Expression of membrane protein disulphide isomerase A1 (PDIA1) disrupt a reducing microenvironment in endometrial epithelium for embryo implantation

Sudini R Fernando[1,2], Cheuk-Lun Lee[1,3], Benancy PC Wong[1], Kiu-Wai Cheng[1], Yin-Lau Lee [1,3], Ernest HY Ng[1,3], William SB Yeung[1,3], Kai-Fai Lee[1,3,*]

1 Department of Obstetrics and Gynaecology, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong SAR, China

2 Department of Animal Science, Faculty of Animal Science & Export Agriculture, Uva Wellassa University, Badulla, 50000, Sri Lanka

3 Shenzhen Key Laboratory of Fertility Regulation, The University of Hong Kong-Shenzhen Hospital, Haiyuan 1st Road, Futian District, Shenzhen, 518053, China

* Corresponding author: Kai-Fai Lee (PhD), Department of Obstetrics and Gynaecology, The University of Hong Kong, Pokfulam, Hong Kong. Fax: 852-28161947 and E-mail: ckflee@hku.hk

Abstract

Various proteins in the endometrial epithelium are differentially expressed in the receptive phase and play a pivotal role in embryo implantation. The Protein Disulphide Isomerase (PDI) family contains 21 members that function as chaperone proteins through their redox activities. Although total PDIA1 protein expression was high in four common receptive (Ishikawa and RL95-2) and non-receptive (HEC1-B and AN3CA) endometrial epithelial cell lines, significantly higher membrane PDIA1 expression was found in non-receptive AN3CA cells. In Ishikawa cells, oestrogen up-regulated while progesterone down-regulated membrane PDIA1 expression. Moreover, mid-luteal phase hormone treatment down-regulated membrane PDIA1 expression. Furthermore, oestrogen at 10 nM reduced spheroid attachment on Ishikawa cells. Interestingly, inhibition of PDIA1 function by bacitracin or 16F16 increased the spheroid attachment rate onto non-receptive AN3CA cells. Over-expression of PDIA1 in receptive Ishikawa cells reduced the spheroid attachment rate and significantly down-regulated integrin β3 levels, but not integrin αV and E-cadherin. Addition of reducing agent TCEP induced a sulphydryl-rich microenvironment and increased spheroid attachment onto AN3CA cells and human primary endometrial epithelial cells collected at LH+7/8 days. The luminal epithelial cells from human endometrial biopsies had higher PDIA1 protein expression in the proliferative phase than in the secretory phase. Our findings suggest oestrogen and progesterone regulate PDIA1 expression, resulting in the differential expressions of membrane PDIA1 protein to modulate endometrial receptivity. This suggests that membrane PDIA1 expression prior to embryo transfer could be used to predict endometrial receptivity and embryo implantation in women undergoing assisted reproduction treatment.

Key words: Protein Disulphide Isomerase/Oestrogen/Progesterone/Membrane Protein/Oestrogen receptor/Progesterone receptor/ Spheroid attachment/Endometrial receptivity

Introduction

The human endometrium becomes receptive to the developing embryo during a specific period between Days 19 and 24 of the menstrual cycle, known as the Window of Implantation (Lessey, 2002). Structural and functional remodelling of the endometrium in the secretory phase of the cycle creates a receptive environment for the blastocyst. In humans, embryo implantation starts with the apposition and adhesion of a blastocyst onto the luminal epithelium of the endometrium. These processes are associated with the increased expression of several adhesion molecules in endometrial epithelial cells, such as integrins, cadherins, immunoglobulins, and selectins (Achache & Revel, 2006). Many other proteins are also involved in the adhesion process, but their identities remain to be determined (Bhagwat et al., 2014).

Membrane receptors on endometrial epithelial cells are involved in the transduction of exogenous signals to the cytoplasm and nucleus through the binding of ligands such as hormones, drugs, and other signalling molecules (Apodaca et al., 2012; Gatenby, 2019). Moreover, the functional status of cellular membranes relies on the expression of different proteins, lipids, and carbohydrates (Cao et al., 2012). In fact, the polar organization of the cell membrane creates an unfavourable environment for blastocyst adhesion (Singh & Aplin, 2009). Therefore, the composition and expression of cell membrane proteins first need to be modified to create a receptive endometrium for embryo implantation(Kakar-Bhanot et al., 2020). Oestrogen and progesterone play a vital role in regulating the expression of cell adhesion molecules and receptivity markers in the human endometrium (Lessey et al., 2006). However, the high levels of serum oestradiol after ovarian stimulation affects endometrial receptivity, resulting in lower implantation and pregnancy rates in IVF cycles (Arslan et al., 2007; Liu et al., 2009; Shapiro et al., 2011; Toner et al., 1991; Yu Ng et al., 2000).

Protein Disulphide Isomerase (PDI), also known as the beta-subunit of prolyl 4-hydroxylase (P4HB), belong to the thioredoxin superfamily (Ali Khan & Mutus, 2014). The PDI family contains 21 members that are mainly localized in the endoplasmic reticulum of cells. The PDI protein consists of a polypeptide chain made up of 508 amino acids. It functions as a chaperone protein, a redox-dependent chaperone, a disulphide isomerase, and a redox regulator (Kozlov et al., 2010). The disulphide interchange and enzymatic redox activity of PDI have been extensively

studied. For example, PDI regulates disulphide bond formation in cys374 of actin in the cytoskeleton (Sobierajska et al., 2014). It has been reported that PDI is present in the nucleus and cell surface (Terada et al., 1995). The major route of PDI translocation from the inner cell to other locations is through the Golgi (Araujo et al., 2017b). In patients with unexplained infertility, the expression of PDIA3 in the endometrium is higher in the mid-secretory phase than in the early-secretory phase (Manohar et al., 2014). Consistently, the expression of PDIA3 is higher on the cell surface of receptive RL95-2 cells compared to non-receptive HEC1-A endometrial cells (Bhagwat et al., 2014). Oestrogen treatment was reported to up-regulate PDI expression in ovariectomised mice and in vascular endothelial cells (Yuan et al., 2014). Depending on the disulphide bonds between two cysteine residues, PDI exists in an oxidized or reduced form (Parakh & Atkin, 2015). Studies have shown that PDI can be reduced by DTT and oxidized by Diamide (Jessop & Bulleid, 2004; Kranz et al., 2017). Interestingly, sulphydryl group formation favours sperm motility (Kumar et al., 1990; Nivsarkar et al., 1996) and a reducing microenvironment in the endometrium favours embryo implantation (Nivsarkar et al., 2001; Thomas et al., 1994). However, no studies have reported the role of PDI in regulating endometrial receptivity.

