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Research Article

Human Blastocyst Secreted microRNA Regulate Endometrial Epithelial Cell Adhesion

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ABSTRACT

Successful embryo implantation requires synchronous development and communication between the blastocyst and the endometrium, however the mechanisms of communication in humans are virtually unknown. Recent studies have revealed that microRNAs (miRs) are present in bodily fluids and secreted by cells in culture. We have identified that human blastocysts differentially secrete miRs in a pattern associated with their implantation outcome. miR-661 was the most highly expressed miR in blastocyst culture media (BCM) from blastocysts that failed to implant (non-implanted) compared to blastocysts that implanted (implanted). Our results indicate a possible role for Argonaute 1 in the transport of miR-661 in non-implanted BCM and taken up by primary human endometrial epithelial cells (HEECs). miR-661 uptake by HEEC reduced trophoblast cell line spheroid attachment to HEEC via PVRL1. Our results suggest that human blastocysts alter the endometrial epithelial adhesion, the initiating event of implantation, via the secretion of miR, abnormalities in which result in implantation failure.

1. Introduction

Embryo–endometrial interactions are critical for implantation and subsequent placental development. During the early stages of implantation, the blastocyst enters the uterine cavity, apposes and then adheres to an adequately prepared or 'receptive' endometrial uterine luminal epithelium to initiate implantation. Abnormalities in adhesion during the very early stages of implantation result in implantation failure, which is a major cause of infertility (Dimitriadis et al., 2005; Koot et al., 2012). In humans, very little is known of the blastocyst–endometrial interactions, largely due to the difficulty in studying implantation in humans. The influence of human blastocysts on human endometrial receptivity is largely unknown.

The conceptus enters the uterine cavity up to 72 h prior to implantation (Norwitz et al., 2001) and is thought to act on the endometrium at least in part via soluble factors to facilitate receptivity and implantation (Cuman et al., 2013).We have previously published that human blastocysts release soluble factors that alter primary human endometrial epithelial cell (HEEC) gene expression and adhesion, the initiating event of implantation (Cuman et al., 2013).

miRs are short (~20–22 nucleotides), highly conserved sequences that regulate the expression of 50% of genes in the human genome (Bartel, 2004). Mature miRs act by binding to complementary regions of mRNAs, inhibiting translation or by destabilising the gene, resulting in down regulation of their target genes (Bohnsack et al., 2004; Chen and Rajewsky, 2007; Kim, 2005; Lee et al., 2003). miR can be secreted by cells, via a number of mechanisms including exosomes, apoptotic bodies and bound to lipid or RNA binding complex (RBC) proteins, such as Argonaute (Ago) 1 and 2 (Arroyo et al., 2011; Vickers et al., 2011). MiRs are present not only within cells but also in body fluids such as saliva, urine, blood, plasma and cell culture media (Hanke et al., 2010; Mitchell et al., 2008; Park et al., 2009; Zubakov et al., 2010).

Analysis of human endometrium and trophectoderm has identified the expression of a large number of miRs (Dior et al., 2014; Galliano and Pellicer, 2014; Kresowik et al., 2014; Rosenbluth et al., 2013), with more recent studies demonstrating that miRs are secreted by human and bovine embryos in culture (Kropp et al., 2014; Rosenbluth et al., 2014). We hypothesised that miRs are released by human blastocysts and are taken up by endometrial surface epithelial cells to regulated endometrial receptivity and implantation. The aim was to identify miR

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profiles of spent culture media (BCM) from embryos that successfully implanted compared to those that failed to implant. Furthermore, we aimed to determine miR uptake by human endometrial epithelial cells and the effect on adhesion and therefore identify the possible functional consequences relevant to endometrial receptivity and implantation.

2. Materials and Methods

2.1. Ethical Approval

Human ethical approval was obtained for all the studies in this manuscript as follows:

Endometrium collection

Written informed consent was obtained from each patient, before surgery in the case of women with primary infertility, and protocols were approved by the Southern Health Human Research Ethics Committee, Melbourne, Australia.

Blastocyst media collection

Written informed consent was obtained from each patient and the study was approved by the Monash Surgical Private Hospital Human Research Ethics Committee, Melbourne, Australia.

Trophectoderm collection

Written informed consent was obtained from each patient and the study was approved by Monash Health (#12,101) and the Embryo Research Licencing Committee, National Health and Medical Research Council of Australia (#309722).

2.2. Endometrial Collection

Endometrial biopsies (n = 33) were collected at curettage from women with regular menstrual cycles throughout the proliferative and secretory phases of the menstrual cycle (Cuman et al., 2013; Paiva et al., 2009; Van Sinderen et al., 2013). The women had no steroid treatment for at least 2 months prior to tissue collection. An experienced gynaecological pathologist confirmed biopsies showed no evidence of possible endometrial dysfunction. Biopsies were either placed into DMEM F/12 media for further isolation or fixed in Formalin. See supplemental experimental procedures for further details on endometrial isolation.

2.2.1. Spent Conditioned Media (BCM)

Spent blastocyst conditioned media (BCM) were collected from embryos (fertilised by ICSI only) that had been cultured from days 3 to 5 and stored at −80 °C. Control culture media (not exposed to an embryo) were also collected. BCM were collected from two groups: 1. Blastocysts that successfully implanted (clinical pregnancy carried to term >36 weeks) (Implanted) and 2. Blastocysts that did not implant and did not result in pregnancy (no biochemical or clinical indications) (non-implanted).

