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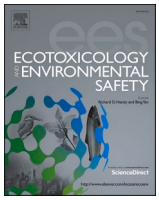

Research paper

The fungicide Mancozeb reduces spheroid attachment onto endometrial epithelial cells through downregulation of estrogen receptor β and integrin β3 in Ishikawa cells

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ABSTRACT

Mancozeb is a metal-containing ethylene bis-dithiocarbamate fungicide widely used in agriculture. Ethylene thiourea (ETU) is the primary metabolite of Mancozeb. Mancozeb has been associated with spontaneous abortions and abnormal menstruation in women. However, the effects of Mancozeb and ETU on embryo attachment remain unknown. The human blastocyst surrogate trophoblastic spheroids (JEG-3), endometrial epithelial surrogate adenocarcinoma cells (Ishikawa), or human primary endometrial epithelial cells (EECs) monolayer were used in the spheroid attachment models. Ishikawa and EECs were pretreated with different concentrations of Mancozeb or ETU for 48 h before the attachment assay. Gene expression profiles of Ishikawa cells were examined to understand how Mancozeb modulates endometrial receptivity with Microarray. The genes altered by Mancozeb were confirmed by qPCR and compared with the ETU treated groups. Mancozeb and ETU treatment inhibited cell viability at 10 μg/mL and 5000 μg/mL, respectively. At non-cytotoxic concentrations, Mancozeb at 3 μg/mL and ETU at 300 μg/mL reduced JEG-3 spheroid attachment onto Ishikawa cells. A similar result was observed with human primary endometrial epithelial cells. Mancozeb at 3 μg/mL modified the transcription of 158 genes by at least 1.5-fold in Microarray analysis. The expression of 10 differentially expressed genes were confirmed by qPCR. Furthermore, Mancozeb decreased spheroid attachment possibly through downregulating the expression of endometrial estrogen receptor β and integrin β3, but not mucin 1. These results were confirmed in both overexpression and knockdown experiments and co-culture assay. Mancozeb but not its metabolite ETU reduced spheroid attachment through modulating gene expression profile and decreasing estrogen receptor β and integrin β3 expression of endometrial epithelial cells.

1. Introduction

Mancozeb (MZ) is manganese- and zinc-containing fungicide belonging to the ethylene bis-dithiocarbamate (EBDC) family. It has a wide range of agricultural applications, including protecting nuts, crops, fruits, vegetables, and ornamentals from fungal diseases. Mancozeb has been used for over 70 years since it was first registered in 1948 and accounts for more than 20% of the current global fungicide market (Runkle et al., 2017). Moreover, Mancozeb is also used as a slimicide in water cooling systems, as a metal scavenger in sewage treatment, and as an accelerator for vulcanization in the rubber industry (Tsang and Trombetta, 2007).

In the past, Mancozeb was regarded as a safe reagent because it has short environmental persistence and low reported acute toxicity. The

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half-life of Mancozeb is 1–3 days on fruits and vegetables. The residue of Mancozeb became undetectable in the environment within a week after application (Paramasivam and Chandrasekaran, 2013; Devi et al., 2015). Mancozeb has been detected at a concentration of 39 μg/L in environmental water near farms (Archer and Van Wyk, 2015). The acceptable daily intake (ADI) of Mancozeb in humans is 50 μg/kg body weight/day and the acute reference dose is 600 μg/kg body weight/day (Schmidt et al., 2013). However, no study has directly reported the dose of Mancozeb in human fluids after exposure. In humans, the main metabolite of Mancozeb is ethylene thiourea (ETU), which has been used to evaluate Mancozeb exposure among agricultural and industrial workers (Frakes, 1988; Lindh et al., 2008). The concentration of ETU was found to be around 0.8–61.4 μg/L in the urine of workers exposed to EBDCs (Aprea et al., 1996; Colosio et al., 2002; Mandic-Rajcevic et al., 2018). However, ETU persists longer in the soil for 5–10 weeks and has higher water solubility than Mancozeb (Dearfield, 1994).

Recently, Mancozeb has been identified as an environmental endocrine disrupting chemical (EDC). Mancozeb was found to regulate the endocrine system, disrupt mononuclear cells and thymocytes, impair the hypothalamus-pituitary-thyroid axis, and interfere with nervous system development, and also has elevated carcinogenic potential (Shukla et al., 1990; Srivastava et al., 2012; Harrison et al., 2013; Pandey and Mohanty, 2015). Growing epidemiologic evidence indicates that Mancozeb may affect the reproductive system by impairing female fertility. Several studies reported that women exposed to Mancozeb were more likely to experience abnormal menstrual cycles and spontaneous abortions (Arbuckle et al., 2001; Garry et al., 2002; Farr et al., 2004). In line with this, some in vivo and in vitro studies revealed Mancozeb exposure disrupted the structure of human granulosa cells, decreased healthy follicles in rats, blocked ovulation by inhibiting luteinizing hormone (LH) secretion, reduced oocyte fertilization, declined progesterone synthesis in bovine luteal cells, and caused mouse fetal malformation (Bhaskar and Mohanty, 2014; Miranda-Contreras et al., 2005; Palmerini et al., 2018; Rossi et al., 2006a). In our previous work, Mancozeb was found to impair the embryo implantation, but no detail mechanism of Mancozeb on endometrial cells was described (Akthar et al., 2020). However, the effects of Mancozeb exposure on human reproductive processes are largely unknown, particularly its impacts on the endometrium during embryo implantation.

In the present study, we tried to delineate the molecular mechanisms behind the Mancozeb induced disruption of the embryo implantation. It is practically infeasible to study embryo implantation in humans, thus we adopted an in vitro spheroid-endometrial co-culture model to mimic the embryo implantation process (Kodithuwakku et al., 2011; Tsang et al., 2012; Li et al., 2017). We also investigated the effects of Mancozeb on gene expression in endometrial Ishikawa cells in relation to the signaling pathways governing endometrial receptivity, considering successful embryo implantation requires a receptive endometrium (Achache and Revel, 2006).

2. Materials and methods

2.1. Cell lines and treatment preparation

Human endometrial adenocarcinoma cells (Ishikawa, ECACC, 99040201; RL95-2, ATCC, CRL-1671) and choriocarcinoma cells (JEG-3, ATCC, HTB-36) were maintained in Dulbecco's Modified Eagle's Medium DMEM/F12 (Sigma) at 37 °C in 5% CO_2. The endometrial cells were starved for 24 h before treatment with Mancozeb/ETU in phenol red-free DMEM/F12 (Sigma) supplemented with 10% charcoal/dextran-stripped fetal bovine serum (csFBS, Hyclone).

Mancozeb (Cat no. 45553, Sigma) was dissolved in dimethyl sulfoxide (DMSO, Sigma) and used within 6 months (Lopez-Fernandez et al., 2017), whereas ETU (Cat no. 45531, Sigma) was dissolved in phenol red-free DMEM/F12 without FBS and filtered with sterile syringe filters (Millex, MERCK). The controls for Mancozeb and ETU were 0.1% DMSO and complete culture medium, respectively.

2.2. Cell viability assay

Ishikawa and JEG-3 cells were seeded in 96-well plates at a density of 5×10^3 cells per well. The cells were treated with different concentrations of Mancozeb or ETU for 48 h. Cell viability was measured using the CyQUANT NF Cell Proliferation Assay Kit (Invitrogen) as instructed. The cell viability was expressed as Viability (%) = A (test)/A (control) × 100%, where A is the absorbance with emission detection at 535 nm and excitation at 485 nm.

