The effects of oocytes on Smad signaling pathways in granulosa cells

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A thesis submitted to the Victoria University of Wellington in partial fulfillment of the requirements for the degree of Master of Biomedical Science

TE WHARE WĀNANGA O TE ŪPOKO O TE IKA A MĀUI

VICTORIA
UNIVERSITY OF WELLINGTON

Victoria University of Wellington

Te Whare Wānanga o te Ūpoko o te Ika a Māui

2017

Abstract

It is well established that two oocyte-secreted growth factors (OSF), namely growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) regulate the growth of ovarian follicles and ovulation rate. Moreover, it has been reported that the gene expression ratio of *Gdf9:Bmp15* varies between species and may, at least in part, influence litter size in mammals. Generally, poly-ovulatory and mono-ovulatory species express a high and low ratio of *Gdf9:Bmp15,* respectively. Intriguingly pigs, a poly-ovulatory species, have a low *GDF9: BMP15* ratio and are the exception to this rule. To further our understanding of species differences in oocyte-secreted growth factors (OSF), and how this impacts follicular growth, oocytes and follicular cells (granulosa cells; GC) from a range of species that differ in litter size were cultured together. The aims of this study were to perform same- and cross-species co-incubations of oocytes with GCs to elucidate species-specific effects of OSF on (i) GC proliferation rate, (ii) expression levels of GC type I and type II receptors and (ii) activation of Smad signaling pathways in rats, sheep, pigs and red deer. The methodologies utilised were tritiated thymidine incorporation assays, qPCR and Western blotting, respectively.

The co-incubation studies revealed in general, that rat GC are more proliferative than GCs from any other species tested, especially with time in culture. Moreover deer oocytes, which express negligible *GDF9* mRNA, are unable to stimulate proliferation in rat GCs to the same extent as oocytes from the other species. This supports previous studies that suggest that rat GC only require GDF9 to proliferate, whilst both BMP15 and GDF9 are able to stimulate the GCs of sheep. This may illustrate that these two OSF play divergent roles in folliculogenesis dependant on the species.

Interestingly, deer oocytes decreased *Bmpr1b* mRNA expression in rat GC but increased *BMPR1B* mRNA expression in pig GC. This may suggest that whilst the rat and pig are both poly-ovulatory species, their GCs respond in different ways to negligible amounts of GDF9, compared to BMP15. Although deer oocytes differentially affected receptor expression levels in GCs of various species, they were able to increase Smad 1/5/8 levels in both rat and deer GCs, illustrating that the BMP15 secreted by deer oocytes utilise this signaling pathway.

The cross-species incubations of rat GCs with either rat, sheep or pig oocytes stimulated proliferation to a high extent. However, sheep oocytes concomitantly increased *Tgfβr1* expression, and increased Smad 2/3 in rat GCs, whilst rat and pig oocytes did not. This suggests that OSF from different species may elicit proliferation by utilising different type I receptors and signaling pathways.

In summary, this study illustrated that GCs of rat were different to that of the other species tested due to their suspected inability to respond to the proliferative actions of BMP15. Moreover, species-specific differences exist in the ability of OSFs to stimulate GC proliferation and activate downstream signaling pathways.

Acknowledgements

Last but not least, I would like to thank my friends, my cousins and my aunties and uncles, for their encouragement and understanding when things got busy. I would especially like to thank my parents, Demetrius and Maria, for encouraging my study and their unfailing support.

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ActRI	Activin receptor type I
ActRIB	Activin receptor type I B
ActRII	Activin receptor type II
ActRIIB	Activin receptor type II B
ACVR1	Activin A receptor type 1
ACVR1B	Activin A receptor type 1B
ACVR1C	Activin A receptor type 1C
ACVRL1	Activin A receptor type II like I
ACVR2A	Activin A receptor type IIA
ACVR2B	Activin A receptor type IIB
ALK	Activin receptor like kinase
ALK5	Activin receptor like kinase-5 (aka TGFβR1)
ALK6	Activin receptor like kinase-6 (aka BMPR1B)
AMH	Anti-Mullerian hormone
ANOVA	Analysis of variance
AROM	Aromatase
BAMBI	BMP and activin membrane bound inhibitor
BIM_{EL}	BCL-2-interacting mediator of cell death-extra long
BMP15	Bone morphogenetic protein 15
BMP1B	Bone morphogenetic protein 1B
BMPR2	Bone morphogenetic protein receptor 2
cDNA	Complimentary deoxyribonucleic acid
COC	Cumulus-oocyte complex
Co-Smad	Common mediator Smad
CPM	Counts per minute
C_T	Cycle threshold
DTT	Dithiothreitol
ERK1/2	Extracellular signal-regulated kinases
FAST	Forkhead activin signaling transducer
$FecX^I$	Fecundity gene Inverdale on X chromosome
GC	Granulosa cell
GDF9	Growth differentiation factor 9
GDNF	Glial cell-derived neurotrophic factor
hCG	Human chorionic gonadotropin
I-Smad	Inhibitory Smad
IVF	*In vitro* fertilisation
IVM	*In vitro* maturation
LAP	Latency-associated peptide
LH	Luteinising hormone
LSD	Least significant difference
MAPK	Mitogen-activated protein kinase
MH	Mad-homology domain
mRNA	messenger Ribonucleic acid
NCBI	National Center for Biotechnology Information
NF-κB	Nuclear factor- κB
OOX	Oocytectomised-cumulus cell complex

OSF	Oocyte secreted factor
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PI3K	Phosphatidylinositol-3-kinase
qPCR	Quantitative polymerase chain reaction
RPL19	Ribosomal protein L19
R-Smad	Receptor activated Smad
RTK	Receptor protein tyrosine kinase
SARA	Smad anchor for receptor activation
StAR	Steroidogenic acute regulatory protein
SDS	Sodium dodecyl sulfate
SBE	Smad binding element
SEM	Standard error of the mean
SMAD	Similar to mothers against decapentaplegic protein
Smurf	Smad ubiquitination regulatory factor
TGFβ	Transforming growth factor β

1.0 Introduction

Growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) are members of the transforming growth factor-β (TGFβ) superfamily. Interestingly, they appear to utilise distinct signaling pathways, firstly binding a common Type 2 receptor and then recruiting unique Type 1 receptors to activate different down-stream Smad proteins. The biological actions of these two oocyte-secreted growth factors facilitate ovarian follicular development and regulate ovulation rate and litter size in mammals. The expression, secretion and biological activity of both GDF9 and BMP15 have been reported to be specific to each mammalian species tested. The aim of this study was to cross-incubate oocytes with granulosa cells of different species to observe the regulation of granulosa cell proliferation and downstream signaling by species-specific oocyte secreted factors, namely GDF9 and BMP15.

1.1 The TGFβ Superfamily

1.1.1 Molecular Forms of the TGFβ Superfamily Members

The transforming growth factor-β (TGFβ) superfamily includes over 30 structurally related polypeptides such as TGF-βs, activins, inhibins, growth differentiation factors and bone morphogenetic proteins, which are conserved across species (Massague, 1998, Chang et al, 2002, ten Dijke et al, 2002, Shi and Massague, 2003, Mueller and Nickel, 2012). The cellular processes that this superfamily of structurally-related ligands are involved in include development (Jones et al, 1996, Dudley et al, 1995, Luo et al, 1995, Winnier et al, 1995, Celá et al, 2016), wound healing (Carrington et al, 2006, Sefat et al, 2014, Yang et al, 2016), immune surveillance (Rich et al, 1995, Tiemessen et al, 2003, Takai et al, 2010), and reproductive function (Yan et al, 2001, Juengel et al, 2009, Gao et al, 2015). Using possible phylogenetic relationships, the members of the superfamily have been classified into five groups (INHα, MIS, TGFβs, BMPs and INHβs), as well as several subgroups (Burt, 1992). As reviewed by Knight and Glister (2006), analysis of additional structural characteristics allows the further classification of subfamilies. These include the TGFβ (including TGFβ1, TGFβ2, and TGFβ3), the activin/inhibin, the glial cell-derived neurotrophic factor (GDNF), the bone morphogenetic protein (BMP)

and the growth and differentiation factor (GDF) subfamilies. The BMP and GDF subfamilies comprise of at least 20 and nine members, respectively.

TGFβ superfamily members are translated as prepropeptide precursors prior to their processing and secretion (Chang et al, 2002). Several experiments have detected the secretion of latent TGFβ, with its biological activation being observed upon release from its latent form through acidification (Pircher et al, 1984, Lawrence et al, 1985, Pircher et al, 1986). Thus, it was hypothesised that latent TGFβ is comprised of TGFβ non-covalently associated with its precursor (Lawrence et al, 1985, Wakefield et al, 1988), a dimer now known as the latency associated peptide (LAP) (Chang et al, 2002). Inactive TGFβ also contains a latent TGFβ binding protein (LTBP), which is comprised of TGFβ1 bound to a complex of components which are disulfide-linked (Miyazono et al, 1988). The integrin $\alpha_v\beta_6$ is able to recognise an amino acid sequence on LAP. However for $\alpha_v\beta_6$-mediated activation of TGFβ, LTBP-1 is required, with this process being LTBP isoform specific (Annes et al, 2004).

As reviewed by Taylor et al (2003), proprotein convertases (PC) are a family of endoproteases, which cleave at basic motifs, changing the precursor molecule to an active protein. Such proprotein convertases include PC1, PC2, PC4, PC6B, furin and LPC, with the convertases' predicted substrates including TGFβ superfamily members. The expression of the proprotein convertases PC1 and PC2 in a recombinant vaccinia virus vector allows the processing of proopiomelanocortin at pairs of basic residues (Benjannet et al, 1991). The pro-protein convertase furin is able to convert pro-TGFβ1 into its mature monomer and pro-region (Dubois et al, 1995), having been verified as an endogenous TGFβ1 convertase (Dubois et al, 2001). In comparison, furin inefficiently cleaves TGFβ2, with the tertiary structure of the LAP regions influencing the ability of furin to to cleave TGFβs (Kusakabe et al, 2007).

The prodomains of the TGFβ family are described as having low to unrelated sequence homology, with their length varying from 169 to 433 residues (Shi et al, 2011). Although the pro-domain of TGFβ is poorly conserved across various family members, the mature

region is more highly conserved. Seven cysteine residues within the mature region are present in almost all members of the family (Lee, 1990, Daopin et al, 1992, Kingsley, 1994). The pro-regions of several members of the TGFβ superfamily, such as activin A and TGFβ1, are important in aiding the folding, disulfide bond formation and export of their homodimers (Gray and Mason, 1990). Analysis of the crystal structure of TGFβ2 illustrates that this protein's overall structure consists of two finger-like β strands and loops, attached to a α3 helix. The core structure is expected to be shared amongst members of the TGFβ superfamily (Daopin et al, 1992), with BMP2, BMP7 and the TGFβs sharing a cysteine-knot motif core, described as a palm, and finger-like β sheets (Scheufler et al, 1999).

1.2 Receptors of the TGFβ Superfamily Members

Members of the TGFβ superfamily act through the binding of receptors that have intrinsic serine/threonine kinase activity (Chang et al, 2002). As reviewed in Massague, (1998), and Chang et al, (2002), the receptors of the TGFβ superfamily have two subfamilies, the type I and type II receptors. In mammalian species, the type I receptors are subdivided into three groups. The three groups include: 1) activin receptor-like kinase 5 (ALK5; also known as TGFβR1), ALK4 (also known as ACVR1B) and ALK7; 2) ALK3 (also known as BMPR1A) and ALK6 (also known as BMPR1B) and: 3) ALK1 and ALK2.

Cloning of the TGFβ type I receptor revealed an open reading frame of 509 amino acids, divided into a 363-and 123-amino acid cytoplasmic and extracellular domain, respectively. The core protein of this receptor is around 55 kDa (Ebner et al, 1993). The TGFβ type II receptor has also been isolated and cloned, illustrating that it is a ~ 80 kDa protein (Lin et al, 1992). The type I and II receptors share sequence similarity in their extracellular domains, both having three cysteines upstream of their transmembrane domain (Ebner et al, 1993). TGFβ receptor I has a unique glycine/serine-rich sequence known as the GS-domain. Mutational analysis has demonstrated that the presence of the amino acids serine and threonine in the GS-domain of TGFβ receptor I functions in phosphorylation and signaling (Wrana et al, 1994, Wieser et al, 1995). For example,

TGFβ1 is able to elicit phosphorylation of Ser165 of TGFβ receptor I, and the GS domain of this receptor, which is required for signaling (Souchelnytskyi et al, 1996).

The TGFβ type II receptor contains functional kinase domains, and is constitutively active, evidenced by high phosphorylation without the addition of TGFβ (Lin et al, 1992, Wrana et al, 1994, Souchelnytskyi et al, 1996). Thus, its autophosphorylating activity is not activated through ligand binding (Wrana et al, 1994). TGFβ binds to receptor II; it is recognised by receptor I, which in turn is phosphorylated by receptor II. A signaling cascade then begins after receptor I propagates the signal to the following substrates (Wrana et al, 1994). Furthermore, the binding of TGFβ to the type II receptors may require the type I receptor, which could also be important for transport of the type II receptor to the cell surface. Stoichiometric ratios between the type I and II TGFβ receptors may further mediate TGFβ binding (Ebner et al, 1993).

TGFβIII (betaglycan) has membrane proteoglycan properties and its binding site resides in the 100- 120 kDa core polypeptide (Cheifetz et al, 1988). The TGFβ receptor type III does not have a recognisable kinase domain, however, it does mediate TGFβ binding to the type II receptor, which is especially important for TGFβ2 (Lin et al, 1992, Brown et al, 1999, Shi and Massague, 2003). Betaglycan also acts as a co-receptor with ActRII for inhibin. It is able to bind inhibin with high affinity, as well as forming crosslinked complexes, mediating the functional antagonism of activin signaling (Lewis et al, 2000).

1.3 Growth Differentiation Factor 9 & Bone Morphogenetic Protein 15

1.3.1 Protein Structures of GDF9 & BMP15

Human, murine and ovine GDF9 spans approximately 2.5 kb, containing 1 intron and 2 exons (Incerti et al, 1996; Bodensteiner et al, 1999). Overall, the structure of BMP15 is similar to that of other TGFβ superfamily members, and is most closely related to GDF9, with the two sharing high sequence homology (Dube et al, 1998). BMP15 also has two exons, separated by one intron, encoding a pre-propeptide of 393 amino acids. Its secondary structure is comprised of one α-helix and two fingers, with two anti-parallel β-strand segments (Liao et al, 2003, Galloway et al, 2000).

GDF9 shows close homology to other known members of the TGFβ superfamily, with the presence of a COOH-terminal region. The COOH-terminal region of BMP15 shares 53% homology with that of GDF9. GDF9 however lacks the conserved cysteine residue common between other TGFβ family members, instead having six cysteine residues and four potential N-glycosylation sites (McPheron and Lee, 1993, Bodensteiner et al, 1999). Similarly to GDF9, BMP15 also lacks this cysteine, with a serine residue being present instead of the fourth conserved cysteine (Laitinen et al, 1998). The fourth cysteine residue in most TGFβ superfamily members forms a disulfide bond, covalently linking the two subunits (Vitt et al, 2001).

The post-translational processing of GDF9 and BMP15 affect the biological activity of these proteins. BMP signaling is likely regulated through the activity of proprotein convertase activity (Constam and Robertson, 1999). For example, GDF9 and BMP15 are more susceptible to proteolytic cleavage as individual homodimers compared to proprotein heterodimers. This is illustrated as the co-expression of these two proteins significantly impaired the processing of these proproteins. This may be due to endopeptidases having a decreased ability to cleave the proproteins (Liao et al, 2003).

In the mouse and human, a possible five N-linked glycosylation sites have been observed for BMP15. Between the two species, three of these glycosylation sites are conserved. However, BMP15 differs between the two species in that upstream of the first conserved cysteine, mouse BMP15 has two additional cysteines (Dube et al, 1998). Discrepancies in BMP15 glycosylation occur between species, which may also affect their biological activity. For example, the treatment of the mature proteins with the endoglycosidase PNGase F, which releases N-linked oligosaccharides, has revealed that whilst mouse BMP15 contains N-glycosylation sites, the mature protein of human BMP15 does not (Hashimoto et al, 2005). The biological activity of GDF9 and BMP15 is also affected by protein phosphorylation, with GDF9 and BMP15 being the first phosphoproteins to be observed in the TGFβ superfamily, as phosphorylation is needed for their bioactivity (McMahon et al, 2008b).

Interactions between the pro and mature regions of TGFβ superfamily proteins are crucial in the assembly of homodimers and protein secretion (Gray and Mason, 1990, Walton et al, 2009). When transfected 293H cells secreted BMP15 and GDF9, GDF9 homodimers were observed, however BMP15 homodimers were absent, with BMP15 being observed as monomeric proregion, mature, and multimeric protein forms (McIntosh et al, 2008). Previous studies demonstrated that when expressed individually, BMP15 and GDF9 were able to form non-covalent homodimers, however when co-expressed, GDF9: BMP15 heterodimers were observed (Liao et al, 2003). Utilising granulosa cell (GC) and cumulus cell expansion assays, Peng et al (2013) showed that GDF9: BMP15 heterodimers were the most bioactive ligands in humans and mice, compared with mouse GDF9 homodimers, or human BMP15 homodimers. These heterodimers are thought to act through ALK 4/5/7 and bone morphogenetic receptor type 2 (BMPR2), activating the Smad2/3 pathway. However, due to BMP15 and GDF9 mature regions not forming covalent dimers, equilibrium between monomers, heterodimers and homodimers may occur, influencing results. Thus it can also not be assumed that cooperative effects of GDF9 and BMP15 in studies monitor the actions of two homodimers (Mottershead et al, 2013).

1.3.2 Localisation of GDF9 & BMP15

Expression of *GDF9* and *BMP15* mRNA is present exclusively in oocytes in sheep and murine species (Bodensteiner et al, 1999, Galloway et al, 2000, Crawford et al, 2012, Laitinen et al, 1998, Mester et al, 2014). Conversely, studies indicate the expression of *GDF9* and *BMP15* in the GC of several other species such as the pig, goat and primates (Duffy, 2003, Silva et al, 2005, Prochazka et al, 2004, Paradis et al, 2009). In rat oocytes, *Gdf9* is expressed from the one-layer primary follicle stage, however the most intensive immunostaining was observed in primary and preantral follicles, compared to antral follicles (Jaatinen et al, 1999). In sheep however, *GDF9* is first expressed in primordial (type 1) follicles (Bodensteiner et al, 1999). Immunohistochemistry studies indicate the presence of porcine GDF9 in follicles at all developmental stages of folliculogenesis (Sun et al, 2010).

It is demonstrated through the use of Northern blot and *in situ* hybridisation that the mRNA expression pattern for *Bmp15* is similar to that of *Gdf9* in mice, whereby *Bmp15* expression also begins at the one layer primary follicle stage, and is expressed throughout follicular development to ovulation (Dube et al, 1998, Jaatinen et al, 1999). In sheep, *BMP15* mRNA is expressed exclusively in the oocyte from the type II, or primary stage of folliculogenesis (Galloway et al, 2000). Furthermore in the porcine follicle, *BMP15* mRNA expression is present in primordial follicles onward (Sun et al, 2010), with the abundance of *BMP15* mRNA decreasing from the recruitment phase of folliculogenesis to the post-LH surge (Paradis et al, 2009). Thus in summary, these studies together demonstrate that the timing of expression of *GDF9* and *BMP15* during folliculogenesis is species-specific.

GDF9 and BMP15 both bind BMPR2 (Vitt et al, 2002), with oocytes and GC from all mammalian species studied expressing *BMPR2* mRNA, indicating the presence of an autocrine/paracrine system in the ovary (Shimasaki et al, 1999, Wilson et al, 2001, Glister et al, 2004, Paradis et al, 2009). Through *in situ* hybridisation, it was observed that *BMPR1B* (*ALK6*) is expressed in the oocyte and granulosa cells in sheep (Wilson et al, 2001). This localisation pattern of receptor gene expression is similar in the rat; with *in situ* hybridisation experiments illustrating the localisation of *BMPR1B* mRNA to both oocytes and granulosa cells in the rat follicle (Shimasaki et al, 1999). The *TGFBR1 (ALK5)* gene is expressed in the oocyte, granulosa cells and theca interna of sheep (Feary et al, 2007). Rat GC express *Tgfβr1*, which is required for GDF9 signaling. This is demonstrated through *Tgfβr1*, siRNA transfection into GCs decreasing GDF9 signaling (Mazerbourg et al, 2004). Both *TGFβR1*, and *BMPR1B* have been found in porcine oocytes and GC (Paradis et al, 2009, Sun et al, 2010). Thus, as seen through localisation patterns in the follicle, BMP15 and GDF9 and their receptors are crucial for ovarian function.

1.4 Function of GDF9 & BMP15

1.4.1 Information from Sheep Mutants and Mouse Knockouts

There are species differences in the required dose of BMP15 and GDF9 for folliculogenesis. The importance of BMP15 on GC function differs between high and low ovulation rate species. For example, ovine GC require ovine GDF9 and ovine BMP15 to stimulate GC proliferation. In comparison, rat GC require only rat GDF9 and not BMP15 to proliferate (Lin et al, 2012). This illustrates how low ovulation rate species such as sheep require both GDF9 and BMP15 for folliculogenesis, however BMP15 is not as crucial for rodents. The species differences in the required dose of BMP15 and GDF9 have been demonstrated in a variety of experiments using animals with natural mutations, knockout models and immunisation.

The importance of GDF9 in high ovulation rate species, and in female fertility, has been demonstrated in knockout mice models. Homozygous GDF9-knockout (GDF9$^{-/-}$) mice, with a targeted deletion of exon 2 encoding the mature region of GDF9, are infertile, with hypoplastic ovaries. Infertility is due to an arrest in folliculogenesis; however mice are hypergonadotrophic. Due to altered negative feedback of gonadal hormones, a three-fold elevation in FSH serum levels and a two-fold elevatation in luteinising hormone (LH) serum levels were measured in these mice compared to the wild type (Dong et al, 1996). GDF9 is required for the formation of corpora lutea and the formation of normal follicles past the primary one layer follicle stage (Dong et al, 1996). However, despite follicle growth arresting at the type 3b stage, mouse oocytes deficient in GDF9 grow at a faster rate compared to control oocytes. The oocytes from these GDF9 homozygous mutant mice also acquired meiotic competence in a manner similar to heterozygous control mice. Many of these GDF9 deficient oocytes were unable to complete maturation *in vitro,* whilst spontaneously activating, suggesting their inability to maintain meiotic arrest (Carabatsos et al, 1998)*.* In summary, these results illustrate that in the mouse, which is a poly-ovulatory species, GDF9 is a requirement for early folliculogenesis (Dong et al, 1996).

Whilst GDF9 is a requirement for fertility in females, demonstrated in homozygous mutant mice, homozygous BMP15-knockout (BMP15$^{-/-}$) mice were subfertile, showing a reduced ovulation rate. Whilst ovaries from GDF9$^{-/-}$ mice were small due to early perturbation in folliculogenesis, in comparison, the ovarian histology of BMP15$^{-/-}$ mice is normal. Thus, while BMP15 is important in mice fertility, it is not as crucial as GDF9 (Yan et al, 2001). In contrast to this, oocyte-specific overexpression of BMP15 in mice led to accelerated follicle development, with GCs displaying a higher mitotic index compared to wild type mice. Interestingly, these transgenic mice had a normal litter size (McMahon et al, 2008a). To determine the importance of BMP15 on ovulation quota, the molecular characteristics of BMP15 have been compared in high and low ovulating species. Mouse BMP15 was not detected in conditioned media when cells were transfected with a BMP15 expression vector. However, this absence may be due to the post-translational processing of mouse BMP15, as mouse BMP15 proprotein was present in transfected cells similarly to the expression of human BMP15 proprotein. This lack of secreted mouse BMP15 is likely due to the pro-region impairing secretion through defective post-translational processing (Hashimoto et al, 2005). However, the secretion of mouse BMP15 from transfected 293H cells has been observed in another study (McIntosh et al, 2008), which may be due to differences in experimental protocol. Therefore mouse knockout models illustrating the lesser importance of BMP15 compared to GDF9 in mouse folliculogenesis are supported by a lack of secreted mouse BMP15 mature protein being observed.

Similarly to mice, GDF9 is also required for folliculogenesis in low ovulation rate species, such as the sheep. This has been concluded through the discovery of several genetic lines of sheep that exhibit natural mutations in GDF9. In both Cambridge and Belclare breeds, the mutation of GDF9 in these ewes is associated with sterility. The sterility phenotype is observed in sheep homozygous for a variant in GDF9 (*FecG^H*), illustrating the importance of GDF9 in sheep fertility (Hanrahan et al, 2004). Natural mutations in BMP15 have been discovered in sheep also, including the Hanna, Inverdale, Belclare and Cambridge sheep (Davis et al, 1991, Galloway et al, 2000, Hanrahan et al, 2004). Similarly to natural mutations in GDF9, homozygous mutations in BMP15 in Belclare and Cambridge sheep also cause sterility (Hanrahan et al, 2004). The Inverdale gene in

sheep, with the locus named "*FecX*", is an X-linked prolifacy gene. As the *FecX^I* locus is orthologous to human BMP15 and that such mutations are reported to lead to a loss of BMP15 function, BMP15 was concluded to be essential in sheep fertility (Galloway et al, 2000). Indeed, homozygous carriers of the Inverdale gene have nonfunctional ovaries, with no follicular activity observed (Davis et al, 1991). Some ewes may also develop abnormal nodular structures, which contain granulosa-like cells due to BMP15 deficiencies (Braw-Tal et al, 1993). Moreover, this was also observed in Hanna sheep, which are unrelated but also exhibit a BMP15 mutation resulting in the same phenotype (Galloway et al, 2000). Moreover, the immunisation of ewes against either GDF9 or BMP15 peptide both cause an arrest in folliculogenesis and induced anovulation (Juengel et al, 2002), suggesting that this immunisation induces a similar phenotype to ewes with natural homozygous mutations in GDF9 and BMP15, as described previously. Thus, in mammalian species such as the sheep, which display a low ovulation rate phenotype, both GDF9 and BMP15 are required for normal follicular development.

Sheep with heterozygous mutations in *GDF9* and *BMP15* however display a contrasting phenotype to their homozygous counterparts. Heterozygous mutations in *GDF9* and *BMP15* increasing ovulation rate in Cambridge and Belclare sheep (Hanrahan et al, 2004). Furthermore, sheep heterozygous for both *BMP15* and *GDF9* mutations have an increased ovulation rate, suggesting the additive effects of BMP15 and GDF9 (Hanrahan et al, 2004). An increased ovulation rate is also observed in Inverdale sheep that are heterozygous for *FecX^I* (Galloway et al, 2000). Heterozygous Inverdale ewes have a significantly larger proportion of nonatretic follicles with granulosa cells responsive to LH/human chorionic gonadotropin (hCG) compared to wild type ewes. In contrast, the follicular sensitivity to FSH does not differ between the heterozygous and wild type genotypes (McNatty et al, 2009). Thus, the *BMP15* mutation in heterozygous Inverdale ewes leads to a higher ovulation rate due to earlier acquisition of LH responsiveness by GC (McNatty et al, 2009). In a similar manner to natural heterozygous mutations in sheep, ovulation rate can be increased in ewes through immunisation also. Antibodies blocking the N terminal region of both BMP15 and GDF9 can inhibit the paracrine actions of these proteins. In this manner, decreasing the amount of BMP15 or GDF9 increases ovulation rate up to a minimum threshold (McNatty et al, 2007). An increased

ovulation rate is also observed using short-term (partial) immunisation against BMP15 and GDF9, without effecting fertilisation or embryo survival (Juengel et al, 2004b).

In addition to the natural mutations in *GDF9* and *BMP15* observed in sheep, the mutation of these proteins' receptors also impact fertility in ewes. Booroola ewes have a segregating autosomal gene (*FecB*), which is present on sheep chromosome 6 (Montgomery et al, 1993, Wilson et al, 2001). In humans this corresponds to chromosome 4q22-23, which contains the *BMPR1B* gene. Thus BMPR1B, which is the type I receptor for BMP15, is a candidate gene for *FecB* in sheep (Montgomery et al, 1993, Mulsant et al, 2001, Wilson et al, 2001). The presence of this mutation in sheep increases ovulation and has an additive effect, whereby ovulation rate increases by approximately 1.6 corpora lutea per cycle with each copy an ewe has (Wilson et al, 2001).

1.4.2 Roles of GDF9 and BMP15 in Folliculogenesis

Several studies illustrate the importance of GDF9 in early follicle growth and differentiation. Treatment with GDF9 increases follicular diameter of preantral follicles from rats. Conversely, this was not observed when non-bioactive amino-terminal tagged GDF9 was used (Hayashi et al, 1999). Furthermore in mice, the deletion of an oocyte-specific homeobox gene, *Nobox,* caused a down-regulation of *Gdf9* mRNA expression*,* and an inability of follicles to transition from the primordial stage, in addition to accelerated oocyte loss (Rajkovic et al, 2004). Studies in *Bmp15$^{-/-}$* and *Gdf9$^{+/-}$* mice demonstrate that BMP15 and GDF9 also play important roles in both cumulus expansion and maintaining cohesive interaction between cumulus cells and oocytes (Yan et al, 2001). During folliculogenesis, GDF9 and BMP15 are both potent mitogenic stimulators of GC proliferation. This has been demonstrated through mitogenic bioassays, where GDF9 promoted a dose-dependent increase in DNA synthesis in GC (Gilchrist et al, 2006). BMP15 is reported to stimulate cumulus cell expansion, as well as being a potent stimulator of DNA synthesis and kit ligand (KL) expression in GC (Otsuka and Shimasaki, 2002).

In addition to their proliferative effects during follicular development, GDF9 and BMP15 also impact steroidogenesis in the follicle. Whilst GDF9 stimulates the proliferation of GC, it simultaneously inhibits GC differentiation induced by FSH and suppresses FSH-stimulated oestradiol and progesterone production (Vitt et al, 2000). In human GC cell lines, BMP15, but not GDF9, treatment suppressed progesterone production through the suppression of steroidogenic acute regulatory protein (StAR) and Smad 1/5/8 signaling pathways. Thus, the oocyte prevents premature luteinisation through its regulation of progesterone production (Chang et al, 2013). Other studies support the role of BMP15 in reducing both basal progesterone secretion and basal oestradiol levels in human GC (Prapa et al, 2015). In comparison, GDF9 does not affect basal progesterone production, but does inhibit 8-Br-cAMP-stimulated StAR protein expression and progesterone synthesis (Yamamoto et al, 2002). Therefore, GDF9 and BMP15 stimulate follicular proliferation whilst simultaneously preventing premature differentiation of the follicle, as implicated through their actions on steroidogenesis.

Whilst stimulating follicular development, oocyte-secreted factors also display anti-apoptotic actions in the ovarian follicle, thus promoting follicular survival. In GCs at an early stage of follicular development, GDF9 is able to attenuate apoptosis induced by ceramide (Orisaka et al, 2006). A similar effect is observed with BMP15, which is able to decrease apoptosis in porcine cumulus cells in a dose-dependent manner (Zhai et al, 2013). The secretion of BMPs from the oocyte itself also decreased cumulus cell apoptosis, illustrated through an increased cumulus cell apoptosis following oocyte removal. Thus the oocyte creates an anti-apoptotic gradient, with apoptosis within the cumulus cells of a cumulus cell-oocyte complex (COC) being greater in the outer layers, farthest from the oocyte (Hussein et al, 2005). Oocyte-secreted factors decreased the expression of the proapoptotic BCL-2-interacting mediator of cell death-extra long (BIM$_{EL}$) protein, in the cumulus cells of pigs. In particular, treatment of oocytectomised-cumulus cell complexes (OOX) with GDF9 produced similar BIM$_{EL}$ protein levels (Wang et al, 2013a). These anti-apoptotic capabilities displayed by TGFβ superfamily members have also been studied through their signaling proteins, the Smads. In this manner, the targeting of the inhibitory Smad, *Smad7,* through siRNA increases *Bcl-2* and decreases

Bax RNA levels, which is indicative of the inhibition of apoptosis in porcine GCs (Liu et al, 2014).

As discussed in an earlier section, the importance of BMP15 to folliculogenesis and fertility differs in high or low ovulation species. Similarly, the expression levels of *GDF9* mRNA also differ in low and high ovulating species, with ratios of *GDF9: BMP15* being the same within species, but different between species. For example, *GDF9* mRNA expression levels were found to be higher in high ovulation rate species such as the mouse and rat, compared to low ovulation rate species, such as cows and deer. Conversely, the pig, which is a high ovulation rate species, had a low ratio of *GDF9: BMP15* mRNA in oocytes (Crawford and McNatty, 2012). Other studies also indicate that the oocyte itself is of importance in the determination of ovulation rate in mammalian species. For example, high ovulation rate mutant female mice were generated through an oocyte-specific deletion of T synthase, an enzyme that generates core 1-derived O-glycans (Williams and Stanley, 2008). Furthermore, the high ovulation rate phenotype of mutant mice deficient in core 1-derived *O*-glycans was associated with an increase in the *GDF9:BMP15* expression ratio at diestrus (Grasa et al, 2015).

