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Embryotropic actions of follistatin: Paracrine and autocrine mediators of oocyte competence and embryo developmental progression*

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Abstract

Despite several decades since the birth of the first test tube baby and the first calf derived from an in vitro fertilized embryo, the efficiency of assisted reproductive technologies remains less than ideal. Poor oocyte competence is a major factor limiting efficiency of in vitro embryo production. Developmental competence obtained during oocyte growth and maturation establishes the foundation of successful fertilization and pre-implantation embryonic development. Regulation of molecular and cellular events during fertilization and embryo development is mediated in part by oocyte derived factors acquired during oocyte growth and maturation and programmed by factors of follicular somatic cell origin. Available evidence supports an important intrinsic role for oocyte-derived follistatin and JY-1 proteins in mediating embryo developmental progression post fertilization and suggests that paracrine and autocrine actions of oocyte-derived GDF9 and BMP15 and follicular somatic cell derived members of the fibroblast growth factor family impact oocyte competence and subsequent embryo developmental progression post fertilization. An increased understanding of molecular mechanisms mediating oocyte competence and stage specific developmental events during early embryogenesis is crucial to further improvements in assisted reproductive technologies.

Introduction

Over the last five decades, several assisted reproductive technologies (ART) have been introduced to help overcome fertility problems in humans and to facilitate propagation of high genetic merit animals and biotechnology applications in farm animals (Mapletoft and Hasler 2005; Amiridis and Cseh 2012). Success rates of ART procedures are influenced by the quality of oocyte and sperm used, and the formulation of the culture media provided

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during oocyte maturation, fertilization and subsequent stages of embryonic development (Peura *et al.* 2003; Borowczyk *et al.* 2006; Lee *et al.* 2009). Production of an embryo via ART that is suitable for transfer requires successful completion of several developmental endpoints including but not limited to: meiotic maturation of the oocyte, fertilization, reprogramming of nucleus and transcriptional activation of the embryonic genome, and blastocyst formation. Developmental competence acquired during the oocyte growth and maturation process establishes the foundation of successful fertilization and pre-implantation embryonic development (Krisher 2004). While great progress has been made in the field of ART since its inception, efficiency of in vitro embryo production remains low and less than desirable. An increased understanding of molecular mechanisms mediating oocyte competence and stage specific developmental events during early embryogenesis is crucial to further improvements in ART.

A number of studies have been carried out to investigate the molecular mechanisms and factors involved in the maturation process that contribute to oocyte competence (Heikinheimo and Gibbons 1998). These studies revealed that development of competent oocytes requires successful nuclear and cytoplasmic maturation events such as breakdown of nuclear envelope, cytoskeleton rearrangement, assembly of meiotic spindle, chromatin remodeling, post-transcriptional and post-translational modifications of the oocyte mRNA and protein pool and glutathione production (Calarco *et al.* 1972; Sutovsky and Schatten 1997; Laurincik *et al.* 1998; Liang *et al.* 2007; Kang and Han 2011; Cheng *et al.* 2013). Simultaneous coordination of these developmental events requires an enormous amount of metabolic input from various substrates such as glucose, amino acid, lipids and vitamins (Downs and Mastropolo 1994; Leese 1995; Sturmey *et al.* 2009), and can be influenced by intrinsic and extrinsic factors including numerous growth factors released from the oocyte and (or) somatic cells resident in ovarian follicles.

Improvements in understanding of nutritional requirements of oocytes and paracrine, autocrine and endocrine regulation of meiotic maturation have led to improvements of in vitro oocyte maturation protocols (culture conditions and media components) which allow achievement of > 90% oocytes progressing to metaphase II stage with resulting > 80% cleavage rate after fertilization in the majority of farm animal species including cattle 92.2% (Prentice-Biensch *et al.* 2012), buffalo 80.4% (Mehmood *et al.* 2007), sheep 93.4 % and goat 82.4 % (Cox and Alfaro 2007). Despite achieving high rates of progression to metaphase II stage and first cleavage after fertilization, rates of further development progressively decrease, with a significant proportion of embryos permanently arrested at the 2–4 cell stage during in vitro culture (Betts and Madan 2008) and the vast majority of embryos fail to reach a transferable stage. The reasons for this high rate of embryonic loss during early stage of development are not clear but could include chromosomal abnormalities, poor quality oocytes, lack of essential maternal effect proteins and suboptimal culture conditions (Bavister 1995; Munne *et al.* 1995). Several oocyte derived maternal-effect genes/proteins have been identified in mammals, which are critically important for progression of early embryonic development (Gurtu *et al.* 2002; Burns *et al.* 2003; Payer *et al.* 2003; Wu *et al.* 2003; Bultman *et al.* 2006; Ma *et al.* 2006; Li *et al.* 2008; Zheng and Dean 2009). Expression and stability of these proteins is influenced by the autocrine-

paracrine actions of factors released from the oocyte and surrounding somatic (cumulus) cells. Interestingly, cumulus cells play a critical role in development of oocyte competence and share an indirect but large contribution to successful embryonic development (Zhang *et al.* 1995).