Preliminary studies from our laboratory showed that membrane PDIA1 is highly expressed in non-receptive AN3CA human endometrial epithelial cells compared to receptive Ishikawa cells (unpublished data). In this study, we hypothesized that PDIA1 is differentially expressed and regulated by steroid hormones in endometrial cells. We further hypothesized that the membrane expression of PDIA1 protein regulates endometrial receptivity. The present study aimed to understand the hormonal regulation of PDIA1 expression and its functional role on human endometrial receptivity using a trophoblast spheroid-endometrial cell co-culture model.

Materials and methods

Patients

Infertile women attending the Assisted Reproduction Unit of the Department of Obstetrics and Gynaecology, Queen Mary Hospital, Hong Kong for assisted reproduction treatment were recruited in this study. Endometrial biopsies were collected from women with regular menstrual cycles and male factor infertility, and with no steroid or other treatments taken within 3 months. The study was approved by Institutional Review Board of the University of Hong Kong and the Hospital Authority of Hong Kong (UW17-458 and UW14-153). Written consent was obtained from all the participants before the start of the study. Tissues samples were collected during the proliferative phase (Day 1-14, n=12) and the secretory phase (Day 15-23, n=24). Tissue samples were also collected from the mid-luteal phase (LH+7/8 days, n=10) for the co-culture assay.

Immunohistochemistry

Human endometrial biopsies were fixed in 4% paraformaldehyde and embedded in paraffin. The 5-μm thick sections were mounted on polylysine-coated slides and de-waxed followed by antigen retrieval as reported previously (Lee et al., 2006). Monoclonal rabbit anti-PDIA1 antibody (1:100) and negative control with omitted primary antibody were used. Positive signals were obtained by 3,3′-diaminobenzidine (Dako Cytomation) staining and nuclei were counter-stained with haematoxylin. The stained sections were observed under a Zeiss Axioskop microscope (Photometrics Sensys, AZ, USA) with bright-field optics.

Cell culture

Human endometrial epithelial Ishikawa (ECACC 99040201), RL95-2 (ATCC CRL1671), AN3CA (ATCC HTB-111), and HEC1-B (ATCC HTB-113) cells, and human trophoblastic choriocarcinoma Jeg-3 cells (ATCC HTB-36) were used in this study. Ishikawa, AN3CA, and HEC1-B cells were maintained in Minimum Essential Medium (MEM, Sigma M0268), whereas RL95-2 and Jeg-3 cells were maintained in Dulbecco's Modified Eagle Medium nutrient mixture F12 (DMEM F12, Sigma D8900) supplemented with 1% penicillin/streptomycin, 1% L-glutamine, and 10% foetal bovine serum (FBS) (Thermo Fisher). All the cells were sub-cultured every 2-3 days and maintained at 37°C in 5% CO_2.

Membrane protein labelling for mass spectrometry analysis

We compared surface protein expressions between endometrial receptive (Ishikawa) and non-receptive (AN3CA) cells. Cell surface proteins were labelled with EZ-Link^{TM} Sulpho-NHS-SS-Biotin (Thermo Fisher Scientific). In brief, cells in T75 flask (90% confluency) were washed with ice cold PBS twice. The cells were labelled with 10 mL Sulfo-NHS-SS-Biotin (0.25mg/mL) for 30 minutes at 4°C. The reaction was stopped by addition of 500μL quenching solution. The labelled cells were washed with TBS and then collected by cell scraping. The cells were lysed in lysis buffer and then extracted using 500μL of NeutrAvidin agarose in a spin column. Proteins were separated by SDS-PAGE followed by silver staining (GE Healthcare). Gel bands were excised, reduced, and alkylated with DTT and iodoacetamide in 50 mM ammonium bicarbonate. After trypsin digestion, extracted peptides were purified by C18 zip-tips and analysed by reverse phase nano-liquid chromatography mass spectrometry (Mass Spectrometry Proteomics Services, The University of Hong Kong). Data obtained were searched against SWISSPROT and NCBI by MASCOT (Matrix Science Ltd) using the Refseq human sequence database. A probability-based MOWSE score was used to identify and screen proteins exceeding the threshold ($p<0.05$) with a minimum of two peptide matches.

Treatment of endometrial epithelial cells with steroids, steroid receptor antagonists, and siRNA

Ishikawa cells expressing oestrogen receptor (ER) and progesterone receptor (PR) were used in this part of the study. The cells were treated with 5% charcoal-dextran stripped FBS (csFBS, Hyclone) in phenol red-free MEM for 24 hours before treatment with oestrogen (0.01-100 nM; Sigma E2758-1G), progesterone (0.01-1 μM; Sigma P7873-5G), or combined oestrogen and progesterone. After 24 hours of treatment, total membrane proteins were extracted (ProteoExtract-Native Membrane Protein Extraction Kit, Calbiochem 444810) and PDIA1 expression was determined by Western blotting. For inhibitor studies, Ishikawa cells were cultured as described above and treated with oestrogen receptor alpha antagonist (MPP), oestrogen receptor beta antagonist (PHTPP), or progesterone receptor antagonist (RU486) with or without oestrogen (10 nM) or progesterone (0.1 μM) for 24 hours. Total and membrane proteins were extracted and PDIA1 expressions were determined by Western blotting. Ishikawa cells were also treated with ERalpha siRNA (ESR1 siRNA, Dharmacon J003401-11 & J003401-13) or non-target siRNA

alone or with oestrogen for 24 hours, and PDIA1 expression levels were then detected by Western blotting.

Isolation and culture of human endometrial epithelial cells from tissue samples

Human primary endometrial epithelial cells were isolated as described previously (Li et al., 2017). Briefly, endometrial tissues were minced and suspended in 0.5 mg/mL Type IA collagenase (Sigma) and 150 μg/mL deoxyribonuclease I (Worthington) in DMEM/F12 containing 1% BSA, and digested in a shaking water bath at 37°C for 1 hour. The mixture was filtered through a 100 μM strainer, and cells were collected by centrifugation at 600 x g for 5 minutes. The cells were resuspended in DMEM-F12 medium with 10% FBS and filtered through a 40 μM strainer, and then centrifuged at 600 x g for 5 minutes. Cells were resuspended in DMEM/F12 complete medium and seeded in 48-well plated coated with BD Matrigel. Cells were cultured in phenol red-free DMEM/F12 medium with 10% csFBS and 500 pM 17β-oestradiol and 40 nM progesterone. The medium was changed every 2 days. Cells at 80%-90% confluence were treated with 1 mM Tris (2 carboxyethyl) phosphine (TCEP) for 24 hours before the co-culture assay.