TGFβ superfamily member expression, as well as expression of their receptors and signaling molecules have also been evaluated in human follicles. Expression levels of *GDF9, BMP15, BMPR2,* and *Smad4* mRNA have differed in primordial to late secondary stage follicles, illustrating their importance in early human folliculogenesis (Kristensen et al, 2014). The importance of these oocyte-secreted factors in follicular development is further validated through the study of humans displaying natural mutations in these TGFβ superfamily members. It has been hypothesised that similarly to mutations of BMP15 in sheep causing hyperproliferacy phenotypes (Galloway et al, 2000, Hanrahan et al, 2004), mutations in BMP15 may cause dizygotic twinning. However, although there was evidence between a *BMP15* variant and dizygotic twinning, after correction for multiple testing, there was no significant association (Zhao et al, 2008). Mutations in *GDF9* have been identified in humans also, such as insertion/deletions and missense alterations, which are related to a higher incidence of dizygotic twinning compared to controls (Montgomery et al, 2004, Palmer et al, 2006). Taken together, these studies

demonstrate the likely importance of GDF9, compared to BMP15, in regulating twinning in humans. As well as increasing ovulation rate in humans, *GDF9* and *BMP15* mutations can also negatively impact fertility. Heterozygous non-conservative substitutions in the pro-region of BMP15 have been identified to cause a reduction in GC growth, and be associated with familial ovarian dysgenesis (Pasquale et al, 2004). Alterations in GDF9 and BMP15 have also been associated with premature ovarian failure in women (Laissue et al, 2006).

In human GC, BMP15 also preferentially binds the BMPR1B and BMPR2 receptors, illustrated through the use of affinity-labeling with [^{125}I]-BMP15 (Pulkki et al, 2012). A homozygous mutation that causes loss of function of BMPR1B has been attributed to causing ovarian dysfunction in a human patient (Demirhan et al, 2005). Furthermore, recently it has been shown that a decrease in BMPR1B receptor density is correlated with an age-induced decrease of the ovarian reserve in IVF patients during pre-ovulatory follicle maturation and dominant follicle selection (Regan et al, 2016). Thus BMPR1B, being the receptor for BMP15 signaling, also plays a concomitant role in human fertility.

1.5 Receptors of GDF9 & BMP15

Crystallographic structural analysis predicts that TGFβ superfamily ligand dimers bind two type I and two type II receptors forming a complex (Nickel et al, 2001, Hart et al, 2002, Allendorph et al, 2006). To aid in the understanding of ligand: receptor interactions, the structures of the type I and type II receptors have been assessed through protein crystallography. The BMPR2 core structure is comprised of a three-finger toxin fold, similar to other TGFβ family receptor ectodomains (Mace et al, 2006). The N-glycosylation pattern of BMPRII has also been studied, showing that asparagines in the ligand-binding domain of BMPRII are glycosylated, aiding in the ability of this receptor to bind BMP2 (Lowery et al, 2014). The TGFβR1 core region of the catalytic domain is comprised of a canonical protein kinase fold, a C lobe involved in substrate recognition and an N lobe, which is required for ATP binding. Structural analysis of TGFβR1's unphosphorylated cytoplasmic domain in complex with the immunophilin

FKBP12 illustrates that FKBP12, an inhibitor of TGFβ signaling, is able to bind to two highly conserved residues, Leu-195 and Leu-196, located at the N-terminal of the GS2 helix (Huse et al, 1999). The specificity of the TGFβ superfamily ligands to their type I receptors is dependent upon ligand and receptor structure, as illustrated with a single residue in the pre-helix loop of GDF5 being responsible for this ligand's specificity to its receptor, BMPRIB (Nickel et al, 2005).

Both GDF9 and BMP15 require BMPR2 for their downstream signaling (Moore et al, 2003, Vitt et al, 2002). This has been validated through a co-precipitation study, as the BMPR2 ectodomain directly interacts with GDF9 (Vitt et al, 2002). Furthermore, the BMPR2 extracellular domain is the most effective in the inhibition of BMP15 bioactivity (Soyun et al, 2001). In addition to GDF9 and BMP15 several other TGFβ superfamily members such as inhibins, BMP2, BMP6, BMP10, GDF2, GDF5, GDF6 and GDF7 also display an affinity for BMPR2 (Ebisawa et al, 1999, Wiater et al, 2003, Mazerbourg et al, 2005, Scharpfenecker et al, 2007, Kirkbride et al, 2008), illustrating the promiscuity of this receptor.
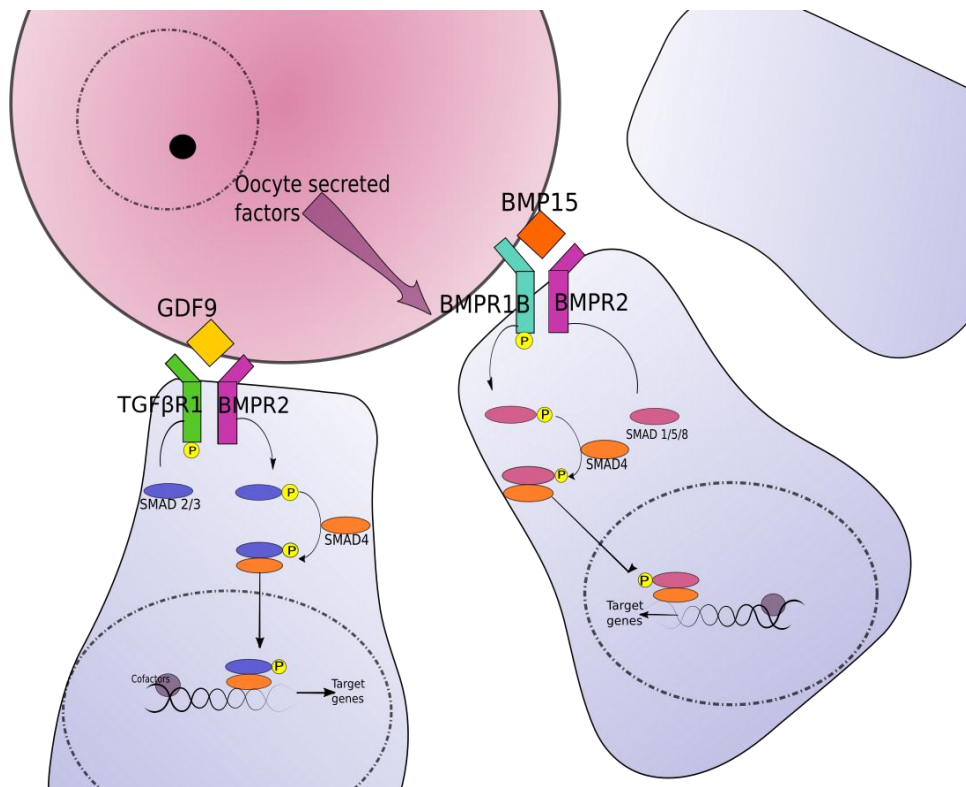
Figure 1.1: Paracrine signaling between oocyte secreted factors and follicular somatic cells.

Paracrine signaling between oocytes and GCs involves interactions between oocyte secreted factors, their receptors, and downstream signaling molecules. Oocytes secrete a range of oocyte secreted factors, two of which are the TGFβ superfamily members GDF9 and BMP15. Follicular somatic cells such as cumulus cells and GCs express several type I (including TGFβR1 and BMPR1B) and type II (including BMPR2) receptors. Smads are intracellular signaling proteins, involved in the signal transduction of oocyte secreted factors. Ligands bind receptor serine/threonine kinases, forming a hetero-tetrameric receptor complex. GDF9 binds BMPR2/TGFβR1, whilst BMP15 binds BMPR2/BMR1B. The GS-domain of the type I receptors is phosphorylated by the type II receptor, creating a pS-x-pS motif which acts as a docking site for receptor-regulated Smads (Smads 2/3 and Smads 1/5/8). In the basal state, Smads are constantly being shuttled in and out of the nucleus. The activation of TGFβR1 and BMPR1B leads to phosphorylation at the C terminus of Smad 2/3 and Smad 1/5/8 respectively. Both Smad 2/3 and Smad 1/5/8 associate with the common Smad4. This complex moves into the nucleus, whereby they interact with specific DNA motifs and encode transcriptional activators. Figure modified from Gilchrist et al (2008) and descriptions from Massague et al (2005) and Gilchrist et al (2008).

Using a CAGA-luciferase reporter, it has been illustrated that the action of GDF9 is mediated by the type I receptor, TGFβR1 (Mazerbourg et al, 2004, Kaivo-Oja et al, 2005). Conversely, the type I receptor for BMP15 has been demonstrated to be BMPR1B, as it's extracellular domain co-immunoprecipitates with BMP15 (Moore et al, 2003). However much cross-reactivity is observed among various BMP ligands and their type-I receptors, as reviewed in Shimasaki et al (2004). For example, whilst TGFβR1 binds GDF9, this receptor also interacts with several other ligands including GDF8, GDF11, TGFβ1 and TGFβ2 (Goumans et al, 2003, Rebbapragada et al, 2003, Andersson et al, 2006, Townsend et al, 2011). Similarly, BMPR1B also binds GDF5, GDF6, GDF7, BMP4, BMP10 and AMH in addition to BMP15 (ten Dijke et al, 1994, Nishitoh et al, 1996, Gouedard et al, 2000, Mazerbourg et al, 2005), illustrating the overall promiscuity of these type I and type II receptors.

1.6 Cellular Signaling Pathways of GDF9 & BMP15

1.6.1 Smad Signaling Pathways

In 1995, the gene *Mothers against decapentaplegic (Mad)* was identified in *Drosophila melanogaster* (Sekelsky et al, 1995). Similarly in *Caenorhabditis elegans, sma-2, sma-3* and *sma-4* genes are homologous to the *Mad* gene product in Drosophila (Savage et al, 1996). These molecules were described as having a relative molecular mass of 42-62 kDa (Chen et al, 1997, Wu et al, 2001b, Hu et al, 2003, Nahar-Gohad et al, 2015). Subsequently, related genes were identified in invertebrates also, and through merging the terms *Sma* and *Mad*, were given the nomenclature of *Smad* (Derynck et al, 1996). Smad proteins are crucial constituents of the signaling cascade, which are activated by members of the TGFβ family. They are recruited to the TGFβ receptor complex as the first step in the TGFβ/Smad signalling pathway, and accumulate in the nucleus, where they are able to regulate the expression of genes (Dennler et al, 1999, ten Dijke et al, 2002, Schmierer and Hill, 2005). Thus, Smads are responsible for signal transmission from transmembrane serine/threonine kinase receptors to the nucleus (Tsukazaki et al, 1998). The nucleocytoplasmic shuttling of Smads is dynamic, with the shuttling of Smads to the nucleus quantitatively reflecting the strength of the extracellular TGFβ signal (Schmierer et al, 2008).
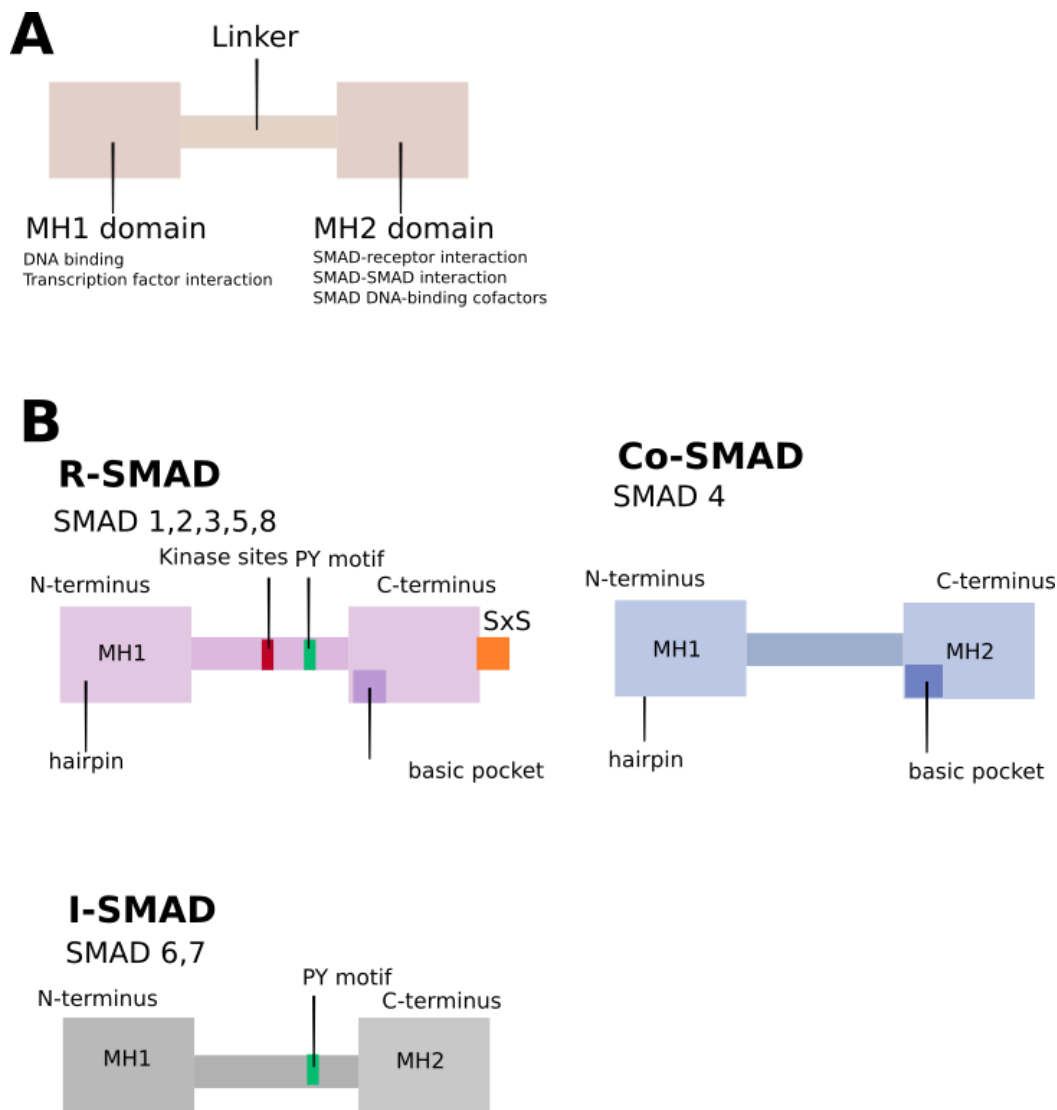
Figure 1.2: Smad structure and function.

(A) The basic structure of Smad proteins consists of an N-terminal and C-terminal, known as Mad-homology domains, MH1 and MH2 domains respectively. A proline-rich linker region, which is variable in sequence and size, links the MH1 and MH2 domains together. Whilst the MH1 domain is involved in DNA binding, the MH2 domain is involved in protein-protein interactions. (B) R-Smad, Co-Smad and I-Smad structure: There are three categories of SMADs including inhibitory, common-mediator and receptor-activaed SMADs, with seven mammalian Smad proteins falling under these categories. The β-hairpin present on the MH1 domain of R-Smads and Co-Smads is involved in DNA binding. The linker region of R-Smads contains kinase sites allowing phosphorylation by CDKs, MAPKs and various other kinases. The PY motif (PPXY) on R and I-Smads is involved in interactions with Smurf ubiquitin ligases. On the MH2 domain in R-Smads a basic pocket interacts with type I receptors and the SxS motif (orange square), whilst the basic pocket in Co-Smads interacts with the SxS motif only. Modified from Massague and Wotton (2000) and Massague et al, (2005).

Three classes of Smad proteins have been identified: the inhibitory Smads (I-Smads), the common-mediator Smads (Co-Smads), and the receptor-activated Smads (R-Smads, including Smad1, 2, 3, 5, and 8) (Shia and Massague, 2003). Apart from the I-Smads (Smad6 and Smad7), two regions of homology exists at the amino terminals, known as the Mad-homology domain (MH1), and carboxy terminals (MH2) (Heldin et al, 1997, Massague, 1998). Co-Smads and R-Smads (except Smad2) recognise Smad binding elements via MH1 domains (ten Dijke et al, 2002).

Once TGFβ superfamily members, such as TGFβ bind to heteromeric complexes of transmembrane Ser/Thr kinases, Smad2 and Smad3 are phosphorylated on their C terminus residues (Dennler et al, 1999). SARA (Smad anchor for receptor activation) is a protein that recruits Smad2 to the TGFβ receptor, directly interacting with the MH2 domain of Smad2 and 3 (Tsukazaki et al, 1998). A cytoplasmic protein, promyelocytic leukaemia tumour suppressor, is essential in modulating TGFβ signaling, as it is required for the association of Smad2/3 with SARA (Lin et al, 2004). Once phosphorylated, receptor regulated Smads form heterocomplexes with the co-mediator Smad; For example Smad2 forms a heterodimer with Smad4 (Wu et al, 2001a). When Smad3 is phosphorylated, it has a greater propensity for the formation of a heterotrimer with Smad4 (Chacko et al, 2001).

The MH1 domain of Smad3 and Smad4 is able to specifically bind DNA. Smad2 and Smad4 are able to recognise an 8 bp palindromic sequence termed the Smad binding element (SBE). Tandem repeats of SBE allow for transcriptional activation (Zawel et al, 1998). Whilst Smad3 acts as a transcription factor, binding to the CAGA box, Smad2 does not bind (Dennler et al, 1998). This difference may be due to differences in the N-terminal MH1 domain, where Smad2 contains two more stretches of amino acids (Dennler et al, 1999). Smads also bind GC-rich sequences, which confer BMP responsiveness. Multimerisation of GGCGCC palindromic sequence as well as the CAGC and CGCC motifs is required to generate a BMP/Smad-dependent specific enhancer (Korchynskyi and ten Dijke, 2002).

As reviewed in Massagué and Wotton (2000), Smads interact with DNA-binding Smad cofactors, allowing high-affinity binding to DNA. There are a multitude of Smad cofactors; some of which are specific for TGF-β superfamily member signaling. A 30-zinc finger protein (OAZ) exclusively cooperates with BMP-activated Smads, leading to the activation of the *Xvent-2* promoter (Hata et al, 2000). Furthermore, Sox5 is also involved in the activation of BMP target genes, acting as a cofactor for R-Smads in embryos and explants (Nordin and LaBonne, 2014). In TGF-β signaling, Olig1 is a helix-loop-helix Smad cofactor, which interacts with Smad3 (Motizuk et al, 2013). Conversely, in Nodal signaling, E proteins act as signaling cofactors, associating with Smad 2 and 3 (Yoon et al, 2011). Not all Smad cofactors are likely to be involved in GC proliferation, as cofactor expression is often tissue-specific. For example, OAZ is not usually expressed in smooth muscle cells and myoblasts (Ku et al, 2005). Thus, the specificity of the Smad-cofactor complex formation in various tissues acts as a determinant of TGFβ signaling specificity (Massagué and Wotton, 2000).

The signaling of the TGFβ superfamily can be modified at several levels. Extracellular antagonists can block ligand binding, and include noggin, gremlin and chordin (Kaivo-oja et al, 2006). BMP and activin membrane bound inhibitor (BAMBI) is an antagonist that associates with TGFβ family receptors, inhibiting BMP, activin and TGFβ signaling. Inhibition is mediated by its intracellular domain, which prevents the formation of receptor complexes (Onichtchouk et al, 1999).

Targeted ubiquitination of Smads acts as a control of cellular responses to TGFβ signals. Smurf1 is an E3 ubiquitin ligase, which interacts with Smads that are specific for the BMP pathway. It is able to trigger ubiquitination, inactivating Smads (Zhu et al, 1999). Furthermore, Smurf2 is involved in the ubiquitin-mediated degradation of Smad2, thereby also regulating TGFβ signaling (Lin et al, 2000). Epidermal growth factor and hepatocyte growth factor receptor protein tyrosine kinases (RTKs) cause Erk-mediated phosphorylation, which is inhibitory, preventing nuclear accumulation of Smad1 (Kretzschmar, 1997). Oncogenic Ras is able to inhibit TGFβ and Smad signaling through phosphorylation, inhibiting the nuclear accumulation of Smad2 and Smad3 (Kretzschmar et al, 1999). Lastly, Smad6 and Smad7 (I-Smads) are inhibitors of TGFβ

signaling, interacting with the TGFβ receptor, and preventing the phosphorylation and activation of Smads (Hayashi et al, 1997, Imamura et al, 1997).

The Smad proteins are crucial in the cellular signaling pathways of both GDF9 and BMP15. GDF9 stimulates the phosphorylation of both Smad2 and Smad3 (Mazerbourg et al, 2004). Addition of BMP15 to rat and human GC causes transient phosphorylation and activation of the Smad1/5/8 pathway. In comparison, weak activation of Smad2 was observed with BMP15 stimulation (Moore et al, 2003). Smad5, 1 and 8 are the downstream signal mediators for several BMPs (Chang and Matzuk, 2001).

The importance of these cellular pathways in fertility and folliculogenesis is demonstrated experimentally through the use of knockout mice. A null mutation in the TGFβ1 gene leads to almost complete infertility in mice, with disrupted ovulation, oocyte development, and corpus luteum function being observed. Furthermore, the proestrus surge in LH was not observed in these mice (Ingman et al, 2006). Using a Smad3-deficient mouse model, it was observed that these mice had decreased fertility in comparison to wild type mice. Thus, Smad3 may regulate the growth of primordial follicles to the antral stage, as fewer preantral follicles were present in the ovaries of Smad 3$^{-/-}$ mice. This reiterates the importance of Smad3 in the growth of ovarian follicles and in female fertility (Tomic et al, 2002). An ovarian conditional knockout of Smad4 shows subfertility in mice and disrupted steroidogenesis resulting in increased serum progesterone levels. Premature luteinisation of granulosa cells and cumulus cell defects were also observed (Pangas et al, 2006). Thus, these studies illustrate the essential role the Smad signaling pathway plays in transmitting the signal induced by oocyte secreted factors during folliculogenesis.

1.6.2 Non-Smad signaling pathways

In addition to the Smad signaling pathway, oocyte secreted factors also act through several non-Smad signaling pathways, described herein. Similarly to the Smad signaling pathway, these pathways are also involved in folliculogenesis and GC proliferation (Yang and Roy, 2006, Reader et al, 2008). The TGFβ/BMP signaling pathway is dependent

upon the cross-communication with several signaling pathways (Engel et al, 1999, Reader et al, 2011 Sasseville et al, 2010), one of which is the mitogen-activated protein kinase (MAPK) pathway (Kretzschmar et al, 1997). MAPKs are evolutionary conserved signal transducing enzymes. Regulation of MAPK activity occurs through MAPK, MAPK kinase and MAPKK kinase, which form a cascade (Chang and Karin, 2001). Four signaling cascades share the MAPK signaling pathway; these include Jun amino-terminal kinases (JNK1/2/3), the extracellular signal-related kinases (ERK1/2), p38-MAPK and ERK5 (Sun et al, 2015).

Cross-communication between Smad and non-Smad signaling pathways principally occurs due to Smad proteins making several contacts with other proteins as well as the various targets the TGFβ superfamily members have (Guo and Wang, 2009). Smad1 is a mediator of BMP signaling, but is also targeted by mitogenic growth factor signaling. The ERK family of MAPKs phosphorylates Smad1, inhibiting its nuclear accumulation. ERK phosphorylation of Smad1 occurs at the linker region, in contrast to BMP stimulated phosphorylation at the C-terminal serines, which causes Smad1 nuclear accumulation (Kretzschmar et al, 1997). Smad2 activates gene transcription by cooperating with DNA binding proteins such as forkhead activin signaling transducer (FAST). Activation of the JNK cascade blocks the ability of Smad2 to activate FAST proteins (Pessah et al, 2001). However, the interconnection between the Smad and MAPK pathway is more complex than MAPK having an antagonistic effect. For example, phosphorylation of Smad3 mediated by JNK facilitates Smad3 nuclear accumulation as well as its phosphorylation by TβRI (Engel et al, 1999). Furthermore, the transcriptional responses caused by TGFβ induced Smad3 phosphorylation is decreased by p38 inhibition, suggesting that the p38 cascade and Smad signaling pathway act in unison to activate gene expression in the nucleus (Abécassis et al, 2004).

Epidermal growth factor (EGF) is able to induce the activation of the MAPK signaling pathway (Wu et al, 1993). Oocyte secreted factors, such as GDF9 and BMP15, promote epidermal growth factor receptor (EGFR) expression in cumulus cells. This is illustrated as the removal of oocytes from COCs decreases *Egfr* expression in cumulus cells and reduces MAPK3/1 activation (Su et al, 2008). Conversely, TGFβ1 has a negative

interaction with EGF, whereby alone, TGFβ1 and EGF stimulate GC DNA synthesis, however together an attenuation of DNA synthesis is observed. EGF with TGFβ1 causes an attenuation of TGFβ1-induced activation Smad3 phosphorylation. Conversely TGFβ1 or EGF1 stimulate MAPK1 activation, implicating both of these pathways in GC DNA synthesis (Yang and Roy, 2006). The MAPK pathway is also required for BMP15-induced mitosis of GC, as upon the addition of an ERK1/2 phosphorylation inhibitor GC proliferation was decreased (Moore et al, 2003). In a similar manner to BMP15, MAPK signaling has also been implicated in GDF9-induced GC proliferation. The action of GDF9, which induced GC proliferation and cumulus cell expansion, can be inhibited through the use of an MEK inhibitor (Su et al, 2002, Sasseville et al, 2010). The ERK1/2 pathway is important in maintaining the Ser208 phosphorylation of Smad3 at its linker region, illustrating an interaction between these two pathways (Sasseville et al, 2010). Thus the MAPK pathway is likely to interact with the Smad signaling pathway and is required for BMP15 and GDF9 induced folliculogenesis. However, a p38 MAPK inhibitor, although causing a partial inhibition of tritiated thymidine uptake by ovine GDF9 and BMP15, also suppressed thymidine uptake in control cultures, suggesting that this signaling pathway is non-specific (Reader et al, 2011).

As reviewed in Pahl (1999), the Rel/NF-κB family of transcription factors includes RelA (p54), RelB, c-Rel, p50/p105, and p52/p100. These dimers bind to κB sites and regulate gene expression. Members of the TGFβ superfamily directly can elicit NF-κB pathway activation in a variety of cells. For example, TGFβ, Mullerian Inhibiting Substance (MIS), BMP2 and BMP4 are all capable of stimulating the activation of NF-κB in osteoclasts, cancer cells and the human cornea, respectively (Gingery et al, 2008, Hoshiya et al, 2003, Mohan et al, 1998). In several types of cells the activation of NF-κB blocks apoptosis. However, NF-κB may inhibit or promote apoptosis depending on the cell type and the inducer type (Barkett and Gilmore, 1999). In particular, the NF-κB pathway plays a critical role in the regulation of GC apoptosis. Early growth response (EGR1) modulates apoptosis, with its expression increasing in the aging mouse ovary and with follicular atresia. This overexpression of EGR1 elicits the promotion of apoptosis in GC, which occurs through the NF-κB pathway (Yuan et al, 2016). However, not all studies are in concordanance with the pro-apoptotic capabilities of NF-κB in GC. Activation of the NF-

κB pathway is implicated in cadmium-induced apoptosis in GC. However, inhibition of the NF-κB pathway increases cadmium induced apoptosis, indicating that the NF-κB may prevent porcine GC apoptosis under these conditions (Wang et al, 2013b).

1.6.3 Synergistic Effects of GDF9 & BMP15

BMP15 and GDF9 are known to have cooperative effects during folliculogenesis. A synergistic effect is observed when adding low doses of both GDF9 and BMP15, as observed by GC proliferation, compared to the addition of GDF9 and BMP15 separately. This synergistic effect was also demonstrated in cumulus cells, and in suppressing FSH-stimulated progesterone production (McNatty, 2005bc, Mottershead et al, 2012). The cooperative effects of GDF9 and BMP15 in stimulating GC proliferation are inhibited by blocking the extracellular domain of BMPR2 by fusing to the IgG Fc region, seen as an inhibition in thymidine incorporation. This suggests that this receptor modulates these cooperative effects (Edwards et al, 2008, McIntosh et al, 2008). Furthermore, a BMP15 proregion antibody was able to immunoneutralise BMP15/GDF9 synergism, demonstrating that the cooperative actions of recombinant mouse GDF9 and BMP15 involve the proregion of BMP15 (McIntosh et al, 2008).

The synergistic effects of GDF9 and BMP15 have also been studied by observing which cellular signaling pathways these proteins activate. GDF9 and BMP15 alone showed minimal Smad3 activation. However, synergistically there was a substantial activation. In comparison, Smad1/5/8 showed high activation with BMP15 alone, but no synergistic effect was observed in activation. Therefore, GDF9 and BMP15 synergism is seen when observing Smad3 pathway activation, and this response was inhibited using the SB431542 inhibitor (Mottershead et al, 2012). This was also demonstrated by Reader et al (2011) through an inhibitor of the SMAD2/3 pathway, which prevented ^{3}H-thymidine incorporation by granulosa cells by ovine and murine GDF9 and BMP15. This was not observed with the Smad 1/5/8 pathway inhibitor. Thus, ovine and murine GDF9 and BMP15 stimulated ^{3}H-thymidine uptake in GC is dependent on the Smad 2/3 signalling pathway, rather than the Smad 1/5/8 pathway. The synergistic effects of GDF9 and BMP15 also activate a variety of non-Smad signaling pathways. Using recombinant mouse GDF9 and human BMP15 together with pathway inhibitors,

Mottershead et al, (2012) observed that the synergistic actions of these proteins were dependent on ERK1/2 and SRC kinase signaling. In rat GC, recombinant ovine GDF9 and BMP15 act through the p38-MAPK and NF-κB pathways. In contrast, recombinant murine GDF9 and BMP15 cause rat GC proliferation through the ERK-MAPK and JNK signaling pathways (Reader et al, 2011). Human recombinant GDF9 and BMP15 act through the NF-κB and JNK signaling pathways (Reader et al, 2016). Thus, the non-Smad signaling pathways activated through the synergistic actions of GDF9 and BMP15 vary depending on the species.

In summary, GDF9 and BMP15 are crucial in folliculogenesis, however as illustrated, there are differences in the importance of expression of these two proteins in high and low ovulating species. This may also be studied through the cellular signaling pathways utilised by these proteins, namely the Smad proteins (Smads 1, 2, 3, 5 and 8), which are a fundamental part of signaling.

2.0 Methodology

2.1 Study Aims

The factors that regulate ovulation rate in species are unknown, but several studies point towards the oocyte itself being of importance (Williams and Stanley, 2008, Crawford and McNatty, 2012, Grasa et al, 2015). Although GDF9 and BMP15 both impact folliculogenesis, differences in the importance of expression of these two proteins in high and low ovulating species have been illustrated (Crawford and McNatty, 2012). GDF9 and BMP15 signal through intracellular signaling proteins, known as the Smads (Dennler et al, 1999, ten Dijke et al, 2002, Schmierer and Hill, 2005). Thus, the difference in the expression and importance of GDF9 and BMP15 in mono-ovulatory and poly-ovulatory species may be indirectly studied through the Smads (Smad 1, 2, 3, 5 and 8). Oocyte secreted factors, such as GDF9, have been implicated in the management of fertility, influencing embryo generation (Li et al, 2014). New insights into the interactions between oocyte secreted factors and their signaling pathways may ultimately improve *in vitro* maturation (IVM) methodologies, which are currently less efficient than hormone-stimulated *in vitro* fertilisation (IVF) systems (Gilchrist, 2011). This study will further our understanding of differences in ovulation rate in various species with the inclusion of both the pig, a non-rodent poly-ovulatory species, and the deer, a strict mono-ovulatory species. Despite these ovulation rate differences, both species express high levels of BMP15 in the oocyte (Crawford and McNatty, 2012). This study will investigate the species differences in the bioactivity of oocyte secreted factors (OSFs) on GCs.

The aims of this study were, through cross-species co-incubations of oocytes and GC, to determine the (i) proliferative effects, (ii) expression levels of key genes and (iii) Smad signaling activation of oocyte secreted factors on GC of rat, sheep, pig and red deer.

Specifically, the cross-species effects of oocyte secreted factors on (i) GC proliferation rate were determined using a tritiated thymidine incorporation assay (ii) GC expression

levels of *BMPR2, TGFBR1,* and *BMPR1B* mRNA were determined using qPCR and (iii) GC levels of unphosphorylated and phosphorylated Smads 1, 2, 3, 5 and 8 were measured using Western blotting.

