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Research paper

Tissue organization alters gene expression in equine induced trophectoderm cells

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ABSTRACT

Rapid morphological and gene expression changes occur during the early formation of a mammalian blastocyst. Critical to successful retention of the blastocyst and pregnancy is a functional trophectoderm (TE) that supplies the developing embryo with paracrine factors and hormones. The contribution of TE conformational changes to gene expression was examined in equine induced trophoblast (iTr) cells. Equine iTr cells were cultured as monolayers or in suspension to form spheres. The spheres are hollow and structurally reminiscent of native equine blastocysts. Total RNA was isolated from iTr monolayers and spheres and analyzed by RNA sequencing. An average of 32.2 and 31 million aligned reads were analyzed for the spheres and monolayers, respectively. Forty-four genes were unique to monolayers and 45 genes were expressed only in spheres. Conformation did not affect expression of *CDX2*, *POU5F1*, *TEAD4*, *ETS2*, *ELF3*, *GATA2* or *TFAP2A*, the core gene network of native TE. Bioinformatic analysis was used to identify classes of genes differentially expressed in response to changes in tissue shape. In both iTr spheres and monolayers, the majority of the differentially expressed genes were associated with binding activity in cellular, developmental and metabolic processes. Inherent to protein:protein interactions, several receptor-ligand families were identified in iTr cells with enrichment of genes coding for PI3-kinase and MAPK signaling intermediates. Our results provide evidence for ligand initiated kinase signaling pathways that underlie early trophectoderm structural changes.

1. Introduction (2000)

Reproduction and embryogenesis in the horse contains several unique features that set it apart from other domestic livestock, rodents, and humans (Betteridge, 2007). The inner cell mass (ICM) of blastocysts is tightly clustered in blastocysts of most mammals, but in equids the ICM contains relatively large and loosely packed embryonic cells (Tremoleda et al., 2003). Also, as the embryo emerges into the uterus, the trophectoderm (TE) secretes glycoproteins that creates a capsule that protects the conceptus and prevents its adhesion to the uterus (Klein and Troedsson, 2012; Oriol et al., 1993b). Similar to other species, conceptus-secreted factors prevent corpus luteum regression by suppressing endometrial prostaglandin F2α secretion (Ealy et al., 2010; Stout and Allen, 2002). However, the identity of the embryonic factors necessary for pregnancy recognition by the mare remains unknown.

Trophectoderm formation begins during the first 6-days post-ovulation in the mare with the blastocyst emerging from the oviduct with a loosely defined inner cell mass surrounded by a layer of trophoblast cells (Betteridge, 2007). Shortly after emerging into the uterus, the blastocyst becomes encased in a protective glycocalyx capsule that allows for motility throughout the uterus until fixation in the uterine horn (Stout et al., 2005; Tachibana et al., 2014). The TE transcriptome at embryonic day (ED) 8 includes CDX2, TEAD4, ELF3 and TFAP2A, genetic signatures reported for mouse, human and bovine TE (Golos et al., 2013; Iqbal et al., 2014; Latos and Hemberger, 2014; Roberts et al., 2004; Sakurai et al., 2012). Unlike the mouse, equine TE also expresses the pluripotency factor, POU5F1 (OCT4), arguing species-specific gene networks control formation of the placenta precursor (Choi et al., 2009; Desmarais et al., 2011). The signals emanating from the TE that initiate maternal recognition of pregnancy remain elusive. Factor(s) produced by the conceptus (ED14) down-regulate uterine endometrial prostaglandin H synthetase expression thus preventing prostaglandin synthesis and the return to estrus (Ealy et al., 2010). An enzyme critical to progesterone synthesis, CYP17A1, is abundant in the ED16 conceptus suggesting that the endocrine systems necessary for a

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successful pregnancy are present (Klein, 2015). Chorionic girdle formation begins at ED25 culminating with an increase in GCM1 expression at ED34 and subsequent production of chorionic gonadotrophin beta (de Mestre et al., 2009). The gondatrophin contains both LH and FSH-like activities which allows for the formation of secondary corpora lutea (Allen and Wilsher, 2009).

Tissue morphology can drive changes in gene expression. The fluid-filled blastocoel cavity pushes the inner cell mass against the outer zona pelucida with the resulting mechanical forces responsible for establishment of the core pluripotent gene network (Mammoto et al., 2012). Culture of human embryonic stem (ES) cells in suspension leads to the formation of embryoid bodies capable of expressing genes associated with the three primary germ layers as well as the trophectoderm (Giakoumopoulos and Golos, 2013). In a similar manner, trophoblast spheroids are created by suspension culture with the resulting structures morphologically and biochemically reminiscent of native blastocysts. Human trophoblast spheroids demonstrate an increase in HCG production, attach to receptive endometrial cells and invade the monolayer (Lee et al., 2015). Thus, cultivation in a conformation typical of a native blastocyst may allow for a more complete representation of core transcriptional networks.

The objective of the experiment was to identify genes that are differentially expressed as a function of tissue configuration in induced equine trophoblast cells (iTr). Results may assist with discerning the mechanisms underlying maternal recognition of pregnancy and the establishment of a successful pregnancy.

