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EGF, IGF-I, VEGF and CSF2: Effects on Trophectoderm of Porcine Conceptus

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ABSTRACT

The majority of early embryonic mortality in pregnancy occurs during the peri-implantation stage, suggesting that this period is important for conceptus viability and the establishment of pregnancy. Successful establishment of pregnancy in all mammalian species depends on the orchestrated molecular events that transpire at the conceptus- uterine interface during the peri-implantation period. This maternal-conceptus interaction is especially crucial in pigs because in them non-invasive epitheliochorial placentation occurs, in which the pre-implantation phase is prolonged. During the pre-implantation period, conceptus survival and the establishment of pregnancy are known to depend on the developing conceptus receiving an adequate supply of histotroph, which contains a wide range of nutrients and growth factors. Evidence links growth factors including epidermal growth factor (EGF), insulin-like growth factor-I (IGF-I), vascular endothelial growth factor (VEGF), and colony-stimulating factor 2 (CSF2) to embryogenesis or implantation in various mammalian species; however, in the case of pig, little is known about such functions of these growth factors, especially their regulatory mechanisms at the maternal-conceptus interface. Our research group has presented evidence for promising growth factors affecting cellular activities of primary porcine trophectoderm (pTr) cells, and we have identified potential intracellular signaling pathways responsible for the activities induced by these factors. Therefore, this review focuses on promising growth factors at the maternal-conceptus interface regulating the development of the porcine conceptus and playing pivotal roles in implantation events during early pregnancy in pigs.
(Key words : Peri-implantation, EGF, IGF-I, VEGF, CSF2)

INTRODUCTION

During the pre-implantation period, temporal synchrony between early embryonic development and uterine receptivity, and the establishment of a well-organized reciprocal dialog between the conceptus (embryo-fetus and associated extraembryonic membranes) and the maternal uterus is indispensable for successful implantation in mammals (Tranguch *et al*., 2005). In this period, a substantial number of conceptuses die because of asynchronous development of the uterus and conceptus, failure of pregnancy recognition signaling, and inappropriate implantation, which eventually leads to poor pregnancy rates and low reproductive efficacy across species (Lawson *et al*., 1983; Pope, 1988).

The developing conceptus signals its presence to the endometrium and thereby prolongs the life span of the corpus luteum (CL) and secretes a variety of molecules including interferons (INFs), prostaglandins (PGs), growth factors, cytokines, and other as yet unknown factors (Geisert and Yelich, 1997; Jaeger *et al*., 2001; Lefevre *et al*., 1998; Tuo *et al*., 1996). In response to these factors, the uterine endometrium undergoes rapid morphological changes and produces various molecules that are either secreted by uterine epithelia or are transported into the uterine lumen and are collectively referred to as histotroph, which is required for conceptus development and the uterus's receptivity for implantation (Bazer, 1975; Kane *et al*., 1997). Histotroph includes numerous factors including lipids, ions, amino acids, sugars, growth factors, cytokines, hormones, enzymes, adhesion proteins, and transport proteins. Developing preimplantation embryos ultimately depend on a sufficient supply of histotroph for survival, growth, and development. Evidence from studies conducted using the ute-

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rine-gland knockout ewe (UGKO) model emphasizes that secretions of the uterine glands (GE) are crucial for early conceptus survival, development and implantation during the early pregnancy (Gray *et al*., 2002; Gray *et al*., 2001).

The aforementioned conceptus-uterine dialog is especially critical in pigs because non-invasive epitheliochorial implantation occurs in them, during which the preattachment phase is prolonged (Aplin and Kimber, 2004). In contrast to species that have the invasive type of implantation characterized by the nidation of the blastocyst into the uterine stroma, in pigs, implantation is superficial and the blastocyst remains within the uterine lumen. Before attaching to uterine epithelia, porcine blastocysts rapidly elongate by a process involving the transformation of a spherical blastocyst into a filamentous conceptus (Bazer *et al*., 2009; Guillomot, 1995). Typically, the conceptuses that elongate the most maximize the area of contact with the uterine epithelia to exchange nutrients and gases and thus have the highest chance of survival, emphasizing the importance of conceptus development during the peri-implantation period for establishment of pregnancy in pigs (Burton *et al*., 2007; Pope, 1988; Pope *et al*., 1986).