Identification of the molecular characteristics of competent oocytes and their functional significance to early embryonic development and therapeutic potential has been a major area of emphasis in our laboratory in recent years and led to discovery of an intrinsic regulatory role for the TGF β growth factor superfamily binding protein follistatin and the novel oocyte specific protein JY-1 in promoting embryo developmental progression. Evidence supporting a role for such factors and other select paracrine and autocrine growth factors such as GDF9 and BMP15 and members of the fibroblast growth factor (FGF) family in promoting oocyte competence and successful progression through early embryogenesis in the bovine is the focus of this review.

Key developmental endpoints in early embryogenesis

After fertilization, an embryo undergoes a species specific and critical cascade of complex cytological and molecular changes in a precisely orchestrated manner and such changes are influenced by intrinsic and extrinsic factors. Errors in one step can negatively impact subsequent steps, potentially resulting in embryo lethality (Heikinheimo and Gibbons 1998). Following fusion of the metaphase II stage oocyte with fertilizing sperm, a series of morphological and biochemical changes occur to produce a one-cell embryo with a diploid component of chromosomes. These events include completion of meiotic maturation, decondensation of sperm nucleus, formation of pronuclei and microtubule organizing center (MTOC), and subsequent packaging of DNA into diploid chromosomes (Li *et al.* 2010; Ward 2010). Fertilization triggers the proteolytic degradation of c-mos and cyclin-B, and thus cessation of maturation promoting factor activity, which allows the pre-embryo to complete meiotic maturation and resume regular cell divisions (Heikinheimo and Gibbons 1998). The pool of oocyte glutathione, which is synthesized from cysteine precursor during meiotic maturation, serves as an endogenous reducing agent and promotes the decondensation of sperm nucleus by reduction of disulfide bonds present in sperm protamines. After destabilization of disulfide bonds, oocyte-derived histones replace the protamines, and the sperm nucleus develops into the male pronucleus (Sutovsky and Schatten 1997; McLay and Clarke 2003).

Moreover, within sperm, the centriole present in the mitochondrial sheath and the striated columns of the sperm connecting piece contain abundant disulfide bonds, which are disassembled by glutathione in the oocyte cytoplasm and reconstituted into an active zygotic microtubule organizing center using ψ -tubulin and other centrosomal proteins present in the ooplasm (Battaglia *et al.* 1996). Further, packaging of DNA of male and female pronuclei into chromosomes is catalyzed by phosphorylation of histone H1 by histone H1 kinases, which are activated by calcium oscillations after fertilization (He *et al.* 1997). The one cell embryo then undergoes a coordinated series of cleavage divisions, progressing through 2-cell, 4-cell, 8-cell and 16-cell stages. The cells in cleavage stage embryos are known as blastomeres which start to form tight junctions with one another after 8- or 16-cell stage

(depending upon the species) and results in development of a mulberry shaped structure called a morula (Sheth *et al.* 1997; Miller *et al.* 2003) and subsequent cavitation and blastocyst formation.

In addition to these cytological and morphological changes in the developing embryo, several molecular changes occur during early embryonic development allowing progression from a transcriptionally repressed to a transcriptionally permissive state. Studies in the mouse, bovine and human have shown that regulation of gene expression during early stages of embryonic development involves a complex regulatory mechanism in which initial rounds of DNA replication change the chromatin structure via epigenetic modifications and make enhancers and promoters accessible for maternally derived transcription factors (Davis and Schultz 1997). Therefore, maternal transcripts in DNA replication, chromatin remodeling and transcription factor categories play a critical functional role during early stages of embryonic development. Moreover, gene expression profiling of early bovine embryos showed a characteristic depletion of maternal RNAs, and revealed that embryonic genome activation (EGA) occurs in two phases. The first phase is minor genome activation, in which several hundred genes are transcribed between the zygote and 4 cell stage (Viuff *et al.* 1996) related to translation of maternal transcripts and presumably for complete activation of the zygotic genome. The second phase is a major genome activation at approximately the 8-cell stage (Barnes and First 1991), which activates the transcription of genes essential for subsequent stages of embryonic development (De Sousa *et al.* 1998). Successful early embryonic development is the outcome of highly orchestrated molecular and regulatory mechanisms occurring during meiotic maturation, fertilization, first cleavage and embryonic genome activation. These molecular mechanisms are dependent upon oocyte derived factors including RNAs and proteins as well as their synergistic interaction and cross-talk with factors release from nearby somatic cells during follicular development

