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Endometrial androgen signaling and decidualization regulate trophoblast expansion and invasion in co-culture: A time-lapse study

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ABSTRACT

Introduction: To elucidate whether trophoblast expansion and invasion are modulated by androgen signaling in an *in vitro* co-culture model system with decidualizing endometrial stromal cells (ESCs).
Methods: We employed an *in vitro* co-culture model of early embryo implantation, consisting of human ESCs (EtsT499 cells) and spheroids generated by extravillous trophoblast (EVT) derived HTR8/Svneo. The ESCs were decidualized with 8-bromo-cAMP (8-br-cAMP) in the presence or absence of dihydrotestosterone (DHT) at various concentrations for 5 days before co-culture with EVT spheroids. Trophoblast expansion was monitored by fluorescent time-lapse imaging microscopy. ESCs motility was visualized by using CellTracker™ Orange CMRA fluorescent probe. Apoptosis of ESCs was detected by CellEvent™ Caspase-3/7® green detection reagent. Invasion assays were performed to quantify EVT invasion through a chemotaxis cell membrane.
Results: Expansion of EVT spheroids was significantly enhanced by decidualized compared to undifferentiated ESCs. This process was further stimulated if ESCs were first decidualized in the presence of DHT. In contrast to decidualized ESCs, undifferentiated cells actively migrated away from expanding EVT spheroids. Invasiveness of EVT toward decidualized ESCs was significantly attenuated in comparison to undifferentiated ESCs. DHT had no effect on EVT invasion. However, an inhibitor of intercellular gap junction communication significantly enhanced EVT invasion towards decidualized ESCs.
Conclusions: These results indicate distinct roles for androgen signaling and gap junction formation in decidual cells in regulating trophoblast expansion and invasion.

1. Introduction

Establishment of pregnancy requires coordination of three interdependent processes, embryo development, endometrial decidualization, and placenta formation. Interactions between the invasive trophoblast and the maternal uterine decidual environment are critical for pregnancy [1]. Decidualization denotes the morphologic and biological differentiation process in the endometrial stromal compartment, initiated in the mid-to late-secretory phase of the cycle. It is characterized by the induction of marker genes, such as *PRL* and *IGFBP1*, in differentiating endometrial stromal cells (ESCs) and coincides with vascular remodeling and influx of specialized immune cells, mainly uterine natural killer cells and macrophages, into the stroma [2,3]. Upon breaching of the luminal endometrial epithelium, the implanting embryo is rapidly surrounded and encapsulated by migrating decidualizing ESCs. As the

Abbreviation: EVT, extravillous trophoblast; AR, androgen receptor; ESC, endometrial stromal cell; 8-br-cAMP, 8-bromoadenocine 3′,5′-cyclic monophosphate; DHT, dihydrotestosterone; CX, connexin; GJIC, gap junction intercellular communications; AGA, 18α-glycyrrhetinic acid; OcOH, Octanol; IUGR, intrauterine growth restriction; EMT, epithelial-mesenchymal transition; MET, mesenchymal-epithelial transition.

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differentiation process unfolds, gap and tight junctions form between decidualizing cells [4,5]. Consequently, the conceptus becomes anchored in the endometrium and forms a matrix that enables coordinated trophoblast invasion. Although the decidua is believed to provide a cytokine/chemokine environment that attracts invading extravillous trophoblast (EVT), it also serves as a physical barrier on the way to gaining access to the maternal blood supply [6]. Impaired decidualizing process is considered to link with a variety of reproductive disorders including, infertility, recurrent miscarriages, utero-placental disorders, and endometriosis [3,7,8].

Decidual transformation of estrogen-primed endometrium is mediated by the postovulatory rise in circulating progesterone and rising intracellular cAMP levels. The effects of sex steroids are mediated predominantly by their cognate nuclear receptor, i.e. the estrogen and progesterone receptors. The androgen receptor (AR) is expressed in both male and female reproductive organs [9,10]. In women, ovarian AR is essential for folliculogenesis; and its ablation in mice leads to premature ovarian failure [9]. Serum androgen levels fluctuate throughout the normal menstrual cycle and peak around ovulation. In the endometrium, AR expression is expressed in endometrial stromal cells (ESCs) but not in epithelial cells [11,12]. Notably, tissue androgen levels and conversion of androstenedione to testosterone are higher in secretory when compared to proliferative endometrium [13,14]. Recently, we demonstrated that androgens enhance morphological transformation of decidualizing ESCs and upregulate the expression of the gap junction protein Connexin43 (Cx43) [15,16]. These observations suggest that androgen may also coordinate decidual-trophoblast interactions in early pregnancy. However, the effects of androgens, if any, on trophoblast expansion and invasion in early pregnancy are poorly understood.