Cell transfection

One day before transfection, Ishikawa cells were seeded in 12-well plates at $1x10^5$cells per well or AN3CA cells were seeded at $3x10^5$cells per well. Transfection was performed using lipofectamine 2000 (Thermo Fisher 11668-019) when cells were at 70% confluence. The culture medium was replaced with 900 μL of Opti-MEM (Thermo Fisher 31985-070) before the transfection. Briefly, hPDIA1-pCMV-FLAG (1 μg, Addgene 31384) and pCMV1 control plasmids (1 μg), 25 nM siRNA (ESR1-Dharmacon J003401-11 and J003401-13, PDIA1-Dharmacon J003690-05-0002 and J003690-06-0002, and non-target siRNA) were diluted in 50 μL of Opti-MEM and mixed with 3 μL of lipofectamine in 50 μL of Opti-MEM. After incubation at room temperature for 20 minutes, 100 μL of the transfection mixture was added dropwise to Ishikawa or AN3CA cells. After 6 hours, the medium containing lipofectamine was replaced with fresh culture medium and the transfected cells were further incubated for 48 hours before the Western blot analysis, immunofluorescence staining, and co-culture assay.

Trophoblast spheroid-endometrial cell co-culture assay

The attachment of trophoblast (Jeg-3) spheroids onto endometrial epithelial cells was quantified by a spheroid-endometrial cell co-culture assay, as described previously (Hohn et al., 2000; Kodithuwakku et al., 2010; Kottawatta et al., 2015; So et al., 2015) with modifications. Briefly, multicellular spheroids were generated from trypsinized Jeg-3 cells by shaking at 4 x g overnight. Spheroids with a diameter of 60-200 µm were transferred onto a confluent monolayer of Ishikawa or AN3CA cells with or without prior treatments, and co-cultured for 1 hour at 37°C in a 5% CO_2 and humidified environment. Unattached spheroids were removed by shaking at 8 x g for 10 minutes. Attached spheroids were counted under a light microscope and expressed as a percentage of the total number of spheroids used (% adhesion). For hormone studies, Ishikawa cells were treated with oestrogen (10 nM), progesterone (0.1 µM), or mid-luteal phase oestrogen (743 pM) and progesterone (52.6 nM) for 24 hours before the co-culture study. For the control experiments, AN3CA and Ishikawa cells were treated with or without DMSO solvent. For the inhibition studies, AN3CA and the Ishikawa cells were pre-treated with PDI inhibitor 16F16 (1 and 10 µM) for 24 hours before co-culture, or AN3CA cells were transfected with PDIA1 siRNA for 48 hours before co-culture. For the over-expression studies, Ishikawa cells were transfected with hPDIA1-pFLAG-CMV1 plasmid or pCMV1 empty plasmid for 48 hours before co-culture, AN3CA and Ishikawa cells were pre-treated with reducing agent TCEP (0.01, 0.1, and 1 mM) for 24 hours before co-culture, and human primary endometrial epithelial cells (EECs) were treated with TCEP (1 mM) for 24 hours before co-culture.

Extraction of total, membrane, and cytoplasmic proteins

Total cell protein from Ishikawa, RL95-2, AN3CA, and HEC1-B cells were extracted using RIPA buffer containing 1X phosphate-buffered saline (PBS), 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitors. Membrane proteins were extracted using a native membrane protein extraction kit (Calbiochem 444810). Briefly, $3x10^5$ cells were collected, washed two times with 1X Tris-buffered saline (TBS) and centrifuged at 600 x g for 10 minutes after each wash. Cytoplasmic proteins were extracted by adding 2 mL of lysis buffer I with 10 µL of protease inhibitor cocktail. After 15 minutes incubation, the cytoplasmic protein fraction was collected by centrifugation at 16000 x g for 30 minutes without disturbing the pellet. The pellet was mixed with 1000 µL of lysis buffer II and 5 µL of protease inhibitor cocktail for 30 minutes with intermittent

shaking. The extracted protein was centrifuged at 16000 x g for 30 minutes and the supernatant was collected.

Western blotting

The purity of the extracted membrane protein fraction was confirmed by Western blotting for the presence of membrane protein markers E-cadherin, integrin beta 3, and sodium/potassium-ATPase; and for the absence of cytoplasmic markers GAPDH and β-actin. Protein concentration was measured using a Pierce BCA assay (Thermo Fisher 23225). The extracted proteins were denatured and separated by SDS-PAGE and transferred to PVDF membranes and blocked in 5% skimmed milk in PBST for 1 hour. Rabbit monoclonal anti-PDIA1 antibody (1:1000), mouse monoclonal anti-E-cadherin antibody (1:1000), rabbit monoclonal anti-integrin beta 3 antibody (1:1000), or mouse monoclonal anti Sodium/potassium-ATPase antibody (1:250) in blocking buffer was added and incubated overnight at 4°C (supplementary table 1). After washing with PBST, anti-rabbit or anti-mouse secondary antibody conjugated with horse-radish peroxidase (1:5000, GE Healthcare) was added. The amount of protein loaded into each lane was normalized to β-actin expression or total protein by Coomassie blue staining.

Immunofluorescence staining

Treated or untreated Ishikawa and AN3CA cells were fixed in 4% paraformaldehyde for 15 minutes and washed with 1X PBS five times for 5 minutes each. For intracytoplasmic staining, the cells were permeabilized with permeabilization buffer (0.4% Triton-X in PBS) for 15 minutes, and then washed with 1X PBS five times for 5 minutes each. The cells were then blocked with blocking buffer (5% normal serum from the same species as secondary antibody in PBS) for 1 hour. Primary antibodies in blocking solution were added and cells were incubated overnight at 4°C. On the following day, unbound primary antibodies were washed with 1X PBS five times for 5 minutes each. Alexa flour 448- or 555-conjugated anti-mouse or anti-rabbit secondary antibodies in blocking buffer were added and incubated for 1 hour. After washing off unbound antibodies with 1X PBS five times for 5 minutes each, 4’,6-diamidino-2-phenylindole (DAPI) (1:1000) in PBS was added and incubated for 15 minutes. The cells were washed with 1X PBS three times before mounting with fluorescence mounting medium.

Confocal microscopy and Total Internal Reflection Fluorescence (TIRF) microscopy

For cell surface staining, treated or untreated cells were fixed in 2% paraformaldehyde with 120 mM sucrose for 10 minutes. The fixed cells were washed with 1X PBS five times for 5 minutes each. After the fixed cells were blocked in blocking buffer for 1 hour, the same steps as the intracytoplasmic immunofluorescence staining were performed, but without the cell permeabilization step. The fluorescent signals were observed by a Carl Zeiss LSM 700 Laser scanning confocal microscope (Jena) or by a Total Internal Reflection Fluorescence (TIRF) microscope (Zeiss) installed at the Centre for PanorOmic Sciences (CPOS), The University of Hong Kong.

