted from the aspirated fluid by use of a 10 µL micro-pipette and washed twice by transfer to fresh Medium C.

Subsequently, both the bovine or ovine oocytes were washed once in incubation medium (i.e. Medium D; M199 containing Glutamax, PenStrep and NaHCO₃; pH 7.4). Thereafter, pools of 100-130 oocytes in 50 µL incubation medium were either immediately frozen at -20°C (hereafter, referred to as freshly collected oocyte lysate) or cultured for 18 h in 1.7 mL microcentrifuge tubes at 37°C in 5% CO₂. To help maintain sterility whilst ensuring gas exchange, the microcentrifuge tube was uncapped and the top was covered in a single layer of parafilm (Bemis flexible packaging, Neenah, WI, USA) that was punctured twice with a 20G needle. Following culture, the incubation medium was removed from the incubation tube containing the cultured oocytes and stored separately. This contains the BMP15 and GDF9 forms secreted by the oocytes. To keep storage conditions the same for all samples, 50 µL of fresh Medium D was added back to the cultured oocytes, containing BMP15 and GDF9 forms present in oocyte lysates following culture. Samples were stored at -20°C until analysed. Prior to freezing at -20°C, all samples had 2 µL of a 26x protease inhibitor cocktail (Appendix 5) added. In all instances, oocytes that were either frozen or placed in culture were done so within 5 hours of collecting ovaries from the abattoir.

4.2.3 Antibody specificity

The specificity of Western blot immunostaining was tested by using antibodies that had been pre-adsorbed with their respective antigens. For clone MN2-61A (anti-BMP15) and clone 37A (anti-GDF9), *E. coli*-derived mature ovine BMP15 and ovine GDF9 were used, respectively. On the day before use in Western blotting, a 20x antibody solution was incubated overnight at 4°C with 20x mass of the respective antigen (e.g. 10 µg Clone MN2-61A was incubated with 200 µg antigen). Immediately before use, the pre-adsorbed antibody was centrifuged at 6000 g for 5 min and the supernatant diluted 1:20 with blocking buffer prior to being incubated with the nitrocellulose membrane (see Section 2.4.3). Western blots for the blocking studies had previously been used to identify the molecular forms of BMP15 or GDF9. These were chemically stripped of the previous antibodies used, prior to the Western blotting technique being repeated, using pre-adsorbed antibodies. Chemical stripping consisted of incubating the membrane for 30 min in 50 mL of 62.5 mM Tris-HCl containing 2% (w/v) sodium dodecyl sulphate and 0.75 M 2-

mercaptoethanol, pH 6.7 at 50°C. At room temperature, the membrane was washed vigorously by manual shaking three times for 20 seconds, and then on a rocking platform (15° incline, 30rpm) three times for 10 min, replacing the low salt buffer after each wash step (Appendix 5). Thereafter, membranes were subjected to the previously described Western blotting procedure (see Section 2.3.3).

4.2.4 Western blot band determination

Figure 4.1 Use of ImageJ to identify the presence of faint bands in Western blots. Density plots for each lane in the Western blot (using the same image as described in the Results section, see Figure 4.4A, upper image). Conditioned medium from HEK293 cells transfected with either ovine GDF9 (lane 1), wild-type ovine proregion/ovine mature BMP15 (o/oBMP15, lane 2), mutant human proregion/ovine mature (S356C) (h/oBMP15 (S356C), lane 3) lysates from freshly collected oocytes (lane 4), medium from incubated oocytes (lane 5) and lysates from incubated oocytes (lane 6). The area enclosed by the dashed lines enlarged (lower image) used to objectively define a peak from a faint band (*)

It became apparent that some Western blots contained immunoreactive bands that were visible when viewed on a monitor screen, but on some occasions became less clear or barely visible when printed. This was despite attempts to maximise contrast and brightness of the images. Therefore, the freeware ImageJ (<http://rsb.info.nih.gov/ij/index.html>) was used to objectively identify and record the presence of these faint bands. The method used was that outlined by Miller (2010). Briefly, identical-sized areas were selected from each

lane of the Western blot. From these, a density plot was generated corresponding to the changing density along the length of each lane. The peaks within this plot corresponded to each band within the lane (Figure 4.1).

4.3 Results

4.3.1 Specificity of the monoclonal antibodies MN2-61A (anti BMP15) and 37A (anti GDF9)

Figure 4.2 Immuno-blocking of monoclonal MN2-61A by *E. coli*-derived mature oBMP15. Western immunoblots under cross-linking/reducing conditions (A, B) and reducing conditions (C, D) showing the molecular forms of BMP15 produced and secreted by bovine oocytes and recombinant proteins. For A and C, lane 1 = o/oGDF9; lane 2 = wild-type ovine proregion/ovine mature BMP15 (o/oBMP15); lane 3 = mutant human proregion/ovine mature (S356C) [h/oBMP15 (S356C)]; lane 4 = o/oBMP15 + o/oGDF9; lane 5 = h/oBMP15 (S356C) + o/oGDF9; lane 6 = bovine oocyte lysate at the start of incubation; lane 7 = oocyte lysate at the end of incubation; and lane 8 = medium in which oocytes were incubated and for B and D, the corresponding immunoblots where the BMP15 antibody (clone MN2-61A) was preabsorbed with *E. coli*-derived mature ovine BMP15. . Order of lanes for C and D were allocated so as to match lanes in A and B. Molecular sizes are shown to the left of the image (kDa).

As mentioned earlier, clones MN2-61A and 37A had previously been validated for use on ovine oocytes (Lin *et al.* 2012) but not for the bovine. The specificity of MN2-61A, using the Western blotting procedure, was tested by preadsorption of the antibody with an *E. coli*-derived, mature oBMP15. This was undertaken once under reducing (Figure 4.2A and B) and once under cross-linking/reducing conditions (Figure 4.2C and D).

Likewise the specificity of clone 37A was established by preadsorption with an *E. coli*-derived, mature GDF9 (Figure 4.3A and B). Generally, the immunostained bands were abolished by pre-incubation with the specific antigen. However, when the blots were incubated with pre-absorbed anti BMP15 (MN2-61A), some residual immunostaining was present in the mature band in Figure 4.2C, lane 5 (i.e. co-incubated BMP15 (S356C) and GDF9). Also, under reducing conditions, non-specific binding to a band within BMP15 (S356C), larger than the promature band, was detected (Figure 4.2D, lane 3).

Figure 4.3 Immuno-blocking of monoclonal 37A by *E. coli*-derived mature oGDF9. Western immunoblots under reducing conditions showing the molecular forms of BMP15 produced and secreted by bovine oocytes and recombinant proteins. For image A: Lane 1=o/oGDF9; Lane 2=o/oBMP15; Lane 3=h/oBMP15 (S356C); Lane 4=bovine oocyte lysate, at the beginning of incubation; Lane 5=oocyte lysate at the end of incubation and; Lane 6=medium in which oocytes were incubated (incubation medium) Image B shows the corresponding immunoblots where the GDF9 antibody (clone 37A) was preabsorbed with *E. coli*-derived mature ovine GDF9. Molecular sizes are shown to the left of the image (kDa).

Residual immunostaining was also detected when blots were reprobed with pre-absorbed anti GDF9 (clone 37A) within the mature band of recombinant oGDF9. Nevertheless, all forms of BMP15 and GDF9 that were secreted and/or produced by bovine oocytes were abolished by pre-incubation with the specific antigens for each antibody.

Table 4.1: Frequency of immuno-reactive BMP15 Western blot (WB) bands of varying molecular sizes in oocyte lysates and incubation medium under non-reducing, reducing and cross-linking conditions. Brackets [] refer to the number of samples where band was observed/total number of samples].

WB condition	Molecular form	COC lysate Freshly collected		COC lysate Post incubation		Oocyte incubation medium	
		Bovine	Ovine	Bovine	Ovine	Bovine	Ovine
Non-reducing	>100 kDa	[3/3]	[3/3]	[3/3]	[3/3]	[3/3]	[2/3]
	Promature	[0/3]	[0/3]	[0/3]	[0/3]	[0/3]	[0/3]
	~40 kDa	[3/3]	[3/3]	[2/3]	[3/3]	[0/3]	[1/3]
	mature monomer	[0/3]	[2/3]	[0/3]	[1/3]	[0/3]	[0/3]
Reducing	>100 kDa	[0/3]	[1/3]	[1/4]	[0/3]	[0/4]	[0/3]
	Promature	[3/3]	[3/3]	[4/4]	[3/3]	[4/4]	[1/3]
	~40 kDa	[0/3]	[1/3]	[0/4]	[1/3]	[0/4]	[0/3]
	mature monomer	[2/3]	[2/3]	[2/4]	[3/3]	[2/4]	[0/3]
Cross-linked	>100 kDa	[3/3]	[3/3]	[2/3]	[3/3]	[3/3]	[2/3]
	Promature	[3/3]	[3/3]	[3/3]	[3/3]	[3/3]	[1/3]
	~40 kDa	[1/3]	[2/3]	[0/3]	[2/3]	[0/3]	[0/3]
	mature monomer	[2/3]	[3/3]	[3/3]	[3/3]	[3/3]	[1/3]

4.3.2 Molecular forms of BMP15 produced by ovine and bovine oocytes

Under non-reducing conditions, the only immuno-reactive BMP15 form that was consistently detected in all replicate experiments from both bovine (Figure 4.4A) and ovine (Figure 4.4B) oocytes was a form(s) present at the >100 kDa band size. Thus, the molecular forms of BMP15 secreted from bovine and ovine oocytes were predominately detected as high molecular sized bands (Figure 4.4A & B, Lane 5; Table 4.1). However, in one ovine replicate, an additional band of ~40 kDa was observed in secreted media (Figure 4.4B, Lane 5). This ~40 kDa band was also observed in all oocyte lysates from freshly

collected oocytes and in three out of three (ovine), and two out of three (bovine) oocyte lysates collected from oocytes following culture for 18 h. An additional band of 15 kDa, corresponding to the mature BMP15 monomer was observed in two out three freshly-collected ovine oocyte lysates (data not shown), and in one out of three ovine oocyte lysate samples following an 18 hr incubation (Figure 4.4B, Lane 6), but was not observed in any bovine oocyte lysate samples.

Figure 4.4 Western immunoblots under non-reducing conditions showing the molecular forms of BMP15 in lysates and incubation medium of (A) bovine and (B) ovine oocytes. The blots depict bands from conditioned medium from HEK293 cells transfected with either: ovine GDF9 (Lane 1); o/oBMP15 (Lane 2); or h/oBMP15 (S356C) (Lane 3); as well as lysates from freshly collected oocytes (Lane 4), medium from incubated oocytes (Lane 5) and lysates from incubated oocytes (Lane 6). Molecular sizes are shown to the left of the image (kDa).

Under reducing conditions, the predominant form of immunoreactive BMP15 produced by both ovine and bovine oocytes was in bands corresponding to the unprocessed promature form. A small amount of this unprocessed promature form had been cleaved to release the mature monomer. The 40 kDa band was still observed although not consistently and only within pools of freshly-collected or incubated ovine oocyte lysates (Figures 4.5B, Lane 5 and Figure 4.5C, Lane4). Only one out of three replicates under reducing conditions detected a BMP15 form in secreted media from ovine oocytes, which was the uncleaved promature form (Figure 4.5C, Lane 6). In contrast, the uncleaved promature form was consistently observed in secretions from bovine oocytes, and the mature monomer was also identified in two out of four replicates (Figure 4.5A).

Figure 4.5 Western immunoblots under reducing conditions showing the molecular forms of BMP15 in lysates and incubation medium of (A) bovine and (B, C) ovine oocytes. The blots depict bands from conditioned medium from HEK293 cells transfected with either: ovine GDF9 (Lane 1); o/oBMP15 (Lane 2); or h/oBMP15 (S356C) (Lane 3); as well as lysates from freshly collected oocytes (Lane 4), lysates from incubated oocytes (Lane 5), medium from incubated oocytes (Lane 6), lysates from incubated oocytes devoid of cumulus cells (naked oocytes) (Lane 7), medium from incubated naked oocytes (Lane 8), lysates from freshly collected cumulus cell-oocyte complexes (COCs) (Lane 9). Molecular sizes are shown to the left of the images (kDa).

Note: The use of “oocytes” in lanes 4, 5 and 6 refers to a mix of both naked oocytes and COCs as mentioned in Section 4.2.2.

To determine if forms of BMP15 were being bound within the ECM of cumulus oocyte complexes (COCs), a comparison was made between secretions in incubation medium that had cultured oocytes with cumulus cells still intact (COCs) and oocytes devoid of cumulus cells (naked oocytes) (Figure 4.5A). However, no discernible differences were observed between the lysates from incubated COCs and naked oocytes (Figure 4.5A; Lanes 10 and 7, respectively) or between the secreted forms detected in the medium following incubation of COCs and naked oocytes (Figure 4.5A; Lanes 11 and 8 respectively).

Figure 4.6 Western immunoblots under reducing conditions showing the molecular forms of BMP15 in cross-linked samples of lysates and incubation medium of (A) bovine and (B) ovine oocytes. The blots depict bands from conditioned medium from HEK293 cells transfected with either: ovine GDF9 (Lane 1); o/oBMP15 (Lane 2); h/oBMP15 (S356C) (Lane 3); co-incubated h/oBMP15 (S356C) and oGDF9 (Lane 4); or co-incubated o/oBMP15 and oGDF9 (Lane 5), as well as lysates from freshly collected oocytes (Lane 6), lysates from incubated oocytes (Lane 7) and medium from incubated oocytes (Lane 8). Molecular sizes are shown to the left of the image (kDa).

The cross-linking of proteins prior to their addition on a reducing gel revealed a similar pattern to that observed in non-cross-linked samples. Generally, for both the bovine and ovine oocyte lysates, BMP15 was located within bands corresponding to the promature and free mature monomer forms, with the ~40 kDa band being present in some, but not all, oocyte lysates (Figures 4.6A, 4.6B and 4.7A, Lanes 6 and 7). In one instance, the lysate from freshly collected bovine oocytes contained additional bands immediately above and below the promature band and did not contain any mature monomer (Figure 4.6A, Lane 6). Also present in this gel were high molecular weight complexes in both the bovine oocyte lysates and incubation media (Figure 4.6A, Lanes 6 - 8). The size of these bands appears lower than that found in other bovine replicates or in ovine samples, perhaps indicating incomplete cross-linking. Mature, promature and high molecular weight forms, were consistently observed secreted by bovine oocytes (Figure 4.6A, Lane 8). Secreted BMP15 from ovine oocytes was present in much lower quantities than that observed in bovine. In one replicate no discernible forms were detected. High molecular weight complexes were observed in two of the three replicates (Figure 4.6B and 4.7A, Lane 8). Also, in only one out of three replicates of medium following incubation of ovine oocytes, a band corresponding to the promature and mature monomer was detected (Figure 4.7A & B, Lane 8).

Figure 4.7 Western immunoblots under reducing conditions showing the molecular forms of BMP15 in cross-linked samples of lysates and incubation medium of (A, B) ovine oocytes. (A) The blots depict bands from conditioned medium from HEK293 cells transfected with either: ovine GDF9 (Lane 1); o/oBMP15 (Lane 2); h/oBMP15 (S356C) (Lane 3); co-incubated o/oBMP15 and oGDF9 (Lane 4); or co-incubated h/oBMP15 (S356C) and oGDF9 (Lane 5), as well as lysates from freshly collected oocytes (Lane 6), lysates from incubated oocytes (Lane 7) and medium from incubated oocytes (Lane 8). Molecular sizes are shown to the left of the image (kDa). (B) Density plots of aforementioned lanes in (A). The faint bands in Lane 8 at high molecular sizes and those corresponding to promature and mature domains are indicated (↓).

4.3.3 Molecular forms of recombinant oBMP15 secreted by HEK-293 cells

Under non-reducing conditions, wild-type oBMP15 (o/oBMP15) was present mainly as high molecular size forms >100 kDa (Figures 4.4A and 4.4B, Lane 2). The cysteine mutant form [h/oBMP15 (S356C)] was present as high molecular weight forms as well as with molecular weight bands corresponding to promature forms and dimeric mature growth factor domains, and on one occasion, a faint band corresponding to the mature monomer was also observed (Figures 4.4A and 4.4B, Lane 3). Under reducing conditions (both with and without cross-linking), wild-type o/oBMP15 was present as promature and mature growth factor domains, (Figures 4.5A, 4.5B, 4.5C, 4.6A, 4.6B, Lane 2). In contrast, h/oBMP15 (S356C) was found predominantly in bands corresponding to the promature, mature dimer and mature monomer domains along with some higher molecular weight forms (Figures 4.5A, 4.5B, 4.6A, 4.6B, Lane 3). With cross-linking, an additional band of ~17 kDa was observed just above the mature monomer in h/oBMP15 (S356C). Also with cross-linking, a greater proportion of o/oBMP15 as well as the h/oBMP15 (S356C) proteins were in the promature form. Cross-linking of h/oBMP15 (S356C) or o/oBMP15, in either the presence (Figures 4.6A and B, Lanes 4 and 5, respectively) or absence (Figures 4.6A and B, Lanes 3 and 2 respectively) of o/oGDF9 did not change the position of the pro-mature, mature dimer or mature monomer bands although more material appeared to be incorporated in the high molecular weight bands of h/oBMP15 (S356C).

Table 4.2 Frequency of immuno-reactive GDF9 bands of varying molecular sizes in oocyte lysates and incubation media under non-reducing, reducing and cross-linking conditions. Brackets [] refer to the number of samples where a band was observed /total number of samples].

WB condition	Size (kDa)	COC lysate Freshly collected		COC lysate Post incubation		Incubation medium	
		Bovine	Ovine	Bovine	Ovine	Bovine	Ovine
Non-reducing	>100 kDa	[2/3]	[3/3]	[2/3]	[3/3]	[3/3]	[2/3]
	promature	[3/3]	[3/3]	[3/3]	[3/3]	[1/3]	[2/3]
	mature monomer	[3/3]	[3/3]	[2/3]	[3/3]	[1/3]	[3/3]
	6-10 kDa	[3/3]	[3/3]	[2/3]	[3/3]	[1/3]	[0/3]
Reducing	>100 kDa	[0/3]	[0/3]	[0/4]	[0/3]	[0/4]	[0/3]
	promature	[3/3]	[3/3]	[4/4]	[3/3]	[4/4]	[1/3]
	mature monomer	[3/3]	[3/3]	[3/4]	[3/3]	[1/4]	[2/3]
	6-10 kDa	[3/3]	[3/3]	[3/4]	[3/3]	[1/4]	[2/3]
Cross-linked	>100 kDa	[2/3]	[2/3]	[2/3]	[2/3]	[2/3]	[2/3]
	promature	[3/3]	[3/3]	[3/3]	[3/3]	[2/3]	[2/3]
	mature monomer	[2/3]	[3/3]	[1/3]	[2/3]	[0/3]	[2/3]
	6-10 kDa	[2/3]	[3/3]	[2/3]	[2/3]	[0/3]	[1/3]

4.3.4 Molecular forms of GDF9 produced by ovine and bovine oocytes.

Under non-reducing conditions, the forms of immuno-reactive GDF9 present in lysates from both freshly-collected and incubated bovine and ovine oocytes were at molecular sizes corresponding to the promature and mature monomer domains, as well as in bands of higher and lower (6-10 kDa) molecular sizes (Figures 4.8A and B, Lanes 4 and 5). Secreted forms of GDF9 from bovine oocytes were consistently observed in high molecular weight complexes (Figures 4.8A, Lanes 6a, 6b & 6c). However, in one replicate, secreted bovine GDF9 was also found as promature and fully processed mature monomers

(Figures 4.8A & C, Lanes 6a). Secreted forms of GDF9 from ovine oocytes were consistently found as fully processed mature monomers and in two replicates were also observed in high molecular size bands (>100 kDa) as well as a band corresponding to the promature domain (Figures 4.8B, Lane 6).

Figure 4.8 Western immunoblots under non-reducing conditions showing the molecular forms of GDF9 in lysates and incubation medium of (A) bovine and (B) ovine oocytes. The blots depict bands from conditioned medium from HEK293 cells transfected with either: ovine GDF9 (Lane 1); o/oBMP15 (Lane 2); or h/oBMP15 (S356C) (Lane 3); as well as lysates from freshly collected oocytes (Lane 4), lysates from incubated oocytes (Lane 5), medium from incubated oocytes (Lane 6 or 6a; and replicate Western blots, Lanes 6b and 6c). (C) Density plots of lanes 5 and 6a. Lane 5 shown to aid orientation relative to (A). The faint bands in Lane 6a at high molecular weights and those corresponding to promature and mature domains are indicated (↑). Molecular sizes are shown to the left of the image (kDa).