In pigs, most oocytes undergo fertilization, which initiates development, but 20～30% of early embryonic deaths occur during early pregnancy. How implantation fails because of inappropriately developed conceptuses is not well-understood, and elucidating the molecular and cellular processes required for these events should provide key insights into human and animal reproduction. Insufficient delivery of histotroph to the developing conceptus results in intrauterine growth restriction, a major social and economic problem of global importance (Bazer *et al*., 2012). Among the components of histotroph, growth factors are known to orchestrate various changes in the endometrium and/or conceptus to establish an optimal uterine microenvironment required for appropriate conceptus development (Kane *et al*., 1997; Schultz and Heyner, 1993).

This review was focused on promising growth factors involved in conceptus-maternal interaction. Recently, we have attempted to determine the molecular mechanisms by which the four selected uterine factors activate the intracellular signaling cascades involved in orchestrating conceptus development during the peri-implantation period; epidermal growth factor (EGF), insulin-like growth factor I (IGF-I), vascular endothelial growth factor (VEGF), and colony-stimulating factor 2 (CSF2). These factors have been hypothesized to be involved in regulating diverse aspects of reproductive physiology, and to either directly or indirectly regulate the growth and development of conceptus during the peri-implantation period. Knockout mice featuring a disrupted VEGF or CSF2 system exhibited embryonic developmental abnormalities and/or early embryonic lethality (Carmeliet *et al*., 1996; Ferrara *et al*., 1996; Fong *et al*., 1995; Pollard, 1997; Robertson *et al*., 2001). Furthermore, providing recombinant EGF, VEGF, or CSF2 to embryos in *in vitro* cultures enhanced their developmental ability and implantation rate after embryo transfer and also rescued deficiencies in placental structure and fetal growth (Biswas *et al*., 2011; Einspanier *et al*., 2002; Hannan *et al*., 2011; Robertson *et al*., 2001; Sjoblom *et al*., 2005). Despite these hypothesized roles and functions of these promising growth factors, little is known about the cellular signaling pathways that are stimulated by the growth factors in porcine trophectoderm (pTr) cells and about how these factors stimulate conceptus development during early pregnancy. Therefore, this review addresses the general characteristics of the early pregnancy, the developmental process of the peri-implantation porcine embryo, various factors at maternal-conceptus interface, and the promising growth factors including EGF, IGF-I, VEGF and CSF2 that affect conceptus development in pigs.

EVENTS DURING THE EARLY PREGNANCY

Pre-Implantation Period

During the period between fertilization and implantation, the pre-implantation embryo undergoes numerous mitotic cell divisions and morphological changes. The pre-implantation embryo is unique in that it develops in a fluid environment in a free-floating state, in the absence of direct cellular contact with the uterus for 1～2 weeks (depending on the species) before implantation. Abnormal regulation of the events before and during implantation may often be a cause of substantial loss of pre-implantation embryos and poor pregnancy rates in eutherian mammals.

The fertilized single-cell ovum undergoes cleavages which generate 2, 4, 8, and finally 16 blastomeres that form a compact ball of cells known as the morula. The blastomere compaction that occurs in this stage enables greater cell-cell interaction, and this process is a prerequisite for the segregation of the internal cells that form the inner cell mass (ICM). Eventually, a differentiated tissue called as the blastocyst is formed before implantation is initiated. At the early blastocyst stage, cells first undergo a process that ultimately decides cell fates; the pluripotent ICM is the bundle of cells that will generate future cell lineages leading to the endo-

derm, mesoderm, or ectoderm, eventually giving rise to the fetus. Conversely, a thin outer layer surrounding the ICM, the epithelial trophectoderm, establishes the connection with the luminal epithelia (LE) of the maternal uterus and forms the placenta interface or other extra-embryonic membranes. The trophectoderm is necessary for the transfer of nutrients from the mother to the embryo. Before implantation, the embryo at the blastocyst stage escapes from the zona pellucida, and the exposed trophectoderm cells become capable of attaching to the endometrial epithelium that lines the uterus, and this results in implantation being initiated (Wang and Dey, 2006).

Opening of the Implantation Window and Implantation

Shortly before implantation, the uterus undergoes alterations that are necessary for the attachment of the conceptus trophectoderm (Paria *et al*., 1993). This embryo reception-ready phase of the endometrium, a limited period during the initiation of implantation, is termed "implantation window" (Psychoyos, 1973). The implantation window is initiated by changes in the endometrium of the uterus that help transform not only the lining of the uterus, but also the composition and pattern of endometrial secretions (Wilcox *et al*., 1999). During this period, the blastocyst approaches the endometrium and the endometrium is primed for blastocyst attachment, given that it has acquired the accurate morphological and functional state (Finn and Martin, 1974). The implantation window is a critical period in that it requires the establishment of reciprocal interactions between the maternal endometrium and the developing conceptus; dysregulation on the part of either the conceptus or the uterine endometrium results in the inability to establish pregnancy (Fazleabas *et al*., 2004; Spencer *et al*., 2007).