Influence of oocyte and follicular somatic cell secreted factors on oocyte competence and subsequent embryo developmental progression

In the past several decades, most of the research on mammalian preimplantation development has focused on mouse oocytes and embryos. Discoveries made possible using functional genomics and gene targeting technologies have greatly increased understanding of the individual maternally derived molecules and factors critical to the maternal-to-embryonic transition in the mouse and insight into maternal control of early embryogenesis (Li *et al.* 2010). However, inherent species specific differences exist in the ovulation quota, follicular waves, duration of the ovarian cycle and the number of embryonic cell cycles required for embryonic genome activation between the traditional polyovulatory mouse model versus monotocous species like cattle and primates including humans (Bettegowda *et al.* 2008). The developmental potential of the oocyte is reflected by its molecular and biochemical state which allow the oocyte to mature correctly and to undergo successful fertilization and embryo development. Increasingly, it is recognized that regulation of molecular and cellular events during fertilization and embryo development is mediated by oocyte derived factors acquired during oocyte growth and maturation in close association with follicular somatic cells (Gilchrist *et al.* 2008). Understanding the molecular basis of

regulation of these developmental events is of great fundamental and practical importance relative to improvements in ART in humans and economically important farm animals.

Oocyte developmental competence is defined as the capacity of the oocyte to resume meiosis, cleave after fertilization, help promote embryonic development and implantation, and bring a pregnancy to term in good health (Sirard *et al.* 2006). From a practical perspective, oocyte competence is the key limiting factor in the efficiency of in vitro embryo production in cattle (Lonergan and Fair 2008). Despite advancements in human ART, ratios of oocytes collected to live babies born have remained as high as 25:1 (Inge *et al.* 2005) with the low efficiency attributed primarily to poor oocyte quality (Gosden and Lee 2010). However, functional understanding of the molecular determinants of oocyte competence is highly lacking and impedes development of new strategies to diagnose or enhance oocyte competence and thus increase efficiency of ART in a clinical setting. A large body of evidence suggests the quality of an oocyte and subsequent fate of an embryo is influenced by the milieu of maternal mRNAs and proteins accumulated during oogenesis that are present during meiotic maturation, fertilization and initial cleavage divisions and via cross talk with surrounding somatic cells occurring during oocyte growth, development and maturation (Bettegowda *et al.* 2008; Lechniak *et al.* 2008).

A large body of evidence indicates the oocyte actively participates in the regulation of the surrounding somatic (cumulus cell) functions and this helps distinguish them from steroid-producing mural granulosa cells (Thibault *et al.* 1975; Wiesen and Midgley 1993; Eppig *et al.* 2002; Richards 2005). The oocyte communicates with cumulus cells via both paracrine oocyte-secreted factors and cell-cell contact mediated communication, affecting a broad range of follicular cell functions, including cumulus expansion, expression of cumulus cell markers, luteinization, proliferation, and cellular apoptosis (Dong *et al.* 1996; McKenzie *et al.* 2004; Su *et al.* 2004; Sugiura *et al.* 2007; Gilchrist *et al.* 2008). More recently, a few landmark studies have been carried out in human, bovine and other farm animals to understand the role of crucial growth factors derived from the oocyte and follicular somatic cells in promoting early stages of embryo development. Such studies are summarized below.

Previously it was assumed that overall competence of oocyte is influenced primarily by factors derived from follicular somatic cells (cumulus, granulosa and theca cells) which are themselves modulated by gonadotropins, nutrients and growth factors. However, it has become evident now that the oocyte is a key modulator of follicular somatic cell functions and thereby plays a central role in the regulation of folliculogenesis, oogenesis and several other oocyte related milestones required to achieve developmental competence (Gilchrist *et al.* 2008). During oogenesis, soluble growth factors (OSFs) are released from the oocyte which regulate the functions of neighboring cumulus cells surrounding the oocyte, and mural granulosa cells (MGCs) lining the wall of antral follicle. These somatic cell types are not only phenotypically different from each other but also exhibit large functional differences (Eppig 2001). Cumulus cells are metabolically linked with the oocyte and communicate through bidirectional interaction to promote the growth and developmental competence of the oocyte. In the presence of gonadotropins, murine cumulus cells produce hyaluronic acid required for cumulus expansion whereas mural granulosa cells primarily perform endocrine functions including steroidogenesis as indicated by higher levels of

mRNA expression for *Lhcgr*, *Cyp11a1* and *Cd34* and other steroidogenic enzymes in mural granulosa cells (Diaz *et al.* 2007). Moreover, differences in a variety of growth factors and hormone receptors present on cumulus versus mural granulosa cells is considered one of the major reasons for their functional differences (Camp *et al.* 1991; Manova *et al.* 1993; Canipari *et al.* 1995).