Under reducing conditions, the predominant forms of immunoreactive GDF9 produced by both bovine (Figure 4.9A) and ovine (Figure 4.9B) oocytes was in unprocessed promature and mature monomer forms, as well as an uncharacterised band at 6-10 kDa. Both bovine and ovine oocytes secreted all three of these forms. Bovine oocytes secreted an unprocessed promature form consistently and in one out of four replicates a mature form was also observed. However, in ovine, the promature form was only observed in one out of three replicates and the mature form in two out of three replicates.

Figure 4.9 Western immunoblots under reducing conditions showing the molecular forms of GDF9 in lysates and incubation medium of (A) bovine and (B) ovine oocytes. The figures show conditioned medium from HEK293 cells transfected with either: ovine GDF9 in (Lane 1); o/oBMP15 in (Lane 2); and h/oBMP15 (S356C) (Lane 3); and in lysates from freshly collected oocytes (Lane 4), lysates from incubated oocytes (Lane 5), and medium from incubated oocytes (Lane 6). Molecular sizes are shown to the left of the image (kDa).

The oocyte lysates and incubation medium subjected to cross-linking prior to their addition on a Western gel under reducing conditions resulted in more immunoreactive GDF9 being retained within the high molecular weight bands, compared to that in the non cross-linked samples. Oocyte lysates, both before (Figure 4.10A and 4.10B, Lane 6) and after (Figure 4.10A and 4.10B, Lane 7) incubation showed GDF9 was present in a promature form and in high molecular weight complexes. Although not found in all replicate samples, the mature monomer form was also detected in oocyte lysates of both species before (Figure 4.10A and B, Lane 6) and after incubation (Figure 4.10B, Lane 7; not shown in the representative blot from bovine oocytes). Mature monomers in ovine and bovine oocyte lysates, before culture were detected in three out of three and two out of three replicates, respectively. Following culture, mature monomers were detected in two out of three, and one out of three, ovine and bovine oocyte lysates, respectively. High molecular weight complexes were consistently detected secreted into media during the incubation period (Figure 4.10A, B & C, Lane 8). However, for both ovine and bovine oocytes, promature forms were detected secreted into media in two of the three replicates. Mature monomers were only detected secreted into the incubation media in two out of three ovine replicates but were not detected in media in which bovine oocytes were cultured.

Figure 4.10 Western immunoblots under reducing conditions showing the molecular forms of GDF9 in cross-linked samples of lysates and incubation medium of (A) bovine and (B) ovine oocytes. The figures show the results from conditioned medium from HEK293 cells transfected with either: ovine GDF9 (Lane 1); o/oBMP15 (Lane 2); h/oBMP15 (S356C) (Lane 3); co-incubated h/oBMP15 (S356C) and oGDF9 (Lane 4a and 4b); and co-incubated o/oBMP15 and oGDF9 (Lane 5a and 5b); as well as lysates from freshly collected oocytes (Lane 6); lysates from incubated oocytes (Lane 7); and medium from incubated oocytes (Lane 8). Lanes 1-5a (A; from a different Western blot than that for lanes 4b – 8) were included to better illustrate the effect of cross-linking on recombinant proteins. Molecular sizes are shown to the left of the image (kDa). (C) Density plots of Lanes 6, 7 and 8. Lane 6 shown to aid orientation relative to (A). The faint bands in Lane 8 at high molecular sizes and those corresponding to promature domains are indicated (↓).

4.3.5 Molecular forms of recombinant oGDF9 secreted by HEK-293 cells

Bands corresponding to the pro-mature and mature monomeric forms of GDF9 as well as bands of 6-10 kDa were present under all conditions tested, non-reducing (Figure 4.8A and B, lane 1), reducing (Figure 4.9A and B, lane 1) and cross-linking/reducing (Figure 4.10A and B, lane 1). A mature dimeric form of GDF9 was only observed under non-reducing (Figure 4.8A and B, lane 1) and cross-linking/reducing (Figure 4.10A and B, lane 1) conditions. Bands of molecular sizes greater than that of the pro-mature domain were not observed under reducing conditions (Figure 4.9A and B, lane 1), however they were

present under both non-reducing and cross-linking/reducing conditions (Figures 4.8A and B and 4.10B, lane 1). Moreover, under cross-linking/reducing conditions, there was a larger number of bands which comprised a greater proportion of the total immunoreactive GDF9 found in these high molecular weight bands. Cross-linking of oGDF9 in either the presence (Figures 4.10A and B, lanes 4b and 5b respectively) or absence of h/oBMP15 (S356C) or o/oBMP15 (Figures 4.10A and B, lane 1) did not change the position of the pro-mature, mature dimer or mature monomer bands of oGDF9.

4.4 Discussion

As mentioned elsewhere the anti-BMP15 monoclonal antibody (clone KM2-61A) used in this study binds to residues within the sequence SEVPGPSREHDGPES (McNatty *et al.* 2005a). This sequence is located in a highly flexible area at the N terminal end of the mature growth factor domain. This flexibility has resulted in a failure to successfully model this region of the mature growth factor domain for any member of the TGFB family.

However a three-dimensional structure model of BMP15 using promature TGFB as a template (Figure 5.6A), predicts this region is exposed on the surface of the protein and would be expected to be freely available to mab MN2-61A (Figure 4.11A). Furthermore, this region is adjacent to the furin-like proprotein convertase cleavage site, RXXR, an area that would need to be available to such an enzyme. These theoretical expectations were confirmed for recombinant BMP15 by the Western blotting results presented in Chapter 3. These results showed the ability of MN2-61A to recognise both promature and mature BMP15 both in the monomeric and dimeric forms, under both reducing and non-reducing conditions. The blocking experiments with *E. coli*-derived BMP15 provided evidence for the specificity of this antibody for bovine oocytes (Figure 4.1). The anti GDF9 monoclonal antibody (clone 37A) was raised against *E. coli*-derived ovine mature GDF9 and was subsequently screened and subcloned for its ability to recognise a N-terminal peptide sequence (Dr K. P. McNatty, personal communication). Blocking of immunostaining by preincubating clone 37A with excess *E. coli*-derived oGDF9 mature domains (Figures 4.3A & B) confirmed the specificity of immunostaining to the bands within Western blots (both of recombinant GDF9 and that produced and secreted by bovine oocytes) corresponding to both the promature and mature forms. Importantly, antibodies raised to any of three overlapping sequences covering the 34-amino acid length of the N-terminal end of ovine GDF9 or the sequence against which mab MN2-61A recognises, have been shown to block, or largely inhibit the biological activities of GDF9 or BMP15 *in vivo*

(McNatty *et al.* 2007). However, it was not determined whether this was due to blocking the action of either pro-mature or mature forms or both.

Figure 4.11 Location of antibody binding sites.

Homology models of (A) dimeric promature ovine BMP15 and (B) dimeric promature ovine GDF9. Proregion depicted in blue and mature growth factor domain in cyan, with sequences, within which the epitope recognised by (A) clone MN2-61A or (B) clone 37A are located, is shown in orange. The dimer partner is shown as proregion (red) and mature domain (pink).

Under non-reducing conditions, the majority of immunoreactive BMP15 resides within bands of >100 kDa. One of these bands corresponds in size to what may represent a dimer of promature BMP15 (110-115 kDa) and was observed in the recombinant proteins o/oBMP15 and h/oBMP15 (S356C), as well as in those proteins produced and secreted by both bovine and ovine oocytes (Figure 4.4A & B). Under reducing conditions, these high molecular weight complexes resolve into promature and mature growth factor domains (Figures 4.5A & B and 4.6A & B). Interestingly, cross-linking of proteins prior to their electrophoresis under reducing conditions (Figure 4.6A & B), failed to show the same high molecular weight band(s) that were observed under non-reducing conditions (Figure 4.4A & B). The cross-linker chemical used in this study (bis(sulfosuccinimidyl)suberate) links proteins by stable amide bonds between primary amines. This might potentially disrupt the antigenic site recognised by clone MN2-61A (located near the N-terminal end of mature BMP15). However, bands of high molecular weight and those corresponding to the promature and mature forms of BMP15 were observed. These high molecular weight bands (different from that seen under non-reducing conditions) suggest these complexes may contain proteins other than just the promature and mature domains, one likely

candidate protein is free prodomains (Edwards *et al.* 2008), and/or that some forms of BMP15 are aggregating into high molecular weight complexes.

A ~40 kDa band which was intermediate in size to the pro-mature and the dimeric mature form of BMP15 (S356C) was observed in some, but not all, oocyte lysates and was only observed once in incubation medium. There was no consistent pattern that might explain the appearance of this band. It was observed in both bovine and ovine oocyte lysates and in lysates from both freshly-collected and cultured oocytes. If this band was correlated with the health status of follicles, then the way in which these pools of oocytes were collected may provide a potential explanation. Oocytes were retrieved from antral follicles without the health of these follicles first being accessed. As such, the proportion of oocytes from healthy follicles will vary between pools.

Figure 4.12 Location of ~40 kDa band relative to homodimers of BMP15 and GDF9.

Western immunoblots under reducing conditions showing the molecular forms of (A) BMP15 and (B) GDF9 following cross-linking. In image A, the Western blot for GDF9 was stripped before being re-probed for BMP15 Lane 1 = co-incubated h/oBMP15 (S356C) and oGDF9, lane 2 = co-incubated o/oBMP15 and oGDF9, lane 3 = lysate from freshly collected oocytes. Molecular sizes are shown to the left of the image (kDa).

It is worth noting that the ~40 kDa band was located between the sizes of dimeric mature BMP15 and dimeric mature GDF9 (Figure 4.12A & B) which may be evidence for a heterodimer of BMP15 and GDF9. However, this band was only seen when immuno-stained for BMP15 and never when immuno-stained for GDF9. Although the ability of clone MN2-61A and clone 37A to detect heterodimers of BMP15 and GDF9 has not been established, both MN2-61A and 37A are capable of detecting mature homodimers of BMP15 (Figure 4.12A, Lane 1) and GDF9 (Figure 4.12B, Lanes 1 & 2), respectively.

Further evidence against this band being a heterodimer is its continued presence under reducing conditions (Figure 4.5B, Lane 5). Alternatively, this band may be the result of cleavage of the promature BMP15 at an alternative site to the one that releases the mature domain. There are three potential furin-like (RXXR) sites that are conserved between bovine and ovine BMP15. In ovine BMP15, these cleavage sites are located at amino acid positions 57-60 (RKPR), 84-87 (RENK) and 95-98 (RLVR). Cleavage at these sites would result in theoretical molecular sizes of 38.1, 34.9 and 33.7 kDa, respectively, which may be further modified by post-translational processing. Such translational modifications have previously been reported for human BMP15 (Saito *et al.* 2008).

Under reducing conditions, the forms of BMP15 detected in expression media from transfected HEK293 cells were mainly mature monomers along with an uncleaved promature form. Likewise, the forms of BMP15 secreted by bovine oocytes were also consistently observed in bands corresponding to both promature and mature forms. The amount of BMP15 secreted by ovine oocytes often proved to be at levels close to, or below, the detection limit of the Western blot protocol used in this study. However, both the promature and the mature forms were detected in at least one replicate (Table 4.1). Lysates from both bovine and ovine oocytes contained a large amount of promature, and a lesser amount of mature, domains (Figures 4.5A & B). Therefore, it would seem that ruminant oocytes both produce and secrete promature BMP15 largely in an uncleaved form together with some mature domains. These mature growth factor monomers were almost entirely located within high molecular weight multimeric complexes (Figure 4.5A & B), which are also likely to contain the prodomain (Edwards *et al.* 2008). Likewise, mBMP15 was also found in expression media in both mature monomer and promature forms (Otsuka *et al.* 2000). When the normally conserved cysteine (involved in dimer formation) was re-introduced to either human (Pulkki *et al.* 2012) or ovine BMP15 (Figures 4.4A & B, Lane 3; 4.6A & B, Lane 3), dimeric mature growth factor forms were produced. However, no bands corresponding to mature dimers were observed for recombinant wild-type o/oBMP15 or among the proteins secreted and/or produced by either bovine or ovine oocytes (Figure 4.6A & B). However forms of mature hBMP15, with either a N-terminal FLAG tag (Liao *et al.* 2004, Hashimoto *et al.* 2005) or a N-terminal MycHis tag (Rossetti *et al.* 2009), have been shown to include mature growth factor dimers. Likewise, highly purified recombinant hBMP15, under cross-linking conditions, has also been reported to exist as mature dimers (Pulkki *et al.* 2012).

Recombinant oGDF9 expressed from HEK293 cells and GDF9 produced and secreted by bovine and ovine oocytes was found in high molecular size complexes (Figure 4.7A & B). These complexes may include dimeric promature forms and also uncleaved promature and mature domains (Figure 4.8A & B). A low molecular size protein band of 6-10 kDa was produced by recombinant oGDF9 expressing HEK293 cells and by both bovine and ovine oocytes (Table 4.2). However, (Lin *et al.* 2012) who also used an antibody raised against a near N terminal sequence of ovine GDF9, did not report the presence of this band in Western blotting using either recombinant mGDF9 or lysed rat oocytes. This protein band is likely the result of either a cross-reacting protein or a breakdown product of GDF9 and is possibly species-specific. In contrast to oBMP15, both monomeric and dimeric mature forms of recombinant oGDF9 were detected under cross-linking conditions: this finding is in agreement with McIntosh *et al.* (2008). Interestingly, under the same conditions, no such dimers were detected in GDF9 produced or secreted by either bovine or ovine oocytes (Figures 4.9A & B). This may be a consequence of differences in protein folding and/or post-translational modifications by HEK293 cells and by oocytes. Furthermore, cross-linking experiments with oBMP15 and oGDF9 failed to show the presence of either dimeric mature BMP15 or heterodimeric GDF9/BMP15 however dimeric mature GDF9 was observed (Figures 4.6A & B, 4.9A & B, 4.11 A & B; Edwards *et al.* 2008). It would appear that while ovine and bovine BMP15 mature growth factor monomers may be capable of forming dimers, probably during synthesis and secretion, the wild-type BMP15 is unable to maintain these dimeric forms (Edwards *et al.* 2008). Moreover, using the same antibodies as used in the present study, (McNatty *et al.* 2006) reported that ovine oocytes secrete uncleaved promature BMP15 and uncleaved promature GDF9 into the follicular fluid. Likewise, the detection of both promature and mature domains of BMP15 and GDF9 has been detected in bovine follicular fluid (Behrouzi *et al.* 2016), with the mature domains detected at 25 kDa. These *in vivo* results are in agreement with the present study.

These results establish that under *in vitro* conditions, BMP15 and GDF9 are produced and secreted by both bovine and ovine oocytes. Moreover, the results show that BMP15 and GDF9 are present in high molecular weight complexes composed of promature and mature domains, and that these mature domains do not interact to form homo- or hetero-dimers that were detectable using Western blotting.

CHAPTER 5: *IN SILICO* MODELLING OF BMP15 AND GDF9 AND THEIR INTERACTIONS WITH CELL SURFACE RECEPTORS

5.1 Introduction

Generally TGFB superfamily members are thought to signal via a cell-surface receptor-ligand complex following the binding of mature growth factor domain dimers to two type I, and two type II, receptors. This in turn leads to the phosphorylation of receptor regulated Smads (Sections 1.3.3 and 1.3.4). However, as mentioned previously (Section 4.1), BMP15 and GDF9 belong to a small sub-family of TGFB proteins unable to form a disulphide bridge between monomers and thus are unable to form stable dimers of the mature growth factor domains. As discussed in Chapter 4, the molecular forms of BMP15 and GDF9 produced and secreted (Table 5.1) by ovine and bovine oocytes were identified, as were the recombinant forms secreted by mammalian cells. These results support published evidence (McNatty *et al.* 2006; McIntosh *et al.* 2008; Lin *et al.* 2012) that suggests that both BMP15 and GDF9 are secreted in a partially unprocessed form as both cleaved and/or uncleaved promature proteins along with some mature growth factor domain. Other studies have reported a dimeric form of mature ovine and mouse GDF9 *in vitro* (Edwards *et al.* 2008; McIntosh *et al.* 2008). Results from Chapter 4 also reports the presence of recombinant GDF9 dimers (Figure 4.8 and 4.10) but not oocyte-secreted GDF9 dimers (Table 5.1). Currently however, only promature forms of both BMP15 and GDF9 have been identified *in vivo* (McNatty *et al.* 2006). Table 5.1 below provides a summary of the native ovine and bovine molecular forms of GDF9 and BMP15 that have been detected to date.

Notwithstanding the synergistic effects of BMP15 and GDF9 reported in both *in vivo* (Yan *et al.* 2001; Hanrahan *et al.* 2004) and *in vitro* (McNatty *et al.* 2005a,b; Edwards *et al.* 2008; McIntosh *et al.* 2008; Reader *et al.* 2011; Mottershead *et al.* 2012) studies, there is some debate as to whether BMP15-GDF9 heterodimers exist *in vivo* (Mottershead *et al.* 2013; Peng *et al.* 2013a,b). Nevertheless, a biologically-active recombinant heterodimer of BMP15 and GDF9, termed cumulin, has been produced by transfection of mammalian cells with both BMP15 and GDF9 in which the cysteine residue involved in heterodimerisation in other TGFB members has been re-introduced (Mottershead *et al.* 2015). However, the mechanism(s) to explain how BMP15 co-operates with GDF9 and/or with its receptors to regulate somatic cell responses has proven elusive. The aim of this study was to: (i) use protein modelling techniques to visualise the various molecular forms

of BMP15 and GDF9 and; (ii) utilise these models, in conjunction with the known forms secreted by oocytes (Table 5.1) and published information, to provide a hypothetical model of how oocyte-secreted forms of BMP15, GDF9 and their cell surface receptors interact.

Table 5.1 Summary of BMP15 and GDF9 molecular forms secreted by ovine and bovine oocytes

Growth factor	Molecular form	Western blot conditions					
		Non-reducing		Reducing		Cross-linking	
		Ovine	Bovine	Ovine	Bovine	Ovine	Bovine
BMP15	High MW complexes	✓	✓	ND	ND	✓	✓
	Promature	ND	ND	✓	✓	✓	✓
	Mature dimer	ND	ND	ND	ND	ND	ND
	Mature monomer	ND	ND	✓	ND	✓	✓
GDF9	High MW complexes	✓	✓	ND	ND	✓	✓
	Promature	✓	ND	✓	✓	✓	✓
	Mature dimer	ND	ND	ND	ND	ND	ND
	Mature monomer	✓	✓	✓	✓	ND	✓

Present (✓), not detected (ND).

5.2 Methods

5.2.1 Amino acid hydrophobicity

Amino acids were allocated a hydrophobicity score as determined by Kyte and Doolittle (1982). For the purposes of this study, the truly hydrophobic amino acids, (I, V, L, F, C, M and W) and the less hydrophobic or indifferent amino acids (A, Y, T, S, P, and G) which have a hydrophobicity score of >-2.0 were defined as being hydrophobic while those amino acids with a hydrophobicity score of <-2.0 (H, E, Q, D, N, K and R) were defined as hydrophilic (Table 5.2).

Table 5.2 Amino acid names, codes and hydrophobicity scores

Amino acid			Hydrophobicity score	Hydrophobic (H) Hydrophilic (Y)
Full name	3 letter Code	1 letter Code		
Isoleucine	Ile	I	4.5	H
Valine	Val	V	4.2	H
Leucine	Leu	L	3.8	H
Phenylalanine	Phe	F	2.8	H
Cysteine	Cys	C	2.5	H
Methionine	Met	M	1.9	H
Alanine	Ala	A	1.8	H
Glycine	Gly	G	-0.4	H
Threonine	Thr	T	-0.7	H
Serine	Ser	S	-0.8	H
Tryptophan	Trp	W	-0.9	H
Tyrosine	Tyr	Y	-1.3	H
Proline	Pro	P	-1.6	H
Histidine	His	H	-3.2	Y
Glutamate	Glu	E	-3.5	Y
Glutamine	Gln	Q	-3.5	Y
Aspartate	Asp	D	-3.5	Y
Asparagine	Asn	N	-3.5	Y
Lysine	Lys	K	-3.9	Y
Arginine	Arg	R	-4.5	Y

5.2.2 Molecular modelling

Two methods were utilized to generate three-dimensional structural models of promature BMP15 and GDF9. The protein homology/analogY recognition engine v2 (Phyre²) (Kelley & Sternberg 2009) was used to create models of oBMP15 (Figure 6.6A) and oGDF9 (Figure 6.6B). Using the Protein Data Bank (www.pdb.org), the pTGFB1 (Accession number 3RJR; (Shi *et al.* 2011b) and GDF5-BMPRII complex (Accession number 3EVS; (Kotzsch *et al.* 2009) were used as templates for the promature and receptor-bound forms, respectively. Additionally, a manual approach was used to model both oBMP15 and oGDF9. Sequence alignments for ovine promature BMP15 and GDF9 with mouse BMP9 were prepared using the software CRUSTALW (Thompson *et al.* 1994). Utilizing the software package Quanta2008 (Molecular Dynamics, San Diego, CA), amino acids were then replaced manually in the mBMP9 template (Accession number 4YCG; (Mi *et al.* 2015) to build models of promature oBMP15 and oGDF9. The models were then modified where necessary, by assigning rotamer positions, such that side chain proximities did not produce van der Waals interference. Subsequently, energy minimisations were run to determine the lowest energy conformation. Initial energy minimisations were run with the backbone atoms being fixed in place and where only side chain atoms were allowed to move.

The UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIH P41 RR-01081; Pettersen *et al.* 2004) was used to produce molecular graphics images and to create models of the hetero-oligomeric signalling complexes comprised of BMP15 or GDF9 dimers and their receptors. To visualise the positions of both type I and type II receptors relative to a dimer of pro-mature oBMP15 or oGDF9, a composite template was created. The BMP2 dimer-BMPRII complex (Accession number 1ES7; Kirsch *et al.* 2000b) gave a base template for positioning the mature growth factor domains into a dimer. Aligning BMP2 associated with both BMPRII and ACTRII (Accession number 2GOO; (Allendorph *et al.* 2006) with each of the BMP2 mature monomers present in 1ES7 enabled visualisation of the positioning of two type I, and two type II, receptors relative to the mature growth factor dimer. These receptor locations were then in turn used to position the type I and II receptors relevant for BMP15, namely BMPRII (Accession number 3EVS; Kotzsch *et al.* 2009) and BMPRII (Accession number 2HLR; (Mace *et al.* 2006), respectively. Based on these criteria, promature and mature oBMP15 and oGDF9 (modelled on BMP9 or TGFB1) could then be positioned within this template.

5.3 Results

5.3.1 Non-covalent interaction between the pro- and mature domains of oBMP15 and oGDF9

5.3.1.1 Hydrophobic residues in the proregion of human BMP15 and GDF9 differ from the conserved hydrophobic motif found in most other TGFB family members

Walton *et al.* (2009) have identified a hydrophobic motif (HHxxHxH; H = hydrophobic, x = any amino acid), present in the pro-region of most human TGFB family members, which is involved in a non-covalent interaction with the mature region. This motif has been identified in 28/33 (84.8%) of the human TGFB family members (Figure 5.1). For the remaining 5/33 (15.2%) family members, there is an incomplete or modified motif (HHxxHxY; Y= hydrophilic amino acid). All of the modified motifs are unique with a different hydrophilic amino acid being substituted in each growth factor (hBMP15, glutamate; hGDF3, aspartate; hGDF9, lysine; hGDF10, glutamine and hMIS, arginine; Figure 5.1).

5.3.1.2 A hydrophobic motif is conserved across all mammalian species of BMP15 and GDF9 proregion

The HHxxHxY (H = hydrophobic, x = any amino acid, Y= hydrophilic; Table 5.2) motif is found in 11/11 (100%) of mammalian BMP15 sequences. This can be extended to become HHHHHHxxHHY (BMP15 motif; Figure 5.2). Mouse and rat BMP15 sequences differ from those of other mammalian species with the 7th amino acid within the extended motif being hydrophobic, while for all other species this residue is hydrophilic. Alignment of the motif for all mammalian species of BMP15 reveals a 3/11 (27%) sequence homology, however this increases to 8/11 (73%) when mouse and rat sequences are excluded.

As GDF9 is closely related to BMP15, and is also an oocyte derived growth factor, the conservation of this motif across mammalian species of GDF9 was also investigated. The HHxxHxY motif is found in 13/13 (100%) of mammalian GDF9 sequences. This can be extended to become YYxHHHHHHHHY (GDF9 motif; Figure 5.2). There is a 2/9 (22%) sequence similarity within the extended motif between all mammalian species of GDF9. A common hydrophobic motif (HHHHxxHHY) is found in all mammalian species of both BMP15 and GDF9 pro-domains (24/24), (common motif; Figure 5.2). In BMP15 and GDF9, this motif is preceded by hydrophobic or hydrophilic residues respectively.

			HH-xxHxH	
hINH α	22	LELARELVLAKVRAL	FL-DALGPPAV-46	
hActA	47	VPNSQPEMVEAVKKH	IL-NMLHLKKR-71	
hActB	67	LGRVDGDFLEAVKRH	IL-SRLQMRGR-91	
hActC	36	LESQRELLLDLAKRS	IL-DKLHLTQR-60	
hActE	36	PQAERALVLELAKQQ	IL-DGLHLTSR-60	
hTGF β 1	38	MELVKRKRIEAIRGQ	IL-SKLRLASP-62	
hTGF β 2	29	MDQFMRKRIEAIRGQ	IL-SKLKLTSP-53	
hTGF β 3	32	FGHIKKKRVEAIRGQ	IL-SKLRLTSP-56	
hBMP2	34	AAASSGRPSSQPSDEV	IL-SEFELRLL-58	
hBMP3	164	DLSAWTLKFSRNQSQ	LL-GHLSVDMA-188	
hBMP4	46	RSGQSHELLRDFEAT	LL-QMFGLRRR-70	
hBMP5	41	-RRLRNHERREIQRE	IL-SILGLPHR-64	
hBMP6	69	YRRLKTQEKREMQKE	IL-SVLGLPHR-92	
hBMP7	44	-RRLRSQERREMQRE	IL-SILGLPHR-67	
hBMP8a	34	-RRLGARERRDVQRE	IL-AVLGLPGR-57	
hBMP8b	34	-RRLGARERRDVQRE	IL-AVLGLPGR-57	
hBMP10	47	DGVDFNTLLQSMKDE	FL-KTLNLSDI-71	
hBMP15	29	GQSSIALLAEAPTLP	LI-EELLEESP-53	
hNODAL	42	AYMLSLYRDPLPRAD	II-RSLQAEDV-66	
hGDF1	29	-----PVPPGPAAAL	LL-QALGLRDE-47	
hGDF2	51	HTFNLKMFLENVKVD	FL-RSLNLSGV-75	
hGDF3	67	SRDLCYVKELGVRGN	VL-RFLPDQGF-91	
hGDF5	220	QRYVFDISAL-EKDGL	LLGAELRLLRK-244	
hGDF6	146	QKYLFDVSMUSDKEE	LVGAELRLFRQ-171	
hGDF7	139	QSFLFDVSSLNDADEV	VVGAELRVLRR-164	
hGDF8	45	RQNTKSSRIEAIKIQ	IL-SKLRLETA-61	
hGDF9	46	PWSLLQHIDERDRAG	LL-PALFKVLS-70	
hGDF10	158	SGRPLPLGPPTREQHL	LF-RSLSQNTA-182	
hGDF11	68	RQHSRELRLSEIKSQ	IL-SKLRLKEA-92	
hGDF15	48	TEDSRFRELKRYED	LL-TRLRANQS-72	
hMIS	68	SPLRVVGALSAYEQAF	FL-GAVQRARW-92	
hLefty1	22	-----LTGEQLLGS	LL-RQLQLKEV-40	
hLefty2	22	-----LTEEQLLGS	LL-RQLQLSEV-40	

Figure 5.1 Sequence alignment of prodomains of human (h) TGF β family members. Adapted from Walton *et al.* (2009). Prodomains of human TGF β family members were aligned using ClustalW. (Thompson *et al.* 1994) The amino acids are numbered according to the first amino acid of the signal peptide. The conserved hydrophobic motif (top of alignment) is marked; hydrophobic amino acids are highlighted in green, hydrophilic amino acids in blue. Act, activin, INH α , inhibin alpha subunit.

The 6th residue within the common motif is always hydrophilic in BMP15 and always hydrophobic in GDF9. Mouse and rat BMP15 sequences have an intermediate hydrophobicity pattern that falls between that of the remaining BMP15 sequences and the GDF9 sequences (Figure 5.2).

Original motif			HHxxHxY																
BMP15 motif			HHHHHHxYHHY																
GDF9 motif			YYxHHHHHHHHY																
Common motif			HHHHxxHHY																
mBMP15	29	WPSTALLAD	D	P	T	L	P	S	I	L	D	L	A	K	E	A	P	52	
rBMP15	29	WPSTTLAE	N	P	T	L	P	S	S	L	D	L	A	K	E	A	P	52	
pBMP15	30	QPSVALLPE	A	C	T	L	P	L	I	R	E	L	L	E	E	A	P	53	
cBMP15	29	QPSNALMAD	A	P	S	L	P	L	I	R	E	L	L	E	E	G	A	P	52
oBMP15	29	QPSIAHLPE	A	P	T	L	P	L	I	Q	E	L	L	E	E	A	P	52	
gBMP15	30	QPSIAHLPE	A	P	T	L	P	L	I	Q	E	L	L	E	E	A	P	53	
bBMP15	30	QPSIAHLPE	A	P	T	L	P	L	I	Q	E	L	L	E	E	A	P	53	
buBMP15	30	QPSIAHLPE	A	P	T	L	P	L	I	Q	E	L	L	E	E	A	P	53	
hBMP15	30	QSSIALLA	E	A	P	T	L	P	L	I	E	E	L	L	E	E	S	P	53
chBMP15	30	QSSIALLA	E	A	P	T	L	P	L	I	E	E	L	L	E	E	S	P	53
rhBMP15	30	QSSIALLA	E	A	P	T	L	P	L	I	E	E	L	L	E	E	S	P	53
mGDF9	46	WSLLLPVDG	T	D	R	S	G	L	L	P	P	L	F	K	V	L	S	70	
rGDF9	46	WSLLLPVDG	T	D	R	S	G	L	L	P	P	L	F	K	V	L	S	70	
pGDF9	46	WSLLRPPDE	R	H	R	S	G	L	P	S	P	L	F	N	V	L	Y	70	
cGDF9	45	WSLVQPLDE	K	D	R	L	G	F	L	P	P	L	F	K	V	L	Y	69	
shGDF9	45	WSLLQPLDG	R	E	R	A	G	L	L	P	P	L	F	K	V	L	Y	69	
oGDF9	46	WSLLNHLGG	R	H	R	P	G	L	L	S	P	L	L	E	V	L	Y	70	
gGDF9	46	WSLLNHLGG	R	H	R	P	G	P	L	S	P	L	L	K	V	L	Y	70	
bGDF9	46	WSLLKHLDG	R	H	R	P	G	L	L	S	P	L	L	N	V	L	Y	70	
buGDF9	46	WSFLKHLDG	R	H	R	P	G	L	L	S	P	L	L	K	V	L	Y	70	
hGDF9	46	WSLLQHIDE	R	D	R	A	G	L	L	P	A	L	F	K	V	L	S	70	
cmGDF9	47	WSLLQPIDE	R	D	R	A	G	L	L	P	L	L	F	K	V	L	S	71	
btGDF9	46	WSLLQPLDG	K	D	R	A	G	L	F	P	P	L	F	K	V	L	Y	70	
ogGDF9	46	WSLLQPADR	R	E	S	S	G	L	L	P	P	L	F	K	V	L	S	70	

Figure 5.2 Sequence alignments of mammalian BMP15 and GDF9 prodomains. The conserved hydrophobic motif(s) (top of alignment) are marked; hydrophobic amino acids (H) are highlighted in green, hydrophilic amino acids (Y) in blue. mouse (m), rat (r), porcine (g), cat (c), ovine (o), caprine (g), bovine (b), water buffalo (bu), human (h), chimpanzee (ch), rhesus monkey (rh), shrew (sh), *Callicebus moloch* (cm), bat (bt), *Otolemur garnettii* (og). The amino acids are numbered according to the first amino acid of the signal peptide.

hINH α	269	ISFQELGWERWIVYPPS	285	335	MRPLHVRTTSDGGYSF	350
mBMP15	298	VSFHQLGWDHWIIPRL	314	362	FLPMSILLIETNGSIL	378
rBMP15	297	VSFHQLGWDHWIIPRL	313	361	FLPMSILLIEANGSIL	377
pBMP15	300	VSFHQLGWDHWIIPHF	316	364	YVPISILLIEANGSIL	380
cBMP15	300	VSFHQLGWDHWIIPHL	316	363	YVPISILLVEANGSIL	379
gBMP15	300	VSFQQLGWDHWIIPHL	316	364	YVPISILLIEANGSIL	380
bBMP15	300	VSFQQLGWDHWIIPHL	316	364	YVPISILLIEANGSIL	380
buBMP15	300	VSFQQLGWDHWIIPHL	315	364	YVPISILLIEANGSIL	380
hBMP15	298	ISFRQLGWDHWIIPPF	314	362	YVPISVLMIEANGSIL	378
chBMP15	298	ISFRQLGWDHWIIPHF	314	363	YVPISVLMIEASGSIL	379
rhBMP15	298	VSFRQLGWDHWIIPPF	314	363	YVPISVLMIEANGSIL	379
oBMP15	299	VSFQQLGWDHWIIPHL	315	363	YVPISILLIEANGSIL	379
mGDF9	347	LSFSQLKWDNWIIVAPHR	363	411	YSPLSVLTIEPDGSIA	426
rGDF9	346	LSFSQLKWDNWIIVAPHR	362	410	YSPLSVLTIEPDGSIA	425
pGDF9	350	LSFSQLKWDNWIIVAPHK	366	414	YSPLSVLAIEPDGSIA	429
cGDF9	358	LSFSQLKWDNWIIVAPHR	374	422	YSPLSVLTIESDGSIA	437
gGDF9	359	LSFSQLKWDNWIIVAPHK	375	423	YSPLSVLAIEPDGSIA	438
bGDF9	359	LSFSQLKWDNWIIVAPHK	375	423	YSPLSVLAIEPDGSIA	438
buGDF9	359	LSFSQLKWDNWIIVAPHK	375	423	YSPLSVLAIEPDGSIA	438
hGDF9	360	LSFSQLKWDNWIIVAPHR	376	424	YSPLSVLTIEPDGSIA	439
cmGDF9	362	LSFSQLKWDNWIIVAPHR	378	426	YSPLSVLTIEPDGSIA	441
btGDF9	361	LSFSQLKWDNWIIVAPQR	377	425	YSPLSVLTIEPDGSIA	440
ogGDF9	358	LSFSQLKWDNWIIVAPHR	374	422	YSPLSVLTIEPDGSIA	437
oGDF9	359	LSFSQLKWDNWIIVAPHK	375	423	YSPLSVLAIEPDGSIA	438

Finger 1

Finger 2

Figure 5.3 Sequence alignments of human inhibin alpha (hINH α) and mammalian species of BMP15 and GDF9 mature domains. The conserved hydrophobic amino acids are located within the two finger domains (Figure 1.3) of the mature region are highlighted in green. mouse (m), rat (r), porcine (p), cat (c), ovine (o), caprine (g), bovine (b), water buffalo (bu), human (h), chimpanzee (ch), rhesus monkey (rh), bat (bt) and *Otolemur garnettii* (og). The amino acids are numbered according to the first amino acid of the signal peptide.

5.3.1.3 Hydrophobic residues in the mature region are conserved across all mammalian species of BMP15 and GDF9

Through targeted point mutations, five specific residues have been identified within the inhibin α mature subunit which are essential for correct interaction between the pro- and mature domains as well as subsequently, for successful dimerisation and secretion (Walton

et al. 2009). These five amino acids (F271, I280, P283, L388 and V340; numbering based on human inhibin α subunit) are all hydrophobic residues (Figure 5.3). Of the 33 human TGFB family members tested, the residues F271, I280, P283, L338 and V340 are conserved in 29/33 (87.9%), 25/33 (75.8%), 33/33 (100.0%), 10/33 (30.3%) and 11/33 (33.3%) of members, respectively (Walton *et al.* 2009). Within mammalian BMP15, amino acids Phe (F), Ile (I), Pro (P), Leu (L) and Val (V) are conserved in 11/11 (100.0%), 11/11 (100.0%), 11/11 (100.0%), 0/11 (0%) and 3/11 (27.3%) respectively (Figure 5.3). All substitutions are with replacement hydrophobic amino acids. All GDF9 species of mammalian origin showed complete agreement with the human inhibin α subunit (Figure 5.3). The residues F271, I280, P283 are located on finger 1 of the mature region and the residues L388 and V340 on finger 2 (See Figure 1.2 for position of fingers) and together, these make a hydrophobic packing core (pocket).

5.3.2 Identification of potential BMP15 and GDF9 receptor binding sites

5.3.2.1 Type II receptor

The amino acids comprising some or all of the type II receptor binding sites have been reported for the following ligand:ECD-receptor complexes: BMP2-ACVR2B (Weber *et al.* 2007); BMP2-BMPR2 (Kirsch *et al.* 2000b), BMP7-ACVR2A (Greenwald *et al.* 2003; Allendorph *et al.* 2006); activin β A-ACVR2B (Thompson *et al.* 2003) and; TGFB3-TGFBRII (Hart *et al.* 2002). The proteins BMP15, GDF9 and BMP2 all bind to the same type II receptor, BMPR2 (Vitt *et al.* 2002; Moore *et al.* 2003; Weber *et al.* 2007). As such, and for the purposes of this study, any residues which comprise the type II receptor binding site in BMP2, which also align with those in BMP7 or activin β A, were used to infer a type II binding site in oBMP15 and oGDF9 (Figure 5.4A). The type II receptor binding site of TGFB3 does not align with the binding sites of the other family members (Figure 5.4A), being nearer to the tips of the fingers than it is for BMPR2 and the activin type II receptors. Consequently, the TGFB3 alignment was not used to infer the type II binding sites of oBMP15 or oGDF9. Amino acids P313 and I366 (numbering based on oBMP15) are common to both the type II receptor binding site and the hydrophobic pocket.

A.

TGFB3	318	RPLYIDFRQDLGW-KWVHEPKSYIANF	343
hBMP2	299	HPLYVDFS-DVGWNDWIVAPPGYHAFY	324
hBMP7 ⁰	333	HELYVSFR-DLGWQDWIIAPEGYAAYY	358
hBMP7 ^ψ	333	HELYVSFR-DLGWQDWIIAPEGYAAYY	358
hActA	324	KQFVVSFK-DIGWNDWIIAPSGYHANY	349
oBMP15	295	HPFQVSFQ-QLGWDHWI IAPHLYTPNY	320
oGDF9	359	HDFRLSFS-QLKWDNWIVAPHKYNPRY	375
TGFB3	377	CCVPQDLEPLTILYYVG-RTPKVEQLSNMVVKSCCKCS	412
hBMP2	360	CCVPTELSAISMLYLDENEKVV LKNYQDMVVEGCGCR	396
hBMP7 ⁰	395	CCAPTQLNAISVLYFDDSSNVILKKYRNMVVRACGCH	431
hBMP7 ^ψ	395	CCAPTQLNAISVLYFDDSSNVILKKYRNMVVRACGCH	431
hActA	390	CCVPTKLRPMSMLYDDGQNIKKDIQNMIVEECGCS	426
oBMP15	357	SCVPYKYVPIISILLIEANGSILYKEYEGMIAQSCTCR	393
oGDF9	417	SCVPAKYSPLSLVLAIEPDGSIAYKEYEDMIATKCTCR	453

B.

hBMP2	299	HPLYVDFS-DVGWNDWIVAPPGYHAFY	324
Belclare	295	HPFQVSFQ-QLGWDHWI IAPHLYTPNY	320
Thoka	359	HDFRLSFS-QLKWDNWIVAPHKYNPRY	375
hBMP2	360	CCVPTELSAISMLYLDENEKVV LKNYQDMVVEGCGCR	396
Belclare	357	SCVPYKYVPIISILLIEANGSILYKEYEGMIAQSCTCR	393
Thoka	417	SCVPAKYSPLSLVLAIEPDGSIAYKEYEDMIATKCTCR	453

Figure 5.4 Sequence alignments of areas of type II receptor binding.