RNA extraction, reverse transcription, and real-time PCR

Total RNA were extracted from human endometrial samples using the MirVANA PARIS kit (Thermo Fisher) following the manufacturer's instructions. TaqMan reverse transcription reagents (TaqMan 2X Universal PCR Master Mix, Life Technologies) were used for the reverse transcription of RNA to cDNA. Real-time PCR was carried out in a QuantStudio™ 5 real-time PCR System. Human PDIA1 assay (Applied Biosystems Hs01050257_m1) TaqMan probe was used to study the PDIA1 expression, and Eukaryotic 18S (Applied Biosystems 4318839) TaqMan probe was used as the internal control. The 2-ΔΔCt method was used to calculate the relative mRNA expressions.

Flow cytometric analysis of cell surface PDIA1

Treated or untreated Ishikawa and AN3CA cells ($2x10^5$) were collected after trypsinization, and then washed with 1X PBS containing 2% serum. After blocking with blocking solution for 30 minutes at 4°C, cells were incubated for 1.5 hours with Alexa Fluor 488-Recombinant Anti-P4HB antibody [EPR9499] at 1:50 dilution. Unstained cells and cells labelled with Recombinant Rabbit IgG and monoclonal [EPR25A]-Isotype Control (Alexa Fluor- 488) were used as the controls. After incubation with primary antibodies, cells were washed twice with 2% serum in 1X PBS and analysed by flow cytometry (CytoFLEX). The percentage of positive cells was analysed by FlowJo software.

Statistical analysis

All results were expressed as means ± SEM. Statistical comparisons were performed by two-tailed t-test or one-way ANOVA with Tukey's post-hoc test using SPSS 20 (IBM) and GraphPad Prism5 (GraphPad Software Inc) where appropriate. A non-parametric analysis test was used when the data were not normally distributed. A probability of $p<0.05$ was used to indicate a significant difference.

Results

Mass spectrometry detection of PDIA1 proteins in the membrane fraction of endometrial epithelial cells

We first investigated the expression of membrane proteins in receptive Ishikawa and non-receptive AN3CA cells. The labelled membrane protein fractions were separated by SDS-PAGE and visualized by silver staining (Supporting Info. Figure 1A). A band at around 55kDa that was highly up-regulated in non-receptive AN3CA cells was subjected to mass spectrometry analysis. The protein with protein score of 287 was identified as chain A of human Protein Disulphide Isomerase (PDIA1) or prolyl 4-hydroxylase subunit beta (P4HB).

Total, cytoplasmic, and membrane protein expressions in Ishikawa, RL95-2, AN3CA, and HEC1-B cells

We first isolated total, membrane, and cytoplasmic protein fractions from endometrial Ishikawa cells and measured the enrichment of membrane (E-cadherin, Na/K-ATPase and integrin β3) and cytoplasmic (GAPDH and β-actin) proteins (Suppl. Figure 1B) by Western blotting. Membrane proteins (MP) E-cadherin, Na/K-ATPase and integrin β3 were highly expressed in the membrane fraction, whereas GAPDH and β-actin proteins were highly enriched in the cytoplasmic fraction. Next, we studied the expression of PDIA1 proteins in Ishikawa, RL95-2, AN3CA and HEC1-B cells. Total PDIA1 protein was significantly lower in HEC1-B cells than in the other three cell lines (Figure 1A). Interestingly, a much higher expression of PDIA1 protein was found in the membrane fraction of AN3CA cells compared to the other cell lines (Figure 1B). Immunofluorescence staining of permeabilized Ishikawa and AN3CA cells did not reveal any differences in PDIA1 expression (Figure 1C). We analysed cell surface protein expressions by TIRF microscopy, which can detect minimal intracellular fluorescence signals (Mattheyses et al., 2010). The expression of PDIA1 protein on the cell surface of non-permeabilized AN3CA cells was much higher than in Ishikawa cells. E-Cadherin membrane protein expression was detected in receptive endometrial Ishikawa, but not in non-receptive AN3CA cells (Figure 1D). Furthermore, flow cytometric analysis of live non-permeabilized Ishikawa and AN3CA cells showed a significantly higher percentage of PDIA1 expression in AN3CA cells than in Ishikawa cells (77.8±11.2% in AN3CA vs. 39.8±7.3% in Ishikawa cells, $p<0.05$) (Figure 1E).

Oestrogen and progesterone action in the regulation of PDIA1 expression

Ishikawa cells were used to study the effect of oestrogen and progesterone on PDIA1 expression. Oestrogen (0.1-100 nM) treatment for 24 hours significantly up-regulated total and membrane PDIA1 protein expressions compared to the vehicle control (Figure 2A). No significant change in β-actin protein expression was observed. Progesterone (0.1–1 μM) treatment for 24 hours significantly down-regulated total and membrane PDIA1 protein expressions (Figure 2B). We used hormone levels in the mid-secretory phase of the cycle to study the combined effects of physiological doses of oestrogen and progesterone (E2+P4) in the receptive endometrium. Ishikawa cells were treated with oestrogen (743 pM) and progesterone (52.6 nM) for 24 hours. Western blot analysis showed the total PDIA1 protein expression level in the E2+P4 group was similar to the DMSO control group. However, the membrane PDIA1 protein expression level was significantly down-regulated ($p<0.05$), and cytoplasmic PDIA1 protein expression was significantly up-regulated (Figure 2C). The protein loading was normalized by β-actin expression or by Coomassie blue protein staining. Total internal reflection microscopy was used to analyse surface PDI expression from non-permeabilized Ishikawa cells treated with 10 nM oestrogen, 0.1 μM progesterone, or luteal phase E2+P4 (Figure 2D). A higher fluorescent signal was found on the cell surface of non-permeabilized oestrogen-treated Ishikawa cells compared to the vehicle control, whereas a lower fluorescent signal was found in progesterone-treated and E2+P4-treated Ishikawa cells, suggesting lower membrane PDIA1 expressions with progesterone treatment. Furthermore, flow cytometric analysis of PDIA1 expressions in Ishikawa cells treated with oestrogen (10 nM) showed significantly higher surface PDIA1 expression (72.2±6.5%) compared to the solvent control (38.4±7.7%, 0.1% ethanol, Figure 2E). In addition, the percentage of cells expressing surface PDIA1 was significantly reduced with progesterone (0.1 μM, 13.8±4.3%) or oestrogen plus progesterone (E2+P4, 9.0±3.4%) compared to the solvent control (Figure 2E). We further studied the effect of 10 nM oestrogen, 0.1 μM progesterone, and E2+P4 on spheroid attachment on Ishikawa cells. Oestrogen (10 nM), but not progesterone or E2+P4, resulted in significantly reduced spheroid attachment on Ishikawa cells (Figure 2F).