2.2 Experimental design

Preliminary experiments were conducted to determine the incubation time that produced the maximum proliferation rate (i.e. tritiated thymidine uptake) in rat, sheep, pig and deer GC. Activin A, which is known to act through the Smad2/3 pathway (Baker and Harland, 1996, Dennler et al, 1998), was used as a positive control. Three to five replicate experiments were performed for each species, whereby oocytes from each species were incubated with GC from their corresponding species.

Following the preliminary experiments, same- and cross-species co-incubations using GC and oocytes from rats, sheep, pig and red deer were conducted in every possible combination (Figure 2.1). For each incubation run, GC samples were collected following incubation with ooctyes for determination of proliferation rate, expression levels of key growth factor receptor genes and relative amounts of phosphorylated and unphosphorylated Smad proteins. The number of replicate experiments performed for the proliferation assays, gene expression and protein measurements were three to five, five to seven, and three, respectively.

GC	Rat	Rat	Rat	Rat	Sheep	Sheep	Sheep	Sheep
Oocytes	Rat	Sheep	Pig	Deer	Rat	Sheep	Pig	Deer

GC	Pig	Pig	Pig	Pig	Deer	Deer	Deer	Deer
Oocytes	Rat	Sheep	Pig	Deer	Rat	Sheep	Pig	Deer

Figure 2.1: Experimental design of cross-incubations of granulosa cells and oocytes from the four test species.

2.3 Tissue collection

In accordance with the 1999 Animal Welfare Act (Part 6) of New Zealand, ovaries were collected from prepubertal female Sprague Dawley rats obtained from the Psychology Department (Victoria University of Wellington). Rats were euthanised with CO_2 gas, and thereafter, cervical dislocation was performed. To maintain sterility, the abdomen was dowsed in 70% ethanol prior to dissection. Upon removal, rat ovaries were placed in dissection media (see Appendix One) that had been previously warmed to 37 °C for transport back to the laboratory. Sheep, deer and pig ovaries were obtained from Ngauranga Meatworks (Wellington, NZ), Venison Packers Feilding Ltd (Feilding, NZ), and Cabernet Foods, Kintyre Meats Ltd (Gladstone, NZ), respectively. Sheep ovaries were transported in a plastic bag at room temperature to the laboratory. Pig and deer ovaries were transported in 0.9% saline pre-warmed to 37 °C in a flask, with transportation taking two and three hours, respectively. Upon arrival, extraneous connective and fatty tissues were removed from the ovaries. For sterilisation purposes, rat ovaries were briefly placed in pre-warmed ethanol and washed twice in dissection media. Sheep, pig and deer ovaries were also immersed briefly in 70% ethanol to rid the ovarian surface of possible bacterial contamination and then washed twice in 0.9% saline. During oocyte retrieval, rat ovaries were kept in dissection media, and sheep, deer and pig ovaries were kept in 0.9% saline, in a 37 °C water bath.

2.3.1 Denuded oocyte collection

Rat ovaries were placed in 3.5 cm petri dishes whilst sheep, deer and pig ovaries were placed in petri dishes filled with dissection media. Using a dissection microscope, visible follicles on rat ovaries were punctured using a 30-gauge needle. For sheep, pig and deer ovaries, a 20-gauge needle attached to a 1 mL syringe was used to puncture visible follicles. Media was often injected into the follicles of sheep, pig and deer ovaries to encourage detachment of the COC from its follicular surroundings. Isolated denuded oocytes and COCs were transferred into a clean petri dish containing dissection media. COCs were denuded by aspirating with a 1 mL syringe, or vigorous pipetting, until the majority of cumulus cells were removed. When collecting large quantities of oocytes, oocytes were temporarily stored in a petri dish with incubation media (see Appendix

One) in a 37 °C (for rat) or 39 °C (for sheep, pig and deer) incubator whilst the remaining oocytes were collected.

2.3.2 GC collection

The GCs of rats, sheep, pigs and deer were collected once all COCs and denuded oocytes had been collected. It is possible that the cells remaining contained a small proportion of cell types other than GC, such as theca cells. The GC preparations were aspirated twice with a 20-gauge needle attached to a 1 mL syringe to disperse any clumps of GC and transferred into a 10 mL falcon tube and centrifuged at 453 g for 5 minutes, at 20 °C. Collection media was removed taking care not to disrupt the pellet containing GC. Cells were washed once in 1mL incubation media (Appendix 1.1). After the final wash, the GC were then evenly resuspended by pipetting in incubation media, ensuring no clumps were visible in the media. Thereafter, a 50 µL aliquot of GCs was removed and mixed gently with 5 µL of 0.4% trypan blue (Gibco Life Technologies, USA). An aliquot of 10 µL was pipetted under a cover slip on a hemocytometer with a 0.1 mm depth (Improved Neubauer, BS 74B Hawksley). Under a Leica DM1000 microscope, the number of live (clear) and dead (blue) cells with GC morphology were counted and recorded. The morphological characteristics used to distinguish GCs were the presence of a nucleus and a cytoplasm with a granular appearance. The concentration of cells within the preparation was determined using the equation below and the cell suspension was diluted further in incubation media so that 1,000 live cells were present in 1µL (20,000 in 20 µL).

Number of cells/mL = live cell count x 10,000 x dilution factor x volume

If fewer than 1,000 live cells per µL were present, the falcon tube containing the GCs was centrifuged again and a smaller volume of incubation media was added so that the cell concentration was 20,000 viable cells/20 µL.

2.4 Incubation Experiments

All incubation experiments were performed in an incubator with 5% CO_2 at 37 °C (for rat) or 39 °C (for sheep, pig and deer). From this point onwards, the term CO_2 incubator will be used.

2.4.1 Preliminary time series incubation experiments

The 96-well plates (Jetbiofil) were used for all incubation experiments. For the preliminary time series experiments, 55 µL of incubation media was added to three to five wells within the first row of each plate and used as the baseline from which each result was normalised against. Three to five wells within the second row of each plate contained 20 µL of 20,000 viable rat GC together with 35 µL of incubation media. Three to five wells within the third row of each plate contained 20 µL of 20,000 viable rat GCs together with 32 rat denuded oocytes, with a final volume of 55 µL. As the positive control, three to five wells in the final row of each plate contained 20 µL of 20,000 live rat GCs, 34 µL of incubation and 1 µL of Activin A (final concentration of 10 ng/mL; Accession # P08476, R & D systems). This plate set-up regimen was repeated for GC and denuded oocyte of the other species also (i.e. sheep, pig and deer). Following the addition of cells, plates were placed in a CO_2 incubator. The rat cells were incubated for either 0, 4, 8, 12 or 16 hours, whilst sheep, pig and deer cells were incubated for either 8, 12 or 16 hours. The rationale behind the incubation times for the rat was determined by previous literature, whereby an 18 hour incubation has been previously used in similar studies employing tritiated thymidine uptake in GC (McNatty et al, 2005b, McNatty et al, 2005c). Therefore, a maximum incubation time of 16 hours was used for all species. However, shorter incubation times of four hour increments were also tested in the time series experiments to determine both basal proliferation rate at 0 hours and the relationship of time in culture and proliferation rate. Fewer time points were used for the sheep, pig and deer experiments due to time limitations in oocyte retrieval.

2.4.2 Same- and cross-species incubation experiments

For the experiments involving cross-incubations of GC and denuded oocyte from different species, the first row of each plate contained 55 µL of incubation media. Three

to five wells within the second, third and fourth rows of each plate contained 20 µL of 20,000 viable GCs from one species in 35 µL of incubation media. In the third and fourth rows, 32 denuded oocytes of either of the four species were added to the wells containing the GCs and the second row contained wells with GCs alone. Whilst all experiments were set up in an identical manner, the experiments destined for gene expression and Western blot analyses were performed on separate 96-well plates than those destined for proliferation analyses.

2.5 Proliferation Assay

Following the stipulated incubation period (variable for the preliminary experiments and 16 hours for all other experiments), 96-well plates were removed from the incubator for the addition of tritiated thymidine (NET35501MC, Perkin Elmer). Tritiated thymidine was diluted 1:25-fold in incubation media and 10 µL ($\sim$ 0.4 µCi) was added to each well. The plate was returned to the CO_2 incubator for a further 6 hours. Thereafter, the plate was removed for cell harvesting. Briefly, the radiolabelled cellular contents of the 96-well plates were transferred onto a glass fibre filter mat (1450 microβ fibre filter, 90 x 120 mm printed filter mat, Perkin Elmer, MA, USA) using the 'Pulse Wash' programme of a Wallac Harvester (TOMTEC). The filter mat was carefully dried in a microwave oven (1500W, 2-3 minutes), and then placed into a sample bag (Perkin Elmer, MA, USA). An aliquot of 4.5 ml of β-scintillation fluid (SC/9200.21, Perkin Elmer Life Sciences) was added inside the sample bag and the bag edges were completely sealed with heat (Wallac heat sealer; Perkin Elmer). The bag was maneuvered such that the filter mat was evenly immersed in the β-scintillation fluid. The bag containing the filter mat was placed into the Wallac microβ trillux scintillation counter (1450 LSC and Luminescence counter, Perkin Elmer) and β particle emissions at positions corresponding to a 96-well plate format were measured on the filter mat in counts per minute (CPM).

2.6 GC sample preparation for qPCR and Western Blots

Following a 16 hour incubation period, 96-well plates were removed from the CO_2 incubator and placed on ice to dislodge the cells from the well bottom. This was aided by

mechanical pipetting of media over the well bottom and the resultant cell suspension was placed into a 3.5 cm petri dish. Any ruptured (absent) denuded oocytes were noted to account for any discrepancies in results, and oocytes were removed from GC. For gene expression analyses, the GC were then transferred into eppendorf tubes and pelleted by centrifugation at 800 g for four minutes. The pellet was washed with 200 µL of PBS, pelleted by centrifugation and excess PBS was removed.

For Western blot samples, the media was carefully removed without disturbing the pellet. An aliquot of 1 µl of 10x protease inhibitor (Ref # 04693159001, Roche) was added to the GC pellet. All qPCR and Western blot samples were snap frozen and stored at -80 °C until further use.

2.7 Quantitative PCR

2.7.1 Total RNA Extraction

Total RNA was isolated from samples containing 20,000 GC using an ArrayPure™ Nano-scale RNA Purification Kit (Cat # MCR85102, Epicentre), following the manufacturer's instructions. For each GC sample, 1 µL of 5 µg/µL Proteinase K was diluted into 30 µL of Nano-scale Lysis Solution. Thirty µL of this solution was added to each GC sample and then incubated at 65 °C for 15 minutes. After samples were placed on ice for 5 minutes, 18 µL of MPC Protein Precipitation Reagent was added and briefly vortexed. Samples were centrifuged at 12,000 g for 7 minutes at 4 °C and the supernatant was transferred to a clean Eppendorf tube. Total RNA was precipitated with the addition of 50 µL of isopropanol. Following a 30 second vortex, the RNA was pelleted by centrifugation at 12,000 g for 5 minutes at 4 °C. Residual isopropanol was removed and the RNA pellet was air-dried. The pellet was then re-suspended in 20 µL of Dnase I solution, which was prepared using 1 µL of Rnase-Free Dnase I with 40 µL of 1 X Dnase Buffer. Thereafter samples were incubated for 30 minutes at 37 °C. After the addition of 20 µL of 2X Nano-scale Lysis Solution and brief vortexing, 20 µL of MPC Protein Precipitation reaction was added. Following centrifugation at 12,000 g for 5 minutes at 4 °C, the supernatant was transferred to a clean Eppendorf tube. Total RNA was again precipitated with the addition of 50 µL of isopropanol and the RNA was pelleted by centrifuging at 12,000 g

for 5 minutes at 4 °C. The pellet was washed with 50 μL of 70% ethanol, and centrifuged at 12,000 g for 3 minutes. The residual ethanol was removed and the pellet air-dried for 5 minutes. Total RNA was resuspended in 10μL Dnase free water and vortexed for one minute. The RNA samples were then either frozen at -80 °C for further processing, or used immediately for cDNA synthesis.

2.7.2 cDNA Synthesis

Complementary DNA was synthesised from GC RNA using the SuperScript VILO cDNA Synthesis Kit (Cat # 11754-250, Invitrogen, Life Technologies) following the manufacturer's instructions. Briefly, in a 200 μL PCR tube (Scientific Specialities Inc, USA), 4 μL of 5X VILO reaction mix, 2 μL of 10X SuperScript enzyme mix, 10 μL of isolated GC RNA and 4 μL of DEPC-treated water were added and mixed thoroughly. The synthesis of cDNA was performed on a Corbett Rotor-Gene 6000 (Corbett Research Ltd) under the following conditions: 25 °C for 10 minutes, 42 °C for 120 minutes and 85 °C for 5 minutes.

2.7.3 qPCR optimisation

Primers and Taqman probes (for some genes) for rat, sheep, pig and deer *Tgfβr1, Bmpr1b, Bmpr2*, *Rpl19* (reference) and *Ppia* (reference) genes were designed using Beacon Designer 8.13 (Premier Biosoft; see Table 2.7.3.1). Primers were synthesised by Life Technologies (New Zealand) and Taqman probes were manufactured by Sigma Proligo (Australia).

Optimisation of the qPCR was completed using various concentrations of forward and reverse primers for each gene to determine the optimal primer concentrations. Combinations of 100, 200, 300 and 500 nM forward and reverse primers were tested. Reaction mixes containing 26 μL Brilliant SYBR Green qPCR master mix (Cat # 600828, Agilent technologies), the appropriate concentration of forward and reverse primers (Table 2.7.3.2), 1.04 μL of GC cDNA, and UltraPure dH$_2$O to a final volume of 52 μL were prepared in 0.2mL PCR tubes. For those genes in which Taqman probe chemistry were used, 100, 200, 300 and 500 nM concentrations of probes were tested. Reaction mixes

containing 26 µL Brilliant Multiplex qPCR master mix (Cat # 600553, Agilent technologies), the optimal concentrations of forward and reverse primers (Table 2), the appropriate concentrations of Taqman probe (Table 2.7.3.2), 1.04 µL of GC cDNA, and UltraPure dH$_2$O to a final volume of 52 µL were prepared in 0.2mL PCR tubes. Duplicates of 25 µL reactions were then added to strip-tubes (QartaBio), loaded into a RotorGene 6000 (Corbett) and subjected to the following conditions: 95 °C for 10 minutes, and then 40 cycles of 95 °C for 15 seconds, and 60 °C for 60 seconds.

PCR efficiencies were determined using serial dilutions (1 to 1:128) with 02.08 µL of cDNA, to generate an efficiency value and an R^2 value (Table 2.7.3.3). The PCR efficiency of all genes measured in this study was >75%.

Following optimisation of all gene sets, gene expression levels were then measured in samples collected for qPCR. Similar reaction mixes were prepared as described above in accordance with genes either quantified using SYBR Green or Taqman qPCR chemistry. SYBR Green qPCR chemistry was utilised for the genes *PPIA, RPL19* of all species and deer *BMPR2*. Taqman qPCR chemistry was utilised for every other gene as well as the *RPL19* gene of the sheep, pig and rat. Taqman reactions were performed in quadraplex (rat and sheep *TGFβR1, BMPR1B, BMPR2, RPL19*), triplex (pig *BMPR1B, BMPR2, RPL19*), duplex (deer *TGFβR1, BMPR1B*) and singleplex (pig *TGFβR1*). For SYBR green chemistry, 26 µL Brill SYBR Green qPCR master mix, 1.04 µL of cDNA, forward and reverse primers at the optimised concentrations (Table 2.7.3.2) were added to a 200 µL PCR tubes, and UltraPure H$_2$O was added to a final volume of 52 µL. For Taqman chemistry, 26 µL Brill SYBR Green qPCR master mix, 1.04 µL of cDNA, forward and reverse primers at the optimised concentrations (Table 2.7.3.2), and Taqman probe(s) at the optimised concentration(s) (Table 2.7.3.2) were added to 200 µL PCR tubes, and UltraPure H$_2$O was added to a final volume of 52 µL. Again, for each sample, 25 µL of the reaction mix was aliquoted in duplicate into strip-tubes. Gene expression levels from five-seven replicates of each experimental group (collected randomly over multiple experiments) were analysed. The gene, *RPL19,* was used as the reference gene for sample normalisation however an additional reference gene, *PPIA*, was also measured to ensure parallelism of the two reference genes for each sample. Additionally a calibrator sample

was present at the beginning and end of every reaction run. Thus, the Ct value of *Rpl19* for each sample was subtracted from every sample's Ct value for each gene, obtaining a ΔCt value. Thereafter, the Ct value of the calibrator sample for each gene was subtracted from the ΔCt value resulting in the ΔΔCt value. To deduce the relative difference in mRNA expression levels for each gene between GC samples, the equation of $2^{(-\Delta\Delta CT)}$ was calculated (Livak & Schmittgen, 2001).

Table 2.7.3.1: Nucleotide sequences and NCBI accession numbers for forward and reverse rat, sheep, pig and deer primers and TaqMan probes.

Gene	Accession Number	Primers	Taqman probe
Rat			
Bmpr2	NM_080407	F-CGTCAACTTCACTGAGAA	CCACCTCCTGATACAACACCACTC
		R-TGCCAAAGCAATGATTATAG	
Tgfβr1	NM_012775	F-TGCTGATGGTCTATATCTG	TAACCGCACTGTCATTCACCACC
		R-TGCCCTCTGAAATGAAAG	
Bmpr1b	NM_001024259	F-GGACAGAGATTTTGTTGATG	AGCCTTACTCATATCTGTGACTGTCTGT
		R-CCTGAAGTAACAGAATAAAATAATG	
Rpl19	NM_031103	F-CAGGAAGCTGATCAAAGA	CGGAAGCCTGTGACTGTCCAT
		R-CTTCCCTATGCCCATATG	
Ppia	NM_017101	F-CTTTGACTTGCGGGCATTT	-
		R-GAACTTCAGTGAGAGCAGAGAT	
Sheep			
BMPR2	NM_001306123	F-GGACTTGTGTAATGTCAAC	CACCTCCAGACACAACACCACTC
		R-GATGCCAATGCAATGATTA	
TGFβR1	XM_012120354	F-GCCAGTTGTATCTTCCATA	AGCACTATCATTACAGCATAACCACAGG
		R-CACTGTATCCTGACTCAAG	
BMPR1B	NM_001009431	F-GGCGCTTTAAAATAGAGATAA	TACCTCCATCCTCACCGCCT
		R-CGCATACCTAAGACACAA	
RPL19	XM_004012836	F-GACGATACCGTGAATCTAA	CATGTATCACAGCCTGTACCTGAAGG
		R-CAGCTTGTGGATATGTTC	
PPIA	XM_015095354	F-TTACTTAACCACCAGACCAT	-
		R-CGAGAGCACAAAGATTATAGG	
Pig			
BMPR2	NM_001204900	F-CACAGATTTGTGTAATGTCA	CCTCCAGACACAACACCACTCAG
		R-ATGCCAGAGCAATGATTA	
TGFβR1	AF_317296	F-CACTCATGTTGATGGTCTA	TGCCATAACCGTACAGTCATTCACC
		R-AAGGGCGATCTAATGAAG	
BMPR1B	AF_432128		CATCCAGAAGTGACCACAGGCAT

RPL19	XM_003131509	F-GAAGCTGATCAAAGATGG	ACCCTTCCGCTTACCGATGC
		R-CCAAGTTACCTTCTCAGG	
PPIA	XM_013985800	F-ACCGTCTTCTTCGACATC	-
		R-TGAGCAGTCACTTCTTGG	
Deer			
BMPR2	N/A	F-GCAACAAAGATAAATAACTATTTG	CCAGACAGAAGAAGAGTTGCCTTACATT
		R-CCCAAACACAGTGTAGTA	
TGFβR1	N/A	F-GATCCTTAGGTTTACCATTG	AACAGCAACTTCTTCTCCTCTCCATT
		R-CCATGAACGTTCTTCTCTA	
BMPR1B	N/A	F-CTGAGTTTAGAAGGTGAATC	TGGTCAGCAGCCTCAGCATT
		R-CACAGTTCTTGGATGTTG	
RPL19	N/A	F-CTGGATGAGGAGGATGAGA	-
		R-ATGTGGCGGTCAATCTTC	
PPIA	N/A	F- TCAGCAGAGGAGCAAGAC	-
		R-CACTGTCCTTTGTCCATTAGAG	

Table 2.7.3.2: Optimised forward and reverse rat, sheep, pig and deer primer concentrations and Taqman probes.

Gene		Primer (nM)		TaqMan Probe (nM)
		Forward	Reverse	
Rat	*Bmpr2*	300	300	200
	Tgfβr1	300	500	100
	Bmpr1b	300	500	100
	Rpl19	300	300	200
	Ppia	300	300	-
Sheep	*BMPR2*	500	200	100
	TGFβR1	300	200	300
	BMPR1B	300	300	100
	RPL19	300	500	100
	PPIA	500	300	-
Pig	*BMPR2*	300	200	200
	TGFβR1	300	200	200
	BMPR1B	300	200	200
	RPL19	200	200	100
	PPIA	300	300	-
Deer	*BMPR2*	500	300	300
	TGFβR1	300	200	200
	BMPR1B	200	200	300
	RPL19	300	200	-
	PPIA	300	500	-

Table 2.7.3.3: Amplification efficiencies of rat, sheep, pig and deer genes.

	Gene	R^2	Efficiency
Rat	*Bmpr2*	0.98	0.90
	Tgfβr1	0.98	0.93
	Bmpr1b	0.98	0.87
	Rpl19	0.99	0.84
	Ppia	0.99	0.99
Sheep	*BMPR2*	0.97	0.78
	TGFβR1	0.98	0.85
	BMPR1B	0.98	0.77
	RPL19	0.99	0.81
	PPIA	0.98	0.97
	BMPR2	0.99	0.81
Pig	*TGFβR1*	0.99	0.79
	BMPR1B	0.97	0.86
	RPL19	0.99	0.79
	PPIA	0.99	0.87
Deer	*BMPR2*	0.99	0.89
	TGFβR1	0.98	0.97
	BMPR1B	0.99	0.86
	RPL19	0.99	0.93
	PPIA	0.98	0.99

2.8 Western Blots

2.8.1 SDS PAGE Gel Preparation

A 13.5% acrylamide gel was prepared by combining the components in Table 2.8.1 (reagents listed in Appendix 2). The Mini-PROTEAN Tetra Electrophoresis System (Bio-Rad) was used in gel preparation. After the gel solution was thoroughly mixed, it was pipetted between two glass plates, with an in-built 0.75 mm spacer, held in place by a Tetra Cell casting stand and clamps. A layer of water was pipetted above the solution, which was left to polymerise for at least one hour at room temperature. The top layer of water was then removed and the gel was blotted dry. Thereafter, the stacking gel was prepared (Table 2.8.1) and pipetted on top of the separating gel. A 10-well comb was inserted, and the gel was left to polymerise for over 30 minutes. Following complete

polymerisation, the gels were either used immediately or stored at 4°C for later use in a pre-wet plastic bag with the combs in place and wrapped in damp paper towels.

Table 2.8.1: Table of reagents for 13.5% separating gel and 4% stacking gel recipes.

Components	13.5% Separating gel	4% Stacking gel
Milli Q water	2.85 mL	3.0 mL
1.5 M Tris-HCl (pH 8.8)	2.5 mL	-
0.5 M Tris-HCl (pH 6.8)	-	1.25 mL
10% (w/v) SDS	100 μL	50 μL
30% Bis-acrylamide mix	4.5 mL	0.65 mL
10% (w/v) Ammonium persulphate	50 mL	25 μL
TEMED	5 μL	5 μL

2.8.2 Sample Preparation

The GC samples collected for Western Blotting were removed from -80 °C storage and placed on ice to ensure the proteins were maintained in their phosphorylated state. The negative control present in all Western blots was a recombinant deer GDF9 preparation that had been previously produced in-house (Swinerd, 2016). Two positive controls that were present in all Western blots were 20,000 live sheep GCs treated with either recombinant deer GDF9 or deer BMP15 (produced in-house; Swinerd, 2016). Both the negative and positive controls were prepared in a manner similar to that of the samples. For each GC sample, phosphatase inhibitor (4 μl; final concentration of 1x, Appendix 1.3), was immediately added to each GC sample which contained ~ 3 μl of media, followed by 12 μl of lysis buffer (Appendix 1.3) and a 1:1 ratio of reducing loading buffer (Appendix 1.3), for a final volume of 40 μl. Each GC sample was then thoroughly vortexed to complete cell lysis, briefly centrifuged and split into two aliquots, so that each sample contained ~10,000 previously-viable GCs. Negative controls were prepared in a similar manner, with 1 μl of GDF9, 1 μl McCoy's media, 2 μl phosphatase inhibitor, 6

μL lysis buffer, and reducing loading buffer in a 1:1 ratio per sample. Samples were firmly sealed in eppendorf tubes and placed in a 95 °C water bath for 5 minutes for denaturation.

2.8.3 SDS PAGE Gel electrophoresis

Gels were assembled inside a running cassette (BioRad), and placed in a cell, which was filled with 1x running buffer (Appendix 1.3). Aliquots (20 µl) of sample were loaded into each well and 3 µl of ladder (Precision Plus Proteins Dual Colour Standards, 1610374, Bio-Rad) was added into the two outer wells. The gel was electrophoresed at 150V for approximately 1 hour, or until the blue dye had reached the bottom of the gel.

2.8.4 SDS PAGE Gel transfer

A Criterion™ Blotter (Bio-Rad) was used to transfer proteins from the gel onto a nitrocellulose membrane (Cat# 10600004, Amersham Protran, GE Healthcare).

The stacking gel was removed from the PAGE gel and thereafter the transfer cassette was assembled in the following manner:

Black side of cassette (bottom)

Scotch-brite pad

2x Blotting paper

Gel

Membrane

2x Blotting paper

Scotch-brite pad

Red side of cassette (top)

The scotch-brite pads, blotting papers and membrane were presoaked in transfer buffer (Appendix 1.3) prior to assembly. The cassette was placed into an electrode module in

the correct orientation with a stirrer and ice pack placed inside, and the tank filled with transfer buffer. The protein was transferred at 100 V for one hour at 4 °C. Following the transfer, the cassette was disassembled, and the membrane was placed in a square petri dish. The ladder bands that were visible to the naked eye were marked with a pencil, and the membrane was washed twice for 10 minutes in membrane washing buffer (Appendix 1.3).

2.8.5 Membrane blocking

A blocking step was used to prevent non-specific binding of antibodies to the membrane. For blots on which antibodies for phosphorylated proteins would be used, 5% w/v BSA (Cat # ABRE, MP Biomedicals) in membrane washing buffer was used. For blots on which antibodies for unphosphorylated proteins would be used, 5% w/v milk powder in membrane washing buffer was used. Each membrane was incubated in the blocking solution for one hour with gentle agitation at room temperature.

2.8.6 Primary antibody incubation

Each membrane, which contained proteins from 10,000 previously-viable GC per lane or the controls or the ladder, were firstly incubated with phosphorylated Smad 2/3 (Santa Cruz, Lot# D1216) and 1/5/8 (Cell Signaling, Lot#13820) antibodies, prior to being stripped and then being incubated with unphosphorylated Smad 2/3 (Santa Cruz, Lot# B1313) and 1/5/8 (Santa Cruz, Lot # C2216) antibodies (see Appendix 3 for antibody details). Finally an additional stripping step was performed and each membrane was incubated with an antibody for the reference protein β-actin (Sigma-Aldrich, Cat# A5441). All antibodies for phosphorylated and unphosphorylated Smad proteins were added to washing buffer containing 5% w/v BSA or 5% w/v milk powder, respectively. Antibody concentrations were optimised by titration experiments and final optimised antibody concentrations are listed in Table 2.8.6. The antibody solution was added to the membrane and incubated overnight at 4 °C with gentle agitation.

Table 2.8.6: Optimised antibody concentrations.

Antibody	Concentration
Phosphorylated Smad 2/3	1: 500
Phosphorylated Smad 1/5/8	1:500
Smad 2/3	1:250
Smad 1/5/8	1: 250
β -actin	1: 1,000

2.8.7 Secondary antibody incubation

Upon removal of the primary antibody, membranes were washed with agitation three times for ten minutes in membrane washing buffer. Thereafter, the appropriate secondary antibody was added to membrane washing buffer containing 5% w/v BSA or 5% w/v milk powder for antibodies for phosphorylated and unphosphorylated proteins, respectively. Whilst a Horse Radish Peroxidase AffiniPure Rabbit anti-Goat IgG secondary antibody (Jackson Immunoresearch, Lot# 117560), was used to probe for the primary unphosphorylated Smad2/3 antibody, a Horse Radish Peroxidase Goat anti-Rabbit IgG secondary antibody (Abcam, Ab97051), was used to probe all other Smad primary antibodies. A Horse Radish Peroxidase AffiniPure Rabbit anti-Mouse secondary antibody (Jackson Immunoresearch, Lot# 119936) was utilised to probe the β-actin primary antibody at a 1:2,000 concentration. The optimised concentration for all other secondary antibodies used was 1:3000. Membranes were incubated with gentle agitation in the secondary antibody solution for two hours at room temperature.

2.8.8 Western blot development

After the antibody incubation steps, membranes were washed three times for 10 minutes in washing buffer. A Luminal:H_2O_2 solution (Western Bright™ ECL-Spray; Advantsa, K-12049-D50) was sprayed onto the membrane surface and left for three minutes. The membrane was then blotted dry and placed into an Omega Lum G Imaging

System (Aplegen) for image capture. Membranes probed with Smad antibodies were exposed for 15 minutes, whilst those probed with β actin were exposed for 10 minutes.

2.8.9 Antibody stripping

In order to strip the membranes of antibodies to allow for protein detection with another antibody, 100 mL of stripping buffer was prepared immediately prior to use (Appendix 1.3), and heated for 5 minutes in a plastic container placed in a water bath at 55°C. Thereafter membranes were added to the stripping buffer and incubated for 30 minutes at 55°C and the container was agitated every five minutes to evenly disperse the stripping buffer over the membrane. The membranes were then washed three times for 10 minutes in washing buffer, and the blocking, primary and secondary antibody incubation steps were performed, as described previously.

2.8.10 Antibody preabsorption

To validate the specificity of the antibodies used in these experiments, antibodies were preabsorbed with their specific blocking peptides (Appendix 3). The same primary and secondary antibody concentrations described in sections 2.6.5 and 2.6.6 were used. The membrane was probed with its appropriate antibody, which was subject to the same conditions as the antibodies utilised for the preabsorption steps. After membrane development, the membrane was stripped for preabsorption. For preabsorption, primary antibodies were incubated with blocking peptide at a 5:1 (blocking peptide: antibody) ratio, based on molecular weight, in a total volume of 200 μL of membrane washing buffer for 24 hours at 4°C with gentle agitation. Thereafter, the blocking peptide: antibody mixture was centrifuged for 5 minutes at 600 g, to remove any precipitate, and the supernatant was added to the appropriate blocking buffer and developed as described previously.

2.8.11 Western Blot Analysis

Densitometric analysis was performed using ImageJ software (version 1.50i with Java 1.6.0_41 (64-bit), National Inustitutes of Health (NIH), USA). Images were obtained from the Omega Lum G Imaging System (Aplegen), saved as *.tif* files and transferred onto a PC.

Semi-quantitative densitometric analysis was performed on each specific band using ImageJ. The mean grey value of each band was measured using a rectangle of uniform size. A total of 2-5 mean grey values of the background were measured across the blot, and the average of these values were subtracted from the value for each band to standardise the data. Thus, each band value is represented as a fold-change relative to β-actin.

2.9 Smad Homology Comparison

A Smad homology comparison was performed between the four test species (rat, sheep, pig and deer), in order to compare the similarity between these proteins for Western blotting. Accession numbers were obtained from National Center for Biotechnology (NCBI), apart from deer sequences, which were obtained from Brauning et al, 2015. The homology comparison was completed using NCBI BLAST.

2.10 Statistical Analysis

All statistical analysis was completed using the IBM software package SPSS statistics (version 22). *P* values of < 0.05 were considered statistically significant. Graphs were created using GraphPad Prism (Version 7.02). Prior to analyses, all data were tested for normal distribution, as determined by p-p plotting. Any data that was not normally distributed underwent either a natural log or square root transformation to ensure the data exhibited a normal distribution. All graphs show mean values ± SEM.

Analyses of raw CPM values and fold-changes indicating GC proliferation was performed using a two-way ANOVA. If significant differences were observed, a Bonferroni post-hoc analysis test was performed.

