Additive Effects Among Uterine Paracrine Factors in Promoting Bovine Trophoblast Cell Proliferation

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ABSTRACT

Several uterine-derived paracrine factors have been implicated as critical regulators of conceptus development in cattle, but it remains unclear how these factors work together to establish and maintain pregnancies. The primary objectives of this work were to establish if cooperative interactions between epidermal growth factor (EGF), fibroblast growth factor-2 (FGF2) and insulin-like growth factor-1 (IGF1) promote bovine trophoblast cell proliferation, and to decipher the intracellular signaling mechanisms employed by these growth factors to regulate cell proliferation. Pilot studies established effective concentrations for each growth factor on a bovine trophoblast cell line (CT1). The first set of studies examined how each factor worked individually or in conjunction with each other to impact CT1 proliferation. Mitotic index (percentage of EdU-positive nuclei after a 45 min challenge) was increased ($P<0.05$) by supplementation with 10 ng/ml EGF, 10 ng/ml FGF2, or 50 ng/ml IGF1 when compared with non-treated controls. In addition, a greater increase ($P<0.05$) was detected when all three factors were supplemented together. A follow-up study determined that supplementation of any two growth factors could not replicate the cooperative effect noted when all three factors were provided. A second set of studies was undertaken to examine how mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase/AKT (PI3K/AKT) signaling systems mediate the independent and cooperative effects of these paracrine factors. Both EGF and IGF1 transiently activated mitogen-activated protein kinase3/1 (MAPK3/1) in

CT1 cells as determined by Western Blot analysis. By contrast, FGF2 did not affect MAPK3/1 phosphorylation status, but increased AKT phosphorylation status. Neither EGF nor IGF1 impacted AKT activity. Supplementation with a pharmacological inhibitor of MAPK3/1 (PD98059) prevented EGF-, IGF1-, and FGF2-dependent increases in CT1 cell proliferation. This inhibitor also completely abolished the increases in cell proliferation observed when all three factors were supplemented together. Supplementation with a pharmacological inhibitor of AKT (Wortmannin) reduced FGF2-stimulated CT1 proliferation, but did not impact EGF- and IGF1 effects. The AKT inhibitor partially attenuated the cooperative effects of all three factors on CT1 cell proliferation. A final study examined how the combination of EGF, FGF2, and IGF1 affect bovine embryo development. In vitro produced bovine blastocysts were cultured either with the combination of growth factors or vehicle only from day 8 to day 12 post-in vitro fertilization (IVF). The combination of EGF, FGF2, and IGF1 increased ($P<0.05$) the percentage of hatched blastocysts and outgrowth formation versus controls. Increased ($P<0.05$) diameters were detected in blastocysts treated with the combination of three growth factors on day 12 post-IVF when compared to controls. Treatment with the combination of EGF, FGF2, and also IGF1 increased ($P<0.05$) the change of diameter from day 8 to 12 post-IVF. In summary, these observations provide evidence that cooperative interactions of uterine-derived factors promote trophoblast proliferation and conceptus development in ways that may promote the establishment and maintenance of pregnancy in cattle. The mechanisms utilized for these activities remain unresolved, but MAPK3/1 and PI3K/AKT signaling systems appear to play integral roles in some of these processes.

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LIST OF ABBREVIATIONS

BCS	Body Condition Score
CDX2	Caudal type homeobox 2
CL	Corpora Lutea
CT1	Cow Trophoblast Cell Line 1
EGF	Epidermal Growth Factor
FGF	Fibroblast Growth Factor
FRT	Female Reproductive Tract
GATA3	GATA family transcription factor 3
HGF	Hepatocyte Growth Factor
HMG	High Mobility Group
ICM	Inner Cell Mass
IGF1	Insulin-like Growth Factor-1
IFNT	Interferon tau
IVF	In Vitro Fertilization
KLF5	Kruppel-Like Factor 5
LIF	Leukemia Inhibitory Factor
MAPK	Mitogen-Activated Protein Kinase
MMP9	Matrix Metallopeptidase 9
MUC1	Mucin 1
OCT4	Octamer-binding transcription factor 4
PE	Primitive Endoderm
PGF2α	Prostaglandin F2alpha

PI3K/AKT	Phosphoinositide 3-Kinase/AKT
PRL	Prolactin
RNA	Ribonucleic Acid
SHP-2	SRC homology region 2 domain-containing phosphatase-2
SOX2	SRY-box 2
TE	Trophectoderm
TEAD4	TEA domain family member 4
TGF-β	Transforming Growth Factor beta
UGKO	Uterine Gland Knockout phenotype
VEGFA	Vascular Endothelial Growth Factor A
YAP	Yes-Associated Protein

CHAPTER I: LITERATURE REVIEW

PREGNANCY LOSS IN CATTLE

In the past several decades, dairy cow fertility has declined coincident with increased milk production (Lucy, 2001). Many factors, such as genetics, physiology, farm management, and nutrition are thought to be responsible for this declining fertility. Fertilization rates of cows and heifers right after insemination are 85% and 95% (Walsh et al., 2011). However, the high rate of early embryo mortality produces pregnancy rates of only approximately 55 to 65% in cows and 75% of heifers at day 7 after breeding. Pregnancy rates are further reduced to 40 to 45% on day 21 (Figure 1), and 35% at term in lactating cows, as compared with 60% pregnancy rates at term in heifers (Lucy, 2001; Walsh et al., 2011). Based on these data, early pregnancy loss is a major contributor to infertility in cattle, and especially in lactating dairy cows. Pregnancy loss also is evident in beef cattle, with losses ranging from 10 to 30%, depending on management system and other factors (Lamb et al., 2008).

Early pregnancy loss in cattle results from various physiological factors. Body condition score (BCS) reveals the nutritional and health status of cows, and cows in low BCS have reduced fertilization rate and increased pregnancy loss (Buckley et al., 2003; Banos et al., 2007; Roche et al., 2009). Heat stress reduces the appetite, thereby reducing BCS of lactating cows (Shehab-El-Deen et al., 2010). Heat stress also has direct effects on oocyte and embryo viability (Ealy et al., 1993). Body condition changes are associated with compromised immunity and the potential presence of disease or parasites (Loeffler et al., 1999). Periparturient diseases are directly related with cattle reproduction as reduced conception and increased fetal loss are found in cows with clinical mastitis (Grohn and Rajala-Schultz, 2000; Santos et al., 2004a; Santos et al., 2004b). Decreased

conception rates are detected in cows with diseases such as retained placenta and milk fever (Chebel et al., 2004). Retained placentae, for example, increases the risk of fetal losses by 1.8 fold (Lopez-Gatius et al., 1996).

Pregnancy loss in cows has a profound economic impact on producers. Approximately $555 of income is lost for each pregnancy loss in dairy cows (De Vries, 2006). In beef cattle, cows not pregnant at the end of the breeding season incur an added expense of approximately $94, and their value as an open cow is approximately $600 less than a pregnant cow (Dahlen et al., 2014). Failure of pregnancy results in approximately 4.5% of beef cattle being culled annually (Bellows et al., 2002).

The magnitude and severity of pregnancy loss in cattle has prompted scientists to identify ways to limit pregnancy losses. Unfortunately, opportunities to limit pregnancy failures remain undefined. The basis of this work revolves around the notion that more insights into the developmental events that control early events of pregnancy are needed so that new schemes for preventing pregnancy loss can be developed and tested. The following review will highlight several key times in embryonic and placental development in cattle.

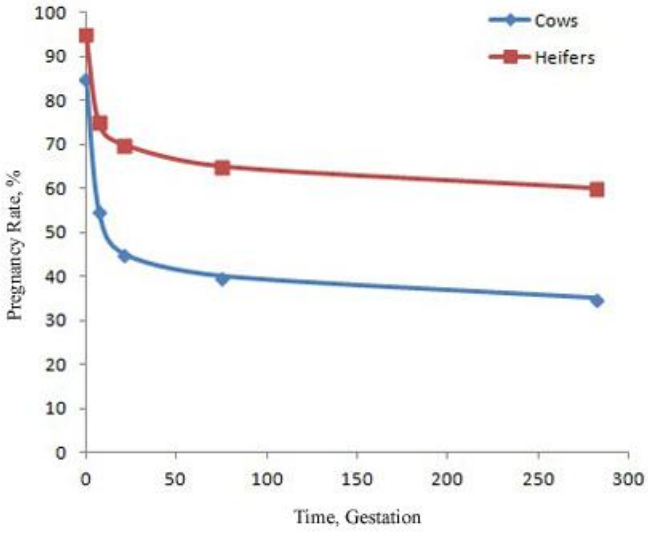

Figure 1: Percentage pregnancy from day 1 to 300 of gestation for lactating dairy cows and heifers (Adapted from Walsh et al., 2011).

EMBRYOGENESIS AND PLACENTAL DEVELOPMENT

Several key development events are indispensible for pregnancy success in mammals. To follow is an overview of key features of embryo development and early placental development that appear essential for the establishment and maintenance of pregnancy.

Embryo development

In mammals, preimplantation development is a process that involves cellular division and cell fate determination into the embryonic and extraembryonic tissues that will eventually form the fetus and placenta. Several key developmental events must occur for development to continue. After fertilization, maternally-derived transcripts and proteins control embryonic development, but by the 2-cell stage in mice, 4-cell stage in pigs, and 8-cell stage in cattle, horses and dogs, the embryonic genome becomes transcriptionally active and takes over control of embryonic processes (Kues et al., 2008; Hall et al., 2013). Following genome activation, compaction occurs via reorganization to achieve apical-basal polarization of the outer-lying blastomeres to promote formation of a blastocyst (Ducibella et al., 1977; Maro et al., 1985; Li et al., 2009). The outer cells of the embryo take on a polarized epithelial phenotype (Fleming and Johnson, 1988), and they gain intercellular junctions, via e-cadherin and other adhesion molecules (Ohsugi et al., 1997; De Vries et al., 2004).

A fluid-filled cavity, called a blastocoel, begins as small luminal spaces combine to form a vesicle (Motosugi et al., 2005). An osmotic gradient of sodium ion (Na^+) concentration is much higher inside the blastocyst cavity, and this leads to water influx (Manejwala et al., 1989; Watson and Barcroft, 2001). Many Na^+/K^+ exchangers are

located at the apical membrane of these outer-lying cells, which will become trophectoderm. They also contain water channels (aquaporins; AQPs) to further control water influx (Borland et al., 1977; Wiley, 1984; Biggers et al., 1988; Barcroft et al., 2003; Bell et al., 2009; Marikawa and Alarcon, 2012). The paracellular sealing of tight junctions is the last step of blastocyst cavity formation (Marikawa and Alarcon, 2012). This will help to maintain the proper concentration of Na^+ inside the cavity and, thereby, maintain fluid (Eckert and Fleming, 2008).

During the time when compaction and cavitation occurs, the blastomeres begin to differentiate into specific lineages. Specifically, the outermost layer of cells forms the trophectoderm, which will develop into the placenta, and the inner-lying cells form the inner cell mass (ICM), which will give rise to the embryo proper and other extraembryonic membranes. The molecular events that control these processes are described below.

Trophoblast lineage specification

Trophoblast is the first cell lineage formed in the embryo. These cells are blastomeres in the outer layer of the embryo. A series of key transcription factors are essential for trophectoderm (TE) formation. Knockout models in the mouse have provided valuable insights into the developmental process of TE and this will be described here. A major player is caudal type homeobox 2 (CDX2). Mouse conceptuses with defects in *CDX2* function fail to produce a placenta and do not implant into the uterus (Strumpf et al., 2005). Interestingly, *CDX2* knockout embryos can generate TE, but this tissue does not proliferate normally (Strumpf et al., 2005; Ralston and Rossant,

2008). *CDX2* blocks *OCT4* and *NANOG* expression in mice, factors needed for maintenance of pluripotency in the ICM (Chen et al., 2009).

Another key transcriptional regulator is TEAD4, a member of TEA domain transcription factor family. Its primary function is to regulate CDX2 expression. *TEAD4* null mouse embryos do not form a blastocoel cavity and do not produce TE (Nishioka et al., 2008). *TEAD4* expression is mediated by another transcriptional controller, termed Yes-associated protein (YAP) (Nishioka et al., 2009). This factor is expressed in all early embryonic blastomeres in mice. It exhibits a nuclear-localization in the ICM but is transported out of the nuclei in the outer cells (i.e. future TE cells) (Nishioka et al., 2009; Marikawa and Alarcon, 2012). This movement is controlled by activation of the Hippo signaling pathway, and specifically Lats1/2 kinases (Nishioka et al., 2009). Lats1/2 in the inner cells blocks the activation of YAP which leads to inactivation of TEAD4 and subsequently inhibits the expression of CDX2 in the inner cells (Nishioka et al., 2009). This Hippo signaling pathway induced restriction of YAP and CDX2 makes the inner cells commit to ICM instead of TE.

Several other transcriptional regulators appear to help control CDX2 expression in mouse embryos. GATA3, a member of GATA family transcription factors characterized by their ability to bind with the DNA sequence “GATA” (Ko and Engel, 1993), is detected early in embryogenesis (e.g. 4-cell stage in mice), and later it is localized in TE (Home et al., 2009; Ralston et al., 2010). Knockdown of *GATA3* in mouse embryos leads to reduced *CDX2* expression and defects in cavitation (Home et al., 2009). Another is SOX2, a HMG (High Mobility Group) domain transcription factor. It is expressed both in TE and ICM, and in mouse knockout models loss of *SOX2* limits blastocyst development

(Keramari et al., 2010). Moreover, knockdown of *SOX2* reduces expression of *CDX2* and *YAP* (Keramari et al., 2010). A kruppel-like zinc-finger transcription factor, KLF5, is reported as an important regulator of blastocyst development (Ema et al., 2008; Lin et al., 2010). KLF5 is expressed in the outer cells of blastocyst and the expression of *CDX2* is reduced in *KLF5* knockout mouse embryos (Lin et al., 2010).