Oestrogen receptor alpha and progesterone receptors regulate steroid-mediated PDIA1 expression

We also studied the expression of oestrogen receptor alpha (ESR1), oestrogen receptor beta (ESR2), and progesterone receptor (PR) in the four endometrial epithelial cell lines. Ishikawa and RL95-2 cells expressed ESR1 and PR, whereas all four cell lines expressed ESR2 (Figure 3A). No significant change in PDIA1 expression was observed in Ishikawa cells treated with oestrogen receptor alpha antagonist MPP and oestrogen when compared to cells treated with oestrogen alone (Figure 3B). On the other hand, up-regulation of PDIA1 was observed in Ishikawa cells treated with oestrogen receptor beta antagonist PHTPP and oestrogen compared to cells treated with PHTPP alone. To further evaluate the role of oestrogen receptor alpha on PDIA1 expression, we knocked down ER alpha with siRNA and treated the cells with oestrogen. Similar to the ER alpha antagonist, ER alpha knockdown did not change PDIA1 expression in cells treated with 10 nM oestrogen compared with cells treated with non-target siRNA and oestrogen (Figure 3C). Similarly, we used progesterone receptor antagonist RU486 (10 μM) to study the regulation of PDIA1 expression by a progesterone receptor-mediated signalling pathway. We found RU486, but not solvent control (0.1% ethanol), nullified the down-regulation of PDIA1 expression by progesterone in Ishikawa cells (Figure 3D). In addition, putative Oestrogen Responsive Elements (ERE) and Progesterone Responsive Elements (PRE) were found on the promoter region of PDIA1 (Supportive Infor Figure 2A-C) detected in the Eukaryotic Promoter Database (https://epd.epfl.ch//index.php).

Modulation of PDIA1 expression affects spheroid attachment on endometrial epithelial cells

We studied if PDIA1 inhibitor 16F16, strong reducing agent TCEP, or PDIA1 siRNA could enhance the receptivity of non-receptive endometrial AN3CA cells for spheroid attachment. Treatment with 16F16 (1 and 10 μM) for 24 hours in AN3CA cells significantly enhanced Jeg-3 spheroid attachment (Figure 4A). The viability (90%-95% viable) of AN3CA cells was determined by trypan blue staining (data not shown) and was comparable to that of the control. To investigate if a reducing environment in endometrial cells enhances endometrial receptivity, we treated AN3CA cells with TCEP (0.01 -1 mM) for 24 hours, which resulted in a significant increase in spheroid attachment at 1 mM TCEP compared to the vehicle control (Figure 4B). Treatment of AN3CA cells with DNA methylation inhibitor 5-Aza-2'-deoxycytidine (AZA, 20 μM) for 48

hours significantly increased the spheroid attachment rate from 26% to 60% compared to the vehicle control. On the other hand, AN3CA cells with knockdown of PDIA1 by siRNA decreased PDIA1 expression (Figure 4D), but increased the spheroid attachment rate from 35.2% to 49.6% ($p<0.05$) (Figure 4C).

Receptive Ishikawa cells treated with 16F16 (1 μM) for 24 hours did not significantly change Jeg-3 spheroid attachment after 1 hour of co-culture (Figure 4E). Treatment with 16F16 (1 μM) did not affect the cell viability of Ishikawa cells (data not shown). Treatment of receptive Ishikawa cells with TCEP (0.01-1 mM) did not significantly change Jeg-3 spheroid attachment after 1 hour of co-culture (Figure 4F). To determine if hPDIA1 over-expression would increase the total PDIA1 expression and reduce spheroid attachment in vitro, Ishikawa cells were transfected with pCMV1-hPDIA1-FLAG over-expressing plasmid (Figure 4G). Co-culture assay showed the over-expression of PDIA1 lowered the spheroid attachment rate from 88% to 62.4% compared with the control (Figure 4H). To study whether TCEP could nullify the suppressive effect of PDIA1 over-expression, Ishikawa cells transfected with PDIA1 over-expressing vector were treated with 1 mM TCEP for 24 hours. Co-culture assay showed that TCEP treatment partially nullified the suppressive effect of PDIA1 over-expression in transfected Ishikawa cells (Figure 4I). The Wnt-pathway inhibitor PRI 724 (10 μM) was used as a positive control to suppress spheroid attachment in Ishikawa cells ($p<0.05$).

Effects of PDIA1 over-expression on E-Cadherin, Integrin beta 3, and Integrin alpha V expression in transfected Ishikawa cells

We next investigated whether the over-expression of PDIA1 affected endometrial receptivity through modulation of endometrial receptivity markers such as E-cadherin, integrin beta 3, and integrin αV (Kakar-Bhanot et al., 2018). Ishikawa cells were transfected with PDIA1 expression vector, and expression of membrane PDIA1 was determined by immunofluorescent staining (Figure 5A). Over-expression PDIA1 total protein and membrane proteins significantly reduced total and membrane expression of integrin beta 3, but not E-cadherin or integrin alpha V (Figure 5B and C), whereas β-actin protein was absent in the membrane fraction of the transfected cells.

PDIA1 expression at proliferative and secretory phases of the human menstrual cycle

To investigate if PDIA1 plays a role in endometrial receptivity, we measured PDIA1 mRNA and protein expressions in human endometrial samples collected throughout the menstrual cycle. The expression of PDIA1 transcripts in the endometrial aspirates remained stable throughout the menstrual cycle (Figure 6A). However, the immunohistochemical staining suggested a stronger PDIA1 signal in luminal and glandular epithelium of human endometrium (Figure 6B). H-scoring showed that the expression of PDIA1 protein was significantly higher in luminal epithelium in the proliferative phase compared to the secretory phase of the cycle. Such changes were not observed in glandular epithelium throughout the menstrual cycle (Figure 6B). In human primary endometrial epithelial cell cultures, treatment with the reducing agent TCEP (1 mM) for 24 hours enhanced Jeg-3 spheroid attachment compared to untreated controls (Figure 6C).

Discussion

In this study, we found membrane PDIA1 was highly expressed in non-receptive AN3CA endometrial epithelial cell lines and was involved in the regulation of spheroid attachment. Inhibitors of PDIA1 (bacitracin and 16F16) or PDIA1 siRNA treatment in non-receptive AN3CA cells increased spheroid attachment, whereas over-expression of hPDIA1 in receptive Ishikawa cells decreased spheroid attachment. In Ishikawa cells, PDIA1 transcript was up-regulated by oestrogen but down-regulated by progesterone, whereas physiological oestrogen plus progesterone (E2+P4) did not affect total PDIA1 expression but suppressed membrane PDIA1 expression. The reducing agent TCEP enhanced spheroid attachment in non-receptive AN3CA cells, whereas TCEP in receptive Ishikawa cells partially nullified the suppressive effects of PDIA1 over-expression on spheroid attachment. Over-expression of PDIA1 was associated with a lower expression of membrane and total integrin β3, but not integrin αV and E-cadherin proteins. In human endometrial tissue samples, the expression of PDIA1 transcript remained stable throughout the menstrual cycle, but the expression of PDIA1 protein in luminal epithelial cells was higher in the proliferative phase than in the secretory phase. Addition of TCEP enhanced spheroid attachment onto human primary endometrial epithelial cells, suggesting a reducing environment could abolish membrane PDIA1 function and favour embryo implantation in vivo.

