The impact of infertility diagnosis on embryo-endometrial dialogue

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Abstract

Initial stages of implantation involve bi-directional molecular crosstalk between the blastocyst and endometrium. This study investigated an association between infertility etiologies, specifically advanced maternal age (AMA) and endometriosis, on the embryo-endometrial molecular dialogue prior to implantation. Co-culture experiments were performed with endometrial epithelial cells (EEC) and cryopreserved day 5 blastocysts (n = 41 $\geq$ Grade 3BB) donated from patients presenting with AMA or endometriosis, compared to fertile donor oocyte controls. Extracellular vesicles isolated from co-culture supernatant were analyzed for miRNA expression and revealed significant alterations correlating to AMA or endometriosis. Specifically, AMA resulted in 16 miRNAs with increased expression ($P \leq$ 0.05) and strong evidence for negative regulation toward 206 target genes. *VEGFA*, a known activator of cell adhesion, displayed decreased expression ($P \leq$ 0.05), validating negative regulation by 4 of these increased miRNAs: miR-126; 150; 29a; 29b ($P \leq$ 0.05). In endometriosis patients, a total of 10 significantly altered miRNAs displayed increased expression compared to controls (miR-7b; 9; 24; 34b; 106a; 191; 200b; 200c; 342-3p; 484) ($P \leq$ 0.05), targeting 1014 strong evidence-based genes. Three target genes of miR-106a (*CDKN1A*, *E2F1* and *RUNX1*) were independently validated. Functional annotation analysis of miRNA-target genes revealed enriched pathways for both infertility etiologies, including disrupted cell cycle regulation and proliferation ($P \leq$ 0.05). These extracellular vesicle-bound secreted miRNAs are key transcriptional regulators in embryo-endometrial dialogue and may be prospective biomarkers of implantation success. One of the limitations of this study is that it was a stimulated, *in vitro* model and therefore may not accurately reflect the *in-vivo* environment.

Introduction

The World Health Organization estimates that 10% of couples worldwide will struggle with infertility. Although a small number of these reproductive-age couples may succeed using conventional methods over many months and possibly years, most will require the use of assisted reproductive technologies (ARTs) and often *in vitro* fertilization (IVF) to establish a pregnancy (Thoma *et al.* 2013). Although considered relatively successful, 70% of IVF cycles will not result in a live birth (Weimar *et al.* 2013).

An intricate, bi-directional molecular dialogue between embryo and endometrium during the window of implantation, approximately 6–12 days post ovulation, is crucial for success (Wilcox *et al.* 1999). Without a precise interchange between the two, implantation will ultimately fail. Key genes involved with cell cycle regulation, as well as ion-binding and signal-transporting proteins, have been identified as contributors to the molecular dialogue between the embryo and a receptive endometrium by sampling the uterine microenvironment (Hannan *et al.* 2012, Ruiz- Alonso *et al.* 2012). MicroRNAs (miRNAs) also play an important role during the window of implantation and are expressed by both the implanting embryo and the receptive endometrium (Blakaj & Lin 2008, Laurent 2008). Altered expression of miRNAs (including miR- 30b, miR-30d and miR-494) have been shown to regulate endometrial receptivity (Altmae *et al.* 2013).

An *in vitro* co-culture system can provide valuable insight into the initial bi-directional dialogue (Simon *et al.* 1998). *In vitro* studies utilizing an embryo/ endometrial co-culture system have observed key components of the molecular crosstalk between the embryo and endometrial epithelial cells (EEC). However, limited information regarding this interaction is known (Barmat *et al.* 1999, Simon *et al.* 1999, Rubio *et al.* 2000).

Our unique study utilized a human uterine luminal endometrial epithelial cell and human blastocyst co-culture system to investigate the molecular dialogue at the time of adhesion, in association with two major causes of female factor infertility (endometriosis and advanced maternal age). Advanced maternal age (AMA) is associated with a substantial linear decline in reproductive potential and is the major cause of female infertility (Rosenwaks *et al.* 1995). There are many factors contributing to the significant decrease in live births as women approach their 5th decade, including diminishing ovarian reserve (Zhang 2015), and the increase in oocyte chromosomal aneuploidy (Harton *et al.* 2013). Aneuploidy screening in IVF cycles has allowed for the transfer of euploid blastocysts, resulting in higher implantation rates, independent of maternal age (Schoolcraft *et al.* 2011). However, there are other variables beyond the oocyte/embryo chromosome constitution that impact the ability of a euploid blastocyst to successfully implant. Traditionally, AMA patients are shown to have a longer infertility duration and diminished ovarian reserve and require a higher dose of gonadotropins in controlled ovarian hyperstimulation, all resulting in poorer oocyte quality (Ocal *et al.* 2012).

Another pathological cause of infertility, widely diagnosed and treated within the field of reproductive medicine, is endometriosis. Endometriosis is a disease which causes tissue normally found within the uterus, to migrate and grow outside of it (Buck Louis *et al.* 2011). This debilitating disease is often painful, impacting a woman's quality of life, and greatly reduces the chances of conception. It is estimated that nearly half of all women with this diagnosis will have difficulty achieving a pregnancy (Practice Committee of the American Society for Reproductive Medicine 2006, Ozkan *et al.* 2008). The causes of endometriosis remain unclear; however a genetic predisposition is suspected as a common risk factor, including a family history of the condition (Wenzl *et al.* 2003). Several pathways have also been

identified within patients diagnosed with endometriosis which may contribute to the poor oocyte quality associated with the disease (Shebl *et al.* 2017). Within infertile populations, it is estimated that the prevalence of patients diagnosed with endometriosis is approximately 26.13% in women undergoing laparoscopy (Garcia- Velasco & Quea 2005, Rizk *et al.* 2015).

The aim of this study is to investigate these different primary infertility etiologies within a controlled co-culture system, to elucidate differences within the secretome. We found significant differences in the miRNA content within the extracellular vesicles of the supernatant, as well as differences in gene expression within the co-cultured endometrial cells and blastocysts which may impact endometrial function during the window of implantation.