(A) Amino acids comprising the type II receptor binding sites for TGFB3 (Hart *et al.* 2002), hBMP2 (Weber *et al.* 2007), hBMP7⁰ (Greenwald *et al.* 2003), hBMP7^ψ (Allendorph *et al.* 2006), hActA (Thompson *et al.* 2003) are marked in blue, with corresponding areas in oBMP15 and oGDF9 marked in pink. The residues involved in forming the hydrophobic pocket in oBMP15 and oGDF9 are marked in green, with residues also common to the type II receptor binding area marked in yellow. (B) Amino acids, that when mutated in hBMP2 (Kirsch *et al.* 2000a), have either decreased (red) or no change (grey) in binding affinity for the type II receptor. The location of Belclare (oBMP15, S367I) and Thoka (oGDF9, S427R) mutations are marked in orange. The amino acids are numbered according to the first amino acid of the signal peptide.

5.3.2.2 Type I receptor

Amino acids comprising both the GDF5:BMPr1B (Kotzsch *et al.* 2009) and the BMP2: TGFB1 (Radaev *et al.* 2010) receptor binding interface were used to infer a potential type

I binding site for both BMP15 and GDF9 (Figure 5.5A). Ten of 21 amino acids (F409, M412, W414, W417, L437, H446, I476, K488, Y490, E491; 47.6%) making up the GDF5:BMPR1B binding site shared sequence similarity, based on conservative intra-family amino acid substitutions (Figure 5.5B), for both BMP15 and GDF9. Moreover, BMP15 and GDF9 showed additional sequence similarity at positions R438 & G413 and H440 & D492 respectively. Likewise, both BMP15 and GDF9 shared sequence similarity with 13 of 29 amino acids (V308, W310, W313, L333, I344, L348, I368, S369, M370, L371, V380, K383, Y385; 44.8%) making up theTGFB1 binding site of BMP2. BMP15 exhibited sequence similarity at positions G309, N341 and V352, and GDF9 showed additional sequence similarity with S306, H336 and S353. Overall, both BMP15 and GDF9 shared sequence similarity with 12 of 21 (57.1%) and 16 of 29 (55.2%) amino acids making up the GDF5 and BMP2 type I binding sites, respectively. The F301, I366 and I368 amino acids in BMP15 and the F361, L426 and V428 amino acids in GDF9 are common to both the potential type I binding site and the hydrophobic pocket (Figures 5.3 and 5.5A).

5.3.3 Molecular modelling of promature oBMP15 and oGDF9

As mentioned earlier, three-dimensional structural models for promature ovine GDF9 and promature ovine BMP15 were generated using either protein homology/analogy recognition engine v2 (Phyre²), (Figure 5.6) or manually using the software Quanta2008 (Figure 5.7). Models of oBMP15 and oGDF9 utilising promature pTGFB1 as the template (hereafter referred to as the TGFB model) showed the pro-domain arching above and over the mature domain (Figure5.6) with dimeric promature BMP15 and GDF9 forming a ring-like configuration, (oBMP15; Figure 5.8A). Utilising promature mBMP9 as the template (hereafter referred to as the BMP model), the pro-domain rotated away from the mature domain (Figure 5.7) and dimeric promature forms adopting a more open armed configuration (oBMP15; Figure 5.8B). It should be noted that due to the very flexible nature of the region encompassing the RRXR cleavage site in both the TGFB and BMP promature models, this region has not been modelled. This, together with cleavage by a furin-like proprotein convertase, make it uncertain as to which pro- and mature-domain derive from the same polypeptide chain. Shi *et al.* (2011) concluded that the pro-mature pair was likely to be the pair closest to each other (30Å versus 50Å distant) based on the lack of conformational change between cleaved and uncleaved forms and the maximising of access to furin. This convention was also applied to the BMP based models (Figure 5.8A and B; prodomain blue or red and corresponding mature domain cyan or pink

respectively). In this arrangement, the TGFB based model shows the conserved hydrophobic motif on the prodomain (HHHHxxHH) coming into close association with the conserved hydrophobic amino acids located on the mature domain of its dimer partner (Figure 5.9). The N-terminal region of the prodomain encompassing this motif was not modelled for promature BMP9 [Accession number 4YCG]; (Mi *et al.* 2015). Thus, it is not known if this motif, located on the α 1-helix, also interacts with the mature domain in BMP9. However hydrophobic residues on α 5 helix are in close proximity to the hydrophobic motif on the mature domain (Figure 5.10).

B

Amino acid	Description	Colour
I, L, V, A, M	Aliphatic/hydrophobic	
F, W, Y	Aromatic	
K, R, H	Positive	
D, E	Negative	
S, T, N, Q	Hydrophilic	
P, G	Proline/Glycine (conformationally special)	

Figure 5.5 Sequence alignment of areas of type I receptor binding..

(A) Alignment of amino acid sequences showing the type I receptor binding sites for BMP2 and GDF5 and the potential binding sites for BMP15 and GDF9 and in (B) they are marked in colours according to amino acid families, as described. Mutational analysis of GDF5 shows (R438L; ↓) increases bioactivity while (L441P; ↓) renders GDF5 almost inactive (Seemann *et al.* 2005).

Figure 5.6 The ribbon structure of monomeric promature oBMP15 (A) and oGDF9 (B) based on the TGFB model. The proregion is depicted in blue and the mature growth factor domain is depicted in cyan. The oBMP15 and oGDF9 models were created using the protein homology/analogy recognition engine v2 (Phyre²) using pro-mature porcine TGFβ1 (Accession number 3RJR; Shi *et al.* 2011b) as a template.

Figure 5.7 The ribbon structure of monomeric promature oBMP15 (A) and oGDF9 (B) based on the BMP model. The proregion is depicted in blue and the mature growth factor domain is depicted in cyan. The oBMP15 and oGDF9 were created manually using the software Quanta2008 using pro-mature mouse BMP9 (Accession number 4YCG; Mi *et al.* 2015) as a template.

Figure 5.8 The ribbon structure of dimeric promature forms of BMP15 (A) when utilising promature porcine TGFβ1 (Accession number 3RJR; Shi *et al.* 2011b) or (B) when utilising human BMP9 (Accession number 4YCG; (Mi *et al.* 2015) as the template. The prodomain (blue) and mature growth factor (cyan) are presumed to derive from a single polypeptide chain, while the remaining prodomain (red) and mature growth factor (pink) are presumed to derive from another single polypeptide chain (Shi *et al.* 2011b).

Figure 5.9 Conserved hydrophobic motifs on pro- and mature domains of oBMP15 in the TGFB-based model.

(A) Location of hydrophobic residues based on the TGFB model (for clarity, one mature domain is not shown). (B) Viewing orientation is rotated forward towards the reader, such that the angle of view is directly along the axis of the $\alpha 1$ helix of the prodomain, (C) as depicted by arrow. The prodomain (blue) and mature growth factor (cyan) are presumed to derive from a single polypeptide chain, while the prodomain (red) and mature growth factor (pink) are presumed to derive from another single polypeptide chain. The hydrophobic motif located on the prodomain is depicted in orange, whilst the conserved hydrophobic amino acids on the mature domain are depicted in green.

Figure 5.10 Conserved hydrophobic motifs on the mature domain of oBMP15 in the BMP-based model.

(A) Location of hydrophobic residues based on the BMP model. (B) Viewing orientation is rotated forward towards the reader, such that the angle of view is directly along the axis of the $\alpha 5$ helix of the prodomain, (C) as depicted by arrow. The prodomain (blue) and mature growth factor (cyan) are presumed to derive from a single polypeptide chain, while the prodomain (red) and mature growth factor (pink) are presumed to derive from another single polypeptide chain. The hydrophobic motif located on the prodomain is not modelled however, hydrophobic residues located on the $\alpha 5$ helix (depicted in orange) are in close proximity to the conserved hydrophobic amino acids on the mature domain (depicted in green).

5.3.3.1 Conformational differences between prodomain and receptor bound mature growth factor domains

Another major difference noted between the two models is the conformation of the mature growth factor domain while in a complex with the prodomain. The BMP model shows the mature growth factor domain having good conformational similarity to other BMP domains when associated with their type I receptor (Figure 5.11A-C). However the TGFB model shows, the prodomain would hold the mature growth factor domain in an alternative conformation compared to that when associated with its receptors (Figure 5.11D).

Figure 5.11 Receptor versus promature bound forms of mature BMP15.

Comparisons between the mature growth factor domain of oBMP15 when associated with its prodomain modelled on BMP9 (Accession number 4YCG; (Mi *et al.* 2015) (A-C; yellow), or TGFβ1 (Accession number 3RJR; Shi *et al.* 2011b) (D-F; gold) or its receptor complex (cyan; A-F; modelled on GDF5:BMPR1B complex (Accession number 3EVS; (Kotzsch *et al.* 2009). View of the full mature domain (A & D). Partial view of the mature domain as seen in A or D, but with the “palm” and “finger” regions not shown for simplicity (B & E respectively) and partial view of the mature domain as seen in B or E, but rotated 90° into a transverse orientation looking from the “wrist” towards the” finger tips” (C and F).

Note: (D – F) The views of the full mature domain together with the locations of Y331 (pink) and Q351 (blue) in the prodomain-associated conformation and of Y331 (red) and Q351 (green) in the receptor-associated conformation Y331 and Q351 are illustrated simply to accentuate the degree of movement required to move from one conformation to the other.

From this conformation, the wrist region and the pre-helical loop must rotate almost 180° in the transverse plane (Figure 5.11F) before it is in a conformation which contains a binding site for the type I receptors. While this large conformational change occurs at the wrist, relatively small changes in conformation occur at the “palm” and “finger” regions (Figure 5.11D)

Figure 5.12 The prodomains of dimeric promature oBMP15 sterically hinder access to the binding sites of both type I and type II receptors. In both the TGFB (A) and BMP (B) models the prodomain (red and blue) cover the receptor binding sites for the type I receptor (yellow) of its associated mature domains (pink and cyan, respectively), whilst simultaneously blocking the binding site for the type II receptor (green) of its dimer partner.

5.3.3.2 Prodomains block access to type II receptors in the dimeric but not monomeric promature form

A composite template was created (see Section 5.2.2) to visualise the positions of both type I and type II receptors relative to dimers of pro-mature oBMP15 or oGDF9. Promature and mature BMP15 and GDF9 (modelled on BMP or TGFB templates) could then be positioned within this template. Both models show that a consequence of forming dimeric promature BMP15 or oGDF9 complexes *in vivo* would be the blocking of both the type I and type II receptor binding sites. Each prodomain blocks access to the type I receptor binding site of its own mature domain by steric hindrance. In the TGFB model, the prodomain also holds the mature domain in an alternative confirmation whereby the type I receptor binding site cannot form (Figure 5.11D-F). At the same time, the prodomain covers the type II receptor binding site of its dimer partner (Figure 5.12A and 5.12B).

5.3.3.3 Naturally-occurring inactivating mutations of oBMP15 and oGDF9

Naturally-occurring inactivating mutations in both oBMP15 and oGDF9 have been identified, which are located close to the hydrophobic motif (Figure 5.13). The point mutations of V299S (Inverdale mutation; (Galloway *et al.* 2000), S367I (Belclare mutation (Hanrahan *et al.* 2004), and C321Y (Lacaune mutation; (Bodin *et al.* 2007) have been identified in oBMP15, and a point mutation of S427R (Nicol *et al.* 2009) has been identified in oGDF9. The Inverdale mutation is located two amino acids downstream of the first hydrophobic residues on finger 1 and the Lacaune mutation is located eight amino acids upstream of the last hydrophobic residue on finger 1 (Figure 5.13A). The Belclare and Thoka mutations are located between the two residues comprising the hydrophobic motif on finger 2, (Figure 5.13B & C respectively) and within the type II receptor binding site (Figure 5.4B). Models of mature oBMP15 and oGDF9, with and without these point mutations, were generated using the Swiss model comparative protein modelling server (Schwede *et al.* 2003; Arnold *et al.* 2006; Kopp and Schwede 2006; Kiefer *et al.* 2009) with GDF5-BMPRII complex [3EVS] (Kotzsch *et al.* 2009) as the template. These were then over-layed onto the composite models described in Section 6.3.3.2 comprised of prodomains of BMP15 or GDF9, (both TGFB and BMP models, type I and II receptors).

The inactivating mutations mentioned above, share a common phenotype, heterozygotes have increased prolificacy while homozygotes exhibit ovarian hypoplasia. Another inactivating oGDF9 mutation R351C identified in Vacaria sheep (Souza *et al.* 2014), also shares this phenotype. This R351C point mutation disrupts the cleavage site of promature

GDF9, preventing the release of the mature domain. This demonstrates the absolute requirement of cleaving the promature domain from the mature protein for biological activity. In contrast, BMP point mutations in other sheep breeds (e.g. N337H in Olkaska and T317I in Grivette; Demars *et al.* 2013) or in oGDF9, F345C in Santa Inês sheep (Embrapa; Silva *et al.* 2010) and V371M in Norwegian White sheep, (Våge *et al.* 2013) show an additive effect on prolificacy. Interestingly, the V371M mutation showed an additive effect for increased litter size in Norwegian White Sheep but was not associated with prolificacy in Belclare or Camdridge ewes (Hanrahan *et al.* 2004). However, both the Belclare and Cambridge ewes carry a range of mutations, including inactivating mutations, in both GDF9 and BMP15. This results in extreme variation in ovulation rate between individuals (Hanrahan *et al.* 2004), potentially masking and/or confounding any effects of V371M on prolificacy. The dose-responsive manner of these mutations indicates they are still, at least partially, biologically-active.

Figure 5.13 Sequence alignments of inactivating mutations in oBMP15 and oGDF9 mature domains.

Inverdale (V299D) and Lacaune (C321Y) point mutations within finger 1 and (B) Belclare (S367I) point mutation within finger 2 of oBMP15. (C) Thoka point mutation (S427R) within finger 2 of oGDF9 The conserved hydrophobic amino acids located within the two finger domains of the mature region are highlighted in green. The amino acids are numbered according to the first amino acid of the signal peptide.

In agreement with the result for human inhibin α subunit (Walton *et al.* 2009), the hydrophobic residues in both oBMP15 and oGDF9 (Figure 5.3) formed a hydrophobic pocket located at the interface between the two fingers. Despite its close proximity to these

hydrophobic residues, the Inverdale mutation (V299D) did not appear to have any noticeable effect on the hydrophobic pocket itself (Figure 5.14). The promature BMP9 based model showed it to be located in close proximity to the dimer interface (Figure 5.14B-E). However, the TGFB-based model did not show the location of the Inverdale mutation to be close to the dimer interface, due to the alternative conformation of the mature domains $\alpha 1$ helix and pre-helical loop (Figure 5.11A-C). Nevertheless, upon separation from the prodomain and adoption of the receptor bound form, the Inverdale mutation did also become located within, or immediately adjacent, to the dimer interface (Figure 5.14C-E). The TGFB-based model shows a potential for the Inverdale mutation to interact with the $\alpha 1$ helix of the prodomain of its dimer partner (Figure 5.14A). The Inverdale mutation also lies within the type I receptor binding site (Figure 5.5). In both the TGFB and BMP based models, the Inverdale mutation lies in a critical location, and therefore has the potential to affect dimerization and/or interaction with the type I receptor.

Both the Belclare (S267I) and Thoka (S4237R) mutations are located with their side chain protruding outwards from the “knuckle” side of finger 2 and away from those residues which comprise the hydrophobic pocket on the mature domain. However, both mutations lie within residues making up the presumptive type II binding site (Figure 5.4B). This area is depicted for Belclare and Thoka in Figure 5.15C and 5.16C, respectively. When oBMP15 and oGDF9 are in a dimeric promature form, the TGFB- (Figure 5.15A and 5.16A, respectively) and BMP- (Figure 5.15B and 5.16B, respectively) based models both show that the Belclare and Thoka mutations will be in close association with $\alpha 2$ helix of the proregion of their dimer partner.

The point mutation F345C (Silva *et al.* 2013) falls within the un-modelled, N-terminal portion of mature GDF9. However, the N337H (Demars *et al.* 2013) point mutation, found in BMP15, comprises part of the presumptive type I receptor binding site (Figure 5.5A). Furthermore, both hBMP2 and hGDF5 also contain an asparagine at this location (Figure 5.5A). Similarly, the mutations V371M in GDF9 (Våge *et al.* 2013) and T317I in BMP15 (Demars *et al.* 2013) also lie within residues making up the presumptive type II receptor binding site (Figure 5.4B). Moreover, the Tyr317 residue corresponds to the His321 residue in hBMP2, which when mutated to alanine showed decreased binding affinity for the type II receptor (Kirsch *et al.* 2000a; Figure 5.4B). It seems likely N337H, V371M and T317I all diminish, without blocking, ligand-receptor interaction.

A

Figure 5.14 Inverdale mutation, oBMP15 (V299D).

Legend applies to the figures on this and the facing page. The figure shows the position of the Inverdale mutation (V299D) relative to the pro- and mature domains of its dimeric partner when superimposed onto TGFB (A, this page) or BMP (B and C, opposite page) based models. A surface view of C shown from below (D, opposite page) and above (E, opposite page). The oBMP15 mature region is shown in cyan with the Inverdale mutation (V299D) in orange and the amino acids comprising the hydrophobic pocket in green. The oBMP15 mature dimer partner is shown in pink and the prodomain of the dimer partner in red.

A

Figure 5.15 Belclare mutation, oBMP15 (S267I).

Legend applies to the figures on this and the facing page. The figure shows the position of the Belclare mutation (S267I) relative to its type II receptor and the pro- and mature domains of its dimeric partner when superimposed onto the TGFB (A, this page) or BMP (B and C, opposite page) based models. The images represent oBMP15 mature region (cyan) with the Belclare mutation (S267I) shown in orange and the amino acids comprising the hydrophobic pocket in green. The oBMP15 mature dimer partner is in pink, the prodomain of dimer partner is in red and; BMPR2 is in blue.

B

C

A

Figure 5.16 Thoka mutation, oGDF9 (S427R).

Legend applies to figures on this and the facing page. The figure shows the position of the Thoka mutation (S427R) relative to its type II receptor and the pro- and mature domains of its dimeric partner when superimposed onto the TGF β (A; this page) or BMP (B and C; opposite page) based models. The image represents the oGDF9 mature region (cyan) with the Thoka mutation (S427R; orange) and amino acids comprising the hydrophobic pocket (green). The oGDF9 mature dimer partner is shown in pink, the prodomain of the dimer partner in red and BMPR2 in blue.

B

C

5.4 Discussion

Currently, three-dimensional structural models of BMP15 or GDF9 are unavailable, however models for two closely-related proteins, namely promature pTGFB1 (Shi *et al.* 2011b) and mBMP9 (Mi *et al.* 2015), have been reported. Using these related proteins as templates, two structural models of oBMP15 and oGDF9 were produced to help visualise and explain our current understanding of the molecular forms of BMP15 and GDF9 and how together, they might interact with their cell surface receptors.

Hydrophobic motifs located in both the pro- and mature domains are highly conserved within the human TGFB superfamily members (Walton *et al.* 2009) as well as within BMP15 and GDF9 of known mammalian species (Figure 5.2 and 5.3). If the published information is correct on which pro and mature domains derive from the same polypeptide chain (Shi *et al.* 2011b), the motif on the prodomain can only come in close proximity to the conserved hydrophobic pocket on the mature domain with the assembly of dimeric promature forms using the TGFB-based model (Figure 5.9). However, the hydrophobic motif on the proregion of BMP9 does not fall within those residues making up the structural model of BMP9 (Mi *et al.* 2015) hence the location of this motif is unknown. Mutation of residues within the hydrophobic motifs in inhibin α leads to lowered *in vitro* production and secretion of inhibin A (Walton *et al.* 2009). This emphasises the importance of these motifs and raises the possibility that they may influence protein folding and thereby, the ability to form dimers prior to secretion by the cell. The complex assembly of multi-subunits is one of the checks performed via the endoplasmic reticulum (ER)- associated protein degradation (ERAD) system within the ER lumen of all eukaryotic cells (Ali *et al.* 2011).

It is thought that BMPs must dimerise, prior to cleavage by a furin-like proprotein convertase and secretion (Hogan 1996). The presence of dimeric oBMP15 (S356C) (Figure 4.4A & B) shows that BMP15 is brought together in an orientation to allow homodimers to form. However, based on the results of the studies reported in Chapter 4 and elsewhere (Edwards *et al.* 2008; McIntosh *et al.* 2008), it would appear that oBMP15 (lacking this cysteine) is unable to maintain dimeric forms following secretion from the cell.

As mentioned above, several naturally-occurring mutations of oBMP15 and oGDF9 are known in sheep. Perhaps the most severe of these is in the Galway (Q239>stop; Hanrahan *et al.* 2004) and Hanna (Q291>stop; Galloway *et al.* 2000) sheep. These mutations with the

introduction of premature stop codons, results in the absence of or severely truncated mature domains respectively, most likely causing complete loss of function (Galloway *et al.* 2000). Ewes homozygous for either of the BMP15 mutations Galway, Hanna, Inverdale, Belclare and Lacaune or the GDF9 mutations Thoka and FecG^H (Hanrahan *et al.* 2004) are sterile. It is unknown if any of these mutated proteins are secreted by oocytes.

