PRODUCTION AND FUNCTIONAL ANALYSIS OF RECOMBINANT BOVINE MORPHOGENIC PROTEIN 15

A THESIS

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

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December 2013

Major Subject: Biomedical Science

ABSTRACT

After 40 years of research, *in vitro* systems for mammalian embryo production produce lower quality embryos than those derived from *in vivo* sources. Recent reports have demonstrated that *in vitro* bovine oocyte maturation systems benefit from the addition of oocyte secreted factors, specifically Bone Morphogenic Protein 15 (BMP15) from heterologous sources. However, known amino acid sequence variation and species-specific patterns of post-translational glycosylation lead us to hypothesize that utilization of bovine-specific oocyte secreted factors would be more beneficial than the observed effects of heterologous factors.

To test this hypothesis, wild type, bovine BMP15 was cloned using reverse transcriptase PCR from RNA obtained from bovine ovarian tissue. For improved detection and purification of the biologically active recombinant protein, a FLAG tag peptide sequence (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) was incorporated into the wild type BMP15 gene by PCR. This modified protein was cloned into the pCDNA 3 mammalian expression vector. HEK-293 (human embryonic kidney 293) and FBK (fetal bovine kidney) cell lines were transfected via electroporation and then selected to homogeneity.

Collection and purification of rbFL-BMP15 from conditioned medium was accomplished by incubation with anti-FLAG affinity gel and the use of 3X FLAG peptide for elution. Peptides of 15.4 kDa and 17 kDa were noted from the human HEK-293 transfected cell line, while in contrast, bovine FBK cells produced a

single 17 kDa protein. Bioactivity and BMP receptor signaling specificity were ascertained using *in vitro* treatment of HeLa cells and Western blotting for the BMP signaling molecule phosphorylated-SMAD 1/5. Inhibition of this signaling cascade using dorsomorphin, a selective bone morphogenic protein receptor I inhibitor, demonstrated the purified proteins served as BMP15-like agonists.

To examine the impact of our purified, bovine-specific peptides on oocyte maturation, cumulus oocyte complexes were *in vitro* matured for 24 hours in the presence of 100 ng/ml recombinant human BMP15 or rbFL-BMP15 from human or bovine cell lines. Real time quantitative PCR analysis of BMP15 stimulated genes, PTGS2 and TSG6, revealed statistically significant increases in transcript level for treatment with human BMP15 by a Dunnett's test ($p<0.05$). In this report, however, we failed to detect a significant affect of rbFL-BMP15 on the gene expression of *in vitro* mature cumulus oocyte complexes at 24 hours with 100 ng/ml rbFL-BMP15. Future studies including differing time points and concentrations, along with the addition of GDF9 to form a possible heterodimer should be investigated for the possibility of improving bovine oocyte *in vitro* maturation.

I wish to dedicate this thesis to my family, who has supported me through everything, and without their love and encouragement I would not have made it nearly this far.

To my wife, Kelly:

Your love and daily encouragement has allowed me to accomplish things that I never could have done alone.

ACKNOWLEDGEMENTS

I wish to thank the members of my committee: Dr. Charles Long for the opportunity to pursue a career in research, and Drs. Michael Golding and Qinglei Li for advice and encouragement along the way.

To my mentor, Dr. Paulette Suchodolski: thank you for sharing your scientific knowledge and skills with me throughout the learning process. You have instilled in me a careful attention to detail and patience that I know will prove useful in many areas of life.

I would also like to thank all of the current and past members of the Reproductive Sciences Lab (Dr. Duane Kraemer, Dr. Kim Tessane, Dr. Paulette Suchodolski, Gayle Williamson, Kim Green, Mike Peoples, Lisbeth Ramirez-Carvajal, Chelsie Burroughs, Kylee Veazey and Jane Pryor) for their guidance and support throughout this project.

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CHAPTER I

INTRODUCTION AND REVIEW OF THE LITERATURE

Utilization of ART in Cattle

From the first successful embryo transfer in 1951, the use of assisted reproductive technologies (ART) in the bovine industry has expanded to become commonplace (Willett, Black, *et al.*, 1951). Over 990,000 bovine embryos were transferred worldwide in 2010 as reported by the International Embryo Transfer Society's (IETS) data retrieval committee. *In vitro* produced embryos are increasing their presence within the group with almost 340,000 transfers in 2010. This represents an increase of 11% over 2009, demonstrating an increasing trend for the use of *in vitro* maturation and fertilization in the bovine industry.

According to the IETS, the United States produced almost 35,000 bovine embryos *in vitro* by transvaginal ultrasound-guided ovum pick up (OPU) and *in vitro* fertilization (IVF) in 2010 that resulted in more than 26,000 embryo transfers. These numbers followed the worldwide trend of increased *in vitro* embryo production. The 35,000 bovine embryos represent 6,876 OPU sessions that yielded 109,615 oocytes for subsequent *in vitro* maturation (IVM) followed by IVF. Based on these data, the efficiency of producing transferrable bovine embryos was only 32% in the United States, leaving ample room for improvement.

Following OPU, the oocytes must be taken through IVM, IVF and subsequent culture prior to embryo transfer. These processes include IVM, IVF and subsequent culture. Medium for IVF has developed into a relatively complex system that is customizable even to the individual sire used for fertilization (Parrish, Susko-Parrish, *et al.*, 1988; Fukui, Sonoyama, *et al.*, 1990). Work by Hansen et al. showed that fertilization rates remained unchanged in both Bos taurus and Bos indicus breeds exposed to heat stress despite a drastic drop in embryo production (Hansen, Drost, *et al.*, 2001). This evidence points to fertilization rate as an unreliable measure of the developmental capacity of the mature oocyte. The percentage of oocytes developing beyond the resumption of gene transcription at the maternal to zygotic transition, occurring at the 8- to 16-cell embryo, and into blastocysts is a direct measurement of developmental competence (Telford, Watson, *et al.*, 1990; Memili, Dominko, *et al.*, 1998; King, Niar, *et al.*, 1988). This competence is acquired in the antral phase of folliculogenesis prior to ovulation or during the period of IVM (Eppig, Schultz, *et al.*, 1994). In contrast to medium developed for IVF, which can be customized for the sire, standard maturation medium is basic. Interest in improving *in vitro* maturation efficiency has recently increased with the development of a procedure entitled simulated physiological oocyte maturation or SPOM with two culture medias corresponding to peri-ovulatory (pre-IVM) and post-ovulatory (extended-IVM) development (Albuz, Sasseville, *et al.*, 2010). During this time of

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This thesis follows the style of *Reproduction*

maturation, the oocyte receives nutrients and regulatory signals that are required for proper nuclear and cytoplasmic maturation (Ka, Sawai, *et al.*, 1997; Chian & Sirard, 1995). Developmental competence of *in vitro* produced embryos is lower than those produced *in vivo*, in part due to inadequacies in the *in vitro* maturation culture system (Sutton, 2003; Rizos, Ward, *et al.*, 2002).

Recent work by Hussein et al. demonstrated the addition of oocyte secreted factors (OSFs) BMP15 (bone morphogenetic protein 15) and GDF9 (growth differentiation factor 9) to maturation medium increased the percentage of oocytes developing into blastocysts without affecting cleavage rates, providing evidence that these factors are capable of increasing developmental competence(Hussein, Thompson, *et al.*, 2006). Following an observation that co-culture with denuded oocytes during IVM was beneficial for the COCs, exogenous ovine BMP15 was observed to have a similar effect with the intention of more closely mimicking the complex microenvironment of the follicle during the latter stages of folliculogenesis.

In Vivo Follicular Development

During a single estrous cycle antral follicles, which are marked by the formation of a fluid filled antrum, develop in several waves. The number of waves per cycle differs by species, but the preovulatory wave that begins with luteolysis is the only wave that will result in ovulation (Savio, Keenan, *et al.*, 1988; RAJAKOSKI, 1960). This final growth and development of an antral follicle consists of four major stages: recruitment, development, selection and atresia

(Eppig, Schultz, *et al.*, 1994). This process begins when the hypothalamus with releases of gonadotropin releasing hormone (GnRH), which stimulates the anterior lobe of the pituitary gland to secrete the glycoprotein hormones follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Roche, 1996).

Recruitment is marked by the growth of a group of follicles under the influence of increasing levels of FSH (Sunderland, Crowe, *et al.*, 1994; Adams, Matteri, *et al.*, 1992). FSH increases proliferation of the granulosa cells and is also responsible for directing the expression of luteinizing hormone (LH) receptors (Eppig, Wigglesworth, *et al.*, 1997). These follicles begin to produce low levels of inhibin and estrogen. Inhibin negatively feeds back on the anterior lobe of the pituitary and selectively decreases the level of FSH, while estrogen increases the release of GnRH (Ginther, Bergfelt, *et al.*, 2000b; Ginther, Bergfelt, *et al.*, 2000a; Hillier, 1994; Sunderland, Crowe, *et al.*, 1994). Recruited follicles will either continue to grow to dominance or become atretic and regress. (Fig. 1)

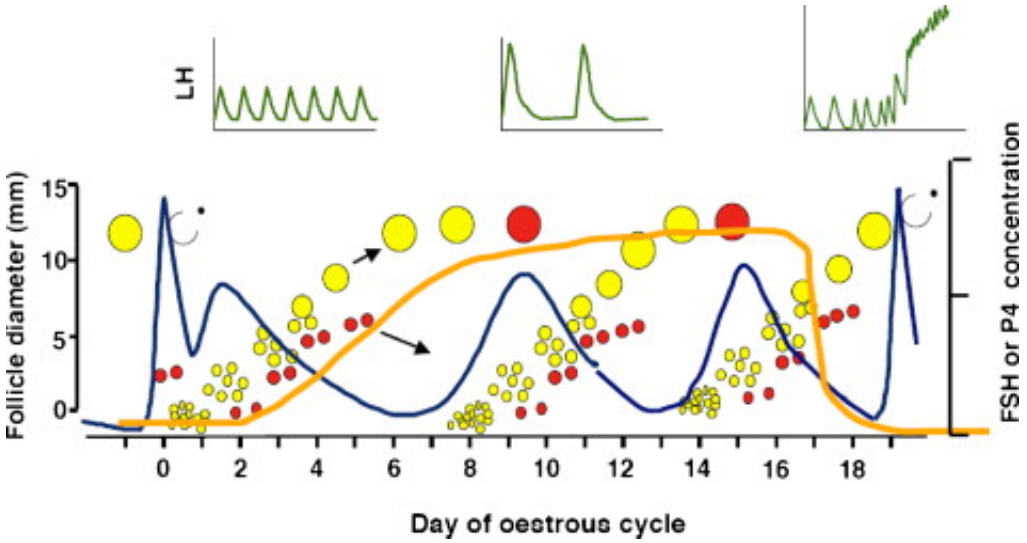


Figure 1 The pattern of FSH (blue), LH (green) and progesterone (yellow, P_4) during the estrus cycle of Bos taurus cattle. Each wave of follicular growth is preceded by a transient rise in FSH concentrations. Healthy growing follicles are shaded in yellow, atretic follicles are shaded red. A surge in LH and FSH concentrations occurs at the onset of estrus and induces ovulation. The pattern of secretion of LH pulses during an 8 hour window early in the luteal phase (greater frequency, lesser amplitude), the mid-luteal phase (lesser frequency, lesser amplitude) and the follicular phase (high frequency) is indicated in the top panel. (Forde, Beltman, *et al.*, 2011)

The increasing level of estrogen produced by the dominant follicle reaches a threshold and stimulates the surge center of the hypothalamus to change the pulsitility of GnRH resulting in the preovulatory LH surge from the anterior pituitary (Sunderland, Crowe, *et al.*, 1994; Forde, Beltman, *et al.*, 2011). This LH surge decreases the release of estrogen by inducing a switch from the production of estrogen to progesterone in the dominant follicle and triggers the production of histamine and prostaglandin E2 (PGE2) (Acosta, Berisha, *et al.*, 1999; Espey, 1980). Under the influence of PGE2 blood flow increases locally

and histamine increases vascular permeability (Espey, 1980). As a result, the theca interna becomes edematous and follicular pressure increases. The surge ends proliferation of thecal and granulosa cells concurrent with the theca interna cellular transition to progesterone producing luteal cells and the formation of a corpus luteum (Gonzalez-Robayna, Falender, *et al.*, 2000). Collagenase begins to break down the connective tissue and weakening the follicle at the same time as follicular pressure is increasing (Espey, 1980). Finally, the LH surge causes the ovary to produce prostaglandin F2α (PGF2α), which increases contractions of the smooth muscle of the ovary (Acosta, Berisha, *et al.*, 1999). The stage is now set for ovulation, but there are significant and complex paracrine interactions occurring inside the follicle during this development in which the oocyte plays an active role in preparing for ovulation (Li, Norman, *et al.*, 2000; Eppig, Wigglesworth, *et al.*, 1997; Gilchrist, Ritter, *et al.*, 2004).

Bidirectional Cell Signaling in the Cumulus Oocyte Complex

The importance of the entire cumulus oocyte complex became apparent in 1967 when Biggers et al. reported the requirement for the metabolites from follicular cells for oocytes and zygotes (Biggers, Whittingham, *et al.*, 1967). Gap junctions containing connexin43 channel proteins proved to be the lifeline between the cumulus somatic cells and the oocyte allowing for direct communication from birth through ovulation (Ackert, Gittens, *et al.*, 2001). In fact, cumulus cell glycolysis is regulated by mRNAs expressed by the oocyte that encode glycolytic enzymes (Sugiura, Pendola, *et al.*, 2005). With the

discovery of autocrine and juxtracrine growth factors such EREG (epiregulin) and AREG (amphiregulin), an increasingly complex microenvironment with two-way communication began to emerge (Conti, Hsieh, *et al.*, 2006).

Oocyte secreted factors (OSFs) direct the actions of cumulus cells via a paracrine mechanism. In fact, folliculogenesis fails without these factors based on evidence provided by inactivating mutations of OSFs in sheep and mice (Gilchrist, Ritter, *et al.*, 2004). These factors include members of the TGFβ (transforming growth factor β) and FGF (fibroblast growth factor) families including BMP6 (bone morphogenetic protein 6), BMP15, GDF9, and FGF8 (fibroblast growth factor 8). Members of the TGFβ family, GDF9 and BMP15 have proven to be important and are required for normal fertility (Galloway, McNatty, *et al.*, 2000; Dong, Albertini, *et al.*, 1996).