In bovine TE, these transcriptional factors are also expressed and their functions are very similar to these in mice. *TEAD4* RNA expression is detected in cattle morula which means *TEAD4* is activated by Hippo signaling pathway to begin the specification of TE lineage and this is also conserved in mouse (Berg et al., 2011). *CDX2* is also very important for TE lineage specification in cattle, however, *CDX2* in cattle does not repress *OCT4* expression, which is repressed in mouse TE (Berg et al., 2011). *OCT4* expression in bovine TE remains strong even when the expression of *CDX2* is very high (Berg et al., 2011).

The ICM component of the embryo

The inner cell mass is maintained by two transcriptional regulators, OCT4 and NANOG. NANOG maintains these cells in a pluripotent state, as evidenced by *NANOG*-deficient ICM failing to generate an epiblast, the pluripotent cell lineage that gives rise to the embryo proper (Mitsui et al., 2003; Xenopoulos et al., 2012). The expression of *NANOG* is found in all undifferentiated cells of the embryo at the 8-cell stage, and its expression persists within the ICM at the blastocyst stages, then declines until its reappearance in primordial germ cells in mouse (Chambers et al., 2003; Mitsui et al., 2003). Deletion of *NANOG* in mouse embryos leads to loss of pluripotency of both the ICM and embryonic stem cells (Mitsui et al., 2003).

The other transcriptional regulator of pluripotency is OCT4 (Octamer-binding transcription factor 4), which is a POU-domain transcription factor expressed in all cells in early stage embryos (Chambers and Smith, 2004). The expression pattern of *OCT4* is similar to that of *NANOG* in mice, as both are restrictively expressed within the ICM at the blastocyst stage. Later, *OCT4* expression occurs in both epiblast and primitive endoderm cells, a lineage that will produce the yolk sac (Palmieri et al., 1994). Deletion of *OCT4* in mouse embryos results in failed ICM formation. Instead, the interior blastomeres acquire a TE identity (Nichols et al., 1998). Conversely, dominant expression of *OCT4* in ICM inhibits the expression of trophectoderm specific genes (Niwa et al., 2005). However, these same pluripotency systems may not be used by cattle. In cattle, *OCT4* is not restricted to the ICM of even expanded blastocysts (Kurosaka et al., 2004). RNA and protein expressions of *OCT4* are found to be restricted to the epiblast in the late bovine blastocyst stage (Degrelle et al., 2005; Vejlsted et al., 2005). And the expression of *OCT4* remains high in the TE during late blastocyst stage in cattle even *CDX2* is enriched for 4 to 5 days after blastocyst formation (Berg et al., 2011). The expression and function of NANOG in cattle is similar to that in mice which maintains the pluripotency of ICM cells (Yang et al., 2011a).

Conceptus elongation and implantation in farm animals

Unlike rodents, primates, and many other mammals, ruminants do not implant right after the blastocyst hatches from the zona pellucida. Rather, this blastocyst continues to develop while floating freely in the uterine lumen. A process termed elongation occurs prior to implantation (Blomberg et al., 2008). These hatched blastocysts develop from a small spherical shape to one that is ovoid and then tubular. In

cattle, this occurs between days 12 and 14 of pregnancy. By day 16 to 18, the elongating bovine conceptus will occupy the majority of one entire uterine horn and span a length of 10 to 30 cm (Betteridge and Flechon, 1988). The growth results from the rapid expansion of TE numbers. The ICM also is active during this time. Gastrulation occurs while conceptuses are free-floating (Dey et al., 2004). Attachment occurs soon after elongation in farm species. In cattle, this event begins on or after day 19 of pregnancy (Hue et al., 2012).

Loss of anti-adhesive components on the uterine epithelia apical surface is required for the attachment of the conceptus to the uterus (Bazer et al., 2009). One main factor of the anti-adhesive components is Mucin 1 (MUC1), which is considered as a barrier of implantation while elongation in proceeding (Bazer et al., 2009). Down-regulation of MUC1 is associated with a loss of reduction of progesterone receptors in the uterine epithelium, an event that occurs whether the animal is pregnant or open (Johnson et al., 2001). This pattern of decreased MUC1 has not been delineated in cattle, but in ewes this occurs between days 9 and 17 of pregnancy (Johnson et al., 2001). Removal of MUC1 allows the exposure of various uterine epithelial cell surface agents that are involved with the adhesion of trophoblast and uterine epithelium (Bazer et al., 2009). These surface endometrial proteins include integrins, galectin-15, and osteopontin (Johnson et al., 2001; Burghardt et al., 2002; MacIntyre et al., 2002; Johnson et al., 2003).

Maternal recognition of pregnancy in cattle

Corpora Lutea (CL) secreted progesterone plays essential roles in supporting secretory functions of the uterine endometrium which are indispensible for conceptus

development and implantation (Bazer et al., 2009). Maternal recognition of pregnancy needs to maintain the functional CL for production of progesterone and this leads to the uterine receptivity of implantation in most mammals (Fazleabas et al., 2004).

In cattle, interferon tau (IFNT) is very essential as it regulates implantation and maintains early pregnancy (Demmers et al., 2001; Spencer et al., 2004). During pregnancy, IFNT prevents luteolysis through blocking the production of PGF2α of ruminants (Demmers et al., 2001; Spencer et al., 2004). The result of IFNT induced blocking of PGF2α is the maintenance of a functional CL for progesterone, which is permissive to actions of uterine secretions (Soares, 2004; Spencer et al., 2007; Bazer et al., 2009). Other roles of IFNT are found in inducing uterine factors which promote conceptus development (Asselin et al., 1997; Arosh et al., 2004).

UTERUS DEVELOPMENT AND FUNCTION

Successful pregnancy cannot happen without an appropriate uterine environment for the survival and development of embryo and extraembryonic tissues. The uterus has several functions, and these will be described below.

Uterine function and its involvement with infertility

The primary function of the uterus is to provide an environment that supports pregnancy. These supportive actions of the uterus can be manipulated by several physiological states. Lactation has a profound effect on uterine functions. Food intake also impacts uterine function. For example, in one study, dietary restriction decreased fetal size in sheep with a restriction between day 50 and day 155 (Faichney and White, 1987). One way that lactation and nutrition impact uterine function is by modifying systemic progesterone concentrations. Progesterone is crucial for uterine gene expression during implantation and the maintenance of pregnancy thereafter (Bazer et al., 2008), and

alterations in the pattern of progesterone production or overall amounts of progesterone can greatly impact pregnancy outcomes. Lactating cows and animals that are undernourished usually contain lower progesterone concentrations (Kenyon et al., 2013), and lower progesterone concentrations are associated with reduced pregnancy rates in cattle (Kenyon et al., 2013). The cause of reduced circulating progesterone concentration is not necessarily caused by reduced progesterone synthesis. In most cases, luteal progesterone production is increased with lactation or nutrient deprivation (Sangsritavong et al., 2002). Instead, it appears that lactation and undernutrition impact progesterone metabolism. Liver metabolism of progesterone increases in these conditions, and this reduces circulating progesterone concentrations (Sangsritavong et al., 2002; L. F. Ferraretto, 2014).

Fetal uterine development

During gastrulation, the intermediate mesoderm generates the urogenital system. The female reproductive tract (FRT) is initially formed from this system (Cunha, 1975; Kobayashi and Behringer, 2003; Spencer et al., 2012). The urogenital system will differentiate into kidneys, gonads, urinary and reproductive tracts. After that, the embryonic intermediate mesoderm proliferates and some mesenchymal cells will transit to epithelial cells, which then generate the tubules that compose the male and female reproductive tracts, kidneys and gonads (Kurita, 2011). The FRT system develops primarily from Müllerian ducts and arises as invaginations of thickened epithelium at the urogenital ridge (Spencer et al., 2012). The epithelial invagination then extends to form the primordium of FRT. Then the right and left Müllerian ducts cross the Wolffian ducts when they begin to fuse with each other. The fusion of the Müllerian ducts, the Wolffian

ducts and the urogenital sinus forms the sinovaginal bulbs as they are projections of solid epithelia cords of urogenital sinus (Spencer et al., 2012).

Prior to sex determination, fetal reproductive tracts are bipotential and have the ability to form male and female reproductive systems. The Müllerian ducts differentiate into the FRT which include oviducts, cervix, and the upper portion of the vagina as well as uterus (Cunha, 1975; Kurita, 2011; Spencer et al., 2012). In females, the bipotential gonad differentiates into an ovary and this new set of ovaries secrets hormones that promote female sexual tremination (Jordan and Vilain, 2002). The differentiating ovaries in the females do not produce Müllerian inhibiting substance, and the lack of this male-determining factor prevents differentiation and development of Wolffian ducts, the ducts needed for development of male reproductive tract organs. Rather, Müllerian ducts will develop and eventually form the oviduct, uterus, cervix and vagina (Cunha, 1975; Kurita, 2011; Spencer et al., 2012). In farm animals, the Müllerian ducts fuse more posteriorly compared with rodents or higher primates, which leads to an extended bicornuate uterus with a small common corpus with a single cervix and vagina (Mossman, 1987).

Postnatal uterine development

Unlike most organs, the development of the uterus is not complete at birth. Specifically, neonatal uteri lack the endometrial glands required for sustaining a pregnancy to term. In the sheep, a well studied model for uterine gland development, or uterine adenogenesis, these processes are not complete until postnatal day 15 (P15) in mice, P56 in sheep, and P120 in pigs (Bartol et al., 1999; Gray et al., 2001a; Hu et al., 2004a; Hu et al., 2004b; Spencer et al., 2012). The timeline of uterine adenogenesis has

not been defined in cattle, but these events are well defined in sheep, and this species will be highlighted below to describe postnatal uterine development in ruminant species.

Postnatal adenogenesis involves the organization, proliferation, and differentiation of the uterine endometrial epithelium (Spencer et al., 2012). The major steps of adenogenesis begin between P0 and P7 in sheep with noted invagination, or pitting, of endometrial epithelia into the underlying stroma. Soon thereafter, between P7 and P21, these small buds proliferate further into the surrounding stroma (Gray et al., 2001a; Spencer et al., 2012). After P21, these endometrial glands further coil and branch to develop deeper into stroma, and by P56 the uterus is similar to what is observed in adult. The speed in progression through these events differs among species.

Several cellular and molecular mechanisms are required to regulate postnatal uterine morphogenesis. These include paracrine and endocrine factors, steroids, and other factors. Estrogens play an important role in adenogenesis. Neonatal sheep have circulating estrogens which promote both stroma and epithelial cell proliferation (Quarmby and Korach, 1984). Estrogen acts on uterine development through its receptor (ER). Two types of this receptor exist, ERα and ERβ. Both the endometrial epithelium and stroma contain ERα. Estrogen induced cell proliferation acts through ERα. This conclusion is based on observing that *ERα* knockout mice lack an endometrial epithelium proliferation response (Lubahn et al., 1993). Interestingly, this proliferation response appears to be mediated by the stroma, since mice lacking *ERα* only in the endometrial epithelium can still undergo adenogenesis (Cooke et al., 1997; Winuthayanon et al., 2010). The proliferative response can be disrupted by progesterone (Kurita et al., 1998). Exposure to progesterone in the early postnatal period inhibits endometrial gland

development in mice and sheep (Gray et al., 2001b; Filant et al., 2012). This occurs by progesterone down-regulating endometrial ERα expression.

Several growth factors are needed for adenogenesis. One large class of paracrine factors thought to play important roles in adenogenesis are the fibroblast growth factors (FGFs). One of the key FGFs is FGF10, which is secreted from the stroma and acts on the epithelial layer through its receptor FGFRIIIb. FGF10 mRNA expression level is constant between P1 and P21 and increases between P21 and P28, which is the period underlying coiling and branching morphogenesis of endometrial glands. It remains abundant thereafter in neonatal uterus of ewes (Taylor et al., 2001; Spencer et al., 2012). A paracrine factor that usually works in concert with FGF10 is Hepatocyte Growth Factor (HGF). This factor is also expressed in stromal cells and interacts with its receptor, MET, on endometrial epithelia, to regulate adenogenesis. Similar to FGF10 mRNA expression, HGF mRNA levels increase between P21 and P28, and remain high thereafter (Taylor et al., 2001). The close association of these two factors with uterine gland coiling and branching is consistent with the activities of these factors in other organs, like intestines and lung, where their activities are essential for organogenesis (Weidner et al., 1993; Bellusci et al., 1997). Another class of growth factors implicated in adenogenesis is insulin-like growth factors (IGFs). In the neonatal ovine uterus, both stromal produced IGF1 and IGF2 increase in expression between P1 and P28 and remain in high thereafter (Taylor et al., 2001). Interestingly, mRNA expression of the primary receptor for IGF1, IGF1R, is present in high concentrations on P1, but decreases at P14 and then increases thereafter P56. The reason for this gene expression pattern is still unclear.

The specific contributions of FGF10, HGF, and the IGFs with adenogenesis are inferred based on the timing of their expression and because of the need for these factors with establishing branching epithelial morphogenesis in other organs (Weidner et al., 1993; Bellusci et al., 1997; Nayak and Giudice, 2003). Inappropriate estrogen or progesterone exposure disrupts the expression of HGF, FGF10, IGF1, IGF2 and their receptors, and this leads to abnormal (i.e. sub-standard) endometrial gland formation (Hayashi et al., 2004).

