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Epidermal growth factor: Porcine uterine luminal epithelial cell migratory signal during the peri-implantation period of pregnancy

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ABSTRACT

The majority of early conceptus mortality in pregnancy occurs during the peri-implantation period, 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 of pregnancy. This maternal-conceptus interaction is especially crucial in pigs because they have a non-invasive epitheliochorial placentation during a protracted peri-implantation period. During the pre-implantation period of pregnancy, conceptus survival and the establishment of pregnancy depend on the developing conceptus receiving an adequate supply of histotroph which contains a wide range of nutrients and growth factors. Evidence links epidermal growth factor (EGF) to embryogenesis or implantation in various mammalian species. EGF exhibits potential growth-promoting activities on the conceptus and endometrium; however, in the case of pigs, little is known its functions, especially their regulatory mechanisms at the maternal-conceptus interface. EGF receptor (EGFR) mRNA and protein are abundant in endometrial luminal (LE) and glandular (GE) epithelia and conceptus trophectoderm on Days 13–14 of pregnancy, suggesting that EGF provides an autocrine signal to uterine LE and GE just prior to implantation. Therefore, the objectives of this study were to determine: 1) the potential intracellular signaling pathways responsible for the activities of EGF in porcine uterine LE (pLE) cells; and 2) the changes in cellular activities induced by EGF. EGF treatment of pLE cells increased the abundance of phosphorylated (p)-ERK1/2, p-P70RSK and p-RPS6 compared to that for control cells. Furthermore, EGF-stimulated phosphorylation of ERK1/2 MAPK was inhibited in pLE cells transfected with an EGFR siRNA compared with control siRNA-transfected pLE cells. Moreover, EGF stimulated migration of pLE cells, but this stimulatory effect was blocked by U0126, a pharmacological inhibitor or ERK1/2 MAPK. Collectively, these results provide new insights into mechanisms whereby EGF regulates development of the peri-implantation uterine LE at the fetal–maternal interface. These results indicate that endometrial- and/or conceptus derived EGF effects migration of uterine LE and that those stimulatory effects are regulated via the ERK1/2 MAPK pathway during early pregnancy in pigs.

1. Introduction

Implantation is the process by which an activated blastocyst establishes a close physical and physiological interaction with the maternal endometrium to form the placenta that serves as the interface for exchange of nutrients and gases between the fetal-placental and maternal circulations. Successful implantation depends on the embryo developing to the blastocyst stage in synchrony with differentiation of the uterus to the receptive state,

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which is followed by two-way interactions between the blastocyst and the uterine luminal epithelium (LE) (Psychoyos, 1973; Tranguch et al., 2005). The aforementioned coordinated interactions of various factors derived from the conceptus or the uterus are especially critical in pigs because they have a true non-invasive epitheliochorial type of implantation and placentation during protracted peri-implantation and placentation phases of pregnancy (Aplin and Kimber, 2004). Also in pigs, 20–30% of embryonic deaths occur during early pregnancy, indicating that the pre-implantation period is critical for establishment and maintenance of pregnancy (Pope, 1988; Pope et al., 1986).