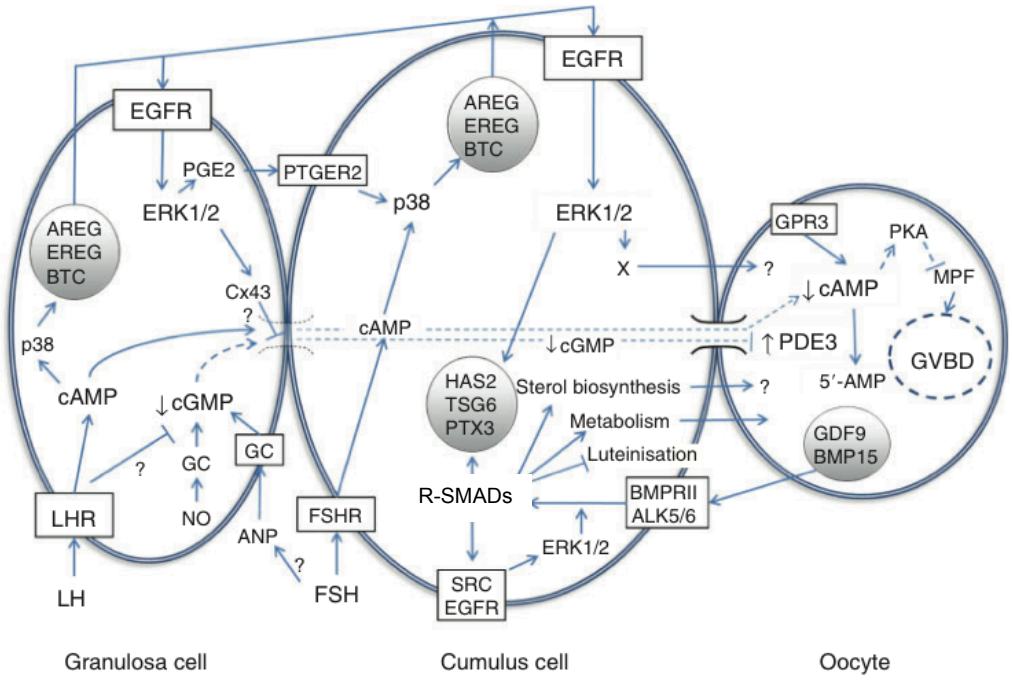


Figure 2 Model of oocyte-follicle cell interactions. How these interactions may interact to regulate oocyte maturation *in vivo*. The resumption of oocyte meiosis *in vivo* is a highly orchestrated, complex process that integrates oocyte paracrine signals with an array of maternal endocrine, granulosa/cumulus paracrine and gap-junctional signals. This highly integrated interaction between oocyte and follicular somatic cells is unlikely to occur during spontaneous IVM. Dotted arrows indicate signals of diminishing intensity. 'X' represents a hypothetical meiotic-inducing factor. R-SMADs represent receptor SMAD signaling molecules. Modified from (Gilchrist, 2010)

Signaling gradients of OSFs GDF9 and BMP15, are crucial for maintaining the phenotypes of the granulosa and cumulus cells (Conti, Hsieh, *et al.*, 2006; Gilchrist, Ritter, *et al.*, 2004). These growth factors act in a paracrine fashion through BMP Receptor type II and ALK5/6 (transforming growth factor beta receptor 1, bone morphogenetic protein receptor type IB) receptor complex on cumulus cells followed by activation of the intracellular transcription factors

SMAD 1/5/8 (mothers against decapentaplegic homolog) or 2/3 by phosphorylation (Figure 2) (Kaivo-oja, Jeffery, *et al.*, 2006). Specifically, in contrast to GDF9 signaling through SMAD 2/3, BMP15 activation of SMAD 1/5/8 occurs by phosphorylation at serine residues 463 and 465 (Moore, Otsuka, *et al.*, 2003). The activated glycosyl-SMAD 1/5/8 associates with the co-SMAD4 to form a complex that translocates to the nucleus and affects the expression profile of several genes through a variety of other transcription factors, co-activators and co-repressors (Fig. 3) (Anderson & Darshan, 2008; Kaivo-oja, Jeffery, *et al.*, 2006). These genes are associated with differentiation of granulosa cells to cumulus cells, maintenance of this phenotype by prevention of luteinization, and expansion of the COC in the developing antral follicle following the LH surge (Richards, Russell, *et al.*, 2002). Cumulus cell expansion mediated by OSFs is carried out with the expression of multiple genes including prostaglandin-endoperoxide synthase 2 (PTGS2), hyaluronan synthase 2 (HAS2), tumor necrosis factor, alpha-induced protein 6 (TNFAIP6) and pentraxin 3 (PTX3) (Richards, Russell, *et al.*, 2002; Li, Rajanahally, *et al.*, 2009; Dragovic, Ritter, *et al.*, 2005; Elvin, Clark, *et al.*, 1999). It has also been shown that BMPs secreted by the oocyte actively prevent apoptosis in the cumulus cell layers (Hussein, Froiland, *et al.*, 2005).

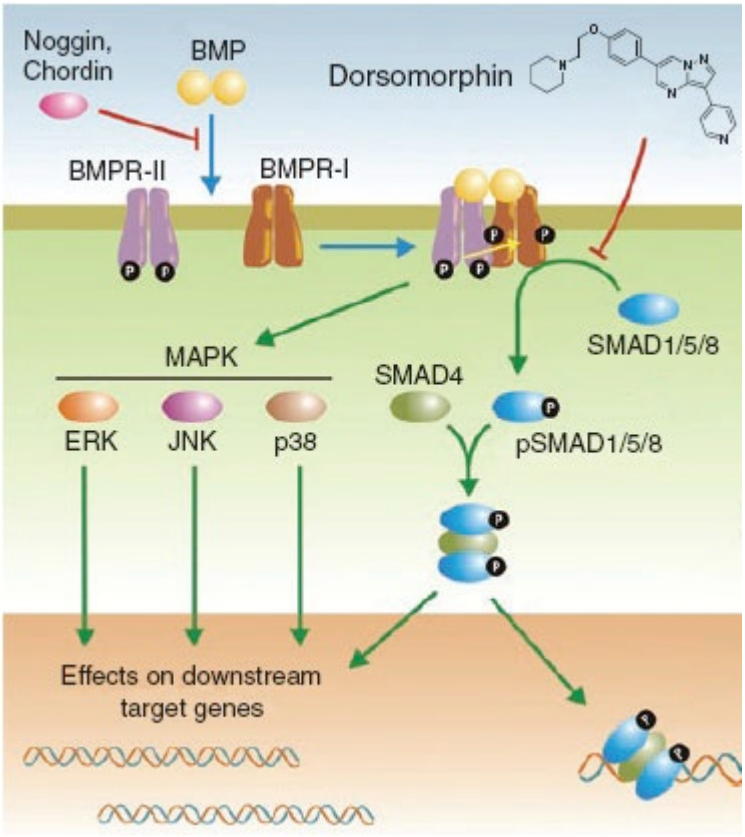


Figure 3 Bone morphogenic protein signaling. The BMPs bind to type I and II receptors and facilitate their association. The constitutively active kinase domains of type II receptors phosphorylate type I receptors, and this in turn activates the SMAD signaling pathway through phosphorylation of receptor SMADs (SMAD1, SMAD5 and SMAD8). These associate with co-SMADs (SMAD4) to form a heteromeric complex that translocates to the nucleus and stimulates the expression of a wide range of target genes. Modified from (Anderson & Darshan, 2008).

PTGS2, also known as COX2 (cyclooxygenase 2), is the rate-limiting step in the synthesis of prostaglandins and is involved in the inflammatory response (Smith, DeWitt, *et al.*, 2000). Along with PTX3 and TNFAIP6, which are also involved in the inflammation, these genes contribute to the expansion of the cumulus cells prior to ovulation (Richards, Russell, *et al.*, 2002). HAS2 is a

component of the extracellular matrix and provides a framework for angiogenesis during wound healing along with inflammation. TNFAIP6 and HAS2 are two components of the extracellular matrix that the cumulus cells migrate on during expansion (Salustri, Camaioni, *et al.*, 1999). The activation of these genes by OSF signaling pathways further demonstrates the active role the oocyte plays in folliculogenesis.

Increased levels of cAMP have been shown to be important for the development of mammalian oocytes, particularly by maintaining meiotic arrest (Cho, Stern, *et al.*, 1974). Shortly after the LH surge, gap junctions between somatic cumulus cells and the oocyte degrade. This degradation prevents the flow of cAMP and cGMP (3'-5'-cyclic adenosine monophosphate; cyclic guanosine monophosphate) from the cumulus cells from entering the oocyte. Natriuretic peptide type C (NPPC) produced by the mural granulosa cells binds to its associated receptor natriuretic peptide receptor 2 (NPR2) expressed by the cumulus cells to increase production of cGMP which is transferred to the oocyte by gap junctions to maintain meiotic arrest (Lee, Zhang, *et al.*, 2013). The decrease in cGMP leads to an increase in PDE3 (phosphodiesterase 3A) in the oocyte, which hydrolyzes the remaining cAMP (Tsafriri, Chun, *et al.*, 1996; Han, Vaccari, *et al.*, 2006). Lower levels of cAMP, decrease the activity of phosphokinase A (PKA) (Tsafriri, Chun, *et al.*, 1996). PKA normally keeps the phosphatase cell division cycle 25 homolog B (CDK25B) inactive and WEE1/myelin transcription factor 1 (WEE1/MYT1) actively phosphorylating

meiotic promoting factor (MPF) complex to keep it inactive (Han, Chen, *et al.*, 2005). Decreased PKA frees CDK25 to dephosphorylate the cyclin-dependent kinase 1 (CDK1) subunit of MPF. MPF then becomes active and releases the oocyte from meiotic arrest at the dictyate stage in late prophase I and it proceeds to metaphase II until fertilization (Duckworth, Weaver, *et al.*, 2002; Han & Conti, 2006). Throughout this process the oocyte actively participates by not only directly influencing the development of the follicle but also maintaining the undifferentiated state of the surrounding cumulus cells (Li, Norman, *et al.*, 2000; Hussein, Froiland, *et al.*, 2005). The OSFs also regulate CC amino acid and energy substrate uptake and transport to the oocyte preserving the health of the oocyte itself (Makabe, Naguro, *et al.*, 2006; Albertini, Combelles, *et al.*, 2001; Sugiura, Pendola, *et al.*, 2005)

In Vitro Maturation

Assisted reproductive technologies are commonplace in the bovine industry, but current *in vitro* systems for mammalian embryo production are inefficient when considering yield of transferrable embryos and produce embryos with lower viability than their *in vivo* derived counterparts (Greve, Xu, *et al.*, 1987; Sutton, 2003; Rizos, Ward, *et al.*, 2002). These developmental differences likely arise from the vastly different environments during final oocyte maturation. Upon removal from the dynamic follicular environment, the *in vitro* matured oocyte is placed in static culture medium that supplies only the critical components necessary for survival. In fact within 9 hours of *in vitro* culture the gap junction

communication between the oocyte and cumulus cells is lost, and the rate at which the connections are lost is dependent on the IVM medium (Thomas, Armstrong, *et al.*, 2004; Thomas, Thompson, *et al.*, 2004). As a result, the viability of the *in vitro* matured oocytes is compromised with only around 10% of the total cultured oocytes continuing development into an offspring as compared to almost 25% of *in vivo* matured oocytes (Gilchrist, 2010). Thus there is tremendous room for improvement of the culture media components and increased efficiency of the embryo production systems.

The nuclear maturation process *in vitro* occurs spontaneously when the cumulus oocyte complex (COC) is removed from the restrictive environment of the follicle (Handel & Eppig, 1998; Eppig, 2001). The typical maturation medium consists of a tissue culture base with sodium pyruvate as an energy source, FBS as a protein source, FSH and LH. This medium is in stark contrast to the complexities of *in vivo* maturation described earlier. Generally, the immature oocyte is placed in this basic medium for a period of 20-24 hours during which time the cumulus cells begin to expand and the gap junctions break down. This accelerated process leads to a decrease in cAMP and a progression by the oocyte to metaphase II. Opportunity is decreased for the oocyte to attain proper cytoplasmic maturation and therefore the necessary RNAs and proteins to continue to develop until gene transcription resumes.

The process of maturation is complex, requiring the participation of both the oocyte and its somatic neighbors (Handel & Eppig, 1998; Buccione, Schroeder,

et al., 1990). It is apparent that the oocyte's role in the micro-environment of the developing follicle is important. For example, BMP15 and GDF9 null animals have been reported to have decreased fertility or be sterile(Dong, Albertini, *et al.*, 1996; Davis, McEwan, *et al.*, 1992; Galloway, McNatty, *et al.*, 2000; Yan, Wang, *et al.*, 2001) Gilchrist et al. reported a 1.4 fold increase in blastocyst rate in response to the addition of exogenous BMP15 to bovine maturation medium (Gilchrist & Thompson, 2007). In another study, the addition of two OSFs, BMP15 and GDF9, during bovine oocyte maturation *in vitro* also increased developmental competence, reflected by a 1.5 fold increase in blastocyst rate (Hussein, Thompson, *et al.*, 2006).

Post-Translational Modifications

Protein synthesis involves translation of the mRNA sequence into a chain of amino acids known as a polypeptide through the utilization of trinucleotide codons (Wold, 1981). To initiate translation the small 40S subunit of the ribosome binds to the mRNA and scans specifically for the start codon sequence of AUG (Merrick & Hershey, 1996). The first transfer RNA (tRNA), which contains the antisense start codon bound to a methionine residue, is present in the 40S ribosome along with several eukaryotic initiation factors (eIFs) and is termed the 43S ribosome unit (Pestova, Borukhov, *et al.*, 1998; Kozak, 1978). Following binding at the AUG start codon several eIFs are released and the 60S subunit joins the complex (Kozak, 1989). The ribosome complex consists of three sites for tRNA molecules termed A for aminoacyl, P for peptidyl and E for

exit. The first tRNA is in the P site when second tRNA bound to eukaryotic elongation factor 1α (eEF1α) and containing the antisense sequence to the next codon binds to the mRNA in the A site (Pestova, Borukhov, *et al.*, 1998; Cigan, Feng, *et al.*, 1988). The amino acid bound to the tRNA in the A site is now next to the methionine residue on the initiating tRNA. A peptide bond is formed at the carbonyl carbon of the residue in the P site with the amino terminus of the residue in the A site. The result is a dipeptide bound to the tRNA in the A site and a tRNA lacking an amino acid in the P site.

The entire ribosome shifts 5' to 3' one 3 nucleotide frame and free tRNA is now located in the E site and can exit and be recycled. Another tRNA matching the next codon and bound to eEF1α moves into the A site and the process continues until the ribosome encounters a codon that has no complementary tRNA known as a stop codon (Valle & Morch, 1988). The stop codon can be one of three sequences: UAA, UAG or UGA. Eukaryotic release factor 1 (eRF1) enters the A site and termination of the peptide begins. eRF1 acts in combination with eRF3 to disassemble the ribosome complex and release the polypeptide (Stansfield, Jones, *et al.*, 1995).