Recent transcriptomic and proteomic studies have identified various signature molecules in the human receptive endometrium. A microarray approach identified a signature of 238 transcripts that could predict endometrial receptivity (Díaz-Gimeno et al., 2011). However, the detailed molecular mechanism of how the transcripts affect embryo implantation is still far from clear. We therefore investigated if endometrial membrane proteins could be used as candidate marker(s) for the prediction of endometrial receptivity. Mass spectrometry analysis of the labelled membrane proteins from endometrial cell lines identified PDIA1 as a candidate protein that was highly expressed in non-receptive AN3CA cells compared with receptive Ishikawa cells. Although PDI proteins have been identified in receptive endometrial RL95-2 cells by a proteomic approach (Bhagwat et al., 2014), results from this study suggested that low membrane PDIA1 protein expression was found in receptive RL95-2 cells when compared to non-receptive AN3CA cells.

We further investigated the expression of PDIA1 in four human endometrial epithelial cell lines. Ishikawa and RL95-2 cells are moderately differentiated endometrial epithelial adenocarcinoma cell lines that are receptive to spheroids (blastocyst surrogates), whereas AN3CA and HEC1-B endometrial epithelial cells have poor adhesiveness resembling a non-receptive endometrium (Hannan et al., 2010). Interestingly, we observed a higher membrane PDIA1 expression in AN3CA cells compared to Ishikawa, RL95-2, and HEC1-B cells. Although a lower membrane PDIA1 expression was found in another non-receptive HEC1-B cell, the total PDIA1 expression in this cell line is also low. It is possible that the ratio of membrane to total PDIA1 proteins may play a role in regulating endometrial receptivity, and the expression of steroid receptors (estrogen and progesterone receptors) and downstream signaling and receptivity molecules would also regulate receptivity of the endometrial cells (Fernando et al., 2021).

Endometrial receptivity is tightly regulated by ovarian steroids (oestrogen and progesterone) that are secreted in a cyclical manner (Teklenburg & Macklon, 2009; Vasquez & Demayo, 2013; Hernández-Vargas et al., 2020). Oestrogen stimulates the growth of the endometrium in the proliferative phase, but its effects are diminished in the secretory phase, which is more dominated by progesterone. High progesterone levels in the secretory phase inhibit epithelial cell proliferation and stimulate decidualization (Wetendorf & Demayo, 2012). In this study, we found oestrogen stimulated total PDIA1 expression in Ishikawa cells, whereas progesterone suppressed total PDIA1 expression. In line with this, oestrogen (0.1 and 10 μM) treatment of bovine aortic endothelial cells was shown to up-regulate PDI (Ejima et al., 1999), and Agr2, a putative PDI gene on breast cancer epithelial cell proliferation and lobuloalveolar development in the mammary gland (Verma et al., 2012). In our study, we found Ishikawa cells treated with mid-luteal phase levels of oestrogen and progesterone (E2+P4) did not affect total protein expression, but reduced membrane and increased cytoplasmic PDIA1 expressions. This regulation could be modulated by putative ERE and PRE elements in the promoter region of the PDIA1 gene.

We used human endometrial and trophoblastic cell lines in the co-culture assay (Hannan et al., 2010; Kakar-Bhanot et al., 2018; Kodithuwakku et al., 2010; Kodithuwakku et al., 2012; Kottawatta et al., 2015; So et al., 2015) to understand the function of PDIA1 on embryo implantation. In this study, receptive Ishikawa cells showed higher Jeg-3 spheroid attachment than

non-receptive AN3CA cells. Addition of PDI inhibitor bacitracin or 16F16 in AN3CA cells increased spheroid attachment. Bacitracin is a non-specific and cell impermeable PDI inhibitor that acts by binding to the functional site of PDI (Dickerhof et al., 2011; Jasuja et al., 2012), whereas 16F16 is a small irreversible PDI inhibitor molecule that can permeate the cell and inhibit the function of both membrane and cytoplasmic PDI (Ge et al., 2013; Kaplan et al., 2015). Similarly, knockdown of PDIA1 in AN3CA increased the attachment of trophoblast spheroids in the co-culture assay. Conversely, over-expression of PDIA1 proteins in Ishikawa cells reduced Jeg-3 spheroid attachment, demonstrating a functional role of membrane PDIA1 expression on endometrial receptivity and spheroid attachment. In fact, over-expression of PDIA1 in Ishikawa cells down-regulated the expression of receptivity marker integrin β3. The down-regulation of integrin β3 reduced embryo implantation (Bronson & Fusi, 1996), and integrin β3 antibody reduced Jeg-3 spheroid attachment on Ishikawa cells (Kakar-Bhanot et al., 2018). Clinically, women with repeated implantation failure have aberrant expressions of integrins including integrin β3 (Germeyer et al., 2014). Thus, the association of membrane PDIA1 expression and down-regulation of integrin β3 needs to be explored in relation to the receptivity of women undergoing assisted reproduction treatment.

The PDI family consists of 21 members, with PDIA1 as the founding member. The PDI proteins function as chaperones and in disulphide bond oxidation, reduction, and isomerization (Ellgaard & Ruddock, 2005). Early studies on PDIA1 in endothelial cells showed it was associated with thrombus formation (Kim et al., 2013; Manickam et al., 2008) and vascular remodelling (Araujo et al., 2017a; Tanaka et al., 2016). We also found that a reducing microenvironment increased spheroid attachment on endometrial epithelial cells. Indeed, embryo implantation was reported to be favoured in a reduced microenvironment (Nivsarkar et al., 2001). The underlying molecular mechanism of how PDIA1 suppresses spheroid attachment remains largely unknown. However, PDIA1 is a thioredoxin family protein that promotes thiol-disulphide exchange (Darby & Creighton, 1995; Xu et al., 2014), and the presence of high levels of membrane PDIA1 may create a disulphide-rich environment that is non-conducive to embryo implantation.

In human endometrial aspirates, although the expression of PDIA1 transcripts largely remained the same throughout the menstrual cycle, PDIA1 protein expression was higher in the apical region

of endometrial luminal epithelial cells in the proliferative phase compared to the secretory phase of menstrual cycle, suggesting differential expressions of membrane proteins irrespective of the PDIA1 transcript level. Also, apical expression of other endometrial proteins also play important roles for embryo implantation (Paule et al., 2021). How these proteins interact with PDIA1 and contributed to increased endometrial receptivity warrants further investigations. In sum, the current study mainly focused on the findings from in vitro experiments, future studies would use in vivo and primary endometrial sample to address the role of PDIA1 on predicting pregnancy outcome in IVF setting. Recent studies suggested that PDI could be transported from and into the cells through various pathway (Araujo et al., 2017b), the mechanism on how PDI transport needs further investigations.