A group of developmental regulators known as the *WNT* genes are indispensable during adenogenesis. *WNT* genes are homologous to the *Drosophila* segment polarity gene *wingless,* which has roles in cell proliferation, differentiation and growth. *WNT5A* and *WNT7A* genes have essential roles in both prenatal and postnatal uterine development (Miller et al., 1998; Parr and McMahon, 1998; Mericskay et al., 2004). The developing neonatal ovine uterus exhibits *WNT5A*, *WNT7A*, and *WNT11* expression in the endometrial epithelia at birth and thereafter, whereas *WNT2B* localizes to the stroma from P14 to P56 (Hayashi and Spencer, 2006). Reducing or ablating mRNA expression of *WNT2B*, *WNT7A*, and *WNT11* prevents gland development in sheep (Hayashi and Spencer, 2006). These *WNT* factors act through a class of receptors known as frizzled receptors, which are expressed in all uterine cells (Lee et al., 2004).

Prolactin (PRL) is another important regulator of adenogenesis. It is a multifunctional hormone that acts on numerous organs and organ systems to regulate growth, viability and differentiation (Bole-Feysot et al., 1998; Freeman et al., 2000). Its receptor, PRLR, has both short and long forms, and both forms are expressed mainly in glandular endometrial epithelium (Taylor et al., 2000). Circulating levels of PRL in

neonatal ewes are high on P1, increase further by P14, and then decline slightly by P56 (Taylor et al., 2000; Carpenter et al., 2003a; Carpenter et al., 2003b). Exogenous supplementation of PRL from P0 to P56 in sheep increased the number of endometrial glands on P14 and P56 whereas inhibition of PRL from P0 to P56 reduced gland number (Carpenter et al., 2003b).

Recently, a lactocrine mechanism has been proposed to regulate adenogenesis. The term "lactocrine" refers to the transfer of bioactive factors to offspring through the milk. This response is best studied in pigs. Retarded endometrial development and adenogenesis are found in gilts fed with milk replacer within 48 h after birth (Miller et al., 2013). Reduced expression of endometrial ERα is observed on P2 to P14 in these gilts fed with milk replacer (Chen et al., 2011; Miller et al., 2013). One factor in milk that may mediate these lactocrine responses is relaxin (Hausman et al., 2006; Ivell et al., 2007). Relaxin is found in porcine colostrum, and supplementation with additional relaxin increases uterine ERα and VEGFA mRNA expression (Chen et al., 2011). Moreover, milk replacer reduces uterine protein expression of ERα, VEGFA and MMP9 within 48 hours after birth in neonatal pigs (Chen et al., 2011). The uterine adenogenesis of pigs is an estrogen dependent process as early uterine epithelial cell proliferation is induced by estrogen and its type 1 receptor ERα (Spencer and Bazer, 2004). Increasing the expression of ERα by relaxin means it helps with uterine cell proliferation and gland development in the neonatal uterus of pigs. And the increased level of VEGFA which is directly related with angiogenesis is associated with the uterine vascular development (Shibuya, 2013).

Information gathered from a uterine gland knockout (UGKO) phenotype

The postnatal endometrial gland morphogenesis described previously can be inhibited in sheep by chronic exposure to a synthetic progestin, norgestomet after birth (Bartol et al., 1988; Spencer and Gray, 2006). Exposure of lambs to norgestomet from birth to P13 completely inhibits endometrial gland development in many cases (Bartol et al., 1988). Removal of norgestomet at P13 will permit glands to develop, but these glands are abnormal in conformation and usually underdeveloped (Bartol et al., 1988). The presumptive target for progestins is ERα, where they prevent ERα expression, thereby preventing estrogens from promoting adenogenesis (Allison Gray et al., 2000). Progestins also inhibit endometrial epithelial development directly, by limiting epithelial cell cycle progression (Stewart et al., 2011). These progestinized ewes result in what is best described as a uterine gland knockout (UGKO) phenotype in adulthood (Bartol et al., 1999; Gray et al., 2001a; Cooke et al., 2013). This progestin-induced uterine knockout phenotype also can be generated in mice, cattle and pigs (Bartol et al., 1993; Bartol et al., 1995; Cooke et al., 2012; Filant et al., 2012). The disruption of endometrial gland development by progestins does not retard or inhibit development of other reproductive organs, including the ovary and oviduct. It also has little impact on the hypothalamic-pituitary-ovarian axis (Spencer et al., 2012). The expression of estrogen, progesterone and oxytocin receptors as well as ovarian function is not different in UGKO ewes as compared with controls (Allison Gray et al., 2000). However, the UGKO ewes lack a 16-17d estrous cycle interval because luteolysis is interrupted and often delayed. This occurs because the endometrial epithelium, the primary uterine source for PGF2α, is not present in sufficient quantities to generate a luteolytic signal. Providing exogenous $PGF_{2\alpha}$ can

induce luteolysis and return to estrus (Allison Gray et al., 2000; Gray et al., 2001b; Gray et al., 2002).

The pregnancy outcomes of UGKO ewes have provided much insight into the necessity of uterine secretions during early pregnancy. Ewes will breed normally, but at D25 pregnancies are not detected in UGKO ewes (Gray et al., 2001b). Fertilization and early embryogenesis processes appear to occur normally in UGKO ewes. The progression to the blastocyst stage, for example, occurs similarly in UGKO and control ewes (Gray et al., 2001b; Gray et al., 2001c; Gray et al., 2002). However, by Day14, the conceptus is either absent or growth retarded and fails to undergo sufficient maternal recognition of pregnancy (Gray et al., 2001c; Gray et al., 2002). The current theory for failed peri-implantation development in UGKO ewes is that the absence of sufficient quantities of uterine gland secretions prevents normal post-blastocyst stage conceptus development (Spencer and Gray, 2006). Uterine secretions are indispensible for conceptus elongation and implantation.

Other lines of evidence demonstrate the necessity for proper uterine gland development and function for optimal pregnancy outcomes. Birth weight of swine fetus is directly associates with maternal uterine gland number and placental interactions with these glands (Knight et al., 1977; van Rens and van der Lende, 2002). If schemes for improving adenogenesis can be created, animal producers may be able to increase litter size and potentially reduce embryonic loss. Also, uterine gland dysfunction may underlie human infertility. Between 50% to 75% of all pregnancies in women fail soon after embryo transfer, when uterine secretions are presumably essential for sustaining embryonic development (Fazleabas, 2007).

Uterine secretions

The composition of secretions produced by uterine glands has been investigated extensively in the past several decades (Bell, 1988; Roberts and Bazer, 1988; Beier-Hellwig et al., 1989). These secretions are comprised of various metabolic compounds (lipids, carbohydrates, and amino acids), enzymes, and hormones. Collectively, these uterine gland secretions are known as histotroph. Histotroph is essential for the development of conceptus as well as fetal-placental growth in the uterus (Spencer and Bazer, 2004). One way histotroph affects fetus development is through inducing the growth of uterine blood vessels (Spencer and Bazer, 2004). The nutrients contained in uterine histotroph provide a valuable source of energy to support conceptus development before it attaches to the uterine epithelium. In addition, the importance of uterine gland secretions continues after implantation in farm animals. Cows, sheep and pigs contain placentally-derived structures termed areolae, which form around the openings of uterine glands and presumably aid in the uptake of uterine gland secretions (Song et al., 2010; Bazer and Johnson, 2014).

In the human, the histotroph also regulates maternal immune responses with placental tissues (Hempstock et al., 2004). Glycodelin acts as an immunosuppressive factor in the intervillous space and inhibits T-cell. Gestation promotes stromal decidual cells migration to the basal lamina of the glandular epithelium (Seppala et al., 1998; Rachmilewitz et al., 1999; Hempstock et al., 2004). It is not clear if glycodelin plays the same roles in domestic farm animals.

PARACRINE FACTORS IN THE UTERUS

Several uterine-derived hormones and cytokines have been implicated as critical regulators of conceptus development in mice, sheep, cattle, pigs and humans. This thesis

will focus on the importance of three of these factors; epidermal growth factor (EGF), fibroblast growth factor 2 (FGF2), and insulin-like growth factor 1(IGF1).

Epidermal growth factor

Approximately 50 years ago, Dr. Stanley Cohen discovered EGF (Cohen, 1962). It is expressed in various organs, such as the mammary, kidney, intestine, ovary and placenta (Carpenter and Cohen, 1979; Read et al., 1984; Fisher et al., 1989; Fisher and Lakshmanan, 1990; Hofmann and Abramowicz, 1990; Hsieh and Conti, 2005; Nair et al., 2008). EGF is best known as a mitogen (Dackor et al., 2007; Hambruch et al., 2010; Jeong et al., 2013; Cha et al., 2014; Fujihara et al., 2014). It also has effects in stem cell expansion and differentiation (Garcez et al., 2009).

The actions of EGF are controlled by its interaction with a specific tyrosine kinase receptor, termed EGFR (Schneider and Wolf, 2009). This receptor is a member of the EGFR/ERBB superfamily. All of these receptors regulate cell signals after forming dimers, undergoing autophosphorylation events, and thereafter mediating multiple intracellular signaling pathways (Schneider and Wolf, 2009). The MAPK and PI3K/AKT are the most common signaling pathways of EGF. Both of these pathways are implicated in controlling EGF induced cell proliferation in pig and cow trophoblast cells (Hambruch et al., 2010; Jeong et al., 2013).

During embryogenesis, EGF promotes early embryo growth both pre- and post-implantation in mouse and pig (Nielsen et al., 1991; Wei et al., 2001; Dadi et al., 2007). In mice, reduced maternal EGF retards fetal growth (Kamei et al., 1999). Also, TE fails to proliferate in *EGFR* knockout mice (Wiley et al., 1992; Wei et al., 2001; Dackor et al., 2007). EGF also plays roles as potent apoptosis inhibitor as it stimulates cell survival

(Smith et al., 2002; Johnstone et al., 2005). TNFα-induced human cytotrophoblast apoptosis is inhibited with EGF supplementation (Johnstone et al., 2005).

The mitogenic effects of EGF also exist in TE from farm animals. Proliferation of a bovine placental trophoblast cell line is stimulated by EGF supplementation, and this activity appears to be transduced through Ras and MAPK3/1 dependent systems (Hambruch et al., 2010). EGF also stimulates the proliferation and migration of porcine trophectoderm cells during early pregnancy (Jeong et al., 2013). PI3K/AKT1 and ERK1/2 MAPK-P90RSK are activated in EGF induced porcine trophoblast proliferation and migration (Jeong et al., 2013). However, it remains unclear if EGF functions early during TE development to mediate proliferation and gene expression.

Fibroblast growth factor 2

Fibroblast growth factors (FGFs) are an extensive family of paracrine, autocrine and endocrine factors that control various biological systems (Burgess and Maciag, 1989; Bottcher and Niehrs, 2005). There are 22 FGFs in mice and humans (Itoh and Ornitz, 2004). Most of the FGFs contain proliferative, differentiative, and mutagenic activities (Abud et al., 1996; Goldfarb, 1996; Itoh and Ornitz, 2004, 2008). These proteins act through FGF receptors (FGFRs), which are comprised of 4 tyrosine kinase receptor isoforms. Spliced variant modifications in ligand binding domains provide specificity for ligand binding. Different FGFs bind to single or multiple different receptors, depending on the ligand. For example, FGF1 binds to all FGFRs while FGF7 only binds to one specific subtype of one receptor (termed FGFR2b) (Powers et al., 2000; Itoh and Ornitz, 2004).

Fibroblast growth factor 2 (FGF2), also known as basic FGF, was first isolated in late 1970s (Gospodarowicz, 1974). FGF2 can bind to several FGFRs (Werner et al., 1992; Johnson and Williams, 1993; Ornitz and Itoh, 2001; Eswarakumar et al., 2005). It is best known as an angiogenesis factor in many organs (Weylie et al., 2006), including the uterus where it helps to control the increase in uterine blood flow observed during early pregnancy in ruminants (Krishnamurthy et al., 1999; Mata-Greenwood et al., 2010). FGF2 is expressed in both luminal and glandular epithelium in ewes and cows throughout the estrous cycle and early pregnancy (Carlone and Rider, 1993; Grundker and Kirchner, 1996; Gupta et al., 1997; Itoh and Ornitz, 2004; Ocon-Grove et al., 2008). Moreover, each of the 4 FGFRs is transcribed active throughout early bovine embryogenesis (Cooke et al., 2009). Specifically, each FGFR is expressed in TE at the blastocyst stage and thereafter (Cooke et al., 2009; Ozawa et al., 2013).

FGF2 plays essential roles in regulating cell proliferation and differentiation during pregnancy. FGF2 promotes bovine primitive endoderm (PE) formation through inducing PE proliferation (Yang et al., 2011a). Moreover, FGF2 stimulates migration in both ovine and bovine trophoblast cells (Yang et al., 2011b). Supplementation with FGF2 of high concentrations also increases bovine blastocyst formation (Fields et al., 2011). Another study shows that FGF2 increases the production of interferon-tau (IFNT) which is a trophoblast-secreted pregnancy signal in bovine blastocyst (Yang et al., 2011c).

Insulin-like growth factor1

Insulin-like growth factor1 (IGF1) is so named because of its structural similarity to insulin. It is well known for controlling various growth mediating activities throughout the body. The circulating form of IGF1 is produced primarily by the liver and promotes

growth through the pituitary growth hormone (Laron, 2004). Various other organs and tissues also produce IGF1, and these sources are normally associated with local (i.e. paracrine) responses. In general, the endocrine actions of IGF1 usually are growth hormone dependent, whereas the autocrine and paracrine actions of IGF1 are growth hormone independent (Lupu et al., 2001). The primary receptor for IGF1 is termed IGF1R. It is a receptor tyrosine kinase that is structurally similar to the insulin receptor (Worrall et al., 2013). Different signaling pathways are involved in IGF1 induced cell development. MAPK and PI3K/AKT are the main signaling pathways activated by IGF1 in its different actions (Forbes et al., 2008; Jeong et al., 2014).