Many proteins require modification, often cleavage by specific proteolytic enzymes, following translation to become biologically active (Schimke & Doyle, 1970). These post translational modifications (PTMs), including phosphorylation, glycosylation, and methylation, require correct conformation of the polypeptide substrate to fit the binding sites of the enzymes (Neurath & Walsh, 1976;

Ottesen, 1967). Kinases attach phosphate groups to the hydroxyl groups of the amino acids serine, threonine and tyrosine (Wold, 1981). The phosphate groups are negatively charged and usually confer a change in the protein's conformation. This conformation change often reversibly changes the activity of a protein and thus is a common mechanism for the regulation of protein activity (Taborsky, 1974; Wold, 1981)

Glycosylation of the side chains of an amino acid with covalently bonded oligosaccharides is another type of PTM and is essential in yeast and higher eukaryotes with around 50% of human proteins glycosylated (Gentzsch & Tanner, 1996; Metzler, Gertz, *et al.*, 1994; te Heesen, Janetzky, *et al.*, 1992; Wong, 2005). These covalently bonded oligosaccharides can cause profound effects in a biological system (Wold, 1981). For example blood type antigens are determined by the difference in oligosaccharides on cell surfaces (Kabat, Bassett, *et al.*, 1965; Anderson, Seno, *et al.*, 1964; Adams, 1965; Creeth & Knight, 1967). Humans and old world monkeys lack 1,3-galactosyltransferase present in other mammals, thus the cells expressing α1,3-galactose glycosylation are seen as foreign and cause a hyperacute rejection in pig to human organ transplantation (Galili, Shohet, *et al.*, 1988; Galili, 2001; Cooper, Koren, *et al.*, 1993; Good, Cooper, *et al.*, 1992). Many proteins that are membrane associated or secreted in eukaryotes are glycosylated in one of two fashions, N or O-linked (Ecker, Mrsa, *et al.*, 2003). N-linked glycosylations are added co-translationally in the lumen of the endoplasmic reticulum (ER) while O-

linked forms primarily manufactured in the Golgi apparatus (GA) (Schachter & Williams, 1982).

N-Acetylglucosamine is linked to the amide of asparagine followed by any amino acid except proline or aspartate ahead of a serene or threonine (Helenius & Aebi, 2001; Kornfeld & Kornfeld, 1985). Peptides that have N-linked glycosylations are glycosylated beginning at the cytosolic face of the ER and continues as the peptide is transported through the ER and to the GA where glycosylation continues (Kornfeld & Kornfeld, 1985; Ungar, 2009).

O-linked glycosylation generally involves β-galactosyl-α-N-acetylgalactosamine covalently bonded to the hydroxyl group of serene or threonine, but can also be galactose, mannose or xylose (Schachter & Williams, 1982). These modifications vary in size from a single galactose up to 1000 disaccharide units. In the golgi apparatus, monosaccharide units are added to the side chains with corresponding glycosyltransferases. The locations of O-linked oligosaccharides are not determined by amino acid sequence as in the N-linked form but are specified by the secondary and tertiary structure of the protein (Bause & Lehle, 1979). The secondary structure results from the pattern of hydrogen bonds between the backbone amino and carboxyl group and consists most often of α helices and β sheets while the tertiary structure refers to the three-dimensional arrangement of the secondary structures (Perutz, 1976; Urry, 1968; Nobbs, Watson, *et al.*, 1966).

A single protein can have multiple N and O-linked glycosylation events. In some cases glycoproteins that lack a certain modification still appear to be functional but other times the oligosaccharide bonds affect structure, stability and activity. Since N-linked glycosylations are added co-translationally, the structures often determine the folding of the processed protein and can add stability by decreasing the protein's flexibility. O-linked glycosylations may stiffen the peptide backbone of the protein and cause extension of the overall length.

Since glycosylation occurs without a template, unlike RNA transcription or proteins translation, and the GA contains over 200 glycosylation enzymes, the production of glycosylated proteins is heterogeneous (Ungar, 2009). The heterogeneity poses an important problem if their interaction with another molecule, such as an enzyme, requires a specific interaction. In order to decrease the heterogeneity of the modified proteins, many cell types express a restricted subset of enzymes for glycosylation that are further segregated throughout the GA to form production lines (Dunphy, Fries, *et al.*, 1981; Spooncer, Fukuda, *et al.*, 1984). The cell specific expression of post-translational enzymes together with the difference in modifications between species points to the importance of cell type selection producing biologically active recombinant proteins. Of particular note, a recent report by Hashimoto, Moore and Shimaski provided evidence of N-linked glycosylation of recombinant mouse BMP15 but not human BMP15 produced in HEK-293 cells (Hashimoto, Moore, et al., 2005).

Figure 4 Sequence alignment of human, bovine and ovine BMP15 using Clustal Omega (Conway Institute, University College Dublin, Dublin, Ireland). Bovine and human sequences share 75% identity. BMP15 Cleavage site is highlighted red. Identical residues (*), conserved residues with similar properties (:) conserved residues with weakly similar properties (.)

Research Rationale

After over 40 years of research, current *in vitro* systems for mammalian embryo production produce lower quality embryos than those derived *in vivo* (Sutton, 2003; Rizos, Ward, *et al.*, 2002). Recent reports demonstrate that *in vitro* bovine oocyte maturation systems benefit from the addition of oocyte secreted factors, specifically GDF9 and BMP15 from heterologous from sources (sheep or mouse) (Hussein, Thompson, *et al.*, 2006; Gilchrist & Thompson, 2007). The difficulty of acquiring OSFs has been a hindrance for this area of research. There is currently one company that markets recombinant human BMP15 and mouse GDF9. In contrast to other members of the TGFβ family of proteins, BMP15 and GDF9 have relatively low homology across species and the differences are located in structurally significant locations (Al-Musawi, Walton, *et al.*, 2013). In fact, differences in amino acid sequence of the pro-region have been shown to affect the expression and processing of GDF9 and BMP15 (Simpson, Stanton, *et al.*, 2012; Al-Musawi, Walton, *et al.*, 2013). Due to known amino acid sequence variation (Fig. 4) and species-specific alterations in post-translational glycosylation of mature proteins, we hypothesize that utilization of species-specific oocyte secreted factors will be more beneficial than the observed effects of heterologous factors. We propose to produce purified recombinant bovine BMP15 from homologous and heterologous cell lines and test the efficiency against commercially available human BMP15. The choice to target BMP15 is based on the results of blastocyst rate after IVM with the

addition of recombinant OSFs where exogenous BMP15 increased the yield by 1.5 fold, but the addition of GDF9 alone or in combination did not increase the blastocyst rate, and therefore, developmental competence. The long-term goal of this work is to produce species-specific recombinant oocyte secreted factors capable of improving mammalian embryo production *in vitro*. Furthermore, the utilization of purified, recombinant protein will allow careful evaluation of cumulus and oocyte specific responses to these factors during a critical time of gamete and embryo development.

CHAPTER II

CONSTRUCTION OF RECOMBINANT BOVINE FLAG-BONE MORPHOGENIC PROTEIN 15

Introduction

After over 40 years of research, current *in vitro* systems for mammalian embryo production have been shown to produce lower quality embryos than those derived from *in vivo* sources (Sutton, 2003; Rizos, Ward, *et al.*, 2002). Recent reports have demonstrated that *in vitro* bovine oocyte maturation systems benefit from the addition of oocyte secreted factors, specifically GDF9 and BMP15 from heterologous from sources (sheep or mouse) (Gilchrist & Thompson, 2007; Hussein, Sutton-McDowall, *et al.*, 2011; Hussein, Thompson, *et al.*, 2006). However, known amino acid sequence variation and species-specific alterations in post-translational glycosylation of mature proteins lead us to hypothesize that utilization of species-specific oocyte secreted factors would be more beneficial than the observed effects of heterologous factors (Simpson, Stanton, *et al.*, 2012; Al-Musawi, Walton, *et al.*, 2013). Commercial availability of recombinant BMP15 was limited to human origin. In order to test the efficacy *in vitro*, the development of a system for the production and purification of recombinant bovine BMP15 was essential.

The goal of this work was to produce a species-specific recombinant oocyte secreted factor with the potential to improve mammalian embryo

production *in vitro*. We hypothesized that due to amino acid sequence divergence and species-specific post-translational glycosylation, homologous BMP15 will be more effective at directing bovine oocyte development during *in vitro* maturation than heterologous BMP15. To test this hypothesis, we first needed to devise a production and purification system for the recombinant bovine BMP15 (rbBMP15). To accomplish this objective we collected mRNA, amplified and modified the transcript via PCR site-directed mutagenesis, subcloned the sequence into an expression vector and transfected mammalian cell lines. Protein was then collected from the cell lines' supernatants and purified using affinity chromatography.

Based on work published by Li, Rajanahally, *et al.*, (2009) we made several modifications to the wild-type transcript to help ensure effective and complete production of the mature form of recombinant bovine BMP15(Li, Rajanahally, *et al.*, 2009). BMP15 is a member of the TGFβ super family of proteins and is first produced as a prepropeptide that must be enzymatically cleaved separating the proregion, containing the signal sequence, from the mature region, which contains the biologically active domain. To ensure efficient transcription of the exogenous gene, a Kozak consensus sequence was inserted 5' of the start ATG. In order to improve the detection and purification of the mature region, a detectable FLAG tag sequence (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) was inserted before the cleavage site. Further, the cleavage domain was modified for recognition by PACE/furin (Paired basic Amino acid Cleaving

Enzyme). This modification was made to ensure the efficient cleavage of the proprotein to form the mature peptide *in vitro* by a proprotein convertase endogenous to HEK-293 cells.

This modified transcript was subcloned into the PCDNA 3 (Life Technologies) mammalian expression vector and transfected via electroporation into HEK-293 (human embryonic kidney 293) and FBK (fetal bovine kidney) cell lines. Following selection with geneticin (G418), an aminoglycoside antibiotic, cell lines were cultured at confluence for 48-72 hours before the culture medium was collected and filtered for purification. rbFL-BMP15 was purified from both cell lines using affinity chromatography with aseptic technique. Concentrations of the purified rbFL-BMP15 samples were then calculated based on densitometric measurements from Western blots using an anti-FLAG antibody.

Materials and Methods

RNA Isolation

RNA was isolated from bovine ovarian tissue using an Rneasy kit (Qiagen), according to the manufacturer's instructions, after thorough homogenization in buffer RLT. Following centrifugation, 70% ethanol was added to the lysate in order to allow RNA binding to the spin column. Contamination from genomic DNA was eliminated with an on-column Dnase I digestion for 15 minutes at room temperature followed by several wash steps. The RNA was then collected with the addition of Rnase free water directly to the column and centrifugation to collect the eluate.

Reverse Transcription

Complementary DNA (cDNA) was produced by reverse transcription with SuperScript II (Life Technologies) according to the manufacturer's instructions. A 12 µl reaction was first set up containing random primers, RNA, and dNTPs which was heated to a temperature of 65°C for 5 minutes before being placed on ice. First-strand buffer (5X) and dithiothreitol (DTT) were then added and the reaction was incubated at room temperature for 2 minutes before the addition of SuperScript II enzyme. The entire 20 µl reaction was incubated for 50 minutes at 42°C followed by inactivation at 70°C for 15 minutes.

FLAG Epitope Tag PCR

A set of PCR directed mutagenesis reactions were designed to modify the wild type transcript in two segments (A&B) separated at the cleavage site. Segment A was amplified from 1 µg bovine ovarian cDNA using primers termed Kozak forward and cleavage reverse (Table 1) with AccuPrime™ *Pfx* DNA Polymerase (Life Technologies) in the following reaction conditions: 90°C for 2 minutes; 30 cycles of 90°C for 30 seconds, 50°C for 30 seconds and 68°C for 1 minute and 30 seconds; with a final extension of 68°C for 2 minutes. Segment B was amplified in similar fashion with FLAG forward and end reverse primers under the same reaction conditions. (Fig. 5)

Table 1 Primer sequences used for amplification and sequencing of rbFL-BMP15.

Name	Sequence
Kozak Forward	TAATGAATTCGCCGCCACCATGGTCCTTCTGAGCATC
Cleavage Reverse	ATTACCCGGGGCCGCCGCTCAGGCTCCGCTTGCTCCGGTGGAGAAGAGAAGGGTCTTTTTC
Flag Forward	TAATCCCGGGGGAGACTACAAGGACGATGACGACAAGCAAGCAGGCAGTATTGCATC
End Reverse	ATTAGGATCCTCACCTGCATGTGCAGGA
T7	TAATACGACTCACTATAGGG
SP6	ATTTAGGTGACACTATAG

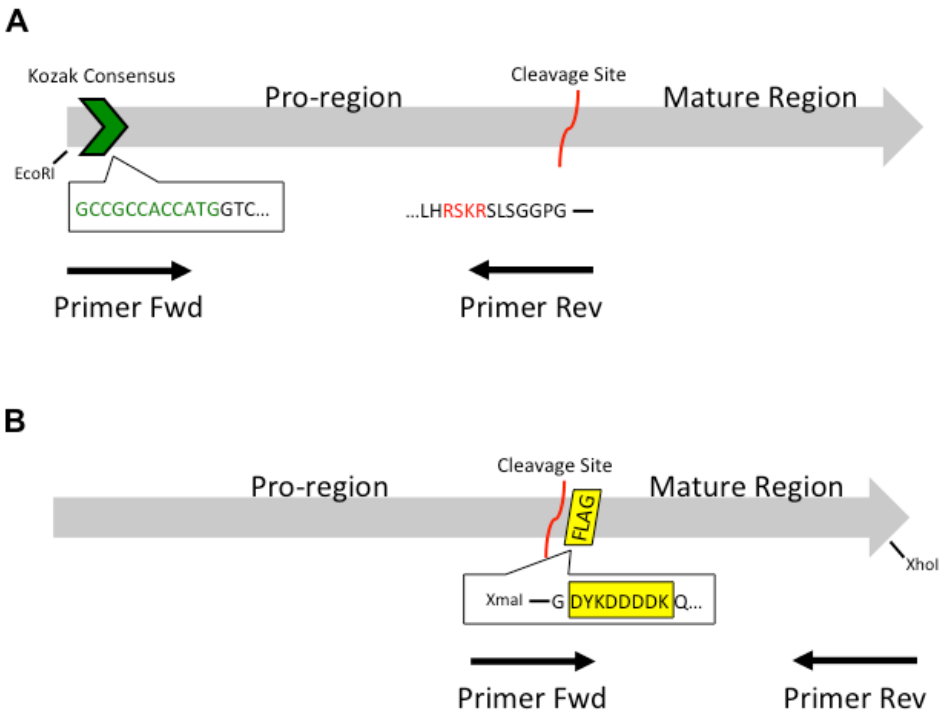


Figure 5 Diagram of rbFL-BMP15 PCR strategy. A) A set of PCR directed mutagenesis reactions were designed to modify the wild type transcript in two segments (A&B) separated at the cleavage site. Kozak forward and cleavage reverse primers (Table 1) were used for the amplification of segment A. B) FLAG forward and end reverse primers were used to amplify segment B. Segments were digested with XmaI restriction enzymes and ligated to form rbFL-BMP15 (recombinant bovine FLAG-bone morphogenic protein 15)

rbFL-BMP15 Assembly

The products from PCR reactions for segments A and B were separated by electrophoresis in a 1% agarose in TAE (Tris-acetate-EDTA) gel containing ethidium bromide. Visualized bands were excised from the gel using a razor blade on an ultraviolet light box. Qiagen's QIAquick gel extraction kit was used to collect purified PCR products from solubilized gel slices following the

manufacturer's protocol. Concentration of the eluted DNA was ascertained using a NanoVue spectrophotometer (GE Healthcare). 500 ng DNA from segments A and B was digested in 50 µl with 10 units XmaI restriction enzyme (New England Biolabs) in the presence of BSA for 1 hour at 37°C. Restriction enzymes were removed using the QIAquick PCR Purification Kit (Qiagen). Digestion products were mixed with 5 volumes of buffer PB and centrifuged at 17,900 x g for 30 seconds to bind DNA to independent columns. Following the addition of wash buffer and centrifugation, DNA was eluted with 30 µl of provided elution buffer.