In the bovine, oocyte secreted factors have been demonstrated to be crucial determinants of cumulus versus mural granulosa cell phenotype and promote cell growth and attenuate progesterone production in granulosa cells. Consequently, cumulus cells of cumulus oocyte complexes exhibit higher growth rates than mural granulosa cells when cultured in the presence or absence of IGF1 due to close association with oocyte derived factors whereas, mural granulosa cells produce 13 fold more progesterone than cumulus cells of cumulus oocyte complexes under FSH+IGF treatment due to absence of these oocyte secreted factors. Another important role of oocyte secreted factors is modulating gonadotropin mediated effects on cumulus cell function. In vitro treatment with FSH + IGF-1 dramatically reduced growth of cumulus cells in cumulus oocyte complexes, but inhibitory effects were not seen when cumulus cells were removed from the cumulus oocyte complex and cultured in the absence of the oocyte (Li *et al.* 2000).

GDF9 and BMP15

Above studies support an active role of the oocyte in regulation of follicular somatic cell function. Several subsequent studies have been carried out in bovine to identify the oocyte secreted factors with an important role in paracrine regulation of cumulus and mural granulosa cell functions to control folliculogenesis. GDF9 and BMP15 are members of the TGF β superfamily secreted from the oocyte throughout most stages of folliculogenesis and regulate functions of cumulus cells required for the appropriate oocyte development (Matzuk *et al.* 2002; Gilchrist *et al.* 2008; Su *et al.* 2009). Mutation or targeted deletion of *GDF9* and *BMP15* leads to altered reproductive functions and infertility in humans, sheep, cattle and mice (Dong *et al.* 1996; Galloway *et al.* 2000; Otsuka *et al.* 2011). It has become evident that these two members of the TGF β superfamily are largely responsible for a majority of the paracrine actions of the oocyte and known to regulate the distinctive functions of cumulus cells, including steroidogenesis (Miyoshi *et al.* 2007; Spicer *et al.* 2008), proliferation (Gilchrist *et al.* 2004b; McNatty *et al.* 2005), differentiation (Kathirvel *et al.* 2013), expansion (Gilchrist *et al.* 2004a; Dragovic *et al.* 2005), apoptosis (Hussein *et al.* 2005), metabolism (Eppig *et al.* 2005; Sugiura *et al.* 2005) and several others (Otsuka *et al.* 2011). Considering the demonstrated biological actions of these growth factors, several studies have been carried out using supplementation of different doses and combinations of GDF9 and BMP15 during oocyte in vitro maturation to determine effects on embryo production and viable offspring after embryo transfer. Addition of either GDF9 or BMP15 or both to the bovine and mice cumulus oocyte complex culture media (during in vitro maturation) mimics the effects of native oocyte secreted factor supplementation on improving the blastocyst yield and quality (Hussein *et al.* 2006; Yeo *et al.* 2008). Exogenous supplementation of these two oocyte secreted factors has a major influence on the oocyte developmental competence if added during first 9 hours of in vitro maturation whereas native oocyte secreted factors exert their effects throughout the maturation process (Hussein

et al. 2011). Declining expression of *GDF9* and *BMP15*, and breakdown of oocyte-cumulus cell gap junctional communication after 9 hours of bovine oocyte maturation might be the possible explanations for the decreased sensitivity of cumulus cells to BMP15 and GDF9 signaling after this time point (Pennetier *et al.* 2004; Thomas *et al.* 2004). However, the positive effect mediated by native oocyte secreted factors beyond 9 hours of in vitro maturation suggests the presence of other oocyte derived factors which promote oocyte competence.