Materials and methods

Embryos

Surplus, cryopreserved, hatching, transfer-grade blastocysts as identified by the Gardner & Schoolcraft grading system (Gardner & Schoolcraft 1999) as ≥Grade 3BB on day 5 of embryonic development (n = 41) were donated with patient consent and IRB approval. Blastocysts were derived from IVF cycles: young fertile oocyte donor controls (fertile control group) with no history of female or male factor infertility, based on WHO guidelines (maternal age ≤32 years old, BMI <29, non-smoker, non-drug user, with normal ovarian reserve and regular menstrual cycles, n = 15), infertile women of AMA group without pre-implantation genetic diagnosis (≥39 years or older, with no other infertility diagnosis, n = 14), and younger female patients diagnosed with endometriosis as the only cause of infertility (mean maternal age of 34.3, with 11 of the 12 patients ≤33 years old, n = 12). Subjects underwent an ovarian stimulation protocol based on clinical discretion which included gonadotropin-releasing hormone (GnRH) agonist down-regulation, microdose GnRH agonist flare or GnRH antagonist with Menopur (Ferring Pharmaceuticals, Saint-Prex, Switzerland) or Bravelle (Ferring Pharmaceuticals) with or without clomiphene citrate. Final oocyte maturation trigger was induced when the lead follicle reached 20 mm mean diameter using an intramuscular injection of hCG, a subcutaneous injection of leuprolide acetate, or a combination of both, based on clinical judgment. Routine oocyte retrieval was performed transvaginally under ultrasound guidance 35 h post trigger. Patients within the endometriosis group were asymptomatic at the time of their oocyte retrieval and biopsy. Patients were excluded from the study if they had any other infertility comorbidities including polycystic ovary syndrome and depleted ovarian reserve. Embryos were vitrified and warmed according to routine laboratory procedures described previously (Kuwayama *et al.* 2005). Total time spent frozen varied, with an average time of approximately 4 years.

Endometrial biopsies

Endometrial biopsies were collected from either young, fertile oocyte donors (fertile control group, n = 7) or from patients diagnosed with endometriosis who were currently asymptomatic at the time of their oocyte retrieval (n = 2). Subjects were stimulated as described earlier. Endometrial biopsies measured 1.5–3.0 cm in length and approximately 0.5 cm in diameter. For every endometrial biopsy collected, tissue was minced into <1 mm sections

and incubated at 4°C overnight in a 0.1% collagenase solution (Sigma-Aldrich). The primary epithelial endometrial cells (EEC) were isolated by vigorously vortexing and rinsing the cell milieu in 10 mL of room temperature DMEM (Sigma-Aldrich), prior to resting the cells for 5–10 min as they settled into distinct layers of EEC and stromal cells. The supernatant containing stromal cells was then removed and the process was repeated three times until no further stromal cells remained. 500 µL of the EEC/DMEM was then added to 6 wells of a Falcon Multiwell 24-well Tissue Culture Plate (Corning Incorporated) (Mercader *et al.* 2003). Cells were cultured in a media composed of 75% Dulbecco Modified Eagle Medium (Sigma) and 25% MCDB- 105 (Sigma) containing antibiotics, 5 µg/mL insulin (Sigma) and supplemented with 10% exosome-depleted fetal bovine serum (System Biosciences, Palo Alto, CA, USA) and observed daily to monitor the development of an EEC monolayer. The monolayer reached acceptable levels of well coverage at >65% after 4–6 days. The growth medium was then replaced with a serum-free medium for embryo development and supplemented with 4% recombinant human serum albumin (Vitrolife, Englewood, CO, USA). For each endometrial biopsy processed, 4–6 wells received an individual thawed blastocyst which was co-cultured for 48 h at 5% O2 and 6% CO2 at 37°C based on current laboratory practices for human blastocyst culture (Kovacic 2012).

Co-culture collection

Blastocysts were co-cultured together for 48 h prior to collection. AMA or fertile control donor blastocysts were co-cultured with fertile control donor endometrial tissue. Additionally, the endometriosis subjects were divided into three groups: fertile control donor blastocysts co-cultured with fertile control donor endometrium (CD), fertile control donor blastocysts co-cultured with endometrial cells from patients diagnosed with endometriosis (ED) and blastocysts and endometrial cells that were both obtained from patients diagnosed with endometriosis (EE). Adhesion by the blastocyst to the endometrial monolayer was observed twice in the AMA group, twice in the fertile control group and once in both of the endometriosis groups. Adhered blastocysts were gently scraped off the monolayer prior to collection. Following co-culture, blastocysts were graded and rinsed in 10% phosphate buffered saline-bovine serum albumin (PBS-BSA) before lysis in 10 µL of extraction buffer (PicoPureRNA Isolation Kit, Thermo Fisher Scientific). Co-culture supernatant was collected (500 µL) and snap-frozen in liquid nitrogen. The monolayer was visually measured for 100% confluence, then rinsed with DPBS (Thermo Fisher) and incubated at 37°C with 1× diluted TrypLESelect (10×) (Thermo Fisher) until cells lifted from the bottom of the plate (5–15 min). Each well of EEC were collected individually. The pelleted EEC were lysed with 50 µL Extraction Buffer (PicoPureRNA Isolation Kit, Thermo Fisher) and snap-frozen with liquid nitrogen. All samples were stored at −80°C until further analysis (Fig. 1).

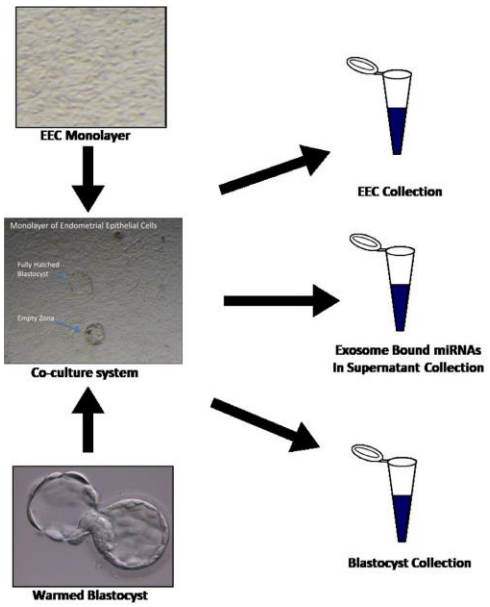


Figure 1 Experimental design for embryo co-culture with cultured endometrial cells.