Segments A and B were ligated in a 20 µl reaction with the Rapid DNA Ligation kit (Roche). 4.5 µl of each segment's eluate, the maximum volume for a 20 µl reaction, was ligated for 5 minutes at 25°C before being placed on ice. Remaining enzyme was removed using a PCR purification kit reaction (Qiagen) to produce full-length rbFL-BMP15.

Sub-cloning rbFL-BMP15 into PCDNA 3

Full-length rbFL-BMP15 and PCDNA 3 (Life Technologies) were digested with EcoRI and XhoI restriction enzymes (NEB) in EcoRI buffer with BSA (bovine serum albumin) prior to ligation of the segments to produce the vector PCDNA rbFL-BMP15.

The full-length rbFL-BM15 ligation product, 260 ng, was digested with 10 units each of EcoRI and XhoI for 1 hour at 37°C and heat inactivated at 65°C for 20 minutes before being cooled to 4°C. Approximately 1 µg of PCDNA 3 was restriction digested with 15 units each of EcoRI and XhoI under the same

conditions. Both products were mixed with loading dye and separated by electrophoresis in a 1% agarose in TAE gel containing ethidium bromide. The 1.3 kb rbFL-BMP15 and 5.4 kb PCDNA 3 bands were excised with a razor blade and extracted with a QIAquick gel extraction kit and column according to the manufacturer's instructions.

A ligation reaction was set up, using the Rapid DNA Ligation kit (Roche), for PCDNA rbFL-BMP15 in a 3:1 insert to vector molar ratio as follows: 2 µl PCDNA, 7 µl rbFL-BMP15 or water, 10 µl 2x buffer, 1 µl ligase. A control ligation to measure background was conducted by replacing the rbFL-BMP15 insert with 7 µl water. Following a 5 minute incubation at 25°C, the product was placed on ice and introduced into 5-alpha competent E. coli (High Efficiency) from New England BioLabs using a standard transformation protocol.

A vial of 5-alpha competent E. coli were removed from -80°C and thawed on ice during the ligation reactions. 25 µl of E. coli was added directly to each 20 µl ligation reaction mix and stored on ice for 30 minutes. The mixture was placed in a 42°C water bath for 1 minute and returned to the ice for 5 minutes before 950 µl 2XYT was added and incubated at 37°C for 45 minutes on a shaking platform at 250 RPM. 500 µl of each broth was seeded onto a 10 cm petri dish containing 2XYT with 1.5% agarose and 50 µg/ml ampicillin and incubated overnight at 37°C.

Briefly, colonies were selected using a sterile 200 µl pipet tip, placed in a 0.2 ml PCR tube containing 10 µl water and agitated before being removed. The

tubes were heated at 95°C for 10 minutes. A master mix containing 0.5 μM T7 and SP6 sequencing primers, 0.2 μM dNTP mixture, 1x PCR buffer and taq DNA polymerase (Life Technologies) was added to a final volume of 20 μl. A PCR reaction to amplify the target region was run for 35 cycles using the following parameters: 95°C for 2 minutes; 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 2 minutes, and final extension at 72°C for 7 minutes. The PCR product was mixed with loading dye and separated in a 1% agarose in TAE gel to determine positive colonies. The colonies that were positive for the ~1.3 kb rbFL-BMP15 band were plucked from the agar plate and placed in 3 ml 2XYT broth containing 50 μg/ml ampicillin. The broth was grown overnight at 37°C on a rotating platform at 300 RPM. Prior to DNA collection with Qiaprep miniprep kit (Qiagen) a glycerol stock of the colony was prepared by mixing a small amount of the broth with 20% glycerol and storing the solution at -80°C.

Bacteria were harvested by centrifugation at 6,800 xg for 3 minutes at 25°C and lysed with alkaline buffer included with the Qiaprep miniprep kit. DNA from the lysate was then bound to a QIAprep spin column by centrifugation. Endonucleases and salts were removed by washing with buffer PB before DNA was collected with elution buffer heated to 60°C.

Sequencing

The sequence of PCDNA rbFL-BMP15 was verified from 5’ and 3’ directions using T7 and SP6 primers respectively. Plasmid DNA collected from

the minipreps was used as input for sequencing reactions as follows: 2 µl BigDye Terminator Ready Reaction Mix (Applied Biosystems), 1.7 µM primer T7 or SP6, 300ng DNA, water to a final volume of 6 µl. Samples were placed in a thermal cycler and a program was run with the following conditions: 96°C for 2 minutes; 30 cycles of 96°C for 15 seconds, 50°C for 15 seconds and 60°C for 4 minutes, and a final step of 60°C for 5 minutes. Excess terminator dye and dNTPs were removed by centrifuging the sequencing product through a sephadex spin column hydrated with water (BioMax).

Samples were sequenced by the DNA Technologies Core Lab (Texas A&M University) with 3130*xl* Genetic Analyzer (Applied Biosystems) and analyzed with Sequencher DNA Sequence Analysis software (Gene Codes Corporation). Overlapping sequences originating from T7 and Sp6 sites were analyzed and aligned with wild-type bovine BMP15 (NM_001031752.1).

Transfection of HEK-293 and Fetal Bovine Kidney Cell Lines

Human embryonic kidney 293 (HEK-293) and primary fetal bovine kidney (FBK) cells were cultured in T75 tissue culture flasks (BD Falcon) to 85% confluence at 37°C and 5% CO_2 in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) with 10% fetal bovine serum (FBS) and 1X antibiotic-antimycotic (Life Technologies). Cells were transfected using the Neon transfection system (Life Technologies) following the manufacturers guideline for preparation of adherent cells. HEK-293 or FBK cells (9*10^6) were suspended in 600 µl provided resuspension buffer. For each cell line, 300 µl of cell suspension

was added to two 1.5 ml microcentrifuge tubes and 7.5 µg of PCDNA3 rbFL-BMP15 or FL-GFP (Dr. Mike Golding) plasmid DNA was added. Cells were electroporated with one pulse of 1650 V for 20 ms and deposited into a 6-well plate (BD Falcon) containing equilibrated DMEM/F12 culture medium with 10% FBS and no antibiotics. At 24 hours post-transfection, the media was changed to DMEM/F12 with 10% FBS and 1X ABAM (DMEM/F12 complete). FL-GFP transfected cells were visualized for fluorescence and estimates of transfection efficiency were recorded at >50%.

Cell Selection and Protein Expression

At 90% confluence, cells transfected with rbFL-BMP15 were washed with 2 ml PBS, incubated with 1 ml 1X trypsin (Gibco) for 5 minutes at 37°C and suspended in DMEM/F12 complete to a volume of 10 ml and moved into a T75 tissue culture flask for expansion and selection. Following cell adherence, 0.3 mg/ml geneticin (Gibco) was added for selection. At 90% confluency, media was aspirated from flasks, cells were washed with 10 ml PBS and incubated with 4 ml 1X trypsin for 5 minutes at 37°C. Cells were suspended with the addition of 8 ml DMEM/F12 complete. 4 ml of cell suspension was added to a T75 tissue culture flask containing 6 ml DMEM/F12 complete with 0.3 mg/ml geneticin. FBK rbFL-BMP15 and HEK rbFL-BMP15 cell lines were passaged under selection once more before protein collection began.

FBK rbFL-BMP15 and HEK rbFL-BMP15 cell lines were allowed to reach confluence in DMEM/F12 complete with 0.3 mg/ml geneticin before the media

was changed to Opti-MEM reduced serum media (Life Technologies) containing 2% FBS and 1X ABAM for 2-3 days of production. Media was removed with a serological pipette and filtered with 0.22 µm syringe filter (Tiffen) and Air-Tite syringe before addition of HALT protease inhibitor cocktail (Pierce).

Purification of rbFL-BMP15

Anti-FLAG M2 affinity gel (Sigma) was prepared according to the manufacturers' instructions for FLAG Fusion Protein Immunoprecipitation. 100 µl anti-FLAG M2 affinity gel suspension was washed with the addition of 500 µl TBS (Tris-buffered saline) in a 1.5 ml microcentrifuge tube. The affinity gel was centrifuged for 30 seconds at 8,000 x g and the supernatant removed by pipetting. The pellet was suspended in 500 µl TBS before centrifugation and another wash with TBS. The resin was suspended in 500 µl TBS and incubated overnight on a tube rotator with 10 ml cell-conditioned and filtered Opti-MEM media containing protease inhibitor. The affinity resin was pelleted by centrifugation at 4,000 x g for 45 seconds and moved to a clean 1.5 ml microcentrifuge tube. The resin pellet was suspended in 500 µl TBS and centrifuged at 8,000 x g for 30 seconds three times. rbFL-BMP15 was eluted by incubation with 100 µl TBS 1% BSA + 200 ng/µl 3X FLAG peptide (Sigma) for 45 minutes at room. The resin was pelleted by centrifugation at 8,000 x g for 30 seconds and the supernatant containing rbFL-BMP15 was removed and stored at -80°C.

Anti-FLAG Western blot

Concentration of the purified rbFL-BMP15 was calculated using densitometric measurements in comparison to a standard curve of amino terminal FLAG-E. coli bacterial alkaline phosphatase (BAP) standards (Sigma). 15 µl purified rbFL-BMP15 was mixed with 15 µl 2X Laemmli buffer (Bio-Rad) containing 2M Focus DTT (G Biosciences) and heated for 5 minutes at 95°C. FLAG-BAP standards of 200, 100, 50 and 25 ng were diluted in a total volume of 10 µl TBS and mixed with an equal volume of 2X Laemmli buffer containing 2M DTT and heated for 5 minutes at 95°C. The complete sample and 20 µl of each standard was loaded into 4-20% or 15% Mini-PROTEAN TGX precast polyacrylamide gels (Bio-Rad) in chilled Tris/glycine/SDS buffer (25 mM Tris, 192 mM glycine, 0.1% sodium dodecyl sulfate). Electrophoresis was conducted at 110 V for 45 minutes until the dye front was within 2 cm of the bottom of the gel cassette. Proteins were transferred to Polyvinylidene fluoride (PVDF) membrane (Amresco), pre-soaked in methanol, at 100 V for 48 minutes in ice cold Towbin's transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol).

The membrane was blocked at 37°C for 1 hour with TBS + 0.1% Tween20 + 5% non-fat dry milk and 20 µl/ml goat serum on a rocking platform. Monoclonal mouse anti-FLAG M2 antibody (Sigma) was diluted in fresh blocking buffer at 1:1000 and incubated with the membrane overnight at 4°C on a rocking platform. Excess wash buffer (TBS + 0.1% Tween20) was added to the membrane and incubated for 15 minutes at 25°C. The wash step was completed

3 times. Goat anti-Mouse IgG HRP (Jackson ImmunoResearch Laboratories) was diluted 1:20,000 in TBS and incubated with the membrane for 1 hour at 25°C. Three 15 minute washes with excess wash buffer were completed at 25°C after the secondary antibody dilution was decanted. 2 ml of pre-mixed ChemiGlow substrate (Protein Simple) was added to the blot and allowed to incubate for 3 minutes before being imaged on the FlourChem E (Protein Simple) camera dock.

Image analysis was conducted using AlphaView Stand Alone software (Protein Simple) with lane profile densitometry. A standard curve was created using the measurements from the FL-BAP samples and the unknown concentrations were fit to the curve to determine the amount of rbFL-BMP15 present at 17 kDa.

Results

The Matzuk laboratory at Baylor College of medicine reported success with modification and production of recombinant human BMP15 (Li et al. 2009). For efficient translation, a Kozak sequence was added to the beginning of the transcript (Kozak, 1987; Kozak, 1986). The cleavage site was modified to match the von Willebrand factor (vWF) cleavage site, which is efficiently processed by PACE/furin. HEK-293 cells highly express the proprotein convertase PACE/furin making it a logical selection. A FLAG epitope sequence was added 3’ of the cleavage site and 5’ to the mature region of the sequence for purification and identification by Western blotting. We produced, with these modifications, an

expression vector capable of being processed efficiently *in vitro*, secreted into the supernatant and containing a FLAG sequence at the N-terminus of the mature peptide. Once cell lines were established, the supernatant could be collected from cultured cells and purified using FLAG affinity gel for downstream testing.

A PCR strategy was designed based on the modifications of the transcript at the N-terminus end, cleavage site and C-terminus. Two segments, named A and B, were amplified in separate reactions from bovine ovarian cDNA. We designed primers based on the wild type sequence of bovine BMP15 with modification to the cleavage site and the addition of specific restriction enzyme sites as well as a Kozak consensus sequence. Following amplification of the fragments, products were ligated after a restriction digest with Xma1 to form full-length rbFL-BMP15. (Fig. 6)

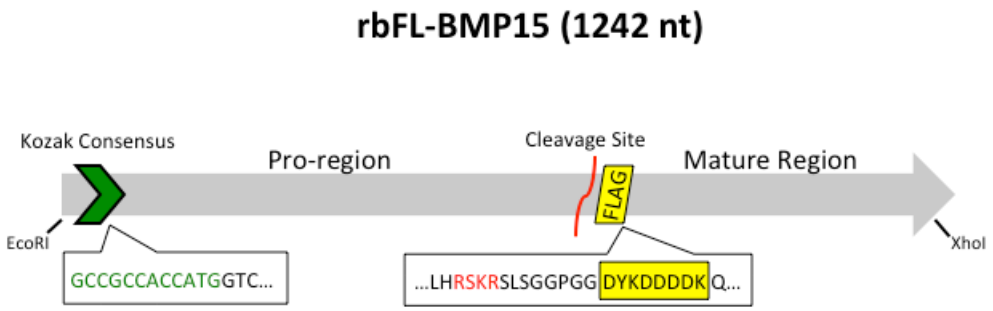


Figure 6 Representation of rbFL-BMP15 as modified from the wild type gene. A Kozak consensus sequence was added for efficient production *in vitro*. The native cleavage site was modified to match the vWF site cleaved by PACE/furin which is abundantly expressed by HEK-293 cells. A FLAG epitope tag was added downstream of the cleavage site for purification by FLAG affinity gel and detection by anti-FLAG Western blotting.