The Inverdale mutation (V299D) has been suggested to interfere with dimer formation (Galloway *et al.* 2000). In the BMP-based model, the V299D mutation would result in the introduction of a negatively-charged asparagine residue into, or adjacent to, the dimer interface (Figure 5.14B and C). This may be sufficient to prevent the creation of homodimers or heterodimers with GDF9, either in solution or at the level of the receptor. However, the TGFB-based model, shows that the mature domain is held in an alternative configuration when associated with its prodomain, and consequently the V299D mutation would not then comprise part of the dimer interface. Nevertheless, it would come into close proximity (3.6Å) to the hydrophobic isoleucine (I43) residue present in the middle of the conserved hydrophobic motif located within the $\alpha 1$ helix of its dimer partners prodomain (Figure 5.14A). Interference with the correct functioning of the hydrophobic motif may result in lowered production and/or secretion, as is found with inhibin α (Walton *et al.* 2009). Indeed, transfection of mammalian cells with the Inverdale (V299D) sequence resulted in lowered secretion compared to wild-type oBMP15, while co-expression of Inverdale BMP15 (V299D) and GDF9 resulted in less GDF9 being secreted and the abolition of secreted BMP15 (V299D) (Liao *et al.* 2003). Once the mature domain is freed from any conformational constraints placed upon it by the prodomain, both models show that the V299D mutation would be located within, or adjacent to, the dimer interface (Figure 5.14C). Similarly the secretion of the Lacaune BMP15 protein was also impaired under *in vitro* conditions (Bodin *et al.* 2007). The Lacaune (C321Y) mutation lacks one of the cysteines required to form the characteristic cysteine knot holding the mature domains in the correct conformation. It is therefore likely that oBMP15 (C321Y) protein is misfolded and directed into ERAD pathway.

The Belclare (S367I) and Thoka (S427I) mutations in BMP15 and GDF9, respectively are located within the hydrophobic pocket on the mature domain however, neither appear likely to interfere directly with the functioning of this motif. This is due largely to the orientation of their side chains being positioned away from the hydrophobic pocket and towards the “knuckle” side of the fingers. However positioned as it is, within the two β

sheets comprising finger 1 of the mature domain, it may disrupt the interaction with the $\alpha 2$ helix of its dimer partners proregion: this was the case for both the TGFB and BMP models (Figures 5.15A & B and 5.16A & B respectively). In turn, this could therefore interfere with dimer formation and subsequent secretion. Alternatively, this same area falls within a predicted type II receptor binding site (Figure 5.4B) and both mutations might exert their effect by inhibiting binding to the receptor or by preventing signalling by the resultant receptor complex. The Ser367 residue in oBMP15 mutated in Belclare and the Ser427 residue in oGDF9 mutated in Thoka, correspond to the Ser370 residue in hBMP2, which when mutated to alanine decreases its binding affinity for the type II receptor (Kirsch *et al.* 2000a; Figure 5.4B). In this study, hBMP2 promature forms were not produced and only a mature dimer-receptor interaction was assessed.

Humans produce and secrete a latent form of promature GDF9, whereas polyovular species such as the mouse secrete an active form (Mottershead *et al.* 2008; Simpson *et al.* 2012; Li *et al.* 2015). This is the result of a few key amino acid differences which determine the ease with which the proregion can be displaced (Mottershead *et al.* 2015). Under *in vitro* conditions, the recombinant human BMP15 secreted is biologically active whilst recombinant mouse BMP15 is not expressed (Mottershead *et al.* 2015). The ratios of *GDF9:BMP15* mRNA expression are also different across species, with monoovular species expressing similar or greater quantities of BMP15 compared to GDF9, and rodent species such as the mouse and rat expressing greater quantities of GDF9 (Crawford and McNatty 2012). Moreover, BMP15 knockout mice are still fertile (Yan *et al.* 2001) therefore BMP15 does not appear to be essential in polyovular species. However in mono-ovular species, both GDF9 and BMP15 are essential for fertility. Indeed ewes which are heterozygous carriers for inactivating BMP15 mutations (e.g. Inverdale), have reduced levels of bioactive BMP15 and consequently, express a phenotype with an increased ovulation rate. Thus in all mammalian species, it appears that GDF9 drives the ovulation rate, while BMP15 acts as a constraint on this system. It is uncertain how this relationship is applied. However, it may be through differences in the extent to which granulosa cells are driven to proliferate relative to the developmental stage of the follicle. For example, on average, follicles from heterozygous Inverdale ewes (with lowered BMP15 levels) develop functional LH receptors on granulosa cells from smaller-sized follicles compared to that in wild-type ewes (McNatty *et al.* 2009). This, in turn, requires more follicles to produce the necessary level of steroid to signal the appropriate feedback messages to the pituitary gland and thus trigger a pre-ovulatory LH surge. In so doing, more follicles with functional LH

receptors on the granulosa cells (pre-ovulatory follicles) will be present, and capable of ovulating in response to the LH surge. Thus, the extent to which granulosa cells proliferate appears inversely related to their subsequent ovulation rate. In mono-ovulatory species, such as the sheep, the mechanism by which BMP15 causes this increase in proliferation and therefore a decrease in ovulation rate may be by a synergistic response with GDF9. Thus, perhaps increasing levels of BMP15 in the presence of GDF9 results in a greater acquisition of granulosa cells before LH receptor acquisition occurs in this cell-type, leading to a corresponding decrease in ovulation rate. For example, heterozygous carriers of the Inverdale gene have both reduced bioactive BMP15 levels and higher ovulation rates than their wild-type counterparts, which produce greater quantities of bioactive BMP15 and have a lower ovulation rate. When GDF9 is bound with its type I receptor (TGFRI) in the presence of BMP15, greater quantities of phosphorylated Smad2/3 are produced than by GDF9 alone (Mottershead *et al.* 2012) and as such, BMP15 is acting as a pseudo-TGFB. These data provide additional support for applying the TGFB-based model to BMP15.

5.4.1 Interaction with receptors

Most evidence suggests that members of the TGF- β family capable of forming covalently bound dimers, do so before binding with their specific receptors (Yardin *et al.* 2016). However, it is uncertain if those TGF- β members incapable of forming covalently bound dimers, such as BMP15 and GDF9, utilise a similar strategy. The presence of dimeric oBMP15 (S356C) (Figure 4.4A & B) shows that BMP15 is brought together in an orientation to allow homodimers to form. However, mature homodimers of BMP15 were not detected amongst the molecular forms secreted by either bovine or ovine oocytes or by HEK cells transfected with oBMP15. Based on the results of the studies reported in Chapter 4, and elsewhere (Edwards *et al.* 2008; McIntosh *et al.* 2008), it would appear that oBMP15 (lacking this cysteine) is unable to maintain detectable dimeric forms following secretion from the cell. Despite this lack of detectable dimers, it is possible that the antibodies used in these studies were incapable of: (i) detecting low levels of homodimers of BMP15; or (ii) recognising heterodimeric forms of oBMP15 and oGDF9. Thus, it is not possible to completely discount the possibility that mature dimers/heterodimers form prior to binding to their respective receptors. However, based on the evidence, it seems more likely that such dimers/heterodimers do not form before receptor interaction but instead at the level of their receptors.

While some mature monomers of BMP15 were observed in the present studies, the predominant molecular forms of BMP15, following secretion by ruminant oocytes into the media, were the cleaved and uncleaved promature forms. It therefore seems likely that these forms play an important role in the signalling of BMP15. Promature hBMP15, in the form of pro-cumulin, has been reported to be biologically active (Mottershead *et al.* 2015), while pro-BMP2 binding to its receptors has been shown to act as an antagonist for mature BMP2 by binding to the type I receptor BMPRIA (Hauburger *et al.* 2009). Binding of pro-BMP2 has also been shown to result in internalisation, intracellular cleavage and subsequent secretion of mature BMP2 (von Einem *et al.* 2011).

The biological activities of recombinant oBMP15 and oGDF9 alone, as well as their synergistic effect on ³H-thymidine incorporation by granulosa cells, have been shown to be inhibited by a BMPR2-extra cellular domain (ECD) fused to a human IgG-Fc domain (BMPR2-ECD-Fc). Whereas all other type II, and all type I, receptor ECD's had no effect (Edwards *et al.* 2008). Likewise, ³H-thymidine incorporation by rat granulosa cells in response to hBMP15 can also be blocked by a BMPR2-ECD-Fc (Moore *et al.* 2003). However, in contrast to oBMP15, hBMP15 was also able to associate with the BMPR1B-ECD. The most likely reason for the discrepancy in receptivity between oBMP15 and hBMP15 are species differences in protein sequence. Indeed, Al-Musawi *et al.* (2013) has identified two residues (R329 and D330) within the pre-helical loop of hBMP15 which contributes to the affinity towards BMPR1B and subsequently, the bioactivity of hBMP15. The substitution of the two corresponding residues in oBMP15 into hBMP15 reduced the bioactivity of hBMP15 by almost 100-fold (Al-Musawi *et al.* 2013).

Moreover, BMPR2 has been reported to undertake two conformations and it is suggested that these conformations represent the receptor in a ligand-bound or free (unbound) state (Mace *et al.* 2006). This fits well with the concept that monomeric promature BMP15 could be presented to the type II receptor causing conformational changes in both the receptor and mature growth factor domains.

Figure 5.17 A proposed sequence of events involved in the formation of a BMP15/GDF9 heterodimer at the level of the receptor:

(A) A monomer of cleaved promature BMP15 (cyan proregion and blue mature domain) binds to BMPR2 (red ribbon structure); (B) this induces an allosteric change in the mature growth factor domain (blue); (C) resulting in the release of the prodomain (cyan) and exposure of the type I binding site; (D) thus allowing binding to BMPR1B (green); (E) BMPR2 (red ribbon) then recruits an occupied type I receptor (yellow) bound to a mature GDF9 (pink) – Type II receptor (red) complex; (F) resulting in a functional heteromeric complex capable of activating signalling pathways.

Figure 5.18 A proposed sequence of events involved in the formation of a BMP15/GDF9 heterodimer at the level of the receptor: a monomer of a cleaved promature (A) BMP15 (cyan proregion and blue mature domain) or (B) GDF9 (purple proregion and pink mature domain) binds to BMPR2 (red ribbon structure); this in turn, induces an allosteric change in the mature growth factor domain of (C) BMP15 (blue) or (D) GDF9 (pink); resulting in the release of the prodomain of (E) BMP15 (cyan) or (F) GDF9 (purple); the concurrence of E and F results in (G) the formation of a BMP15:GDF9 heterodimer at the level of the receptor along with the formation of the complete type I binding sites allowing (H) BMPR1B (green) and TGFBR1 (yellow) to associate. Thereby permitting a functional heteromeric complex capable of activate intracellular signalling pathways.

The conformational change at the type II binding site of BMP15, although relatively small, may trigger a larger allosteric change in the $\alpha 1$ helix and prehelical loop (Figure 5.11, 5.17B). It is proposed that such a conformational change would cause the prodomain to be released (Figure 5.17C), while simultaneously exposing the type I binding site to allow binding of BMP15 to BMPR1B (Figure 5.17D). This binding to BMPR1B would complete a complex comprised of a monomer of mature BMP15, a single type II receptor and a single type I receptor (Figure 5.17E). In the case of BMP15 and GDF9, the evidence suggests that the ligands bind first to a type II receptor which then recruits a type I receptor to the complex (Wrana *et al.* 1992; Edwards *et al.* 2008). If this recruited type I receptor was part of another type II-ligand monomer-type I complex, a complex typical of a TGF β superfamily member would be created. This would be comprised of a mature growth factor domain dimer incorporated into a tetramer receptor complex of two type I and two type II receptors (Figure 5.17F).

An alternative model worthy of consideration is one that is initiated following the binding of both promature forms of BMP15 and GDF9 to two type II receptors (Figure 5.18). The orientations of the mature domains of BMP15 (Figure 5.18A) and GDF9 (Figure 5.18B) to BMPR2 by their prodomains would result in a conformational change in the mature domains (Figures 5.18C & D) and release of the prodomains (Figures 5.18 E & F). This in turn, would result in the formation of a BMP15:GDF9 heterodimer at the level of the BMPR2 receptors. The release of the prodomains and the conformational changes to BMP15 and GDF9 would then allow full exposure the Type I ligand binding domains for BMPR1B and TGFBR1 respectively (Figure 5.18G) and the recruitment of the type I receptors (Figure 5.18H) into an oligomeric receptor complex capable of intracellular signalling. This model is a better fit of the data given that disulfide-linked homodimers of BMPR2-ECD-Fc but not dimeric ECD-Type 1 receptors or monomeric BMPR2-ECD-Fc or dimeric ECD-Type 1 receptors are required for inhibition of biological activity of both recombinant GDF9 and BMP15 when inducing synergistic effects on granulosa cells *in vitro* (Edwards *et al.* 2008). This suggests that stable dimers may form if both partners are also bound to BMPR2

While both models depicted in Figures 5.17 and 5.18 refer to the cleaved promature form presenting the mature domain to the type II receptor, it is also conceivable that the uncleaved promature form also plays a role. Conformational changes to the uncleaved promature form, upon BMPR2 binding, may allow for the mature domain to bind both its

receptors while still attached covalently to the prodomain. Indeed Mi *et al.* (2015) has postulated that the prodomain of BMP9 may rotate between two positions, one being active while the other is latent. Furthermore, it has been reported that receptor bound pro-BMP2 results in internalisation, intracellular cleavage and subsequent secretion of mature BMP2 (von Einem *et al.* 2011). Alternatively, an uncleaved promature form may act as an antagonist, sequestering BMPR2 in a non-signalling complex, similar to that described for pro-BMP2 (Hauburger *et al.* 2009). It is also possible that it might be the target cells ability to enzymatically cleave promature BMP15 that influences how they respond to the oocytes signal (Pankhurst *et al.* 2016).

It is worth noting that purified mature domains of recombinant hBMP15, in the absence of a proregion, possesses biological activity in cultured ovine granulosa cells (Pulkki *et al.* 2012). This indicates that the prodomain is not essential for a direct biological action of BMP15 *in vitro*. Nevertheless it is likely to be of physiological importance as immunisation against either of the promature sequences of mBMP15 or mGDF9 reduced litter size in mice (McIntosh *et al.* 2012). Furthermore, promature cumulin was able to improve oocyte quality whereas mature cumulin did not (Mottershead *et al.* 2015). BMP2 can bind to preformed receptor complexes of type I and II receptors or by binding first to the type I and then recruiting the type II receptor (Gilboa *et al.* 2000; Hassel *et al.* 2003; Sieber *et al.* 2009), resulting in activation of either Smad or non-Smad signalling pathways respectively (Sieber *et al.* 2009). A similar mechanism may exist with BMP15 and/or GDF9 whereby promature forms bind first to a type II receptor while free mature growth factor domains are not so constrained. Indeed, mixtures of recombinant oBMP15 and oGDF9 containing both promature and mature forms were able to stimulate both the Smad and non-Smad pathways (Reader *et al.* 2011). Of the two models, the TGFB-based model would better enforce such constraints, by holding the mature domain in a conformation lacking the type I receptor binding site.

The disassociation of the non-covalently bound prodomain from the mature domain may be sufficient to enable more appropriate mature protein conformations, allowing exposed binding sites for both the type I and type II receptors to form. This disassociation may be facilitated by chemical means during purification methodologies or by the presence of insertions such as a FLAG tag- which may decrease the strength of the non-covalent association between pro- and mature domains. The existence of both monomeric and dimeric forms of mature GDF9 in expression media raises the possibility that GDF9 may associate with its receptors in more than one way. While monomeric GDF9 may become

receptor bound in a similar fashion to that postulated for BMP15 above, dimeric GDF9 would associate with its receptors in much the same way as other traditional TGFB family members comprised of covalently-bound dimers.

Notwithstanding the significant synergistic effects of BMP15 and GDF9 on granulosa cells, there is no evidence of heterodimer formation between any forms of GDF9 and BMP15 secreted by oocytes (Table 5.1) or when expression media is combined (McNatty *et al.* 2005a, b, Edwards *et al.* 2008; McIntosh *et al.* 2008). Thus, when BMP15 and GDF9 are both added to a granulosa cell culture, the aforementioned model suggests that a monomer of BMP15 along with a monomer of GDF9 would form a heterodimer at the level of the receptor. Finally if the tightly-controlled ratio and species specific expression ratio of *GDF9* and *BMP15* mRNA levels (Crawford and McNatty 2012) is also maintained at the protein level, then the relative amounts of hetero- and homodimer receptor complexes and thereby control of the synergistic response will be maintained by the oocyte.

Chapter 6: GENERAL DISCUSSION

6.1 Introduction

From the time a primary follicle begins to grow towards ovulation, the oocyte co-expresses BMP15 and GDF9. Their actions play essential roles during development, in determining ovulation rate and in influencing oocyte “quality”. Indeed, in mono-ovulatory species, such as the sheep or cow, the absence of BMP15 or GDF9 results in an anovulatory and therefore, sterile phenotype (Galloway *et al.* 2000; Juengel *et al.* 2002; Hanrahan *et al.* 2004; Juengel *et al.* 2009). Furthermore, these co-expressed, closely-related growth factors, exert important synergistic actions both *in vivo* (Yan *et al.* 2001; Hanrahan *et al.* 2004) and *in vitro* (Figure 3.12; McNatty *et al.* 2005b; Edwards *et al.* 2008; McIntosh *et al.* 2008; Mottershead *et al.* 2011; Reader *et al.* 2011). However, the manner in which BMP15 and GDF9 interact with one another, and with their cell surface receptors remains unclear. The purpose of this chapter is to evaluate the strengths and weaknesses of the approach taken in these studies to identify the different forms of BMP15 and GDF9 produced and secreted by ovine and bovine oocytes. It also aims to examine how these molecular forms fit with published knowledge and to speculate on potential areas of future research. It then further develops the model of BMP15-GDF9 heterodimerisation, described in Chapter 5, to propose a mechanism for the synergism observed in the granulosa cell responses to the presence of both BMP15 and GDF9.

6.2 Recombinant BMP15 and GDF9

In the present study, recombinant o/oBMP15, h/oBMP15 (S356C) and o/oGDF9 proteins were produced with a 8His-tag at the N-terminus of the proregion. A human embryonic kidney cell line (HEK293) was used to produce these proteins as these cells contain most of the machinery required for post translational folding and processing of recombinant proteins (Thomas and Smart 2005). Moreover, HEK293 cells have previously been used to produce different species of biologically-active, BMP15 and GDF9 (Otsuka *et al.* 2000) (Moore *et al.* 2003; Liao *et al.* 2004). Early methods to assist purification of recombinant BMP15 and/or GDF9 involved the transfection of HEK293 cells with constructs with a FLAG tag at the C-terminus of promature BMP15 (Otsuka *et al.* 2000) (Moore *et al.* 2003; Liao *et al.* 2004). This was later thought to impair biological activity. For example, the placement of a 6His tag at the C-terminus of either GDF9 (Mottershead *et al.* 2008) or BMP15 (Pulkki *et al.* 2011) resulted in a loss of biological activity. In contrast, the placement of a 6His tag near the N-terminus of the mature domain of GDF9 retained biological activity. However, these N-tagged proteins did not interact with Ni²⁺ based

IMAC, possibly due to interference by the prodomains, and therefore could not be purified further (Mottershead *et al.* 2008). Subsequently, the His tag was placed at the N-terminus of the prodomain allowing purification of biologically active BMP15 (Sudiman *et al.* 2014) and BMP15-GDF9 heterodimers (Mottershead *et al.* 2015). In this present study, an 8His tag was placed at the N-terminus of the prodomain, allowing for the production of untagged mature domains. These untagged mature domains have the potential to better mimic the *in vivo* interactions between the growth factors and their cell surface receptors.