The uterus produces IGF1 (Taylor et al., 2001; Forbes et al., 2008; Jeong et al., 2014). Post puberty, the transcript expression of *IGF1* increases fivefold in the bovine uterus (Oberbauer et al., 2014). *IGF1R* is detected in bovine preimplantation embryos and it's mainly distributed in the TE but not in the ICM (Wang et al., 2009). Supplementation with IGF1 and several other growth factors accelerates embryonic development with increased hatched blastocyst rate in bovine (Neira et al., 2010).

Little is known about the actions of IGF1 in regulating cow trophoblast development but it's quite clear in humans and pigs. IGF1 promotes human normal placental cytotrophoblast cell proliferation and it also rescues cytotrophoblast from apoptosis (Li and Zhuang, 1997; Forbes et al., 2008). IGF1 induced human cytotrophoblast proliferation as well as syncytial formation through IGF1R-mediated activating MAPK3/1 pathways (Forbes et al., 2008). However, IGF1R activation of PI3K pathway rescues these cytotrophoblast from apoptosis (Forbes et al., 2008). Similarly, IGF1 also promotes porcine trophectoderm proliferation and migration (Jeong et al.,

2014). PI3K/AKT and MAPK3/1 pathways are activated by IGF1R in IGF1 induced porcine trophoblast cell proliferation and migration (Jeong et al., 2014). Supplementation of IGF1to pregnant pigs during mid-gestation increases both placental and fetal weights (Sferruzzi-Perri et al., 2006).

Additive effects of EGF, FGF2 and IGF1

Combining growth factors may improve embryo development during early pregnancy. There is a study which shows the combination of IGF1, FGF2, LIF (leukemia inhibitory factor), TGF-β (transforming growth factor), and IGF2 stimulates bovine embryonic development to a greater extent than when each factor is provided individually (Neira et al., 2010). The combination of growth factors also maximizes embryo hatching rates (blastocyst expulsion from the zona pellucida). Also, the number of TE and ICM cells is greater at day 8 after fertilization in embryo exposed to the set of factors versus synthetic oviduct fluid alone (Neira et al., 2010). It is not known if these and other uterine-derived factors may impact TE development and function after its formation after the blastocyst stage.

SUMMARY OF PREVIOUS LITERATURE

The formation of a bovine blastocyst includes a series of proliferation and differentiation events. At day 7 to 8 post-fertilization, the blastocyst contains an ICM and TE. Thereafter, the blastocyst hatches from its egg shell, termed the zona pellucida, and continues to proliferate and change from a spherical to an elongated, filamentous shape as it begins to interact with a uterine lining. The uterus provides an appropriate environment for the survival, development and growth of the conceptus. The development of uterine glands is initiated after birth. The formation of uterine glands is vital for pregnancy in ruminants. Among the various components of uterine secretions are several hormones,

and three of these hormones, EGF, FGF2 and IGF1, are candidate mediators of early conceptus development. No definitive experiments have tested how these factors impact bovine TE development and function. Moreover, it is not clear if these factors act in additive or synergistic fashions when they are co-administered, as would normally occur in uteri.

CHAPTER II: ADDITIVE EFFECTS AMONG UTERINE PARACRINE FACTORS IN PROMOTING BOVINE TROPHOBLAST CELL PROLIFERATION

INTRODUCTION

Early conceptus development in ruminants is different from rodents, primates and most other mammals, where several post-blastocyst stages of conceptus development occur prior to uterine adhesion and implantation. In cattle, trophoblast adhesion to the uterine endometrium occurs around day 19-21 of pregnancy (Guillomot, 1995; Yang et al., 2011b). Before attachment, morphological changes of the ruminant conceptus occur as it develops from a small, spherical blastocyst into a filamentous structure that will span the length of one uterine horn before implantation begins (Clemente et al., 2011). This elongation phase occurs between days 14 to 16 of pregnancy in cattle, and trophoblast cells proliferate rapidly during this period so that they may increase the early placental surface area contact with the uterine endometrium (Schlafer et al., 2000). Also, the trophoblast must act on the uterus by producing interferon-tau, which prevents luteolysis by modifying PGF2α production, the major controller of corpus luteum regression in ruminants (Demmers et al., 2001; Spencer et al., 2004). This IFNT-induced block in PGF2α maintains high circulating levels of progesterone, which maintains a pregnant uterine state and promotes uterine secretions that control conceptus development (Soares, 2004; Spencer et al., 2007; Bazer et al., 2009). The developmental events occurring at this time are indispensible for the continuation of pregnancy, and miscues in these processes are considered to be a major contributor to pregnancy loss in cattle (Moore and Thatcher, 2006).

The trophectoderm (TE) lineage is the first differentiated cell type created by the early developing embryo. It will form around the outside of the embryo and eventually develop into the outermost layer of the placenta. A series of transcription factors are essential for TE development. Several major players are CDX2, TEAD4 and HIPPO (Strumpf et al., 2005; Nishioka et al., 2009; Berg et al., 2011).

The early events of embryogenesis, including TE lineage specification, can occur normally in vitro without inclusion of any oviductal or uterine factors (Hardy et al., 1989; Narula et al., 1996). Also, blastocysts form normally in ewes lacking uterine glands (Gray et al., 2001c). However, post-blastocyst development requires uterine factors. Also, conceptus elongation is severely compromised in uterine gland knockout ewes (Gray et al., 2001c; Gray et al., 2002). This and other evidence implicates uterine secretions, termed histotroph, as being essential for elongation, maximal IFNT production, and implantation in ruminants (Spencer and Bazer, 2004). Several locally produced hormones exist in histotroph. Three of special interest are EGF, FGF2, and IGF1. EGF stimulates bovine placental trophoblast cell line F3 proliferation and stimulates proliferation and migration of porcine trophectoderm (Hambruch et al., 2010; Jeong et al., 2013). FGF2 promotes bovine primitive endoderm (PE) formation (Yang et al., 2011a). Moreover, FGF2 stimulates migration in both ovine and bovine trophoblast cells and increases IFNT expression (Michael et al., 2006; Cooke et al., 2009; Yang et al., 2011b; Yang et al., 2011c). In bovine embryos, IGF1 blocks the induction of apoptosis and enhances resistance of embryos to various stresses (Jousan and Hansen, 2004, 2007). IGF1 also promotes porcine trophectoderm proliferation and migration (Jeong et al., 2014).

Based on past findings, we propose that EGF, FGF2, and IGF1 promote bovine trophoblast cell proliferation after blastocyst formation, and this activity helps to mediate conceptus development and elongation. There is evidence that combined action of growth factors maximizes bovine blastocyst formation in vitro (Neira et al., 2010), but no work has been completed to examine the specific effects of uterine factors on trophoblast cells. This work will utilize two models for early trophoblast cells. The first is a bovine trophoblast cell line CT1, which was derived from an in vitro-produced bovine blastocyst (Talbot et al., 2000; Talbot et al., 2007). The second is in vitro produced bovine embryos which will generate trophoblast cells around day 4 to 5 post-in vitro fertilization (Berg et al., 2011).

MATERIALS AND METHODS

Trophoblast cell culture

The CT1 bovine trophoblast cell line was isolated by Talbot et al (Talbot et al., 2000; Talbot et al., 2007). The cell line has since been propagated without a feeder-cell layer. Instead, these cells were propogated on Matrigel Basement Membrane Matrix (BD Biosciences, Bedford, MA) as described previously (Michael et al., 2006). Cultures were maintained in Dulbecco's Modified Eagle's medium (DMEM; Invitrogen Corp. Carlsbad, CA) containing supplements (10% [v/v] fetal bovine serum [FBS], 100 µM non-essential amino acids, 55 µM β-mercaptoethanol, 100 U/ml penicillin G, and 100 µg/ml streptomycin sulfate; Invitrogen Corp. Carlsbad, CA) at 38.5 °C with 5% CO2 in air. Cells were passaged manually by separating them from plates with a cell scraper and dissociating them into small clumps with repeated dissociation through a 20-ga needle and syringe.

CT1 cell proliferation assay

Cells were seeded onto 24-well CellBIND plates (Corning Crop. Corning, NY) that lacked Matrigel. After 2 d, medium was removed and replaced with DMEM lacking FBS but containing all other supplements. After 11-16 h, cells were supplemented with 10 ng/ml EGF (R&D Systems, Minneapolis, MN), 10 ng/ml boFGF2 (R&D Systems, Minneapolis, MN), 50 ng/ml IGF1 (Promega Corp. Madison, WI) or vehicle (1% w/v bovine serum albumin [BSA], Invitrogen Corp. Carlsbad, CA) in fresh serum-free medium. After 22-24 h exposure to EGF, FGF2 or IGF1, EdU reagent was added (100 µM) and cells were incubated for 45 min before fixation in 4% paraformaldehyde (Invitrogen Corp. Carlsbad, CA) in phosphate buffered saline (PBS; Invitrogen Corp. Carlsbad, CA) for 15 min. EdU-positive cells were determined by reaction with Alexa Flour 488 azide. All nuclei were counted in 5 representative fields (about 30-150 cells/field)thermo in each well using NIS-Elements Software (Nikon) after capture on an Eclipse Ti-E inverted microscope equipped with an X-Cite 120 epiflourescence illumination system and Nikon DS Camera Control Unit DS-L3 digital camera. The mitogenic index of trophoblast was determined using the Click-It^{TM} EdU (5-ethynyl-2'-deoxyuridine) Cell Proliferation Assay (Life Technologies Corp. Eugene, OR).

For studies examining the effects of pharmacological inhibitors on EGF, FGF2, and IGF1 induced cell proliferation, CT1 cells were serum-starved for 11-16 h and treated with 50 µM PD98059 (pharmacological inhibitor of MAPK3/1, Cell Signaling Technology Corp. Beverly, MA), 0.2 µM Wortmannin (pharmacological inhibitor of AKT, Cell Signaling Technology Corp. Beverly, MA) or carrier only (1% w/v BSA, Invitrogen Corp. Carlsbad, CA) in fresh serum-free medium for the final 2 h of serum-

starvation. Cell medium then was added with 10 ng/ml EGF, 10 ng/ml FGF2, 50 ng/ml IGF1 or vehicle. The proliferation assay was taken after 22-24 h as described previously.

Western blot analyses

CT1 cells were seeded onto 6-well CellBIND plates lacking Matrigel. After 5 days (approximately 30-50% confluency), medium was replaced with DMEM lacking FBS but containing all other supplements. After 11-16 h, cells were treated with fresh serum-free medium with pharmacological inhibitors or carrier only. After 2 h, medium was added that contained vehicle only, 10 ng/ml EGF, 10 ng/ml boFGF2, 50 ng/ml IGF1, or all factors, depending on the study. Cells were collected either immediately before (time 0) or at several time-points (0, 5, 15, 30, 60 or 240 min) after growth factor supplementation. Cells were harvested by rinsing in 0.1 M PBS [pH 7.4], and then lysed in Radioimmunoprecipitation assay buffer (RIPA; 20 mM Tris-HCL pH 7.5, 150 mM NaCl, 1% v/v NP40, Thermo Scientific, Rockford, IL) supplemented with protease and phosphatase inhibitor cocktail (Thermo Scientific, Rockford, IL). Cell lysates were sonicated for three times (10 seconds each). Protein concentrations were determined using a BCA Protein Assay (Fisher Scientific, Fair Lawn, NJ).

Protein samples (10 µg) were loaded onto 10% [w/v] Tris-Glycine Mini Protein Gels (Life Technologies Corp. Carlsbad, CA) and electrophoresed. Proteins were then electro-transferred onto 0.45 µm PVDF. Membranes (Invitrogen Corp. Carlsbad, CA) were blocked 5% [w/v] nonfat dry milk in TBST (50 mM Tris HCl pH 7.6, 150 mM NaCl, 0.1% [v/v] Tween-20, Sigma-Aldrich, St. Louis, MO), then incubated overnight at 4 °C with antibodies against phosphorylated-ERK1/2 (1:2000), total-ERK1/2 (1:2000), phosphorylated-AKT (1:2000) or total-AKT (1:1000) (Cell Signaling Technology Corp.

Beverly, MA). Peroxidase-Goat anti-rabbit IgG were used as secondary antibody (Invitrogen Corp. Carlsbad, CA). After rinsing, the Novex ECC HRP Chemiluminescence Substrate Reagent Kit (Invitrogen Corp. Carlsbad, CA) was used to visualize reactive bands after exposure to BIORAD film. Band intensities were quantified after scanning using Image Lab software (BIORAD, USA). All studies were replicated 3 separate occasions.

For studies examining the effects of pharmacological inhibitors on EGF, FGF2, and IGF1 induced phosphorylation, CT1 cells were serum-starved for 11-16 h and treated with 50 µM PD98059, 0.2 µM Wortmannin or carrier only in fresh serum-free medium for the final 2 hours of serum-starvation. Cell medium then was added with 10 ng/ml EGF, 10 ng/ml FGF2, 50 ng/ml IGF1 or vehicle. Cells were collected either immediately before (time 0) or at several time-points (0, 15, 30, 60 or 240 min) after growth factor supplementation. The Western blot analysis was completed as described previously.