2. Materials and methods

2.1. Animals, estrous synchronization and embryo recovery

The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Virginia Polytechnic Institute and State University. Mares (n = 4) were maintained on a single pasture and remained on pasture throughout the breeding and collection period. Mares were evaluated by transrectal ultrasonography daily to map follicular development and determine stage of estrous cycle. Examinations were conducted once daily until a 35 mm follicle was observed along with appropriate edema. Once a follicle of this size was observed a single dose (1.8 mg) of deslorelin acetate (SucroMate, BioNiche Animal Health, Louisville KY) was administered via intramuscular injection. Mares were bred to a single stallion of known fertility (500 x 10^6 motile spermatozoa) 24 h after deslorelin injection. At the time of insemination, mares were evaluated via transrectal ultrasound to determine ovulation status. Mares that had failed to ovulate were bred again approximately 40 h post deslorelin injection.

Approximately 7–7.5 d post-ovulation, embryos were collected from all mares. Mares were sedated with xylazine (0.6 mg/kg). A sterile Foley catheter (36 French diameter) was inserted transcervically and secured in the uterus by inflation of the cuff. The uterus of the mare was flushed a minimum of 3 times with 1 L of warmed flush media (BioLife Advantage Complete Flush Medium, AgTech Inc., Manhattan, KS). Prior to the final flush, mares were administered a single intravenous dose (20 IU) of oxytocin (AgriLabs, St. Joseph, MO) to ensure optimal fluid removal from the uterus. The outflow from the catheter was connected to an in-line embryo filter (AgTech Inc., Manhattan, KS). The filter was washed with collection medium and the flushings were examined using a stereomicroscope (SMZ1500, Nikon, Melville, NY). Recovered embryos were transferred to Matrigel (Corning, Corning, NY) coated tissue cultureware for the formation of TE outgrowths.

2.2. Cell culture and sphere formation

Equine trophectoderm cells were created, as described (Ezashi et al., 2011). In brief, equine umbilical cord matrix mesenchymal stem cells were transduced with modified Sendai virus coding for human POU5F1, CDX2, SOX2, KLF4 and c-Myc (Cytotune, Life Technologies, Grand Island, NY). Induced trophectoderm (iTr) colonies formed on mitotic-inactive mouse embryonic fibroblasts (STO, ATCC CRL 1503, American Type Culture Collection, Manassas, VA) within 14 d post-transduction, as observed for induced pluripotent stem (iPS) cells (Breton et al., 2013; Whitworth et al., 2014). To eliminate confounding effects of STO cultures, the iTr cells were passaged onto Matrigel coated cultureware (Corning, Corning, NY). The iTr cells have exceeded 50 passages over a 2 yr timeframe and have retained their TE morphological, biochemical and genetic features. The cells are cultured routinely in growth media (GM) comprised of high glucose Dulbecco's modified Eagle medium (DMEM) supplemented with 15% fetal bovine serum (FBS), nonessential amino acids, 2 mM glutamine, 55 μM β-mercaptoethanol, 1% penicillin-streptomycin and 0.5% gentamicin.

Cells are passaged by physical dissociation. In brief, the cells are scraped from the plates with a cell scraper, transferred to a conical tube, and triturated through an 18-gauge needle prior to seeding onto Matrigel coated plates, as described for bovine TE cultures (Michael et al., 2006). All tissue culture reagents were purchased from Invitrogen (Grand Island, NY). For the formation of tissue spheres, the iTr cells are removed from the plate with L7™ hPSC Passaging Solution (Lonza, Walkersville, MD) and passed through a 100 μm cell strainer to remove non-dissociated cell aggregates and seeded as single-cell suspension in ultra-low attachment tissue culture plates (Corning, Corning, NY) in growth medium. After 3 to 7 d, sphere structures had formed and were visualized on an EVOS® cell imaging system (Life Technologies). Outgrowth were formed by transfer of iTr spheres to Matrigel™ (BD Biosciences, Franklin, NJ) coated tissue cultureware in growth media, as described (Yang et al., 2011). For growth factor treatments, iTr spheres (d 7) were cultured in 0.1X GM supplemented with 10 ng/mL recombinant human BMP4, EGF or FGF2 for 48 h prior to lysis and total RNA isolation.

2.3. RNA isolation and PCR

Total RNA was isolated from iTr monolayers and spheres using TRIzol reagent (Life Technologies). The RNA was purified with PureLink® RNA mini kit (Life Technologies), according to manufacturer's directions. Purity and quantity of RNA was determined using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE). Total RNA was treated with amplification grade DNAse I (Life Technologies) to remove genomic DNA contaminates and first-strand cDNA was synthesized using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Thermo Scientific). Real-time PCR amplification was carried out with Power SYBR® Master Mix with 10 pmols of equine gene specific forward and reverse primers (Table 1). All primer sets exhibited efficiencies greater than 90% as measured by standard dilution curves. Real-time reactions were initiated at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 60° for 30 s. All PCR reactions were performed on an Eppendorf Realplex^2 Mastercycler. Non-reverse transcribed RNA samples were included as negative controls. Relative expression level of target genes between treatments was calculated using $2^{\Delta\Delta Ct}$ method, defined as $\Delta\Delta Ct = (\Delta Ct_{spheres}) - (\Delta Ct_{monolayer})$. Calculation of ΔCt was performed by subtracting GAPDH Ct from the Ct for the gene of interest. The Ct values for GAPDH did not vary as a function of treatment.

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