Supernatant extracellular vesicle miRNA analysis

Extracellular vesicles were isolated using Total Exosome Isolation Reagent (from cell culture media) (Thermo Fisher). 350 µL of supernatant was combined with 175 µL of reagent and vortex-mixed prior to an overnight incubation at 4°C. Samples were centrifuged under refrigerated conditions for 1 h, then the supernatant was aspirated and discarded. The resulting pellet was lysed in 10 µL of lysis solution containing DNase (Taqman MicroRNA Cells-to-CT Kit, Thermo Fisher) and incubated at room temperature for 8 min. Lysed miRNA was reverse-transcribed using the Taqman MicroRNA Reverse Transcription Kit with MegaPlex RT Human Primer Pool A (Thermo Fisher) in a final volume of 7.5 µL under the following thermal cycling conditions: 40 cycles at 16°C for 2 min, 42°C for 1 min and 50°C for 1 s followed by a hold for 5 min at 85°C. cDNA was pre-amplified using the Taqman PreAmp Mastermix with MegaPlex PreAmp Human Primer Pool A (Thermo Fisher). 100 µL of the diluted pre-amplified product was added to each well of the Taqman Human MicroRNA Array Card A and was then run on the 7900HT Fast Real-Time PCR System (Thermo Fisher) under the following thermal cycling conditions: 50°C for 2 min, 94.5°C for 10 min and 40 cycles at 97°C for 30 s and 59.7°C for 1 min. Data were analyzed using the RQ Manager 1.2.1 (Thermo Fisher), and statistical analysis was performed using REST 2009 software (Qiagen). miRNAs that displayed significantly altered expression profiles were further investigated for target gene analysis using miRTarBase (http://mirtarbase.mbc.nctu.edu.tw/index.php), a database of experimentally validated miRNA-target interactions that are classified as strong evidence based if there is sufficient published empirical evidence.

Blastocyst gene expression

Individual blastocysts stored in 10 µL of extraction buffer (PicoPure RNA Isolation Kit, Thermo Fisher) were incubated at 42°C for 30 min prior to isolating RNA. This procedure entailed adding 1 volume of 70% EtOH to each sample before transferring to a pre-conditioned purification column. Samples were then DNase-treated using the RNase-Free DNase Set (Qiagen) and eluted in 20 µL following several washes. All 20 µL of purified total RNA was reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher) in a final volume of 40 µL. The reaction occurred in a thermal cycler at 25°C for 10 min followed by 37°C for 2 h. cDNA samples were diluted 1:5 with nuclease-free water and a 5 µL template was used for real-time PCR using the Power SYBR Green PCR Master Mix (Thermo Fisher) and a 5 µM primer mix (forward + reverse) in a 25 µL final volume. Duplicates of each sample were performed on the ABI 7300 Real-Time PCR System under the following thermal cycling conditions: 95°C for 10 min, 40 cycles at 95°C for 15 s and 60°C for 1 min, followed by a dissociation stage to determine melt temperatures. Standard curves were also employed for each gene using Universal Human Reference RNA (Agilent) in 10-fold serial dilutions and an internal housekeeping gene (*PPIA*) was used to normalize each sample.

Endometrial cell molecular analysis

EEC samples were lysed in 75 µL of lysis solution and miRNAs were isolated using the RNaqueous-Micro Kit (Thermo Fisher) which required the addition of 1.25 volumes of 100% EtOH prior to loading onto a micro filter cartridge assembly. Samples were DNase-treated in the same manner as the blastocyst samples, and washed several times before eluting in 10 µL of elution solution previously heated to 75°C (performed twice for maximum recovery, 20 µL final volume). miRNA samples were then concentrated down to a 10 µL volume using a vacuum concentrator (Vacufuge Plus, Eppendorf).

miRNA samples were pre-amplified, diluted and evaluated using the Taqman Human MicroRNA Array Card A (Thermo Fisher) as described in the earlier supernatant exosome miRNA analysis section. Data were analyzed using the RQ Manager 1.2.1 (Thermo Fisher) and statistical analysis was performed as described in the following.

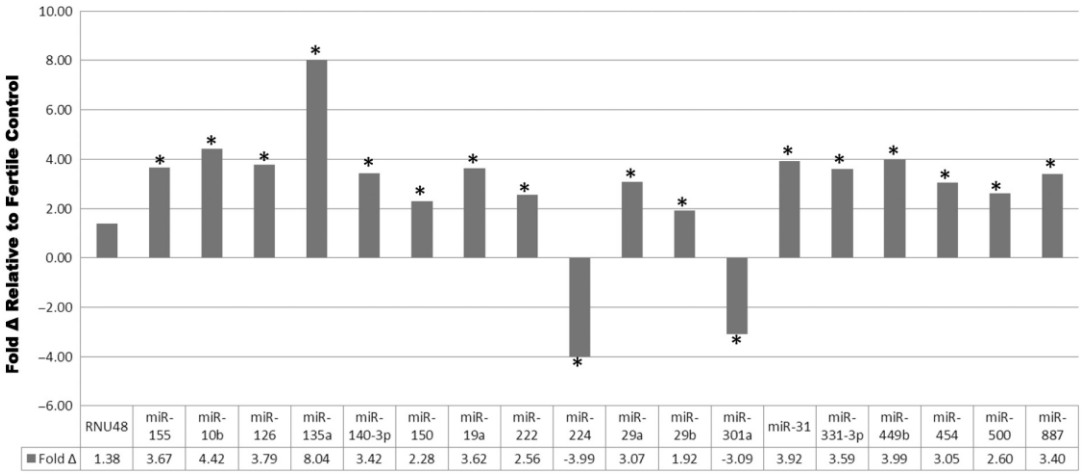
Statistical analysis

A Mann–Whitney *U* test was used to compare differences in the age of the donor women (fertile control, AMA and endometriosis blastocysts), with significance at $P \leq 0.05$. The PCR reaction efficiencies recorded R^2 values ≥0.9 and correlation coefficients were calculated to be >0.99. Statistical analysis was performed with REST-2009 software (Qiagen) which uses bootstrap randomization techniques to correct for exact PCR efficiencies with mean crossing point deviations between sample and control groups to determine an expression ratio that is tested for significance by a pair wise fixed reallocation randomization test. The standard error and the 95% confidence interval (CI) given by REST-2009 software are not derived from Ct values. They are the standard error and the CI for the 50,000 iterations the program runs to calculate the *P* value; thus, no error bars are generated for graphical presentation. Gene expression fold differences with $P \leq 0.05$ were considered statistically significant.