In bovine, the precise molecular mechanism underlying the control of cumulus cell function by oocyte derived GDF9 and BMP15 is not totally understood. Evidence indicates oocyte secreted GDF9 and BMP15 independently act as homodimers in a paracrine mode on cumulus/granulosa cells through binding to cognate receptors on cumulus cells (BMPRII/Alk5 and BMPRII/Alk6, respectively) which activate the SMAD2/3 and SMAD1/5/8 intracellular signaling pathways (Shimasaki *et al.* 2004; Sasseville *et al.* 2010; Pulkki *et al.* 2011). Activation of SMAD intracellular signal transduction regulates a large range of cumulus cell functions, under the influence of FSH and EGF-like peptide, which enhance oocyte growth and development competence during maturation (Kaivo-oja *et al.* 2006). GDF9 and BMP15 have synergistic actions with other ligands, including GDF9 and FGF8 actions in mice (Sugiura *et al.* 2007) and BMP15 and FSH actions in the bovine system (Sutton-McDowall *et al.* 2012). Such actions help promote oocyte developmental competence. Concomitant supplementation of these factors to cumulus oocyte complexes alter the metabolic functions of cumulus cells which in turn transfer the nutrients and other factors to enhance oocyte developmental competence and subsequent development to the blastocyst stage following in vitro fertilization. However, the precise molecular mechanisms underlying these synergistic interactions of TGF-beta superfamily members with other factors are not completely clear.

Fibroblast growth factors

The fibroblast growth factors (FGF) are important autocrine and paracrine factors released from bovine theca and granulosa cells and the oocyte and bind to cognate receptors (FGFR) preferentially expressed on cumulus cells and oocytes during final stages of maturation (Ben-Haroush *et al.* 2005). Studies support an intrinsic role for FGF in bovine early embryonic development as culture of early embryos in the presence of an FGF receptor kinase inhibitor (SU5402) reduced rates of development of bovine embryos to the blastocyst stage (Fields *et al.*, 2011). Furthermore, a growing body of evidence suggests that FGF of uterine origin may also influence embryonic development in vivo (Fields *et al.*, 2011), as FGF2 can induce the expression of interferon- τ (IFNT) mRNA and protein, the maternal recognition of pregnancy signal, in bovine trophoctoderm (Michael *et al.* 2006). This effect is probably mediated by interaction with the FGF2 receptor (FGF2R) which is expressed throughout the early stages of embryo development including the blastocyst stage (Daniels *et al.* 2000; Lazzari *et al.* 2002).

Supplementation of FGF2 during in vitro oocyte maturation revealed a dose dependent effect on cumulus expansion, oocyte maturation and blastocyst development. Oocytes exposed to FGF2 treatment during meiotic maturation displayed increased cumulus

expansion, a 10 % increase in progression to metaphase II stage and an approximate two fold increase in blastocyst yield compared to untreated control oocytes (Zhang *et al.*, 2010b). Furthermore, addition of high concentrations of FGF2 (500 ng/ml) to bovine embryo culture on day 0 or day 4 increased rates of blastocyst development (Fields *et al.*, 2011). Another important function of FGF2 is stimulation of the formation of primitive endoderm (PE) as FGF2 supplementation of blastocyst cultures increases the incidence of blastocyst outgrowth via increasing the mitotic index of primitive endoderm cells on days 13 and 15 after in vitro fertilization. This effect is mediated via FGF2 interaction with FGFR1a and FGFR1b surface receptors, predominantly present on bovine primitive endoderm cells, that induce the expression of GATA4 and GATA6 transcription factors required for the lineage commitment of primitive endoderm cells from the inner cell mass (Yang *et al.* 2011). Therefore, understanding the signaling and regulatory pathways involved in FGF2 mediated effects is of significant fundamental and practical application.

FGF10 is an additional paracrine-acting growth factor produced by theca and granulosa cells and oocytes which binds to its cognate receptor FGFR2b present on cumulus cells and the oocyte (Buratini *et al.* 2007). It has been proposed that during in vitro maturation, competence of the oocyte might be compromised due to absence of theca cell derived factors such as FGF10. Previous studies demonstrated that cumulus oocyte complexes matured in the presence of FGF10 exhibited higher rates of meiotic maturation, and cumulus expansion and increased rates of blastocyst production for in vitro produced bovine embryos (Zhang *et al.* 2010a). The stimulatory effect of FGF10 was not observed when oocytes were cultured in the absence of cumulus cells. Out of four FGF receptors, cumulus cells preferentially expressed FGFR1b whereas FGFR2b was found to be highly abundant in the oocyte. Collectively, these studies suggest that oocyte and theca derived FGF10 binds to the FGFR1b receptor on cumulus cells to induce signaling pathways stimulatory to cumulus expansion, meiotic maturation and embryonic developmental progression post fertilization.