For expression in mammalian cell lines, rbFL-BMP15 was inserted into the multiple cloning site of PCDNA 3 designed with the human cytomegalovirus immediate-early (CMV) promoter for high expression in mammalian cells. Sequence analysis revealed the rbFL-BMP15 clone 1 had the correct and in-frame sequence and was used for all downstream applications. HEK-293 and FBK cell lines were transfected by electroporation using the Neon system. Cell lines were exposed to geneticin during culture to select for cells that expressed neomycin resistance afforded by the PDCNA rbFL-BMP15 plasmid. (Fig. 7)

Conditioned medium from the transfected cell lines was incubated with FLAG affinity gel to collect the FL-BMP15 protein and eluted by competitive binding with a 3X FLAG peptide. The concentration of the purified product was obtained by densitometric analysis of a chemiluminescent Western blot as

compared to a standard curve of FLAG-BAP for each collection. (Fig. 9) The final concentration of purified rbFL-BMP15 (final volume of 100 µl per collection) varied by collection and cell type. Eluate from HEK-293 rbFL-BMP15 cells was consistently higher in concentration (10-15 ng/µl) but interestingly produced 2 distinct immunoreactive bands. rbFL-BMP15 collected from the FBK cell line averaged 5-10 ng/µl and only produced the higher of the bands from the HEK cell line. (Fig. 8)

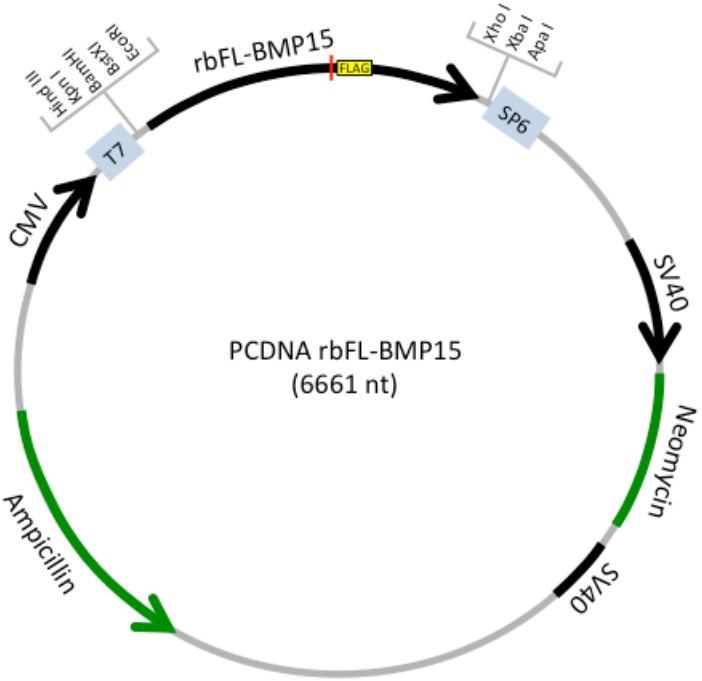


Figure 7 PCDNA rbFL-BMP15 vector map. rbFL-BMP15 was inserted at the multiple cloning site and downstream of the human CMV promoter of PCDNA 3 mammalian expression vector. Locations of T7 and SP6 sites used for sequencing are shown. SV40 promoter drives neomycin resistance upstream of the SV40 origin of replication for selection of eukaryotic cells containing the plasmid with geneticin (G418)

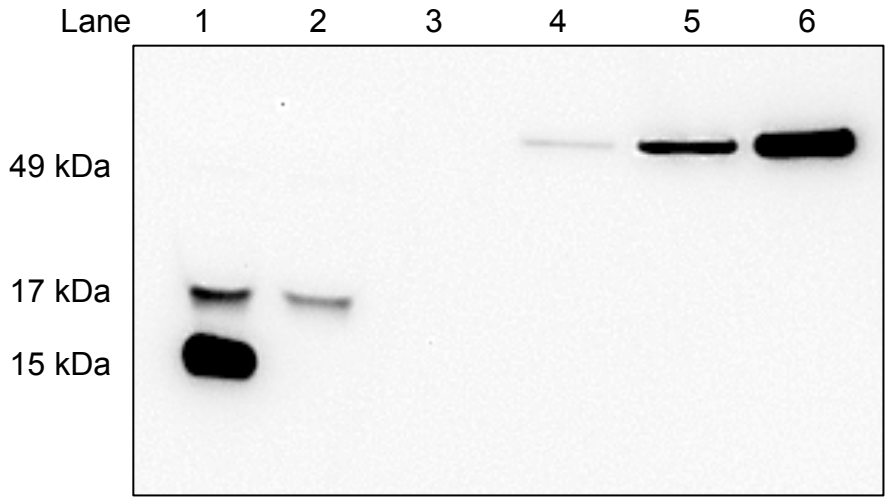


Figure 8 Purified rbFL-BMP15 from HEK-293 and MDBK cell lines. Anti-FLAG Western blot with immunoreactive bands for rbFL-BMP15 from HEK-293 (Lane 1) and FBK (Lane 2) transfected cell lines. Conditioned media was purified using FLAG-affinity gel and eluted with 3X-FLAG peptide in TBS + 1% BSA. FLAG-BAP standards of 50, 100 and 200 ng (Lanes 4-6) were used to create a standard curve for calculation rbFL-BMP15 concentration.

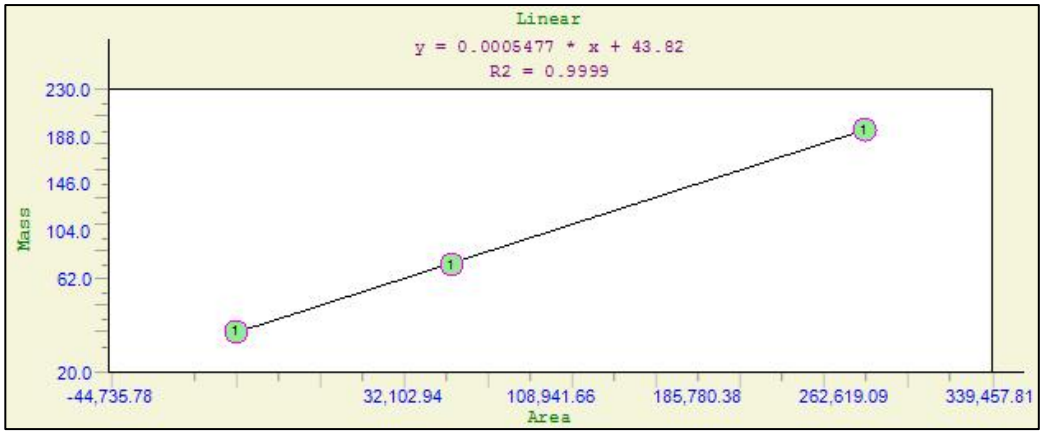


Figure 9 FLAG-BAP Standard curve used to calculate rbFL-BMP15 concentration. Standard curve was produced based on chemiluminescent signal from 50, 100 and 200 ng FLAG-BAP standards.

Discussion

Development of a reliable method for production of a large quantity of highly purified recombinant protein was an essential step in testing our hypothesis. Several methods of protein purification are available including size exclusion chromatography and immunoprecipitation. Due to the high specificity of immunoprecipitation, we chose to produce recombinant bovine BMP15 with a FLAG tag. The FLAG purification system from Sigma has the advantage of elution without denaturing conditions with a 3X FLAG peptide based on the principle of competitive binding.

BMP15, like other members of the TGFβ family, is first produced as a prepropeptide then enzymatically cleaved to resulting in a pro-region and a mature functional protein. Based on this process, there are only two options for the placement of the affinity tag on the final product. The tag could be placed at the C-terminus of the mature region of the sequence, but TGFβ proteins have a conserved cysteine knot structure at the C-terminal end. The addition of a hydrophilic tag could have disrupted this structure and prevented proper dimerization. The other option for a tag is placement 3' of the cleavage site so the tag will be located on the N-terminal end of the mature peptide after processing. Based on previous reports, we chose placement at 3' of the cleavage site (Li, et al. 2009). In addition to purification, protein identity should be verified by Western blotting. This process requires an antibody specific to the protein of interest. There are currently no commercially available antibodies for

bovine BMP15. Monoclonal FLAG antibodies are, however, commercially available and exhibit high specificity. For these reasons combined with the need to purify the mature recombinant protein, we chose to use the FLAG system.

The Matzuk laboratory at Baylor College of medicine reported success with modification and production of recombinant human BMP15 (Li et al. 2009). For efficient translation, a Kozak sequence was added to the beginning of the transcript (Kozak, 1986). The cleavage site was modified to match the von Wildebrand factor (vWF), which is efficiently processed by PACE/furin, based on the efficiency of cleavage *in vitro*. Finally, a FLAG sequence was added 3' of the cleavage site and 5' to the mature region of the sequence. In this manner, we produced a protein transcript capable of being processed *in vitro*, secreted into the extracellular supernatant and containing a FLAG tag at the N-terminus of the mature peptide. Once cell lines were established, the supernatant could be collected from cultured cells and purified with FLAG affinity gel for downstream testing.

In order to produce a stable cell line, we first attempted transduction with a retroviral construct. The rbFL-BMP15 transcript was inserted into the multiple cloning site of the mammalian retroviral expression vector MSCV-PIG (Puro IRES GFP) and combined with VSVG (Vesicular Stomatitis Virus glycoprotein envelope) and PSI retroviral packaging element plasmids for viral production. Collection of recombinant protein from these cell lines proved very difficult despite the presence of GFP labeled cells, which indicated successful

transduction. Analysis of genomic DNA and mRNA from the transduced cell line revealed the presence of the rbFL-BMP15 transgene in the genomic DNA but not in mRNA despite the presence of GFP mRNA, suggesting that rbFL-BMP15 was not being transcribed. The lack of gene transcription could have been due to low expression of the phosphoglycerate kinase promoter utilized in the MSCV-PIG vector by the cell lines. Following several attempts at protein collection, a new strategy was devised using transfection and selection.

Based on previous results, rbFL-BMP15 was then cloned into PCDNA 3, a vector designed for high expression in mammalian hosts with the human cytomegalovirus immediate-early (CMV) promoter. Given the difficulty of transfecting primary cell lines, we chose to use the Neon transfection system from Life Technologies. The FL-GFP control transfected cells were estimated at 50-60% GFP positive 48 hours after electroporation. Following 2-3 days of culture, media was removed from PCDNA rbFL-BMP15 cells lines and tested. This process allowed us to determine the concentration of each collection of recombinant protein for accurate addition to in vitro maturation media. We were able to repeatedly collect protein at a eluate concentration of 5-10 ng/µl, which allowed us to easily add rbFL-BMP15 at a final concentration of 100 ng/ml for in vitro maturation testing. It was noted, however, that the total amount of recombinant protein recovered from the bovine FBK cell line was uniformly lower than that collected from the human HEK-293 cell line. We postulate that this

difference is attributable to the difference in activity of the human CMV promoter driving the transcription of rbFL-BMP15 in the PCDNA 3 vector backbone.

We have developed here two stable cell lines, human and bovine, producing recombinant bovine FLAG tagged bone morphogenic protein 15, allowing us to compare the protein production from the cell lines without the variable of individual transfection efficiency and characterize the products from each cell line. In order to understand the differential production of protein from an identical vector by human and bovine cell lines, recombinant proteins were next characterized and analyzed for bioactivity.

CHAPTER III

CHARACTERIZATION AND FUNCTIONAL ANALYSIS OF RECOMBINANT BOVINE FLAG-BONE MORPHOGENIC PROTEIN 15

Introduction

The objective of the following experiments was to design and test several *in vitro* assays in order to characterize rbFL-BMP15 produced by HEK-293 and FBK cell lines and test bioactivity based on the observed differences in molecular weight of the protein produced by each cell line. These experiments were essential to understanding the differential processing of an identical expression vector by human and bovine cell lines. Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) analysis was utilized to collect accurate molecular mass and charge of rbFL-BMP15 from both cell lines. The human HEK-293 products were of great interest because unlike the bovine FBK cell line, HEK-293 cells produced 2 distinct species of protein by Western blot analysis. BMP15 signals through a BMP receptor type II and (ALK6) TGFBR1 (transforming growth factor, beta receptor 1) heteromeric complex, transducing the signal the interior of the cell by phosphorylation of the receptor SMAD 1/5/8 (mothers against decapentaplegic homolog 1/5/8**)**, a transcription factor (Anderson & Darshan, 2008). We tested the ability of the recombinant proteins to bind to the receptor complex and successfully induce the phosphorylation of SMAD 1/5/8 by immunoreactivity to an antibody for SMAD 1/5 phosphorylated at

serine residues 463 and 465. Receptor binding specificity was determined using dorsomorphin, a selective inhibitor of BMP signaling. Dorsomorphin is an ATP-competitive inhibitor of AMPK (AMP-activated protein kinase) that blocks that action of the tyrosine kinase receptor to phosphorylate receptor SMADs. Primers were then designed for genes known to be upregulated by the action of BMP15: HAS2 (hyaluronan synthase 2), PTX3 (pentraxin 3), TSG6 (tumor necrosis factor, alpha-induced protein 6) and PTGS2 (prostaglandin-endoperoxide synthase 2). Finally, quantitative real time PCR analysis was conducted on groups of cumulus oocyte complexes following *in vitro* maturation with medium containing rhBMP15, homologous (FBK) rbFL-BMP15 or heterologous (HEK-293) rbFL-BMP15.

Materials and Methods

MALDI-TOF (Matrix-assisted laser desorption/ionization-time of flight)

Conditioned medium from HEK-293 and FBK rbFL-BMP15 cell lines was purified using anti-FLAG M2 affinity gel (Sigma) as described previously without the addition of BSA to the elution buffer. 100 µl of purified sample in TBS from each cell line was SpeedVac (Thermo Scientific) concentrated to approximately 10 µl. Acidification of the samples was conducted by the addition of 1/10 volume of 1% trifluoroacetic acid (TFA) (0.1% final concentration). Samples were solid phase extracted with a C4 ZipTip (Millipore) and spotted with alpha-cyano-4-hydroxycinnamic acid as a matrix (6 mg/ml in 50% acetonitrile, 0.1% TFA). Mass analysis was performed using a Shimadzu/Kratos Axima CFR MALDI-TOF mass

spectrometer in linear mode with accelerating voltage of 20,000, pulse extraction voltage of 2,000 and linear detector voltage of 2,950. Spectra were collected with a minimum of fifty laser shots with a mass accuracy at ~0.1%. The analysis was externally calibrated using Cytochrome C.

Phosphorylated-SMAD 1/5 Western Blot

HeLa cells were cultured in 6 well plates (BD Falcon) at 37°C and 5% CO_2 in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) with 10% fetal bovine serum (FBS), 50 units/ml penicillin and 50 µg/ml streptomycin (Life Technologies). Media was changed to OPTIMEM with 2% FBS, 50 units/ml penicillin and 50 µg/ml streptomycin at 80% confluence for 3 hours prior to treatment. Media was removed and wells were washed with PBS before the addition of 100 ng/ml rhBMP15 (recombinant human BMP15, R&D Systems), HEK-293 rbFL-BMP15 conditioned medium or HEK-293 wild type conditioned medium. Conditioned media was collected from confluent cells cultured for 2 or 3 days with OPTIMEM with 2% FBS, 50 units/ml penicillin and 50 µg/ml streptomycin. Media was centrifuged at 20,000 x g for 10 minutes at 4ºC to pellet cellular debris and stored in 1 ml aliquots at -80ºC.

