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Alterations in the distribution of actin and its binding proteins in the porcine endometrium during early pregnancy: Possible role in epithelial remodeling and embryo adhesion

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ABSTRACT

During early pregnancy, uterine epithelial cells undergo major transformations in their cytoskeleton that make the endometrium receptive for conceptus attachment. Actin binding proteins (ABPs) such as cofilin, gelsolin, and vinculin are involved in regulating actin polymerization, severing or crosslinking actin to integrins. However, whether ABPs are involved in epithelial remodeling or embryo adhesion in pigs is unknown. Therefore, the expression and distribution of these proteins were investigated in porcine endometrium on Days 10 and 13 (pre-implantation period), and 16 (attachment phase) of the estrous cycle or pregnancy. While day and pregnancy status had no effect on ABP gene expression, the protein abundance of vinculin was significantly higher on Day 13 than on Day 10 ($p < 0.05$) of the estrous cycle, and its abundance was highest on Day 16 in the pregnant endometrium. Immunofluorescent staining showed alterations in the distribution of these proteins depending on the day of the estrous cycle or early pregnancy examined. Double immunofluorescent staining for the ABPs and actin revealed that while cofilin co-localized with actin in the apical epithelium on Days 13 and 16 of the estrous cycle, in pregnant animals, it was strongly associated with actin in the sub-epithelial stroma of the endometrium. Gelsolin was also co-localized with actin in the apical epithelium on Days 13 and 16 of the estrous cycle, but this association was absent in the pregnant endometrium. Vinculin co-localized with actin in the sub-epithelial stroma on Days 13 and 16 irrespective of the reproductive status, but was additionally associated with actin in the apical epithelium on Day 16 of pregnancy. Vinculin interacted with phosphorylated focal adhesion kinase in the endometrial epithelium, and the interaction was dependent on estradiol-17β, a conceptus-secreted pregnancy-recognition factor in pigs. Furthermore, silencing vinculin in the endometrial epithelial cells negatively affected trophoblast adhesion to them. In conclusion, the influence of stage and reproductive status on the specific localization of actin and its binding proteins in the porcine endometrium suggests that they play a role in regulating the endometrial cytoskeleton. Moreover, vinculin may facilitate conceptus attachment to the epithelium by interacting with focal adhesion kinase.

1. Introduction

Implantation is a complex biological process that requires not only the development of the embryo into an implantation-competent blastocyst, but also a receptive endometrium. As part of achieving receptivity, the endometrium undergoes transformations in response to physiological changes initiated by many peripheral and local factors including ovarian hormones that prepare it for possible pregnancy and implantation [1]. These transformations involve changes to the endometrial epithelium, which is the first site of contact between maternal and placental cells and, for most part, resistant to embryo attachment. It becomes receptive for embryo adhesion only for a transient period, during which it undergoes extensive tissue growth and morphological and biochemical alterations that support rapid conceptus growth and development. In rats, humans, pigs, and few other mammalian species, the luminal uterine epithelial cells of the non-receptive uterus undergo changes involving the loss of apical microvilli, the appearance of apical uterodomes, and an increase in basolateral

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membrane tortuosity [2–5]. These changes are associated with producing the receptive uterine state. Whereas most of the morphological changes are common across species [6], the data regarding changes in the molecular parameters are less consistent.

The luminal epithelium of the uterine endometrium is composed of polarized cells. Whereas the apical region of epithelial cells is orientated toward the uterine lumen, the basal region interacts with the underlying stroma [7]. Cell polarity is imperative for many physiological processes, including cell-cell interactions, transmembrane communication, and molecular transport [8,9]. Establishment or loss of cell polarity is associated with alterations in the actin cytoskeleton [10], the latter being the case during the establishment of pregnancy [11]. Actin-binding proteins (ABPs) are responsible for remodeling the actin cytoskeleton that can occur through the nucleation or severing of actin filaments.

The actin cytoskeleton plays a major role in the formation of many essential cellular structures such as microvilli and cellular junctions, structures that undergo transformations during early pregnancy in mammalian species [2,6]. In humans, the ABP cofilin (CFL) plays an important role in decidualization and blastocyst implantation [12], and its dysregulation leads to endometriosis [7]. Gelsolin (GSN), a key regulator of cellular function, is implicated in actin filament reorganization during cell locomotion [13] and is among the estrogen-responsive genes in human endometrium [14]. Vinculin (VCL), a major component of cell-cell junctions in human endometrium [15], associates with focal adhesion kinase (FAK) and facilitates the anchoring of actin to focal adhesions. Focal adhesion kinase has also recently been implicated in preparing the epithelium to establish endometrial receptivity in mice [16]. Another actin-network protein of importance is filamentous F-actin. In humans, stabilizing F-actin negatively affects decidualization [8], while severing it into its monomers is important for remodeling epithelial cells for adhesion [17].