Conclusion

Our study demonstrated that membrane PDIA1 protein regulates endometrial receptivity, and that down-regulated PDIA1 protein expression or a reducing microenvironment favours embryo implantation. Further studies are needed to elucidate the underlying molecular mechanism of how PDIA1 and other PDI isoforms modulate integrins and other protein expressions to regulate endometrial receptivity, and the use of PDIA1 protein expression to predict pregnancy outcome in a large cohort of IVF patients.

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Author's roles

S.R.F., C.L.L., and K.F.L. participated in the study design, conducted experiments, analysis, manuscript drafting, and revision. Y.L.L., E.H.Y.N., and W.S.B.Y. participated in the study design, data interpretation, and manuscript revision. B.P.C.W. and K.W.C. participated in the experiments and manuscript revision. All authors agreed the final approval of the manuscript for publication.

548

549 **Conflict of interest**

550 None to declare.

551

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727

728 **Figure Legend**

729 **Figure 1 Expression of total and membrane PDIA1 proteins in human endometrial**
730 **epithelial cell lines.** (A) Western blot demonstrating higher expression of total PDIA1 protein in
731 Ishikawa, RL95-2, and AN3CA cells, but lower expression in HEC1-B cells. (B) Western blot of
732 membrane protein fractions of Ishikawa, RL95-2, AN3CA, and HEC1-B cells shows significantly
733 higher PDIA1 expression in AN3CA cells compared to the other three cell lines. The membrane
734 protein fractions were free from β-actin. Protein loading was normalized to total protein
735 determined by Coomassie blue staining. Semi-quantitative analysis of the expressed proteins are
736 shown on the bottom. a-b denotes significant differences between groups at $p<0.05$ (N=5). (C)
737 Immunofluorescence staining of permeabilized Ishikawa and AN3CA cells show similar
738 intracytoplasmic PDIA1 expression (green). The nuclei were counterstained with DAPI (blue).
739 Scale bar = 20 μm. (D) Total Internal Reflection Fluorescence microscopy images of non-
740 permeabilized Ishikawa and AN3CA cells show a higher PDIA1 fluorescence signals (red) on cell
741 membranes of AN3CA cells compared to Ishikawa cells. Scale bar = 10 μm. E-Cadherin was used
742 as a marker for cell membrane protein expression. (E) Flow cytometric analysis of cell surface
743 PDIA1 expression in Ishikawa and AN3CA cells. Isotype antibody was used as negative control
744 in the flow cytometric analysis. There was a significantly higher ($p<0.05$) expression of PDIA1 on
745 the cell surface of AN3CA cells (77.8±11.2%) compared to Ishikawa cells (39.8±7.3%). N=6.
746

747 **Figure 2 Effect of oestrogen and progesterone on the expression of PDIA1 in human**
748 **endometrial epithelial Ishikawa cells.** (A) Western blot images show the up-regulation of PDIA1
749 total protein (TP) and membrane protein (MP) in oestrogen-treated (0.01 – 100 nM) Ishikawa cells
750 (left). Semi-quantitative analysis of Western blot images show a significant up-regulation of
751 PDIA1 TP (middle) and MP (right). (B) Western blot images show down-regulation of PDIA1 TP
752 and MP in progesterone-treated (0.01 – 1 μM) Ishikawa cells (left). Semi-quantitative analysis of
753 Western blot images show a significant down-regulation of PDIA1 TP (middle) and MP (right). a-
754 b denote significant difference between groups at $p<0.05$ (N=4). (C) Western blot images show no
755 significant changes in PDIA1 TP in Ishikawa cells treated with physiological E2+P4 (E2 = 743
756 pM, P4 = 52.6 nM). However, the PDIA1 MP was significantly down-regulated by E2+P4. The
757 PDIA1 cytoplasmic protein (CP) was significantly up-regulated by E2+P4 treatment. The semi-
758 quantitative analysis of Western blot images is shown on the right. * denotes significant difference

759 between groups at $p<0.05$, N=4. (D) Total Internal Reflection Fluorescence Microscopy images of
760 non-permeabilized Ishikawa cells treated with 10 nM oestrogen show higher membrane
761 localization of PDIA1 (red), and co-localization with membrane marker Na/K-ATPase (green).
762 Non-permeabilized Ishikawa cells with 0.1 μM progesterone treatment showed lower membrane
763 localization of PDIA1 protein. Oestrogen and progesterone at physiological doses had lowered
764 expression of PDIA1 membrane protein. Scale bar = 10 μm. (E) Flow cytometric analysis of cell
765 surface PDIA1 in oestrogen (10 nM)-treated Ishikawa cells showed significantly higher
766 percentages of PDIA1 positive cells compared to ethanol-treated cells. Significantly lower
767 percentages of surface PDIA1 positive cells was observed in progesterone (0.1 μM) and E2+P4
768 (E2 = 743 pM, P4 = 52.6 nM)-treated Ishikawa cells compared to 0.1% ethanol treated control
769 (N=5). (F) Jeg-3 spheroid attachment was reduced on Ishikawa cells treated with oestrogen (10
770 nM). However, Ishikawa cells treated with progesterone (0.1 μM) or oestrogen plus progesterone
771 showed no change in Jeg-3 attachment. PRI-724 at 10 μM was used as a positive control for the
772 co-culture assay. a, b and c denote significant differences between groups at $p<0.05$.
773
774 **Figure 3 Role of steroid receptors and their antagonist on PDIA1 expression in Ishikawa**
775 **cells.** (A) Ishikawa and RL95-2 cells, but not HEC1-B and AN3CA cells, expressed oestrogen
776 receptor alpha and progesterone receptors. All four cell lines expressed oestrogen receptor beta.
777 (B) Oestrogen treatment in Ishikawa cells induced PDIA1 expression that was nullified by ERα
778 antagonist MPP (10 nM) but not ERβ antagonist PHTPP (10 nM). (C) ERα siRNA, but not non-
779 target siRNA, suppressed ERα protein expression and nullified oestrogen (10 nM)-induced PDIA1
780 total protein (TP) expression in Ishikawa cells. (D) Progesterone (P4, 0.1 μM) suppressed the
781 expression of PDIA1 TP in treated Ishikawa cells. The progesterone receptor antagonist RU486
782 (10 nM), but not control solvent (0.1% ethanol), nullified the suppressive effect of P4 on PDIA1
783 TP expression in Ishikawa cells. The semi-quantitative analysis of Western blot images are shown
784 on the right. a-b denotes significant difference between groups at $p<0.05$, N=4.
785
786 **Figure 4 Effect of PDI inhibitor 16F16 and PDI siRNA on the attachment of Jeg-3**
787 **spheroids on human endometrial epithelial cells.** The attachment rate of Jeg-3 spheroids on non-
788 receptive AN3CA increased with (A) 16F16, and (B) TCEP treatment. 5'-Aza-2-deoxycytidine
789 (AZA, 20 μM) was used as a positive control to increase Jeg-3 spheroid attachment rate on AN3CA