2.2.2. Trophectoderm Collection

Human embryos consented to medical research (Ethics #12101) were thawed, and allowed to expand with assisted hatching overnight. Using in house technique, the inner cell mass was removed from the embryo and allowed to succumb. The remaining trophectoderm cells were collected directly into lysis buffer for PCR use. MicroRNA was isolated from cells using TaqMan Cell to CT kit (Life Technologies) according to manufactures instructions.

2.2.3. BCM microRNA Real Time PCR Arrays

RNA was isolated from BCM (10 μl) using miRCURY RNA Biofluids isolation Kit (Exiqon, Denmark) according to the manufacturer's instructions. cDNA synthesis and RT qPCR on BCM was performed using the miRCURY LNA™ Universal RT microRNA PCR system (Exiqon, Denmark) according to the manufacturer's instructions. In brief, the RNA was tailed with a poly (A) sequence at their 3′end and then reverse transcribed into cDNA using a universal poly (T) primer with a 3′end degenerate anchor and a 5′end universal tag. The cDNA products were subsequently diluted 125 fold and transferred to the ready-to-use microRNA PCR Human Panels (I + II). The qPCRs were run on a 7900HT thermocycler (ABI) using the thermal-cycling parameters recommended by Exiqon. Raw Ct values were calculated as recommended by Exiqon using the RQ manager software v1.2.1 (ABI) with manual settings for threshold and baseline, i.e. all miRCURY assays were analysed using a ΔRn threshold of 60 and baseline subtraction using cycles 1–14. Analysis was performed using the Gene Ex software.

2.2.4. Primary HEEC Isolation

Endometrial epithelial cells were prepared as previously published (Cuman et al., 2013). Briefly, endometrial tissue was digested with collagenase and the suspension was filtered through 43 and 11 mm nylon mesh to collect endometrial epithelial glands. The cells and epithelial fragments were collected and resuspended in a 1:1 mixture of Dulbecco's modified eagle's medium (DMEM)/Hams F-12 (Gibco) supplemented with 10% foetal calf serum (FCS; Invitrogen), and 1% antibiotic–antimycotic? solution (Gibco, Auckland, NZ) and plated. A purity of 95% was necessary for the cells to be used experimentally.

2.2.5. HTR-8/SVneo Trophoblast Cell Line

The HTR-8/SVneo trophoblast cell line exhibits features of invasive trophoblast cells, such as human leukocyte antigen-G (extravillous trophoblast marker) and cytokeratin-7 expression (Hannan et al., 2010). These cells were cultivated and maintained in RPMI 1640 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FCS, as previously described (Graham et al., 1993).

2.2.6. RNA Isolation and Quantitative PCR

RNA was extracted from cultured cells and conditioned media (excluding BCM and primary trophectoderm cells) using Tri Reagent (Sigma) according to the manufacturer's instructions. Isolated RNA was reversed transcribed into complimentary DNA with M-MLV RT system (Life Technologies) by using the TaqMan primer sets for miRs (Applied Biosystems) or Oligo primers (sigma) for non-miRs. Real time PCR was performed using the TaqMan Fast Universal PCR Master mix (Applied Biosystems) or Power SYBR Green master mix (Applied Biosystems) by using TaqMan probes or specific primer pairs (*MTA1*, F- TAACAAGCCA AATCCGAACC R- TCCTGGCCTCTCTCCATCTA; *MTA2*, F- CGGGTGGGAGAT TACGTCTA R- TGGCTGCTTTGATTCCTCTT*PVRL1* F- AATCGAGAAAGCCAGC TCAA R- CGGATCTCCTGGTACTCTGC; *EPHB2*- F- GATGGGGCAGTACAAG GAGA, R- AGGCAGGTGAATGTCAAACC). miR expression levels were normalised against control snU6 probes. Expression of *MTA2* and *PVRL1*was normalised against 18S and beta-actin.

2.2.7. miR Uptake by Primary HEEC

Fluorescein (FLC) tagged mIR-661 (Sigma) was transfected into HTR8s using Lipofectamine RNAiMax at a concentration of 100 nM, (based on the manufacturer's instructions). HTR8s were washed with culture media 12 h post transfection and incubated with fresh culture media for 12 h further. HTR8-CM was collected and used to treat HEECs. A scramble microRNA sequence (Life Technologies) was used as a control. HTR8 cells and 1 ml-conditioned media were collected for confirmation of overexpression of miR-661 by RT qPCR. HEECs were treated for 8 h and uptake confirmed by RTqPCR and immunofluorescence (method adapted from (Zhou et al., 2013)).

2.2.8. Immunofluorescence

Visualisation of FLC-miR-661 was confirmed using immunofluorescence. Briefly, HEECs were plated onto chamber slides and treated with HTR8-CM as described above. Following treatment, media removed, cells were washed and the chamber slide fixed in 70% ethanol overnight. Nuc-Red (to visualise nuclei;Invitrogen) was applied to the slide prior to fixing with fluorescent mounting media (Dako).