The mechanisms involved in FGF10 enhancement of cumulus expansion and its embryotropic actions are not completely understood. Treatment with FGF10 during in vitro maturation of cumulus oocyte complexes enhanced the cumulus cell mRNA expression of prostaglandin G/H synthase-2 (PTGS2) at 4 hours, pentraxin 3 (PTX3) at 12 hours and tumor necrosis factor-stimulated gene 6 (TSG6) at 22 hours post stimulation (Caixeta *et al.* 2013). *Ptgs2* expression in cumulus cells is rate limiting for prostaglandin E2 (PGE2) production which is a critical mediator of cumulus expansion (Eppig 1981; Hizaki *et al.* 1999). *Ptgs2*^{-/-} mice failed to show any cumulus expansion and also exhibited severe defects in meiotic progression during the preovulatory period (Lim *et al.* 1997; Davis *et al.* 1999; Takahashi *et al.* 2006). Similar defects in cumulus expansion were observed in the bovine system upon partial inhibition of PTGS2 activity during *in-vitro* oocyte maturation. PGE2 binds to the EP2 subtype of PGE2 receptor (PTGER2), which is highly expressed on oocytes, and can activate the MAPK pathway to promote oocyte maturation and subsequent preimplantation embryo development (Nuttinck *et al.* 2011). Moreover, FGF10 induced TSG6 and PTX3 are essential components of the hyaluronan (HA)-enriched extracellular matrix characteristic of an expanded cumulus layer and deficiency of either protein leads to

the defects in cumulus expansion and integrity of the cumulus-oocyte-complex (Fulop *et al.* 2003; Salustri *et al.* 2004).

JY-1

Sequencing of expressed sequence tags from a bovine oocyte cDNA library (Yao *et al.* 2004) led to the discovery of a novel gene (*JY-1*) with an important regulatory role in early embryogenesis (Bettegowda *et al.* 2007). Our published studies (Bettegowda *et al.* 2007) established that the *JY-1* gene encodes for an oocyte specific secreted protein which is a member of a novel protein family. *JY-1* mRNA and protein are present throughout follicular development in primordial through antral follicles and restricted exclusively to the oocyte. *JY-1* like sequences are present at chromosomal locations in other vertebrate species (e.g. mice, rats, humans) that are syntenic to the *JY-1* locus on bovine chromosome 29. But, these syntenic loci in other species do not contain exons 1 and 2 and thus presumably do not encode for a functional protein (Bettegowda *et al.* 2007) suggesting species specificity in evolution of this novel oocyte specific gene.

Our results also established a critical functional role for oocyte derived *JY-1* in promoting early embryogenesis. *JY-1* mRNA present within early embryos is of maternal origin and progressively declines post fertilization to nearly undetectable levels in 16-cell embryos (Bettegowda *et al.* 2007).

Gene knockdown using microinjection of *JY-1* siRNA was utilized to test the functional requirement of JY-1 for oocyte maturation and early embryogenesis. *JY-1* gene knockdown in zygotes decreased *JY-1* mRNA and protein in resulting embryos and significantly reduced rates of development to the 8–16 cell and blastocyst stages versus controls (Bettegowda *et al.* 2007). Furthermore, supplementation with recombinant JY-1 protein rescued development of *JY-1* siRNA injected embryos to the blastocyst stage (Lee *et al.*, unpublished). Thus, results indicate that the novel oocyte specific protein JY-1 is obligatory for bovine early embryonic development. Furthermore, knockdown of *JY-1* in cumulus enclosed germinal vesicle stage oocytes reduced rates of cumulus expansion and progression to metaphase II stage during in vitro maturation (Lee *et al.*, unpublished), suggesting a functional requirement for JY-1 both pre- and post-fertilization. However, it is not yet known whether JY-1 levels are deficient in established models of poor oocyte competence in cattle or whether oocyte JY-1 levels impact fertility in a production (farm) setting.

Follistatin

Our functional genomics studies in the bovine model determined the RNA transcriptome characteristics of oocytes of poor developmental competence (Patel *et al.* 2007). Of the many differences in transcript abundance observed, of particular interest was reduced transcript abundance for follistatin observed in poor quality oocytes obtained from prepubertal animals (Revel *et al.* 1995; Damiani *et al.* 1996) relative to good quality oocytes from adult animals. Follistatin mRNA and protein in early embryos are of oocyte origin and significantly higher in 2-cell stage bovine embryos that cleave early and have higher rates (>40%) of development into blastocysts relative to embryos that cleave late (30–36 h post insemination) and develop to the blastocyst stage at a lower (<10%) rate (Patel *et al.* 2007;

Lee *et al.* 2009). Time to first cleavage is a significant indicator of developmental potential and successful pregnancy in a clinical setting, with > 2-fold higher pregnancy rates observed in single embryo transfers using early cleaving versus later cleaving embryos (Edwards *et al.* 1984; Salumets *et al.* 2003; Van Montfoort *et al.* 2004). Collectively, results support a strong positive relationship between oocyte follistatin levels and oocyte competence.