Steroid hormones serve fundamental roles in the aforementioned changes. The progesterone receptor in the uterine LE is down-regulated immediately before the opening of the implantation window, and this is generally associated with the endometrial transcriptional changes that are necessary for uterine receptivity and lead to implantation in humans (Lessey *et al*., 1988; Lessey *et al*., 1996) and in domestic animals (Geisert *et al*., 1994; Hartt *et al*., 2005; Meikle *et al*., 2001; Spencer and Bazer, 1995). Estrogen stimulation is critical for uterine receptivity because it exerts protective effects related to the CL and induces the endometrial alterations required for conceptus attachment.

For implantation, activated blastocyst establishes a close physical- and physiological contact with the maternal endometrium to form the placenta that serves as the interface between the fetus and the maternal circulation. This process varies between species according to three placental types, the hemochorial (humans, rodents, and non-human primates), epitheliochorial (horses, cows, sheep, and pigs), and endotheliochorial (most carnivores) types. Successful implantation depends on the embryo developing to the blastocyst stage in synchrony with the differentiation of the uterus to the receptive state, which is followed by two-way interactions between the blastocyst and the uterine LE (Psychoyos, 1973).

PHYSIOLOGY OF THE PORCINE CONCEPTUS DURING PERI-IMPLANTATION

Early Conceptus Development

After fertilization, cleavage starts shortly after nuclear division and then subsequent division occurs, allowing the porcine embryo to reach the 4-cell stage within 24 h. Blastomere development beyond the 4-cell stage coincides with the activation of embryonic genome expression (Telford *et al*., 1990; Tomanek *et al*., 1989). Compaction and blastulation occurs within the conceptus by Day 6 after fertilization (Reima *et al*., 1993). The blastocyst forms on Day 8 after fertilization, and the conceptus now features a distinct trophectodermal cell layer and an ICM within the blastocoele (Geisert *et al*., 1982a). Hatching from the zona pellucida on Days 7~8 of pregnancy allows the rapid morphological changes of conceptus into spherical, tubular, and filamentous forms between Days 11 and 12 of pregnancy.

Maternal Recognition of Pregnancy

Early conceptus releases signaling molecules that induce a molecular cascade of events to lengthen the lifespan of the CL (Geisert *et al*., 1990). Protecting the CL promotes continued progesterone production, which allows the maintenance of myometrial quiescence and endometrial histotroph production (Nara *et al*., 1981). Porcine early conceptuses express enzymes that convert steroid precursors into estrogen and secrete estrogen into the uterine lumen on Days 11~12 of pregnancy (Bazer *et al*., 1986; Perry *et al*., 1973; Perry *et al*., 1976). Blastocyst-secreted estrogen activates the mechanism required to direct prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) secretion away from the uterine vasculature and into the uterine lumen, this is called the endocrine-exocrine theory of maternal recognition. In pregnant gilts, $PGF_{2\alpha}$ secreted into the uterine lumen is converted into an inactive form that cannot cause luteolysis. Injections of exogenous estrogen into gilts between Days 11 and 16 of the estrous cycle have been established to prolong the life of the CL and induce psuedopregnancy (Frank *et al*.,

1977; Geisert *et al*., 1982b). In addition to estrogen, Prostaglandin E2 (PGE2) dramatically increases in uterine flushings during the time of maternal recognition of pregnancy in the pig (Geisert *et al*., 1982b). PGE2 has been shown to enhance CL performance and is consi- dered to function as a luteotrophic agent during pregnancy in the pig (Ford and Christenson, 1991).