Results

Co-culture of AMA blastocysts and fertile control blastocysts

The age of the donors in the fertile control blastocyst group were significantly different than those in the AMA blastocyst group ($P \leq 0.05$). Isolated cargo of extracellular vesicles from supernatant collected after co-culture of fertile endometrial epithelial cells with blastocysts from infertile AMA patients revealed altered miRNA expression profiles compared to fertile control blastocysts. A total of 16 miRNAs showed increased expression and 2 exhibited reduced expression in association with the maternal age of the blastocyst relative to the internal housekeeping gene *RNU48* ($P \leq 0.05$, Fig. 2). Target gene investigation of these 18 altered miRNAs using miRTarBase (http://mirtarbase. mbc.nctu.edu.tw/index.php) revealed 576 genes, 206 of which displayed strong evidence as defined by Western blot, qPCR or reporter assay as described previously. Of these strong evidence-based genes, *VEGFA* was identified as a target gene, specifically regulated by four of the altered miRNAs: miR-126, 150, 29a and 29b. Examination of *VEGFA* transcription by qPCR observed decreased expression in both the corresponding endometrial epithelial cells and blastocysts from infertile AMA patients following co-culture, specifically a 50% fold change reduction compared to fertile controls ($P \leq 0.05$; Fig. 3).



	RNU48	miR-155	miR-10b	miR-126	miR-135a	miR-140-3p	miR-150	miR-19a	miR-222	miR-224	miR-29a	miR-29b	miR-301a	miR-31	miR-331-3p	miR-449b	miR-454	miR-500	miR-887
Fold Δ	1.38	3.67	4.42	3.79	8.04	3.42	2.28	3.62	2.56	-3.99	3.07	1.92	-3.09	3.92	3.59	3.99	3.05	2.60	3.40

Figure 2 Differential expression of extracellular vesicle-bound miRNAs in co-culture supernatant in association with maternal age (*$P \leq 0.05$).

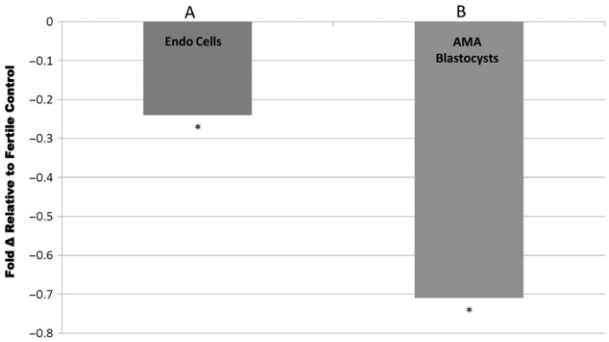


Figure 3 Target gene *VEGFA* expression in endometrial cells (A) and AMA blastocysts (B) compared to young fertile controls (*$P \leq 0.05$).

Co-culture of endometriosis blastocysts or fertile control blastocysts on donor endometrium or endometriosis endometrium

The ages of the donors in the fertile control blastocyst group were not significantly different from those in the endometriosis blastocyst group ($P \geq 0.05$; ns). Isolated extracellular vesicles from supernatant collected after co-culture of endometriosis EEC with blastocysts from endometriosis patients revealed an altered miRNAome compared to fertile controls. Specifically, 10 miRNAs showed significant decreased expression including miR-7b miR-24, miR-34b, miR-200c, miR- 342-3p, miR-9, miR-484, miR-200b, miR-106a and miR-191 ($P \leq 0.05$; Figs 4 and 5) compared to fertile control endometrium co-cultured with fertile control blastocysts. Using mirTarBase, strong evidence-based target genes were revealed, including, among others, 27 target genes for miR-106a. Of these 27 target genes *CDKN1A*, *E2F1* and *RUNX1* were chosen for further investigation because of their known roles in association with implantation in murine (Cdkn1a) (Das 2009) and human models (E2F1 and RUNX1) (Tapia *et al.* 2011, Tapia-Pizarro *et al.* 2014). qPCR analysis observed increased expression of *CDKN1A* in endometriosis blastocysts compared to fertile controls when co-cultured with fertile control derived EEC ($P \leq 0.05$). This expression was further increased when endometriosis derived blastocysts were co-cultured with endometriosis derived EEC ($P \leq 0.05$; Fig. 6A). Endometriosis EEC co-cultured with fertile control blastocysts displayed increased expression of *E2F1* and *RUNX1* ($P \leq 0.05$). This significant difference in expression was further increased when endometriosis derived blastocysts were co-cultured using endometriosis derived EEC($P \leq 0.05$; Fig. 6B).

Gene ontology and pathway analysis

DAVID Bioinformatics Resources 6.8 (https://david. ncifcrf.gov/) was utilized for functional annotation clustering analyses of differentially expressed miRNAs to explore the GO biological processes and pathways, common to both infertility etiologies, and included cellular development, proliferation and cell cycle regulation.

The enriched pathways and processes that were identified as being specific to only the AMA etiology included PI3K-Akt and vascular endothelial growth factor (VEGF) signaling, as well as biological processes involving cellular development and migration, adhesion and cell cycle regulation ($P \leq 0.05$; Supplementary Table 1, see section on supplementary data given at the end of this article).

For the endometriosis etiology group, enriched biological processes and pathways crucial for development and implantation were revealed within functional annotation clusters of the 1014 strong evidence-based target genes, including positive regulation of cell proliferation, G1/S transition of mitotic cell cycle and the p53 signaling pathway and angiogenesis ($P \leq 0.05$). Additionally, cell cycle regulation and arrest, positive regulation of cell proliferation, negative regulation of apoptotic processes and negative regulation of G1/S transition of mitotic cell cycles were also identified (Supplementary Table 2).