In vitro production of bovine embryos

In vitro production of bovine embryos was completed as described previously (Rivera and Hansen, 2001; Zhang et al., 2010), with minor modifications. Ovaries from beef and dairy cattle were obtained from Brown Packing Co. (Gaffney, SC). Cumulus-oocyte-complexes (COCs) were obtained by slashing follicles with a scalpel blade, and then by searching with a Nikon SMZ745 stereomicroscope (Nikon Instruments Inc., Melville, NY). Groups of 20-30 COCs were cultured in 4-well Nunc plates (NUNC, Roskilde, Denmark) with 500 µl oocyte maturation medium (TCM199 containing Earle's salts [Life Technologies Corp. Grand Island, NY], 10% FBS, 25 µg/ml bovine FSH [Bioniche Life Sciences, Bellvill, ON, CA], 2 µg/ml estradiol [Sigma-Aldrich, St. Louis,

MO], 22 µg/ml sodium pyruvate, 1 mM glutamine, and 25 µg/ml gentamicin sulfate [Life Technologies Corp. Grand Island, NY]) each well at 38.5°C in 5% CO_2 in humidified air. After 21-22 h, COCs were transferred to fertilization medium, and exposed to BoviPure™ gradient-purified (Nidacon, Spectrum Technologies, Healdsburg, CA) bovine spermatozoa derived from a pool of frozen semen from 4 Holstein bulls (Select Sires Co. Columbus, OH). After 18 h at 38.5°C in 5% CO_2 in humidified air, cumulus was removed by vortexing, and groups of 20-30 presumptive zygotes were placed in 50 µl drops of SOF containing 20 µg/ml essential amino acids (Sigma-Aldrich, St. Louis, MO), 10 µg/ml nonessential amino acids (Life Technologies Corp. Carlsbad, CA), 4 mg/ml fatty acid free bovine serum albumin (Sigma-Aldrich, St. Louis, MO), and 25 µg/ml gentamicin sulfate (Life Technologies Corp. Grand Island, NY). Presumptive zygotes were cultured at 38.5°C in 5% O_2, 5% CO_2, 90% N_2 until day 8 post-fertilization.

Blastocyst expansion

On day 8 post-in vitro fertilization (IVF), expanded blastocysts were placed individually into 24-well CellBIND plates lacking Matrigel with DMEM containing 10% FBS and other supplements described for CT1 cell culture at 38.5 °C in a 5% CO_2 in humidified air. Medium was changed on days 12 and 16 post-IVF. In one study the treatment of the combination of EGF (10 ng/ml), FGF2 (10 ng/ml) and IGF1 (50 ng/ml) or controls (vehicle only) were provided at the beginning of the culture on day 8 post-IVF. On day 8 and 12 post-IVF each blastocyst was assessed under the EVOS Cell Image System (Life Technologies Corp. Eugene, OR) for its morphology and viability status (hatched or degenerating). Images are taken with the EVOS system. Blastocyst diameters are measured with ImageJ (NIH, USA).

Blastocyst outgrowth determination

The bovine expansion study was continued to day 20 post-IVF to examine whether blastocysts attached to plates and formed outgrowths. On day 16 and 20 post-IVF each blastocyst was assessed with the EVOS Cell Image System to determine outgrowth (attached; outgrowth formation; degenerating). Images are taken with the EVOS system.

Statistical analyses

All analyses were performed by least squares ANOVA using the general linear model of the Statistical Analysis System (SAS Institute, Inc., Cary, NC, USA). Statistical analyses used arcsin-transformed percentage data generated from each replicate (experimental unit = average percentage within each replicate). Differences between individual means were compared using pair-wise comparisons (PDIFF [probability of difference] analysis in SAS). Results were presented as the arithmetic mean ± S.E.M.

RESULTS

EGF, FGF2 and IGF1 stimulate proliferation of bovine trophoblast cells

A pilot study was completed to define the concentrations for EGF, FGF2, and IGF1 required for maximal proliferation responses in CT1 cells (Fig. 2). Mitotic index (percentage of EdU-positive nuclei after a 45 min challenge) was examined in each study. At 10 and 100 ng/ml, EGF increased ($P<0.05$) CT1 cell mitotic index compared with control. For FGF2, maximal responses were observed at 10 ng/ml and 100 ng/ml ($P<0.05$). A maximal IGF1 response was detected at 50 ng/ml ($P<0.05$). Based on these data, we selected 10 ng/ml EGF, 10 ng/ml FGF2, and 50 ng/ml IGF1 for further studies.

The first set of studies examined how each factor worked either individually or in conjunction with other paracrine factors to impact the mitotic index of a bovine trophoblast cell line (CT1). Mitotic index was increased ($P<0.05$) by supplementation with 10 ng/ml EGF, 10 ng/ml FGF2, or 50 ng/ml IGF1 when compared with non-treated controls (Fig. 3). In addition, a further increase in proliferation rate ($P<0.05$) was detected when all three factors were supplemented together.

A follow up study was undertaken to determine whether the supplementation of pairs of growth factors was sufficient to replicate the cooperative effect observed when the three factors were provided (Fig. 4). Supplementation of each pair of growth factors increased ($P<0.05$) mitotic index of CT1 cells when compared with non-treated controls. However, there was a further increase ($P<0.05$) in proliferation rate when all three growth factors were provided.

Involvement of MAPK3/1 and PI3K/AKT signaling systems in growth factor responses

The MAPK3/1 and PI3K/AKT pathways are commonly utilized by these growth factors in porcine, bovine and human trophoblast cells (Johnstone et al., 2005; Forbes et al., 2008; Hambruch et al., 2010; Yang et al., 2011b; Jeong et al., 2013; Jeong et al., 2014). Studies were undertaken to examine whether these signaling molecules control these growth factor responses in CT1 cells.

As illustrated by a representative Western blot (Fig. 5A) and by analysis of densitometric scans (Fig. 6), both EGF and IGF1 transiently increased ($P<0.05$) MAPK3/1 phosphorylation status. EGF activated MAPK3/1 within 5 min of treatment and the stimulation in phosphorylation remained greater ($P<0.05$) than controls for 15 min for MAPK3 and for 240 min for MAPK1. Phosphorylation status was activated by

IGF1 within 15 min for MAPK3 and within 5 min for MAPK1. FGF2 did not affect MAPK3/1 phosphorylation status within the 240 min of examination period.

The next study examined whether these growth factors regulated AKT phosphorylation (Fig. 5B). Activation of AKT was moderately induced by FGF2 (Fig. 7). This increase ($P<0.05$) in AKT phosphorylation was evident within 15 min after initiating FGF2 treatments (Fig. 7). Effects of FGF2 on AKT were short-lived. Neither EGF nor IGF1 impacted AKT activity. As illustrated by analysis of densitometric scans no increase ($P> 0.1$) of phosphorylation status is found in the treatment of EGF or IGF1.

MAPK3/1 and PI3K mediation of EGF, FGF2 and IGF1 effects on CT1 cell proliferation

Specific pharmacological inhibitors against MAPK3/1 (PD98059) and AKT (Wortmannin) were utilized to determine whether these kinases are involved with EGF, FGF2, and/or IGF1 responses in CT1 cells. Concentrations of each inhibitor were chosen based on previous work (Qiu et al., 2004; Yang et al., 2011b). Their ability to prevent growth factor-induced phosphorylation of MAPK3/1 or AKT was verified by Western blot analyses (Fig. 8).

An initial study examined the ability of each inhibitor to interfere with individual growth factor effects on CT1 cells (Fig. 9). In the absence of inhibitors, providing EGF, FGF2 or IGF1 increased ($P<0.05$) CT1 proliferation. Pre-treatment with the MAPK3/1 inhibitor did not affect CT1 proliferation rate in controls, but prevented each of the growth factors from producing maximal proliferative responses. The inhibitory completely blocked EGF- and IGF1-induced proliferation but only partially prevented FGF2-dependent effects. Exposure to the AKT inhibitor did not affect proliferation rate

in controls. It also did not affect proliferation rates in EGF- and IGF1-treated cells. However, the AKT inhibitor prevented ($P<0.05$) FGF2-dependent proliferative responses in these cells.

A second study was completed to examine the involvement of these signaling molecules when all three factors were supplemented to CT1 cells (Fig. 10). Exposure to either the MAPK3/1 or AKT inhibitor, or both, did not affect basal proliferation rates. Exposure to the MAPK3/1 inhibitor blocked ($P<0.01$) growth factor-induced CT1 proliferation. By contrast, the AKT inhibitor partially ($P<0.05$) blocked this activity. Exposure to both inhibitors completely blocked ($P<0.001$) growth factor-induced CT1 proliferation.

Effects of the combination of EGF, FGF2 and IGF1 on blastocyst expansion

An initial study was completed to examine whether the combination of EGF, FGF2 and IGF1 impacts in vitro changes in blastocyst diameter. At day 12 post-IVF, a 4-day exposure to the mix of EGF, FGF2, and IGF1 increased ($P<0.05$) the percentage of hatched blastocysts (Fig. 11). Increased ($P<0.05$) diameters also were detected in blastocysts treated with the mix of three growth factors on day 12 post-IVF (Fig. 12B). Treatment with the combination of EGF, FGF2, and IGF1also increased ($P<0.05$) the diameter changes of the blastocysts from day 8 to 12 post-IVF compared with non-treated controls (Fig. 12C).

Effects of the combination of EGF, FGF2 and IGF1 on blastocyst outgrowth formation

The final study was undertaken to examine whether the combination of EGF, FGF2, and IGF1 impacts bovine blastocyst outgrowth formation in vitro. The major

outgrowths of blastocyst began forming between days 8 and 12 post-IVF. The treatment of the combination of EGF, FGF2, and IGF1 increased ($P<0.05$) the outgrowth percentage by day 16 post-IVF compared with the control group (Fig. 13).

DISCUSSION

This work provides evidence that uterine paracrine factors EGF, FGF2, and IGF1 act in additive fashions on proliferation of CT1 cells through activation of MAPK3/1 and PI3K/AKT cell signaling pathways. Synergism among these growth factors is also found in promoting bovine embryonic development. These findings support our hypothesis that uterine-derived factors play essential roles in developmental events of the bovine conceptus during the early stages of pregnancy.

It is evident that EGF, FGF2, or IGF1 play essential roles in regulating cell proliferation and differentiation during pregnancy in different species such as pig, cattle, sheep, mouse and human (Li and Zhuang, 1997; Wei et al., 2001; Johnstone et al., 2005; Dadi et al., 2007; Forbes et al., 2008; Yang et al., 2011a; Yang et al., 2011b; Jeong et al., 2013; Jeong et al., 2014). Their roles in trophoblast cell development are reported. EGF promotes a bovine placental trophoblast cell proliferation as well as porcine trophectoderm cell proliferation and migration (Hambruch et al., 2010; Jeong et al., 2013). FGF2 stimulates bovine primitive endoderm proliferation, and migration of both ovine and bovine trophoblast cells (Yang et al., 2011a; Yang et al., 2011b). Production of IFNT from bovine trophoblast cell is also induced by FGF2 (Yang et al., 2011c). IGF1 promotes human normal placental cytotrophoblast cell proliferation and it also rescues cytotrophoblast from apoptosis (Li and Zhuang, 1997; Forbes et al., 2008). Similarly, IGF1 also promotes porcine trophectoderm proliferation and migration (Jeong et al.,

2014). In the present study, we demonstrated that EGF, FGF2, and IGF1 promote the proliferation of CT1 cells either individually or cooperatively. Treatment of each pair of the growth factors resulted in a more profound increase in proliferation compared with individual growth factor. However, the supplementation of pairs of growth factors could not replicate the cooperative effect noted when all three factors were provided. To our knowledge, this is the first study to examine the additive effects of combining growth factors of regulating trophoblast cell proliferation in cattle.

The MAPK3/1 and PI3K/AKT signaling pathways are commonly utilized by these growth factors in porcine, bovine and human trophoblast cells (Johnstone et al., 2005; Forbes et al., 2008; Hambruch et al., 2010; Yang et al., 2011b; Jeong et al., 2013; Jeong et al., 2014). The MAPK and PI3K/AKT are the most common signaling pathways of EGF. Both of these pathways are implicated in controlling EGF induced trophoblast cell proliferation in pig and cow (Hambruch et al., 2010; Jeong et al., 2013). FGF2 activates MAPK3/1, p38 MAPK as well as SAPK/JNK pathways to stimulate ovine trophoblast cell migration. IGF1 induced human cytotrophoblast proliferation as well as syncytial formation is regulated through IGF1R-mediated activating MAPK3/1 pathways (Forbes et al., 2008). However, IGF1R activation of PI3K pathway rescues these cytotrophoblast from apoptosis (Forbes et al., 2008). Similarly, IGF1 also promotes porcine trophectoderm proliferation and migration (Jeong et al., 2014). PI3K/AKT and MAPK3/1 pathways are activated by IGF1R in IGF1 induced porcine trophoblast cell proliferation and migration (Jeong et al., 2014). Results of the present study demonstrate that both EGF and IGF1 activated MAPK3/1 through phosphorylation and that PD98059 (pharmacological inhibitor of MAPK3/1) blocked the abilities of EGF and IGF1 to

increase proliferation of CT1 cells. Blocking of MAPK3/1 also partially inhibited FGF2-induced CT1 cell proliferation. Exposure to MAPK3/1 inhibitor also blocked CT1 proliferation induced by the supplementation of all three factors. Results of our study also demonstrated that FGF2 increased AKT phosphorylation status in CT1 cells. Exposure to AKT inhibitor (Wortmannin) prevented FGF2-dependent increase in cell proliferation but not EGF- or IGF1-treated cells. This AKT inhibitor only partially inhibited CT1 proliferation induced by treatment of all three growth factors. These results support our hypothesis that EGF, FGF2, and IGF1 act through two different signaling pathways to maximize CT1 proliferation through paracrine manners. These results also indicate that MAPK3/1is the primary pathway of CT1 proliferation in cattle, which is in accordance with the findings in these growth factors' roles in pig, cattle, and human trophoblast cell proliferation (Forbes et al., 2008; Hambruch et al., 2010; Jeong et al., 2013; Jeong et al., 2014). It is also possible that some other signaling pathways are required for EGF, FGF2, and IGF1to control cell proliferation. The tyrosine phosphatase SHP-2 (SRC homology region 2 domain-containing phosphatase-2), Nodal signaling, and Notch signaling pathways are associated with trophoblast cell proliferation in other studies (Forbes et al., 2009; Fu et al., 2013; Haider et al., 2014). The mechanism for the multiplicity of signaling for controlling proliferative activity in trophoblast cells is not clear. It is possible that multiple uterine factors cooperate together to activate various signaling molecules to govern developmental events of conceptus during the early pregnancy.