The results herein revealed that the forms of secreted recombinant oBMP15 comprised mainly of mature monomers with a lesser amount of the uncleaved pro-mature form (see Chapter 3). These mature growth factor monomers were almost entirely located within high molecular weight multimeric complexes, which are also likely to contain the pro-region (Chapter 3; Edwards *et al.* 2008). Likewise, mBMP15 was also found in expression media in both mature monomer and pro-mature forms (Otsuka *et al.* 2000). In contrast, hBMP15 has been reported to contain mature dimers (Liao *et al.* 2004; Hashimoto *et al.* 2005; Rossetti *et al.* 2009; Pulkki *et al.* 2012). When the normally conserved cysteine (involved in dimer formation) was re-introduced into either human (Pulkki *et al.* 2012; Mottershead *et al.* 2015) or ovine BMP15 (Chapter 3), both dimeric pro-mature and dimeric mature growth factor forms were produced. As mentioned previously, it appears that the mature oBMP15 monomer is capable of forming dimers, probably during synthesis and secretion but that wild-type oBMP15, in contrast to hBMP15, appears unable to maintain the dimeric forms. In this study, Western blotting of cross-linked recombinant oBMP15, did not detect o/oBMP15 mature dimers. However, at least one form of o/oBMP15 expressed by HEK293 cells is biologically active, as assessed by the rat granulosa cell proliferation assay. This suggests that the biologically active form(s) are either a promature form and/or that monomeric mature domains are forming homodimers at the level of the receptor. The current study showed recombinant oGDF9 protein expressed from transfected HEK293 cells was also present in high molecular weight complexes (Chapter 4), comprised of pro-regions, uncleaved pro-mature forms and mature growth factor domains. However unlike oBMP15, dimeric mature forms were also detected (Figure 4.10). This is in agreement with that found for mouse GDF9 (McIntosh *et al.* 2008).

These recombinant proteins enabled the identification of a number of different forms, including homodimers, recognized by monoclonal antibodies MN2-61A (anti-BMP15) and

37A (anti-GDF9). It is worth noting, that whatever forms of BMP15 and GDF9 are bioactive, the aforementioned monoclonal antibodies, or polyclonal antibodies generated against the same target sequences are capable of immuno-neutralising BMP15 and GDF9 bioactivity *in vivo* (Juengel *et al.* 2002; McNatty *et al.* 2007) and *in vitro* (Lin *et al.* 2012). Nevertheless, a weakness in the present study was the inability to confirm if either antibody could detect heterodimers of mature BMP15 and GDF9. It is not clear whether this was due to the inability of either antibody to bind to heterodimers, or if heterodimers in ruminants are unable to form in solution. In Chapter 4, the *in silico* model depicts the locations of the presumed binding sites on homodimers of BMP15 and GDF9 (see Figures 4.10A & B respectively) indicating that they appear to be accessible to the antibodies. It therefore seems likely that epitopes within these regions within a heterodimer would remain available to the antibodies, as they do in a homodimer: this notion would be consistent with the evidence from the immuno-neutralisation studies assuming heterodimers were present albeit at very low concentrations. However, confirmation that the antibodies recognize heterodimers will require binding to recombinant heterodimer proteins (e.g. the cumulin-like proteins). For example, the transformation of HEK293 cells with a construct encoding either o/oGDF9 (S417C) alone or o/oGDF9 (S417C) together with h/oBMP15 (S356C) would enable the production of covalently bound oGDF9 homodimers and BMP15-GDF9 heterodimers.

The inability to retain soluble proteins, following purification of promature and mature forms of both oBMP15 and oGDF9 was another major impediment in this study. The production of oBMP15 prodomains, free of mature domains, was attempted in these studies by inserting the oBMP15 prodomain sequence into the pEFIRE5 vector. However, the *E.coli* transformed with this vector failed to grow colonies (data not shown). Production of soluble, purified prodomain, promature and mature forms would have enabled the different forms to be assessed for biological activity, as well as their interaction(s) with BMPR2 either individually, or in combinations. Such studies would have enabled a more rigorous interrogation of the proposed model of heterodimer formation at the level of the receptor.

6.3 Production and secretion of BMP15 and GDF9 by ovine and bovine oocytes

A major strength of the current study was the identification of the biological forms of both BMP15 and GDF9 produced and/or secreted by ovine and bovine oocytes. BMP15 and GDF9 secreted by ruminant oocytes under *in vitro* conditions were found mainly in an

unprocessed promature form, along with some fully processed mature domains. This is in contrast to another report (Lin *et al.* 2012), where only fully processed oBMP15 and oGDF9 mature forms were reported to be secreted by ovine oocytes. However, Lin *et al.* (2012) only looked at forms under reducing conditions and as such, this study did not preclude the presence of cleaved, non-covalently associated, proregion and mature subunits. Both oBMP15 and oGDF9 have only been found in an uncleaved pro-mature form in ovine follicular fluid (McNatty *et al.* 2006). In bovine (Behrouzi *et al.* 2016) and porcine (Paradis *et al.* 2009) follicular fluid, the forms of BMP15 have been reported to consist of both uncleaved promature and mature domains, although the mature domain was found at 25 kDa instead of a predicted 15-17 kDa. These findings in the pig and cow need further verification as the appropriate specificity studies were not reported. Two of these studies (Behrouzi *et al.* 2016) (Paradis *et al.* 2009) utilized follicular fluid from pre-ovulatory follicles, whereas the earlier study used fluid from 3 month-old lamb ovaries (McNatty *et al.* 2006). Thus the possibility exists that there may be species differences in the oocyte-secreted forms(s) of BMP15 and GDF9, and/or their proportions, may also change with the stage of follicular development. It is known that expression of *BMP15* mRNA begins from the primary stage onwards (Table 1.2) and this was also confirmed by the phenotype in homozygous carriers for inactivating BMP15 mutations. However, using the same antibody as applied in this study (MN2-61A), BMP15 protein has been detected in oocytes during the antral stage of growth (McNatty *et al.* 2006). Evidence from *in situ* hybridization studies revealed that there is an increase in *BMP15* expression levels during preantral development. (Feary *et al.* 2007). This suggests that BMP15 protein production is also likely to increase during follicular development but further studies are needed to confirm this.

Importantly, the results from Chapter 4 show that the BMP15 and GDF9 mature domains, secreted by either bovine or ovine oocytes *in vitro*, do not interact to form discernible mature homodimers or heterodimers. In contrast, Peng *et al.* (2013) reported heterodimers between a MYC-tagged mGDF9 and a FLAG-tagged mBMP15. Similarly, Mottershead *et al.* (2015) showed that promature hBMP15, when immobilized on an affinity column, interacts with both mature hBMP15 and hGDF9 equally. Furthermore, McIntosh *et al.* (2008) reported interactions between mBMP15 pro-domains and mGDF9 mature domains. The later studies of Peng *et al.* (2013) and Mottershead *et al.* (2015) may be explained by either the presence of mature heterodimers, or by interaction between BMP15 prodomains and GDF9 mature domains. In both studies, GDF9 mature domains when complexed with

BMP15, were purified, at least in part, by affinity binding to the BMP15 promature domains. It would be interesting to know if the reverse procedure would also co-purify BMP15.

It is possible that the *in vitro* oocyte incubation conditions employed in the current study results in dimer blocking modifications, during post-translational processing. However, homo- or heterodimeric forms were also undetected in lysates of freshly collected oocytes. If oocyte-secreted ruminant BMP15 or GDF9 are not forming homodimers in solution, then the likelihood of heterodimer formation may be equally low, and especially if ruminant proBMP15 has a similar affinity for both BMP15 and GDF9, as does the human form. Previous publications using the Western blotting procedure to interrogate the forms of BMP15 in follicular fluid (*in vivo* production) have only employed reducing conditions (Behrouzi *et al.* 2016) (Paradis *et al.* 2009) (McNatty *et al.* 2006). As such, it remains uncertain if homodimers or heterodimers may actually form *in vivo*.

6.4 Molecular form and function

The prodomain of related TGFB family members have been shown to play several different roles including: (i) conferring latency on the mature domain (TGFB) (Gentry and Nash 1990); (ii) assembly and secretion (inhibin alpha) (Walton *et al.* 2009); (iii) localisation to the extracellular matrix (BMP7) (Gregory *et al.* 2005) and; (iv) interaction with receptors (Hauburger *et al.* 2009; Sengle *et al.* 2008b). It therefore seems likely, that as with other members of the TGFB family, the continued associations of pro- and mature growth factor domains of BMP15 and GDF9 are important in serving biological functions after secretion (McIntosh *et al.* 2012). Indeed, it has been suggested that the BMP15 prodomain may regulate cooperation between BMP15 and GDF9 (McIntosh *et al.* 2008). For example, the point mutation in Vacaria ewes (R351C; Souza *et al.* 2014) disrupts the cleavage site of promature GDF9, preventing the release of the mature domain and rendering the protein biologically inactive. Therefore, it seems reasonable to assume that an uncleaved promature BMP15 would also be inactive. Both *in vivo* (McNatty *et al.* 2006) (Behrouzi *et al.* 2016) (Paradis *et al.* 2009) and *in vitro* (Figures 4.5A & B and 4.9A & B) studies have reported oocyte secretion of uncleaved promature forms. Therefore, at some point following secretion, it seems evident that both BMP15 and GDF9 require cleavage. This may well occur at it's target cell, as has been proposed for proAMH (McLennan and Pankhurst 2015). It will be of interest to determine the: (i) identity; (ii) originating cell type; (iii) site of action (ECM or level of receptor); and (iv) mechanisms of control, for the

proprotein convertase(s) and/or other proteinases which cleave promature BMP15 and/or GDF9.

An oocyte, through the paracrine action of oocyte secreted factors (OSF), acting upon cumulus cells, creates and maintains the cumulus cell phenotype, and thereby control over its own microenvironment (Gilchrist *et al.* 2008). It is likely that BMP15 and GDF9, as with other TGFB family members, exert their effects through the creation of gradients: being more concentrated near the oocyte (cumulus cells) and becoming less concentrated nearer the basement membrane (mural granulosa cells) (Gilchrist *et al.* 2008). Promature forms of recombinant hBMP15, (Sudiman *et al.* 2014) and human cumulin (Mottershead *et al.* 2015), were more efficacious at promoting blastocyst development in bovine and porcine respectively, than the corresponding mature domains. In contrast, mature covalent cumulin had a greater proliferative effect on mural granulosa cells than did promature-cumulin. Therefore, the secretion of both promature and fully-processed mature forms by ruminant oocytes, may add an additional level of complexity to the control that oocytes exert over the somatic cells of the follicle. The smaller mature domains, relative to the larger promature forms, may better escape the cumulus cell extracellular matrix (ECM) that has been shown to exhibit a morphogenic gradient of OSF, with the highest concentrations closest to the oocyte (Hussein *et al.* 2005). Additionally, the promature forms may interact directly (GDF9), or indirectly (BMP15), with cumulus cell ECM components, e.g. heparin sulphate proteoglycans (Mottershead *et al.* 2015). In this way, the oocyte would be able to maintain higher concentrations of promature forms around the cumulus cells. Indeed these heparin sulphate proteoglycans may act as co-receptors (Mottershead *et al.* 2015).

Uncleaved promature forms of GDF9 (Souza *et al.* 2014) are biologically inactive *in vivo*, yet cleaved promature forms show different biological activities compared to mature domains *in vitro* (Sudiman *et al.* 2014; Mottershead *et al.* 2015). It seems clear that the cleaved promature form must be acting at the level of the receptor. Protein modeling demonstrates, with both the TGFB- and BMP-based models, that dimeric promature forms mask all the receptor binding sites present on the mature domains (Figure 5.12A & B). However, a monomeric promature form, unfettered by the masking actions imposed by a dimer partner, would have an unmasked type II receptor binding site available for interactions with the BMPR2 (Figure 5.17A). As mentioned in Chapter 5 (see Section 5.4.1), the way in which BMP15 and/or GDF9 are presented to, and interact with their

respective receptors, may affect the intra-cellular signal(s) sent to the cell and/or the way the cell uses this signal. For example, the cleaved promature form of BMP15, likely interacts first with BMPR2 before it interacts with BMPR1B and then subsequently this complex recruits a GDF9-TGFBRI-BMPR2 receptor complex (Figure 5.17), to form a biologically-functional oligomeric receptor complex. In turn, this would activate a specific combination of Smad and non-Smad intracellular signaling pathway(s) within the target cell (cumulus). In response to these signals, the cumulus cells provide a micro-environment that is optimal for the the oocyte. In contrast, the mature forms, diffusing beyond the cumulus-oocyte-complex, likely interact with their receptors on mural granulosa cells in a less constrained manner. Consequently, by initiating different combination(s) of intracellular signaling pathways would enable different functional end points to be achieved, i.e. modified proliferation and/or responsiveness to FSH or LH (Figure 1.2). Indeed, within overlapping gradients, the two forms may well compete for both type I and II receptors, thereby acting as antagonists for each other. Changes to such gradients and the relative strengths to each other may explain the mode(s) of action in sheep heterozygous for inactivating mutations. Although producing less bioactive protein than their wild-type counterparts, concentrations of promature forms might be locally elevated, due to being sequestered by the cumulus cell ECM, allowing for an unaltered rate of oocyte maturation. However, the promature and/or mature domains might only reach a lower concentration threshold in follicular fluid and thereby exert different proliferative and/or differentiative effects, on the mural granulosa cells. One such consequence for the heterozygous BMP15 mutants is the acquisition of LH receptors in mural granulosa cells at smaller follicular diameters (i.e. earlier stage of follicular growth) leading to a greater proportion of follicles capable of going on to ovulate and with increased number of offspring (McNatty *et al.* 2009).

It is clear that BMP15 and GDF9, either alone or together, elicit a range of phenotypes in both cumulus and mural granulosa cells (McNatty *et al.* 2005a;b; McIntosh *et al.* 2008; Mottershead *et al.* 2015). The species of growth factor(s) and target cell(s) are also known to affect the downstream cellular signaling responses (Reader *et al.* 2011; Mottershead *et al.* 2012; Reader *et al.* 2014). Presently, there is little consistency between different laboratories researching BMP15 and GDF9. This is due to the use of different cell strains, cell types and constructs for the production of recombinant proteins for different species, different approaches to purify the proteins, different antibodies to detect them, and different “standards” to measure them in relative densitometry assays. The relative

affinities between antibodies, and the different forms of BMP15 or GDF9 (e.g. monomer versus dimer, mature versus promature), are also unknown in these assays. Perhaps, not surprisingly, the present state of research into BMP15 and GDF9, in many ways, mirrors the early research into another TGF β family member, inhibin. For example, inhibin standards originally consisted of charcoal-stripped follicular fluid, containing an unknown mixture of forms, both of inhibins and activins. An early radio-immunoassay, the so called “Monash assay” (McLachlan *et al.* 1986; Robertson 1990), cross-reacted particularly well with the biologically inactive inhibin pro- α C and - α N forms and was unable to distinguish between inhibin A and inhibin B. This resulted in the conclusions derived from these early studies to undergo some revision (de Kretser *et al.* 2002). The importance of determining the correct bioactive form(s) of BMP15 and GDF9 and the ability to detect and measure these forms in a standard manner is of paramount importance to further elucidate the roles and actions of these growth factors.

6.5 Mechanism of synergistic action

The synergistic actions of BMP15 and GDF9 are well documented, with *Bmp15*^{-/-}*Gdf9*^{+/-} mouse knockout models showing more pronounced defects in follicular development than either *Bmp15*^{-/-} or *Gdf9*^{+/-} alone (Yan *et al.* 2001). Conversely, sheep carrying heterozygous mutations in both *GDF9* and *BMP15* have higher ovulation-rates than sheep carrying either of these mutations alone (Hanrahan *et al.* 2004). *In vitro* studies have also shown a synergistic effect on proliferation of granulosa cells by oBMP15 and oGDF9 (Section 3.3.6; McNatty *et al.* 2005a), mBMP15 and mGDF9 (McIntosh *et al.* 2008; Reader *et al.* 2011), as well as a combination of mGDF9 and hBMP15 (Mottershead *et al.* 2012) and oBMP15 and mGDF9 (McNatty *et al.* 2005a). Moreover, these synergistic effects are also evident if progesterone and inhibin production by granulosa cells (McNatty *et al.* 2005a) or expression levels of a range of genes involved in cumulus expansion (i.e. *Ptx3*, *Has2*, and *Ptgs2*; Peng *et al.* 2013b) are used as the end-point. If it is true that heterodimers are not formed prior to receptor binding, GDF9 and BMP15 may exert these synergistic effects at the level of the receptor, and/or by cross-talk, between signalling pathways.

The purified mature domain of a covalently dimerised hBMP15 has a similar bioactivity to monomeric wild-type domains (Pulkki *et al.* 2012). This therefore confirms that BMP15 must act at the level of the receptor in a dimeric form, typical of that for other TGF β family members. The synergistic action of GDF9 and BMP15 on the Smad pathways showed an increased activation of the Smad3 pathway, relative to that for GDF9 alone. In

contrast, the addition of GDF9 failed to stimulate further the Smad1/5/8 pathway compared to that for BMP15 alone (Mottershead *et al.* 2012). Moreover, co-incubation with GDF9 and either covalently-bound hBMP15 (Mottershead *et al.* 2015) or oBMP15 (See Figure 3.12) homodimers, failed to elicit a synergistic response in a mouse or rat granulosa cell ^3H -thymidine incorporation assay respectively.

Figure 6.1 Mechanism of synergistic action.

(A) Hypothesized conformational changes in GDF9 in response to heterodimer formation in comparison to (B) homodimer formation. Small changes in the conformation of GDF9 due to an interaction at the secondary binding site for BMPR1B (B2) may result in allosteric changes in GDF9 at the primary binding site (B3) for TGFBR1. Similarly changes in the relative shape and location of B3 and B4 may improve contact and/or affinity between TGFBR1 and the B3 binding site of GDF9 when part of a heterodimer (A) compared to that created by a GDF9 homodimer (B).

This further supports the notion that this synergism is mediated at the level of the same receptor complex. However, additional crosstalk between non-Smad and/or Smad pathways cannot be discounted. It seems reasonable to assume that the synergistic action is caused by a change in either the way GDF9 interacts with its type I receptor and/or by the way this receptor is activated by BMPR2. Both possibilities could be accommodated by the formation of heterodimers within the receptor complex. Upon formation of the GDF5-BMPR1B complex, GDF5 undergoes an induced change whereby the concave surface of the fingers (secondary type I binding site) move towards the receptor (Kotzsch *et al.* 2009). A similar conformational change occurring between GDF9 and BMPR1B may not only alter the shape of the secondary binding site (Figure 6.1A, binding site B2) but also the potential to fine tune, through allosteric changes, the primary binding site on GDF9 (Figure 6.1A, binding site B3) as well. Such a heterodimer would alter the shape and potentially also alter the relative positions of both the primary and secondary binding sites for GDF9's

type I receptor, (Figure 6.1A, binding sites B3 & B4). In turn, this might result in a greater affinity between GDF9 and its type I receptor leading to an increased activation of the Smad3 pathway. Likewise, a change in the orientation of the type I receptor may also improve its association with, and ability to be phosphorylated by, BMPR2 and/or its association with other intracellular binding partners. Increased bioactivity of heterodimers has been described previously where heterodimers of BMP2 with BMP5, 6 or 7 and heterodimers of BMP4 and BMP7 possessed greater bioactivity than any of the individual homodimers (Israel *et al.* 1996).

6.6 Summary

BMP15 and GDF9 are secreted by ruminant oocytes, largely in an uncleaved promature form, and to date there is no evidence for homodimers or heterodimers forming anywhere but at the level of the receptor *in vivo*. These promature forms, along with mature domains, may well create gradients eliciting different responses from cumulus and mural granulosa cells. It is hypothesised that BMP15 and GDF9 interact as a heterodimer at the level of the receptor. It is proposed that the synergistic increase in phosphorylated Smad3 and subsequent somatic cell responses is the result of subtle changes in the conformation and subsequent orientation between such a heterodimer and its receptors as well as between type I and type II receptors, compared to that of a homodimer complex.

