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Epidermal growth factor stimulates proliferation and migration of porcine trophectoderm cells through protooncogenic protein kinase 1 and extracellular-signal-regulated kinases 1/2 mitogen-activated protein kinase signal transduction cascades during early pregnancy

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ABSTRACT

For successful implantation and establishment of early epitheliochorial placentation, porcine conceptuses require histotroph, including nutrients and growth factors, secreted by or transported into the lumen of the uterus. Epidermal growth factor (EGF), an essential component of histotroph, is known to have potential growth-promoting activities on the conceptus and uterine endometrium. However, little is known about its effects to transactivate cell signaling cascades responsible for proliferation, growth and differentiation of conceptus trophectoderm. In the present study, therefore, we determined that EGFR mRNA and protein were abundant in endometrial luminal and glandular epithelia, stratum compactum stroma and conceptus trophectoderm on days 13–14 of pregnancy, but not in any other cells of the uterus or conceptus. In addition, primary porcine trophectoderm (pTr) cells treated with EGF exhibited increased abundance of phosphorylated (p)-AKT1, p-ERK1/2 MAPK and p-P90RSK over basal levels within 5 min, and effect that was maintained to between 30 and 120 min. Immunofluorescence microscopy revealed abundant amounts of p-ERK1/2 MAPK and p-AKT1 proteins in the nucleus and, to a lesser extent, in the cytoplasm of pTr cells treated with EGF as compared to control cells. Furthermore, the abundance of p-AKT1 and p-ERK1/2 MAPK proteins was inhibited in control and EGF-treated pTr cells transfected with EGFR siRNA. Compared to the control siRNA transfected pTr cells, pTr cells transfected with EGFR siRNA exhibited an increase in expression of *IFND* and *TGFB1*, but there was no effect of expression of *IFNG*. Further, EGF stimulated proliferation and migration of pTr cells through activation of the PI3 K-AKT1 and ERK1/2 MAPK-P90RSK cell signaling pathways. Collectively, these results support the hypothesis that EGF coordinately activates multiple cell signaling pathways critical to proliferation, migration and survival of trophectoderm cells that are critical to development of porcine conceptuses during implantation and placentation.

1. Introduction

Epidermal growth factor (EGF) is a 6 kDa protein consisting of 53 amino acid residues and three intra-molecular disulfide bridges that plays pivotal roles in a variety of biological processes such as cell growth, proliferation, and differentiation (Carpenter and Cohen, 1990). After binding to its cognate EGF receptor (EGFR), which is a transmembrane glycoprotein member of the ERBB family of receptor tyrosine kinases, EGF induces autophosphorylation of EGFR-bound kinases and subsequently relays signals via a series of transcription factors to transactivate expression of target genes (Herbst, 2004). EGF has been implicated in development of the human placental-trophoblast microenvironment in many ways, including trophoblast proliferation, survival, differentiation, invasion and/or migration (Bass et al., 1994; Li and Zhuang, 1997; Biadasiewicz et al., 2011; Barber et al., 2005). Also, it has been shown that the EGF–EGFR system activates the mitogen-activated protein

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kinase (MAPK) and the phosphatidylinositol 3-kinase (PI3 K)/AKT pathways, suggesting that activation of these signaling pathways are important for human placentation and implantation (Squires et al., 2003; Knofler, 2010).

In the porcine uterus, implantation is initiated with apposition and attachment of conceptus (embryo/fetus and associated membranes) trophectoderm to uterine luminal (LE) epithelium (Bazer et al., 2010). Indeed, the porcine conceptus undergoes dramatic morphological changes requiring proliferation, migration and differentiation of trophectoderm cells in preparation for implantation and placentation during the peri-implantation period of pregnancy (Bazer et al., 2009). Following the morphological differentiation and cellular polarization at the blastocyst stage, the porcine conceptus dramatically elongates from 5 to 150 mm in length and also secretes the maternal recognition signal between days 10 and 12 of pregnancy (Bazer and Thatcher, 1977; Geisert et al., 1982). The free-floating filamentous conceptus initiates attachment to the uterine LE by day 13 of pregnancy, and implantation is complete by day 24 of pregnancy (Keys and King, 1990; Geisert and Yelich, 1997). This process requires histotroph, which includes a number of essential molecules such as nutrients, growth factors, hormones, cytokines, lipids, ions, and sugars that are either transported into or secreted into the uterine lumen by uterine epithelia for establishment and maintenance of pregnancy (Bazer et al., 2010). Of these components of histotroph, growth factors are required for many key events such as proliferation, polarity, differentiation, survival and development of the conceptus (Schultz and Heyner, 1993; Simmen and Simmen, 1991). Therefore, deficiencies of these growth factors and nutrients during early pregnancy result in poor pregnancy outcomes, as well as poor postnatal growth and health (Bazer, 1975; Wu et al., 2004). For instance, studies of the uterine gland knockout ewe model demonstrated the requirement for components of histotroph from uterine glandular (GE) epithelium for survival and normal development of the conceptus, as well as pregnancy recognition signaling (Gray et al., 2002). Our previous studies with pigs revealed that nutrients such as glucose, arginine, leucine, and glutamine in the uterine lumen coordinately activate proto-oncogenic protein kinase AKT1 (AKT1), mechanistic target of rapamycin (MTOR), ribosomal protein S6 K (RPS6 K), and ribosomal protein S6 (RPS6) cell signaling to stimulate hypertrophy, hyperplasia, and migration of ovine trophectoderm cells during the peri-implantation period of pregnancy (Kim et al., 2011a,b,c).