Analyses of the qPCR ($2^{(-ddCt)}$ values) and Western blotting (densiometry values) data were analysed using a one-way ANOVA. If significance was observed, either a Bonferroni post-hoc analysis test or a least significant difference (LSD) post-hoc analysis test was performed respectively.

Table 2.9.1: Homology of Smad proteins between species.

Accession number	Smad1	Rat	Sheep	Pig	Deer
NP_037262	**Rat**	100	98	88	98
XP_011952811.1	**Sheep**		100	88	100
XP_013842259	**Pig**			100	98
N/A	**Deer**				100
	Smad2				
XP_006255007	**Rat**	100	99	93	93
XP_011976298	**Sheep**		100	94	94
XP_005659505	**Pig**			100	94
N/A	**Deer**				100
	Smad3				
NP_037227	**Rat**	100	89	100	ND
XP_014959209	**Sheep**		100	90	ND
NP_999302	**Pig**			100	ND
N/A	**Deer**				100
	Smad5				
NP_067724	**Rat**	100	99	99	ND
XP_012033923	**Sheep**		100	99	ND
XP_013842259	**Pig**			100	ND
N/A	**Deer**				100
	Smad8				
XP_017446635	**Rat**	100	95	96	ND
XP_014953594	**Sheep**		100	98	ND
NP_001182439	**Pig**			100	ND
N/A	**Deer**				100

ND – not determined.

3.0 Results

3.1 Effects of incubation time on GC proliferation rate

The proliferation rate of rat, sheep, pig and deer GC following the co-incubation with the same species oocytes or with activin A (control) is illustrated in Figure 3.1.1 as a fold-change relative to baseline GC proliferation rate (normalised values), and is illustrated in Figure 3.1.2 as tritiated-thymidine uptake values of counts per minute (CPM; non-normalised values).

The proliferation rate of rat GC after 0, 4, 8, 12 and 16 hours incubation with denuded oocytes of rats, or with activin A (control) is illustrated as a fold change relative to baseline GC proliferation rate in Figure 3.1.1A. The proliferation rate of sheep, pig, and deer GC after 8, 12 and 16 hours incubation with denuded oocytes of the corresponding species, or with activin A is illustrated as a fold change relative to baseline GC proliferation in Figures 3.1.1B, C and D, respectively.

Overall, the addition of oocytes of the same-species increased rat, sheep, pig, and deer GC proliferation (P < 0.0001, P < 0.0001, P < 0.001 and P < 0.0001, respectively; Figure 3.1.1). The addition of activin A only increased rat (P< 0.0001), and sheep (P < 0.009) GC proliferation levels, but not pig and deer GC proliferation (Figure 3.1.1).

The fold change in proliferation rate of rat GC co-incubated with rat denuded oocytes was higher at 12 hours (P < 0.00, P < 0.001 and P < 0.028, respectively) and 16 hours (P < 0.0001, P < 0.0001 and P < 0.0001, respectively), compared to that at 0, 4, and 8 hours (Figure 3.1.1A). Rat GC cultured with Activin A had a higher proliferation rate at 16 hours compared to 0, 4, and 8 hours (P<0.001, P<0.004 and P<0.002, respectively). The proliferation rate of sheep GC upon sheep oocytes co-incubation was higher (P < 0.019) after 16, compared to 8, hours of incubation. Incubation time did not influence Activin A stimulated sheep GC proliferation rate. Incubation time also did not influence the

proliferation rate of pig or deer GC upon incubation with the same-species oocyte or with Activin A.
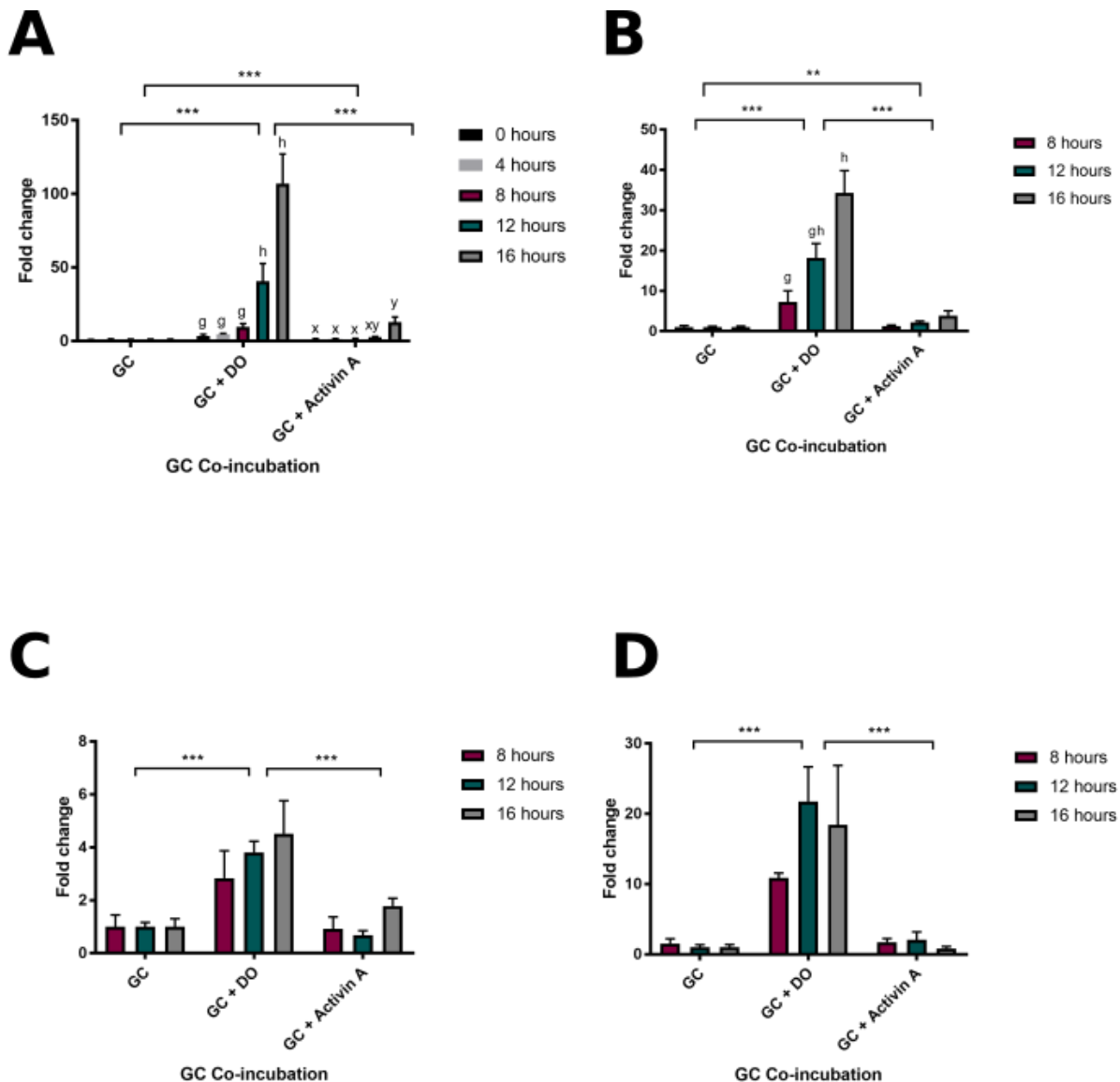
Figure 3.1.1: Fold change in proliferation rate of rat, sheep, pig and deer GC with time in culture.

Mean ± SEM fold change in the proliferation rate of rat (A) or sheep (B) or pig (C) or deer (D) GC co-incubated alone, with denuded oocytes of the corresponding species, or Activin A (10 ng/mL). Values are relative to that of GC in media alone. Mean and SEM of three to five replicates for each treatment group are shown. Different alphabetical superscripts on histograms denote significant difference (P<0.05), whilst asterixes (*) denote significant differences between treatment groups (* P< 0.05, ** P< 0.01, *P< 0.001).

When comparing raw CPM data, the addition of denuded oocytes of the same species increased proliferation of rat, sheep, pig, and deer GC ($P<0.0001$, $P<0.0001$, $P<0.001$ and $P<0.0001$, respectively). The addition of activin A only increased rat GC ($P< 0.0001$) and sheep GC ($P< 0.009$) proliferation, but not sheep, pig and deer GC.

Tritiated thymidine incorporation of rat GC incubated alone decreased after 12 hours ($P<0.001$, $P<0.007$ and $P<0.035$, respectively) and 16 hours ($P<0.0001$, $P<0.001$ and $P<0.004$ respectively) of incubation, compared to that at 0, 4, or 8 hours (Figure 3.1.2A). Tritiated thymidine incorporation was higher ($P<0.0001$) following the addition of rat oocytes but was not affected with incubation time (Figure 3.1.2A). Overall, the addition of Activin A increased tritiated thymidine incorporation however, this decreased after 12 hours incubation ($P<0.047$) (Figure 3.1.2A).

Tritiated thymidine incorporation in sheep GC cultured alone decreased after 12 and 16, compared to 8, hours incubation time ($P<0.01$ and $P<0.002$, respectively) (Figure 3.1.2B). Overall, tritiated thymidine incorporation in sheep GC was increased following the addition sheep denuded oocytes ($P<0.0001$) but was not effected by incubation time (Figure 3.1.2B). Similar to sheep GC cultured alone, the addition of Activin A decreased tritiated thymidine incorporation after 12 and 16 hours, compared to 8 hours, of incubation ($P<0.02$ and $P<0.014$, respectively) (Figure 3.1.2B).

Tritiated thymidine incorporation in pig GC cultured alone decreased at 16 hours, compared to 8 and 12 hours, of incubation ($P<0.009$ and $P<0.035$, respectively) (Figure 3.1.2C). Overall, tritiated thymidine incorporation was higher in pig GC following the addition of pig oocytes, compared to GC alone or with Activin A, however this decreased after 16 hours in culture, compared to that at 8 and 12 hours ($P<0.004$ and $P<0.015$, respectively) (Figure 3.1.2C). Similar to pig GC cultured alone, the addition of Activin A decreased tritiated thymidine incorporation after 16 hours, compared to 8 hours, of incubation ($P < 0.038$) (Figure 3.1.2C).

Overall, tritiated thymidine incorporation was higher (P<0.0001) in deer GC cultured with deer oocytes, compared to media alone or Activin A (Figure 3.1.2D). However, incubation time did not affect tritiated thymidine incorporation levels in any treatment group (Figure 3.1.2D).
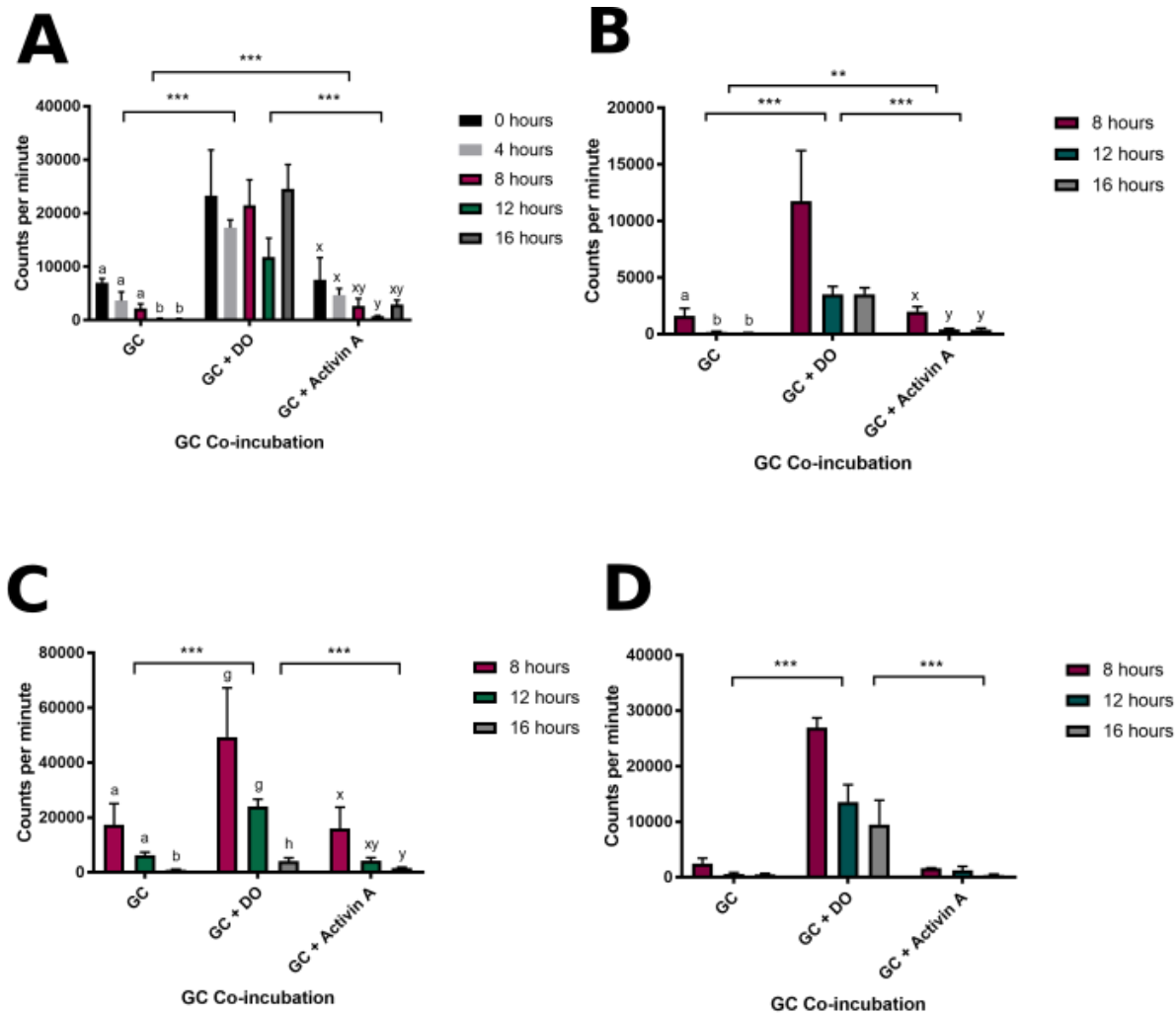
Figure 3.1.2: Tritiated thymidine incorporation of rat, sheep, pig and deer GC with time in culture.

Mean ± SEM tritiated thymidine incorporation (counts per minute; CPM) of rat (A) or sheep (B) or pig (C) or deer (D) GC co-incubated alone, with denuded oocytes of the corresponding species, or Activin A (10 ng/mL). Mean and SEM of three to five replicates for each treatment group are shown. Different alphabetical superscripts on histograms denote significant difference (P<0.05) in treatment groups between times, whilst asterixes (*) denote significant differences between treatment groups (* P< 0.05, ** P< 0.01, *P< 0.001).
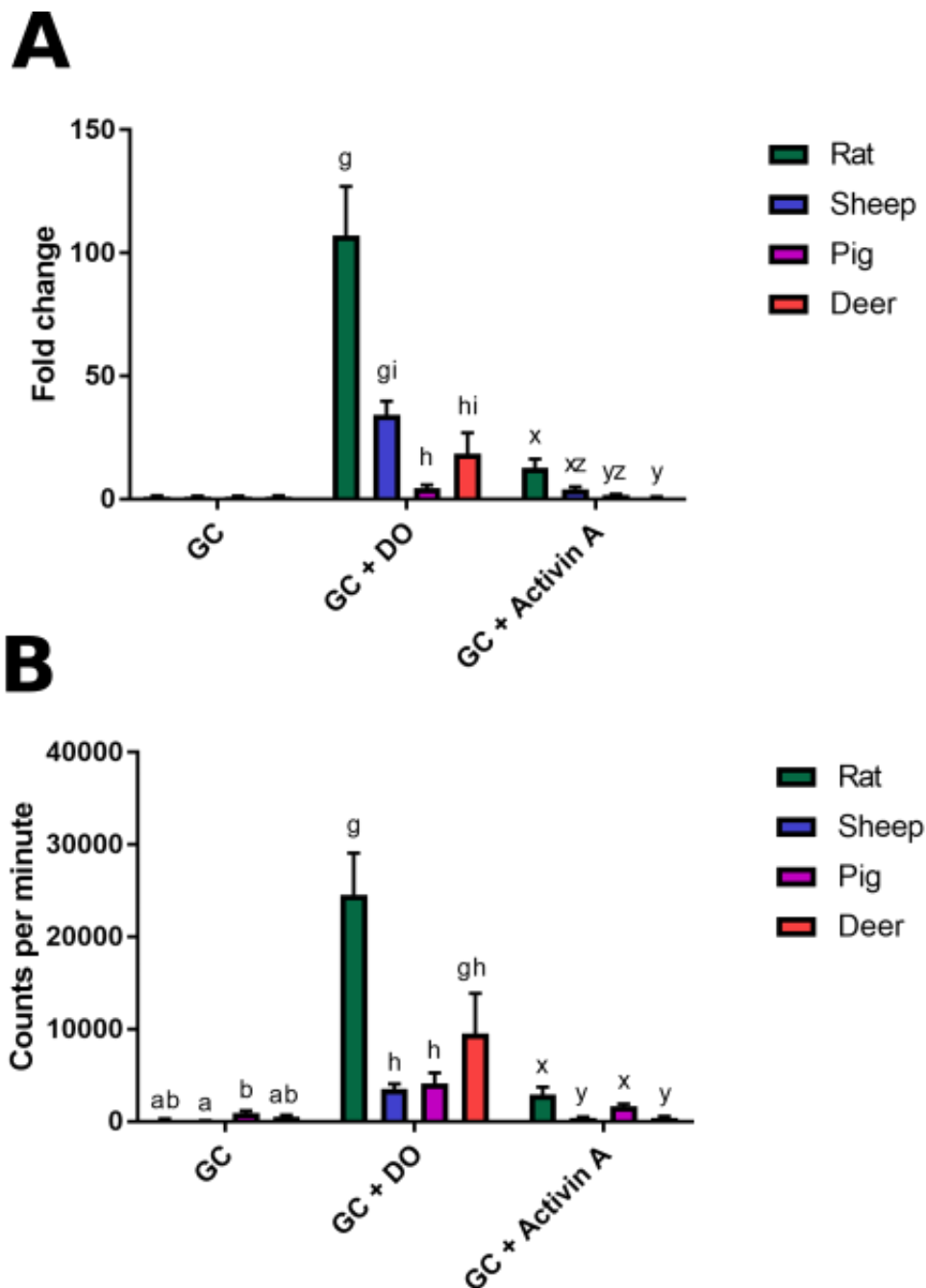
Figure 3.1.3: Proliferation rate of rat, sheep, pig and deer GC after 16 hours in culture

Mean ± SEM fold change (A) or CPM (B) in the proliferation of all species GCs co-incubated alone, with denuded oocytes, or Activin A (10 ng/mL). Mean and SEM of three to five replicates for each treatment group are shown. Different alphabetical superscripts on histograms denote significant difference (P<0.05).

Upon consideration of the proliferation results after the addition of oocytes of GC of all species with time in culture, the optimal incubation time was determined to be 16 hours, as illustrated in Figure 3.1.3. Similar to the graphs shown above, results are illustrated

as a fold change relative to baseline GC proliferation (Figure3.1.3A) or as non-normalised tritiated-thymidine uptake values of counts per minute (CPM) (Figure 3.1.3B).

In regards to proliferation rate relative to that of GC cultured alone (fold-change), proliferation rate of GC co-incubated with denuded oocytes of the same species was highest in rats, compared to that of pigs ($P<0.004$) and deer ($P<0.004$), but similar to that of sheep (Figure 3.1.3A). Furthermore, the co-incubation of sheep GC with sheep oocytes resulted in a higher proliferation rate in comparison to that of the pigs ($P<0.004$) (Figure 3.1.3A). Activin A exhibited a higher fold change in proliferation of rat, compared to, pig ($P<0.004$) and deer ($P<0.001$) GC. Furthermore, Activin A exhibited a higher proliferation in sheep compared to deer, GC ($P<0.029$).

In regards to tritiated thymidine incorporation (CPM), pig GC cultured alone displayed a higher CPM compared to that of sheep GC (Figure 3.1.3A). The co-incubation of GC with oocytes resulted in a higher CPM in rats compared to that in sheep ($P<0.0001$) and pigs ($P<0.005$). The addition of Activin A resulted in a higher tritiated thymidine incorporation of rat and pig GC than to sheep ($P<0.003$ and $P < 0.037$, respectively) and deer ($P<0.003$ and $P<0.048$, respectively) GC.

3.2 Effects of oocyte co-incubations on GC proliferation rate

The effects of co-incubating oocytes of the same or different (cross) species on proliferation rate were examined in GC of each species.

3.2.1 Co-incubations of rat GCs with oocytes

The effects of co-incubating rat GC with rat, sheep, pig and deer denuded oocytes on GC proliferation rate are illustrated in Figure 3.2.1. The co-incubation of rat GC with rat ($P< 0.001$), sheep ($P< 0.001$), pig ($P< 0.001$) and deer ($P< 0.001$) oocytes markedly increased GC proliferation rate compared to rat GC cultured alone. Furthermore, the co-

incubation with rat (P<0.001), sheep (P<0.001) and pig (P<0.003) oocytes stimulated rat GC to proliferate at a greater rate than when co-incubated with deer oocytes.
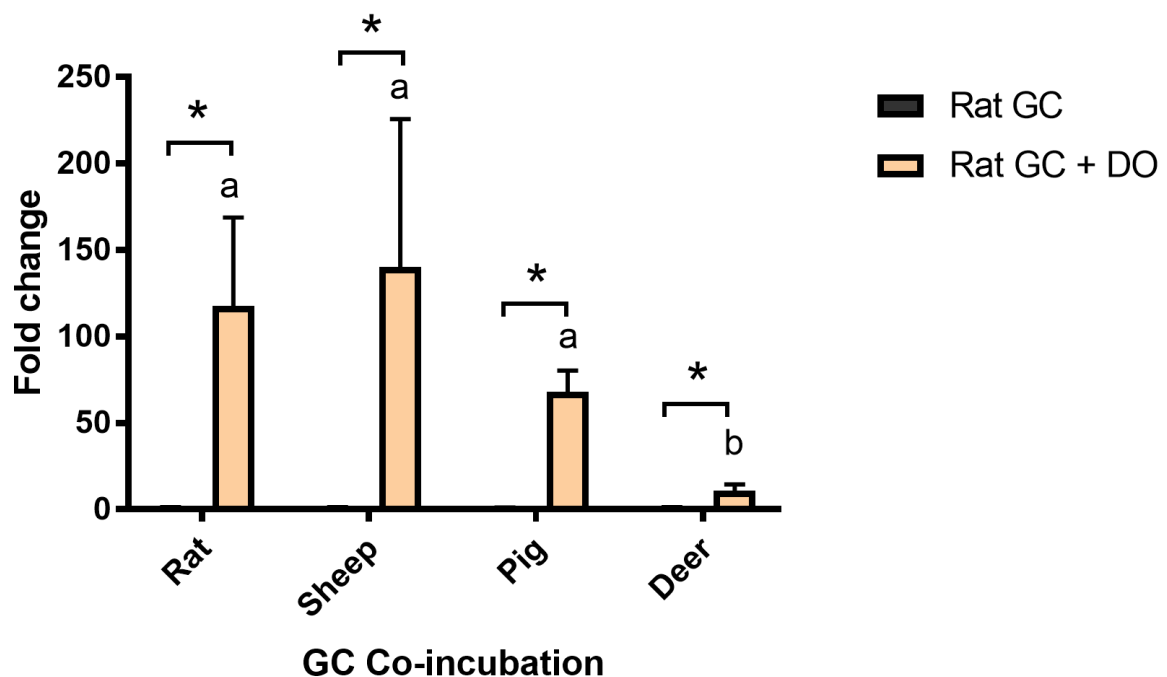
Figure 3.2.1: Proliferation rate of rat GC co-incubated with oocytes.

Mean ± SEM fold change in the proliferation of rat GCs co-incubated with rat, sheep, pig or deer denuded oocytes. Values are relative to rat GCs in media alone. Mean values represent data from five to seven replicates for each treatment group. Different alphabetical superscripts on histograms denote significant differences (P<0.003) between GCs co-incubated with denuded oocytes of different species. Asterisks denote significant differences (P<0.001) between GC incubated alone or with denuded oocytes within each species.

3.2.2 Co-incubations of sheep GCs with oocytes

The effects of co-incubating sheep GC with rat, sheep, pig and deer denuded oocytes on GC proliferation rate are illustrated in Figure 3.2.2. The co-incubation of sheep GC with rat (P< 0.001), sheep (P< 0.001), pig (P< 0.001) and deer (P< 0.001) oocytes markedly increased GC proliferation rate compared to sheep GC cultured alone. However, there were no differences in oocyte-induced GC proliferation between the different species tested.
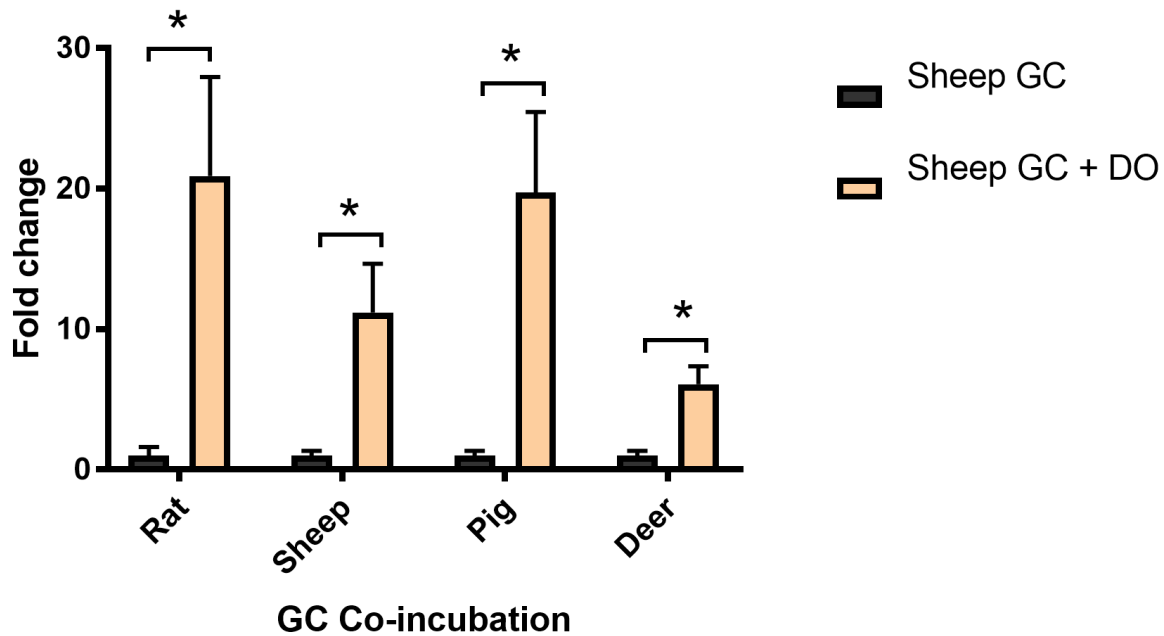
Figure 3.2.2: Proliferation rate of sheep GC co-incubated with oocytes.

Mean ± SEM fold change in the proliferation of sheep GCs co-incubated with rat, sheep, pig, or deer denuded oocytes. Values are relative to sheep GCs in media alone. Mean values represent data from five to seven replicates for each treatment group. Asterisks denote significant differences (P<0.001) between GC incubated alone or with denuded oocytes within each species.

3.2.3 Co-incubations of pig GCs with oocytes

The effects of co-incubating pig GC with rat, sheep, pig and deer denuded oocytes on GC proliferation rate are illustrated in Figure 3.2.3. The co-incubation of pig GC with rat (P< 0.001), sheep (P< 0.001), pig (P< 0.001) and deer (P< 0.001) oocytes markedly increased GC proliferation rate compared to pig GC cultured alone.
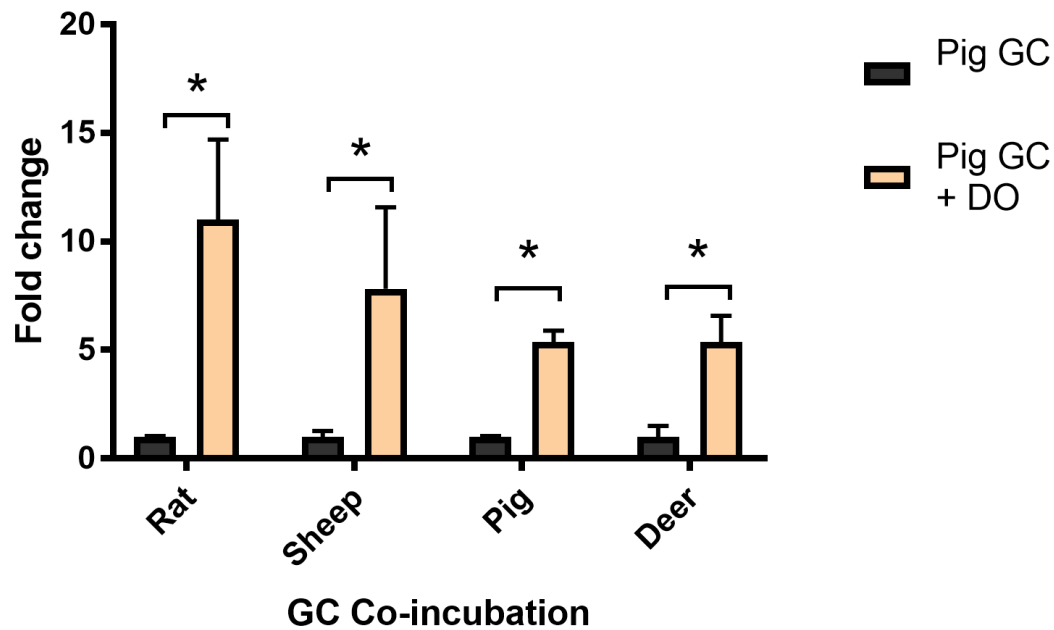
Figure 3.2.3: Proliferation rate of pig GC co-incubated with oocytes.

Mean ± SEM fold change in the proliferation of pig GCs co-incubated with rat, sheep, pig or deer denuded oocytes. Values are relative to pig GCs in media alone. Mean values represent data from five to seven replicates for each treatment group. Asterisks denote significant differences (P<0.001) between GC incubated alone or with denuded oocytes within each species.

3.2.4 Co-incubations of deer GCs with oocytes

The effects of co-incubating deer GC with rat, sheep, pig and deer oocytes on GC proliferation rate are illustrated in Figure 3.2.4. The co-incubation of deer GC with rat (P< 0.001), sheep (P< 0.007), pig (P< 0.001) and deer (P< 0.001) oocytes markedly increased GC proliferation rate compared to deer GC cultured alone. However, there were no differences in oocyte-induced GC proliferation between the different species tested.
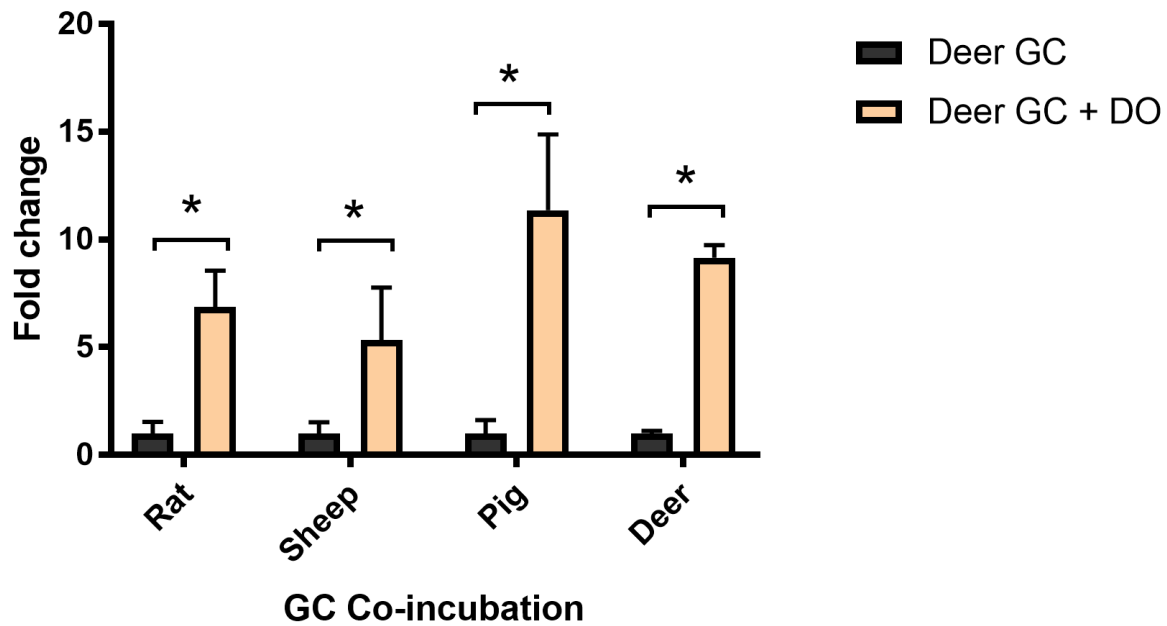
Figure 3.2.4: Proliferation rate of deer GC co-incubated with of deer oocytes.