2.3. JEG-3 spheroid-Ishikawa cell attachment assay

Ishikawa cells were treated with Mancozeb (0.01–3 μg/mL) and ETU (3–300 μg/mL) for 48 h. Methotrexate (MTX) at 5 μM was used as a positive control. The JEG-3 spheroids were generated from trypsinized cells in DMEM/F12 containing 1% bovine serum albumin (BSA, Gibco) rotated at 88 rpm in an orbital shaker at 37 °C overnight. Spheroids with a diameter of 60–200 μm were selected under a dissection microscope. About 25–30 JEG-3 spheroids were transferred onto a confluent pre-treated Ishikawa monolayer. The co-culture system was incubated for 1 h at 37 °C in 5% CO_2. The plates were then shaken at 145 rpm for 10 min and the medium was removed and replaced with PBS. Attached spheroids remaining on the Ishikawa monolayer were counted. Attachment rate was defined as the percentage of attached spheroids out of the total number of spheroids added.

2.4. Isolation of human primary endometrial cells for the co-culture study

Endometrial biopsies were obtained from women visiting the Centre of Assisted Reproduction and Embryology at Queen Mary Hospital, Hong Kong SAR for fertility treatment. These women had a regular menstrual cycle and had not taken hormone replacement therapy in the past 3 months. Thirty-two endometrial biopsies were taken at the early-, mid- and late-proliferative (EP N = 3, MP N = 4, LP N = 6) and secretory (ES N = 6, MS N = 7, LS N = 6) phases of the menstrual cycle. An additional 16 samples were taken during the mid-luteal phase (LH+7/8 days) of the natural menstrual cycle for the co-culture study. This research was approved by the Institutional Review Board of the University of Hong Kong and the Hospital Authority Hong Kong West Cluster (approval number: UW13-118 and UW14-153). Human endometrial stromal cells (ESCs) and endometrial epithelial cells (EECs) were isolated as previously described (Masuda et al., 2016; Li et al., 2017). The EECs were cultured in a 48-well plate with complete DMEM/F12 medium supplemented with 10% csFBS, 500 pM β-estradiol, and 40 nM progesterone (Sigma) for 2–3 days before the treatments. The purity of the isolated endometrial cells was determined by vimentin and cytokeratin (Dako) immunostaining. Next, EECs were treated with 0.1% DMSO or 3 μg/mL Mancozeb for 48 h. The JEG-3 spheroids prepared as above were added onto the EEC monolayer and co-cultured for 3 h at 37 °C under 5% CO_2. The spheroid attachment rate was then determined as above.

2.5. RNA extraction and microarray analysis

After 48 h of Mancozeb (0.1, 1 and 3 μg/mL) treatment, total RNA was extracted from Ishikawa cells in triplicate using the Protein and RNA Isolation System Kit (Invitrogen) according to the manufacturer's instructions. The quality of all RNA samples was determined by RNA Nano 6000 Assay on an Agilent Bioanalyzer 2100 (Agilent Technologies). The microarray hybridization, slide washing and scanning, and data extraction and analysis were performed according to the protocol of GeneChip™ WT PLUS Reagent Kit (Thermo Fisher Scientific) at the Centre for PanorOmic Sciences (CPOS) at the University of Hong Kong, Hong Kong SAR. Details of the protocol are available at (http://www.

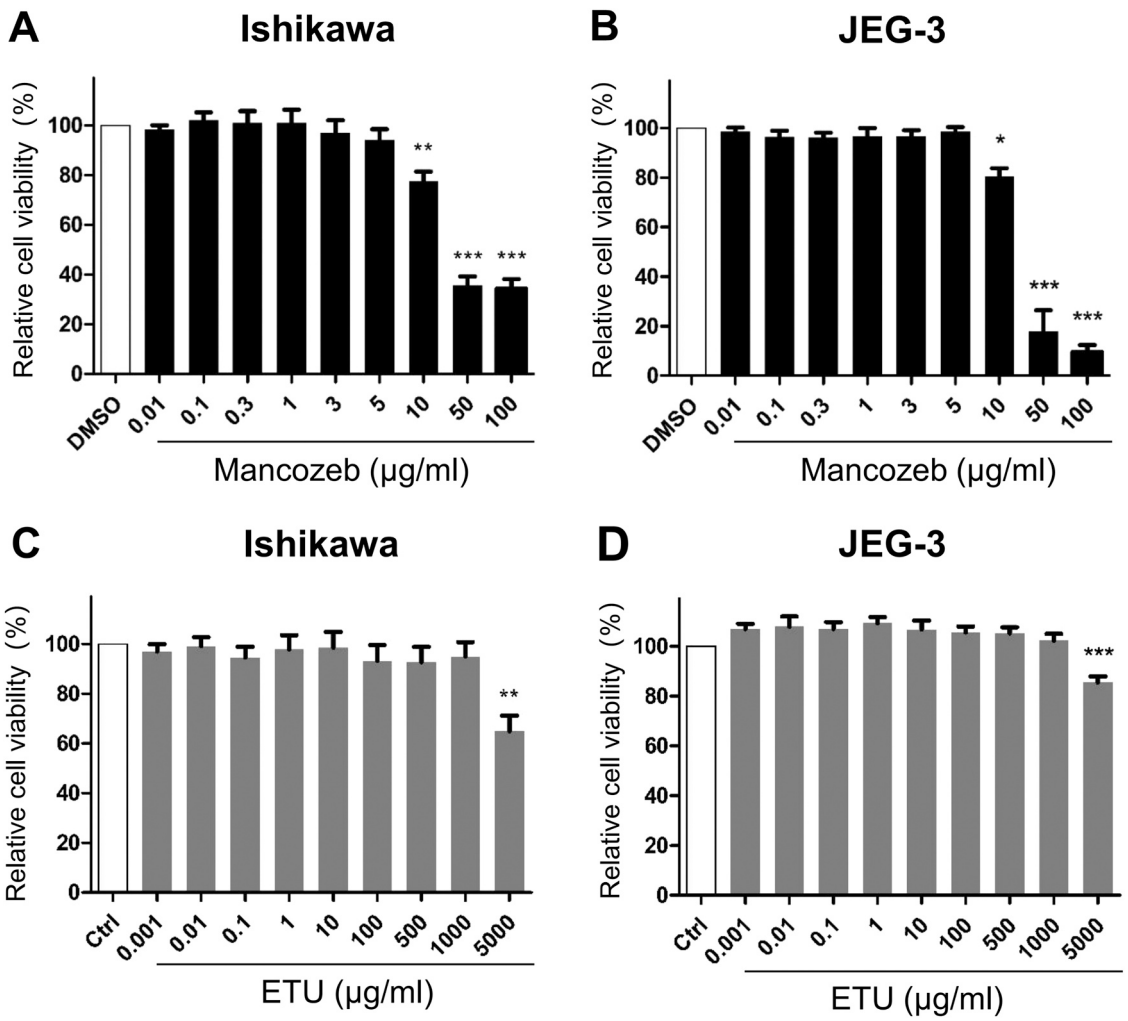

Fig. 1. Effect of Mancozeb and ETU on cell viability. (A-B) Mancozeb and (C-D) ETU on the viability of Ishikawa and JEG-3 cells after 48 h of treatment. The results were compared with 0.1% DMSO (vehicle) control group. Data expressed as mean ± SEM (N = 4; one-way ANOVA, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

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com/catalog/prod770005/AFFY/WT+PLUS+Reagent+Kit#1_1). For the microarray assay, 500 ng of total RNA from each sample was used. Affymetrix Human Gene 2.0 ST Array (Thermo Fisher Scientific) was used for the gene expression profile, in which 53,617 transcripts per sample were presented in the array.