Conceptus Trophoblastic Elongation

In contrast to species that feature the invasive type of implantation, in pigs, which have an epitheliochorial placenta, the trophoblastic elongation is a unique and critical step (Burton *et al*., 2007). On around Day 10 of pregnancy, the 2~3 mm-sized spherical porcine conceptuses grow in diameter (Geisert *et al*., 1982b) and continue to expand until reaching a diameter of approximately 9~10 mm diameter (Geisert *et al*., 1982b; Pusateri *et al*., 1990). Next, the spherical conceptus initiates a process of rapid elongation between Days 11 and 12 of pregnancy (Geisert *et al*., 1982b), which occurs concomitantly with the release of estrogen by the conceptus (Bazer *et al*., 1986; Geisert *et al*., 1982b; Ross *et al*., 2003). The spherical conceptus becomes ovoid and tubular, and eventually filamentous in shape and 150~200 mm long. This transformation results mainly from a process in which the cytoskeletal rearrangements of filamentous actin cause trophectodermal migration (Geisert *et al*., 1982a; Mattson *et al*., 1990; Pusateri *et al*., 1990). Trophoblastic elongation is critical because the conceptus expansion ensures that sufficient uterine space is retained for placentation (Geisert and Yelich, 1997; Stroband and Van der Lende, 1990) and that maternal recognition signal is efficiently delivered throughout the uterine lumen (Dziuk, 1968; Polge *et al*., 1966). Typically, the conceptuses that elongate the most establish the greatest uterine surface area for nutrient and gas exchange and, consequently, have the highest chance of survival.

Conceptus Apposition and Attachment to the Uterine Luminal Epithelium

Following trophoblastic elongation, porcine filamentous conceptuses remain free-floating until Days 13~14 of pregnancy, after which they establish the initial attachment to the uterine LE, and the completion of implantation results in the interdigitation of the uterine LE and the trophectoderm by Day 24 of pregnancy (Dantzer, 1985; Keys and King, 1990; Perry, 1981). Pregnant gilts develop an epitheliochorial placenta that preserves the uterine LE cells, which are not destroyed as in other species and instead contribute to the apposition and attachment of the trophectoderm (Burghardt *et al*., 1997). Carbohydrate ligand-binding molecules such as selectins and galectins mediate the initial low-affinity attachment (Bazer *et al*., 2009; Ziecik *et al*., 2011). More stable adhesions are required between integrins and the extracellular matrix (ECM) expressing specific ligands and receptors necessary for uterine receptivity and conceptus attachment (Hynes, 1992; Lessey, 1995). The attachment of the trophectoderm to the ECM in the uterus involves various factors including fibronectin, secreted phosphoprotein (SPP1), laminin and transforming growth factor (TGF)-β latency-associated peptide (LAP) (Garlow *et al*., 2002; Jaeger *et al*., 2001). Another factor, the cell-surface mucin 1 (MUC-1), can also affect trophectoderm adhesion to the LE through its ability to prevent integrin binding between the trophectoderm and the uterine epithelium (Surveyor *et al*., 1995).

UTERINE MICROENVIRONMENT DURING EARLY PREGNANCY

Maternal-Conceptus Interactions

Successful conceptus development, establishment of a functional placenta and maintenance of pregnancy are the results of effective dialog between an implantation-competent blastocyst and a receptive uterus. The pre-implantation period is critical stage because during that period this maternal-conceptus network is established. Abnormal regulation of the events before implantation may often cause pre-implantation embryonic loss and poor pregnancy rates in eutherian mammals, particularly in livestock species such as the pig; in pigs, non-invasive implantation occurs and a pre-attachment phase is followed by prolonged apposition and attachment, indicating that well-organized intercellular communication is crucial (Aplin and Kimber, 2004). Over the past decades, diverse embryonic- and maternal factors that affect the pre-implantation and implantation processes have been identified, including growth factors, cytokines, ions, glucose, fructose, amino acids, and ovarian hormones (Geisert *et al*., 1982b). In the absence of uterine glands, pregnancy fails early in the peri-implantation period in sheep, indicating that uterine glands and their secretions are essential for the peri-implantation and implantation processes (Allison Gray *et al*., 2000; Gray *et al*., 2001).

Embryonic Factors

Recent advances in molecular biological approaches have led to the discovery of numerous molecules involved in embryo-uterine interactions. Between Days 11 and 12 of pregnancy, the porcine conceptus synthesizes and secretes estrogen, which acts as an initial signal

that enables maternal recognition of pregnancy (Geisert *et al*., 1982b). Conceptus-derived estrogen has been suggested to convert $PGF_{2\alpha}$ secretion from endocrine to exocrine secretion and thereby prevent the development of the endometrial luteolytic mechanism (Spencer and Bazer, 2004). This anti-luteolytic effect of blastocyst-derived estrogen results in the maintenance of a functional CL and the secretion of progesterone, which is required to maintain a uterine environment. Introduction of exogenous estrogen on Days 11~15 of the estrous cycle leads to CL maintenance for a period equivalent to or slightly longer than pregnancy. The changes induced by conceptus-derived estrogen occur concurrently with dramatic gene expression changes and the initiation of phenotypic changes that enable the conceptus to survive in a uterine environment (Choi *et al*., 1996; Yelich *et al*., 1997b).