Based on above results, we conducted studies to determine if maternal (oocyte derived) follistatin abundance is a key determinant of success of bovine early embryonic development in vitro. Follistatin supplementation during first 72 h of embryo culture (until embryonic genome activation) enhanced numbers of early cleaving embryos and embryo numbers developing to the 8- to 16-cell and blastocyst stage in a dose dependent fashion (Lee *et al.* 2009). Follistatin treatment of rhesus monkey embryos significantly enhanced proportion of embryos that cleaved early and proportion of embryos reaching the blastocyst stage (VandeVoort *et al.* 2009) demonstrating translational relevance of data from the bovine model system. Furthermore, follistatin treatment also enhanced total blastocyst cell numbers and trophectoderm cells, with no effect on numbers of inner cell mass cells. An increase in blastocyst mRNA for the trophectoderm specific transcription factor *CDX2* was also observed in response to follistatin treatment (Lee *et al.* 2009). Furthermore siRNA mediated follistatin knockdown in bovine zygotes decreased 8- to 16-cell and blastocyst development and total and trophectoderm cell numbers in resulting blastocysts (Lee *et al.* 2009) and effects of follistatin knockdown were ameliorated by addition of exogenous follistatin to culture media (Lee *et al.* 2009).

Above studies clearly demonstrate stimulatory effects of exogenous follistatin on early embryonic development and a requirement for endogenous (oocyte derived) follistatin for embryo developmental progression in vitro. However, the mechanisms responsible for embryotropic actions of follistatin remain elusive. Follistatin can also bind and regulate activity of multiple additional TGF β superfamily members such as inhibins and select BMPs (Otsuka *et al.* 2001; Balemans and Van Hul 2002; Lin *et al.* 2003). Follistatin binding blocks interactions with respective type I and type II serine threonine kinase receptors thus inhibiting ligand induced signaling through SMAD2/3 (activin, TGF β , nodal) or SMAD1/5/8 (BMPs). Based on results to date, the potential mechanism of action of follistatin in regulation of bovine early embryogenesis seems paradoxical. As stated above, follistatin functions as a high affinity binding protein for activin, but also binds at a lower affinity and inhibits activity of certain BMPs (e.g. BMP4, BMP7 and BMP15; (Lin *et al.* 2003; Glister *et al.* 2004)).

We have previously compared (relative to follistatin treatment) effects of treatment with activin or SB431542 (Lee *et al.* 2009), an inhibitor of phosphorylation of ALK 4, 5 and 7 (type I receptors for activin, TGF β and nodal) and signaling through SMAD2/3. However, results did not clarify follistatin mechanism of action. Although less potent, treatment with exogenous activin mimicked effects of follistatin treatment on time to first cleavage and development to the blastocyst stage, whereas inhibitory effects of SB431542 on such parameters were noted. Treatment with SB431542 muted, but did not totally block stimulatory actions of follistatin on bovine early embryogenesis (Lee *et al.* 2009).

Implications of above studies to overall understanding of follistatin mechanism of action in

regulation of bovine early embryogenesis are unclear because: 1) SB431542 inhibits signaling through type 1 receptors for TGF β and nodal in addition to activin, 2) efficacy of doses of SB431542 utilized in completely blocking activin action were not directly tested, 3) dose of SB431542 tested was lower than maximal dose known to specifically inhibit downstream signaling pathway (Inman *et al.* 2002) and 4) treatments do not control for levels of endogenous growth factors and basal activity of individual signaling components in early embryos during embryo culture. We also tested the effects of treatment during initial 72 h of bovine embryo culture with recombinant human noggin (1, 10 and 100 ng/ml; same doses as used in follistatin supplementation studies) on time to first cleavage and embryonic development to the blastocyst stage. The binding affinity and inhibitory activity of the TGF β superfamily binding protein noggin appears specific to certain members of the BMP family (e.g. BMP2, 4, 5, 7; (Krause *et al.* 2011) which signal through SMAD1/5/8. In contrast to stimulatory effects seen with exogenous follistatin treatment, treatment with exogenous BMP binding protein noggin decreased proportion of embryos that cleaved early (by 50% and 75% at 10 and 100 ng/ml dose respectively) and rates of development to the blastocyst stage in a dose dependent fashion (Folger *et al.*, 2013). Results support antagonistic actions of follistatin versus noggin regulated pathways in early bovine embryos and suggest follistatin mechanism of action is not linked to inhibition of the same growth factors inhibited by noggin which signal through SMAD1/5/8. However, further study will be required to elucidate the intracellular mechanism responsible for embryotropic actions of follistatin on early embryos.