2.6. Confirmation of gene expression by quantitative RT-PCR

Total RNA from endometrial tissues or cells were extracted as described above and 300 ng was used for the reverse transcription in 20 μL of reaction volume in TaqMan RT reagents (ABI Biosystems). Quantitative PCR (qPCR) was performed using TaqMan 2X Universal PCR Master Mix (Life Technologies) in an Applied Biosystems 7500 Detection System (Applied Biosystems) according to the standard protocols for mRNA. The TaqMan probes of human ATP1A2 (Hs00265131_m1), CLDN16 (Hs00198134_m1), KRTAP2-2 (Hs01661623_s1), MUC1 (Hs00159357_m1), ERα (Hs00174860_m1), ERβ (Hs00230957_m1), AR (Hs00171172_m1), PR (Hs00168730_m1), MMP-14 (Hs01037003_m1), ITGB3 (Hs01001469_m1), and 18S (4318839, internal control) were purchased from Applied Biosystems, USA. The PCR conditions were 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression of mRNA (Livak and Schmittgen, 2001).

2.7. Protein extraction and Western blotting

Ishikawa and RL95-2 cells were lysed in a cell disruption buffer (Invitrogen) containing 0.5% protease inhibitor cocktail (Calbiochem) at 4 °C. Proteins were mixed within SDS loading buffer, denatured at 95 °C and separated by 10% SDS-PAGE gel and then transferred onto nitrocellulose membranes. Membranes were probed with the primary antibodies ERβ (1:1000, Abcam), ERβ (1:500, Invitrogen), MUC1 (1:1000, Santa Cruz), or integrin β3 (1:1000, Abcam) overnight at 4 °C. After blocking and washing, membranes were incubated with the HRP-conjugated anti-mouse IgG (1:5000, GE Healthcare) or HRP-conjugated anti-rabbit IgG (1:5000, GE Healthcare) at room temperature for 1 h. The immunoreactive signals were detected using a Western Bright ECL kit (Advansta) according to the manufacturers' protocol. Protein levels were normalized against β-actin (1:5000, Santa Cruz) expression and signal intensity were analyzed by Image J software (NIH, USA).

2.8. Cell transfection for overexpression and knockdown studies

Ishikawa cells in the mid-log growth phase were seeded in a 12-well culture plate at a density of 1×10^5 cells per well. The Ishikawa cells at 80–90% confluency were transfected with 40 pM siRNA, negative control siRNA, or 1.6 μg plasmids with 2 μL lipofectamine 2000 (Invitrogen) in 200 μL Opti-MEM medium for 6 h at 37 °C. The medium was changed to complete culture medium and cultured for 48 h before the co-culture

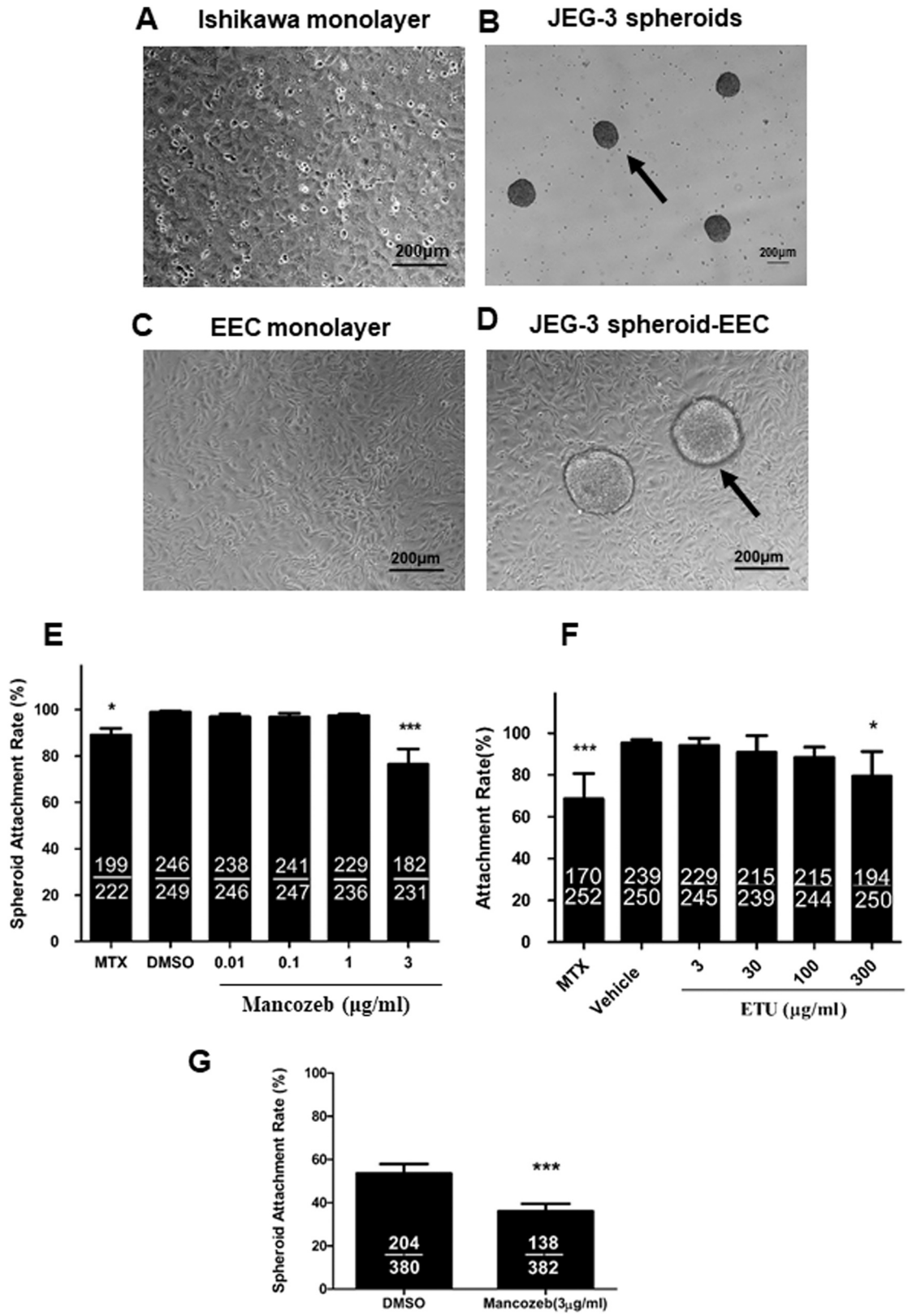

Fig. 2. Mancozeb suppresses spheroid attachment on both Ishikawa cells and human primary endometrial epithelial cells (EECs). Photomicrographs of (A) Ishikawa monolayer, (B) JEG-3 spheroids, (C) EEC monolayer, and (D) JEG-3 spheroids cocultured with EECs monolayer (200X, scale bar 200 μm). Effects of (E) Mancozeb and (F) ETU on JEG-3 spheroid attachment onto Ishikawa cells (N = 5; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$). Methotrexate (MTX) suppresses spheroid attachment in the co-culture assay. (G) Effects of Mancozeb on JEG-3 spheroid attachment onto human primary endometrial epithelial cells (N = 16; LH+7/8, Paired *t*-test followed by the normality test).