Available results indicate that two interferons (IFNs) derived from the porcine trophoblast, IFNG (IFN-γ) and IFND (IFN-δ), play critical roles during early pregnancy in pigs, by stimulating the remodeling and/or depolarization of endometrial epithelial cells, a prerequisite for implantation and the establishment of a functional placenta (Cencic *et al*., 2003). Moreover, during the peri-implantation period, the conceptus also synthesizes and secretes a variety of molecules such as growth factors (e.g. IGFs, EGF, TGFs), cytokines (e.g., interleukins (ILs), CSFs), protease (e.g., matrix metalloprotein protease, tissue inhibitor of metalloproteinase), prostaglandins, hormones (e.g., corticotrophin-releasing hormone), and other unknown factors (Geisert and Yelich, 1997; Lefevre *et al*., 1998; Tuo *et al*., 1996). In response to these factors, the uterine endometrium undergoes morphological- and functional changes and secretes various factors to induce the development of conceptus and to become receptive to it.

Uterine Factors

Coinciding with conceptus changes, numerous genes are expressed in a spatiotemporally specific manner by uterine LE, GE and stromal cells; these genes encode secretory molecules and transporters of nutrients secreted within the uterine lumen. The complex mixture of uterine luminal secretions and molecules transported into the uterine lumen is referred to as histotroph, which orchestrate embryonic cellular activities including cell division, gene expression, and metabolism during the peri-implantation period of pregnancy. The pre-implantation embryo develops in a fluid environment that contains histotroph, and the development occurs in free-floating state, in absence of direct cellular contact with the uterus. Conceptuses may fail to develop because of failing to respond to histotroph, which includes a variety of molecules such as hormones, cytokines, growth factors, proteins, ions, lymphokines, enzymes, amino acids, glucose, vitamins, and other molecules. Histotroph components increase in the uterine lumen immediately following the release of estrogens from the conceptus on Day 11 of pregnancy (Geisert *et al*., 1982c).

GROWTH FACTORS AND CYTOKINES REGULATING CONCEPTUS CELLULAR PROCESSES

Among histotroph components, growth factors are known be required for numerous key cellular events such as proliferation, polarity, differentiation, and survival and for the development of the conceptus (Kane *et al*., 1997; Schultz and Heyner, 1993). IGFs have been well characterized throughout early pregnancy in the pig. The expression of the porcine conceptus gene encoding IGF-I increases steadily during the pre-elongation stages and peaks at the time of conceptus elongation (Letcher *et al*., 1989). Kim *et al*. demonstrated that IGF-II markedly increased the migration of ovine trophectoderm cells (Kim *et al*., 2008). EGF and TGFα are additional growth factors that can affect conceptus development (Vaughan *et al*., 1992). Another family of growth factors that have been extensively investigated during conceptus-maternal interaction is the TGFβ family, which contains three isoforms (TGFβ-1, -2 and -3). The expression of genes encoding all three TGFβ isoforms and TGFβ receptors tends to increase in the porcine conceptus trophectoderm and uterine LE during the period of rapid morphological change in conceptus development (Gupta *et al*., 1996; Gupta *et al*., 1998; Yelich *et al*., 1997a). Placental estrogens act on the endometrial epithelia to increase the expression of another specific growth factor, fibroblast growth factor-7 (FGF-7), that acts on the trophectoderm to stimulate cell proliferation as well as conceptus development (Ka *et al*., 2001).

Several cytokines have also been reported to be involved in regulating conceptus development and the establishment of pregnancy. IL-1β is known to induce the gene expression for the increase of PGE and cell membrane fluidity necessary for trophectoderm remodeling (Guan *et al*., 1998; Kol *et al*., 2002). Modric *et al*. further indicated that the expression of IL-6 gene in the pre-implantation porcine conceptus peaked on Day 12 (Modric *et al*., 2000). CSF-1 is a factor that is expressed in conceptuses as early as Days 10~12 of pregnancy (Tuo *et al*., 1995). A previous study conducted using mice lacking the CSF-1 gene indicated that conceptus-produced CSF-1 is required for successful female fertility (Wiktor-Jedrzejczak *et al*., 1990). Leukaemia-in-

hibitory factor (LIF) is another cytokine that has been proposed to facilitate conceptus-uterine communication (Anegon *et al*., 1994). Modric *et al*. indicated that LIF levels in porcine uterine-luminal flushings peaks at the time that rapid trophoblastic elongation is initiated and that LIF likely exerts direct effects through LIF-receptor β on both pre- and post-elongation conceptuses (Modric *et al*., 2000).