Following 1 hour of incubation at 37ºC and 5% CO_2 with conditioned medium containing rbFL-BMP15, HeLa cells were washed twice with PBS collected for nuclear extraction. 1 ml of buffer A (10 mM HEPES, pH 7.5; 10 mM KCl; 0.1 mM EDTA) was prepared by the addition of 25 µl 10% IGEPAL CA-630 (Sigma-Aldrich), 1 µl 2M Focus DTT (G Biosciences) and 10 µl 100X HALT

protease/phosphatase inhibitor (Thermo Scientific). 200 µl was added to each well of treated HeLa cells and the plate was rocked at room temperature for 10 minutes. Cells were scraped with a pipet tip bent at a 90º angle, transferred to a 1.5 ml microcentrifuge tube and centrifuged at 20,000 x g for 3 minutes at 4ºC. Tubes were immediately placed on ice and the cellular lysate removed in the supernatant. 0.5 ml buffer B (20 mM HEPES, 0.4 M NaCl, 1 mM EDTA, 10% glycerol) was prepared by the addition of 5 µl 100X HALT protease/phosphatase and 0.5 µl 2M Focus DTT. 30 µl of prepared buffer B was added to each pellet and shaken at 1,000 RPM in an Eppendorf thermomixer at 4ºC for 2 hours. The lysates were centrifuged for 5 minutes at 20,000 x g for 5 minutes and the supernatant collected as nuclear lysate.

Protein content of the nuclear extracts was determined by a Bradford assay. 5 standards were prepared within the range of 100 to 1000 µg/ml BSA and mixed with 1X Bradford Reagent (BioRad) before measurement of absorbance at 595 nm with a GE NanoVue spectrophotometer. 2.5 µl of each nuclear extract was mixed with 1X Bradford reagent, absorbance was measured and the concentration was determined by its placement on the standard curve.

Nuclear extract from HeLa cells (2 µg) treated with 100 ng/ml rhBMP15, conditioned medium containing rbFL-BMP15 or control conditioned medium was mixed with an equal volume 2X Laemmli buffer (Bio-Rad) containing 2M DTT (focus) and heated for 5 minutes at 95°C. The complete sample was loaded into a 10% Mini-PROTEAN TGX precast polyacrylamide gels (Bio-Rad) in chilled

Tris/glycine/SDS buffer (25 mM Tris, 192 mM glycine, 0.1% sodium dodecyl sulfate). Electrophoresis was conducted at 200 V for 40 minutes until the dye front was within 2 cm of the bottom of the gel cassette. Proteins were transferred to PVDF (Polyvinylidene fluoride) membrane (Amresco), pre-soaked in methanol, at 100 V for 60 minutes in ice cold Towbin's transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol).

The membrane was blocked at 25°C for 1 hour with TBS + 0.1% Tween20 + 5% non-fat dry milk and 20 µl/ml goat serum on a rocking platform. Monoclonal rabbit Phospho-Smad1/5 (Ser463/465) antibody (Cell Signaling Technology) was diluted in TBS, 0.1% Tween20, 5% w/v BSA at 1:1,000 and incubated with the membrane overnight at 4°C on a rocking platform. Excess wash buffer (TBS + 0.1% Tween20) was added to the membrane and incubated for 15 minutes at 25°C. The wash step was completed 3 times. Mouse anti-Rabbit IgG HRP (Jackson ImmunoResearch Laboratories) was diluted 1:5,000 in TBS and incubated with the membrane for 1 hour at 25°C. Three 15 minute washes with excess wash buffer were completed at 25°C after the secondary antibody dilution was decanted. 2 ml of pre-mixed SuperSignal West Pico chemiluminescent substrate (Thermo Scientific) was added to the blot and allowed to incubate for 3 minutes before being imaged on the AlphaFlour (Alpha Innotech) camera dock.

BMPRI (Bone Morphogenic Protein Receptor I) Inhibition

HeLa cells were cultured in 6-well plates (BD Falcon) with DMEM F/12 complete in 5% CO_2 at 37ºC to 80% confluence. Dorsomorphin (Sigma Aldrich) is a selective BMP inhibitor and acts as an ATP-competitive inhibitor of AMPK. Dorsomorphin was added to HeLa cells for 1 hour at a final concentration of 4 µM. Cells were left untreated, or 100 ng/ml rhBMP15, rbFL-BMP15 from HEK-293 or FBK cell lines was added for 1 hour to cells treated with dorsomorphin or untreated. Nuclear lysates were collected for phosphorylated SMAD 1/5 Western blotting as previously described.

In Vitro Maturation

In vitro maturation medium was prepared by adding 10% FBS, 50 units/ml penicillin and 50 µg/ml streptomycin to M199 Earl's salts buffered with sodium bicarbonate (Gibco). rhBMP15, rbFL-BMP15 from HEK-293 or FBK cell lines was added at a final concentration of 100 ng/ml to the medium before overnight shipment to TransOva (Long Prarie, MN) at 4°C. Control medium was sent without addition of recombinant protein. Upon arrival, the medium was equilibrated to 5% CO_2 at 37ºC. bFSH and bLH (bovine follicle stimulating hormone, bovine luteinizing hormone) was added by TransOva according to protocol. Cumulus-oocyte complexes were aspirated from abattoir derived ovaries and shipped overnight to the Reproductive Sciences Complex with a portable incubator containing tubes with approximately 1.5 ml of each media and 50 oocytes/tube (treatment). Tubes were uncapped and placed in a 5% CO_2 and

37.5ºC incubator when the shipment arrived to ensure proper buffering until a maturation time of 24 hours was reached. Three separate rounds of *in vitro* maturation were completed as individual biological replicates.

RNA Collections

Groups of approximately 50 cumulus oocyte complexes were lysed in 350 µl buffer RLT (Rneasy kit (Qiagen)) and vortexed for 2 minutes to homogenize the sample. The lysate was loaded onto a QIAshredder spin column (Qiagen) and centrifuged at 20,000 x g for 2 minutes. The flow-through was collected, 350 µl of 70% ethanol was added and the sample mixed by pipetting and loaded onto a Qiagen Rneasy spin column. RNA was bound to the column by centrifugation at 8,000 x g for 15 seconds. The column was washed with 350 µl RW1 before on-column Dnase treatment. 10 µl Dnase I (Qiagen) was mixed with 70 µl buffer RDD for each reaction. 80 µl was added to the membrane of each column and incubated for 15 minutes at room temperature. 350 µl buffer RW1 was added to the column and centrifuged at 8,000 x g for 15 seconds. Two wash steps with 500 µl buffer RPE were completed before 30 µl Rnase-free water was added directly to the membrane for elution at 8,000 x g for 1 minute.

Reverse Transcription

RNA, 100 ng, as measured by a GE NanoVue spectrophotometer, was used as the input for a reverse transcription using the Q-Script cDNA supermix (Quata Biosciences). 4 µl Q-Script cDNA supermix (5X) was mixed with 100 ng RNA and water to a total volume of 20 µl and incubated under the following

reaction conditions: 25ºC for 5 minutes, 42ºC for 30 minutes, 85ºC for 5 minutes and 4ºC hold. No reverse transcription (no-RT) control reactions were set up with the 5X supermix replaced with water and incubated using the same program.

Primer Design

Primers were designed and tested for HAS2, PTX3, TSG6 and PTGS2 using published sequences from NCBI with assistance from Primer3 Plus (http://www.bioinformatics.nl) and NetPrimer (Premier Biosoft). An optimum primer was described as 18-22 nt in length, T_m of 60ºC, a minimum of secondary structure or dimerization, GC content of 50%, and a product of 100-200 bp spanning an intron boundary. Oligonucleotides were synthesized by Life Technologies and reconstituted from lyophilized pellets at 100 nM with molecular grade water. Working stocks of primers were prepared by dilution 1:10 to a 10 nM concentration.

Primer Testing

RNA was collected from cumulus cells post-fertilization and reverse transcribed with previously described methods. Standard curves of cDNA dilutions were prepared for testing the linear response of primer pairs at 1:10 (2 ng cDNA/reaction), 1:50 (0.4 ng), 1:250 (80 pg), 1:1250 (16 pg), and 1:6250 (3.2 pg). Reactions were set up in triplicate as follows: 6 µl DyNAmo Flash SYBR green mastermix (Finnzyme), 0.25 µl 50X ROX reference dye, 0.2 µl (0.2 nM final concentration each) primers, 2 µl H2O, and 4 µl cumulus cell cDNA.

Samples were run on a StepOnePlus Real-Time PCR machine (Applied Biosystems) under the following reaction conditions: 95ºC for 5 minutes; 40 cycles of 95ºC for 30 seconds, 58ºC for seconds and 72ºC for 1 minute; followed by a melt curve analysis. Standard curves were generated and R^2 values calculated by StepOne software (Applied Biosytems).

Quantitative Real-Time PCR

cDNA and no-RT reactions from treated cumulus oocyte complexes were diluted at 1:50 for a final concentration of 0.4 ng cDNA per qPCR reaction. Reactions were set up as described for primer testing with no-RT sample, water or triplicates of cDNA from each treatment. Samples were run on a CFX384 Touch Real-Time machine (BioRad). Expression of target genes was normalized to the mean of GAPDH and YWHAZ Cq using the $\Delta\Delta C^T$ method with CFX software (BioRad). At least two technical replicates were completed for each of the three biological replicates.

Statistical Analysis

Statistical analysis of gene expression was performed with JMP 9 (SAS Institute). Expression, as calculated by the CFX manager software with GAPDH and YWHAZ as reference genes and normalized to the untreated control for each replicate, was tested for statistically significant differences in treatment and within the gene by ANOVA (analysis of variance). If $p<0.05$ for the ANOVA test, a Wilcoxon/Kruskal Wallis nonparametric test was used to verify rejection of the null hypothesis ($H_0= \mu_1=\mu_2=\mu_3=\mu_4$). Control expression values were excluded for

a Brown Forsythe test for unequal variances with $p>0.05$. The assumption of normality was checked by plotting the distribution of residual values, testing for Goodness of Fit with $p>0.05$ and checking for a linear pattern on the Normal Q/Q plot. Within each gene, every pair of expression values was compared with a Tukey/Kramer HSD test and a Dunnett's test with control was used to assess a statistical difference between a treatment and the control with a $p<0.05$ threshold for significance.

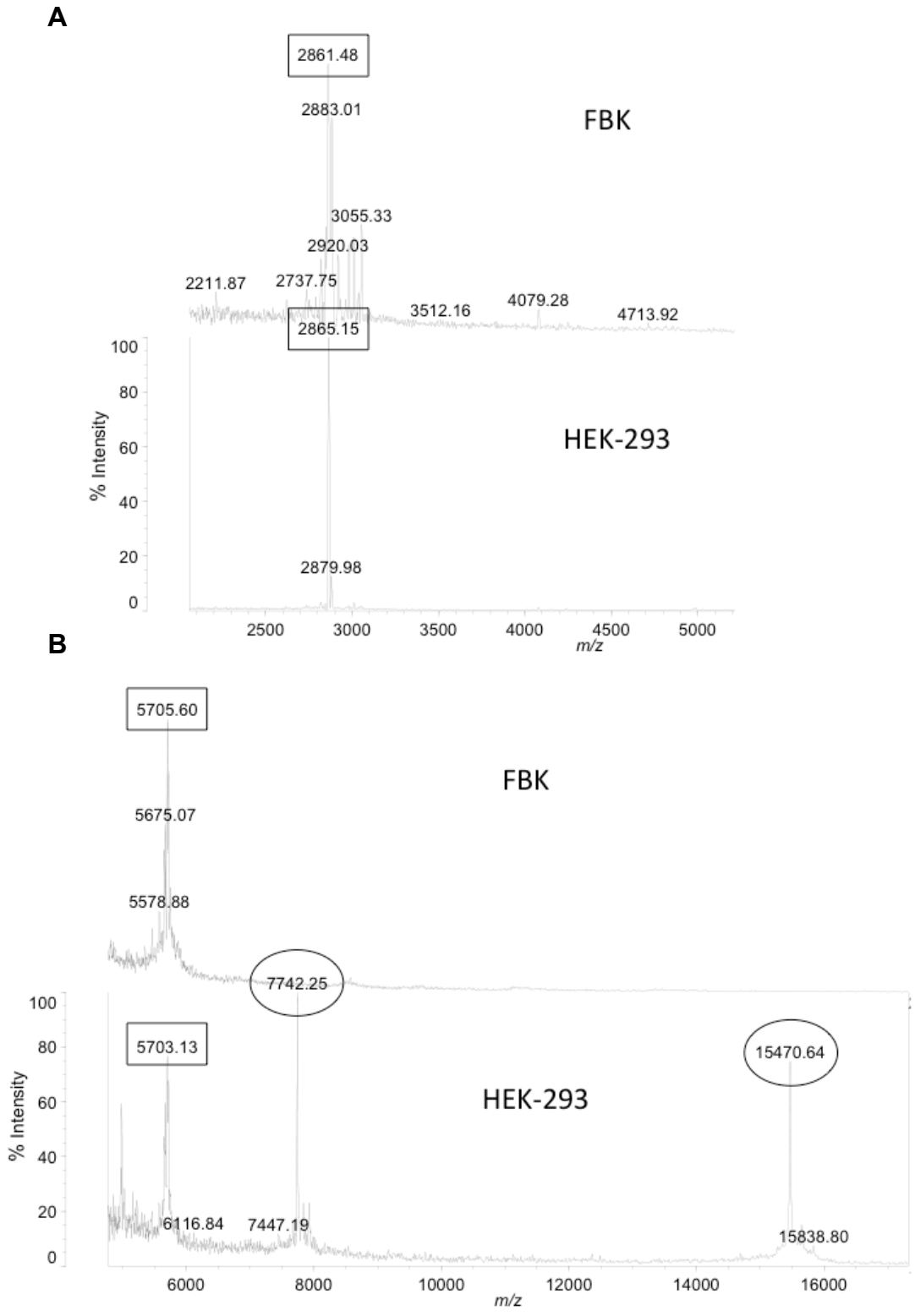
Results

Western blots of purified samples matched a previous report of two distinct immunoreacitve bands of recombinant human BMP15 at approximately 15 and 17 kDa from HEK-293 cells (Fig. 8) (Li, Rajanahally, *et al.*, 2009). Mature rbFL-BMP15 was predicted to be 15.5 kDa based on amino acid sequence. Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) analysis can give mass accuracies better than 0.1 kDa and was used to collect accurate molecular weights of the protein; in particular to ensure the lower 15 kDa band was not a degraded product. Samples are ionized by laser pulses and separated by a mass to charge ratio and plotted versus absolute intensity to generate a spectrum. MALDI-TOF analysis resulted in multiple mass/charge peaks corresponding to the two masses of 15.5 and 17.1 kDa from the HEK-293 cell line and a single peak corresponding to 17.1 kDa from the FBK cell line. (Fig. 10) The results were reported as mass over charge and therefore the mass has to be interpreted based on a number of charges. A total of four major peaks

were noted with two unique to the HEK-293 cell line matching the 15.5 kDa immunoreactive band. These results suggest that the 17.1 kDa protein from the FBK cell line is a result of post-translationally added side chains, likely N-glycosylated, that exist in multiple charge forms and that the 15.5 kDa protein is unmodified and not a degraded product.