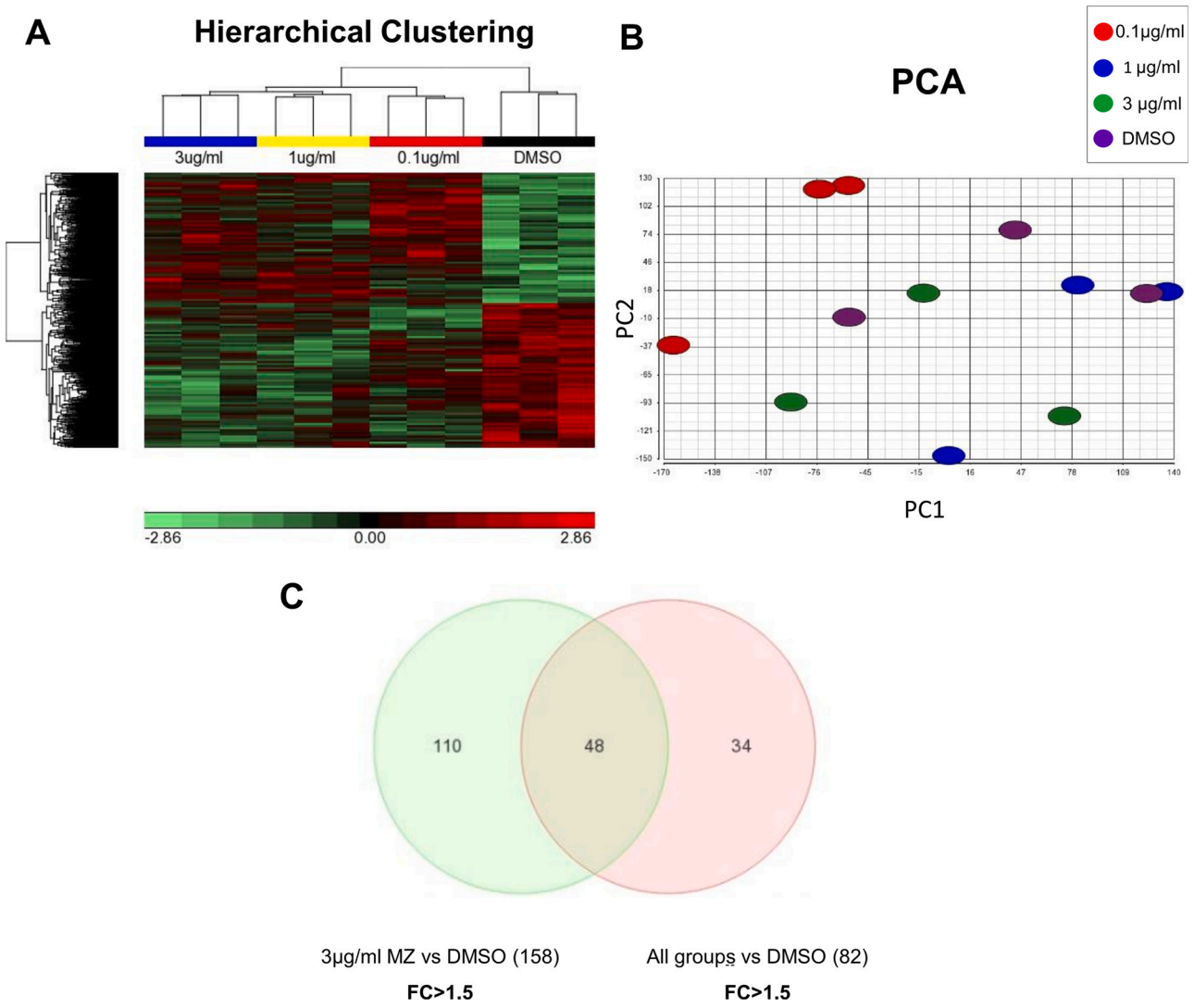

Fig. 3. Microarray analysis of Ishikawa cells treated with different concentrations of Mancozeb. (A) Hierarchical clustering showed the downregulated (green) and upregulated genes (red) in Ishikawa cells after 48 h of Mancozeb (0.1, 1 and 3 μg/mL) treatment (N = 3; fold change > 1, $p < 0.05$). (B) Principal component analysis (PCA) grouped samples by similar transcriptome patterns. (C) Venn diagram represented the genes differentially regulated by 3 μg/mL Mancozeb (3 vs. DMSO) and all Mancozeb groups (all vs. DMSO; FC, fold change).(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

assay. The ITGB3 and MUC1 siRNA (Dharmacon), ERβ and control siRNA (Santa Cruz), plasmid pcDNA-Flag-ERβ and β3-integrin-YFP plasmids (Addgene) were used in this set of experiments. The expression of target proteins after transfection was confirmed by Western blot analysis.

2.9. Data analysis

The expression data of samples were analyzed with the Partek Genomics Suite 6.6 software (Partek). Genes with significant expression changes were filtered, with $p < 0.05$ considered significant compared with the control group. Genes with a fold change setting at > 1.5 in the statistical analysis were recorded separately. The other data were analyzed using the SPSS statistic software version 22.0 (IBM) and GraphPad Prism5. One-way analysis of variation (ANOVA) or paired student's *t*-test was used to analyze the differences between the various Mancozeb groups and the control group as appropriate. The results were expressed as mean ± SEM. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of Mancozeb and ETU on the viability of Ishikawa and JEG-3 cells

Mancozeb exhibited similar cytotoxic effects on both Ishikawa and JEG-3 cells. Mancozeb treatment of 10, 50, and 100 μg/mL significantly decreased the viability of Ishikawa and JEG-3 cells in a dose-dependent manner (Fig. 1A & B). However, ETU reduced cell viability only at the concentration of 5000 μg/mL, which was a hundred-fold higher than Mancozeb (Fig. 1C & D).

3.2. Mancozeb reduced spheroid attachment onto endometrial cells

We used trophoblastic spheroid-endometrial epithelial cell co-culture assay to study the effects of Mancozeb on spheroid attachment onto the endometrial epithelial cells in vitro. In this study, both Ishikawa cells and purified human primary endometrial epithelial cells (EECs) were used in the co-culture assays (Fig. 2A–D). The purity of endometrial epithelial and stromal cells was determined to be over 95% by

Table 1
List of genes regulated by Mancozeb (3 μg/mL) with fold change > 1.5 and p < 0.05.

Gene symbol	*Gene description*	*Fold change*
KRTAP2-2	Keratin associated protein 2-2	−1.94
CLDN16	Claudin 16	−1.91
CFAP126	Cilia and flagella associated protein 126	−1.83
EFCAB12	EF-hand calcium binding domain 12	−1.72
IFITM2	Interferon induced transmembrane protein 2	−1.68
HNRNPA3P1	Heterogeneous nuclear ribonucleoprotein A3 pseudogene 1	−1.67
SCX	Scleraxis bhlh transcription factor	−1.66
SLC14A1	Solute carrier family 14 member 1	−1.66
IGHV3-38	Immunoglobulin heavy variable 3-38 (non-functional)	−1.66
GLYATL3	Glycine-N-acyltransferase-like 3	−1.64
ATP1A2	Atpase, Na^+/K^+ transporting, alpha 2 polypeptide	−1.63
SULT2B1	Sulfotransferase family 2B member 1	−1.63
CCDC106	Coiled-coil domain containing 106	−1.62
SERPINF1	Serpin family F member 1	−1.58
CTF1	Cardiotrophin 1	−1.56
MIR3685	Microrna 3685	−1.56
C19orf54	Chromosome 19 open reading frame 54	−1.56
ANGPTL1	Angiopoietin like 1	−1.55
PCDHB14	Protocadherin beta 14	−1.54
ZNF358	Zinc finger protein 358	−1.53
NHLRC1	NHL repeat containing E3 ubiquitin protein ligase 1	−1.53
LINC00441	Long intergenic non-protein coding RNA 441	−1.52
TGFB1I1	Transforming growth factor beta 1 induced transcript 1	−1.51
OR2L3	Olfactory receptor, family 2, subfamily L, member 3	−1.51
11-Sep	Septin 11	1.51
MIR421	Microrna 421	1.51
OR1S1	Olfactory receptor, family 1, subfamily S, member 1	1.53
LTB4R2	Leukotriene B4 receptor 2	1.54
IGHM	Immunoglobulin heavy constant mu	1.55
LOC101928188	Uncharacterized LOC101928188	1.57
NPIPB11	Nuclear pore complex interacting protein family, member B11	1.58
MIR4267	MicroRNA 4267	1.59
SNORD82	Small nucleolar RNA, C/D box 82	1.72