A collection of growth factors and cytokines produced locally at the maternal-conceptus interface has been implicated in regulating trophoblast migration and/or invasion (Cohen and Bischof, 2007); however, the mechanisms that link these factors to intracellular signal transduction are still only partially understood. A few studies have linked growth factor-induced intracellular signaling pathways to cellular activities in the trophoblast. IGF-II is known to stimulate the migration of human extravillous trophoblast (EVT) cells by activating the MAPK signaling pathway (Gleeson *et al*., 2001; McKinnon *et al*., 2001). Moreover, Kim *et al*. demonstrated that ovine IGF-II stimulates the migration of trophectoderm cells by activating both PI3K and MAPK signaling pathways (Kim *et al*., 2008). Furthermore, hepatocyte growth factor (HGF) has been demonstrated to induce PI3K-dependent migration in the SGHPL-4 human trophoblast (Cartwright *et al*., 2002), and Qiu *et al*. suggested that both PI3K and MAPK pathways are required in EGF-induced EVT migration in human (Qiu *et al*., 2004).

MECHANISMS OF EGF, IGF-I, VEGF AND CSF2 ON DEVELOPMENT OF PORCINE CONCEPTUS TROPHECTODERM

Recently, we reported novel insight into the mechanisms by which EGF, IGF-I, VEGF and CSF2 in histotroph regulate the conceptus trophoblastic properties and

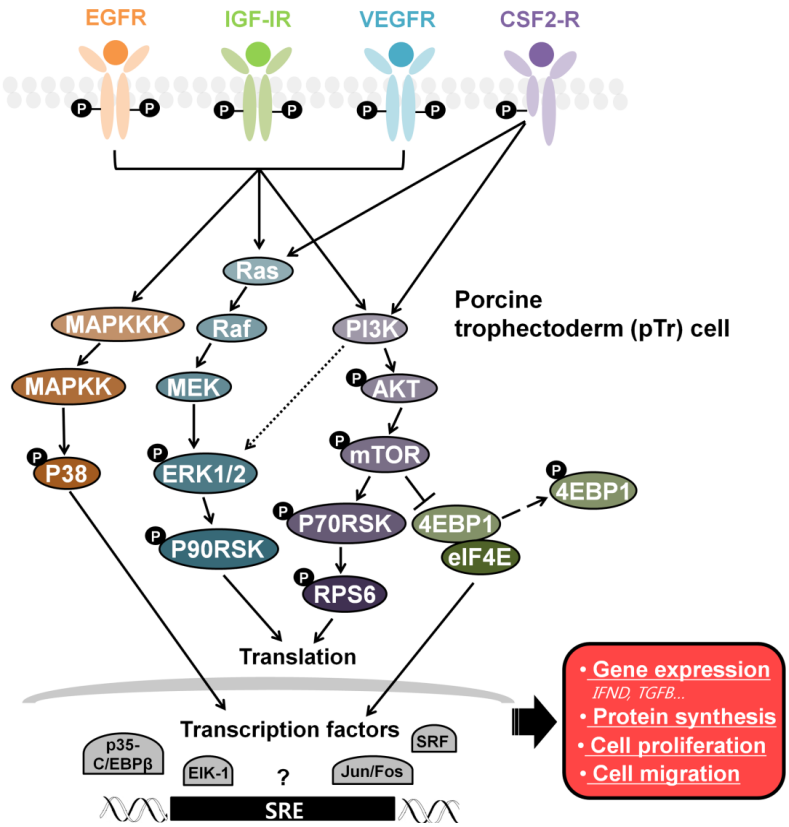

Fig. 1. A schematic illustration of the mechanisms by which the selected growth factors affect various intracellular signaling cascades responsible for the cellular activities of pTr cells during the peri-implantation period. Endometrium- and/or trophoblast-derived factors commonly induce the phosphorylation of MTOR-RPS6 through the PI3K-AKT1 signaling pathway. The ERK1/2 and P38 MAPK signaling cascades act in parallel to transduce the signals from EGF, IGF-I, and VEGF. These activated signaling pathways are likely involved in regulating gene expression and protein synthesis in the case of growth- and/or development-related genes that affect the proliferation and/or migration of the porcine trophectoderm.