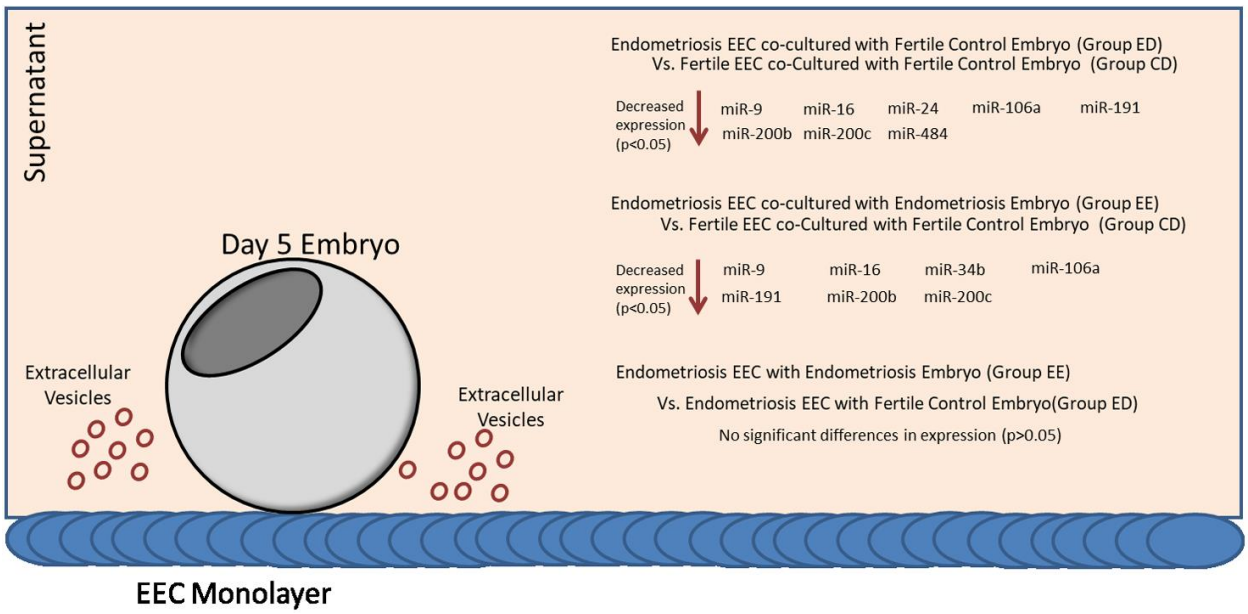


Figure 4 Experimental groups (CD = fertile control endometrium/fertile control blastocyst; ED = endometriosis endometrium/fertile control blastocyst; EE = endometriosis endometrium/endometriosis blastocyst) and the affected miRNAs for embryos and endometrial cells derived from patients diagnosed with endometriosis compared with fertile control.

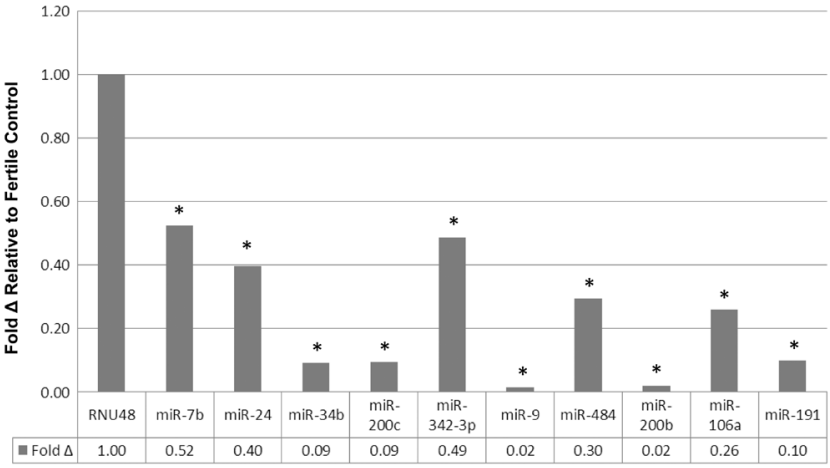


Figure 5 Extracellular vesicle-bound microRNA expression of co-culture supernatant. Endometriosis derived blastocysts cultured on a monolayer of endometriosis derived endometrial tissue and fertile control derived endometrial tissue, fertile control oocyte derived blastocysts (*$P \leq 0.05$).

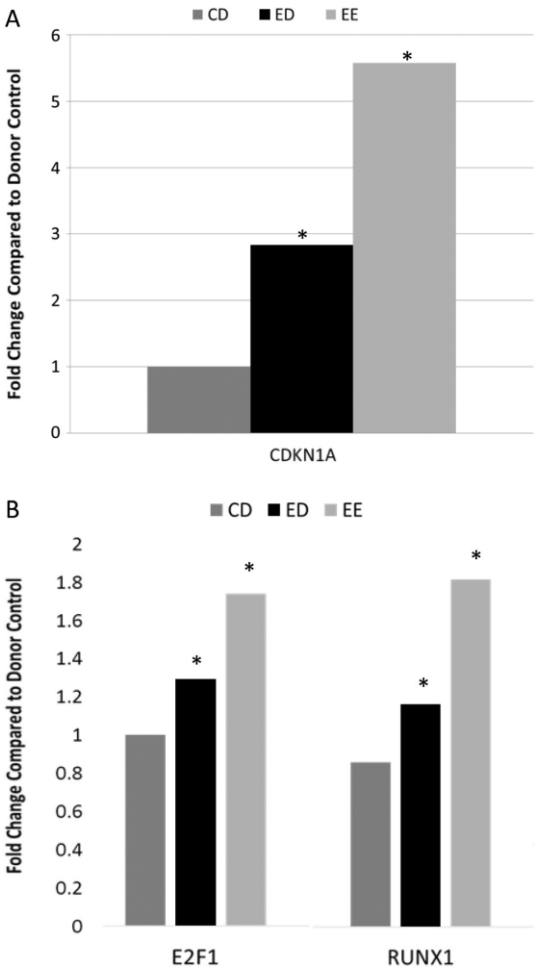


Figure 6 (A) miR-106a target gene validation of endometriosis derived blastocysts revealed significantly increased expression of *CDKN1A* when co-cultured with endometriosis derived endometrial cells compared to fertile control (CD = fertile control endometrium/ fertile control blastocyst; ED = endometriosis endometrium/fertile control blastocyst; EE = endometriosis endometrium/endometriosis blastocyst) (*$P \leq 0.05$). (B) miR-106a target gene validation of endometriosis derived endometrial cells co-cultured with endometriosis derived blastocysts revealed significantly increased expression of *E2F1* and *RUNX1* compared to fertile control derived controls (CD = fertile control endometrium/fertile control blastocyst; ED = endometriosis endometrium/fertile control blastocyst; EE = endometriosis endometrium/endometriosis blastocyst) (*$P \leq 0.05$).

Discussion

This is a novel study investigating the bi-directional molecular communication at the time of implantation in association with infertility etiologies. Blastocysts from patients presenting with AMA or endometriosis were co-cultured on a monolayer of endometrial cells to capture the molecular dialogue between a blastocyst and the endometrium during the initial stages of implantation. Specific extracellular vesicle-bound miRNAs derived from co-culture supernatant were identified to have significantly altered expression in association with AMA or endometriosis, highlighted by the differences within embryo-endometrial communication depending on the source of the embryo or endometrium. This impacted downstream target gene expression in either blastocysts or endometrial cells during the window of implantation.