immunostaining (Fig. S1). Endometrial epithelial cells were treated with various concentrations of Mancozeb before co-culture. Ishikawa cells pretreated with Mancozeb at concentrations of 0.01, 0.1 and 1 μg/mL did not have significant effects ($p > 0.05$) on JEG-3 spheroid attachment onto Ishikawa cells compared with the 0.1% DMSO control group. However, a higher concentration of 3 μg/mL Mancozeb significantly decreased the attachment rate from 98.8% to 76.4% ($p < 0.05$) (Fig. 2E). In contrast, Ishikawa cells pretreated with ETU at 3, 30 and 100 μg/mL had no effects on JEG-3 spheroid attachment onto Ishikawa cells, but a higher concentration of 300 μg/mL ETU significantly reduced the attachment rate compared with the control group (Fig. 2F). Primary EECs treated with Mancozeb at 3 μg/mL also significantly suppressed JEG-3 spheroid attachment when compared to the control group (36.1% vs. 53.7%, $p < 0.001$; Fig. 2G).

3.3. Effects of Mancozeb on modulating the endometrial transcriptome

Total RNA from Ishikawa cells treated with Mancozeb was subjected to microarray analysis. Hierarchical Clustering and Principal Component Analysis (PCA) were used to display general gene expression patterns of the different treatment groups (Fig. 3A & B), and samples from the same concentration of Mancozeb were clustered together. The higher Mancozeb concentration induced more differentially expressed transcripts when compared to the DMSO control. Mancozeb treatments (0.1, 1 and 3 μg/mL) induced a total of 82 differentially expressed transcripts compared to the control group (> 1.5-fold change, $p < 0.05$). Mancozeb at 3 μg/mL induced 158 differentially expressed transcripts, including 72 downregulated and 86 upregulated transcripts, and 33 were distinct genes compared to the control group (Fig. 3C, Table 1). Pathway analysis with Gene Ontology (GO) enrichment was used to determine the molecular, cellular, and biological functions of these 158 transcripts (Fig. S2A–C). The functional annotations indicated the majority of the differently expressed genes regulated by 3 μg/mL regulated the transporter activity, cell junctions, and biological adhesion, but the functional category was not significant when enrichment score was over 3 ($p > 0.05$). Moreover, the most enriched pathway modulated by 3 μg/mL Mancozeb was proximal tubule bicarbonate reclamation ($p = 0.032$), which involves a key gene ATPase Na^+/K^+ transporting subunit alpha 2 (ATP1A2).

3.4. Confirmation of Mancozeb-induced gene expressions by quantitative RT-PCR

We used qPCR to confirm candidate genes ATP1A2, CLDN16, KRTAP2-2 which had the highest fold change in the microarray after treatment with 3 μg/mL Mancozeb. ITGB3, MUC1, and MMP14 were studies since they are already known to be endometrial receptivity markers and they were significantly downregulated by 3 μg/mL Mancozeb in the microarray result. We also selected steroid hormone receptors encoding genes ERα, ERβ, AR and PR for this study since there are potential Mancozeb interacting partners (Kjeldsen et al., 2013). The qPCR results for Mancozeb-treated Ishikawa cells agreed with the microarray data (Fig. 4). Interestingly, qPCR results of ETU-treated samples did not show similar gene expression patterns to Mancozeb, albeit a 100-fold increase in the concentrations of ETU was used (Fig. S3). ATP1A2 was the only gene downregulated by both Mancozeb and ETU (Fig. 4A & S3A).

3.5. Mancozeb but not ETU reduced spheroid attachment via decreased expression of ERβ and integrin β3 in endometrial cells

We further investigated the effects of ERβ, ITGB3, and MUC1, which were reported to modulate endometrial receptivity. The expressions of these molecules in human endometrial samples were analyzed (Fig. 5A-C). The expression of ERβ transcript was found to increase from EP to LP and then decrease from ES to LS. Similar observations were found for ITGB3 expression, which was highest at the ES stage. Interestingly, the MUC1 transcripts increased from EP to LP and then decreased to very low levels at the MS stage, suggesting low levels of MUC1 transcript favor embryo implantation on a receptive endometrium. Mancozeb treatment was found to decrease ERβ, ITGB3, and MUC1 protein expressions in treated Ishikawa cells. Western blot analysis showed 3 μg/mL Mancozeb decreased protein expressions of ERβ, ITGB3, and MUC1 in treated Ishikawa cells (Fig. 5D-F), whereas 300 μg/mL ETU did not have any effect (Fig. S4A). Similar effects were observed on ITGB3 expression when using another receptive endometrial epithelial cell line RL95-2 (Fig. S4B).

Knockdown of ERβ, ITGB3, and MUC1 by siRNA transfection into Ishikawa cells was performed to investigate their involvement in the suppressive effects of Mancozeb on spheroid attachment in the co-culture study. Our results showed that the downregulation of ERβ and ITGB3, but not MUC1, significantly decreased JEG-3 spheroid attachment when compared with siRNA controls (Fig. 6A & B). In contrast, overexpression of ERβ and ITGB3 in transfected Ishikawa cells nullified the inhibitory effect of Mancozeb on spheroid attachment (Fig. 6C & D).

4. Discussion

Accumulating evidence suggests that exposure to the fungicide Mancozeb lengthens the menstrual cycle and increases the risk of miscarriage and spontaneous abortion (Garry et al., 2002; Farr et al., 2004). However, the underlying mechanisms of how Mancozeb modulates embryo implantation and pregnancy outcomes still remain unclear. We demonstrated that endometrial Ishikawa cells treated with