Mean ± SEM fold change in the proliferation of deer GCs co-incubated with rat, or sheep, or pig, or deer denuded oocytes. Values are relative to deer GCs in media alone. Mean values represent data from five to seven replicates for each treatment group. Asterisks denote significant differences (P<0.007) between GC incubated alone or with denuded oocytes within each species.

3.5 Effects of cell viability and species on GC proliferation rate

The effects of differences in GC viability, and basal GC proliferation rate between species were tested.

3.5.1 Differences in GC viability between species

The proportion (%) of live GC present within the GC sample at the time of collection (GC viability) for each species is illustrated in Figure 3.5.1. Whilst the GC viability of rat, sheep and pig were similar (17- 25%), the viability of deer GC was lower (11%; P<0.002) than that of sheep and pig.
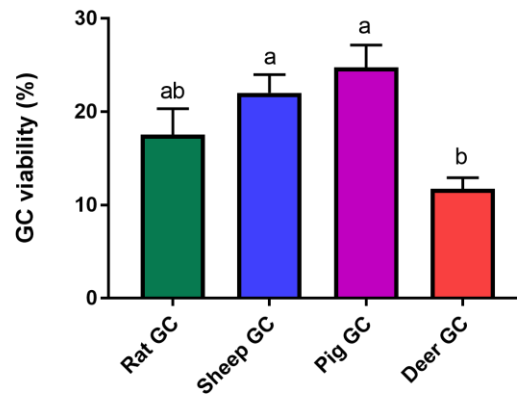
Figure 3.5.1: Cell viability of rat, sheep, pig and deer GC at time of collection.

Mean ± SEM (%) values represent 15 to 21 replicate samples for each species. Different alphabetical superscripts on histograms denote significant differences (P<0.05) between species.

3.5.2 Differences in basal GC proliferation rate between species

Comparisons in tritiated thymidine incorporation rate of rat, sheep, pig and deer GC incubated alone for 16 hours are illustrated in Figure 3.5.2. Whilst basal proliferation rates of GC were similar in rat, sheep and deer (623- 888 CPM), pig GC had a higher proliferation rate than that of rats (P< 0.009) and deer (P< 0.03).
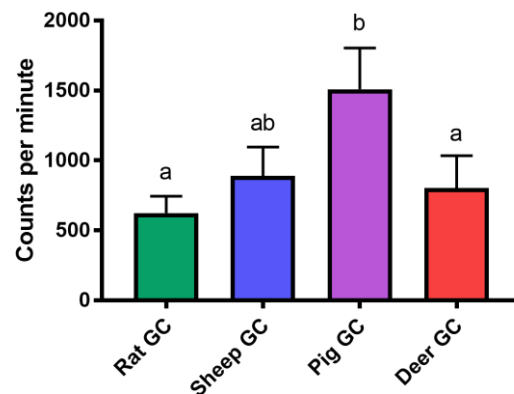
Figure 3.5.2: Basal proliferation rate of rat, sheep, pig and deer GC.

Mean ± SEM counts per minute (CPM; proliferation rate) of rat, sheep, pig or deer GCs incubated for 16 hours in media alone. Mean values represent data from three to five replicate experiments for each treatment group. Different alphabetical superscripts on histograms denote significant differences (P<0.03) between species.

3.5.3 Effects of GC viability on basal proliferation rate

The correlations between cell viability (%) and proliferation rate (CPM) in rat, sheep, pig and deer GC are illustrated in Figure 3.5.3. Due to the low r^2 value calculated, it was deemed that there was no linear relationship between GC viability and basal proliferation rate in any of the species tested.
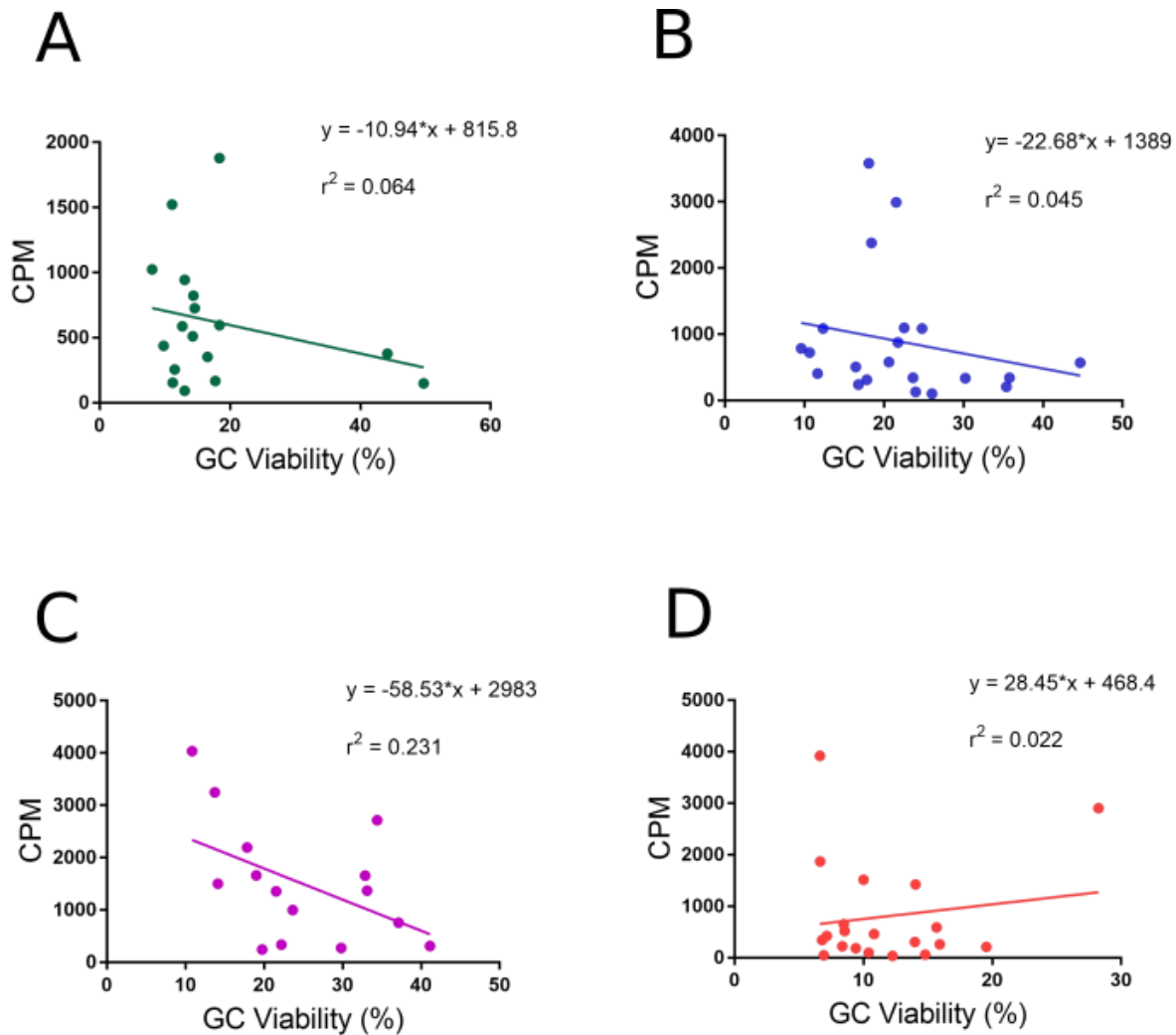
Figure 3.5.3: Correlation between GC viability and basal proliferation rate.

The correlation between the proportion of viable cells (%) and basal proliferation rate (counts per minute; CPM) in rat (A), sheep (B), pig (C) and deer (D) GC. Each dot (circle) within each plot represents an individual replicate. R^2 values depict the coefficient of determination and measure the closeness of the data to the fitted regression line.

3.6 Effects of oocyte co-incubation on GC-derived gene expression

The effects of co-incubating oocytes of the same or different (cross) species on expression levels of key genes in GC of each species were examined and are each presented below. The key genes tested were those that are responsible for the transcription of the Type I (*TGFBR1, BMPRIB*) and Type II (*BMPR2*) receptors for GDF9 and BMP15. The suitability of the chosen reference genes (*RPL19, PPIA*) was also examined for each species.

3.6.1 Validation of reference genes

Rat, sheep, pig and deer GC were co-incubated either alone or with denuded oocytes of rat, sheep, pig or deer. Comparisons between gene expression values (cycle threshold; Ct) for *RPL19* and *PPIA* mRNA in rat, sheep, pig and deer GC samples are illustrated in Figure 3.6.1A, B, C and D, respectively.
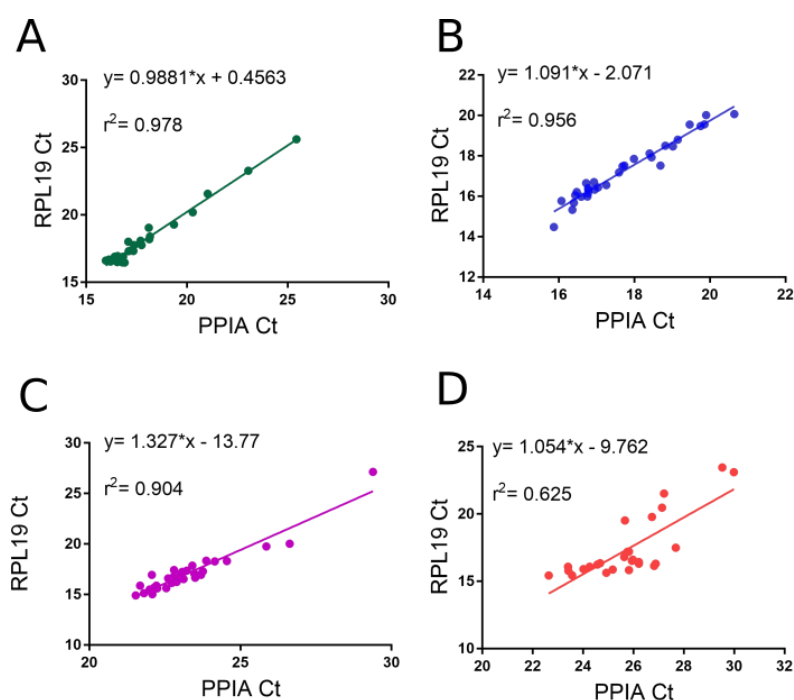
Figure 3.6.1: PPIA and RPL19 expression in rat, sheep, pig and deer GC samples.

The correlation between RPL19 and PPIA mRNA levels, expressed as a C_t values, in rat (A), sheep (B), pig (C), or deer (D) GC samples, that have been incubated alone, or with rat, or sheep, or pig or deer denuded oocyte. Each point represents a single sample consisting of two technical replicates. R^2 values depict the coefficient of determination and measure the closeness of the data to the fitted regression line.

3.6.2 Relative expression of key genes in rat GCs

The effects of co-incubating rat GC with denuded ooytes of rat, sheep, pig and deer on relative expression levels of *Tgfbr1, Bmpr1b and Bmpr2* mRNA are illustrated in Figure 3.6.2. The relative expression levels of *Tgfbr1* mRNA in rat GC were elevated (P<0.05) after co-incubation with sheep ooytes, in comparison to co-incubation with media alone or with rat, pig or deer oocytes (Figure 3.6.2A). The relative expression level of *Bmpr1b* mRNA in rat GC was reduced (P<0.03) following co-incubation with oocytes of deer, compared to that of all other species and the controls (Figure 3.6.2B). Furthermore, *Bmpr1b* mRNA levels of rat GC co-cultured with rat oocytes were higher (P<0.021) than with pig oocytes (Figure 3.6.2B), although neither differed from controls. Similarly in comparison to controls (media alone), the relative expression level of *Bmpr2* mRNA in rat GC was higher (P<0.001) following co-incubation with rat oocytes and lower (P<0.001) following co-incubation with deer oocytes (Figure 3.6.2C). The co-incubation of rat GC with sheep or pig oocytes did not alter *Bmpr2* mRNA levels from control values (Figure 3.6.2C).

3.6.3 Relative expression of key genes in sheep GCs

The effects of co-incubating sheep GC with rat, sheep, pig and deer denuded oocytes on relative expression levels of *Tgfbr1, Bmpr1b and Bmpr2* mRNA are illustrated in Figure 3.6.3. The relative expression levels of *Tgfbr1* mRNA in sheep GC were elevated (P<0.02) after co-incubation with pig oocytes, in comparison to co-incubation with media alone, or sheep or deer oocytes, but not rat oocytes (Figure 3.6.3A). The addition of oocytes of any species did not alter relative expression level of *Bmpr1b* mRNA in sheep GC (Figure 3.6.3B). The relative expression level of *Bmpr2* mRNA in sheep GC was lower (P<0.001) following co-incubation with deer oocytes (P<0.05), compared to co-incubation with rat and sheep oocytes (Figure 3.6.3C).
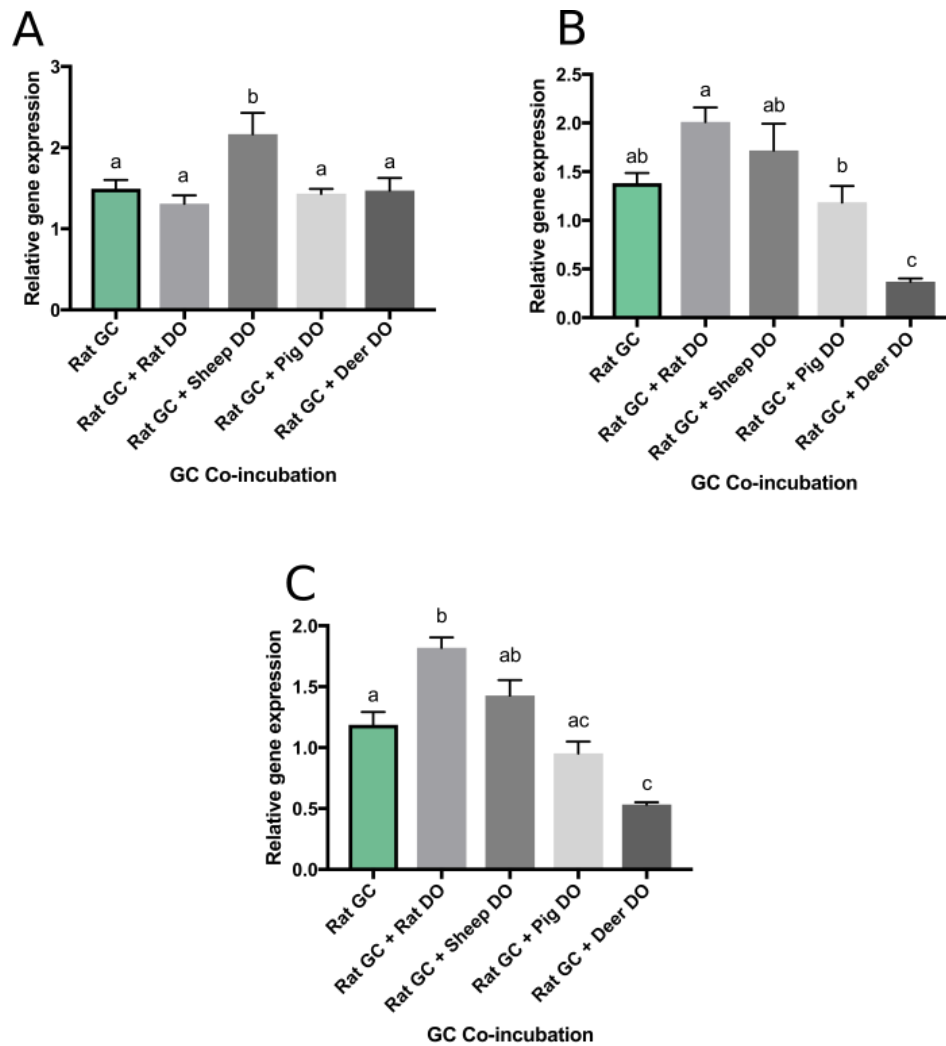
Figure 3.6.2: Gene expression levels in rat GC co-incubated with oocytes.

Mean ± SEM values of relative expression levels of Tgfbr1 (A), Bmpr1b (B) and Bmpr2 (C) mRNA in rat GCs incubated in media alone (controls), or with rat, sheep, pig or deer denuded oocyte. Mean values represent data from five to seven replicate experiments for each GC co-incubation group. Different alphabetical superscripts on histograms denote significant differences (P<0.05) between rat GC incubated alone or with denuded oocyte of different species.
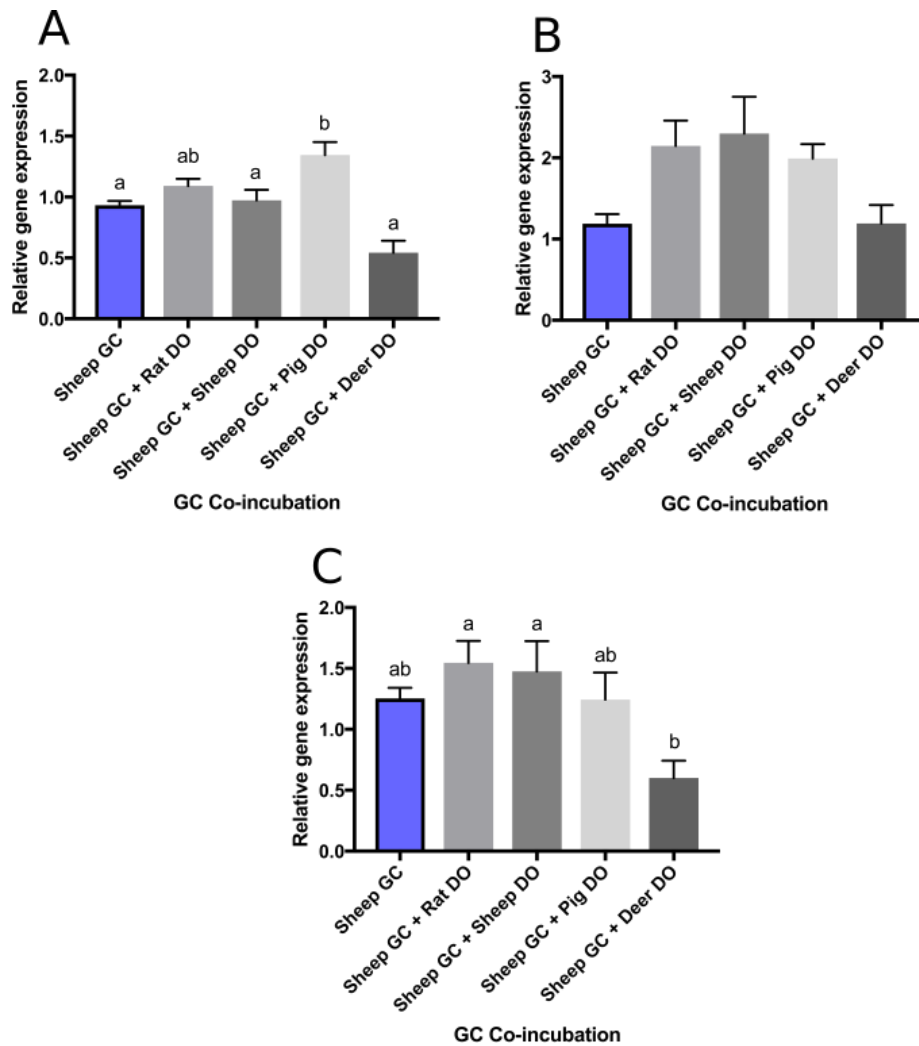
Figure 3.6.3: Gene expression levels in sheep GC co-incubated with oocytes.

Mean ± SEM values of relative expression levels of TGFBR1 (A), BMPR1B (B) and BMPR2 (C) mRNA in sheep GC incubated in media alone (controls), or with rat, sheep, pig or deer denuded oocyte. Mean values represent data from five to seven replicate experiments for each GC co-incubation group. Different alphabetical superscripts on histograms denote significant difference (P<0.05) between sheep GC incubated alone or with denuded oocyte of different species.

3.6.4 Relative expression of key genes in pig GCs

The effects of co-incubating pig GC with rat, sheep, pig and deer denuded oocytes on relative expression levels of *TGFBR1, BMPR1B and BMPR2* mRNA are illustrated in Figure 3.6.4. The relative expression levels of *TGFBR1*mRNA in pig GC were higher (P<0.05) after co-incubation with deer oocytes, in comparison to co-incubation with media alone, or sheep oocytes, but not rat or pig oocytes (Figure 3.6.4A). The relative expression level of *BMPR1B* mRNA in pig GC was higher (P<0.001) following co-

incubation with pig oocytes, compared to control GC (Figure 3.6.4B). Additionally, expression levels of *BMPR1B* mRNA in pig GC was markedly higher (P<0.001) following co-incubation with deer oocytes, compared to control GC, or pig GC co-incubated with oocytes from all other species (Figure 3.6.4B). The addition of oocytes of any species did not alter relative expression levels of *BMPR2* mRNA in pig GC (Figure 3.6.4C).
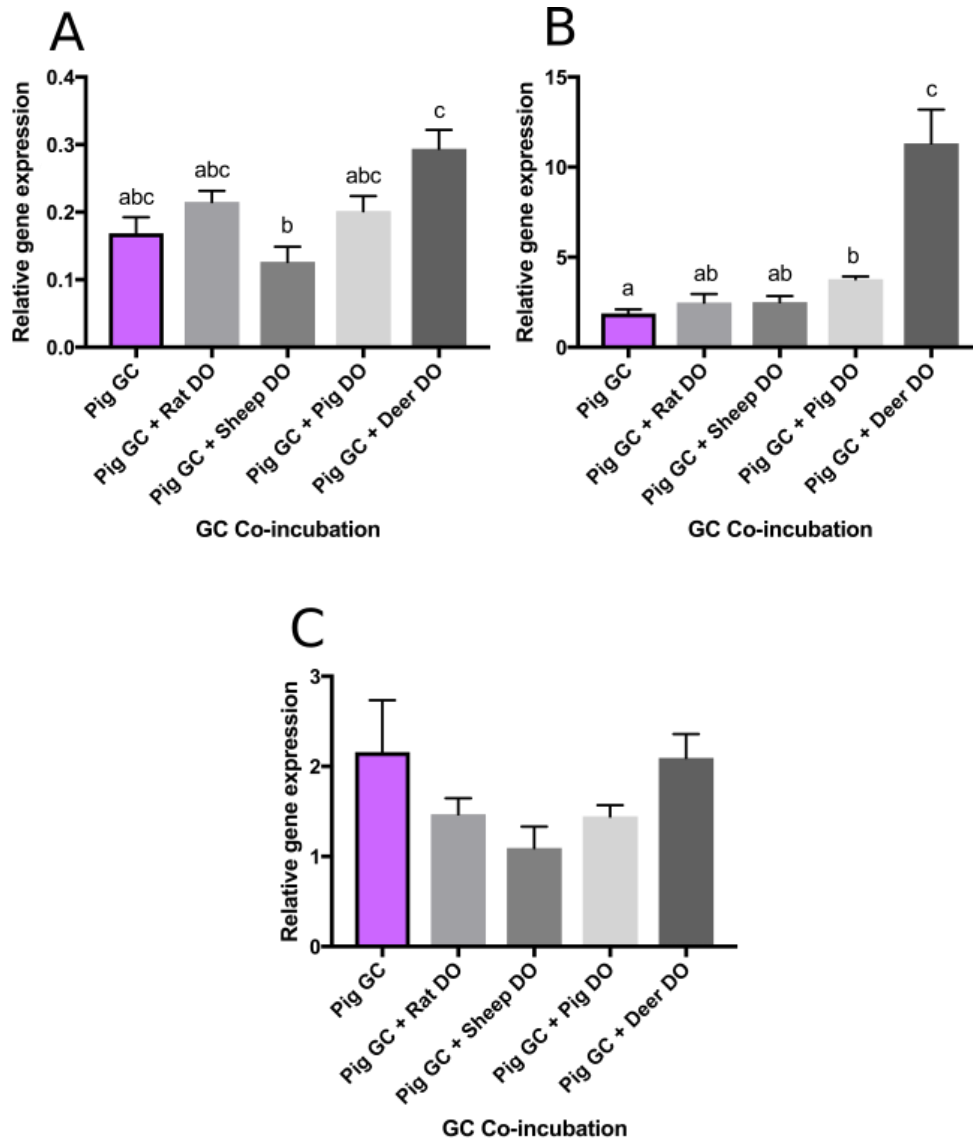

Figure 3.6.4: Gene expression levels in pig GC co-incubated with oocytes.

Mean ± SEM values of relative expression levels of TGFBR1 (A), BMPR1B (B) and BMPR2 (C) mRNA levels in pig GC incubated in media alone (controls), or with rat, sheep, pig or deer denuded oocyte. Mean values represent data from five to seven replicate experiments for each GC co-incubation group. Different alphabetical superscripts on histograms denote significant differences (P<0.05) between pig GC incubated alone or with denuded oocyte of different species.

3.6.5 Relative expression of key genes in deer GCs

The effects of co-incubating deer GC with rat, sheep, pig and deer denuded oocytes on relative expression levels of *TGFBR1, BMPR1B and BMPR2* mRNA are illustrated in Figure 3.6.5. The addition of rat, sheep, pig or deer oocytes to cultured deer GC did not effect expression levels of *TGFBR1* or *BMPR1B* mRNA (Figures 3.6.5A and B, respectively). Conversely compared to control GC, the relative expression level of *BMPR2* mRNA in deer GC was lower (P<0.001) following co-incubation with sheep or pig oocytes, but not rat or deer oocytes (Figure 3.6.5C).
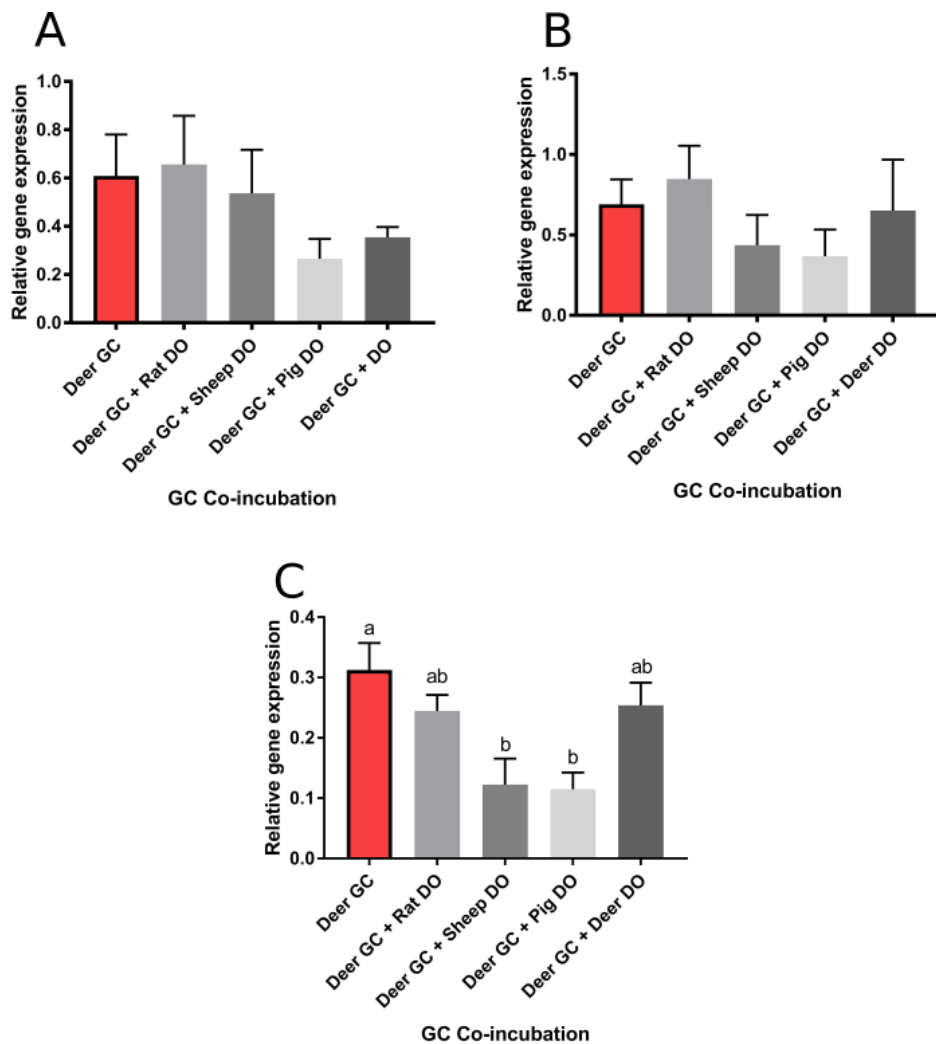
Figure 3.6.5: Gene expression levels in deer GC co-incubated with oocytes.

Mean ± SEM values of relative expression levels of TGFBR1 (A), BMPR1B (B) and BMPR2 (C) mRNA levels in deer GC incubated in media alone (controls), or with rat, sheep, pig or deer denuded oocyte. Mean values represent data from five to seven replicate experiments for each GC co-incubation group. Different alphabetical superscripts on histograms denote significant differences (P<0.05) between deer GC incubated alone or with denuded oocyte of different species.

3.7 Effects of oocyte co-incubation on Smad protein levels in GCs

The effects of co-incubating oocytes of the same or different (cross) species on relative Smad protein amounts in GC of each species were examined and are each presented in sub-sections below. The key Smads tested were the phosphorylated and unphosphorylated forms of Smads 2/3 and 1/5/8. The Smad protein levels presented in the graphs below are relative to the amount of the reference protein β-actin (ACTB) present. All figures presented herein (Figures 3.7.1- 3.7.4) show a representative Western blot of phosphorylated (A1) and unphosphorylated (B1) proteins, and the mean amount of phosphorylated (A2) and unphosphorylated (B2) proteins relative to ACTB protein levels from three replicate Western blots. The positions of the bands depicting the proteins of interest that were visible on the Western blots are denoted by a black box to the right side of each blot.

The negative control added to each blot consisted of secreted media from H293 cells transfected with recombinant deer GDF9. Only very faint or no bands depicting the specific proteins of interest were present on any Western blot and no corresponding densitometry results were collated.

The positive controls added to each blot were sheep GC co-incubated with H293-produced recombinant deer GDF9 and deer BMP15. Previous experiments revealed that the addition of recombinant deer GDF9 and BMP15 to sheep GC cultures modestly stimulated GC proliferation rate (Bibby and Pitman, data unpublished), and were assumed to be facilitated predominantly via stimulation of the Smad2/3 and 1/5/8 pathways, respectively. In these studies, the addition of recombinant deer GDF9 and BMP15 proteins to GC of every species tested resulted in moderate to heavy bands of Smad2/3 and 1/5/8 protein forms. Thus the prospective positive controls in these experiments were used to ensure success of the Western blotting procedure and were eliminated from the analyses.

Several non-specific bands (~150 kDa, and 10- 40 kDa) were observed. These bands represent unknown proteins and were denoted by a black circle to the right side of each blot.

3.7.1 Relative Smad 2/3 protein levels in rat GCs

Representative Western blots and mean relative densiometry results depicting the levels of phosphorylated and unphosphorylated Smad 2/3 proteins in rat GC incubated with media alone, or with rat, sheep, pig or deer oocytes are illustrated in Figure 3.7.1. The addition of oocytes, regardless of species, to rat GC cultures did not affect relative phosphorylated Smad 2/3 protein levels in rat GC (Figure 3.7.1A). The co-culture of sheep, but not rat or deer or pig, oocytes elicited an increase ($P<0.05$) in relative unphosphorylated Smad 2/3 levels in rat GC above that of rat GC cultured alone. (Figure 3.7.1B).