Combination of IGF1, FGF2, LIF (leukemia inhibitory factor), TGF-β (transforming growth factor), and IGF2 stimulates bovine embryonic development to a greater extent than when each factor is provided individually (Neira et al., 2010). The

combination of growth factors also maximizes embryo hatching rates (blastocyst expulsion from the zona pellucida). Also, the number of TE and ICM cells is greater at day 8 after fertilization in embryo exposed to the set of factors versus synthetic oviduct fluid alone (Neira et al., 2010). Our study also demonstrated that combination of uterine-derived EGF, FGF2, and IGF1 increased bovine embryo hatching rates. The diameters of blastocysts are also increased at day 12 post-IVF compared with non-treated controls. This increase of diameters of blastocysts may be directly related with proliferation of TE which is located at the outer layer of blastocyst and increased diameter also means increased surface area of blastocyst and this is important for latterly attachment to uterine endometrium after elongation in cattle. Further investigation is needed to test whether TE proliferation is induced by these uterine-derived growth factors in these in vitro produced blastocysts.

In conclusion, based on the present work, it is reasonable to propose that uterine-derived EGF, FGF2, and IGF1act in additive fashions to regulate bovine TE proliferation and embryonic development. Insight also was made describing signaling mechanisms that respond to these factors and control trophoblast cell proliferation. Further studies are still required to assess the quality of conceptus, pregnancies and offspring in vivo through embryo transfer of these in vitro produced embryos, which are treated with the combination of uterine factors.

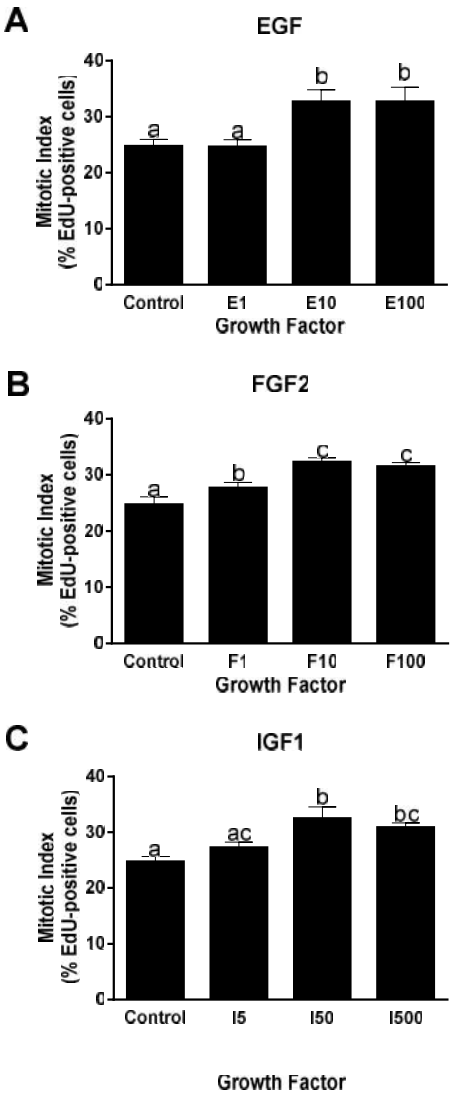

Figure 2: Determination of the ideal concentration for EGF, FGF2, and IGF1 in promoting CT1cell proliferation. Cells were serum-starved overnight, and then were exchanged with serum-free medium containing 0, 1, 10 or 100 ng/ml EGF; 0, 1, 10 or 100 ng/ml FGF2; or 0, 5, 50 or 500 ng/ml IGF1for 24 h. At the end of growth factor treatments, EdU reagent (100 µm) was added to each well for 45 min. Cells were than fixed in 4% paraformaldehyde and mitotic index was examined by epi-fluorescence microscopy. The ratio of EdU positive cells to total number (stained with 5 µg/ml Hoechst 33342) are represented as mean ± SEM (n=2 replicate studies within each panel). Differences ($P<0.05$) are denoted within each panel with different superscripts.

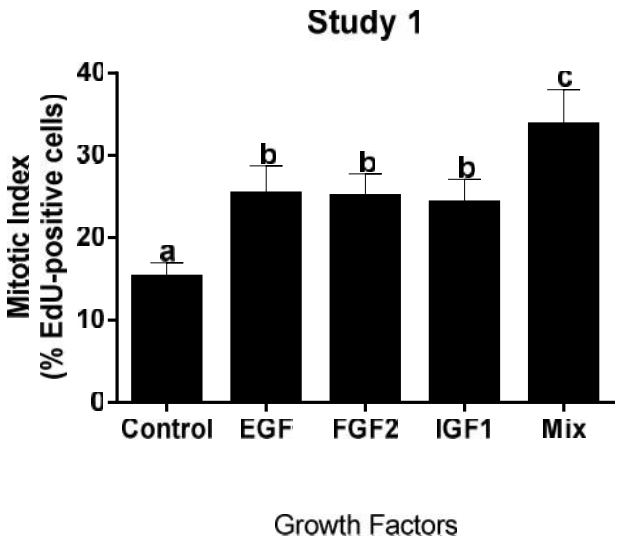

Figure 3: Supplementations with EGF, FGF2, and IGF1 promotes CT1cell proliferation. Cells were serum-starved overnight, then were changed with serum-free medium containing 10 ng/ml EGF, 10 ng/ml FGF2, 50ng/ml IGF1or the combination (Mix) of all three growth factors for 24 h. At the end of growth factor treatments, EdU reagent (100 µm) was added to each well for 45 min. Cells were than fixed in 4% paraformaldehyde and mitotic index was examined with epi-fluorescence microscopy. The ratio of EdU positive cells to total number (stained with 5 µg/ml Hoechst 33342) are represented as mean ± SEM (n=4 replicate studies within each panel). Differences ($P<0.05$) are denoted within the panel with different superscripts.

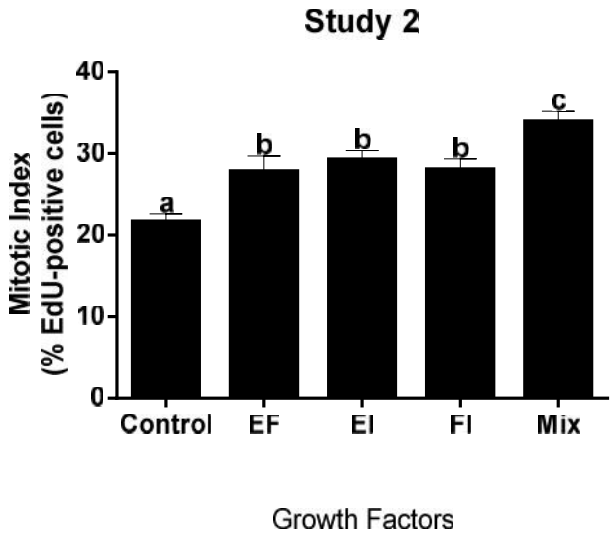

Figure 4: Promotion of CT1 cell proliferation by pairs of growth factors versus the mix of all three factors. Cells were serum-starved overnight, and then were changed with serum-free medium containing pairs of growth factors or all three growth factors (Mix; 10 ng/ml EGF, 10 ng/ml FGF2, and 50ng/ml IGF1) for 24 h. At the end of growth factor treatments, EdU reagent (100 µm) was added to each well for 45 min. Cells were than fixed in 4% paraformaldehyde and mitotic index was examined with epi-fluorescence microscopy. The ratio of EdU positive cells to total number (stained with 5 µg/ml Hoechst 33342) are represented as mean ± SEM (n=5 replicate studies within each panel). Differences ($P<0.05$) are denoted within the panel with different superscripts.

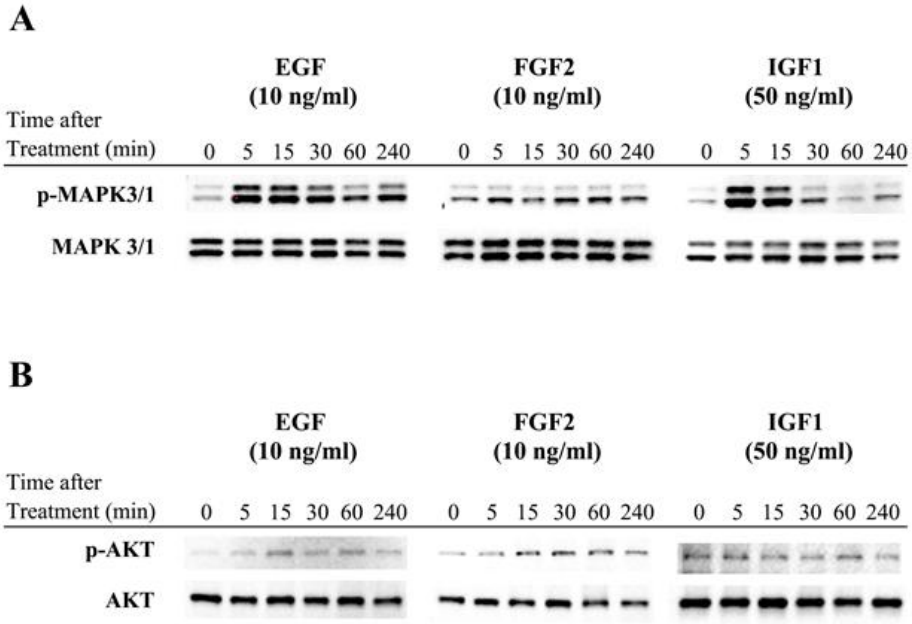

Figure 5: MAPK3/1 phosphorylation status is enhanced by EGF and IGF1 whereas AKT phosphorylation is induced by FGF2 in CT1 cells. Western blotting was used to examine changes in MAPK3/1 and PI3K/AKT phosphorylation statuses. CT1 cells lysates were collected either immediately before (time 0) or at specific periods after treatment with 10 ng/ml EGF, 10 ng/ml FGF2, or 50ng/ml IGF1. Lysates were immunoblotted with antibodies recognizing phosphorylated or total MAPK3/1 or AKT (p-MAPK3/1 and MAPK3/1; p-AKT and AKT). Three independent studies were completed, and a representative blot for outcomes from supplementing EGF, FGF2, and IGF1is provided. Two immunoreactive bands of the correct molecular mass were detected in MAPK3/1or p-MAPK3/1 blots (*Panel A*) whereas a single band was observed in AKT or p-AKT blots (*Panel B*). The higher molecular mass band in Panel A represents MAPK3 and the lower band represents MAPK1.

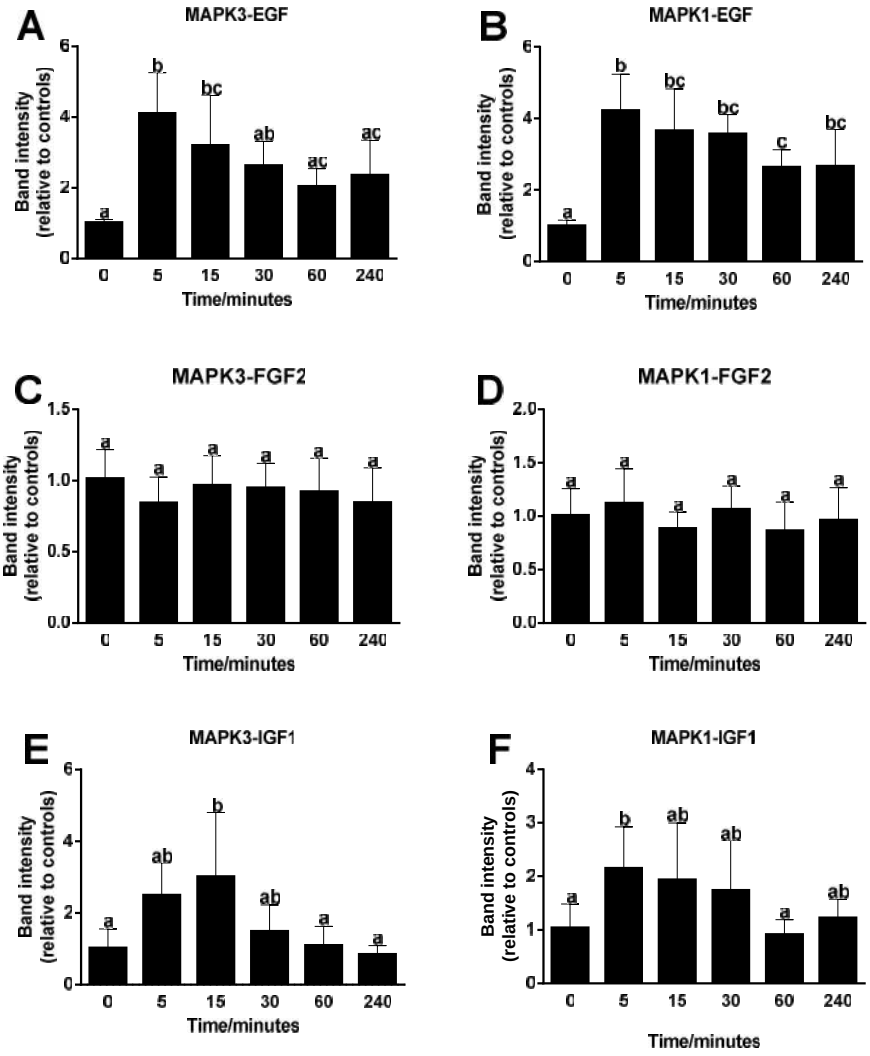

Figure 6: Densitometry of MAPK3/1 immunoblotted bands induced by EGF, FGF2, and IGF1. The relative intensities of MAPK3 and MAPK1 bands over time were quantified and analyzed to determine the effects of treatments with EGF (*Panel A and B*), FGF2 (*Panel C and D*), and IGF1 (*Panel E and F*). Data are presented as mean fold-differences ± SEM from the control value (n=3 replicate studies). Differences ($P<0.05$) are denoted within each panel with different superscripts.