Advanced maternal age

It is well documented that infertile women presenting with advanced maternal age have some of the poorest prognoses with regard to oocyte and blastocyst quality. Additionally, there is some evidence that the endometrium is also affected by advanced age, resulting in embryo-endometrial asynchrony (Shapiro *et al.* 2016). The molecular dialogue between the embryo and endometrium is crucial for successful implantation, and it is believed that miRNA-bound extracellular vesicles are involved in this bi-directional molecular communication.

A total of 18 extracellular vesicle-bound miRNAs displayed altered expression with the presence of AMA blastocysts. One example was miR-150, a critically important miRNA for lineage cell differentiation, which also plays a role in mediating an appropriate inflammatory response within the endometrial environment. This response includes not only the endometrial epithelial cells and stromal cells, but also the immune-related cells and vasculature (Pan & Chegini 2008, Elton *et al.* 2013, Goossens *et al.* 2013). Pregnancy is a pro-inflammatory state and molecular miscommunication may result in an improperly coordinated auto-immune response, leading to inflammation at the implantation site, causing implantation failure and embryonic death. This is a delicate balance, and if the immune response is too great or insufficient, implantation potential may be impacted (Clark 2008). miR-150 is also a regulatory miRNA for the platelet-derived growth factor/VEGF family of genes, responsible for both angiogenesis and vasculogenesis, and is vital for successful embryo implantation. It has also been shown that supplementing embryo culture media with VEFG had beneficial effects with regard to post-compaction mouse embryo development, outgrowth, implantation and fetal development (Binder *et al.* 2014, 2016).

VEGFA is a glycosylated mitogen that specifically acts on endothelial cells with various effects, including mediating vascular permeability, angiogenesis, cell growth and cell migration and inhibiting apoptosis. Treatment of human endometrial cells with recombinant human VEGFA protein has been found to significantly increase endometrial cell adhesion (Hannan *et al.* 2011). In this study, *VEGFA* displayed a significantly lower expression in AMA samples ($P \leq 0.05$), which may lead to dysregulated cellular function and increased apoptosis. This decrease in *VEGFA* expression may be indicative of the compromised communication between the AMA blastocyst and fertile control endometrium.

An additional altered miRNA observed in extracellular vesicles following co-culture was miR-135a with an 8-fold decrease in expression in AMA blastocysts ($P \leq 0.05$). A previous study observed that increased expression of miR-135a during pre-implantation embryo development led to the down-regulation of E3 ubiquitin ligase seven in absentia homologue 1A (*SIAH1A*) expression (Pang *et al.* 2011). The SIAH1A protein is involved in many cellular processes including apoptosis, TNF-alpha signaling and cell cycle regulation (Relaix *et al.* 2000). Nearly a third of the zygotes injected with miR-135a inhibitor were observed to arrest in development. The mechanism for this disruption is believed to be due to dysregulated proteosomal degradation, possibly controlling the expression of chemokines in DNA-binding protein (Pang *et al.* 2011). miR-135a has also been correlated to increased apoptosis resistance in cancer cells by *BCL2* regulation, a protein which determines cell survival and death through the mitochondrial apoptotic signaling pathway. Dysregulation of *BCL2* and disruption of the apoptotic pathway resulted in interference of not only the establishment of pregnancy, but also pregnancy

maintenance if successful implantation was able to occur at all (Mathew *et al.* 2009, Pan *et al.* 2014). The dysregulation of miR-135a observed in our samples suggests a decline in the cellular regulation of blastocysts derived from AMA patients, thus decreasing their viability and implantation potential.

Another miRNA found in the extracellular vesicles of the supernatant that displayed significantly disrupted differential expression was miR-449b. One strong, evidence-based target gene for miR-449b is *NOTCH1* which has demonstrated its ability to act as a promoter of centriole multiplication multiciliogenesis by repressing the Delta/Notch pathway in *Xenopus laevis* embryonic dermis (Marcet *et al.* 2011). It is currently unknown precisely how mammalian cells are affected by this pathway; however it is hypothesized that, rather than the cilia found in *Xenopus* cells, it is the formation of the pseudopodia within the blastocyst trophectoderm that is being directly regulated (Goossens *et al.* 2013). miR-449b showed significantly increased expression in the co-culture supernatant of AMA blastocysts, which may indicate compromised trophectoderm formation, leading to an increase in failed implantation as observed clinically in this patient population.

Endometriosis

Endometriosis is a disease characterized by an abnormal, and in many cases, compromised endometrium. The impact this diagnosis bears on the bi-directional embryo-endometrial dialogue at the time of implantation is largely unexplored. In our study, co-culture of endometriosis endometrium with blastocysts derived from patients diagnosed with endometriosis displayed significantly decreased extracellular miRNA, from within the isolated extracellular vesicles, when compared to fertile control endometrium with fertile control derived blastocysts for 10 key miRNAs ($P \leq 0.05$). Of particular interest was miR-106a, commonly associated with adherens junctions, receptor interactions, metabolic pathways and VEGF signaling. In one study utilizing a uterine cavity lavage, miR-106a was identified in the exosomes found within the mucus near the time implantation may occur. It is suspected that as the blastocyst transitions toward the implantation site and begins to adhere, exosomes trapped within the mucus on the luminal surface of the endometrium release miRNAs which contribute to endometrial-embryo crosstalk by binding to the trophectoderm cells themselves (Ng *et al.* 2013). miR-106a has also revealed itself to be a strong identifying biomarker for various types of cancer where it is often down-regulated (Wang 2016). MiRTarBase target gene analysis of miR-106a identified 29 strong evidence-based target genes, including *CDKN1A*, *E2F1* and *RUNX1*.

CDKN1A works in conjunction with *TP53* and is linked to stress response in the G1 phase. If this stress response continues to cascade, it causes arrested cell cycle, potentially compromising the viability of embryos, and also contributing to possible de-regulation of the endometrium itself (Sharma *et al.* 2016). *CDKN1A* expression has been shown to induce cellular growth, arrest, terminal differentiation or apoptosis (Ying *et al.* 2011). Altered aberrant *CDKN1A* expression is also believed to play a vital role in the pathogenesis of specific cancer types, including ovarian and uterine carcinomas (Elbendary *et al.* 1996). Our results demonstrate altered *CDKN1A* gene expression in blastocysts derived from patients diagnosed

with endometriosis, which may negatively impact their viability, and also contribute to the reduced implantation potential of embryos derived from this patient population.