Nuclear lysates collected from HeLa cells following exposure to recombinant human BMP15 or rbFL-BMP15 from HEK-293 conditioned medium showed an increase in phosphorylated SMAD 1/5 over the untreated group by Western blot with recombinant hBMP15 demonstrating the highest activity. (Fig. 11) This increase demonstrates that rbFL-BMP15 is capable of binding to BMP receptor type II and inducing the SMAD signaling pathway and is therefore biologically active.

Figure 10 Matrix-assisted laser desorption/ionization-time of flight MALDI-TOF analysis to determine accurate mass/charge ratio of purified samples from FBK and HEK-293 cell lines transfected with PCDNA rbFL-BMP15. (Figures A and B represent a continuation of the same spectrum starting from a m/z value of 2,500 in A and continuing from 5,000 to 16,000 in B) Ovals mark charged species of a 15.4 kDa protein and squares mark multiple charged a 17.1 kDa protein, likely N-glycosylated. A) FBK (top) and HEK-203 (bottom) peaks measuring m/z 2.86 representing a 6 charged protein of 17.1 kDa. B) Continuation of A) at larger m/z values. Peaks at 15.47 and 7.74 kDa in the HEK-293 sample correspond to the 15 kDa rbFL-BMP15 with 1 and 2 positive charges, respectively. The 5.7 peak represents a 17.1 kDa protein with 3 positive charges. This figure reveals a difference in the final protein produced by bovine and human cell lines from the same vector.


A
2861.48
2883.01
FBK
3055.33
2920.03
2211.87
2737.75
3512.16
4079.28
4713.92
2865.15
100
80
60
40
20
0
% Intensity
HEK-293
2879.98
2500
3000
3500
4000
4500
5000
m/z
B
5705.60
FBK
5675.07
5578.88
7742.25
100
80
60
40
20
0
% Intensity
5703.13
15470.64
HEK-293
6116.84
7447.19
15838.80
6000
8000
10000
12000
14000
16000
m/z

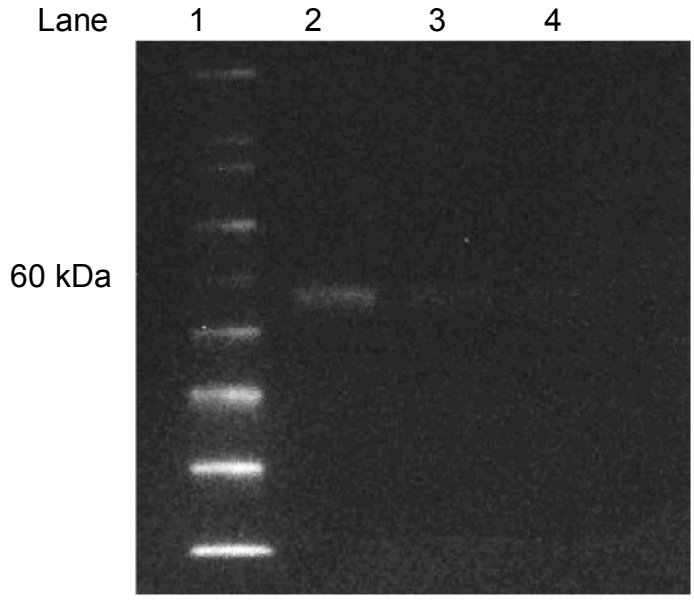


Figure 11 Phosphorylated SMAD 1/5 Western blot from nuclear lysates of treated HeLa cells. Cells were treated for 1 hour with 100 ng/ml rhBMP15 (recombinant human BMP15, R&D Systems), HEK-293 rbFL-BMP15 conditioned medium or HEK-293 wild type conditioned medium. Lane 1: Magic Mark XP (Life Technologies); Lane 2: 100 ng/ml rhBMP15; Lane 3: HEK-293 rbFL-BMP15 conditioned medium; Lane 4: HEK-293 wild type conditioned medium.

Treatment with dorsomorphin, a selective BMP signaling inhibitor, decreased the phosphorylation of SMAD 1/5 in HeLa cells after treatment with recombinant hBMP15 or rbFL-BMP15 from HEK-293 and FBK cell lines. (Fig 12) Importantly, this result shows specific action of rbFL-BMP15 from both cell lines through the classical BMP and TGFBR1 receptor heterodimer and signal transduction by activation (phosphorylation) of SMAD transcription factors. Nuclear lysates from wild-type HeLa cells or treated with dorsomorphin at 4 μM

were collected following exposure to 100 ng/ml hBMP15, rbFL-BMP15 from HEK-203 and FBK cell lines, or TBS + 1% BSA as a vehicle control for the purified proteins. Densotometric measurements corrected locally for background were compared and showed a marked decrease in the amount of immunoreactive phosphorylated SMAD 1/5. (Table 2, Fig. 13) For example, the background corrected average for hBMP15 treatment was decreased by 80% and rbFL-BMP15 from HEK cells was decreased by 47% following dorsomorphin administration. FBK derived rbFL-BMP15 and the untreated group had undetectable levels of phosphorylated SMAD 1/5 after exposure to dorsomorphin.

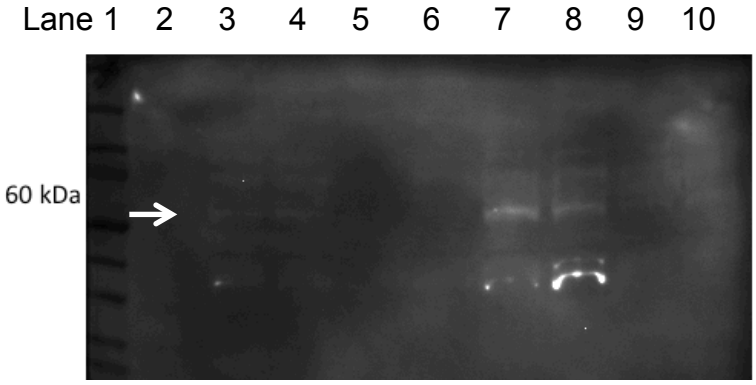


Figure 12 Phosphorylated SMAD 1/5 Western blot from nuclear lysates of treated HeLa cells with or without Dorsophomorphin BMP receptor inhibitor. HeLa cells were treated for 1 hour at a final concentration of 4 μM. Cells were left untreated, or incubated 100 ng/ml of rhBMP15, rbFL-BMP15 from HEK-293 or FBK cell lines before nuclear extraction. Lane 1: Precision Plus Dual Color (BioRad); Lanes 3-6 treated with dorsomorphin. Lane 3: rhBMP15; Lane 4: HEK-293 rbFL-BMP15; Lane 5: FBK rbFL-BMP15; Lane 6: TBS + 1% BSA vehicle control Lanes 7-10: same treatments as lanes 3-6 without dorsomorphin.

Table 2 Densitometry analysis from phosphorylated SMAD 1/5 Western blot of HeLa nuclear lysate following dorsomorphin treatment. Background corrected averages were normalized to untreated cells exposed to TBS + 1% BSA as a control. Dorsomorphin values represent a percentage of expression as compared to its untreated counterpart.

Treatment	No Inhibitor	Dorsomorphin (4 μM)
hBMP15	3.07	0.20
HEK-293 rbFL-BMP15	1.82	0.53
FBK rbFL-BMP15	1.26	0.00
Control	1.00	0.00

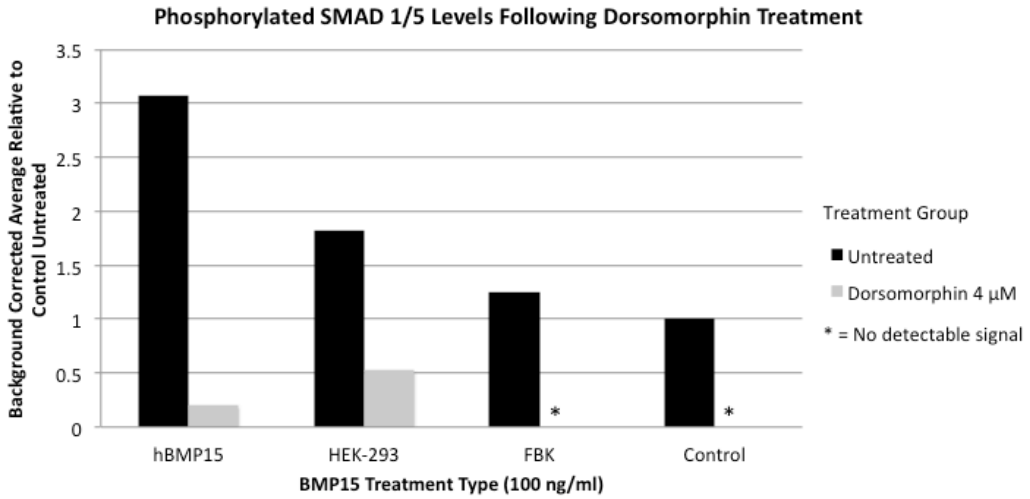


Figure 13 Phosphorylated SMAD 1/5 levels following dorsomorphin treatment. HeLa cells were left untreated or treated with 4 μM dorsomorphin, a selective BMP signaling inhibitor, for 1 hour. Cells were then exposed to 100 ng/ml hBMP15, rbFL-BMP15 from HEK-293 or HEK cells, or TBS + 1% BSA as a vehicle control. Untreated values are background corrected averages relative to control treatment. Dorsomorphin treatment values represent levels of phosphorylated SMAD 1/5 as compared to untreated levels in the same group. hBMP15 stimulation was decreased 80%, HEK-293 decreased by 47% and FBK and Control groups had undetectable levels after treatment with dorsomorphin inhibitor demonstrating that rbFL-BMP15 signals through the canonical BMP signaling pathway.

Multiple primer pairs were designed and tested for four BMP15 stimulated genes: PTX3, HAS2, PTGS2 and TSG6 (Bos taurus). (Table 3) Standard curves created by increasing dilutions of template cDNA. Primer sets were chosen based on linearity of standard curves ($R^2 > 0.99$) and calculated primer efficiency. (Fig. 14) Standard curves using bovine cumulus cell cDNA template for the chosen primer pair for each gene are shown along with their sequences.

Table 3 Real-time quantitative PCR primer sequences for BMP15 stimulated genes. GAPDH and SDHA primers were used for reference genes.

Name	Forward	Reverse
PTX3	GTTTTGTGCGCTCTGGTCTGC	GGTGAAGAGCTTGTCCCACTCG
HAS2	ATCCCATGGTTGGAGGTGTCGG	AGGTCCGCTAATGCACTGGACAC
TSG6	GTCTCAATAGAAGTGAAAGATGGG	GCAGTCACTGAAGCATCACTTAGG
PTGS2	TCCTGTGCCTGATGACTGCC	TTGATGGGTGAAGTGCTGGG
GAPDH	CTGCCCGTTCGACAGATAG	CTCCGACCTTCACCATCTTG
SDHA	ACCTGATGCTTTGTGCTCTG	TCGTACTCGTCAACCCTCTC

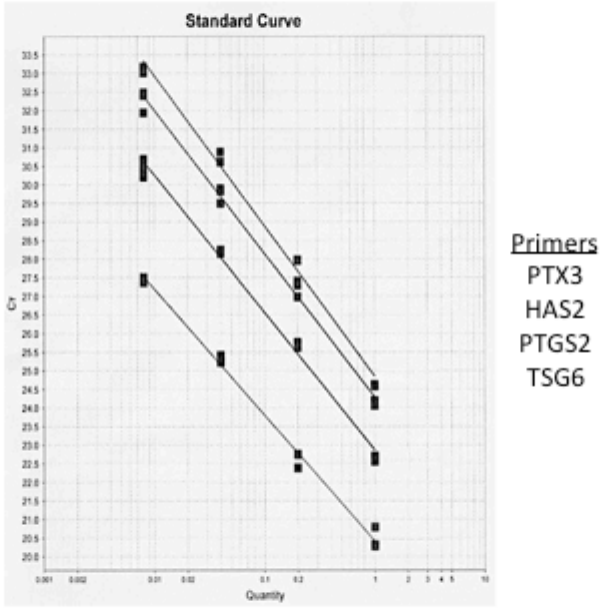


Figure 14 Standard Curves of cDNA dilutions by qPCR for BMP15 stimulated genes. Standard curves of primer sets for PTX3, HAS2, PTGS2 and TSG6 showing a linear response with dilutions of bovine cumulus cell cDNA in a range from 2 ng/reaction to 16 pg/reaction (1:10 – 1:1250). $R^2 \geq 0.99$ for all primers.