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APPENDIX 1: DNA and protein sequence of h/oBMP15 (S356C)

Figure A1.1 Schematic depiction of the h/oBMP15 (S356C) construct. Green arrows depict major components of the sequence including rat albumin signal sequence; 8His tag; human BMP15 prodomain (hBMP15pro) and ovine mature domain (oBMP15 mat). The location of cysteines within the mature domain are labelled. The mutation (S356C; numbered from start of hBMP15pro) is shown as Cysteine 4

Protein	M K W V T F L L L L F I S									
DNA	TGA	GAATTCA	CTCGAG	GCCA	CCATGAAGTG	GGTAACCTTT	CTCCTCCTCC	TCTTCATCTC		60
Protein	G S A F S H	H H H H	H H H	G G E	G G Q S					120
DNA	CGGTCTGCC	TTTCTCACC	ACCATCACCA	CCACCATCAT	GGAGGTGAAG	GAGGGCAGTC				
Protein	S I A L L A	E A P T	L P L	I E E	L L E E					180
DNA	CTCTATTGCC	CTTCTGGCTG	AGGCCCTAC	TTTGCCCTG	ATTGAGGAGC	TGCTAGAAGA				
Protein	S P G E Q P	R K P R	L L G	H S L	R Y M L					240
DNA	ATCCCTGGC	GAACAGCAA	GGAAGCCCCG	GCTCCTAGGG	CATTCACTGC	GGTACATGCT				
Protein	E L Y R R S	A D S H	G H P	R E N	R T I G					300
DNA	GGAGTTGTAC	CGGCGTTCAG	CTGACTCGCA	TGGGCACCCT	AGAGAGAACC	GCACCATTGG				
Protein	A T M V R L	V K P L	T S V	A R P	H R G T					360
DNA	GGCCA	CCATG	G	TGAGGCTGG	TGAAGCCCTT	GACCAGTGTG	GCAAGGCCCTC	ACAGAGGTAC		
Protein	W H I Q I L	G F P L	R P N	R G L	Y Q L V					420
DNA	CTGGCATATA	CAGATCCTGG	GCTTTCCTCT	CAGACCAAAC	CGAGGACTAT	ACCAACTAGT				
Protein	R A T V V Y	R H H L	Q L T	R F N	L S C H					480
DNA	TAGAGCCACT	GTGGTTTACC	GCCATCATCT	CCAACCTAAT	CGCTTCAATC	TCTCTGCCA				
Protein	V E P W V Q	K N P	T N H F	P S S	E G D S					540
DNA	TGTGGAGCCC	TGGGTGCAGA	AAAACCCAAC	CAACCACTTC	CCTTCCTCAG	AAGGAGATTC				
Protein	S K P S L M	S N A W	K E M	D I T	Q L V Q					600
DNA	CTCAAAACCT	TCCCTGATGT	CTAACGCTTG	GAAAGAGATG	GATATCACAC	AACCTGTTCA				
Protein	Q R F W N N	K G H R	I L R	L R F	M C Q Q					660
DNA	GCAAAGGTTT	TGGAATAACA	AGGGACACAG	G	GATCC	TACGA	CTCCGTTTTA	TGTGTCAGCA		
Protein	Q K D S G G	L E L W	H G T	S S L	D I A F					720
DNA	GCAAAAAGAT	AGTGGTGGTC	TTGAGCTCTG	GCATGGCACT	TCATCCTTGG	ACATTGCCTT				

Protein	L L L Y F N D T H K S I R K A K F L P R	
DNA	CTTGTTACTC TATTTC AATG ATACTCATAA AAGCATTCGG AAGGCTAAAT TTCTTCCCAG	780
	G M E E F M E R E S L L R R T R Q A G S	
751	GGGCATGGAG GAGTTCATGG AAAGGGAATC TCTTCTCCGG AGAACCCGAC AAGCAGGCAG	840
Protein	I A S E V P G P S R E H D G P E S N Q C	
DNA	TATTGCATCG GAAGTTCCTG GCCCCTCCAG GGAGCATGAT GGGCCTGAAA GTAACCAAGTG	900
Protein	· S L H P F Q V S F Q Q L G W D H W I I A	
DNA	TTCCCTCCAC CCTTTTCAAG TCAGCTTCCA GCAGCTGGGC TGGGATCACT GGATCATTGC	960
Protein	P H L Y T P N Y C K G V C P R V L H Y G	
DNA	TCCCCATCTC TATACCCCAA ACTACTGTAA GGGAGTATGT CCTCGGGTAC TACACTATGG	1020
Protein	L N S P N H A I I Q N L V S E L V D Q N	
DNA	TCTCAATTCT CCCAATCATG CCATCATCCA GAACCTTGTC AGTGAGCTGG TGGATCAGAA	1080
Protein	V P Q P C V P Y K Y V P I S I L L I E	
DNA	TGTCCCTCAG CCTTGC TGTG TCCCTTATAA GTATGTTCCC ATTAGCATCC TTCTGATTGA	1140
Protein	· A N G S I L Y K E Y E G M I A Q S C T C	
DNA	GGCAAATGGG AGTATCTTGT ACAAGGAGTA TGAGGGTATG ATTGCCCAGT CCTGCACATG	1200
Protein	· R * *	
DNA	CAGGTGATAA TCTAGAGAA TTCAGT	1226

Figure A1.2 The DNA sequence and protein translation for h/oBMP15 (S356C). Restriction enzyme cut sites are highlighted: EcoRI, yellow; XhoI, green; NcoI, blue; BamHI, orange; and; XbaI, red. The location of proprotein convertase cleavage site is shown by a box and the location of mutation (S356C) within the mature domain is highlighted in pink. The number of base pairs is shown on the right of the figure.

APPENDIX 2: DNA and protein sequence of o/oBMP15

Figure A2.1 Schematic depiction of o/oBMP15 construct.

Green arrows depict the major components of the sequence including rat albumin signal sequence, 8His tag, ovine BMP15 prodomain (oBMP15pro) and ovine mature domain (oBMP15 mat). The location of cysteines within the mature domain are labelled.

[illegible]

Protein	Q S V Q K T K P L P K G L K E F T E K D	
DNA	TCAGAGTGTT CAGAAGACCA AACCTCTCCC TAAAGGCCTG AAAGAGTTTA CAGAAAAAGA	840
Protein	P S L L L R R A R Q A G S I A S E V P G	
DNA	CCCTTCTCTT CTCTTGAGGA GGGCTCGTCA AGCAGGCAGT ATTGCATCGG AAGTTCCTGG	900
Protein	P S R E H D G P E S N Q C S L H P F Q V	
DNA	CCCCTCCAGG GAGCATGATG GGCCTGAAAG TAACCAGTGT TCCCTCCACC CTTTTC AAGT	960
Protein	S F Q Q L G W D H W I I A P H L Y T P N	
DNA	CAGCTTCCAG CAGCTGGGCT GGGATCACTG GATCATTGCT CCCCATCTCT ATACCCCAA	1020
Protein	Y C K G V C P R V L H Y G L N S P N H A	
DNA	CTACTGTAAG GGAGTATGTC CTCGGGTACT ACACATATGGT CTCAATTCTC CCAATCATGC	1080
Protein	I I Q N L V S E L V D Q N V P Q P S C V	
DNA	CATCATCCAG AACCTTGTCA GTGAGCTGGT GGATCAGAAT GTCCCTCAGC CT TCC TGTGT	1140
Protein	P Y K Y V P I S I L L I E A N G S I L Y	
DNA	CCCTTATAAG TATGTTCCCA TTAGCATCCT TCTGATTGAG GCAAATGGGA GTATCTTGTA	1200
Protein	K E Y E G M I A Q S C T C R * *	
DNA	CAAGGAGTAT GAGGGTATGA TTGCCCAGTC CTGCACATGC AGGTGATAA T CTAGAA GAAT	1260
Protein		
DNA	TC AGT 1265	

Figure A2.2 DNA sequence and protein translation for o/oBMP15

Restriction enzyme cut sites are highlighted: EcoRI, yellow; XhoI, green; NcoI, blue; and XbaI, red. The location of the proprotein convertase cleavage site is shown in a box and the location of S356 (mutated in h/oBMP15 (S356C); numbered from start of oBMP15pro) within the mature domain is highlighted in pink. The number of base pairs are shown on the right of the figure.

APPENDIX 3: Sequencing results for hBMP15pro

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oBMP15pro_Foward  TGGGCCCCGACGTCGCATGCTCCCGGCCGCCATGG-CGGCC-
GCGGGAATTCGATTACTCG 106
oBMP15pro_Reverse  TGGGCCCCGACGTCGCATGCTCCCGGCCGCCATGGGCGGCCGCGGGAATTCGATTACTCG 178
h/oBMP15 (S356C)   -----TGAATTC-----ACTCG 14
                                     *  * * * * *
                                     *  * * * * *

oBMP15pro_Foward  AGGCCACCATGAAGTGGGTAACCTTTCTCCTCCTCTTCATCTCCGGTTCTGCCTTTT 166
oBMP15pro_Reverse  AGGCCACCATGAAGTGGGTAACCTTTCTCCTCCTCTTCATCTCCGGTTCTGCCTTTT 238
h/oBMP15 (S356C)   AGGCCACCATGAAGTGGGTAACCTTTCTCCTCCTCTTCATCTCCGGTTCTGCCTTTT 74
                   *****

oBMP15pro_Foward  CTCACCACCATCACCACCACCATCATGGAGGTGAAGGAGGGCAGTCCTCTATTGCCCTTC 226
oBMP15pro_Reverse  CTCACCACCATCACCACCACCATCATGGAGGTGAAGGAGGGCAGTCCTCTATTGCCCTTC 298
h/oBMP15 (S356C)   CTCACCACCATCACCACCACCATCATGGAGGTGAAGGAGGGCAGTCCTCTATTGCCCTTC 134
                   *****

oBMP15pro_Foward  TGGCTGAGGCCCTACTTTGCCCTGATTGAGGAGCTGCTAGAAGAATCCCTGGCGAAC 286
oBMP15pro_Reverse  TGGCTGAGGCCCTACTTTGCCCTGATTGAGGAGCTGCTAGAAGAATCCCTGGCGAAC 358
h/oBMP15 (S356C)   TGGCTGAGGCCCTACTTTGCCCTGATTGAGGAGCTGCTAGAAGAATCCCTGGCGAAC 194
                   *****

oBMP15pro_Foward  AGCCAAGGAAGCCCCGGCTCCTAGGGCATTCACTGCGGTACATGCTGGAGTTGTACCGGC 346
oBMP15pro_Reverse  AGCCAAGGAAGCCCCGGCTCCTAGGGCATTCACTGCGGTACATGCTGGAGTTGTACCGGC 418
h/oBMP15 (S356C)   AGCCAAGGAAGCCCCGGCTCCTAGGGCATTCACTGCGGTACATGCTGGAGTTGTACCGGC 254
                   *****

oBMP15pro_Foward  GTTCAGCTGACTCGCATGGGCACCTAGAGAGAACCGCACCATTGGGGCCACCATGGTGA 406
oBMP15pro_Reverse  GTTCAGCTGACTCGCATGGGCACCTAGAGAGAACCGCACCATTGGGGCCACCATGGTGA 478
h/oBMP15 (S356C)   GTTCAGCTGACTCGCATGGGCACCTAGAGAGAACCGCACCATTGGGGCCACCATGGTGA 314
                   *****

oBMP15pro_Foward  GGCTGGTGAAGCCCTTGACCAAGTGTGGCAAGGCCTCACAGAGGTACCTGGCATATACAGA 466
oBMP15pro_Reverse  GGCTGGTGAAGCCCTTGACCAAGTGTGGCAAGGCCTCACAGAGGTACCTGGCATATACAGA 538
h/oBMP15 (S356C)   GGCTGGTGAAGCCCTTGACCAAGTGTGGCAAGGCCTCACAGAGGTACCTGGCATATACAGA 374
                   *****

oBMP15pro_Foward  TCCTGGGCTTTCTCTCAGACCAAACCGAGGACTATACCAACTAGTTAGAGCCACTGTGG 526
oBMP15pro_Reverse  TCCTGGGCTTTCTCTCAGACCAAACCGAGGACTATACCAACTAGTTAGAGCCACTGTGG 598
h/oBMP15 (S356C)   TCCTGGGCTTTCTCTCAGACCAAACCGAGGACTATACCAACTAGTTAGAGCCACTGTGG 434
                   *****

oBMP15pro_Foward  TTTACGCCATCATCTCCAATAACTCGCTTCAATCTCTCCTGCCATGTGGAGCCCTGGG 586
oBMP15pro_Reverse  TTTACGCCATCATCTCCAATAACTCGCTTCAATCTCTCCTGCCATGTGGAGCCCTGGG 658
h/oBMP15 (S356C)   TTTACGCCATCATCTCCAATAACTCGCTTCAATCTCTCCTGCCATGTGGAGCCCTGGG 494
                   *****
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oBMP15pro_Forward  TGCAGAAAAACCCAACCAACCACTTCCCTTCCTCAGAAGGAGATTCCCTCAAAACCTTCCC 646
oBMP15pro_Reverse  TGCAGAAAAACCCAACCAACCACTTCCCTTCCTCAGAAGGAGATTCCCTCAAAACCTTCCC 718
h/oBMP15 (S356C)   TGCAGAAAAACCCAACCAACCACTTCCCTTCCTCAGAAGGAGATTCCCTCAAAACCTTCCC 554
*****

oBMP15pro_Forward  TGATGTCTAACGCTTGGAAGAGATGGATATCACACAACCTTGTTTCAGCAAAGGTTCTGGA 706
oBMP15pro_Reverse  TGATGTCTAACGCTTGGAAGAGATGGATATCACACAACCTTGTTTCAGCAAAGGTTCTGGA 778
h/oBMP15 (S356C)   TGATGTCTAACGCTTGGAAGAGATGGATATCACACAACCTTGTTTCAGCAAAGGTTCTGGA 614
*****

oBMP15pro_Forward  ATAACAAGGGACACAGGATCCTACGACTCCGTTTTATGTGTCAGCAGCAAAAAGATAGTG 766
oBMP15pro_Reverse  ATAACAAGGGACACAGGATCCTACGACTCCGTTTTATGTGTCAGCAGCAAAAAGATAGTG 838
h/oBMP15 (S356C)   ATAACAAGGGACACAGGATCCTACGACTCCGTTTTATGTGTCAGCAGCAAAAAGATAGTG 674
*****

oBMP15pro_Forward  GTGGTCTTGAGCTCTGGCATGGCACTTCATCCTTGGACATTGCCTTCTTGTTACTCTATT 826
oBMP15pro_Reverse  GTGGTCTTGAGCTCTGGCATGGCACTTCATCCTTGGACATTGCCTTCTTGTTACTCTATT 898
h/oBMP15 (S356C)   GTGGTCTTGAGCTCTGGCATGGCACTTCATCCTTGGACATTGCCTTCTTGTTACTCTATT 734
*****

oBMP15pro_Forward  TCAATGATACTCATAAAGCATTTCGGAAGGCTAAATTTCTTCCCAGGGGCATGGAGGAGT 886
oBMP15pro_Reverse  TCAATGATACTCATAAAGCATTTCGGAAGGCTAAATTTCTTCCCAGGGGCATGGAGGAGT 958
h/oBMP15 (S356C)   TCAATGATACTCATAAAGCATTTCGGAAGGCTAAATTTCTTCCCAGGGGCATGGAGGAGT 794
*****

oBMP15pro_Forward  TCATGGAAAGGGAATCTCTTCTCCTGA--ATTC AATCACTAGTGAATTGCGGGCCGCCTG 944
oBMP15pro_Reverse  TCATGGAAAGGGAATCTCTTCTCCTGA--ATTC AATCACTAGTGAATTGCGGGCCGCCTG 1016
h/oBMP15 (S356C)   TCATGGAAAGGGAATCTCTTCTCCGAGAACCCGACAAGCAGGCAGTATTGCATCGGAAG 854
*****

oBMP15pro_Forward  CAGGTCGACCATATGGGAGAGCTCCCAAACGCGTTGGATGCATAGCTTGAGTATTCTATA 1004
oBMP15pro_Reverse  CAGGTCGACCATATGGGAGAGCTCCCAA-CGCGTTGGATGCATAGCTTGAGTATTCTATA 1075
h/oBMP15 (S356C)   TTCTTGCCCTCCAGGGAG---CATGATGGGCCTGAAAG--TAACCAGTGTTCCCTCCA 909
*****

oBMP15pro_Forward  GTGTCACCTAAATAGCTTACGTAATCATGATCATAGCTGTTTCCTGTGTGAATTGTATC- 1063
oBMP15pro_Reverse  GT-TCACCTAAATAGCTGGCG----ATGATT---CCGTTTTTTTGTGAGA--TGGGGC- 1122
h/oBMP15 (S356C)   CCCTTTTCAAGTCAGCTTCCAGCAGCTGGGCTGGGATCACTGGATCATTGCTCCCATCT 969
*****

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FigureA3.1 Sequencing results for hBMP15pro

Alignment (*) of h/oBMP15 (S356C) sequence with results from sequencing of hBMP15pro using oBMP15pro_Forward, 5'primer; [5'-ACTCGAGGCCACCATGAAGTG-3'] and oBMP15pro_Reverse, 3' primer; [5'-GAATTCAGGAGAAGAGATTCCTTTC-3'] Restriction enzyme cut sites are highlighted: EcoRI in yellow and XhoI in green.