As compared with classical hormones, growth factors have unique features in that their sites of synthesis and sites of action are restricted to defined glands or tissues (Carpenter and Cohen, 1990). Since EGF was discovered and characterized by Stanley Cohen, who won Nobel Prize in Physiology and Medicine in 1986, numerous studies in humans and mice have elucidated biological functions of EGF. During the female reproductive cycle, the expression of EGF and EGFR is predominantly in pregnant uteri of various mammalian species such as human (Chegini et al., 1986; Smith et al., 1991) and mouse (Das et al., 1994) and associated with proliferation and differentiation of cells and uterine remodeling for implantation and placentation. However, little is known about EGF and EGFR in the porcine uterus regarding regulatory mechanisms whereby EGF affects uterine functions and development of the conceptus. Although failures of implantation and placentation due to under-developed conceptuses are not well-understood, a better understanding of molecular and cellular processes required for these events will provide important insights into human and animal reproduction. Insufficient delivery of histotroph, including growth factors and nutrients, to the developing conceptus results in intrauterine growth restriction, a significant social and economic problem of global importance (Bazer et al., 2012). Furthermore, despite the hypothesized roles and functions of the EGF–EGFR system, little is known about cell signaling pathways stimulated by EGF in porcine conceptus trophectoderm cells and how EGF stimulates conceptus development during pregnancy. Therefore, the present study was conducted to determine the molecular mechanisms by which EGF activates the PI3 K-AKT1 or ERK1/2 MAPK cell signal transduction pathway for proliferation, migration and gene expression by pig trophectoderm cells.

2. Materials and methods

2.1. Experimental animals and animal care

Sexually mature gilts of similar age, weight, and genetic background were observed daily for estrus (day 0) and exhibited at least two estrous cycles of normal duration (18–21 days) before being used in this study. All experimental and surgical procedures were in compliance with the Guide for Care and Use of Agricultural Animals in Teaching and Research and approved by the Institutional Animal Care and Use Committee of Texas A&M University.

2.2. Experimental design and tissue collection

To evaluate the effects of pregnancy on expression of EGFR, ERK1/2 MAPK-P90RSK and PI3 K-AKT1, gilts were assigned randomly to either cyclic or pregnant status. Those in the pregnant group were bred when detected in estrus and 12 and 24 h later. Gilts were ovariohysterectomized on either days 9, 12, or 15 of the estrous cycle or on days 9, 12, 13, 14, 15 or 20 of pregnancy (n = 3–4 pigs per day per status). For confirmation of pregnancy prior to implantation, the lumen of each uterine horn was flushed with 20 ml of physiological saline on days 9–15 of pregnancy and examined for the presence of morphologically normal conceptuses. Uteri from gilts on days 9–15 of the estrous cycle were processed in the same way. Several sections (~0.5 cm) of the entire uterine wall from the middle of each uterine horn were fixed in fresh 4% paraformaldehyde in PBS (pH 7.2) and embedded in Paraplast-Plus (Oxford Laboratory, St. Louis, MO).

2.3. Cell culture

Mononuclear porcine trophectoderm (pTr) cells from day 12 pig conceptuses were cultured and used in the present *in vitro* studies as described previously (Kim et al., 2011a,b,c). For experiments, monolayer cultures of pTr cells were grown in culture medium to 80% confluence in 100-mm tissue culture dishes. Cells were serum starved for 24 h, and then treated with recombinant human pro-EGF (100 ng/ml; R&D Systems, Inc., Minneapolis, MN) for 0, 5, 15, 30, 60 or 120 min. Based on preliminary dose–response experiments, 100 ng/ml EGF was selected for use in all experiments in the present study. This design was replicated in three independent experiments.

2.4. RNA isolation

Total cellular RNA was isolated from endometrium from cyclic and pregnant gilts using Trizol reagent (Invitrogen, Carlsbad, CA) and purified using an RNeasy Mini Kit (Qiagen) according to the manufacturer's recommendations. The quantity and quality of total RNA was determined by spectrometry and denaturing agarose gel electrophoresis, respectively.

2.5. Cloning of partial cDNA for porcine EGF receptor

Complementary DNA was synthesized using AccuPower® RT PreMix (Bioneer, Daejeon, Korea). A partial cDNA for porcine *EGFR* mRNA was amplified using specific primers based on data for por-