3.7.2 Relative Smad 1/5/8 protein levels in rat GCs

Representative Western blots and mean relative densiometry results depicting the levels of phosphorylated and unphosphorylated Smad1/5/8 proteins in rat GC incubated with media alone, or with rat, sheep, pig or deer oocytes are illustrated in Figure 3.7.2. The addition of oocytes, regardless of species, to rat GC cultures did not influence the relative levels of phosphorylated Smad 1/5/8 in rat GC (Figure 3.7.2A). The addition of deer oocytes to cultured rat GC increased unphosphorylated Smad 1/5/8 levels compared to rat GC incubated with media alone ($P<0.04$), but not rat or sheep or pig oocytes (Figure 3.7.2B).
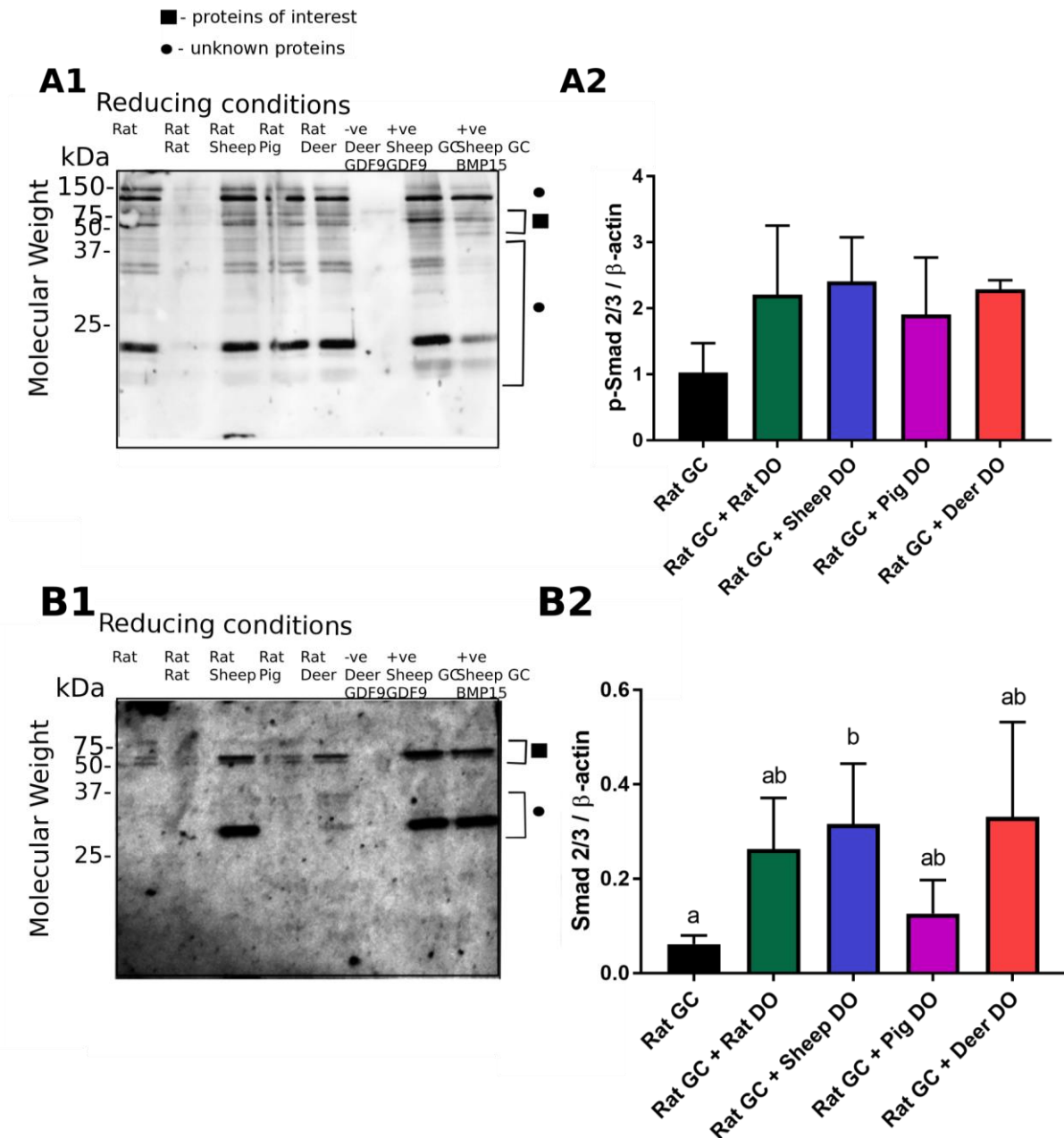
Figure 3.7.1: Smad 2/3 levels in rat GC co-incubated with oocytes.

Representative Western blots (1), and resultant plots of densiometry readings from specific proteins bands relative to ACTB bands (2) for phosphorylated (A) and unphosphorylated (B) Smad2/3 in rat GC incubated in media alone (controls), or with rat, sheep, pig or deer denuded oocyte (Lanes 1-5, respectively). Recombinant deer GDF9 was used as a negative control (Lane 6), and sheep GC treated with either recombinant deer GDF9 (Lane 7) or BMP15 (Lane 8) were used as positive controls. Blots were probed with phosphorylated (p)-Smad 2/3 antibody (A1, A2), and thereafter stripped and reprobed for Smad 2/3 antibody (B1, B2). All blots were performed under reducing conditions. A2 and B2 represent mean ± SEM values from three replicate Western blot experiments. Histograms that do not share at least one similar alphabetical superscript are significantly different (P<0.05) to each other.
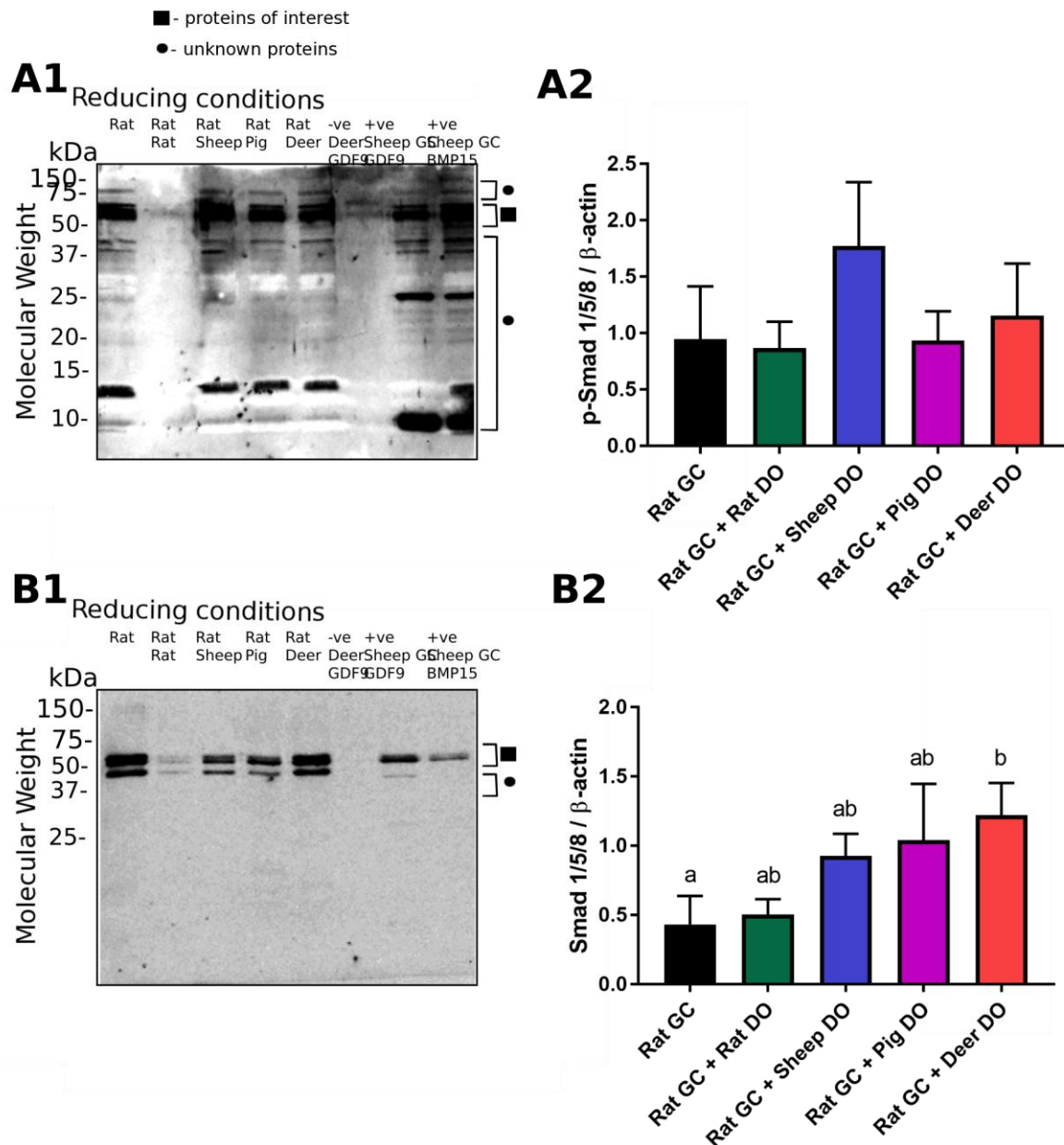
Figure 3.7.2: Smad 1/5/8 levels in rat GC co-incubated with oocytes.

Representative Western blots (1), and resultant plots of densiometry readings from specific proteins bands relative to ACTB bands (2) for phosphorylated (A) and unphosphorylated (B) Smad1/5/8 in rat GC incubated in media alone (controls), or with rat, sheep, pig or deer denuded oocyte (Lanes 1-5, respectively). Recombinant deer GDF9 was used as a negative control (Lane 6), and sheep GC treated with either recombinant deer GDF9 (Lane 7) or BMP15 (Lane 8) were used as positive controls. Blots were probed with phosphorylated (p)-Smad1/5/8 antibody (A1, A2), and thereafter stripped and reprobed for Smad1/5/8 antibody (B1, B2). All blots were performed under reducing conditions. A2 and B2 represent mean ± SEM values from three replicate Western blot experiments.. Histograms that do not share at least one similar alphabetical superscripts are significantly different (P<0.05) to each other.

3.7.3 Relative Smad 2/3 protein levels in deer GCs

Representative Western blots and mean relative densiometry results depicting the levels of phosphorylated and unphosphorylated Smad2/3 proteins in deer GC incubated with media alone, or with rat, sheep, pig or deer oocytes are illustrated in Figure 3.7.3. The addition of deer oocytes to cultured deer GC increased phosphorylated Smad2/3 protein levels above that of deer GC cultured alone (P<0.018 (Figure 3.7.3A). In contrast, there were no effects of oocytes, regardless of species (rat, sheep, pig, deer), on unphosphorylated Smad2/3 levels in deer GC (Figure 3.7.3B).

3.7.4 Relative Smad 1/5/8 in deer GCs

Representative Western blots and mean relative densiometry results depicting the levels of phosphorylated and unphosphorylated Smad1/5/8 proteins in deer GC incubated with media alone, or with rat, sheep, pig or deer oocytes are illustrated in Figure 3.7.4. The addition of oocytes, regardless of species (rat, sheep, pig or deer) to deer GC cultures did not alter phosphorylated Smad1/5/8 levels in these GC (Figure 3.7.4A). In contrast, the addition of deer, but not rat, sheep or pig, oocytes to cultured deer GC increased (P<0.012) unphosphorylated Smad 1/5/8 protein levels compared to deer GC cultured alone (Figure 3.7.4B).
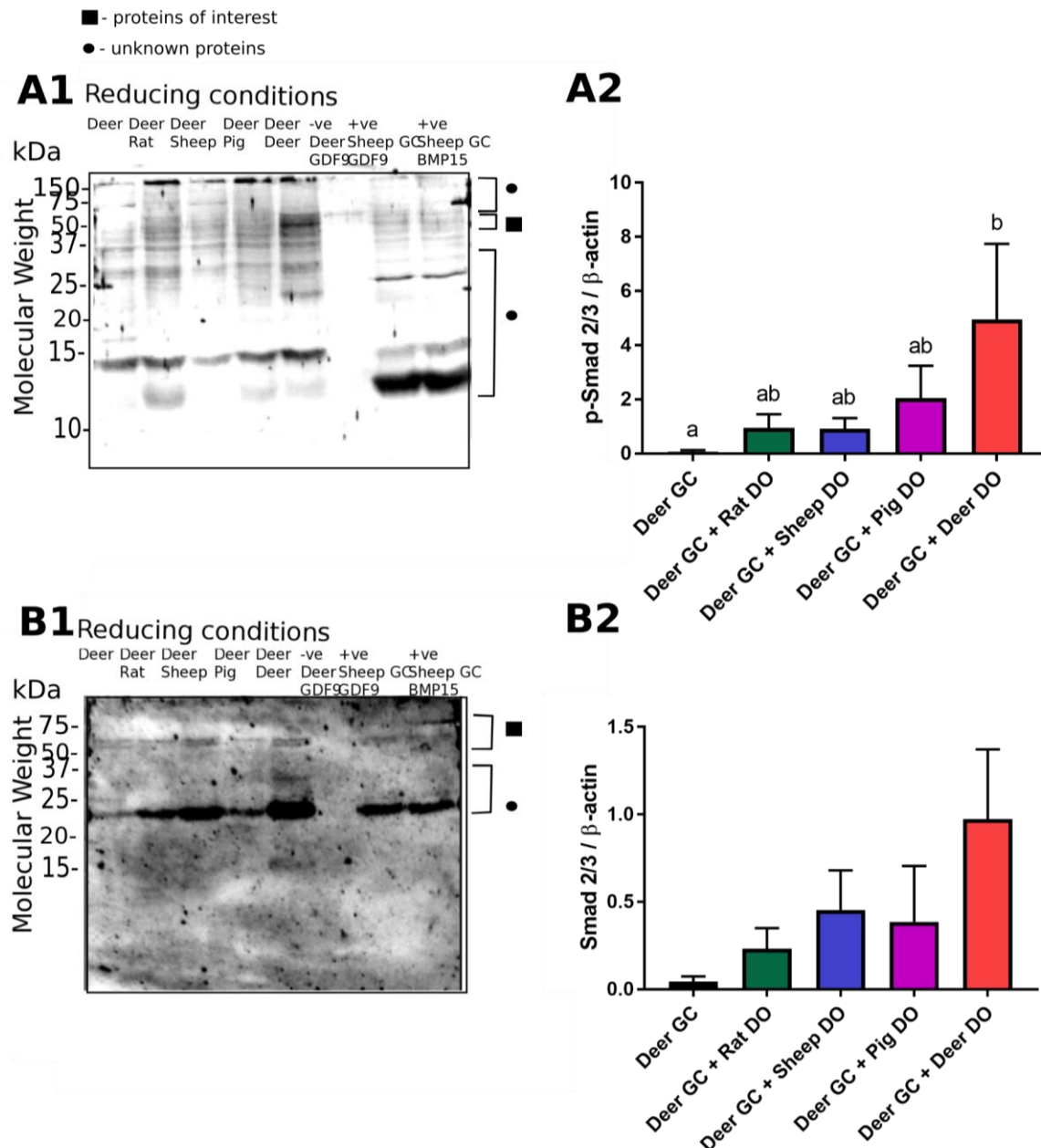
Figure 3.7.3: Smad 2/3 levels in deer GC co-incubated with oocytes.

Representative Western blots (1), and resultant plots of densiometry readings from specific proteins bands relative to ACTB bands (2) for phosphorylated (A) and unphosphorylated (B) Smad2/3 in deer GC incubated in media alone (controls), or with rat, sheep, pig or deer denuded oocyte (Lanes 1-5, respectively). Recombinant deer GDF9 was used as a negative control (Lane 6), and sheep GC treated with either recombinant deer GDF9 (Lane 7) or BMP15 (Lane 8) were used as positive controls. Blots were probed with phosphorylated (p)-Smad 2/3 antibody (A1, A2), and thereafter stripped and reprobed for Smad 2/3 antibody (B1, B2). All blots were performed under reducing conditions. A2 and B2 represent mean ± SEM values from three replicate Western blot experiments. Histograms that do not share at least one similar alphabetical superscripts are significantly different (P<0.05) to each other.

Figure 3.7.4: Smad 1/5/8 levels in deer GC co-incubated with oocytes

Representative Western blots (1), and resultant plots of densiometry readings from specific proteins bands relative to ACTB bands (2) for phosphorylated (A) and unphosphorylated (B) Smad1/5/8 in deer GC incubated in media alone (controls), or with rat, sheep, pig or deer denuded oocyte (Lanes 1-5, respectively). Recombinant deer GDF9 was used as a negative control (Lane 6), and sheep GC treated with either recombinant deer GDF9 (Lane 7) or BMP15 (Lane 8) were used as positive controls. Blots were probed with phosphorylated (p)-Smad1/5/8 antibody (A1, A2), and thereafter stripped and reprobed for Smad1/5/8 antibody (B1, B2). All blots were performed under reducing conditions. A2 and B2 represent mean ± SEM values from three replicate Western blot experiments.. Histograms that do not share at least one similar alphabetical superscripts are significantly different (P<0.05) to each other.

3.8 Validation of Smad 2/3 and Smad 1/5/8 antibodies

Western blots containing preparations of sheep GC treated with a mixture of recombinant GDF9 and BMP15 proteins were probed with phosphorylated and unphosphorylated Smad 2/3 antibodies, resulting in the presence of specific and non-specific bands, as illustrated in Figure 3.8A1 and B1). These bands were ~78, ~32 and ~16 kDa in size (Figure 3.8A1) for phosphorylated Smad 2/3, and ~ 23 kDa in size (Figure 3.6B1), for unphosphorylated Smad 2/3. In comparison, non-specific bands were not observed for unphosphorylated Smad 1/5/8, as illustrated in Figure 3.8C1.

All blots were stripped and reprobed with the same antibody that had been preabsorbed with its corresponding blocking peptide, as illustrated in Figures 3.8A2, B2 and C2. Upon preabsorption, the band representing phosphorylated Smad2/3 (~52 kDa) was only very faint, whilst the bands at ~16 and ~32 kDa are still present, indicating that these bands are non-specific bands, as illustrated in Figure 3.8A2. Similarly, the ~ 51 kDa band, representing unphosphorylated Smad2/3 disappeared upon preabsorption, indicating the specificity of this antibody. Interestingly, the non-specific band at ~ 23 kDa also faded significantly, although it was still visible (Figure 3.8B2). As illustrated in Figure 3.8C2, the band representing unphosphorylated Smad 1/5/8 disappeared, illustrating the specificity of this antibody.
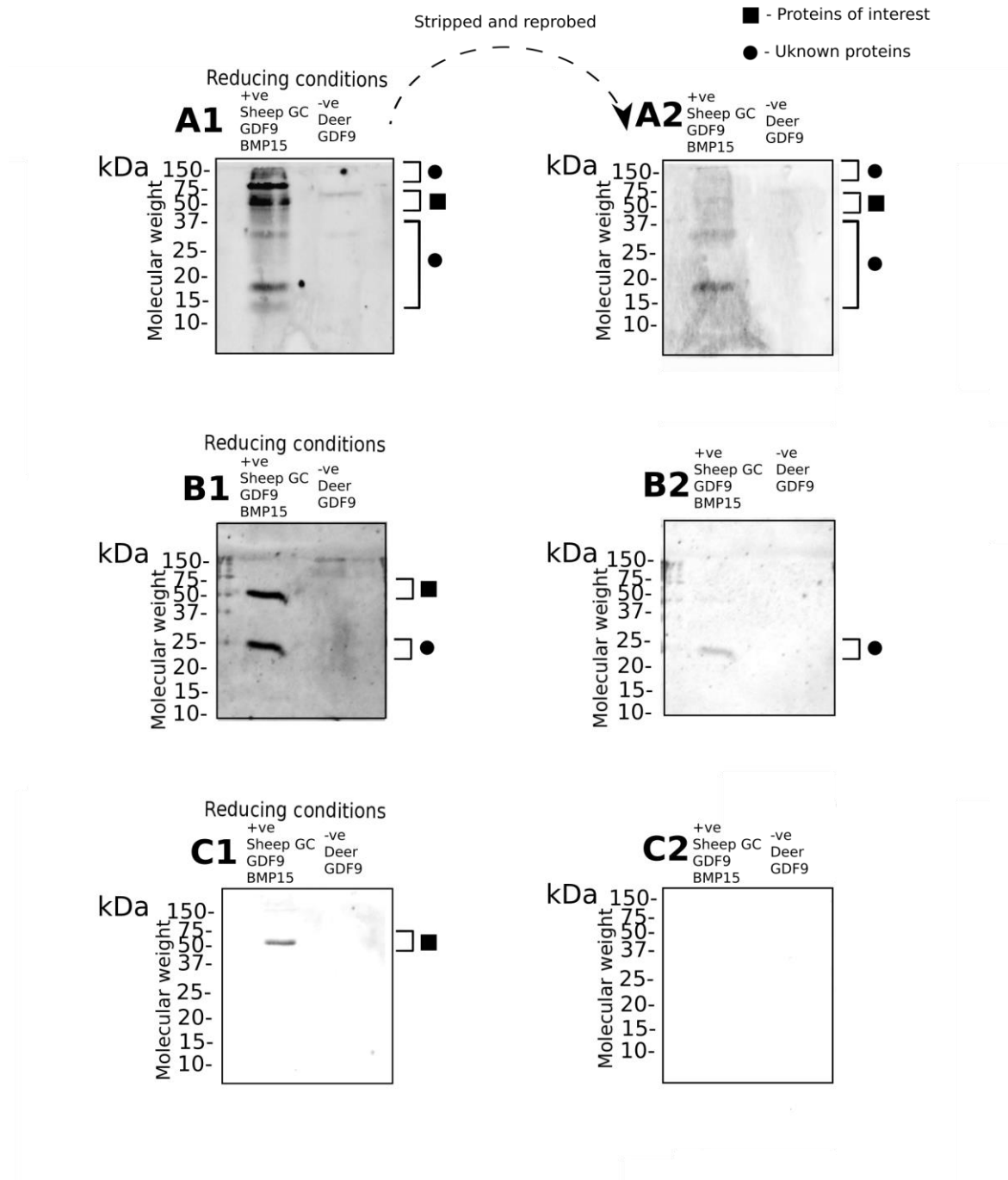
Figure 3.8: Western blots testing Smad2/3 and 1/5/8 antibody specificity.

Sheep GC cultured with recombinant deer GDF9 and BMP15 were probed with antibodies for either phosphorylated Smad2/3 (A1), Smad2/3 (B1) or Smad 1/5/8 (C1). Thereafter, membranes were stripped and reprobed with their appropriate antibodies (phosphorylated Smad 2/3 (A2), Smad 2/3 (B2) and Smad 1/5/8 (C2)) preabsorbed with their specific blocking peptides. Recombinant deer GDF9 was used as a negative control for all blots.

4.0 Discussion

This is the first reported study that compares differences in the ability of OSFs from mono-ovulatory and non-rodent poly-ovulatory species to stimulate GC proliferation. This study evaluated these species differences through the cross-incubation of oocytes and GCs from rats, sheep, pigs and deer. The results described herein revealed that OSFs influenced the capacity of GC to proliferate in a species-specific manner. Additionally, the innate ability of GC to proliferate in the absence of OSF also differed between species. The downstream effects of OSF binding onto GC were also species-specific with differences observed in both the upregulation of target receptors, and activation of downstream Smad signaling pathways

The use of oocytes in this study, rather than specific recombinant proteins, allows only for speculation as to which OSFs are eliciting the proliferation of GC. Several previous studies have illustrated the multitude of factors secreted from oocytes, and the different target receptors that are expressed in GCs of different species (see Table 4.1.1 for a summary). These studies utilised various methods to quantify the protein and gene levels reported and thus, it is difficult to draw comparisons between this data and that presented herein. Furthermore, it is important to note that only a proportion of the OSFs listed in Table 4.1.1. are capable of stimulating GC proliferation. These proliferative factors include TGFβ, GDF9 (Gilchrist et al, 2006, Mottershead et al, 2008, Sasseville et al, 2010), BMP15 (Gilchrist et al, 2006, Mottershead et al, 2008, Sasseville et al, 2010), BMP1 (Lei et al, 2016), BMP7 (Lee et al, 2001), and Activin A (Reader et al, 2011). However, BMP6 (Otsuka et al, 2001, Gilchrist et al, 2006) and BMP2 (Juengel et al, 2006) are not capable of eliciting a proliferative response in GC and other OSF are only proliferative in some species, for example BMP4, 6 and 7 are able to stimulate GC proliferation in rats, but not sheep (Juengel et al, 2006).

The following discussion focusses on GDF9 and BMP15 as the main OSF contributors to the GC proliferation reported herein. The rationale for this is that both recombinant

GDF9 and BMP15 have been reported to elicit GC proliferation (Otsuka and Shimasaki, 2002, McNatty et al, 2005b, Gilchrist et al, 2006) and the immunoneutralisation of either GDF9 or BMP15 in oocyte-GC co-culture experiments revealed that GDF9 and BMP15 are responsible for a significant proportion, if not all, of GC proliferation observed upon cross-incubation of oocytes with GCs (Lin et al., 2012). For example, immunoneutralisation of GDF9 decreased the proliferation of both sheep and rat GC co-incubated with rat or sheep oocytes by 50% and 100%, respectively. Furthermore, the immunoneutralisation of BMP15 reduced the proliferation of sheep GCs co-incubated with sheep oocytes by over 50% (Lin et al, 2012). Thus, the immunoneutralisation of both OSFs in sheep GC co-incubated with sheep oocytes would likely completely inhibit sheep GC proliferation.

Table 4.1.1: Expression of OSFs and GC receptors in the four different species.

	Oocyte	Granulosa cells
GDF9	**Rat:** (Crawford and McNatty, 2012), (Lin et al, 2012), (Jaatinen et al, 1999), (Hung et al, 2012)	**Rat: not expressed** (Jaatinen et al, 1999)
	Sheep: (Bodensteiner et al, 1999), (Crawford and McNatty, 2012), (Hung et al, 2012), (Kyasari et al, 2012), (Lin et al, 2012)	**Sheep: not expressed** (Bodensteiner et al, 1999)
	Pig: (Prochazka et al, 2004), (Zhu et al, 2008), (Paradis et al, 2009), (Sun et al, 2010), (Crawford and McNatty, 2012), (Jackowska et al, 2013)	**Pig:**(Prochazka et al, 2004), (Paradis et al, 2009), (Sun et al, 2010)
	Deer: (Crawford and McNatty, 2012)	**Deer: Unknown**
BMP15	**Rat:** (Erickson and Shimasaki, 2003), (Crawford and McNatty et al, 2012)	**Rat: not expressed** (Erickson and Shimasaki, 2003)
	Sheep: (Galloway et al, 2000), (Kyasari et al, 2012), (Lin et al, 2012)	**Sheep: not expressed** (Galloway et al, 2000)
	Pig: (Zhu et al, 2008), (Paradis et al, 2009), (Sun et al, 2010), (Crawford and McNatty, 2012)	**Pig:** (Paradis et al, 2009), (Sun et al, 2010)
	Deer: (Crawford and McNatty, 2012)	**Deer: unknown**
BMP1	**Rat: unknown**	**Rat: unknown**
	Sheep: uknown	**Sheep:** (Canty-Laird et al, 2010)
	Pig: (Lei et al, 2017)	**Pig:** (Lei et al, 2017)
	Deer: unknown	**Deer: unknown**
BMP2	**Rat: Not expressed** (Erickson and Shimasaki, 2003)	**Rat:** (Hung et al, 2012), (Erickson and Shimasaki, 2003)
	Sheep: Not expressed (Juengel et al, 2006)	**Sheep:** (Juengel et al, 2006), (Hung et al, 2012)
	Pig: (Brankin et al, 2005), (Paradis et al, 2009)	**Pig:** (Brankin et al, 2005), (Paradis et al, 2009)
	Deer: unknown	**Deer: unknown**
BMP3	**Rat: not expressed** (Erickson and Shimasaki, 2003)	**Rat: not expressed** (Erickson and Shimasaki, 2003)
	Sheep: unknown	**Sheep: unknown**
	Pig: unknown	**Pig: unknown**
	Deer: unknown	**Deer: unknown**
BMP4	**Rat: not expressed** (Erickson and Shimasaki, 2003)	**Rat: not expressed** (Erickson and Shimasaki, 2003)
	Sheep: not expressed (Juengel et al, 2006)	**Sheep: not expressed** (Juengel et al, 2006)
	Pig: expressed in ovaries (Shimizu et al, 2004)	**Pig: expressed in ovaries** (Shimizu et al, 2004)
	Deer: unknown	**Deer: unknown**
BMP5	**Rat:** (Pierre et al, 2005)	**Rat:** (Pierre et al, 2005)
	Sheep: unknown	**Sheep: unknown**
	Pig: expressed in ovaries (Shimizu et al, 2004)	**Pig: expressed in ovaries** (Shimizu et al, 2004)
	Deer: unknown	**Deer: unknown**

	Oocyte	Granulosa cells
BMP6	**Rat:** (Erickson and Shimasaki, 2003) **Sheep:** (McNatty et al, 2005a), (Campbell et al, 2006), (Juengel et al, 2006), (Kayasari et al, 2012) **Pig:** (Brankin et al, 2005), (Zhu et al, 2008), (Paradis et al, 2009) **Deer: unknown**	**Rat:** (Erickson and Shimasaki, 2003) **Sheep: Conflicting evidence; expressed** (Campbell et al, 2006), **not expressed** (Juengel et al, 2006) **Pig:** (Branklin et al, 2005) **Deer: unknown**
BMP7	**Rat: not expressed** (Erickson and Shimasaki, 2003) **Sheep: not expressed** (Juengel et al, 2006) **Pig: unknown** **Deer: unknown**	**Rat: not expressed** (Erickson and Shimasaki, 2003) **Sheep:** (Juengel et al, 2006) **Pig: unknown** **Deer: unknown**
BMP9 (GDF2)	**Rat: not expressed** (Hosoya et al, 2015) **Sheep: unknown** **Pig: unknown** **Deer: unknown**	**Rat:** (Hosoya et al, 2015) **Sheep: unknown** **Pig: unknown** **Deer: unknown**
Inhibin α	**Rat:** (Ogawa et al, 1991) **Sheep: not expressed** (Tisdall et al, 1994) **Pig: unknown** **Deer: unknown**	**Rat:** (Woodruff et al, 1987), (Meunier et al, 1988), (Ogawa et al, 1991), (Drummond et al, 1996) **Sheep:** (Tisdall et al, 1994) **Pig:** (Wang et al, 2015) **Deer: unknown**
Inhibin β A	**Rat:** (Ogawa et al, 1991), (Drummond et al, 1996) **Sheep: expressed in antral follicles** (Tisdall et al, 1994) **Pig:** (Kempisty et al, 2015) **Deer: unknown**	**Rat:** (Woodruff et al, 1987), (Meunier et al, 1988), (Ogawa et al, 1991), (Drummond et al, 1996) **Sheep: expressed in antral follicles** (Tisdall et al, 1994) **Pig:** (van den Hurk and van de Pavert, 2001) **Deer: unknown**
Inhibin β B	**Rat:** (Ogawa et al, 1991), (Agca et al, 2013) **Sheep: unknown** **Pig:** (Kempisty et al, 2015) **Deer: unknown**	**Rat:** (Ogawa et al, 1991), (Meunier et al, 1988), (Drummond et al, 1996), **Sheep: unknown** **Pig:** (van den Hurk and van de Pavert, 2001) **Deer: unknown**
TGFβ1	**Rat:** (Teerds and Dorrington, 1992) **Sheep: not expressed** (Juengel et al, 2004a) **Pig:** (Jackowska et al, 2013) **Deer: unknown**	**Rat:** (Teerds and Dorrington, 1992) **Sheep: not expressed** (Juengel et al, 2004a) **Pig:** (May et al, 1996), (Sriperumbudur et al, 2010) **Deer: unknown**