During the peri-implantation period of pregnancy in pigs, the developing conceptus secretes estrogens which are responsible for maternal recognition and establishment of pregnancy. Estradiol from coneptus trophectoderm signals its presence to uterine epithelia by inducing synchronous release of secretions from uterine luminal (LE) and glandular (GE) epithelia and gene expression (Geisert et al., 1982a, 1982b). In response to uterine secretions, pig conceptuses secrete factors including estrogens, interferons (IFNs), prostaglandins (PGs), growth factors, cytokines, and other unidentified factors (Tuo et al., 1996; Geisert and Yelich, 1997; Lefevre et al., 1998; Jaeger et al., 2001), The uterine endometrium undergoes morphological changes and produces various molecules that are either secreted by uterine epithelia or are transported into the uterine lumen by those cells and collectively referred to as histotroph, which is required for conceptus development and uterine receptivity for implantation and placentation (Bazer, 1975; Kane et al., 1997). Studies conducted using the uterine-gland knockout ewe (UGKO) model demonstrated that sufficient secretions of the uterine GE are required for survival, growth and pregnancy-recognition signaling by the peri-implantation conceptus (Gray et al., 2001, 2002). 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 study focused on epidermal growth factor (EGF) that is involved in conceptus-maternal interactions. EGF is a multifunctional growth factor that plays a role in initiating signaling that directs the behavior of epithelial cells including growth, proliferation, and differentiation (Carpenter and Cohen, 1990; Pedersen et al., 1994; Haase et al., 2003; Bass et al., 1994; Engebraaten et al., 1993; Shibata et al., 1996). EGF exerts its biological effects on cells via the EGF receptor (EGFR) identified as a one transmembrane glycoprotein of the ERBB receptor tyrosine kinase family (Das et al., 1977; Salomon et al., 1995). EGF is a potent stimulatory growth factor affecting development of the placenta by inducing the proliferation, survival, differentiation, and invasion/migration of the trophoblast in diverse species (Bass et al., 1994; Li and Zhuang, 1997; Biadasiewicz et al., 2011; Barber et al., 2005; Joslin et al., 2007; Llimargas and Casanova, 1999; Henic et al., 2006). EGF has also 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 of pregnancy (Chegini et al., 1986; Smith et al., 1991; Das et al., 1994). Exogenous estrogen stimulates the binding of EGF to EGFR in immature rats and exerts mitogenic effects in ovariectomized mice, raising the possibility that EGF participates in estrogen-induced uterine growth and differentiation (Nelson et al., 1991; Mukku and Stancel, 1985). Furthermore, including recombinant EGF in *in vitro* culture media for embryos enhances their developmental ability and implantation rate after transfer to the uterus, and a deficiency in EGF results in deficiencies in placental structure and fetal growth (Kamei et al., 1999; Wood and Kaye, 1989; Harper and Brackett, 1993; Wei et al., 2001; Mtango et al., 2003; Dackor et al., 2009). Whereas porcine EGF is considered to contribute to conceptus development because its gene is expressed during the peri-implantation period of pregnancy, little is known about the EGF-EGFR system in the porcine uterus in terms of regulatory mechanisms.

A recent study conducted using pigs revealed that the levels of expression *EGF* and *EGFR* mRNA in endometrial epithelia and/or conceptus trophectoderm increased during the peri-implantation period of pregnancy when the conceptus is elongating, and this increase in EGF secretion was proposed to induce development of peri-implantation conceptuses (Jeong et al., 2013). Evidence for concurrent expression of EGF and its receptor in maternal endometrial cells suggests the possibility of autocrine signaling induced by EGF in uterine epithelia. Despite these hypothesized roles and functions of EGF, little is known about the cell signaling pathways stimulated by EGF in pLE cells and how this signaling stimulates uterine development during early pregnancy. It should be noted that pigs have a central type of implantation and form a true epitheliochorial placenta as the uterine LE remains intact throughout pregnancy and trophectoderm/chorion directly attaches to the uterine LE; however, there is extensive remodeling and folding of chorion attached to the corresponding endometrium to increase the area of uterine–placental association (Bazer and Johnson, 2014). Therefore, future research should examine the functional mechanisms and the signaling pathways by which EGF induces structural- and functional changes in uterine endometria.

In this study, we hypothesized that during the peri-implantation period of pregnancy in pigs, the EGF-EGFR system induces activation of internal signaling cascades and cellular functional changes in pLE cells as for trophectoderm cells of pig conceptuses. We tested this hypothesis by determining: 1) stimulatory effects of EGF on the ERK1/2 MAPK cell signaling cascades in pLE cells; and 2) functional effects of EGF on migration of pLE cells.

2. Materials and methods

2.1. Cell culture

A spontaneously immortalized porcine uterine endometrial luminal epithelial (pLE) cell line (Wang et al., 2000) was cultured and used in the present *in vitro* studies. This cell line was developed by immortalizing primary uterine LE cells by transfection with the replication-defective retrovirus (SV40) vector pLXSN-16E6E7 expressing E6/E7 proteins of human papilloma virus (HPV) type 16 (Wang et al., 2000). The pLE cells have typical epithelial-like cobblestone-shaped morphology, and show positive staining with antibodies to epithelium-specific cytokeratin and negative staining for vimentin (Wang et al., 2000). The pLE form a single monolayer at confluence. All analyses of protein expression and cell migration were performed on pLE cells between passages 25–30. Briefly, monolayer cultures of pLE cells were grown in DME/F12 1:1 culture medium containing 20% fetal bovine serum to 80% confluence in 100-mm tissue culture dishes. For assays, *in vitro* cultured pLE cells were serum starved for 24 h, and then incubated in the presence of various treatments.

2.2. Western blot analyses

Concentrations of protein in whole-cell extracts were determined using the Bradford protein assay (Bio-Rad, Hercules, CA) with bovine serum albumin (BSA) as the standard. Proteins were denatured, separated using SDS-PAGE and transferred to nitrocellulose. Blots were developed using enhanced chemiluminescence detection (SuperSignal West Pico, Pierce, Rockford, IL) and