Real-time quantitative PCR of recombinant BMP15 treated (100 ng/ml) *in vitro* matured bovine cumulus oocyte complex cDNA showed a statistically significant increase in expression of PTGS2 and TSG6 transcripts from the hBMP15 treated groups compared to the untreated control by a post-hoc Dunnett's test. HAS2 and PTX3 showed no significant difference among treatment with $p > 0.05$ for ANOVA tests. With respect to expression of TSG6, there were also differences between treatments as tested by Tukey/Kramer HSD comparison of each pair. Treatments were labeled based on statistical grouping A, B or AB. hBMP15 was the only member of group A and FBK rbFL-BMP15 treated oocytes were grouped into AB. HEK-293 produced rbFL-BMP15 was not

different from the control with both treatments placed in group B. (Fig. 15) (Table 4)

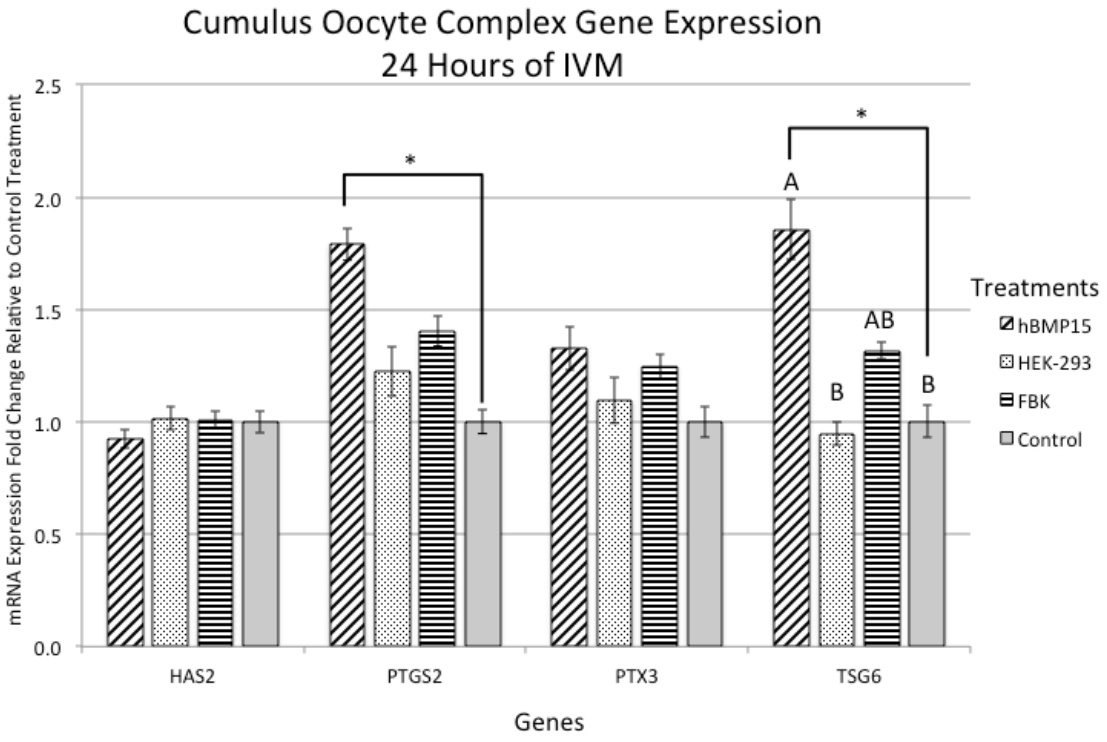


Figure 15 mRNA expression fold change relative to control treatment for BMP15 stimulated genes. Gene Expression at 24 hours of *in vitro* maturation as calculated by $\Delta\Delta C^T$ with GAPDH and YWHAZ as reference genes and untreated samples as a control. Data represents 3 biological replicates of 50 cumulus oocyte complexes per treatment with each analysis of each replicate completed at least two times. Treatment with 100 ng/ml recombinant human BMP15 resulted in statistically significant increases in transcript levels of PTGS2 and TSG6. Treatments marked with * are statistically different from the control treatment by a post-hoc Dunnett's test. Treatments with letters are statistically different from other letter within the same gene. $p < 0.05$ for all tests. Error bars represent +/- SEM of the expression values.

Table 4 mRNA expression results of *in vitro* matured cumulus oocyte complexes relative to control treatment for BMP15 stimulated genes. Values correspond to the bars in Figure 15. Treatments marked with * are statistically different from the control treatment by a post-hoc Dunnett's test.

	mRNA Expression			
Treatment	**HAS2**	**PTGS2**	**PTX3**	**TSG6**
hBMP15	0.92	*1.79	1.33	*1.85
HEK-293	1.02	1.22	1.09	0.95
FBK	1.01	1.40	1.25	1.32
Control	1.00	1.00	1.00	1.00

Discussion

We hypothesized that due to amino acid sequence divergence and species-specific post-translational modifications, homologous BMP15 will be more effective at directing bovine oocyte development during *in vitro* maturation than heterologous BMP15 (Hashimoto, Moore, *et al.*, 2005). Evidence of this difference in post-translational modifications was evident in the results combined from anti-FLAG Western blots and MALDI-TOF. Western blots revealed that rbFL-BMP15 produced by the HEK-293 cell line was present in two distinct forms, 17.1 and 15.4 kDa, with the second band of lower size not present in products from the bovine FBK cell line. The presence of two bands was not unexpected since it had been previously reported that recombinant human BMP15 produced by HEK-293 cells can be visualized as two bands by western blotting (Li, Rajanahally, *et al.*, 2009). It was highly interesting, however, that the

homologous rbFL-BMP15 seemed to only be present at the higher molecular weight.

MALDI-TOF analysis will accurately determine the mass of protein samples in relation to charge. The results are reported as mass over charge and therefore have to be interpreted based on the number of charges. A peak corresponding to 15.5 kDa was noted in the HEK-293 purified protein along with a species with 2 charges at 7.75 kDa. These peaks were absent in the product from the FBK cell line with only multiply charged species corresponding to a 17.1 kDa protein. Where the human cell line produced 2 distinct molecular weight proteins, the bovine cell line produced only the larger. This evidence suggests the bovine (FBK) cell line completes any post-translational modifications, likely N-linked glycosylation (Hashimoto, Moore, *et al.*, 2005), prior to secretion from the cell. Since post-translational modifications can be crucial to a protein's function, consideration should be given to the host and cell type one might use for recombinant protein production.

We have shown evidence of the biological function of rbFL-BMP15 produced by human and bovine cell lines by detection of phosphorylated-SMAD 1/5, as part of the canonical BMP signaling pathway, and inhibition by the selective inhibitor dorsomorphin. Analysis of genes activated by the phosphorylated SMAD 1/5 transcription factor by reverse transcription quantitative real-time PCR (RT-qPCR), however, showed only a modest increase in transcript levels at 24 hours. These results are in contrast to research reported in the mouse with

granulosa cell culture treated with 100 ng/ml rhBMP15 or recombinant N-terminal tagged rhBMP15 (Li, Rajanahally, *et al.*, 2009). This level of rbBMP15 or rhBMP15 may not be sufficient for a similar induction of transcription for downstream targets of phosphorylated SMAD 1/5. The timing of COC RNA collection after IVM for qPCR analysis could have also had an effect, for example Li et al. reported an increase in mRNA expression of mouse granulosa cells following treatment with recombinant human BMP15 at 5 hours of treatment (Li, Rajanahally, *et al.*, 2009). There could have been a difference in mRNA transcript levels prior to the 24 hour time point used in this study that was undetectable at 24 hours. The time point for collection was chosen based on a standard IVM maturation protocol and transit times for the oocytes to arrive from their collection point to the laboratory for analysis.

Gilchrist et al. reported an increase of 17%, from 41% to 58%, blastocyst rate with the addition of 10%, by volume, conditioned medium containing recombinant ovine BMP15 produced in a human cell line (HEK-293) (Gilchrist & Thompson, 2007). In this report, fifteen oocytes were collected from each of the three rounds of *in vitro* maturation were fertilized, cultured and blastocyst rates were recorded. There was no increase in blastocyst rate between the treatments, correlating with the minimal change in transcript levels as measured by RT-qPCR. Based on species differences in OSF activity and the previous report in a bovine system using mouse GDF9 and sheep BMP15, the lack of a strong response could be attributable to a decreased role of endogenous BMP15 in lieu

of GDF9 similar what has been described in the mouse (Yan, Wang, *et al.*, 2001; Yoshino, McMahon, *et al.*, 2006).

It was recently discovered that human GDF9 is secreted in a latent form, that can be activated by modifying the base sequence to result in an single amino acid change, in contrast to secreted active sheep GDF9 (Simpson, Stanton, *et al.*, 2012). Considering the significant differences in activity among species, it is plausible that endogenous bovine GDF9 signaling through SMAD 2/3 may be, in fact, more active during the final stages of maturation than BMP15 in the bovine model. The methods described here were designed to reduced variables, where possible, by using a known concentration of homologous recombinant mature protein purified by affinity chromatography.

We have developed a system for the repeatable production of purified rbFL-BMP15 from two different cell lines. These cell lines are an important resource for understanding the role of homologous BMP15 added to *in vitro* maturation medium and the influence of differential post-translational modifications. In this report, we failed to detect a significant affect of rbFL-BMP15 on the gene expression of *in vitro* mature cumulus oocyte complexes. Due to resource limitations, this study only evaluated one time point, 24 hours, and one concentration, 100 ng/ml, therefore several other experiments could be conducted in the future to more completely ascertain the potential role of rbFL-BMP15 in improving the *in vitro* maturation procedure.

Bone morphogenic proteins produced by the oocyte have been shown to prevent cumulus cell apoptosis (Hussein, Froiland, *et al.*, 2005). A demonstration of this anti-apoptotic affect on cumulus cells by a TUNEL would corroborate the biological function of the recombinant proteins as measured by phosphorylation of SMAD 1/5.

Alternatively, measurement of SMAD 1/5/8 phosphorylation could be detected with a luciferase assay (Mottershead, Pulkki, *et al.*, 2008). This method would allow one to more easily and definitively observe the phosphorylation response from each of the recombinant proteins and their inhibition by dorsomorphin. The possibility of increased responsiveness of SMAD phosphorylation by signaling through a heterodimer of BMP15 with its proregion could also be investigated by comparing the conditioned medium response to the purified rbFL-BMP15 at levels too low for adequate detection by Western blotting of nuclear lysates. The rbFL-BMP15 construct could also be modified to include a FLAG tag on the proregion to purify both fragments for heterodimer testing.

Different concentrations of BMP15 should be explored to determine the optimal response from the cumulus oocyte complexes as well as several time points. Transcripts activated by phosphorylated SMAD 1/5/8 may be detectable at earlier time points than 24 hours. Obtaining abattoir-derived ovaries from a local source and aspirating abattoir-derived oocytes at the laboratory would most easily accomplish the objective of measuring the response of downstream genes

at earlier time points by RT-qPCR. In this manner, several concentrations of rhBMP15 and rbFL-BMP15 from both cell lines could be tested for efficacy.

The systems and information described here have the potential to impact the understanding of *in vitro* maturation of bovine oocytes and well as an understanding of the active role that oocytes play in deciding the fate of the follicle. Even a modest increase in development rates for *in vitro* culture of bovine embryos would increase the number of transferrable embryos available to a producer from a single oocyte collection, reflecting a significant savings of time and money. The possibility of improving culture conditions for oocytes and embryos reaches even to human medicine as the incidence of assisted reproductive technologies become more common. The Barker hypothesis suggests that adverse environmental conditions during fetal development may lead to adult diseases later in life including cardiovascular disease and diabetes (Barker, Bull, *et al.*, 1990; Barker, 2001). Scientific evidence involving human and animal studies suggests that the Barker hypothesis may also pertain to exposure to insults during oocyte growth and maturation, fertilization and/or early embryonic development (Blondin, Farin, *et al.*, 2000; Eppig & O'Brien, 1998; Leese, Donnay, *et al.*, 1998). Further investigation into the optimization *in vitro* culture during the critical stages of oocyte maturation and pre-implantation development will lead to greater culture efficiency and understanding of the important interactions between the developing oocyte/embryo and its environment.

A novel system was recently developed for a more physiological maturation of bovine and mouse cumulus oocyte complexes named SPOM, or stimulated physiological oocyte maturation (Albuz, Sasseville, *et al.*, 2010). This method incorporates a two-step maturation process consisting of a 1-2 hour pre-IVM phase, where COCs are incubated with agents that cause a rapid increase in cAMP to prevent premature maturation, followed by an extended IVM period of 18-22 hours with a phosphodiesterase (PDE) inhibitor and FSH. The results obtained from the SPOM system were comparable to *in vivo* matured and IVF processed embryos with a blastocyst rate of 69% and a fetal yield of 26%. The high levels of cAMP induced in the cumulus cells during the pre-IVM period intend to mimic the environment of the follicle and the continued addition of PDE inhibitors at low concentrations during the extended IVM phase were utilized to slow the pace of meiotic maturation. In context of the SPOM system, the addition of recombinant homologous OSFs to the pre-IVM stage would be appropriate to further mimic the physiological environment of the antral follicle increase the effectiveness of the *in vitro* maturation procedure by assisting in the blockage of cumulus cell apoptosis and contributing to a more complete cytoplasmic maturation.

CHAPTER IV

SUMMARY

In vitro systems for mammalian embryo production have been shown to produce lower quality embryos than those derived from *in vivo* sources. Recent reports have demonstrated that *in vitro* bovine oocyte maturation systems benefit from the addition of oocyte secreted factors, specifically GDF9 and BMP15 from heterologous sources (sheep or mouse) (Gilchrist & Thompson, 2007; Hussein, Sutton-McDowall, *et al.*, 2011; Hussein, Thompson, *et al.*, 2006). The goal of this work was to produce a species-specific recombinant oocyte secreted factor with the potential to improve mammalian embryo production *in vitro*.

Due to the high specificity of immunoprecipitation, we chose to produce recombinant bovine BMP15 with a FLAG tag in bovine and human cell lines. The FLAG purification system from Sigma has the advantage of elution without the use of denaturing. A PCR strategy was designed based on planned modifications of the transcript at the N-terminus end, cleavage site and C-terminus. For expression in mammalian cell lines, rbFL-BMP15 was inserted into the multiple cloning site of PCDNA 3 designed for high expression in mammalian hosts with the human cytomegalovirus immediate-early (CMV) promoter. Following transfection and selection, we were able to repeatedly collect protein at a final concentration of 5-10 ng/µl in 100 µl TBS + 1% BSA, which allowed us

to easily add rbFL-BMP15 at a final concentration of 100 ng/ml media for *in vitro* maturation testing. The concentration and consistency obtained using this approach suggests a system that could be suitable for a number of mammalian recombinant protein targets where species-specific post-translational modifications are required.

We hypothesized that due to amino acid sequence divergence and species-specific post-translational glycosylation, homologous BMP15 would be more effective at directing bovine oocyte development during *in vitro* maturation than heterologous BMP15 derived from human sources. Evidence of this difference in post-translational modifications was evident in the results combined from anti-FLAG Western blots and MALDI-TOF. These observations suggest that the bovine (FBK) cell line completes any post-translational modifications, likely N-linked glycosylation, prior to secretion from the cell. Since post-translational modifications can be crucial to a protein's function, consideration should be given to the host of the cell type one might use for recombinant protein production.

We have shown evidence of the biological function of rbFL-BMP15 by detection of phosphorylated-SMAD 1/5, as part of the canonical BMP signaling pathway, and inhibition by the selective inhibitor dorsomorphin. Analysis of genes activated by the phosphorylated SMAD 1/5 transcription factor by reverse transcription quantitative real-time PCR (RT-qPCR), however, showed no statistically significant increase in transcript levels at 24 hours. Measurable

increases in stimulated gene transcripts may be detectable at earlier time points, so it would be interesting to profile gene expression of the COCs over the time course of IVM in the presence or absence of rbFL-BMP15. Considering the significant differences in activity among species, it is plausible that endogenous GDF9 may be, in fact, more active during the final stages of maturation than BMP15 in the bovine model. As such, the next step to investigation of the role of homologous OSFs in bovine IVM would be the production of recombinant FLAG tagged bovine GDF9 in the same manner as described here. Investigation of the effects of GDF9 alone, or in combination with BMP15, could help us draw conclusions about the bioactivity of these proteins in combination and specifically the possibility of a biologically active heterodimer formed between bovine GDF9 and BMP15.

Here, we have developed a system for the repeatable production of purified rbFL-BMP15 from two different cell lines that could be easily transferred to many other members of the TGFβ family of proteins. The cell lines produced here are an important resource for understanding the role of homologous BMP15 added during *in vitro* maturation of bovine COCs and the influence of differential post-translational modifications on the biological activity of these products, especially if combined with the production of recombinant bovine GDF9.

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