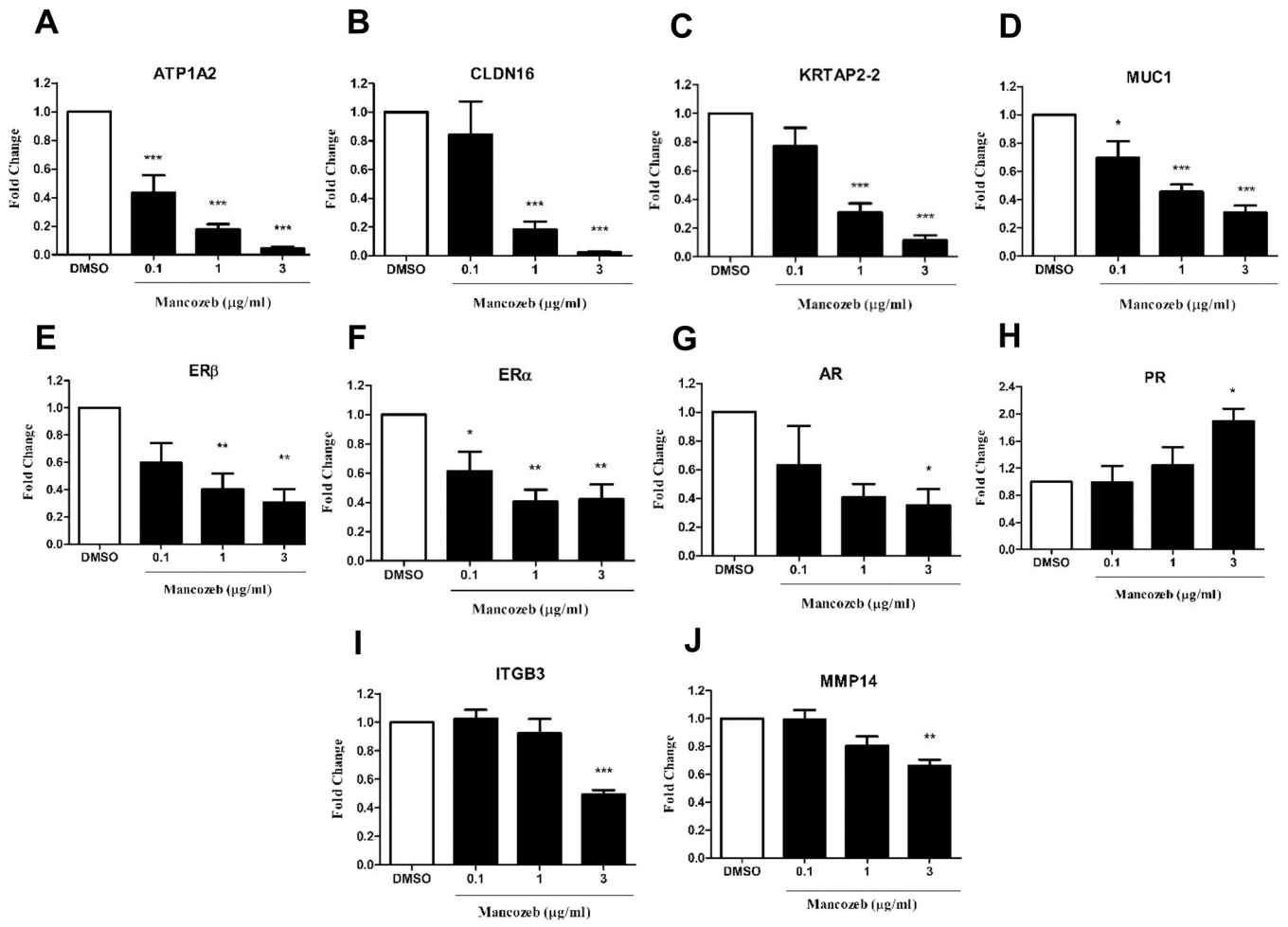

Fig. 4. . Quantitative PCR confirmation of differentially expressed transcripts in Ishikawa cells after Mancozeb treatment. Expressions of ATPase Na^+/K^+ transporting subunit alpha 2 (ATP1A2), claudin 16 (CLDN16), keratin associated protein 2–2 (KRTAP2-2), mucin 1 (MUC1), estrogen receptor beta (ERβ), estrogen receptor alpha (ERα), androgen receptor (AR), progesterone receptor (PR), integrin β3 (ITGB3) and matrix metallopeptidase 14 (MMP14) after Mancozeb treatments were measured by qPCR to confirm the Microarray results. Data were normalized to housekeeping gene 18S and presented as mean ± SEM (N = 5).

Mancozeb at 3 μg/mL suppressed spheroid attachment. Microarray analysis revealed changes in genes involved in endometrial receptivity, including suppression of ERβ, ITGB3, and MUC1 expression. However, similar findings were not observed with exposure to ETU, the metabolite of Mancozeb, suggesting that ETU has lower cytotoxicity than Mancozeb in our current models.

We first studied the effect of Mancozeb and ETU on the viability of Ishikawa and JEG-3 cells. Treatment with ≥ 10 μg/mL Mancozeb for 48 h significantly suppressed the viability of Ishikawa and JEG-3 cells (Lopez-Fernandez et al., 2017). The toxicity of Mancozeb has been reported to vary according to cell types. Mancozeb was found to reduce the viability of liver cancer HepG2 cells at a concentration of 0.1 μg/mL (Pirozzi et al., 2016), and inhibit cell growth of human colon cells at 21.6–64.9 μg/mL (Hoffman and Hardej, 2012), and MCF-7 cells at 10 and 50 μg/mL (Lin and Garry, 2000). Interestingly, cytotoxic effects in primary cells occurred with much lower concentrations of Mancozeb than in cell lines (Domico et al., 2006). In this study, we observed that Mancozeb at 3 μg/mL did not have any cytotoxic effects on primary endometrial cells. In some animal species, it was found that ETU appeared to be more toxic than Mancozeb (Houeto et al., 1995), but few studies have reported the cytotoxicity of ETU in human cells. This study was the first to compare the cytotoxicity of Mancozeb with ETU in human cells and found that ETU had much lower cytotoxic effects (100-fold less) than Mancozeb.

Accumulating evidence suggests that Mancozeb can affect mouse reproduction through disrupting the estrous cycle, reducing the number of healthy follicles, inducing atretic follicles, interfering with fertilization, and inhibiting embryo implantation in vivo (Baligar and Kaliwal, 2001; Bindali and Kaliwal, 2002; Rossi et al., 2006b). In this study, trophoblastic cell-derived JEG-3 spheroids, Ishikawa and primary EECs were chosen to study embryo implantation. We chose to use JEG-3 cells to generate spheroids because they are reported to have high sensitivity to pesticides and acceptable model for trophoblast study (Mesnage et al., 2014). Ishikawa cells are well-differentiated adenocarcinoma cells and commonly used to study the effect of EDCs on the endometrium during embryo implantation (Naciff et al., 2010). In humans, the endometrium is only receptive to the implanting embryo within a limited period from day 19 to 23 (LH+5 to LH+9) of the menstrual cycle. We isolated human primary EECs from endometrial biopsies collected on day LH+ 7/8 for the in vitro co-culture studies. We demonstrated that Ishikawa cells and EECs treated with Mancozeb (3 μg/mL) reduced JEG-3 spheroid attachment when compared with the control groups. However, ETU treatment decreased the spheroid attachment rate only at concentrations of 300 μg/mL or higher, indicating ETU is less effective than Mancozeb in inhibiting spheroid attachment in vitro. This suggests the suppressing effect of Mancozeb on embryo implantation in humans is more significant than its metabolite ETU. Although our unpublished data suggest ETU could mildly induce JEG-3 cell proliferation, however, it was found to disrupt endometrial adenocarcinoma cell proliferation in one other study (Yoshida et al., 1996).