	Oocyte	Granulosa cells
TGFβ2	**Rat:** (Teerds and Dorrington, 1992), (Ergin et al, 2008), (Agca et al, 2013) **Sheep: not expressed** (Juengel et al, 2004a) **Pig:** (Jackowska et al, 2013) **Deer: unknown**	**Rat:** (Mulheron and Schomberg, 1990), (Teerds and Dorrington, 1992) **Sheep: not expressed** (Juengel et al, 2004a) **Pig: not expressed** (Sriperumbudur et al, 2010) **Deer: unknown**
TGFβ3	**Rat: expressed in ovaries** (Rosairo et al, 2008) **Sheep: not expressed** (Juengel et al, 2004a) **Pig:** (Steffl et al, 2008), (Jackowska et al, 2013) **Deer: unknown**	**Rat: expressed in ovaries** (Rosairo et al, 2008) **Sheep: not expressed** (Juengel et al, 2004a) **Pig: not expressed** (Steffl et al, 2008) **Deer: unknown**
AMH	**Rat: not expressed** (Baarends et al, 1995) **Sheep: not expressed** (Bézard et al, 1988), (Campbell et al, 2012) **Pig:** (Almeida et al, 2012) **Deer: unknown**	**Rat:** (Ueno et al, 1989), (Baarends et al, 1995), (Ortega et al, 2012) **Sheep:** (Bézard et al, 1988), (Campbell et al, 2012) **Pig:** (Almeida et al, 2012) **Deer: unknown**
Nodal	**Rat: not expressed** (Wang et al, 2006) **Sheep: unknown** **Pig: unknown** **Deer: unknown**	**Rat:** (Wang et al, 2006) **Sheep: unknown** **Pig: unknown** **Deer: unknown**
BMPR2 (BRK3/T-Alk)	**Rat:** (Shimasaki et al, 1999) **Sheep:** (Wilson et al, 2001), (Bertoldo et al, 2014), (Kyasari et al, 2012) **Pig:** (Quinn et al, 2004), (Zhu et al, 2008), (Paradis et al, 2009), (Quinn et al, 2004), (Sun et al, 2010) **Deer: unknown**	**Rat:** (Shimasaki et al, 1999), (Erickson and Shimasaki, 2003) **Sheep:** (Chen et al, 2009), (Wilson et al, 2001), (Souza et al, 2002), (Bertoldo et al, 2014) **Pig:** (Quinn et al, 2004), (Paradis et al, 2009), (Quinn et al, 2004), (Sun et al, 2010) **Deer: unknown**
ALK1 (TSR1/SKR3/ACVRL1)	**Rat: unknown** **Sheep: unknown** **Pig: unknown** **Deer: unknown**	**Rat:** (Hosoya et al, 2015) **Sheep: unknown** **Pig: unknown** **Deer: unknown**
ALK2 (TSK7L/ActRIA/SKR1)	**Rat:** (Drummond et al, 2002) **Sheep: unknown** **Pig: unknown** **Deer: unknown**	**Rat:** (Drummond et al, 2002) **Sheep: unknown** **Pig: unknown** **Deer: unknown**
ALK3 (BMPRI/BMPRIA/BRK1/Tfr11/ACVRLK3)	**Rat:** (Shimasaki et al, 1999), (Erickson and Shimasaki, 2003) **Sheep:** (Kyasari et al, 2012), (Bertoldo et al, 2014) **Pig:** (Zhu et al, 2008) **Deer: unknown**	**Rat:** (Shimasaki et al, 1999), (Erickson and Shimasaki, 2003) **Sheep:** (Bertoldo et al, 2014) **Pig: unknown** **Deer: unknown**
ALK4 (ActRIB/ACVR1B)	**Rat:** (Drummond et al, 2002) **Sheep: unknown** **Pig: unknown** **Deer: unknown**	**Rat:** (Drummond et al, 2002) **Sheep: unknown** **Pig: unknown** **Deer: unknown**

	Oocyte	Granulosa cells
ALK5 (TGF β R1)	**Rat: unknown** **Sheep:** (Juengel et al, 2004a), (Kyasari et al, 2012) **Pig:** (Paradis et al, 2009) **Deer: unknown**	**Rat:** (Mazerbourg et al, 2004) **Sheep:** (Juengel et al, 2004a), (Chen et al, 2009) **Pig:** (Paradis et al, 2009), (Sun et al, 2010), (Sriperumbudur et al, 2010) **Deer: unknown**
ALK6 (BMPR1B)	**Rat:** (Shimasaki et al, 1999), (Erickson and Shimasaki, 2003) **Sheep:** (Wilson et al, 2001), (Kyasari et al, 2012), (Bertoldo et al, 2014) **Pig:** (Quinn et al, 2004), (Zhu et al, 2008), (Paradis et al, 2009), (Quinn et al, 2004), (Sun et al, 2010) **Deer: unknown**	**Rat:** (Shimasaki et al, 1999), (Erickson and Shimasaki, 2003) **Sheep:** (Chen et al, 2009), (Souza et al, 2002), (Bertoldo et al, 2014) **Pig:** (Quinn et al, 2004), (Paradis et al, 2009), (Quinn et al, 2004), (Sun et al, 2010) **Deer: unknown**
ALK7 (ACVR1C)	**Rat: unknown** **Sheep: unknown** **Pig: unknown** **Deer: unknown**	**Rat:** (Wang et al, 2006) **Sheep: unknown** **Pig: unknown** **Deer: unknown**
ActRII (ACVR2)	**Rat: unknown** **Sheep: unknown** **Pig:** (van den Hurk and van de Pavert, 2001) **conflicting evidence** **Deer: unknown**	**Rat: unknown** **Sheep: unknown** **Pig:** (van den Hurk and van de Pavert, 2001) **Deer: unknown**
ActRIIA (ACVR2A)	**Rat:** (Sadatsuki et al, 1993), (Drummond et al, 2002) **Sheep: unknown** **Pig: unknown** **Deer unknown**	**Rat:** (Drummond et al, 2002) **Sheep: unknown** **Pig: unknown** **Deer: unknown**
ActRIIB (ACVR2B)	**Rat:** (Cameron et al, 1994), (Drummond et al, 2002) **Sheep:** (Kyasari et al, 2012) **Pig: unknown** **Deer: unknown**	**Rat:** (Cameron et al, 1994), (Drummond et al, 2002) **Sheep: unknown** **Pig: unknown** **Deer: unknown**
TGF β RII	**Rat:** (MacConnell et al, 2002) **Sheep: not expressed** (Juengel et al, 2004a) **Pig: unknown** **Deer: unknown**	**Rat:** (MacConnell et al, 2002) **Sheep: not expressed** (Juengel et al, 2004a) **Pig:** (Sriperumbudur et al, 2010) **Deer: unknown**
TGF β RIII	**Rat:** (Drummond et al, 2002), (Agca et al, 2013) **Sheep: unknown** **Pig: unknown** **Deer: unknown**	**Rat:** (Drummond et al, 2002), (Omori et al, 2005) **Sheep: unknown** **Pig:** (Wang et al, 2015) **Deer: unknown**
MIS RII (AMHR2)	**Rat: not expressed** (Baarends et al, 1995) **Sheep: unknown** **Pig:** (Knapczyk-Stwora et al, 2014) **Deer: unknown**	**Rat:** (Baarends et al, 1995) **Sheep:** (Pierre et al, 2016) **Pig: not expressed** (Knapczyk-Stwora et al, 2014) **Deer: unknown**

4.1 Proliferation assay time series

Prior to initiation of the co-culturing experiments, it was critical to determine the timing of maximum GC proliferation after incubation with oocytes or activin A in each of the species studied. This information indicates the time taken for appreciable amounts of stimulatory factors to be secreted from the oocyte into the media to cause GC proliferation. The downside of an extended incubation time is the increasing chance that the GC will lose their GC characteristics as discussed below.

The timing of maximum GC proliferation rate following co-incubation with oocytes or activin A, relative to GCs cultured alone, occurred at 16 hours for the rat and sheep. In contrast, incubation time did not affect GC proliferation rate in pig or deer, which is likely due to high variation between replicates and in the case of the pig, a much lower fold change GC proliferation. Thus, 16 hours was selected as the optimum incubation time for all four species to allow for species comparisons.

As stated above, the increase in GC proliferation rate in the rat and sheep after 16 hours of co-culturing with oocytes is most likely due to increasing OSF concentrations, but an upregulation of their concomitant receptors in GC over time also can't be discounted. Interestingly, a previous study illustrated that sheep COCs cultured *in vitro* had higher *BMPR1B* and *BMPRII* mRNA expression after 27 hours, compared to 3 hours, in culture (Kyasari et al, 2012). Furthermore, mRNA levels of *GDF9* and *BMP15* in rat and sheep oocytes decreased after 24h incubation (Lin et al, 2012). It is possible that whilst the rat and sheep cultures may exhibit changing levels of OSFs and/or receptor numbers, they remain relatively constant in pigs and deer.

Overall, an increase in GC proliferation was observed amongst all species upon oocyte co-incubation. This is presumably due to stimulation by OSF, as members of the TGFβ superfamily have been previously shown to elicit GC proliferation (Otsuka and Shimasaki, 2002, McNatty et al, 2005b, McNatty et al, 2005c, Gilchrist et al, 2006, Spicer et al, 2006). Moreover, the addition of BMP15, GDF9 or denuded oocytes to cumulus cell cultures prevented cumulus cell apoptosis (Hussein et al, 2005; Orisaka et al, 2006). Due

to the culture of rat, sheep or pig GCs alone over time displaying a decrease in CPM, GCs may require OSFs to prevent apoptosis and elicit proliferation under the current culture conditions. Conversely, a decrease in CPM was not observed upon the culture of deer GCs alone over time. The presence of growth factors in GCs themselves has also been illustrated (see Table 4.1.1; Drummond et al, 2000, Erickson an Shimasaki, 2003, Juengel et al, 2006, Hung et al, 2012). Therefore, as deer was the only species whereby a decrease in CPM of GC was not observed with time in culture, it is possible that deer GCs secrete a factor preventing apoptosis

The high CPM values observed in unstimulated (cultured alone) GCs of pigs, compared to the other species, suggests that pig GCs secrete proliferative growth factors or have a higher mitotic rate. Interestingly, the ability of rat OSFs to stimulate rat GCs (CPM values) did not dissipate with time in culture. In contrast, the CPM values of pig GCs co-cultured with oocytes markedly declined with time in culture, and those of sheep and deer showed an apparent decline although this was not significant. This may indicate that rat OSFs may either reduce luteinisation or apoptosis in rat GC.

The *in vitro* culture system used in this study resulted in GCs being adhered to the bottom of a plastic well in a monolayer. This does not provide an adequate 3D-scaffold and enable GC luteinisation to occur (Vigo et al, 2005), as illustrated by increased progesterone, and decreased oestradiol production (Picton et al, 1999). Moreover, the culture of GCs on plastic has been reported to decrease gap junction communication (Ben-Ze'ev and Amsterdam, 1985), with GCs dislaying a 25% survival rate after 48 hours of culture on uncoated wells (Huet et al, 2001). The decrease with time in proliferation rate of cultured GC in this study suggests that the GCs of all species were undergoing at least a degree of luteinisation and/or apoptosis. The addition of components from the ECM, such as laminin or collagen, together with utilising 3D culture systems has been shown to promote survival in cultured GCs (Huet et al, 2001, Bellego et al, 2002, Joo et al, 2016), and may be used in the future to mimic follicular integrity and potentially decrease cell death. Additionally, a decline in GC proliferation rate with time in culture may also be attributed to the inadequate components present in the culture media. To create a media that accurately replicates follicular fluid, proteomic analysis of this

follicular fluid could be undertaken. However, studies utilising mass spectrometry to identify follicular fluid proteins (Zamah et al, 2015), demonstrate how difficult it is to replicate the *in vivo* follicular environment due to the presence of hundreds of proteins in various quantities.

It is unlikely that the decrease in proliferation rate with time in culture is due to tritiated thymidine exposure. Several studies have illustrated that tritiated thymidine in cell proliferation assays causes cell cycle perturbations (Hoy et al, 1990, Solary et al, 1992, Hu et al, 2002), which is largely dependent on the amount of radiation and the length of exposure (Hu et al, 2002). However, in this experiment the amount and time of exposure to tritiated thymidine was kept constant at $\sim$ 0.4 µCi and six hours respectively. However to adequately discount this, alternative methods for measuring cell proliferation may be utilised, including adenosine triphosphate bioluminescence (Crouch et al, 1993), and fluorometric assays (Rak et al, 2015). This would also enable the comparison of intra-assay sensitivity in determining GC proliferation rate. As tritiated thymidine incorporation occurs at the S phase of the cell cycle (Naito et al, 1987), the recorded CPM is likely influenced by the cell cycle stage the GCs are at, although in the current *in vitro* environment, it is unknown how long one GC cycle would take. To verify whether at each time point, the proportion of GCs incorporating tritiated thymidine into their DNA correlates with the number of GCs present, the expression of *Ccnd2,* a cell cycle transcript, could be monitored at each time point.

Activin A is a member of the TGFβ superfamily of granulosa cell origin (Knight and Glister, 2006), which activates the Smad2/3 pathway (Baker and Harland, 1996, Dennler et al, 1998). This growth factor was included as a positive control as it has been previously shown to increase proliferation of rat GC (Reader et al, 2011). In addition to eliciting GC proliferation, activin A is also involved in GC steroidogenesis (Chang et al, 2014, Chang et al, 2015) and embryo development (Kannampuzha-Francis et al, 2016). In this study, rats and sheep were the only species to show an increase in GC proliferation upon activin A addition, with rat GCs being more sensitive ($\sim$ 13-fold versus $\sim$ 4-fold increase in GC proliferation, respectively). In contrast, neither pig nor deer GC responded to activin A, and it appeared that deer GC proliferation was partially

inhibited (~ 0.8 fold change) by activin A at 16 hours. This anti-mitogenic effect may be due to the activin A concentration used (10 ng/mL) being much higher than that observed in the deer's *in vivo* follicular environment, eliciting a toxic effect. The results of this *in vitro* culture may indicate species differences in the importance of activin A in follicular development *in vivo.* It is important to note that in this study, human recombinant activin A was used from a Chinese hamster ovary cell line (R & D systems), rather than the native proteins for each species. Therefore, species' differences in activin A stimulation of GC proliferation may be partially attributed to differences in homology in the ActRII/IIB receptor, whereby the recombinant activin A used had a different affinity to the receptor in each of the species.

Thus, species differences in GC proliferation were observed in GC cultured alone and with oocytes. The co-incubation of rat oocytes with rat GC caused a markedly higher proliferation (~107-fold), compared to deer and pig GC co-incubated with their respective oocytes. This is likely due to differences in amounts and bioactivity of the OSFs of each species' in combination with receptivity differences of GCs. Through the addition of recombinant OSFs to GC culture, it has been illustrated that different OSFs increase the uptake of tritiated thymidine to varying degrees, with activin A promoting higher murine GC proliferation compared to BMP4, FCS and a combination of GDF9 and BMP15 (Reader et al, 2011). As summarised in Table 4.1.1, oocytes from all the species tested secrete a multitude of OSFs, although little is known about the secretions of deer oocytes. These OSFs are likely to be secreted in differing amounts with differing bioactivities which are dependent on the species, and thus have varying capacities of inducing proliferation. Furthermore, the types and levels of target receptors are also likely to differ in the GCs of different species'. These species' differences in OSFs and receptors can be further studied through same and different species cross-incubations, as is discussed in Chapter 4.2 below.

4.2 Effects of oocyte co-incubations on GC proliferation rate

To compare the biological activity of OSFs, together with the receptivity of GC across four species that differed in ovulation rate, cross-species incubations of oocytes with GC

were employed. The study hypothesis that differences in the types, bioactivity and/or amounts of OSFs between species would result in differences in proliferation levels was validated by comparing effects of OSFs from all species on proliferation of GC from each species. Additionally, the study hypothesis that differences in GC receptivity between species would result in differences in proliferation levels was also validated by comparing effects of OSF from each species on proliferation of GC from all species. Thus the species-specific differeneces in proliferation rate are due to both differences in bioactivity of OSFs and the receptivity of GC between species.

The proliferation rate of rat GCs after a 16h incubation with OSFs (excluding deer OSFs) was higher than that of GCs of the other species tested. As discussed in the previous section, this may indicate that OSFs may either reduce luteinisation or apoptosis in rat GC. The higher mitogenicity of rat GC in response to OSF has been previously reported. A four fold increase in DNA synthesis was observed of rat GC following co-culture with GDF9 and BMP15 (McNatty et al, 2005c, Reader et al, 2011), compared to a ~1.5 fold increase in sheep GC (McNatty et al, 2005b). The reason that rat GCs were more proliferative is likely due to differences in receptor expression, with rat GCs potentially expressing higher levels of target receptors in response to OSF. These results may also be indicative of what occurs *in vivo*. Follicular growth in rats is much faster compared to sheep (Peters and McNatty, 1980). Rapid follicular growth may be necessary in this species due to a shorter oestrous cycle (~4 days; Westwood, 2008) compared to sheep (~16 days; Woody et al, 1967), pigs (~21 days; Henricks et al, 1972) and red deer (~18 days; Guiness et al, 1971).

Another interesting observation from this study is that deer oocytes are less stimulatory, compared to other species, on rat GC proliferation rate. Deer oocytes express much lower mRNA levels of *GDF9* compared to rat, sheep and pig oocytes (Crawford and McNatty, 2012). Moreover, the ratio of *GDF9:BMP15* mRNA expression in deer oocytes is very low compared to that in the other species tested (rat > sheep > pig > deer). Thus, the reduced ability of deer oocytes to stimulate GC proliferation may be due to this lower abundance of the *GDF9* gene. It is important to note however that the higher levels of *GDF9* expression in rat and sheep oocytes in this study are unlikely to be

solely responsible for the mitogenic effects on murine GC, as has been previously illustrated through immuno-neutralisation of this OSF (Gilchrist et al, 2004, Lin et al, 2012). Previous studies illustrate that recombinant GDF9 stimulates murine GC proliferation to a greater extent than recombinant BMP15 (Mottershead et al, 2012), and immunoneutralisation studies show that rat GC do not require BMP15 for proliferation (Lin et al, 2012). However the addition of a GDF9 antibody to a rat oocyte-GC culture revealed that immunoneutralisation of GDF9 ablated oocyte-stimulated GC proliferation (Lin et al, 2012). This present study validates the mitogenic responsiveness of rat GC to high GDF9 levels compared to BMP15, as rat oocytes elicit high proliferation, and express higher *Gdf9* mRNA levels, and lower *Bmp15* mRNA levels compared to deer oocytes. It has been further hypothesised that BMP15 may in fact have an inhibitory effect on murine folliculogenesis, due to a reduction in mouse preantral follicle proliferation upon BMP15 culture being observed over time (Fenwick et al, 2013), whilst mutations of BMPR1B or Smads 1 and 5, increase mouse GC proliferation, ultimately leading to tumorigenesis (Middlebrook et al, 2009, Edson et al, 2010). Deer oocytes are unique in that they express negligible *GDF9* mRNA levels but moderate *BMP15* expression levels. Thus, murine GCs, which are known to only require GDF9 for proliferation, would be exposed to mostly BMP15 upon deer oocyte co-incubation, concomitantly stimulating murine GC proliferation to a lesser extent than oocytes from the other species.

It is well acknowledged that the addition of a combination of both GDF9 and BMP15 results in a synergistic effect on GC proliferation, in comparison to the addition of GDF9 and BMP15 separately (McNatty, 2005bc, Mottershead et al, 2012). Thus, it might be expected that any species's oocytes, that secretes appreciable amounts of both GDF9 and BMP15 would stimulate high GC proliferation The sheep and pig both express significant amounts of both *Gdf9* and *Bmp15* mRNA (*GDF9:BMP15* ratio of 1.26 and 0.56, respectively), whilst as stated above, the rat expresses mainly *Gdf9* mRNA (*Gdf9:Bmp15* ratio of 3.65) and the deer expresses relatively more *BMP15* mRNA (*GDF9:BMP15* ratio of 0.10) (Crawford & McNatty, 2012). Thus, it is likely that both the sheep and pig elicit the highest synergistic effect of these two growth factors. It is important to note that whilst the culture of mouse preantral follicles with BMP15 decreases follicle growth

over time, this does not occur with the addition of GDF9 and BMP15 together, or GDF9 alone (Fenwick et al, 2013). This is in accordance with this study, which illustrated increased rat GC proliferation upon incubation with pig and sheep oocytes, which express significant amounts of both *GDF9* and *BMP15*, and incubation with rat oocytes, which express high *Gdf9* mRNA levels. In addition, this raises the possibility that activation of the Smad 2/3 pathway has highly proliferative effects on murine GC, as the synergistic actions of GDF9 and BMP15, secreted from sheep and pig oocytes, would be expected to act through the Smad 2/3 pathway (Mottershead et al, 2012), in addition to the high levels of *Gdf9* in rat oocytes, which would also utilise this pathway (Mazerbourg et al, 2004). In contrast, as deer oocytes express negligible *GDF9* mRNA, the relatively high *BMP15* mRNA levels in deer oocytes would stimulate the Smad 1/5/8 pathway (Moore et al, 2003), which is potentially less proliferative.

In comparison, no species-specific effects were observed on proliferation of sheep, pig, or deer GC following co-culture of oocytes from any of the species tested. This may be due to the proliferation of GC being too low or variable to denote a significant species' differences in cross-species oocyte co-culture. However, given that the proliferation rate of GCs of sheep, pig and deer in response to co-incubation with deer oocytes was similar to that after co-incuation with rat, sheep and pig oocytes, this supports the notion that whilst the addition of BMP15 alone may have an inhibitory effect on GC proliferation in murine species (Fenwick et al, 2013), it has a stimulatory effect in other species. This is strongly supported by studies in sheep, whereby natural mutations in BMP15 (Davis et al, 1992, Galloway et al, 2000) or BMPR1B (Wilson et al, 2001) increased ovulation rate. In mice however, overexpression of BMP15 increases atresia in antral follicles with mice exhibiting early acyclicity. Interestingly however, litter size is not impacted in these transgenic mice (McMahon et al, 2008a).

4.3 Baseline CPM of GC proliferation

Examination of species differences in the baseline (i.e. unstimulated) proliferation rate of GCs after 16 hours in cultures lends an insight into differences in GC function. However several factors must be taken into consideration.

Firstly, deer GCs had a lower viability compared to that of rat, sheep and pigs. The time taken to transport deer ovaries back to the laboratory was longer than for rat and sheep ovaries, but similar to that for the pig. Despite this, differing cell viability between species was unlikely to influence this study's results as only viable GC were counted for inclusion in the proliferation assays. This resulted in the total number of cells utilised in qPCR and western blots to differ between samples, therefore each sample was corrected against *RPL19* and β-actin respectively, to correct for these differences.

Furthermore, basal GC proliferation varied across species also. Unstimulated pig GC had a higher tritiated thymidine incorporation compared to sheep GC, but similar to that of rat and deer GC. There was no correlation between GC viability and basal proliferation rate. Thus, elevated innate proliferation levels in pig GC may be due to the presence of growth factors within the cell culture system, that affect pig, but not sheep, GCs. This may be due to differences in secreted growth factors from pig GC or a factor in the media in which pig GC are more receptive to. The expression of *GDF9* and *BMP15* mRNA is confined to the oocyte in sheep and rats (Bodensteiner et al, 1999, Galloway et al, 2000, Crawford et al, 2012, Laitinen et al, 1998, Mester et al, 2014) but numerous reports indicate that pig GC also expression of *GDF9* and *BMP15* mRNA (Prochazka et al, 2004, Paradis et al, 2009). It is currently not known whether deer GCs express *GDF9 and BMP15* mRNA, however deer cumulus cells do not (Crawford et al., 2012). Therefore, if GDF9 and BMP15 are expressed in pig GC, these growth factors will stimulate proliferation in the absence of oocytes. Alternatively, growth factors such as *IGF1* and *EGF* are also expressed in pig GC (Silva et al, 2011), and have been shown to stimulate GC proliferation (Baranao and Hammond, 1984, Buck and Schomberg, 1988, Gospodarowicz et al, 1977, Mao et al, 2004). As GC proliferation was deduced as a fold change relative to baseline GC proliferation, high basal proliferation levels in GC of pigs influences the calculated fold change such that the pig GCs have a low proliferation rate following oocyte co-culture, in comparison to other species. Therefore, this low fold change may not be only due to pig oocyte secreted factors being unable to elicit proliferation as effectively as other species' oocyte secreted factors, but rather due to growth factors secreted by pig GCs raising the baseline levels.

4.4 Relative expression of key genes in GCs

Species-specific effects of OSF on the transcriptional regulation of target receptors in GCs was also investigated in this study. Cross-species co-cultures of oocytes and GC showed that OSF from different species, elicited species-specific affects on the expression levels of GC-derived type I and type II receptors for GDF9 and BMP15. Despite this, the three receptors investigated in this study, TGFβR1, BMPR1B and BMPR2, are bound and activated by a number of ligands. In addition to GDF9, the ligands for TGFβR1 include GDF8, GDF11, TGFβ1 and TGFβ2 (Goumans et al, 2003, Rebbapragada et al, 2003, Mazerbourg et al, 2004, Kaivo-Oja et al, 2005, Andersson et al, 2006, Townsend et al, 2011). Conversely, in addition to BMP15, the ligands for BMPR1B include GDF5, GDF6, GDF7, BMP4, BMP10 and AMH (ten Dijke et al, 1994, Nishitoh et al, 1996, Gouedard et al, 2000, Wilson et al, 2001, Mazerbourg et al, 2005). Furthermore, BMPR2 binds several ligands including GDF9, BMP15, inhibins, BMP2, BMP4, BMP6, BMP10, GDF2, GDF5, GDF6 and GDF7 also display an affinity for BMPR2 (Nohno et al, 1995, Ebisawa et al, 1999, Vitt et al, 2002, Moore et al, 2003, Wiater et al, 2003, Mazerbourg et al, 2005, Scharpfenecker et al, 2007, Kirkbride et al, 2008). For ease of species comparisons in this study, the gene expression results for the target receptors are summarised in Table 4.4.1, 4.4.2 and 4.4.3.

Table 4.4.1: The effect of oocytes on TGFβR1 mRNA levels in GCs in mono-ovulatory and poly-ovulatory species. Values are relative to GC cultured alone.

		Oocytes			
		Rat	Sheep	Pig	Deer
GCs	Rat	-	↑	-	-
	Sheep	-	-	↑	-
	Pig	-	-	-	↑
	Deer	-	-	-	-

Table 4.4.2: The effect of oocytes on BMPR1B mRNA levels in GCs in mono-ovulatory and poly-ovulatory species. Values are relative to GC cultured alone.

		Oocytes			
		Rat	Sheep	Pig	Deer
GCs	Rat	-	-	-	↓
	Sheep	-	-	-	-
	Pig	-	-	↑	↑↑
	Deer	-	-	-	-

Table 4.4.3: The effect of oocytes on *BMPR2* mRNA levels in GCs in mono-ovulatory and poly-ovulatory species. Values are relative to GC cultured alone.

		Oocytes			
		Rat	Sheep	Pig	Deer
GCs	Rat	↑	-	-	↓
	Sheep	-	-	-	-
	Pig	-	-	-	-
	Deer	-	↓	↓	-

In rat GCs, *Tgfβr1* mRNA levels increased upon the co-incubation of sheep oocytes, whilst in sheep GCs, *TGFβR1* mRNA levels increased upon the co-incubation of pig oocytes. As previously mentioned, the synergistic effects of GDF9 and BMP15, in addition to the biological effects of GDF9 alone, both utilise *TGFβR1* as their receptor (Mazerbourg et al, 2004, Kaivo-Oja et al, 2005, Mottershead et al, 2012). Thus rat and sheep GCs, in response to sheep and pig oocytes, which are both known to express appreciable levels of both *GDF9* and *BMP15* (Crawford and McNatty, 2012), may be responding to the synergistic action of these two OSFs by increasing *TGFβR1* expression. It is interesting to note however, that mouse preantral follicles treated with both GDF9 and BMP15 displayed a decrease in *Tgfβr1* expression (Fenwick et al, 2013), which is not in accordance with this study's results in murine GCs. As this study utilised oocytes, rather than recombinant proteins, affinity differences in the binding of recombinant and native proteins, or interactions between GDF9, BMP15 and other expressed OSFs in sheep oocytes may cause these result disparities. The increase in *TGFβR1* mRNA levels in rat and sheep GCs, upon the respective co-incubation of sheep and pig oocytes may

potentially lead to a concomitant increase in the Smad 2/3 signaling pathway, which has been previously suggested to be highly proliferative.

Interestingly, *TGFβR1* expression in pig GCs increased upon deer oocyte co-incubation. As deer oocytes have a low abundance of *GDF9* mRNA, several other OSFs secreted by deer oocytes, and known to signal through this receptor may be eliciting increased *TGFβR1* mRNA levels. In contrast to other species, pig GCs express *GDF9* (Paradis et al, 2009), which may be acting synergistically with BMP15 secreted from deer oocytes via TGF β R1, thus eliciting an increase in mRNA levels. Although pig and sheep oocytes express relatively higher *BMP15* mRNA levels than deer oocytes, this may implicate an increased bioactivity of deer *BMP15,* or an increased ability of this species' OSF to synergistically interact with pig *GDF9.*

A reduction in *Bmpr1b* mRNA levels was observed in rat GCs upon the co-incubation with deer oocytes. Conversely, whilst rat oocytes appeared to increase *Bmpr1b* mRNA levels, this did not reach significance. Whilst rat oocytes express *Gdf9,* and negligible *Bmp15,* deer oocytes express *BMP15* and negligible *GDF9* (Crawford and McNatty, 2012)*. In vivo,* rat GCs would not usually be exposed to *BMP15* alone. Thus, rat GCs may respond to treatment with *BMP15* alone, from deer oocytes, by reducing *Bmpr1*b expression, which is in accordance with a study whereby treatment of mouse preantral follicles with BMP15 elicited a trend in decreased *Tgfβr1* expression (Fenwick et al, 2013). Murine GCs may respond in such a manner to concomitantly decrease downstream Smad 1/5/8 signaling. Whilst the sheep and pig express higher *BMP15* levels than the deer, there is a disparity between these species, as sheep and pig oocytes also express significant amounts of *GDF9.* Thus *GDF9* and *BMP15* levels from pig and sheep oocytes would activate the *Tgfβr1* receptor, rather than *Bmpr1b.*

In contrast to both rat and pig GCs, sheep and deer GCs did not display altered *BMPR1B* mRNA levels in response to oocyte co-cultures. In pig GCs, co-cultures with pig oocytes increased *BMPR1B* mRNA levels above baseline, whilst deer oocytes dramatically increased *BMPR1B* expression. In particular, this may be an indication of pig GCs

responding to *BMP15* secreted by deer and pig oocytes, through increased *BMPR1B* mRNA levels, and potentially a concomitant increase in Smad 1/5/8 signaling. Low proliferation levels were observed throughout this study when utilising pig GCs, which may be due to this species' innate response to increase *BMP15* signaling.

Bmpr2 expression increased in rat GC upon rat oocyte co-incubation, and conversely decreased upon deer oocyte co-incubation. As rat oocytes express *Gdf9,* whilst deer oocytes express *BMP15,* this may be indicative of *Bmpr2* expression to decrease in murine GC upon exposure to *BMP15* alone and conversely increase *Bmpr2* expression upon exposure to *Gdf9* alone. Furthermore, sheep GCs co-incubated with rat oocytes displayed higher *BMPR2* mRNA levels compared to those co-incubated with deer oocytes. These results are in accordance with a previous study, which illustrated that GDF9 increases *Bmpr2* mRNA levels in murine and ovine GC to a greater extent than BMP15. However, due to large replicate variation, differences in receptor expression with these two different oocyte secreted factors did not reach significance (J Hutchison, personal communication). As deer oocytes elicited less proliferation in murine GC compared to other species, this may be indicative of a correlation between *Bmpr2* expression, and the murine GCs capacity of proliferation.

In contrast to this, *BMPR2* expression was not affected in pig GC upon co-incubation with oocytes from any species. Furthermore, the co-incubation of sheep and pig oocytes with deer GC decreased *BMPR2* expression below that of deer GC incubated alone. In its natural follicular environment *in vivo,* deer oocytes express negligible *GDF9,* and lower *BMP15* mRNA levels than sheep and pig oocytes and thus BMPR2 may not have copious amounts of OSFs activating this receptor. Therefore, in this *in vitro* culture system, deer GCs may respond to these high levels of both *GDF9* and *BMP15* from sheep and pig oocytes by decreasing receptor availability. This is supported by the fact that deer oocytes did not decrease *BMPR2* expression in deer GC, as was observed in rat and sheep GCs.

4.5 Effects of oocyte co-incubation on Smad protein levels in GCs

To determine the signaling pathways involved in the species-specific actions of OSF on GC proliferation, Smad protein levels were investigated. The same- and cross-species co-culture of oocytes with rat or deer GC resulted in the differential upregulation of phosphorylated and unphosphorylated Smad protein levels. This is in accordance with the hypothesis that the cross-species co-incubation of oocytes with GC would differentially stimulate the Smad 2/3 and Smad 1/5/8 pathways in GC, due to the secretion of varying levels, forms and bioactivities of factors secreted by mono-ovulatory and poly-ovulatory species' oocytes. Whilst GDF9 is known to stimulate the phosphorylation of Smad 2/3 (Mazerbourg et al, 2004), conversely BMP15 is reported to stimulate the phosphorylation of the Smad 1/5/8 pathway (Moore et al, 2003). Furthermore, as previously stated, different species' oocytes secrete several other oocyte-secreted factors (as summarised in Table 4.1.1), which are known to differentially stimulate either the Smad 2/3 or Smad 1/5/8 pathway.

Unfortunately due to time constraints, the GCs from every species could be not tested. Rat GCs were utilised for these experiments as they exhibited the highest between species variation in proliferation elicited by OSF and are known to only require GDF9 for this proliferative effect. Conversely, deer GCs were also selected due to the fact that deer oocytes express predominantly *BMP15* mRNA and thus, it is hypothesised that deer GCs may only require BMP15 to proliferate.