Summary and future directions

The negative impact of poor oocyte quality on assisted reproductive technologies is without question. Indeed, poor egg quality may be the single greatest impediment to a successful pregnancy in otherwise healthy women (Gosden and Lee 2010). Poor oocyte quality is also a major factor limiting efficiency of reproductive biotechnologies (in vitro embryo production) in bovine species (Lonergan and Fair 2008) and may also potentially contribute to highly costly bovine embryonic loss and poor reproductive efficiency in a production setting. Acquisition of oocyte competence is controlled by the interaction of genetics, the endocrine milieu and the intrafollicular microenvironment. By contrast, poor oocyte quality is exacerbated by a variety of adverse health conditions and maternal age, often necessitating use of costly assisted reproductive technologies. Despite decades of research, the fundamental questions remain of what makes an egg good or bad and how to improve egg quality in a clinical or laboratory setting?

It has been established that the quality of an oocyte and subsequent fate of an embryo are programmed by the milieu of maternal mRNAs and proteins accumulated during oogenesis and present during meiotic maturation, fertilization and initial cleavage divisions and via bidirectional communication occurring with surrounding somatic cells during oocyte growth, development and maturation (Bettgowda *et al.* 2008; Lechniak *et al.* 2008). Available evidence indicates that factors of somatic cell (e.g. fibroblast growth factors) and oocyte origin (e.g. GDF9, BMP15) can influence oocyte competence with beneficial effects on embryo developmental progression observed following supplementation with exogenous growth factors during in vitro maturation (Zhang *et al.* 2010a; Zhang *et al.*, 2010b; Hussein

et al. 2011). However, the mechanisms and potential regulatory roles of endogenous growth factors pre and post fertilization in promoting embryo developmental progression merit further investigation.

Application of functional genomics and siRNA mediated gene knockdown technologies in our laboratory has provide novel insight on intrinsic oocyte derived factors linked to oocyte competence and embryo developmental progression. Our published results established that the JY-1 gene encodes for a species specific secreted protein belonging to a novel protein family with activity of JY-1 required both pre- (Lee *et al.*, unpublished) and post-fertilization (Bettegowda *et al.* 2007) to ensure normal embryo developmental progression. However, the intracellular mechanisms whereby JY-1 promotes early embryogenesis and the relationship between endogenous oocyte JY-1 levels and oocyte competence and fertility in a production setting are not known, but critical to further understanding the role and significance of JY-1 to the reproductive process in cattle.

We have also demonstrated a positive association of oocyte follistatin expression with oocyte competence (Patel *et al.* 2007) and pronounced tropic actions of maternal (oocyte-derived) follistatin that promote progression through early embryogenesis (increased blastocyst rates) and positively impact indices of embryo quality including blastocyst cell allocation to trophectoderm (Lee *et al.* 2009). Our comparative studies in the rhesus monkey model demonstrated stimulatory actions of exogenous follistatin on rates of blastocyst development (VandeVoort *et al.* 2009) and support potential clinical relevance of results in the bovine model. Critical questions are currently being addressed regarding mechanism of action of follistatin and TGF β superfamily ligands and signaling pathways (e.g. SMAD 2/3 and (or) SMAD 1/5/8) involved in mediating it's above described embryotropic actions (Figure 1) and impact of follistatin treatment during embryo culture on pregnancy rates following embryo transfer limit. Lack of knowledge in such areas limits understanding of the functional significance of follistatin to early embryogenesis and the translational relevance of above findings to improvements in human ART and reproductive biotechnologies (in vitro embryo production) in cattle.

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Figure 1.

Potential influence of follistatin on SMAD signaling pathways in bovine embryos. A functional role for oocyte derived follistatin in regulation of time to first cleavage, rates of development to the 8- to 16-cell and blastocyst stages and blastocyst cell allocation to trophectoderm has been established for bovine embryos (Lee et al., 2009). Elucidation of the effect of follistatin treatment of bovine embryos on SMAD2/3 and or SMAD1/5/8 phosphorylation and downstream signaling components will be critical to determination whether follistatin mechanism of action in early embryos is through classical or nonclassical pathways. (TF: transcription factors; SSXS: Activin/Nodal/TGF-B Signaling specific phosphorylated motif of rSMAD2/3, ST: serine and threonine residues).