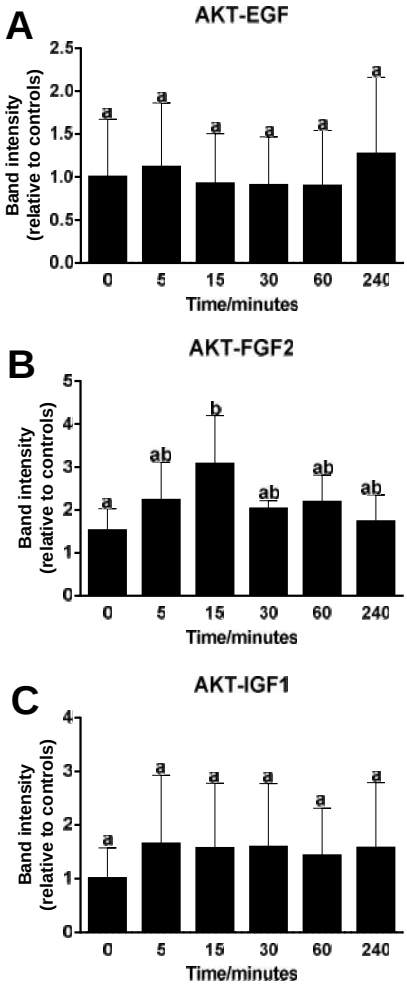

Figure 7: Densitometry of AKT immunoblotted bands induced by EGF, FGF2, and IGF1. The relative intensities of AKT bands over time were quantified and analyzed to determine the effects of treatments with EGF (*Panel A*), FGF2 (*Panel B*), or IGF1 (*Panel C*). Data are presented as mean fold-differences ± SEM from the control value (n=3 replicate studies). Differences ($P<0.05$) are denoted within each panel with different superscripts.

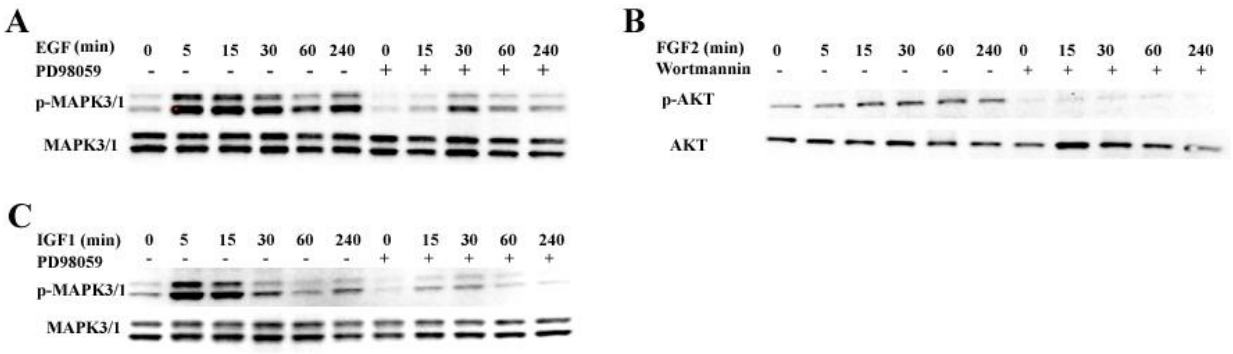

Figure 8: Ability of PD98059 and Wortmannin to prevent growth factor-induced phosphorylation of MAPK3/1 and AKT, respectively, in CT1 cells. Cells were serum-starved overnight, and either 50 µM PD98059, 0.2 µM Wortmannin or Vehicle (1% w/v BSA) was added to cultures for 2 h before exposure to growth factor. Lysates were collected either immediately before (time 0) or at specific periods after treatment with 10 ng/ml EGF, 10 ng/ml FGF2, or 50ng/ml IGF1. Lysates were immunoblotted with antibodies recognizing phosphorylated or total MAPK3/1 or AKT (p-MAPK3/1 and MAPK3/1; p-AKT and AKT). A representative blot for outcomes from supplementing EGF, FGF2, and IGF1is provided. Two immunoreactive bands of the correct molecular mass were detected in MAPK3/1or p-MAPK3/1 blots (*Panel A and Panel C*) whereas a single band was observed in AKT or p-AKT blots (*Panel B*). The higher molecular mass band in Panel A represents MAPK3 and the lower band represents MAPK1.

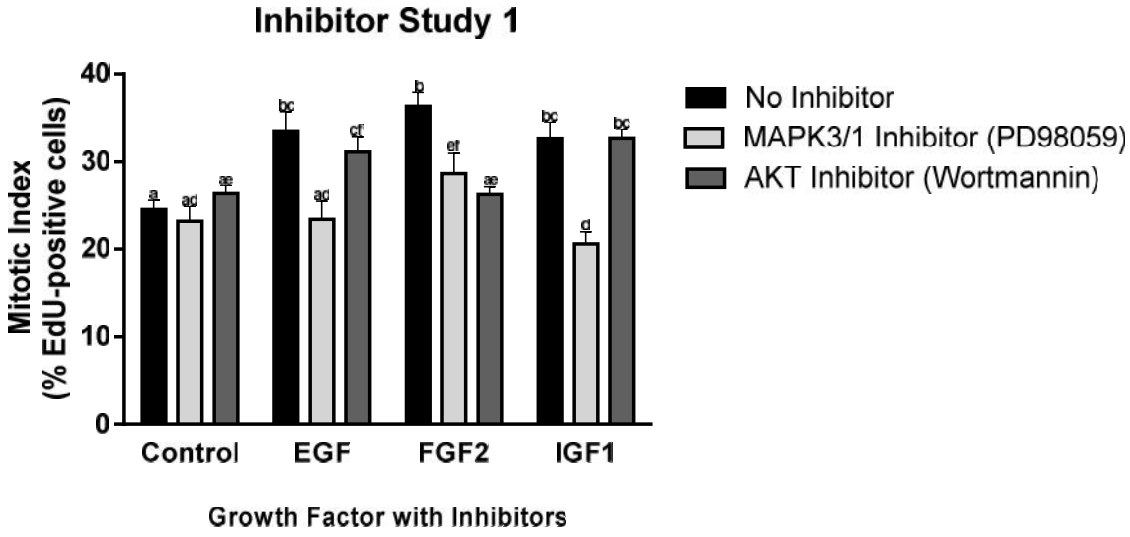

Figure 9: Delineation of MAPK3/1 and AKT molecules involved with EGF-, FGF2-, and IGF1-dependent increases in CT1 cell proliferation. Cells were serum-starved overnight. Either 50 µM PD98059, 0.2 µM Wortmannin or Vehicle (1% w/v BSA) was added to cultures for the last 2 h of the serum starvation in fresh serum-free medium. Cells were added with 10 ng/ml EGF, 10 ng/ml FGF2, or 50ng/ml IGF1 directly to the medium for 24 h. At the end of growth factor treatments, EdU reagent (100 µm) was added to each well for 45 min. Cells were than fixed in 4% paraformaldehyde and mitotic index was examined with epi-fluorescence microscopy. The ratio of EdU positive cells to total number (stained with 5 µg/ml Hoechst 33342) are represented as mean ± SEM (n=4 replicate studies within each panel). Differences ($P<0.05$) are denoted within the panel with different superscripts.

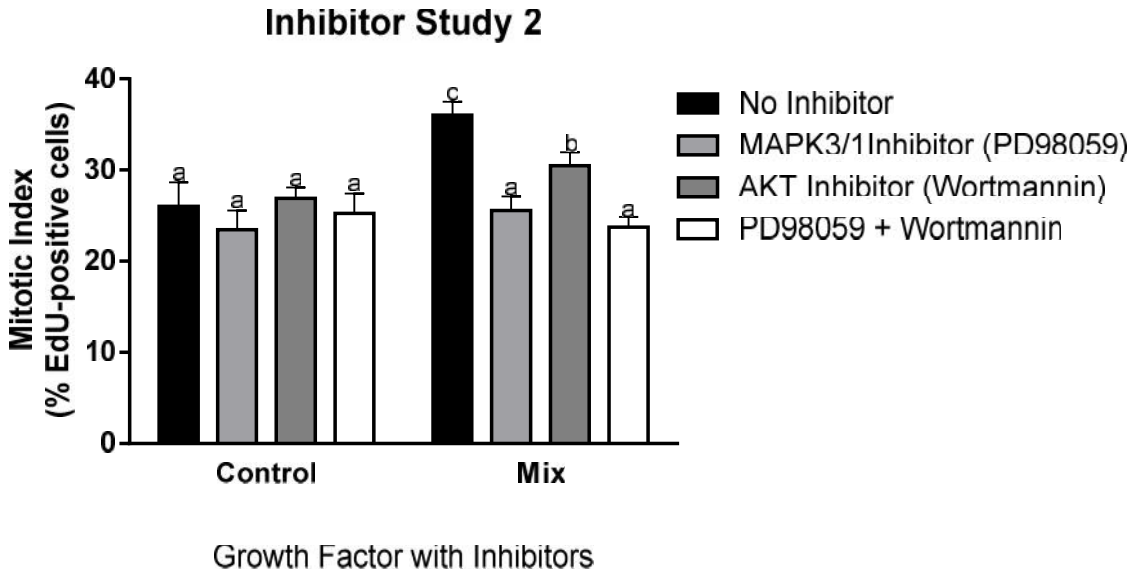

Figure 10: Delineation of MAPK3/1 and AKT molecules involved with the combination of EGF, FGF2, and IGF1dependent increases in CT1 cell proliferation. Cells were serum-starved overnight. Either 50 µM PD98059, 0.2 µM Wortmannin, both PD98059 and Wortmannin or Vehicle (1% w/v BSA) was added to cultures for the last 2 h of the serum starvation in fresh serum-free medium. Cells were added with 10 ng/ml EGF, 10 ng/ml FGF2, and 50ng/ml IGF1 (Mix) for 24 h. At the end of growth factor treatments, EdU reagent (100 µm) was added to each well for 45 min. Cells were than fixed in 4% paraformaldehyde and mitotic index was examined with epi-fluorescence microscopy. The ratio of EdU positive cells to total number (stained with 5 µg/ml Hoechst 33342) are represented as mean ± SEM (n=5 replicate studies within each panel). Differences ($P<0.05$) are denoted within the panel with different superscripts.

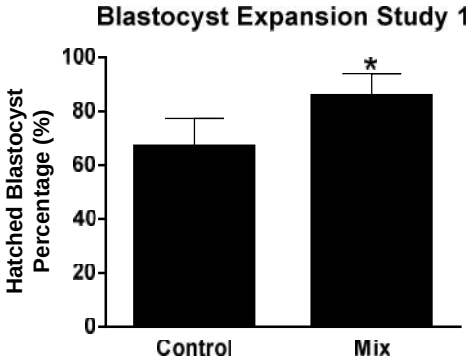

Figure 11: Combination treatment of EGF, FGF2, and IGF1 promotes the zona pellucida hatching rate of bovine blastocysts. On day 8 post-IVF, expanded blastocysts were treated with the mix of three growth factors (10 ng/ml EGF, 10 ng/ml FGF2, and 50ng/ml IGF1) or Vehicle (1% w/v BSA). On day 8 and day 12 post-IVF each blastocyst was assessed under the EVOS Cell Image System and for its morphology and viability status (hatched or degenerating). Images were taken with the EVOS system. The percentage of hatched blastocysts to total blastocyst number are represented as mean ± SEM (n=33-38 blastocysts examined over 4 replicate studies). An asterisk (*) denotes treatment effects that differ significantly from the control value ($P<0.05$).