activate intracellular signaling during the peri-implantation period of pregnancy. Our results revealed that the four uterine growth factors stimulated the proliferation and/or migration of pTr cells and that these effects were regulated in a coordinated manner by the PI3K-AKT and ERK1/2 MAPK signaling cascades during early pregnancy (Fig. 1). EGF, IGF-I, VEGF, and CSF2 present in the uterine cavity have been implicated as promising embryotrophic factors that underpin embryogenesis and regulate implantation in various mammalian species.

EGF has been confirmed to function as a potent stimulatory growth factor in the development of the placenta by inducing the proliferation, survival, differentiation, and invasion/migration of the trophoblast in diverse species (Barber *et al*., 2005; Bass *et al*., 1994; Biadasiewicz *et al*., 2011; Henic *et al*., 2006; Joslin *et al*., 2007; Li and Zhuang, 1997; Llimargas and Casanova, 1999). In various cellular systems, the binding of EGF to EGF receptor (EGFR) activates myriad intracellular signaling pathways including the PLCγ/protein kinase C, MAPK and PI3K pathways, resulting in the transcriptional regulation of target genes involved in numerous cellular activities (Squires *et al*., 2003; Thomas *et al*., 2003; Wieduwilt and Moasser, 2008). Furthermore, administering exogenous estrogen stimulated the binding of EGF to EGFR in immature rats and exerted mitogenic effects in ovariectomized mice, raising the possibility that EGF participates in estrogen-induced uterine growth and differentiation (Mukku and Stancel, 1985; Nelson *et al*., 1991).

IGF-I exerts mitogenic and insulin-like metabolic effects by binding to type I IGF receptor (IGF-IR) and type II IGF receptor (IGF-IIR), respectively (Jones and Clemmons, 1995; Rechler and Nissley, 1985). In pigs, IGF-I and its specific receptors are expressed in the uterine endometrium throughout the peri-implantation period and in the embryo during the pre-elongation stages (Green *et al*., 1995; Letcher *et al*., 1989; Simmen *et al*., 1990). Moreover, the expression of uterine *IGF-I* transcripts and the concentration of secreted IGF-I in uterine flushings both peak when the conceptus elongates rapidly and secretes estrogen (Geisert *et al*., 2001; Green *et al*., 1996; Miese-Looy *et al*., 2012; Simmen *et al*., 1992). However, despite the spatiotemporally matching expression of IGF-I, potential novel functions of IGF-I during the peri-implantation period and molecular mechanisms linked to the IGF-I system are poorly understood.

VEGF, also known as VEGF-A, is a heparin-binding glycoprotein that plays a critical role in angiogenesis in a variety of tissues (Charnock-Jones *et al*., 1993; Cullinan-Bove and Koos, 1993; Das *et al*., 1997; Ferrara *et al*., 2003). VEGF activates intracellular signaling cascades by binding to VEGF receptor (VEGFR)-1 (c-fms-like tyrosine kinase, Flt-1) or VEGFR-2 (fetal liver kinase-1/kinase domain-containing receptor, Flk-1/KDR) (Robinson and Stringer, 2001; Waltenberger *et al*., 1994). VEGF is known to be present in the uterus of various mammals and to participate in the processes of early embryonic development (Charnock-Jones *et al*., 1993; Cullinan-Bove and Koos, 1993; Greb *et al*., 1997; Reynolds *et al*., 1998; Winther *et al*., 1999). Knockout mice featuring a disrupted VEGF or VEGFR system show embryonic developmental abnormalities and/or early embryonic lethality (Carmeliet *et al*., 1996; Ferrara *et al*., 1996; Fong *et al*., 1995). Moreover, adding recombinant VEGF to *in vitro* culture media can stimulate the outgrowth of mouse blastocysts and increase blastocyst cell number (Biswas *et al*., 2011; Einspanier *et al*., 2002; Hannan *et al*., 2011). The VEGF-VEGFR system is detected in peri-implantation trophoblast cells, where no angiogenesis occurring, suggesting that VEGF exhibits other novel functions in blastocysts and conceptuses during the peri-implantation period.