Ethical concerns and technical limitations significantly hinder our understanding of the implantation process in humans. To overcome this hurdle, *in vitro* implantation models involving co-culture of a monolayer human ESCs and trophoblast spheroids, mimicking the trophectoderm of the early conceptus, are widely used [17–19]. In this study, we used time-lapse imaging of trophoblast-decidual cell co-cultures to elucidate the effects of androgens on the implantation process.

2. Material and method

2.1. Cell cultures

We employed the immortalized human endometrial stromal cell (ESC) cell line (EtsT499) [20] and the immortalized first trimester extravillous trophoblast (EVT) cell line (HTR-8/SVneo) which was kindly provided from Dr. Charles H. Graham (Queen's University, Ontario, Canada) and Dr. Eiko Yamamoto (Nagoya University Graduate School of Medicine, Nagoya, Japan) [21]. EtsT499 cells were passaged and cultured in flask with Dulbecco Modified Eagle Medium (DMEM)/F-12 (ThermoFisher Scientific, Waltham, MA) containing 10% dextran-coated charcoal-treated fetal bovine serum and 1% Penstrep® (Penicillin 10,000 U/mL, Streptomycin 10,000 μg/mL; ThermoFisher Scientific). Before starting co-culture, confluent monolayer EtsT499 cells were treated with 0.5 nmol 8-bromoadenocine 3′,5′-cyclic monophosphate (8-br-cAMP; Sigma-Aldrich, St. Louis, MO) in the presence or absence of various concentration of dihydrotestosterone (DHT; Sigma-Aldrich) for 5 days to induce decidualization.

HTR-8/SVneo cells were maintained in RPMI (Roswell Park Memorial Institute) Medium 1640 (ThermoFisher Scientific) with 5% FBS and 1% Penstrep®. Spheroids were prepared from EVT cells on the basis of a procedure described by Korff et al. [22]. In brief, HTR-8/SVneo cells were seeded at 10,000 cells per well with 200 μl condition medium in a 96-well plate with non-adherent round well bottoms (Asahi Techno Glass, Funabashi, Japan). Spheroids of 100 μm in diameter, which were similar size to an implantation blastocyst, were generated after 4 days of culture.

2.2. In vitro implantation model

The co-culture system was set up in wells of six-well plates. EtsT499 cells were either decidualized in the presence or absence of DHT (10^{-8} or 10^{-7} M) for 5 days or left untreated. HTR-8/SVneo spheroids (6 spheroids/well) were carefully transferred onto the confluent EtsT499 monolayers with a pipette tip. The culture medium was then changed to medium, containing no 8-br-cAMP or DHT (DMEM F-12 with 10% FBS and 1% Penstrep®). This time point was designated as day 0. The microphotographs of expanded EVT spheroids after co-culture were captured by a fluorescent microscope (Keyence BZ-9000) using a 4 × objective. The expansion areas of spheroids were measured using ImageJ (NIH Image; http://rsb.info.nih.gov/nih-image/).

2.3. Cell labeling and continuous observation of cell migration

The EtsT499 cells were labeled with CellTracker™ orange fluorescent probe (C34551, Molecular Probes®, ThermoFisher Scientific), and migration monitored by time-lapsed microscopy (KEYENCE fluorescence microscopy BZ-9000, Osaka, Japan). Untreated or differentiating EtsT499 cells were incubated with CellTracker™ Orange CMRA at a concentration of 5 M for 30 min. Then, the labeling medium was aspirated, non-labeled spheroids were placed onto labeled monolayer EtsT499 cells and the cultures refreshed with medium that did not contain either 8-br-cAMP or DHT (DMEM F-12 with 10% FBS and 1% Penstrep®). Cell migration was monitored for 24 h by time-lapsed microscopy.

2.4. SDS-PAGE and Western blotting

Protein fractions from whole-cell extracts were immunoblotted as described previously [23,24]. Primary antibodies were used as follows: anti-androgen receptor (AR; 1:1000 dilution, Biogenex, San Ramon, CA), anti-connexin 43 (CX43; 1:2500 dilution, Cell Signaling Technology, Danvers, MA), anti-α-Tubulin (1:1000 dilution, Santa-Cruz Biotechnology, Dallas, Texas). Blots were exposed to the primary antibody in Tris-buffered saline for 1 h at room temperature and then incubated with secondary peroxidase-labeled antibody for 1 h at room temperature. Protein bands were detected using Amersham ECL Prime detection reagent (GE Healthcare LifeSciences, Pittsburgh, PA). The developed X-ray films were scanned for quantification and analysis was performed on a Macintosh computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available at http://rsb.info.nih.gov/nih-image/). Loading was corrected by the ratio between the detected proteins values and the value of the corresponding α-tubulin protein and was expressed as arbitrary units (AU).

2.5. RNA extraction

Total RNA was purified from EtsT499 cells using miRNeasy Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. RNA quality was verified by measuring the absorbance at 230 nm, 260 nm and 280 nm with a NanoDrop spectrophotometer (ND-1000, ThermoFisher Scientific, Waltham, MA).