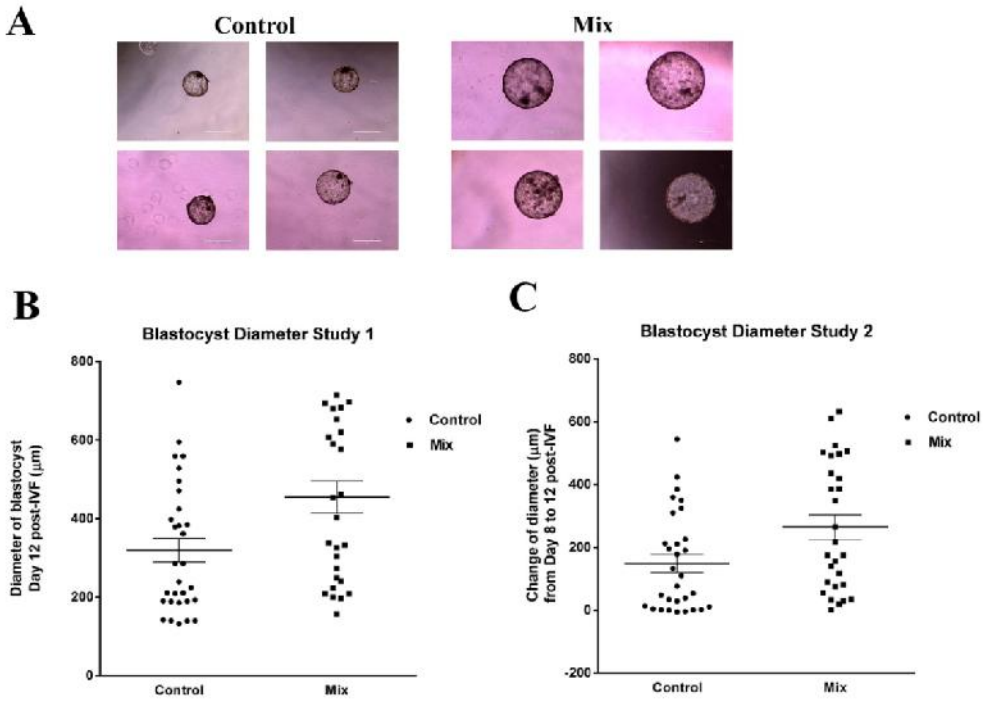

Figure 12: Combination treatment of EGF, FGF2, and IGF1increases bovine blastocyst diameter. On day 8 post-IVF, expanded blastocysts were treated with the mix of three growth factors (10 ng/ml EGF, 10 ng/ml FGF2, and 50ng/ml IGF1) or Vehicle (1% w/v BSA). On day 8 and day 12 post-IVF each blastocyst was assessed under the EVOS Cell Image System. Representative morphological blastocysts on day 12 post-IVF of each group are provided (*Panel A*). The diameter on day 12 post-IVF and the change of diameter from day 8 to day 12 post-IVF of each individual blastocyst (*Panel B* and *Panel C*) are represented as well as group mean ± SEM (n=28-30 blastocysts examined over 4 replicate studies). Significant differences of diameter of blastocyst on day 12 post-IVF and diameter changes from day 8 to day 12 post-IVF are found in the Mix group compared with the control group ($P<0.05$). Bar = 400 µm.

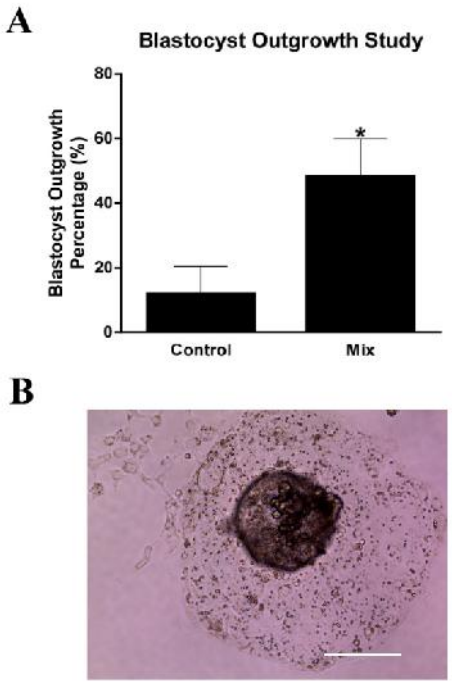

Figure 13: Combination treatment of EGF, FGF2, and IGF1 promotes the outgrowth of bovine embryos. On day 8 post-IVF, expanded blastocysts were treated with the mix of three growth factors (10 ng/ml EGF, 10 ng/ml FGF2, and 50ng/ml IGF1) or Vehicle (1% w/v BSA). On day 12 and day 16 post-IVF each blastocyst was assessed under the EVOS Cell Image System and for its morphology and viability status (outgrowth formation; degenerating). The percentage of outgrowth blastocysts (*Panel A*) to total blastocyst number are represented as mean ± SEM (n=33-38 blastocysts examined over 4 replicate studies). An asterisk (*) denotes treatment effects that differ significantly from the control value ($P<0.05$). Panel B shows an example of blastocyst outgrowth on day 12 post-IVF. Bar = 200 µm.

CHAPTER III: CONCLUSIONS AND IMPLICATIONS

Several uterine-derived paracrine factors have been implicated as critical regulators of conceptus development in cattle. Three of special interest is EGF, FGF2 and IGF1. Previous work provides evidence that these factors likely regulate early placental development and function in cattle. The focus of the present work was to elucidate the underlying mechanisms by which EGF, FGF2, and IGF1 regulate either individually or synergistic proliferation of a bovine trophoblast cell line CT1. In the meanwhile, an attempt was made to discover the roles of these growth factors in regulating early blastocyst development in bovine.

Based on our findings, it is reasonable to propose that uterine-derived growth factors are playing essential roles or even act with additive or synergistic fashions to regulate conceptus development during early pregnancy in cattle. As we only did studies on these factors' effects on TE proliferation, other studies may also needed to figure out whether they can promote trophoblast migration during pregnancy or even their effects on anti-apoptosis. In this study we covered three most interested growth factors and it is possible that some other uterine-derived factors like HGF, IGF2, and TGF-β may also have effects on trophoblast cell development. Further studies are needed to clarify their roles in regulating conceptus development. Studies are also needed to investigate whether there are specific synergisms among all these uterine-derived growth factors in regulating conceptus development, if so, what are the mechanisms responding to them. Findings of the present work may also provide ideas for in vitro produced embryos. Additions of growth factors might be needed for the in vitro produced embryos. Perhaps better

embryos can be generated by the supplementation of growth factors, and with embryo transfer to females, this may help to reduce pregnancy loss in cattle.

APPENDICES

APPENDIX A: USING EDU INCORPORATION TO EXAMINE CT1 CELL PROLIFERATION

This protocol was used to examine the proliferative effects of EGF, FGF2, and IGF1on CT1 cells. This protocol describes the key steps of using the EdU cell proliferation kit (Life Technologies Corp. Eugene, OR).

Preparation of CT1 cells

1. Seed Cell-Bind-coated 24-well plates with approximately a 1:5 split of confluent CT1 cells. Note: the younger the cells, the better. They seem to work in proliferation assays (5-10 days old).

2. After 2 days, aspirate medium and replace with serum-free CT1 medium. Note: cells should be <20% confluent, and barely able or not able to see colonies with your naked eye. Also, may wait until day 3 to start in some instances (depending on cell growth rates).

3. After 8 to 24 h, aspirate medium and replace with 500 µl FBS-free CT1 medium containing the desired treatments. Remember to add a carrier treatment to controls (e.g. unconditioned medium, DMSO, BSA).

4. Incubate for desired time. This usually is either for 24 h or 48 h but potentially could go longer depending on the treatments.

5. Add EdU for the last 45 min of incubation by completing the following steps:

a) The EdU stock in the refrigerator represents a 10X stock (100 µM stock), and 5 µl EdU stock will be used for each well.
b) Remove and warm EdU needed for the study for 30-60 min in an incubator. Remember to take out a small amount more than is needed to compensate for pipetting errors (e.g. remove 125 µl [or 5 µl x 25 wells] if you are using all 24 wells of one plate.
c) After EdU is warmed, add 5 µl to each well.
d) Place into the incubator for 45 min, and then proceed immediately to cell fixation and permeabilization steps that follow.

Note: Slight variations in EdU incubation time may be needed depending on the study.

Cell fixation and permerbilization

1. These and subsequent steps can be completed outside of the biosafety cabinet since sterile conditions are no longer needed.

2. Aspirate medium and gently rinse twice DPBS.

3. Add 0.5 to 1 mL of 4% paraformaldehyde. Incubate for 15 minutes at room temperature.

4. Remove the fixative and rinse once with DPBS.

5. Add 0.5 to 1 mL 0.5% Triton® X-100. Incubate for 20 min at room temperature.

EdU detection

1. After Triton-X 100 exposure, rinse wells twice with 0.5 to 1 ml solution of 3% BSA in PBS.

2. After rinsing, add 0.5 mL of Click-iT® reaction cocktail to each well. Rock the plate briefly to insure that the reaction cocktail is distributed evenly across the bottom of each well.

3. Incubate the plate in a light-proof environment (i.e. closed drawer or foil wrap) for 30 minutes at room temperature.

4. Rinse wells once with 0.5 to 1 mL 3% BSA in PBS.

5. Rinse wells once with DPBS.

DNA staining

1. Add 0.5 mL of 1X Hoechst 33342 solution to each well.

2. Incubate the plate in a light-proof environment for 30 minutes at room temperature.

3. Rinse three times with 1 mL of DPBS

4. Add 1 ml DPBS to each well then proceed to imaging/analysis.

Note: Unlike most other cells, CT1 cells cannot be stored at 4 C for extended periods before imaging. They general last for < 24 h.

Imaging and analysis

1. Place plate on the epifluorescence microscope and focus on cells.

2. Alternate between the DAPI (blue) and GFP (green) filters to detect the Hoechst and EdU positive cells, respectively.

3. Randomly select 5 non-overlapping areas in each well to count. Preference should be given to counting areas on the edges of colonies and not in the middle of colonies.

4. For the approximate fluorescence excitation/emission maxima for Alexa Fluor® dyes and Hoechst 33342 dye bound to DNA.

Reagents needed

4% paraformaldehyde
Combine 1 10 ml vial of 16% formaldehyde (high purity) with 30 ml of DPBS in a 50 ml conical tube. Store at 4 C. Solution is good for up to 2 weeks, although imaging outcomes generally are better when using freshly prepared solution. NOTE: must dispose of formaldehyde in biological waste.

0.5% Triton-X-100
Combine 250 µl Triton-X-100 with 50 ml DPBS. Prepare at least 24 h before the stock is needed as this viscous solution takes time to equilibrate into DPBS. Store at 4 C for up to 1 month.

3% BSA
Combine 1.5 g BSA with 50 ml PBS. Put the powder of BSA at the top of the PBS and leave them combine together. Store at 4 C for up to 2 weeks.

1X Hoechst
Use the 10 mg/ml stock of Hoechst 33342 to prepare a 5 µg/ml working stock. Do this by adding 5 µl of 10 mg/ml Hoechst 33342 to 10 ml DPBS (1:2000 dilution).

Click-iT Reaction Cocktail
Use the following table when preparing this cocktail. Prepare on day of use. Foil wrap (and store in drawer) to protect from light.

Click-iT EdU buffer additive
Dilute the 10 X solution of component F of EdU kit 1:10 using deionized water.

Table 1 Click-iT reaction cocktails.

Reaction Components	**Number of coverslips**						
	1	**2**	**4**	**5**	**10**	**25**	**50**
1x Click-iT reaction buffer Component D	430 ul	860 ul	1.8 ml	2.2 ml	4.3 ml	10.7 ml	21.4 ml
$CuSO_4$ Component E	20 ul	40 ul	80 ul	100 ul	200 ul	500 ul	1 ml
Alexa Fluor azide Component B	1.2 ul	2.5 ul	5 ul	6 ul	12.5 ul	31 ul	62 ul
Reaction buffer additive ***From Component F***	50 ul	100 ul	200 ul	250 ul	500 ul	1.25 ml	2.5 ml
Total Volume	500 ul	1 ml	2 ml	2.5 ml	5 ml	12.5 ml	25 ml

APPENDIX B: IN VITRO PRODUCED EMBRYOS FOR OUTGROWTH STUDIES

The following protocol was used to examine outgrowth formation of bovine in vitro produced embryos.

In vitro produced bovine embryos

1. In vitro production of bovine embryos was completed as described previously (Jousan and Hansen, 2004; Loureiro et al., 2009), with minor modifications. Ovaries from beef and dairy cattle were obtained from Brown Packing Co. Cumulus-oocyte-complexes were obtained by slashing follicles with a scalpel blade, and then by searching with a Nikon SMZ745 stereomicroscope.

2. Groups of 20-30 COCs were cultured in 500 µl/well of oocyte maturation medium covered 4-well nunc plates (TCM199 containing Earle's salts, 10% FBS, 25 µg/ml bovine FSH, 2 µg/ml estradiol, 22 µg/ml sodium pyruvate, 1 mM glutamine, and 25 µg/ml gentamicin sulfate) at 38.5°C in 5% CO2 in humidified air.

3. After 21-22 h, COCs were transferred to fertilization medium, and exposed to BoviPure™ gradient-purified bovine spermatozoa derived from a pool of frozen semen from 4 Holstein bulls.

4. After 18 h at 38.5°C in 5% CO2 in humidified air, cumulus was removed by vortexing, and groups of 20-30 presumptive zygotes were placed in 50 µl drops of SOF containing 20 µg/ml essential amino acids, 10 µg/ml nonessential amino acids 4 mg/ml fatty acid free bovine serum albumin, and 25 µg/ml gentamicin sulfate, and cultured at 38.5°C in 5% O2, 5% CO2, 90% N2 until day 8 post-fertilization.

5. The proportion of cleaved zygotes and proportion of embryos containing 8-16 blastomeres were recorded on day 3 post-fertilization. The proportion and stage of blastocysts (early, regular, expanded, hatching & hatched) was recorded at days 7 and 8 post-fertilization.

Blastocyst expansion culture

1. On day 8 post-in vitro fertilization (IVF), expanded blastocysts were placed individually into 24-well CellBIND plates lacking Matrigel with DMEM containing 10% FBS and other supplements described for CT1 cell culture at 38.5 °C in a 5% CO2 in humidified air. Medium should be changed on days 12 and 16 post-IVF.

2. On day 8, 12 and 16 post-IVF, assess each blastocyst with the EVOS Cell Image System (Life Technologies Corp. Eugene, OR). Examine morphology and viability status (hatched, viable, degenerating).

3. Take images and use ImageJ (NIH, USA) to determine the diameter.

Blastocyst outgrowth culture

1. On day 8 post-in vitro fertilization (IVF), expanded blastocysts should be placed individually into 24-well CellBIND plates lacking Matrigel with DMEM containing 10% FBS and other supplements described for CT1 cell culture at 38.5 °C in a 5% CO2 in humidified air. Medium should be changed on days 12 and 16 post-IVF.

2. On day 12 and 16 post-IVF, assess each blastocyst with the EVOS Cell Image System to determine whether blastocysts have begun to attach, form outgrowths, or have degenerated.

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