APPENDIX 4: Sequence alignment of the sequenced pEFIREs-h/oBMP15 (S356C) **insert against theoretical h/oBMP15 (S356C) sequence**

```

Forward primer A:      ATCCCCCAGGGGGTCCACCTCCCAGTTCAATTACAGCTCTTAAGGCTAGA 50
h/oBMP15(S356C)_theoretical  -----

Forward primer A:      GTACTTAATACGACTCACTATAGGCTAGCCTCGAGGCCACCATGAAGTGG 100
h/oBMP15(S356C)_theoretical  -----TGAGAATTCACTCGAGGCCACCATGAAGTGG 31
                               ** * *****

Forward primer A:      GTAACCTTTCTCCTCCTCCTCTTCATCTCCGGTTCTGCCTTTTCTCACCA 150
h/oBMP15(S356C)_theoretical  GTAACCTTTCTCCTCCTCCTCTTCATCTCCGGTTCTGCCTTTTCTCACCA 81
                               *****

Forward primer A:      CCATCACCACCACCATCATGGAGGTGAAGGAGGGCAGTCTCTATTGCCC 200
h/oBMP15(S356C)_theoretical  CCATCACCACCACCATCATGGAGGTGAAGGAGGGCAGTCTCTATTGCCC 131
                               *****

Forward primer A:      TTCTGGCTGAGGCCCTACTTTGCCCTGATTGAGGAGCTGCTAGAAGAA 250
h/oBMP15(S356C)_theoretical  TTCTGGCTGAGGCCCTACTTTGCCCTGATTGAGGAGCTGCTAGAAGAA 181
                               *****

Forward primer A:      TCCCCTGGCGAACAGCCAAGGAAGCCCCGGCTCCTAGGGCATTCACTGCG 300
h/oBMP15(S356C)_theoretical  TCCCCTGGCGAACAGCCAAGGAAGCCCCGGCTCCTAGGGCATTCACTGCG 231
                               *****

Forward primer A:      GTACATGCTGGAGTTGTACCGGCGTTCAGCTGACTCGCATGGGCACCCTA 350
h/oBMP15(S356C)_theoretical  GTACATGCTGGAGTTGTACCGGCGTTCAGCTGACTCGCATGGGCACCCTA 281
                               *****

Forward primer A:      GAGAGAACCGCACCATTGGGGCCACCATGGTGAGGCTGGTGAAGCCCTTG 400
h/oBMP15(S356C)_theoretical  GAGAGAACCGCACCATTGGGGCCACCATGGTGAGGCTGGTGAAGCCCTTG 331
                               *****

Forward primer A:      ACCAGTGTGGCAAGGCCTCACAGAGGTACCTGGCATATACAGATCCTGGG 450
h/oBMP15(S356C)_theoretical  ACCAGTGTGGCAAGGCCTCACAGAGGTACCTGGCATATACAGATCCTGGG 381
                               *****

Forward primer A:      CTTTCCTCTCAGACCAAACCGAGGACTATACCAACTAGTTAGAGCCACTG 500
h/oBMP15(S356C)_theoretical  CTTTCCTCTCAGACCAAACCGAGGACTATACCAACTAGTTAGAGCCACTG 431
                               *****

Forward primer A:      TGGTTTACCGCCATCATCTCCAATAACTCGCTTCAATCTCTCCTGCCAT 550
h/oBMP15(S356C)_theoretical  TGGTTTACCGCCATCATCTCCAATAACTCGCTTCAATCTCTCCTGCCAT 481
                               *****

Forward primer A:      GTGGAGCCCTGGGTGCAGAAAAACCAACCAACCACTTCCTTCCTCAGA 600
h/oBMP15(S356C)_theoretical  GTGGAGCCCTGGGTGCAGAAAAACCAACCAACCACTTCCTTCCTCAGA 531
                               *****

Forward primer A:      AGGAGATTCTCAAAACCTTCCTGATGTCTAACGCTTGGAAGAGATGG 650
h/oBMP15(S356C)_theoretical  AGGAGATTCTCAAAACCTTCCTGATGTCTAACGCTTGGAAGAGATGG 581
                               *****

Forward primer A:      ATATCACACAACCTTGTTCAGCAAAGGTTCTGGAATAACAAGGGACACAGG 700
h/oBMP15(S356C)_theoretical  ATATCACACAACCTTGTTCAGCAAAGGTTCTGGAATAACAAGGGACACAGG 631
                               *****

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Removed (C)

Forward primer A: ATCCTACGACTCCGTTTATGTGTCAGCAGCAAAAAGATAGTGGTGGTCT 750
h/oBMP15(S356C)_theoretical ATCCTACGACTCCGTTTATGTGTCAGCAGCAAAAAGATAGTGGTGGTCT 681
Forward primer B: GTGTCAGCAGCAAAAAGATAGTGGTGGTCT 46

Inserted (T)

Forward primer A: TGAGCTCTGGCATGGCACTTCATCCTTGGACATTGCCTTCTTGTACTCT 800
h/oBMP15(S356C)_theoretical TGAGCTCTGGCATGGCACTTCATCCTTGGACATTGCCTTCTTGTACTCT 731
Forward primer B: TGAGCTCTGGCATGGCACTTCATCCTTGGACATTGCCTTCTTGTACTCT 66

Forward primer A: ATTTCAATGATACTCATA 818
h/oBMP15(S356C)_theoretical ATTTCAATGATACTCATAAAAGCATTTCGGAAGGCTAAATTTCTTCCCAGG 781
Forward primer B: ATTTCAATGATACTCATAAAAGCATTTCGGAAGGCTAAATTTCTTCCCAGG 116

h/oBMP15(S356C)_theoretical GGCATGGAGGAGTTCATGAAAGGGAATCTTCTCCGAGAAACCCGACA 831
Forward primer B: GGCATGGAGGAGTTCATGAAAGGGAATCTTCTCCGAGAAACCCGACA 166

h/oBMP15(S356C)_theoretical AGCAGGCAGTATTGCATCGGAAGTTCCTGGCCCCCTCCAGGGAGCATGATG 881
Forward primer B: AGCAGGCAGTATTGCATCGGAAGTTCCTGGCCCCCTCCAGGGAGCATGATG 216

h/oBMP15(S356C)_theoretical GGCCTGAAAGTAACCAAGTGTTCCTCCACCCTTTTCAAGTCAGCTTCCAG 931
Forward primer B: GGCCTGAAAGTAACCAAGTGTTCCTCCACCCTTTTCAAGTCAGCTTCCAG 266

h/oBMP15(S356C)_theoretical CAGCTGGGCTGGGATCACTGGATCATTTGCTCCCCATCTCTATACCCCAA 981
Forward primer B: CAGCTGGGCTGGGATCACTGGATCATTTGCTCCCCATCTCTATACCCCAA 316

h/oBMP15(S356C)_theoretical CTACTGTAAGGGAGTATGTCCTCGGGTACTACACTATGGTCTCAATTCTC 1031
Forward primer B: CTACTGTAAGGGAGTATGTCCTCGGGTACTACACTATGGTCTCAATTCTC 366

h/oBMP15(S356C)_theoretical CCAATCATGCCATCATCCAGAACCTTGTCAAGTGAAGCTGGTGGATCAGAAT 1081
Forward primer B: CCAATCATGCCATCATCCAGAACCTTGTCAAGTGAAGCTGGTGGATCAGAAT 416

h/oBMP15(S356C)_theoretical GTCCCTCAGCCTTGCTGTGTCCCTTATAAGTATGTTCCCATAGCATCCT 1131
Forward primer B: GTCCCTCAGCCTTGCTGTGTCCCTTATAAGTATGTTCCCATAGCATCCT 466

h/oBMP15(S356C)_theoretical TCTGATTGAGGCAAAATGGGAGTATCTTGTACAAGGAGTATGAGGGTATGA 1181
Forward primer B: TCTGATTGAGGCAAAATGGGAGTATCTTGTACAAGGAGTATGAGGGTATGA 516

h/oBMP15(S356C)_theoretical TTGCCCAGTCTGCACATGCAGGTGATAATCTAGAGTCGACCCGGGCGGG 1231
Forward primer B: TTGCCCAGTCTGCACATGCAGGTGATAATCTAGAGTCGACCCGGGCGGG 566

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h/oBMP15(S356C)_theoretical -----
Forward primer B: CCGCTCTAGCCCAATCCGCCCCCCCC----- 593
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Figure A4.1 Sequence alignment of the sequenced pEFIRES-h/oBMP15 (S356C) insert against theoretical h/oBMP15 (S356C) sequence.

Alignment (*) of h/oBMP15 (S356C)_theoretical sequence with sequence results of h/oBMP15 (S356C) insert using Forward primer 1, 5'primer; [5' –CTA-TTG-GTC-TTA-CTG-ACA-TCC-A- 3'] and Forward primer 2, 5'primer; [5' –CGT-TCT-GGA-ATA-ACA-AGG-3']. The restriction enzyme cut sites are highlighted: XhoI in yellow and XbaI in green.

Note: Based on the review of the chromatogram files and to correct the auto read of the chromatogram, a cytosine was removed and a thymine inserted at positions 634 and 670 respectively

APPENDIX 5: Materials

A5.1 Reagents

Acetonitrile (MERCK, Darstadt, Germany, Cat. No. 1.00030.2500)

Activin A, rec. Human R&D Systems; Pharmaco, Auckland, New Zealand,
Cat. No. 338-AC

Agarose, 1% (w/v)

0.4 g LE agarose (Seakem; Alphatech, Auckland New Zealand, Cat. No.
CAM50004)

40 mL 1x TAE Buffer

Carefully heated in microwave to melt.

Ammonium persulphate, 10% (w/v)

1 g Ammonium persulphate (Sigma-Aldrich, (SIGMA), Auckland New Zealand,
Cat. No. A3678)

10 mL Distilled H₂O

Stored at -20°C in 1 mL aliquots until required.

Ampicilin (SIGMA, Cat. No. A9393).

Biocore running buffer, 2x

1.99 g	EDTA	} From the laboratory of Dr T. D. Mueller, Department of Plant Physiology and Biophysics, Julius-von Sachs Institute of the University Wuerzburg, D-97082, Wuerzburg, Germany
58.44 g	NaCl	
5.21 g	Hepes	
1.0 ml	Tween 20	
1000 mL	MilliQ water	

The solution was pH to 7.4 with 2 M NaOH and passed through a 0.22 µm filter. 100 mL of this solution was removed and stored as 2x strength. The remaining solution was diluted with 900 mL MilliQ water to give 1x strength.

Bis(sulfosuccinimidyl)suberate (BS³)

(Life Technologies NZ Ltd, Auckland New Zealand, Cat. No. 21580)

Bromophenol blue, 1.0% (w/v)

0.1 g Bromophenol blue (SIGMA; Cat. No. B0126)

Reconstituted in 10 mL of distilled H₂O.

Dissection medium A (Medium A; DMA; for rat granulosa cells)

1x bottle M199 (SIGMA, Cat No.M5017)

4.76 g Hepes (SIGMA, Cat No. H3784)

10 mL PenStrep (Life Technologies Ltd, Cat. No. 15070-063)

900 mL Distilled H₂O

The solution was adjusted to pH 7.3 with 2 M HCl and adjusted to a final volume of 1 L.

The solution was then passed through a 0.22 µm filter into a sterile 1 L glass bottle.

The osmolarity was tested to ensure that it read between 290 and 310 Osm (Fiske OSTM osmomoter; Fiske Associates, Needham Heights, Massachusetts). If required, the osmolarity was adjusted using a sterile solution of 2.5 g/mL NaCl (SIGMA, Cat. No. S3014).

Dissection medium C (Medium C; for oocyte recovery)

500 mL M199 (Life Technologies Ltd, Cat. No. 1150-059)

4.76 g Hepes (SIGMA, Cat No. H3784)

5 mL PenStrep (Life Technologies Ltd, Cat. No. 15070-063)

5 mL Glutamax (100x)

The Hepes was dissolved in 50 mL M199, passed through 0.22 µm filter and added back into remaining 500 mL M199.

EDTA, 0.5M

73.06 g Ethylenediaminetetraacetic acid, Disodium salt (BDH, Global Science, Auckland New Zealand, Cat. No. 100935V)

450 mL Distilled H₂O

The solution was adjusted to pH 8.0 prior to additional distilled H₂O being added to a final volume of 500 mL.

Electical chemi-luminescence (ECL) reagents:**250 mM Luminol**

0.44 g Luminol; 5-amino-2,3-dihydro-1,4-phthalazinedione (SIGMA, Cat. No. 45106182)

10 mL Dimethyl sulphoxide (DMSO), (SIGMA, Cat. No. D2650)

Stored at -20°C in 100 µL aliquots.

90 mM p-coumaric acid

0.15 g p-coumaric acid (SIGMA, Cat. No. C9208)

10 mL Dimethyl Sulphoxide (DMSO), (SIGMA, Cat. No. D2650)

Stored at -20°C in 44 µL aliquots

1M Tris, pH 8.5

30.29 g Tris(hydroxymethyl)aminomethane Life Technologies NZ Ltd, Cat. No. 15504-020)

200 mL Sterile MilliQ water

Adjusted pH to 8.5 with HCl before a final volume of 250 mL was reached with sterile MilliQ water.

Solution A

8.85 mL MilliQ H₂O

1 mL 1 M Tris, pH 8.5

100 µL 250 mM lumiol

44 µL 90 mM p-coumaric acid

Solution B

9.0 mL MilliQ H₂O

1 mL 1 M Tris, pH 8.5

6 µL 30%_(v/v) Hydrogen peroxide (Scharlau, Global Science, Auckland New Zealand, Cat. No. H101361000)

Immediately prior to use, Solutions A & B were mixed together and poured over the nitrocellulose membrane.

Expression medium (HEK293 cells)

100 µg	Heparin (SIGMA, Cat. No. H3149)
0.01 g	Bovine serum albumin (SIGMA, Cat. No. A450)
1 mL	Penicillin (5000 IU/mL) and streptomycin (5mg/mL) (PenStrep; Life Technologies NZ Ltd, Cat. No. 15070063)
1 mL	Glutamax (100x)
98 mL	Dulbecco's Modified Eagle Medium (D-MEM):Hams F12 (1:1), Life Technologies NZ Ltd (Cat. No. 12634010)

Fugene HD Roche, Auckland, New Zealand (Cat No. 04709705001)

Glutamax (100x) L-alanyl-L-glutamine dipeptide; Life Technologies Ltd, (Cat. No. 35050061)

Growth medium (HEK293 cells)

10 mL	Fetal Calf Serum (FCS; Life Technologies NZ Ltd)
10 mL	Penicillin (5000 IU/mL) and streptomycin (5mg/mL) (PenStrep; Life Technologies NZ Ltd, Cat. No. 15070063)
10 mL	100x Glutamax (Life Technologies NZ Ltd, Cat. No. 35050061)
1000 mL	Dulbecco's Modified Eagle Medium (D-MEM; Life Technologies NZ Ltd Cat. No. 11965-092)

Adjusted pH to 7.3 with 5 M HCl, filtered through a 0.2 µm filter into a sterile bottle and stored at 4°C.

Incubation medium D (Medium D; for oocyte incubations)

500 mL	M199 (Life Technologies Ltd, Cat. No. 1150-059)
1.1 g	Sodium bicarbonate (SIGMA Cat. No. S5761)
5 mL	PenStrep (Life Technologies Ltd, Cat. No. 15070-063)
5 mL	Glutamax (100x)

Sodium bicarbonate was dissolved in 50 mL M199 then passed through 0.2 µm filter back into remaining 500 mL M199. Stored at 4°C.

Incubation medium B (Medium B; 3H–thymidine incorporation bioassay)

500 mL McCoy's 5a medium (Life Technologies Ltd, Cat. No.16600-082)

5 mL PenStrep (Life Technologies Ltd, Cat. No. 15070-063)

1.1 g Sodium Bicarbonate (SIGMA Cat. No. S5761)

Sodium bicarbonate was dissolved in 50 mL M199 then passed through 0.2 µm filter back into remaining 500 mL M199. Stored at 4°C.

On day of use:

50 mL McCoy's 5a medium containing NaHCO₃ and PenStrep

0.5 mL 30 mg/mL poly(vinyl alcohol)

Passed through 0.22 µm filter into tissue culture flask. Equilibrated to 37°C and 5% CO₂, for at least 4 hours prior to use.

LB Agar

1 L LB Broth

15 g Agar (Becton Dickinson, Auckland, New Zealand, Cat. No. 214010)

Autoclaved to dissolve and sterilise.

LB Broth (Lysogeny broth; (Bertani 2004)

5 g Yeast extract (Becton Dickinson, Cat. No. 212750)

10 g Bacto-tryptone (Becton Dickinson, Cat. No. 211705)

10 g Sodium Chloride (SIGMA, Cat. No. S3014)

1 L Distilled H₂O

LigaFast™ Rapid DNA Ligation System

(PROMEGA, Sydney, Australia, Cat. No. M8221)

Poly(vinyl alcohol) – 30mg/mL

30 mg Poly(vinyl alcohol) (SIGMA, Cat. No. P8136)

10 mL Distilled H₂O

Dissolved, passed through a 0.2 µm filter and store at 4°C.

Low salt buffer

20 mL 1 M Tris-HCl, pH 7.5
0.876 g NaCl (SIGMA, Cat. No. S3014)
1 mL Tween 20 (SIGMA, Cat. No. P5927)
950 mL Distilled H₂O

Adjust pH if necessary and make up to 1000 mL with distilled H₂O.

Peroxidase conjugated rabbit anti mouse IgG

Jackson Immuno Research Laboratories Ltd, West Grove, PA,
(Cat. No. 315-035-045)

Protease inhibitors (26x)

1x tablet Complete, EDTA-free protease inhibitor cocktail
1.92 mL Distilled Q H₂O

Divided into 50 µL aliquots and stored at -20°C.

Phosphate buffered saline, pH7.4

1x tablet SIGMA, Cat. No. P4417
100 mL Distilled Q H₂O

Sterilised by autoclave and stored at room temperature

Puromycin dihydrochloride (10mg/mL)

25 mg Puromycin dihydrochloride (Sigma-Aldrich, Auckland New Zealand, Cat.
No. P8833)

2.5 mL Sterile distilled water

Stored at -20°C in 500 µL aliquots.

QIAprep spin miniprep kit (QIAGEN Pty. Ltd., Vic, Australia, Cat. No. 27106)**QIAprep gel extraction kit (QIAGEN Pty. Ltd., Vic, Australia, Cat No. 28704)****Restriction enzymes:**

EcoRI (Roche, Auckland, New Zealand, Cat No. 107303737001)

XbaI (Roche, Cat No. 10674257001)

XhoI (Roche, Cat No. 1703770001)

Scintillation fluid (Beta Scint) (Perkin Elmer; Sci-Med, Auckland New Zealand,
Cat. No. SC/9200.21)

SDS PAGE running Buffer, pH 8.3, (5x)

360 g Glycine (Scharlau, Global Science, Auckland New Zealand, Cat. No.
AC04041000)
75 g Tris(hydroxymethyl)aminomethane (SIGMA; Auckland, New Zealand)
25 g Sodium Dodecyl Sulphate.
4500 mL Distilled H₂O
Adjusted pH to 8.3 with HCl and adjusted to a final volume of 5 L. The solution was
diluted 1:5 before use with MilliQ H₂O.

Sodium Dodecyl Sulphate, 10% (w/v)

10 g Sodium dodecyl sulphate (BDH; Global Science, Auckland New Zealand,
Cat. No. 301754L)
100 mL Distilled H₂O

SYBR® Green (Life Technologies NZ Ltd, Auckland, New Zealand
Cat. No. S7567)

TAE Buffer (Tris-Acetate, EDTA, 50x)

242 g Tris(hydroxymethyl)aminomethane Life Technologies NZ Ltd, Cat. No.
15504-020)
57.1 mL 17.4 M Glacial Acetic Acid (Scharlau, Cat. No. AC03442500)
100 mL 0.5 M EDTA, pH 8.0
Make up to 1000 mL with distilled H₂O.
Diluted 1:50 with distilled H₂O before use.

³H-Thymidine (~1 Ci in 1 ml) Perkin Elmer (Cat. No. NET3555001MC; Sci-Med,
Auckland New Zealand)

Used 0.4 µCi in each well (i.e. per 20 000 live cells).

Trifluoroacetic acid (SIGMA-Aldrich, Cat. No. 302031)

Tris-HCl, 0.5M, pH6.8

6.06 g Tris(hydroxymethyl)aminomethane Life Technologies NZ Ltd, Cat. No. 15504-020)

80 mL Distilled H₂O.

Adjusted pH to 6.8 with HCl, then diluted to a final volume of 100 mL.

Tris, 1M, pH 7.5

30.29 g Tris(hydroxymethyl)aminomethane (Life Technologies NZ Ltd, Cat. No. 15504-020)

200 mL Sterile distilled water

Adjusted pH to 7.5 with HCl and diluted to a final volume of 250 mL with sterile MilliQ water.

Tris-HCl, 1.5M, pH8.8

36.34 g Tris(hydroxymethyl)aminomethane Life Technologies NZ Ltd, Cat. No. 15504-020)

160 mL Distilled H₂O.

Adjusted pH to 8.8 with HCl, then diluted to a final volume of 200 ml.

Tris-glycine transfer buffer (10 x)

30.3 g Tris(hydroxymethyl)aminomethane Life Technologies NZ Ltd, Cat. No. 15504-020)

144 g Glycine (Scharlau, Global Science, Auckland New Zealand, Cat. No. AC04041000)

1000 mL Milli Q water.

1x Tris-glycine transfer buffer

300 mL 10 x Tris-glycine stock

600 mL Methanol (VWR; Global Science, Auckland New Zealand, Cat. No. 20847.320)

2100 mL Distilled water.

Buffer was pre-chilled to 4°C before use.

Trypan blue, 0.4% (w/v) SIGMA, Cat. No. T8154

TrypLE™ Express Life Technologies NZ Ltd, Cat. No. 12605010

Water (HPLC grade) MERCK, Cat. No. 1.15333.2500

Western blotting non-reducing sample buffer with SDS, (5x)

1.0 mL 0.5 M Tris-HCl pH 6.8
0.8 mL glycerol (SIGMA, Cat. No. G5516)
4.6 mL 10% (w/v) Sodium dodecyl sulphate
0.2 mL 1.0% (w/v) bromophenol blue
2.28 mL Distilled H₂O

Western blotting reducing sample buffer with SDS, (5x)

1.0 mL 0.5 M Tris-HCl pH 6.8
0.8 mL glycerol (SIGMA, Cat. No. G5516)
4.6 mL 10% (w/v) Sodium dodecyl sulphate
80 mg Dithiothreitol (BDH; Global Science, Auckland New Zealand, Cat. No. 443852A)
0.2 mL 1.0% (w/v) bromophenol blue
0.8 mL Milli Q H₂O

Stored at -20°C in 300 µL aliquots.

Immediately before use, an aliquot was thawed and 60 µL β-mercaptoethanol (SIGMA Cat. No. 1610710) was added to give a final concentration of 2.3 M.

X-Gal 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside,
(Sigma-Aldrich, Auckland New Zealand, Cat. No. B4252).

A5.2 Hardware

Blotting paper Bio-Rad, Auckland, New Zealand, Cat. No. 1703932

Filter (0.22 µm) Corning Inc., Corning, NY, Cat. No. 431118.

Filter mats (90x120 mm, printed Filtermat A) (Perkin Elmer; Sci-Med, Auckland
New Zealand Cat. No. 1450-421)

HPLC reverse phase column (Jupiter, 5 µm, C4, 300Å; Phenomenix, Lane Cove, NSW,
Australia. Cat. No. OOG-4167-EO)

Tissue culture Flasks (Growth flasks)

T25 (Nunc; Life Technologies NZ Ltd, Cat. No.136196)

T80 (Cat. No.178905)

T175 (Cat. No.178883)

Sample bag Perkin Elmer; Sci-Med, Auckland New Zealand, Cat. No.1450-432

Security guard cartridge Wdepore, C4, Phenomenix, Lane Cove, NSW, Australia.
Cat.No. AJO-4330

6 well culture plate NUNC; Life Technologies NZ Ltd, Cat. No 140675

96 well culture plate JET BIOFIL; Interlab, Wellington, New Zealand, Cat. No.
TCP000096)

Nitrocellulose (pore size 0.45 µm) GE Healthcare Ltd, Auckland, New Zealand,
Cat No. 10600016)

X-ray film BIOMAX XAR film, (KODAK) Carestream Health INC.,
Rochester, NY, Cat. No. 165 1454)

A5.3 Equipment

Beta counter Perkin Elmer; 1450-SLC MicroBeta Trilux; Sci-Med, Auckland,
New Zealand

Centrifugal vacuum concentrator Eppendorf®, model 5301, SIGMA