Interestingly in this study, the addition of oocytes (regardless of species) did not affect the levels of either phosphorylated Smad proteins (either p-Smad 2/3 or p-Smad1/5/8) in rat GC. However, the addition of sheep oocytes increased the unphosphorylated Smad 2/3 levels compared to rat GC cultured alone. This observation supports the previous finding that sheep oocytes up-regulated *Tgfbr1* mRNA expression, which is the type 1 receptor for GDF9. Thus, this receptor up-regulation occurs concomitantly with an up-regulation of the Smad signaling pathway for GDF9 (i.e. Smad 2/3). Moreover, as mentioned previously sheep oocytes express significant amounts of both *GDF9* and *BMP15* (Crawford and McNatty, 2012), which cooperatively act through the Smad 2/3

pathway (Reader et al, 2011). Upon comparison of the relative *GDF9* mRNA levels between the studied species, pig oocytes display the highest expression, followed by sheep, rat and deer. Thus, although pig oocytes display higher *GDF9* mRNA levels, known to utilise the Smad 2/3 pathway, compared to sheep oocytes, the latter species increased Smad 2/3 levels whilst pig oocytes did not. This however does not take into account the potential variation in bioactivity of GDF9 and BMP15 across species, which would also influence their ability to activate the Smad 2/3 pathway. Whilst GDF9 and BMP15 are known to be responsible for a high proportion of mitogenicity in sheep and rat GCs (Lin et al, 2012), several other OSFs may be increasing Smad 2/3 signaling, such as the activins, TGFβs, GDF1, GDF8, GDF9, GDF11 and GDF15 (Baker and Harland, 1996, Nakao et al, 1997, Dennler et al, 1998, Oh et al, 2002, Rebbapragada et al, 2003, Xu et al, 2006). Conversely, although rat oocytes appeared to increase Smad 2/3 levels, this did not reach significance, and may be due to the relatively lower *Gdf9* expression compared to sheep oocytes. However, GDF9 also stimulates the MAPK pathway (Su et al, 2002, Sasseville et al, 2010) in addition to the Smad 2/3 pathway. Thus, rat oocytes would potentially activate this pathway also, and perhaps to a greater extent than the Smad 2/3 pathway.

Interestingly, deer oocytes stimulated the phosphorylation of Smad 2/3 proteins in deer GC cultures, despite the extremely low levels of *GDF9* mRNA expressed in deer oocytes. The ability of deer oocytes to activate Smad 2/3 phosphorylation, whilst other species do not, suggests species differences in receptivity of GC, causing a species-specific affinity of deer OSFs to their native receptors, thus eliciting Smad phosphorylation. In addition to the *BMP15* expressed in deer oocytes, it is plausible these oocytes also express higher levels of other OSFs that are capable of stimulating Smad 2/3 in deer GC. . It is interesting to note that of the four species studied, pig oocytes express the highest relative amount of *GDF9* mRNA. Although it appears pig oocytes also slightly increased phosphorylated Smad 2/3 levels in deer GCs, this did not reach significance, further suggesting differences in the bioactivity of GDF9 between species and/or differences in OSF affinity to deer GC receptors.

The effects of OSFs on unphosphorylated Smad 1/5/8 levels were also species-specific, with deer oocytes stimulating higher Smad 1/5/8 levels in rat GC. As mentioned previously, whilst sheep and pig oocytes express higher *BMP15* mRNA levels than deer oocytes, there is a disparity between these species, as sheep and pig oocytes also express significant amounts of *GDF9*. In contrast, deer oocytes express almost only *BMP15* (Crawford and McNatty, 2012), known to utilise the Smad 1/5/8 pathway (Moore et al, 2003), thus validating this study's findings. It is interesting to note that pig oocytes express higher relative levels of *BMP15* mRNA, than sheep or rat oocytes (Crawford and McNatty, 2012), which is in agreement with this study's results, whereby there was a trend in pig oocytes stimulating higher Smad 1/5/8 levels, although this did not reach significance. Moreover, deer oocytes also stimulated unphosphorylated Smad 1/5/8 protein levels in deer GC cultures, illustrating that the *BMP15* from deer oocytes, which is likely eliciting this increase in Smad 1/5/8 is not influenced by a difference in species' GCs. This may illustrate minimal variation in the protein abundance of BMPR1B between rat and deer GC, in addition to minimal differences in the affinity of deer *BMP15* to this receptor. Interestingly, co-incubation of deer oocytes with rat GC increased Smad 1/5/8 levels, but decreased *Bmpr1b* expression in these GC. This suggests that a negative feedback mechanism may exist whereby the stimulation of the Smad 1/5/8 pathway in rat GC under stimulation with *BMP15* alone (i.e.incubation with deer oocytes) may be regulated by a decrease in receptor transcription of *Bmpr1b* mRNA. In the future, it would be interesting to observe whether this decrease in *Bmpr1b* transcription in rat GCs upon deer oocyte co-incubation translates to a decrease in Smad 1/5/8 protein levels over a longer period of time.

Sheep GC treated with deer recombinant GDF9 or BMP15 were used in this study as a positive control, with the expectation that samples treated with GDF9 would lead to increased phosphorylated Smad 2/3 levels, whilst treatment with BMP15 would stimulate phosphorylated Smad 1/5/8 levels. Sheep GC were used as their oocytes are known to express both *GDF9* and *BMP15* (Crawford and McNatty, 2012), therefore their GCs would be expected to respond to both of these OSFs. However, although an increase in unphosphorylated Smad 1/5/8 levels was seen in sheep GC treated with GDF9 or BMP15, no differences in phosphorylated Smad levels were observed in these positive

controls. The concentration of recombinant deer GDF9 and BMP15 utilised may have been unable to stimulate the Smad pathways, however the concentrations of these proteins in the follicular fluid of deer are unknown. Previously, both recombinant deer and recombinant pig GDF9 and BMP15 have been used to increase proliferation rate in sheep and rat GC, with recombinant pig proteins stimulating proliferation to a higher degree (JL Pitman, unpublished results) suggesting a reduced biological activity of the recombinant deer proteins.

Differences in Smad phosphorylation levels were only observed in deer GC upon observation of the Smad 2/3 pathway. This may indicate that the timing of maximum Smad phosphorylation did not coincide with the time of GC sample collection. The time of maximum proliferation rate of GCs was determined as 16 hours. Thus, this incubation time was utilised for proliferation assays, and GC sample collection for qPCR and western blotting, with the assumption that the time of the maximum GC proliferation coincides with the maximum receptor expression and Smad phosphorylation. In addition this enables a direct comparison between all results. Previous studies however have utilised 60-90 minute incubation times, and have illustrated increased Smad phosphorylation in murine GC upon their stimulation with oocytes or recombinant OSFs. (Mazerbourg et al, 2004, Gilchrist et al, 2006). Furthermore, the dynamic and transient nature of Smad signaling has been previously illustrated (Schmierer et al, 2008, Shi et al, 2009), further indicating that in this study, Smad phosphorylation may have occurred at an earlier time point. Smad phosphorylation in GCs may also be species-specific, due to varying bioactivities of OSFs from different species' oocytes. In summary, it is likely that protein phosphorylation is more instantaneous compared to a change in GC receptor transcription and the instigation of GC proliferation by OSFs. In contrast, differences in total Smad protein levels were widely variable in this study upon oocyte co-incubation, despite the regulation of Smad activation being primarily through phosphorylation events. Changes in Smad translation are likely to occur over a longer period of time, explaining why changes in unphosphorylated Smad protein levels were observed. It has been previously validated that stimulation of the Smad signaling pathway may result in changes in the Smad protein levels themselves, as treatment of mice with FSH increases Smad2 protein levels (Guéripel et al, 2004).

The antibodies utilised in this study were for a combination of phosphorylated Smad 2/3 or Smad 1/5/8, through detection of phosphorylation at their respective serine residues. In a similar manner, a combination of unphosphorylated Smad 2/3 and Smad 1/5/8 was detected. On occasion, double bands were observed and measured, although Smad 2/3 and Smad 1/5/8 are extremely homologous, and form homo-oligomers upon their phosphorylation (Kawabata et al, 1998). However, doublets have been observed previously with the same phospho-Smad 1/5/8 antibody used in this study (Zhang et al, 2016). As these antibodies detected phosphorylated Smad complexes, it is not possible to validate which Smad proteins were detected in each band.

Furthermore, rat GCs co-incubated with rat oocytes showed lower protein levels, including a lower abundance of β-actin, compared to other GC samples. This is unlikely to be due to uneven protein transfer, as both Smad 2/3 and Smad 1/5/8 displayed this lower abundance, despite being on separate blots. Therefore, this is indicative of lower cell numbers in these samples. This is plausible, due to the previously discussed variation in GC viability between replicates and species. As each sample contained 20,000 previously viable cells, the total cell number between samples would be expected to vary considerably. Thus, β-actin was utilised as a reference protein, with the assumption that total β-actin levels correspond to the total protein content within each sample. It has been illustrated however, that β-actin antibodies cannot distinguish between varying β-actin protein levels when a high abundance of protein is loaded (Dittmer and Dittmer, 2006). In future studies, varying numbers of GCs may be tested to determine β-actin sensitivity, and the optimum cell number for maximum detection of Smad levels. To determine β-actin levels, each membrane was stripped and reprobed for β-actin. The stripping procedure introduces variability, due to the possibility of uneven stripping making protein quantification variable. Due to the presence of bands near to the area where the β-actin band would be detected, it was not possible to probe for β-actin without stripping blots.

With the exception of phosphorylated Smad 1/5/8 antibodies, which were monoclonal, polyclonal antibodies were utilised in this study. The polyclonal antibodies used are said to detect a wider range of species, which is of use to this study. However, the homology comparison of Smads between species (Table 2.9.1), illustrated high homology between species, with the lowest homology observed being 88%. This suggests that the antibodies utilised in this study were likely capable of probing proteins across all the species studied.

4.6 Validation of reference genes

The mRNA levels of the type I and II receptors in GC samples were displayed as a fold change relative to *RPL19*, which was utilised as a reference gene. In order to validate the use of *RPL19* as a reference gene in this study, mRNA levels of both *RPL19* and *PPIA* were measured in each species' GC cDNA sample. A strong linear relationship between *RPL19* and *PPIA* expression, with R^2 values above 0.9, were observed in rat, sheep, and pig GC. However, in deer GC samples, the relationship between *RPL19* and *PPIA* expression was not as strong as the other species in this study, with an R^2 value of 0.625. This may indicate that in deer GC *RPL19* and/or *PPIA* expression is not as stable compared to the other species GC. Due to the lower cell viability in deer GC, a higher proportion of these cells had undergone apoptosis, leading to a poor regulation of *PPIA* and *RPL19* in these deer samples. Previous studies, have utilised both *RPL19* (Eppig et al, 1997, Otsuka and Shimasaki, 2002) and *PPIA* as reference genes in GC samples (Sasseville et al, 2010), however not in deer GCs. In future studies, further reference genes may be utilised as a comparison to determine the stability of *RPL19* and *PPIA* in deer GCs. Furthermore, more prompt deer GC collection to increase cell viability would verify whether the mRNA degradation in apoptotic deer GC is influencing *PPIA* and *RPL19* mRNA levels.

4.7 Validation of Smad 2/3 and Smad 1/5/8 antibodies

Probing with antibodies to detect Smad proteins led to identification of several non-specific bands, in particular upon the detection of phosphorylated Smad proteins. Whilst milk powder was utilised for blocking and antibody incubation for unphosphorylated

Smad protein detection, BSA was used for phosphorylated Smad protein detection, due to the presence of casein in milk powder, as caseins such as casein kinase II are known to contribute to Smad phosphorylation (Chaverneff and Barett, 2009). Thus, milk powder would be assumed to increase background levels for phosphorylated protein detection. However, it is possible that BSA was not as efficient as milk powder in blocking the binding of antibodies to non-specific protein, as can be seen in the presence of non-specific bands. Acetylation and splice variants of phosphorylated Smads may influence the presence of non-specific bands also. Upon their phosphorylation, acetylation of Smads 2 and 3 has been demonstrated (Simonsson et al, 2006, Inoue et al, 2007, Tu and Luo, 2007). Furthermore, an alternatively spliced variant of Smad2 has been observed, which can be phosphorylated in a similar manner to wild type Smad2 (Yagi et al, 1999), However, this splice variant is of similar size to wild type Smad2. Phosphorylation of Smad2 drives the formation of homotrimers, with a molecular size of 170 kDa (Wu et al, 2001b), raising the possibility that the antibodies utilised were capable of probing Smad homotrimers. Furthermore, previous published studies that utilise these antibodies do not include full blots, presenting only the specific band, thus making it difficult to deduce whether the non-specific bands in this study are due to methodology, or the presence of acetylation, splice variants or homotrimers.

In order to validate the specificity of the detected Smad proteins, the polyclonal antibodies were preabsorbed with their corresponding blocking peptide, which led to the disappearance of all specific bands. Upon preabsorption of phosphorylated Smad 2/3 it appears as if a very light specific band is present. However, it is difficult to deduce band presence due to high background levels. The preabsorption of Smad 2/3 resulted in a lighter non-specific band at ~25 kDa. As this band was not observed at the same affinity as the same band on the non-preabsorbed blot, this suggests that this antibody is capable of binding this non-specific protein, or that this protein may be part of the Smad 2/3 complex. However, complete disappearance of this band was not observed, and it should be noted that blots were stripped prior to preabsorption, which would likely lead to protein loss. A blocking peptide for the phosphorylated Smad 1/5/8 antibody was not available, however, this antibody's specificity has been previously illustrated, as specific

bands were almost absent with a vehicle control and TGFβ treatment on SVOG immortalised human GCs (Zhang et al, 2016).

Preabsorption studies were performed on sheep GC, in comparison to the results obtained from rat and deer GC. Generally, preabsorption experiments showed fewer non-specific bands in comparison to previous western blot results in rat and deer GC. Therefore, it is difficult to determine whether these bands were due to the antibodies recognising non-specific proteins.

4.8 Study limitations

The treatment of GCs with oocytes to stimulate proliferation is advantageous, as GC are subjected to several OSFs simultaneously, and in their native forms, as would occur *in vivo* (Gilchrist et al, 2008). A disadvantage to this methodology includes the difficulty in determining which OSFs, or a combination of these, is causing GC proliferation. Immunoneutralisation of GDF9 and BMP15 in rat and sheep GC has previously illustrated that it is these OSFs that are responsible for a significant proportion of GC proliferation (Lin et al, 2012). Although immunoneutralisation is a useful tool in determining which OSFs induce proliferation, studies using exogenous growth factors and those using oocytes are difficult to compare, as it is unknown at what concentration the OSFs are secreted from the oocyte, and it has not been established whether GC would respond in a similar manner to recombinant, compared to native, forms of OSFs.

It is important to note that the large variation observed in GC proliferation assays is likely due to utilising GCs and oocytes from follicles that are at different developmental stages. The hierarchical nature of follicular development in the ovary of all species means that no follicles are at the same stage of development at any given time (McNatty et al, 2010). Within a replicate, GC aliquots are identical, as they are derived from a pool of cells, however between replicates, variations in GCs would be observed. In this study, each GC replicate would have a different capacity of proliferation, as shown previously through varying proliferating cell nuclear antigen (PCNA) staining varying between GCs within each follicle (Lundy et al, 1999). Furthermore, follicles at different developmental

stages differ in the presence and abundance of GC receptors (Paradis et al, 2009), influencing the gene expression results in this study due to the varying ability of GCs to respond to OSFs. Although effort was made to use only healthy COCs as determined by morphology, each oocyte from an ovary is functionally different, due to the different follicular environment it was obtained from. This would further increase replicate variation, due to the differing capacity of these oocytes to elicit GC proliferation, and activate OSF receptors and signaling pathways. Furthermore, it is important to note the limitations involved in the culture system used, whereby the amounts of oocytes and GCs utilised are not representative of GC and oocyte distribution in the ovary, illustrating the different OSF concentrations present in this culture system compared to an *in vivo* environment.

The methodology utilised to retrieve GCs would have lead to the culture of other cell types, such as theca cells and follicular epithelial cells. Due to the technical difficulty in removing these non-GC from the culture system, these cells were included in the proliferation assays. Therefore it is possible that a proportion of the recorded proliferation may be attributed to the proliferation of the other cell types mentioned. Several methodologies may be utilised in the future to remove the presence of these cells such as a density gradient with Percoll or Ficoll (Beckmann et al, 1991; Kolena et al, 1983; Kwintkiewicz et al, 2010; Liu et al, 2010). Furthermore, immunocytochemistry can be used to validate the presence of GCs compared to other cells (Aghadavod et al, 2015; Chilvers et al, 2012).

Another limitation in this study is the different age and breed of animals used. As sheep, pig and deer ovaries were obtained from local abattoirs, these factors are unknown. In contrast, 3-4 week old prepubertal Sprague Dawley rats were used in this study. Several factors such as season, breed and age in sheep influence folliculogenesis (Cahill, 1981). For example, in this study, during breeding season the quality and quantity of oocytes and GCs would decrease. This, alongside the differing ages and breeds in mammals between different experimental replicates may have influenced GC proliferation, as well as gene and protein phosphorylation and levels, increasing the variation between replicates observed.

4.9 Conclusions

Overall, the results of this study clearly illustrated species differences in GC proliferation, receptor upregulation and Smad activation upon same- and cross-species oocyte co-incubation. These observed differences were dependent on the species from which both the GCs and the oocytes were obtained. This illustrates species differences in OSFs, and differences in growth factors and receptor abundance in GCs in mono-ovulatory and poly-ovulatory species.

Rat GCs demonstrated a high mitogenic capacity compared to other species, however the co-incubation of deer oocytes, which express higher levels of *BMP15,* in relation to *GDF9,* mRNA, stimulated the proliferation of rat GCs to a lesser extent than oocytes from the other species studied. Furthermore, deer oocytes also decreased both *Bmpr1b* and *Bmpr2* expression in rat GC, which may indicate that rat GC are responding to stimulation by *BMP15* alone by decreasing receptor levels, in order to decrease the signaling of this OSF. This is in accordance with previous studies whereby BMP15 has an inhibitory effect on folliculogenesis in mouse preantral follicles (Fenwick et al, 2013). Interestingly in rat GCs, phosphorylated Smad 2/3 levels, and unphosphorylated Smad 1/5/8 levels were increased after deer oocyte co-incubations, suggesting that GCs were negatively regulating the increased activation of the BMP15 signalling pathway by down-regulating its type 1 and type 2 receptor.

A high proliferation rate together with a high expression of *Bmpr2* in rat GC co-incubated with rat denuded oocytes may illustrate that whichever OSFs are eliciting high proliferation in rat GC are also upregulating this receptor. Furthermore, sheep oocytes also elicited high rat GC proliferation and concomitantly increased *Tgfβr1* expression, and increased Smad 2/3 in these GCs. This is indicative of the significant levels of both *GDF9* and *BMP15* levels in sheep oocytes, two OSFs that are known to act cooperatively in GC proliferation and utilise *Tgfβr1* and Smad 2/3 signaling.

Conversely, in pig GCs, there was no species differences in oocytes ability to elicit proliferation, and interestingly deer denuded oocyte increased *BMPR1B* mRNA levels. The rat and the pig are both high-ovulation rate species, however whilst rat oocytes express a high ratio of *Gdf9:Bmp15*, pig oocytes express a low *GDF9:BMP15* mRNA ratio (Crawford and McNatty 2012). This study may indicate further differences between these two species, as BMP15 may influence folliculogenesis, and GC proliferation in particular, differently in these two species. Thus, whilst BMP15 may have an inhibitory effect on GC proliferation in the rat, this may not be the case in the pig, sheep and deer, illustrating that despite these species being poly-ovulatory and mono-ovulatory respectively, they may respond in a similar manner to the stimulation of BMP15 alone.

There were no species differences in proliferation rate of GCs from sheep, pig or deer when cultured with denuded oocytes from the same or different species. Co-incubation of deer oocytes with deer GC however also increased both phosphorylated Smad 2/3 levels and unphosphorylated Smad 1/5/8 levels. Whilst an increase in Smad 1/5/8 levels may be due to BMP15 secreted from deer oocytes, increased phosphorylation of Smad 2/3 may be indicative of deer GC requiring particular OSFs present only in their oocytes to activate this Smad signaling.

5.0 Future directions

As mentioned, a major limitation to using oocytes in this study is that it is unknown which OSFs are eliciting GC proliferation, receptor upregulation and Smad pathway activation. To make direct comparisons between OSFs in rat, sheep, pig and deer oocytes, an unbiased approach such as mass spectroscopy may be employed in the future. Immuno-neutralisation of OSFs upon oocyte co-incubation also offers an indirect means of determining which OSF is acting to promote proliferation, or Smad pathway activation. Due to the differing follicular functions of BMP15 between species, as shown in mutations in sheep and mice, it would be of particular interest to immuno-neutralise BMP15 in these cross-incubations, to elucidate whether this OSF impacts proliferation differently in GCs of each of the species tested.

In the current study it is difficult to attribute the observed GC proliferaton to the activation of certain Smad signaling pathways. Thus signaling pathway inhibitors may be used to enable a direct comparison between proliferation assays and Western blotting. However, due to the redundancy observed in the Smad signaling pathway, this technique would not offer a means of elucidating which OSF is activating these signaling pathways. It would also be of interest to immuno-neutralise non-Smad signaling pathways as a means to compare the differenes in the utilisation of extra-Smad signalling pathways in the different species. Alternatively, the activation of non-Smad signaling pathways in GCs upon oocyte cross-incubation through the use of phospho-antibodies against for example, the MAPK signaling pathway may be utilised. In particular, this may indicate whether rat oocytes signal through non-Smad signaling pathways to a greater extent than sheep oocytes, as both species' oocytes were able to stimulate high levels of GC proliferation, however only sheep oocytes increased Smad 2/3 levels in these GC.

Pig GCs displayed the highest proliferation when incubated alone, which was not related to GC viability. In order to validate whether this is due to the presence of TGFβ superfamily members, or other growth factors secreted by these GCs, it would be of importance to look at the gene expression of candidate growth factors, including *GDF9* and *BMP15*, in this species', and other species' GCs. It is particularly interesting to note that it has been demonstrated that whilst in the pig oocyte, *GDF9* expression is higher than *BMP15* expression (Paradis et al, 2009, Crawford and McNatty, 2012), in GCs, *GDF9* has a 30- fold higher abundance than *BMP15* (Paradis et al, 2009).

Using qPCR methodologies, the results obtained indicate which type I and II receptors are being transcribed in GCs at that point in time (i.e. 16 hours after incubation). However, the ability of OSFs to signal through these receptors depends on the presence of these receptors at the cell surface. In order to validate this study's findings, western blot analysis may be performed in these GCs to deduce the protein levels of the type I and II receptors. In particular, in several of the cross-species incubations, the addition of oocytes decreased receptor mRNA levels compared to basal receptor levels. This may be due to the overstimulation of receptors by OSFs causing a decrease in transcription of

these receptors, however western blot analysis may give a better indication of the dynamics of receptor availability.

In order to verify whether a shorter GC incubation time would elicit a greater Smad phosphorylation, a time series could be performed in future, whereby GCs incubation occurs at various time points, with and without oocytes or recombinant OSFs, to determine the time of maximum Smad phosphorylation compared to controls.

Due to time restrictions, only four mammalian species were included in this study. However, to expand our understanding of the differences in GC proliferation and Smad signaling in mono and poly-ovulatory species, several other species may be added in future studies. For example, the mouse is a poly-ovulatory species that expresses a high ratio of *GDF9: BMP15* mRNA, whilst the cow is a mono-ovulatory species that expresses a low ratio of *GDF9:BMP15* (Crawford and McNatty, 2012).

In addition to GC proliferation and SMAD signaling, OSFs affect several other pathways, including steroidogenesis and apoptosis. In the future, this could be studied through gene expression experiments, by looking at the expression of *Bax* and *Bcl2,* which are pro-apoptotic and anti-apoptotic respectively. Thereby the ratio of these genes may be used to determine the effects of OSFs from various species oocytes on the apoptosis of GCs. OSFs are also known to impact steroidogenesis in follicular development, therefore species differences in the oocyte ability to upregulate genes involved in steroidogenesis, such as *StAR, AROM* and *FSHR.*

Whilst members of the TGFβ superfamily, such as GDF9 and BMP15 are involved in follicular development, to gain a more comprehensive view of the follicular environment, the effect of other growth factors such as IGFs, FGFs, EGFs and gonadotropins may be considered in the future for their expression and impact on folliculogenesis in these cross-incubations.

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Appendices

Appendix 1 – Solutions and reagents

1.1 Solutions for dissection

Saline solution

9g of sodium chloride was dissolved in 1L of dH_2O to make a 0.9% solution, autoclaved and stored at room temperature.

Dissection media

One jar of Medium 199 and 4.766 g of HEPES (20 mM) buffer were dissolved in 800 mL of distilled water and Penicillin-streptamycin was added. This solution was pH adjusted to 7.3 with NaOH and distilled water added for a total volume of 1 L. The media was then filtered and stored at 4 °C.

Incubation media

Polyvinyl alcohol (0.3 mg/mL), sodium bicarbonate (2.2 g/L) and penicillin-streptamycin (100IU/mL) were added to McCoy's 5A (Modified) Medium. The media was filtered and stored at 4 °C.

1.2 Reagents for qPCR

MasterPure RNA Purification Kit

Epicentre, an Illumina company, Catalog number MCR85102

RNASe-Free DNase I, Proteinase K stored at -20 °C; remainder of the kit stored at room temperature

SuperScript VILO cDNA Synthesis Kit

Life technologies, Catalog number 11754-250

Aliquoted and stored at -20 °C

Brilliant Multiplex QPCR Master Mix

Agilent technologies, Catalog number 600553

Stored at 4 °C

Brilliant II SYBR Green QPCR Master Mix

Agilent technologies, Catalog number 600828

Stored at 4 °C

1.3 Solutions for Western Blots

10 X Protease inhibitor stock

1 tablet of complete EDTA-free protease inhibitor cocktail (Ref # 04693159001, Roche) was dissolved in 5 mL of distilled water, and stored at -20 °C.

Phosphatase inhibitor

1 tablet of phosphatase inhibitor (Ref # 04906845001, Roche) was dissolved in 1mL of distilled water for a 10x solution, and stored at 4 °C.

Cell lysis buffer

The following compounds were added:

Compound	Amount
Sodium chloride	150 mM
Sodium deoxycholate	0.5%
Sodium dodecyl sulfate (SDS)	0.1%
Tris (pH 8.0)	50mM

0.5 M Tris HCl

6.06g of Tris base was dissolved in 80ml of distilled water and pH adjusted to 6.8 with HCl, and then made up to a total volume of 100ml.

1 M Tris HCl

30.29g of Tris base was dissolved in 150ml of distilled water and pH adjusted to 8.5 with HCl, and then made up to a total volume of 250ml.

1.5 M Tris HCl

36.34g of Tris base was dissolved in 160ml of distilled water and pH adjusted to 8.8 with HCl, and then made up to a total volume of 200ml.

10x Sodium dodecyl sulfate (SDS)

20g of SDS was dissolved in 200 ml of distilled water.

5x Running Buffer

Compound	Amount
Glycine	60g
Tris	15g
SDS	5g

These chemical compounds were combined with distilled water at a volume below 1L and pH adjusted to 8.3 with HCl in a total volume of 1L. Running buffer was stored at room temperature and diluted to 1x concentration prior to use.

10x Tris-glycine stock

Compound	Amount
Tris base	30.3g
Glycine	144g

These chemical compounds were combined with distilled water in a total volume of 1L and stored at room temperature.

Membrane washing buffer

Compound	Amount
1M Tris HCl	20ml
Sodium chloride	8.76g
Tween20	1ml

These chemical compounds were combined with distilled water in a total volume of 1L and stored at room temperature.

Membrane storage solution

0.05% Sodium azide was combined with 20 ml of membrane washing buffer and membranes were stored in this solution at 4°C.

2x Reducing loading buffer

Compound	Amount
0.5 M Tris HCl pH 6.8	1.0 mL
Glycerol	4.5 mL
10% SDS	4.6 mL
1% (w/v) bromophenol blue	0.4 mL
dH$_2$O	4.5 mL
DTT	80 mg
2-Mercaptoethanol (2-ME)	1.66 mL

These compounds were combined and aliquoted, to be stored at -20°C prior to use (up to 3 months). 2-ME was added fresh prior to use (10% of total volume).

Stripping buffer

Compound	Amount
10% SDS	20 mL
0.5M Tris HCl pH 6.8	12.5 mL
2-Mercaptoethanol (2-ME)	0.7 mL
dH$_2$O	66.8 mL

These compounds were combined, and made fresh prior to use.

Transfer buffer

Compound	Amount
10x Tris-glycine stock	300ml
Methanol	600ml

These compounds were combined with distilled water in a total volume of 3L and stored at 4°C.

Appendix 2 – Reagents

Reagent	Lot/Cat number and Company	Storage conditions
2-Mercaptoethanol (2-ME)	46496KMV, Sigma	Room temperature
30% Acrylamide/Bis solution	161-0158, BioRad	4°C
^{3}H-Thymidine	NET35501MC, Perkin Elmer, Boston, MA, USA	4°C
β-scintillation fluid	SC/9200.21, Perkin Elmer, Sci-Med, Auckland, New Zealand	
Bovine albumin	ABRE, MP Biomedicals	4°C
Bromophenol blue sodium salt	B8026, Sigma-Aldrich	Room temperature
Complete EDTA-free protease inhibitor cocktail tablets	04693159001, Roche	4°C
Glycine	AC04041000, Scharlau	Room temperature
HEK-293 deer BMP15 recombinant conditioned media	N/A	-80°C
HEK-293 deer GDF9 recombinant conditioned media	N/A	-80°C
HEPES	SLBL4126B, Sigma	Room temperature
Ladder – Precision Plus Protein Dual Color Standards	1610374, Bio-Rad	-20°C
McCoy's 5A modified medium	16600082, Gibco (Thermo Fisher Scientific)	4°C
Medium 199	1001954622, Sigma Aldrich	4°C
Methanol	ME03162500, Scharlau	Room temperature
Penicillin-Streptomycin	15140122, Gibco (Thermo Fisher Scientific)	-20°C
Phosphate buffered saline (PBS)	1694258, Gibco (Life Technologies)	4°C
Protease inhibitor	04693159001, Roche	4°C
Polyvinyl alcohol	9002895, Sigma	Room temperature
Sodium azide	26628228, Scharlau	Room temperature
Sodium bicarbonate	SLBF0769V, Sigma	Room temperature
Sodium chloride	7647145, Fisher Scientific	Room temperature
Sodium Dodecyl Sulfate (SDS) dust free pellets	STBG2637V, Sigma	Room temperature
Tris base	BP1521, Fisher Scientific	Room temperature
Tween20	115125, Fisher Scientific	Room temperature
WesternBright ECL-Spray Western Blotting Detection	K-12049-D50, Advantsa	Room temperature

Appendix 3 - Antibodies for Western Blots

Primary antibodies

Antibody	Brand and Lot Number	Storage conditions	Target site/s
SMAD 2/3 (E-20) SC-6033, goat polyclonal IgG	Santa Cruz: Lot#B1313	4°C	Raised against a peptide which maps to the N-terminus of Smad1 of human origin
SMAD 1/5/8 (N-18) – R SC-6031-R, rabbit polyclonal IgG	Santa Cruz: Lot# C2216	4°C	Raised against a peptide which maps to the C-terminus of Smad3 of human origin
p-SMAD 2/3 (Ser423/425)-R SC-11769-R, rabbit polyclonal IgG	Santa Cruz: Lot# D1216	4°C	Recognises Ser 423 and Ser 425 phosphorylated Smad3 and correspondingly phosphorylated Smad2 of mouse, rat and human origin. Raised against an amino acid sequence containing Ser 423 and Ser 425 phosphorylated Smad3 of human origin
p-SMAD 1/5(S463/465)/9(S465/467) (D5B10), rabbit monoclonal IgG	Cell Signaling: Lot#13820	-20°C	Recognises Smad1 and Smad5 pro-protein when phosphorylated at Ser463/465 and Smad8 protein when phosphorylated at Ser465/467

Secondary Antibodies

Antibody	Brand and Lot Number	Storage conditions	Target site/s
Peroxidase-conjugated AffiniPure Rabbit Anti-Goat IgG	Jackson Immunoresearch: Lot# 117560	-20°C	Reacts with whole molecule goat IgG in addition to the light chains of other goat immunoglobulins
Goat polyclonal Ab to Rabbit IgG (HRP)	Abcam: Ab97051	4°C	Reacts with rabbit IgG in addition to the light chains common to other rabbit immunoglobulins

Blocking peptides

Blocking peptide	Brand and Lot Number	Storage conditions
p-Smad 2/3	Santa Cruz: Lot# J0716	4°C
Smad 2/3	Santa Cruz: Lot# D2205	4°C
Smad 1/5/8	Santa Cruz: Lot# E2214	4°C