Epithelial integrity is maintained in pigs, but the attachment of the trophoblast to the apical plasma membrane requires changes to or a redistribution of the cytoskeletal proteins in the apical structure, the modification of transfer routes for metabolite exchange [18], and the regulation of focal adhesions [19]. A proteomic analysis of porcine endometrium found many ABPs such as CFL, GSN, and VCL had differential expression on different days of the estrous cycle and pregnancy [20]. We hypothesized that these ABPs (CFL, GSN and VCL) may be involved in remodeling the actin cytoskeleton of porcine endometrial epithelial cells during early pregnancy.

This study investigated the expression and localization of CFL, GSN and VCL in the porcine endometrium during early pregnancy. Because of VCL's role in cell-cell adhesion and its apical localization in the porcine endometrium, we also investigated whether it is involved in embryo attachment to the endometrial epithelium. Though several ABPs have been previously investigated in the uteri of rats and humans during early pregnancy [21–23], their distribution and role in pigs, which have epitheliochorial placentation, is unknown. To the best of our knowledge, this study is the first report on the association of these proteins with actin in the porcine endometrium during early pregnancy. Furthermore, we also report a possible role for VCL in embryo adhesion.

2. Materials and methods

2.1. Animals

All procedures involving the use of animals were conducted in accordance with the national guidelines for agricultural animal care and were approved by the Animal Ethics Committee, University of Warmia and Mazury, Olsztyn, Poland. Hormonal treatments were used to induce and synchronize the estrous cycle in 30 crossbred gilts (*Sus scrofa domesticus*) that weighed ~100 kg each; the gilts were treated with 750 IU of equine chorionic gonadotropin intramuscularly, followed 72 h later with 500 IU of human chorionic gonadotropin. Following estrus induction, the gilts were randomly divided into two groups that received either a uterine infusion of sterilized PBS (cyclic group; n = 18) or two artificial inseminations with 2.5×10^9 spermatozoa within a 12-h interval (pregnant group; n = 12). The artificial insemination was performed 24 h after treating with human chorionic gonadotropin. The gilts (5–6 per day) were slaughtered at a local abattoir on Days 10, 13 and 16 of the estrous cycle or on Days 13 and 16 of pregnancy. We did not include Day 10 for the pregnant animals because no major differences in ABP abundance have been observed between the estrous cycle and pregnancy on that day [20]. During the pre-implantation stage, both uterine horns were flushed with 20 mL of sterile PBS (pH 7.4) to recover the conceptuses and confirm pregnancy. After collecting the uterus and flushing with PBS, the endometrium was separated from the myometrium by opening the uterus longitudinally on the antimesometrial surface, snap frozen in liquid nitrogen, and stored at −80 °C until further analysis. For the gene expression and western blot studies, the endometria collected from the medial region of each uterine horn were pooled together before snap freezing. For immunofluorescence, approximately three cross sections (1 × 1 cm) of each uterine horn (n = 3) were dissected and fixed overnight.

2.2. RNA isolation and reverse transcription

Total RNA was extracted from frozen endometrial tissue using a Total RNA Prep Plus kit (A&A Biotechnology). The RNA samples (2 μg/reaction) were treated with DNase I (Invitrogen Life Technologies, Inc., Carlsbad, CA, USA) and reverse transcribed using the High-Capacity cDNA Reverse Transcription kit (Life Technologies, Foster City, CA, USA) according to manufacturer instructions. The NormFinder algorithm was applied to select between glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and beta actin as the most stable housekeeping gene. Based on the report, *GAPDH* had higher stability (0.182 vs. 0.247 for beta actin) and was therefore chosen as the housekeeping gene [24].

To evaluate *CFL*, *GSN*, *VCL*, and *GAPDH* gene expression, cDNA (15 ng) was amplified using the *CFL* (Ss03391732_g), *GSN* (Ss03374387_m1), *VCL* (Ss03391018_m1), and *GAPDH* (Ss03375435_u1) TaqMan Gene Expression assays (Life Technologies) using an ABI Prism 7900 Sequence Detection System or a ViiA 7 Real-Time PCR System (Life Technologies). Each PCR reaction (10 μL) was performed in duplicate in a 384-well plate using an initial denaturation of 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 60 s at 60 °C (annealing and extension).

The real-time PCR data were analyzed using the Miner method as described previously [25]. Briefly, the relative mRNA concentration (R0) for each target and control gene was calculated using the equation $R0 = 1/(1 + E)^{Ct}$, where E is the average gene efficiency, and Ct is the cycle number at threshold. The R0 for each gene was normalized to the R0 of the GAPDH control; relative gene expression was calculated as $R0_{\text{target gene}}/R0_{\text{GAPDH}}$ and is expressed in arbitrary units.

2.3. Double immunofluorescence

For the double immunofluorescence studies, uterine tissues were fixed in 4% buffered formalin for 24 h at room temperature (RT), dehydrated in an ethanol gradient, embedded in paraffin, and cut into ~5-μm sections. The sections were deparaffinized and rehydrated before antigen retrieval in a citrate buffer antigen-unmasking solution. The actin and ABPs were visualized in the