CSF2 is a multifunctional cytokine that is hypothesized to be responsible for the survival, proliferation, and differentiation of granulocytes and macrophages (Gasson, 1991; Rapoport *et al*., 1992; Robertson *et al*., 1994). In humans and mice, CSF2 synthesis remains high following fertilization, and then declines at the time of blastocyst implantation (Robertson *et al*., 1996; Tremellen *et al*., 1998). Mice deficient in CSF2 develop blastocysts featuring a reduced numbers of cells, which is linked to impaired placental structure and increased mortality of early conceptuses (Pollard, 1997; Robertson *et al*., 2001). Furthermore, adding CSF to *in vitro* culture media increases the rate at which the blastocyst stage is reached and promotes the subsequent development of *in vitro* cultured blastocysts, and also improves postnatal development of mouse pups (Diaz-Cueto and Gerton, 2001; Hardy and Spanos, 2002; Robertson, 2007; Robertson *et al*., 2001; Sjoblom *et al*., 2005; Sjoblom *et al*., 1999). Evidence suggests that CSF2 promotes the proliferation of bovine trophectoderm cells before and during the peri-implantation period of pregnancy (Michael *et al*., 2006). Moreover, before and after the attachment phase of implantation in cows, conceptus- derived secretions including estrogens and IFN-tau (IFNT) stimulate the expression of CSF2 by the uterine epithelia (Michael *et al*., 2006; Robertson, 2007).

We confirmed that these factors and/or their receptors were expressed in the uterine endometrium and/or the trophectoderm at higher levels around the time of conceptus elongation and the secretion of estrogens by the trophoblast (Jeong *et al*., 2013, 2014a, b; Jeong *et al*., 2014c). All four factors activated PI3K-AKT and MAPK signaling pathways in pTr cells cultured *in vitro*. The

IGF-I, VEGF, and CSF2-stimulated pathways appeared to trigger cross-talk between the PI3K- and ERK1/2 MAPK signaling pathways, which differentially activate common downstream targets associated with protein synthesis, such as MTOR, p70RSK, p90RSK, RPS6, and/or 4EBP1. By contrast, in EGF-stimulated signaling, the PI3K and ERK1/2 MPAK signaling pathways appeared to function independently and probably act on distinct downstream targets in pTr cells. Interestingly, the EGFR knockdown study provides evidence supporting a role of EGF in regulating the mRNA expression of implantation-related genes such as *IFND* and *TGF β-1* during the peri-implantation period. The four factors also exhibited stimulatory effects on proliferation and/or migration of *in vitro* cultured pTr cells, but these effects were abolished upon inhibition of PI3K-, ERK1/2 MAPK-, P38 MAPK-, and MTOR signaling pathways, indicating that these factors coordinately regulate multiple cell signaling pathways that are critical to cell proliferation and migration and gene-expression changes in trophectoderm cells during early pregnancy in pigs.

CONCLUSION

Further investigation is required to elucidate more precisely the roles and signaling mechanisms of the selected factors and/or unknown factors in regulating the development and function of the endometrium and conceptuses during early pregnancy in pigs. The results described herein showing the concurrent expression of the growth factors and their receptor in maternal endometrial cells suggests that autocrine signaling occurs. Future research could examine the functional mechanisms and the signaling pathways by which growth factors induce structural- and functional changes in uterine endometria.

Another key unresolved question is the nature of the endogenous growth-factor systems' response to other molecules. Each several studies have demonstrated only the independent actions of each growth-factor system in pTr cells, but future research will determine the integrated bioavailability of these factors and the correlation between these factors and other molecules at the maternal-conceptus interface. Among these other molecules, maternal steroids are considered to be key regulators of the expression and bioactivity of growth factor systems; moreover, the pTr cells derived from Day 12 blastocysts are a natural source of estrogen and growth factors. Thus, elucidating how circulating hormones and/or other histotroph components affect the expression, secretion, and functions of these growth factor-receptor systems will be useful. Melding the independent- and combined actions of these growth factors will allow a broad and comprehensive understanding of the complex autocrine, paracrine, and juxtacrine interactions that occur in the uterine microenvironment.

More detailed and comprehensive understanding of the physiological models of conceptus development and the conceptus-maternal interaction could be of clinical relevance; unraveling the nature of this mechanism may help identify new strategies to improve embryo culture conditions and alleviate early embryonic losses and implantation failure, and thus ensure improved reproductive health in humans and economically critical livestock.

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