E2F1 is a transcription factor that plays a crucial role in the control of the cell cycle and can mediate apoptosis. Over-expression of *E2F1* in myoblasts has demonstrated proliferation stimulation while also inhibiting differentiation (Luo *et al.* 2016). *E2F1* induces the transcription of several genes involved in cell cycle entry, resulting in either the induction or inhibition of apoptosis. This influence leads to both negative and positive feedback loops, having a critical impact on the outcome of the cell's fate. The *E2F1* protein has also shown increased expression in patients diagnosed with endometrial cancer, where the cell cycle becomes accelerated and irregular (Mints *et al.* 2016). Decreased expression of miR-106a can increase p53 expression in human glioma cells by inhibiting *E2F1*, negatively effecting cell proliferation and inducing apoptosis (Yang *et al.* 2011, Feng *et al.* 2013). Within the endometrium derived from patients diagnosed with endometriosis, irregular feedback loops may lead to inhibited differentiation, compromising the viability of the implantation site.

RUNX1 is also a transcription factor that, when over expressed, increases apoptosis. In a mouse embryo knockout study, embryos with homozygous mutations on Runx1 terminated after 12.5 days of development, and suffered from a lack of fetal liver hematopoiesis as well as hemorrhaging of the central nervous system (Okuda *et al.* 1996, Wang *et al.* 1996). Similar to *E2F1*, over-expression of the *RUNX1* protein has been linked to various forms of cancer, including endometrial and endometrioid carcinomas (Planaguma *et al.* 2004, Abal *et al.* 2006). In mice, *Runx1* also plays an important role in regulating the gene expression of *Ada* in trophoblast cell lineage which is critical for embryo development (Schaubach *et al.* 2006). In our study, endometriosis derived endometrium showed increased expression of *RUNX1* that may lead to decreased implantation potential by compromising hematopoiesis and influencing the gene expression of placental *ADA*.

DAVID functional annotation bioinformatics microarray analysis revealed further dysfunction within the expression of miRNAs for both infertility etiologies, in relation to GO biological processes and pathways. When comparing the three different design groups, we found that miR-24, 34b and 484 were not commonly expressed between the groups of ED vs CD and EE vs CD, highlighting a subset of developmental pathways that were affected by the presence of endometrial cells from patients diagnosed with endometriosis (Supplementary Table 3). Interestingly, there were also no significant differences in miRNA expression between the groups of EE and ED ($P > 0.05$), which may signify that the endometriosis EEC are contributing the majority of the extracellular vesicles to the supernatant and may impact the developmental potential of the implanting blastocyst. One commonality between the endometriosis and AMA groups was the focus on cell cycle regulation. This may be indicative of compromised dialogue within the extracellular matrix. As the window of implantation approaches, specific auto-induction loops must occur to mediate the critical crosstalk between a competent blastocyst and the uterine epithelium at the implantation site (Hamatani *et al.* 2004). If these critical processes and pathways are disrupted, the resulting cell cycle dysregulation may contribute to a harsh environment, unsuitable for implantation and further development necessary for a viable pregnancy by preventing firm adhesion between the blastocyst and the uterine epithelium (Wang *et al.* 2008).

A few limitations of this study include the source of the endometrial biopsy. These biopsies were taken at the time of oocyte retrieval from women undergoing controlled ovarian stimulation. It has been shown that this leads to estrogen-dependent changes in the protein profiles of the endometrial compartments which may affect endometrial receptivity (Ullah *et al.* 2017). Furthermore, due to the scarcity of research materials, these protein profile alterations were unable to be validated further by ELISA analysis.

In conclusion, our study displayed a theme of compromised cellular communication in blastocysts and endometrium derived from patients diagnosed with AMA or endometriosis. These alterations may account for the lower implantation potential and clinical outcomes observed for these infertile patient populations. Any compromise in the delicate embryo-endometrial molecular dialogue, either autocrine or paracrine, may impact transcription levels of key miRNAs and their target signaling molecules, resulting in significantly lower potential for reproductive success. Further investigation of the bi-directional molecular dialogue may help elucidate important implantation biomarkers during this critical time point, and advance our understanding of reproductive success.

Supplementary data

This is linked to the online version of the paper at https://doi.org/10.1530/REP-17-0566.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Supplementary Table I: Enriched pathways of statistically significant miRNA target genes ($p<0.05$) increased or decreased in the Endometriosis group compared to donor controls

hsa-miR-106a

GO Term	Term	# of genes	P-Value
GO:0008284	positive regulation of cell proliferation	4	3.40E-02
GO:2000134	negative regulation of G1/S transition of mitotic cell cycle	3	6.80E-04
GO:0043066	negative regulation of apoptotic process	7	5.60E-05
GO:0051726	regulation of cell cycle	5	3.70E-05

KEGG Pathway	Pathway	# of	P-Value

		Genes	
hsa04110	Cell cycle	5	3.10E-04

hsa-miR-let-7b

GO Term	Term	# of genes	P-Value
GO:2000766	negative regulation of cytoplasmic translation	3	8.30E-05
GO:0071230	cellular response to amino acid stimulus	4	1.16E-04
GO:0000082	G1/S transition of mitotic cell cycle	5	5.17E-05
GO:0051301	cell division	7	6.45E-05
GO:0001934	positive regulation of protein phosphorylation	3	0.027192
GO:0030335	positive regulation of cell migration	6	3.22E-05
GO:0008284	positive regulation of cell proliferation	5	0.014057

KEGG Pathway	Pathway	# of Genes	P-Value
hsa04914	Progesterone-mediated oocyte maturation	7	3.76E-07
hsa04152	AMPK signaling pathway	6	5.13E-05
hsa04914	Progesterone-mediated oocyte maturation	7	3.76E-07
hsa04114	Oocyte meiosis	4	0.006072
hsa04110	Cell cycle	5	7.95E-04
hsa04115	p53 signaling pathway	3	0.022282
hsa04151	PI3K-Akt signaling pathway	10	1.17E-06
hsa04068	FoxO signaling pathway	7	4.80E-06
hsa05213	Endometrial cancer	3	0.013792

hsa-miR-200c

GO Term	Term	# of genes	P-Value
GO:0001525	angiogenesis	7	6.36E-04
GO:0048010	vascular endothelial growth factor receptor signaling pathway	6	2.22E-05
GO:0010595	positive regulation of endothelial cell migration	4	0.001315
GO:0001701	in utero embryonic development	7	2.49E-04
GO:0035924	cellular response to vascular endothelial growth factor stimulus	4	1.66E-04
GO:0045766	positive regulation of angiogenesis	5	0.002113