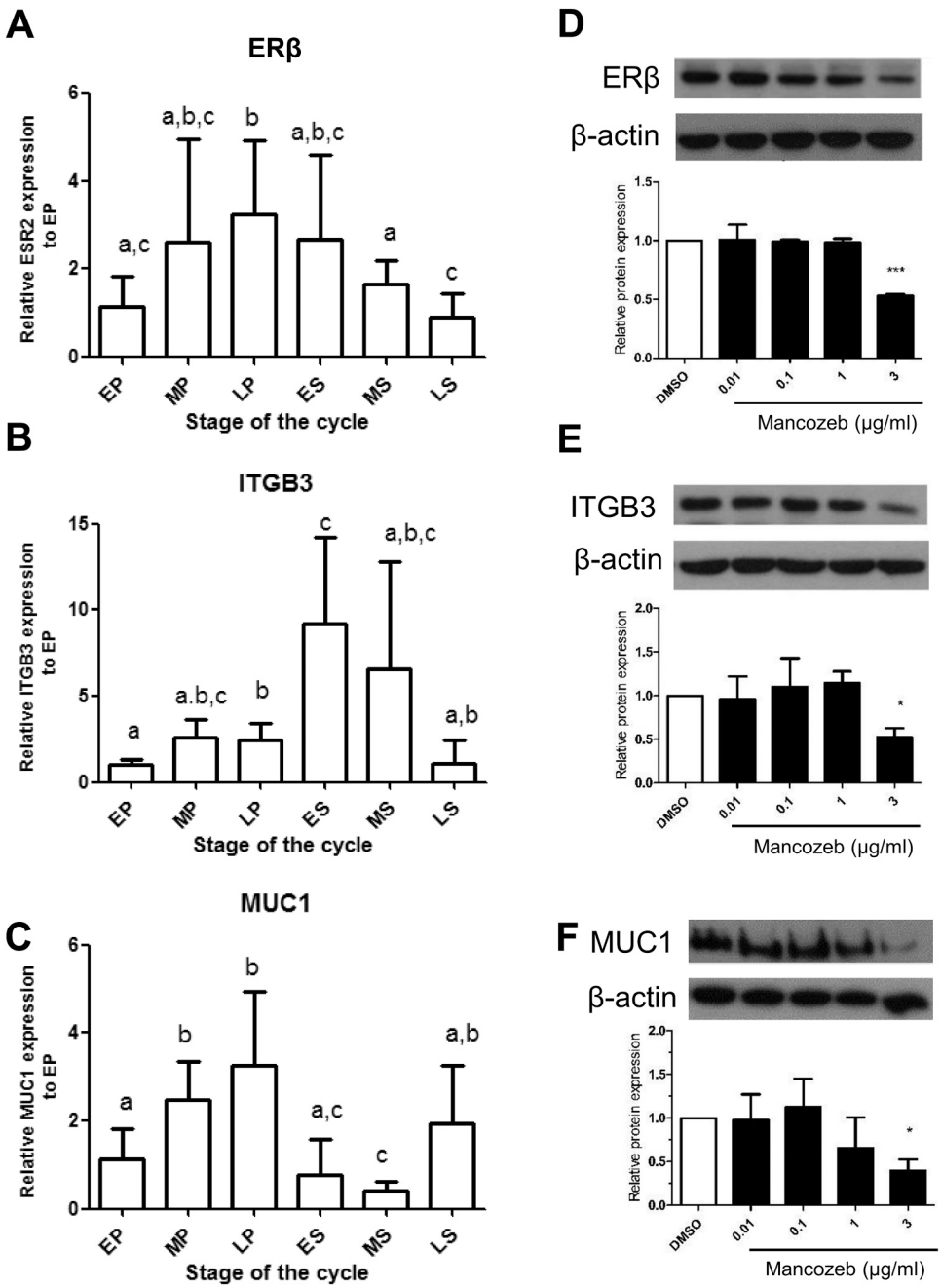

Fig. 5. Expression of endometrial receptivity genes in endometrial tissues and endometrial epithelial cell lines. Expressions of (A) estrogen receptor beta (ERβ), (B) integrin β3 (ITGB3), and (C) mucin 1 (MUC1) in human endometrial tissues (early-, mid- and late-proliferative [EP N = 3, MP N = 4, LP N = 6] and secretory [ES N = 6, MS N = 7, LS N = 6] phases of the menstrual cycle) were analyzed by qPCR. The relative gene expressions were normalized to 18S. a, b, c represent significant differences between groups at $p < 0.05$. (D–F) Western blotting of Ishikawa cells (N = 3) treated with different concentrations of Mancozeb (0.01, 0.1, 1, and 3 μg/mL). Protein expression levels of ERβ, MUC1, and ITGB3 analyzed by Western blotting (*$p < 0.05$, compared with the DMSO control group).

Mancozeb was reported to have multiple mechanisms of action, including the production of reactive oxygen species, suppression of steroidogenesis, and inflammatory responses (Elsharkawy et al., 2019; Weis et al., 2019). Using microarray analysis, we identified ATP1A2, CLDN16 and KRTAP2-2 transcripts were strongly induced by Mancozeb. The ATP1A2 gene encodes the large catalytic α-subunit of the α2 isoform of human Na^+/K^+-ATPase (Isaksen and Lykke-Hartmann, 2016). The α2 isoform is primarily expressed in the central nervous system and in skeletal, heart, and smooth muscles (McGrail et al., 1991). Mutations and deficiencies in ATP1A2 are associated with nervous system diseases (Gritz and Radcliffe, 2013; Ueda et al., 2018). Moreover, ATP1A2 knockout mice displayed malformation of the nervous systems (Monteiro et al., 2020). Prenatal Mancozeb treatment similarly disrupted brain development in postnatal mice (Miranda-Contreras et al., 2005). The CLDN16 gene encodes a tight junction protein in epithelial cells and can be downregulated by Mg^{2+}. (Efrati et al., 2010). In contrast, CLDN16 mutations affect the reabsorption of Mg^{2+} in mice (Breiderhoff et al., 2018). Mancozeb may downregulate CLDN16 through its zinc ion disrupting the regulation of Mg^{2+} (Spencer et al., 1994). The KRTAP2-2 protein is a hair keratin-associated protein that modulates hair follicle differentiation and phenotypic hair diversification (Fujikawa et al., 2012; Khan et al., 2014). The MMP-14 protein is a zinc-dependent endopeptidase molecule that modulates extracellular matrix (ECM) degradation and trophoblast invasion (Itoh and Seiki, 2006; Onogi et al., 2011). Further studies are needed to investigate the functions of these genes in endometrial receptivity and embryo implantation. However, ETU did not show any effect except for the ATP1A2 transcript. The results indicated that the toxicity of Mancozeb was probably owing to the cooperative effect involving both the metal moiety and its chelation with the organic backbone, which had been reported in the colon cells (Hoffman et al., 2016). Thus, the combination effect with metals (zinc and manganese) and organic backbone (ETU) should be studied in the future.