KEGG Pathway	Pathway	# of Genes	P-Value
hsa04510	Focal adhesion	9	6.30E-05

Supplementary Table II: Enriched pathways of statistically significant miRNA target genes ($p<0.05$) increased or decreased in the AMA group

hsa-miR-126

GO Term	Term	# of genes	P-Value
GO:0001938	positive regulation of endothelial cell proliferation	5	3.13E-05
GO:0045766	positive regulation of angiogenesis	5	2.29E-04
GO:0001525	angiogenesis	5	2.70E-03
GO:0048010	vascular endothelial growth factor receptor signaling pathway	3	1.53E-02

KEGG Pathway	Pathway	# of Genes	P-Value
hsa04151	PI3K-Akt signaling pathway	10	1.32E-05
hsa04012	ErbB signaling pathway	6	3.70E-05

hsa04015	Rap1 signaling pathway	7	2.86E-04
hsa04370	VEGF signaling pathway	4	2.46E-03
hsa04210	Apoptosis	4	2.59E-03
hsa04668	TNF signaling pathway	4	1.15E-02
hsa04010	MAPK signaling pathway	5	2.60E-02
hsa04664	Fc epsilon RI signaling pathway	3	3.69E-02
hsa05213	Endometrial cancer	5	7.72E-05
hsa04152	AMPK signaling pathway	5	2.01E-03

miR-150

GO Term	Term	# of genes	P-Value
GO:0043066	negative regulation of apoptotic process	5	3.68E-03
GO:0008284	positive regulation of cell proliferation	5	4.00E-03

KEGG Pathway	Pathway	# of Genes	P-Value
hsa04110	Cell cycle	3	4.90E-02

miR-29a

GO Term	Term	# of genes	P-Value
GO:2000352	negative regulation of endothelial cell apoptotic process	4	2.80E-04
GO:0007160	cell-matrix adhesion	5	7.75E-04
GO:0030335	positive regulation of cell migration	9	1.97E-06
GO:0048010	vascular endothelial growth factor receptor signaling pathway	3	4.32E-02

KEGG Pathway	Pathway	# of Genes	P-Value
hsa04510	Focal adhesion	16	1.81E-11
hsa04151	PI3K-Akt signaling pathway	18	3.14E-10
hsa04068	FoxO signaling pathway	7	5.17E-04
hsa04115	p53 signaling pathway	5	1.65E-03
hsa05213	Endometrial cancer	4	7.22E-03
hsa04370	VEGF signaling pathway	4	1.12E-02

miR-29b

GO Term	Term	# of genes	P-Value
GO:0030335	positive regulation of cell migration	11	8.74E-09
GO:0051781	positive regulation of cell division	7	6.01E-08
GO:0043406	positive regulation of MAP kinase activity	7	2.42E-07
GO:0016477	cell migration	6	1.03E-03
GO:0050680	negative regulation of epithelial cell proliferation	4	2.00E-02
GO:0016049	cell growth	4	2.00E-02
GO:0043065	positive regulation of apoptotic process	7	2.22E-02
GO:0007050	cell cycle arrest	4	2.52E-02
GO:0007160	cell-matrix adhesion	8	1.48E-07
GO:0050714	positive regulation of protein secretion	6	5.25E-07
GO:2000352	negative regulation of endothelial cell apoptotic process	5	6.93E-06
GO:0048010	vascular endothelial growth factor receptor signaling pathway	3	4.11E-02

KEGG Pathway	Pathway	# of	P-Value

		Genes	
hsa04510	Focal adhesion	22	2.71E-19
hsa04151	PI3K-Akt signaling pathway	24	4.96E-17
hsa04014	Ras signaling pathway	9	2.51E-04
hsa04068	FoxO signaling pathway	7	4.65E-04
hsa05213	Endometrial cancer	4	6.85E-03
hsa04110	Cell cycle	6	2.27E-03
hsa04350	TGF-beta signaling pathway	5	3.53E-03
hsa04370	VEGF signaling pathway	4	1.06E-02
hsa04390	Hippo signaling pathway	5	2.64E-02
hsa05213	Endometrial cancer	4	6.85E-03
hsa04370	VEGF signaling pathway	4	1.06E-02
hsa04668	TNF signaling pathway	4	4.51E-02

Supplementary Table III: Enriched pathways of statistically significant miRNA target genes ($p<0.05$) increased or decreased in Combined Differentially expressed miRNA in Association with Endometriosis Diagnosis Endometrial Cells

GO Term	Term	# of genes	P-Value
GO:0000082	G1/S transition of mitotic cell cycle	12	4.01E-12
GO:0051301	cell division	8	0.002112036
GO:0001701	in utero embryonic development	7	3.96E-04
GO:0060070	canonical Wnt signaling pathway	4	0.00887545
GO:0016055	Wnt signaling pathway	4	0.071185563
GO:0048599	oocyte development	3	0.001949408
GO:2001234	negative regulation of apoptotic signaling pathway	3	0.002969315

GO:0045766	positive regulation of angiogenesis	6	3.09E-04
GO:0001525	angiogenesis	6	0.005756441

KEGG Pathway	Pathway	# of Genes	P-Value
hsa04151	PI3K-Akt signaling pathway	16	3.01E-07
hsa04110	Cell cycle	13	8.02E-10
hsa04115	p53 signaling pathway	7	2.92E-05
hsa04390	Hippo signaling pathway	8	4.11E-04
hsa05213	Endometrial cancer	5	0.001224143
hsa04310	Wnt signaling pathway	6	0.008213403
hsa04510	Focal adhesion	9	5.15E-04
hsa04370	VEGF signaling pathway	4	0.017862483
hsa04350	TGF-beta signaling pathway	5	0.007023653
hsa04068	FoxO signaling pathway	5	0.033410186
hsa04010	MAPK signaling pathway	6	0.081996404