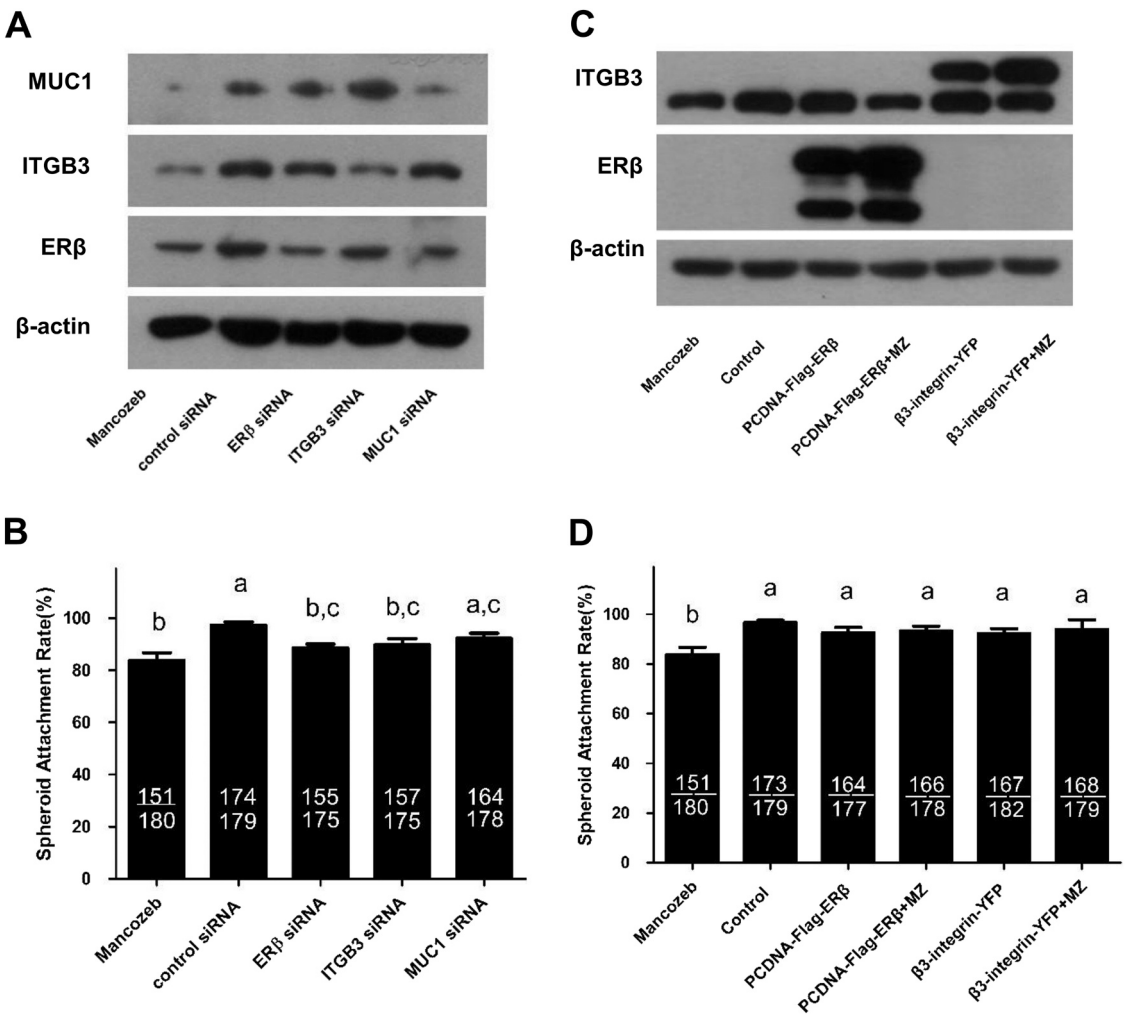

Fig. 6. Effects of ERβ, ITGB3, and MUC1 siRNA and overexpression on spheroid attachment onto Ishikawa cells. (A) ERβ, ITGB3, and MUC1 were knocked down by siRNA transfection in Ishikawa cells. (B) ERβ and ITGB3, but not MUC1 siRNA could recapitulate the suppressive effects of Mancozeb on spheroid attachment. (C-D) Overexpression of ERβ and ITGB3 in Ishikawa cells nullified the suppressive effects of Mancozeb (MZ) on spheroid attachment (N = 6; a, b, c, represent significant differences between groups at $p < 0.05$). Western blot analysis showed the expression of relative proteins after transfection experiments. Protein loading was normalized with β-actin expression in each sample.

In the mid-secretory phase of the menstrual cycle, the human endometrial epithelium acquires a functional and steroidal hormone-dependent status favoring blastocyst implantation, also known as a receptive endometrium (Makker and Singh, 2006). We found Mancozeb decreased the transcription and expression of several receptivity genes in endometrial epithelial cells, including ERβ, ITGB3, and MUC1. These transcripts are known to have cyclical expression in human endometrial tissue. Normally, expressions of ERβ and ITGB3 are increased and expression of MUC1 is decreased in the MS (receptive period) phase of the menstrual cycle. Furthermore, estrogen also functions to generate a receptive endometrial environment via estrogen receptors, ERα and ERβ, and G-protein-coupled estrogen receptor (GPR30) (Hapangama et al., 2015). Of the two isoforms, ERα plays a more crucial role in endometrium differentiation, as ERα knockout mice are infertile and have problems with uterine growth. In contrast, ERβ knockout mice are subfertile with follicular dysplasia (Hamilton et al., 2014). In this study, Mancozeb was found to reduce ERβ, ITGB3, and MUC1 expressions in the Ishikawa cells. Integrin β3 (ITGB3) is a cell adhesion molecule and acts as an endometrial receptivity marker, which is upregulated during the implantation phase (Lessey et al., 1994). Deficiency of ITGB3 in the endometrium causes embryo attachment disorder, implantation failure, and female infertility (Lessey et al., 1995; Sun et al., 2013). Mucin 1 (MUC1) also plays an important role in modulating endometrial receptivity and embryo attachment (Horne et al., 2005; Hattrup and Gendler, 2008). Although we found there was downregulated MUC1 mRNA in the MS phase of the cycle, it has been reported that MUC1 protein is elevated in the peri-implantation period and is removed locally at the blastocyst attachment site for successful implantation in humans (Thathiah and Carson, 2002). Patients with recurrent implantation failure (RIF) exhibit lower MUC1 expression in their endometrium (Aplin et al., 1996; Bastu et al., 2015). Our study supports the notion that Mancozeb can reduce MUC1 and ITGB3 protein expression in endometrial cells, leading to a non-receptive condition resembling RIF in patients. Knockdown of ERβ, MUC1, and ITGB3 expressions using siRNAs was performed to confirm the results and to investigate their involvement in the effects of Mancozeb on spheroid attachment. The knockdown of ERβ and integrin β3 significantly decreased spheroid attachment. Conversely, overexpression of ERβ and integrin β3 reversed the suppressive effects of Mancozeb on spheroid attachment.

Our study had several limitations that need to be addressed. Data on the levels of Mancozeb in the human body is very limited, which means part of the concentrations used our in vitro may not reflect the physiological levels. The concentrations of Mancozeb used in this study are based on previously reported in vitro studies and accidental high-dose exposure to Mancozeb in humans (Aprea et al., 1998; Erro et al., 2011). Moreover, exposure to pesticides typically occurs over a long

period. Thus, future studies looking at prolonged exposure to Mancozeb in vitro may better reflect the in vivo conditions in humans.

5. Conclusion

In conclusion, in vitro exposure of Mancozeb reduced spheroid attachment onto endometrial epithelial cells partly through down-regulating ERβ and ITGB3 expression. Mancozeb was found to be more cytotoxic and inhibit spheroid attachment more than ETU indicating they may have two different signaling pathways in their mode of actions. Further studies are needed to investigate the effect of chronic low dose Mancozeb exposure on reproductive health.

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CRediT authorship contribution statement

Ziyi Wang: Conceptualization, Methodology, Formal analysis, Writing - original draft. **Kottawattage S.A. Kottawatta and Suranga P. Kodithuwakku:** Investigation, Methodology, Writing - review & editing. **Thevarathanthrige S. Fernando:** Methodology, Formal analysis. **Yin-Lau Lee**, **Ernest H.Y. Ng and William S.B. Yeung:** Conceptualization, Writing - review & editing. **Kai-Fai Lee:** Conceptualization, Supervision, Funding acquisition, Methodology, Writing - review & editing.

Declaration of Competing Interest

None to declare.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2020.111606.

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