cells (N=6). (C) Knockdown of PDIA1 by PDIA1 siRNA, but not non-target siRNA, significantly increased the attachment rate of Jeg-3 spheroids (N=7). (D) Knockdown of PDIA1 by siRNA in AN3CA cells showed lower PDIA1 protein expression in the Western blot analysis. Protein loading was normalized to β-actin expression. The attachment rate of Jeg-3 spheroids on receptive Ishikawa cells did not change with (E) 16F16 and (F) TCEP treatment. (G) Receptive Ishikawa cells transfected with human PDIA1 over-expressing plasmid pCMV1-hPDIA1 showed a higher level of total PDIA1 protein expression. The exogenous PDIA1 protein was detected with PDIA1 or FLAG antibodies. Protein loading was normalized to β-actin expression. (H) Over-expression of PDIA1 protein in Ishikawa cells suppressed Jeg-3 spheroid attachment when compared to DMSO or pCMV1 control vector. The Wnt-signalling inhibitor, PRI-724, was used as a positive control for suppressing spheroid attachment (N=5). (I) TCEP at 1 mM partly reversed the suppressive effect of PDIA1 over-expression in Ishikawa cells on spheroid attachment. Neither over-expressing pCMV1 with or without TCEP treatment on Ishikawa cells lowered spheroid attachment (N=6). The numbers in the bar indicate the number of attached spheroids over the total number of spheroids added. a, b and c denotes significant difference between groups at $p<0.05$.

Figure 5 Effect of PDIA1 over-expression in receptive human endometrial Ishikawa cells on the expression of implantation-associated markers. (A) Immunofluorescence staining of pCMV1-hPDIA1 transfected non-permeabilized Ishikawa cells showed increased membrane PDIA1 expression (red) compared to the control. The nuclei were counter stained with DAPI (blue). Scale bar 50 μm (N=4). (B) Western blot images of Ishikawa cells transfected with pCMV1-hPDIA1 plasmid show significantly higher PDIA1 total protein (TP) and membrane protein (MP) expressions. Over-expression of PDIA1 in transfected Ishikawa cells down-regulated integrin β3 expression in TP and MP, but not E-Cadherin and integrin αV. (C) Semi-quantitative analysis of Western blot results (right) show a significantly lower integrin β3 expression in PDIA1 over-expressing Ishikawa cells compared to vector control; no changes were seen in E-cadherin and integrin αV. * denotes a significant difference from the control at $p<0.05$ (N=5).

Figure 6 Human endometrial PDIA1 mRNA and protein expression in the menstrual cycle. Human endometrial tissues were collected in the early- and mid-proliferative (EP+MP), late proliferative (LP), early secretory (ES), mid secretory (MS), and late secretory (LS) phases of the

menstrual cycle. (A) PDIA1 mRNA expression at different menstrual stages were detected by RT-PCR. The relative expression of PDIA1 transcript was normalized by 18S expression. No significant changes were found in the different phases of the cycle. (B) Immunohistochemical staining of PDIA1 protein in human endometrial biopsies. PDIA1 proteins (brown staining) were more abundant in the proliferative phase than in the secretory phase (top panel). H-score analysis (bottom panel) showed significantly higher PDIA1 expression at the apical region of luminal epithelium (LE) in the proliferative phase than in the secretory phase (left). However, no significant change was observed in glandular epithelium (GE, right). a-b denote significant differences between groups at $p<0.05$. Scale bar = 20 μm. (C) Human primary endometrial epithelial cells at mid-luteal phase treated with 1 mM TCEP for 24 hours showed a significant increase in Jeg-3 spheroid attachment (N=10). The numbers above the bar indicate the number of attached spheroids over the total number of spheroids added. * denotes significant difference from the PBS control at $p<0.05$.

Supplementary Figure 1 Identification of PDI proteins in human endometrial epithelial cells by mass spectrometry and the purity of extracted membrane and cytoplasmic protein fractions. (A) Silver staining of SDS-PAGE of membrane protein fractions from human endometrial cell lines (Ishikawa and AN3CA). The band marked with a red rectangle was excised and subjected to mass spectrometry analysis and identified as PDIA1. The table shows the peptides identified by mass spectrometry with protein score of 287. (B) Western blotting showed the membrane protein (MP) fraction was enriched with membrane protein markers E-Cadherin, integrin β3, and Na/K-ATPase, but no cytoplasmic markers GAPDH and β-actin. The cytoplasmic protein (CP) fraction was enriched with GAPDH and β-actin, but not the above membrane protein markers.

Supplementary Figure 2 Presence of Oestrogen Responsive Elements (ERE) and Progesterone Responsive Element (PRE) on the promoter region of PDIA1. (A) Putative ERE- and PRE-binding sites on the proximal promoter region of PDIA1 are shown. The corresponding CCAAT and TATA boxes on the PDIA1 promoter are indicated. The putative (B) ERE and (C)

PRE sequences on the PDIA1 promoter were compared to consensus ERE and PRE sequences. Identical sequences are marked with a vertical bar.

Supplementary Figure 3 Photomicrograph of Ishikawa monolayer, JEG-3 spheroids and JEG-3 spheroids on Ishikawa monolayer. Arrowheads show JEG-3 spheroids. Scale bar=200 μM.

Supplementary Table 1 List of antibodies and inhibitors used in the study.

Antibody/Inhibitor	Company (Catalogue no.)	Dilution
Antibodies		
Na/K ATPase	Santa Cruz (sc-21712)	WB 1:500, IF 1:50
E Cadherin	Abcam (ab1416)	WB 1:1000, IF 1:200
Integrin beta3	Abcam (ab119992)	WB 1:1000
GAPDH	Abcam (ab8245)	WB 1:5000
Beta-actin	ProteinTech (66009)	WB 1:5000
PDIA1	Abcam (ab137110)	WB 1:1000, IHC/IF 1:100
Integrin alpha V	Abcam (ab179475)	WB 1:1000
Oestrogen receptor alpha	Abcam (ab16660)	WB 1:1000
Oestrogen receptor beta	Abcam (ab133467)	WB 1:1000
Progesterone receptor	DAKO (M3569)	WB 1:250
Inhibitors		
MPP	Tocris(1991)	--
PHTPP	Tocris (2662)	--
RU486/Mifepristone	Tocris (1479)	--
Bacitracin	Sigma (B0125-50KU)	--
16F16	Sigma (SML0021)	--
5-Aza-2’-deoxycytidine	Sigma (A3656)	--
PRI-724	Abcam (229168)	--
TCEP	Sigma (4706)	--

WB: Western blotting; IHC: Immunohistochemistry staining; IF: Immunofluorescent staining

Figure 1

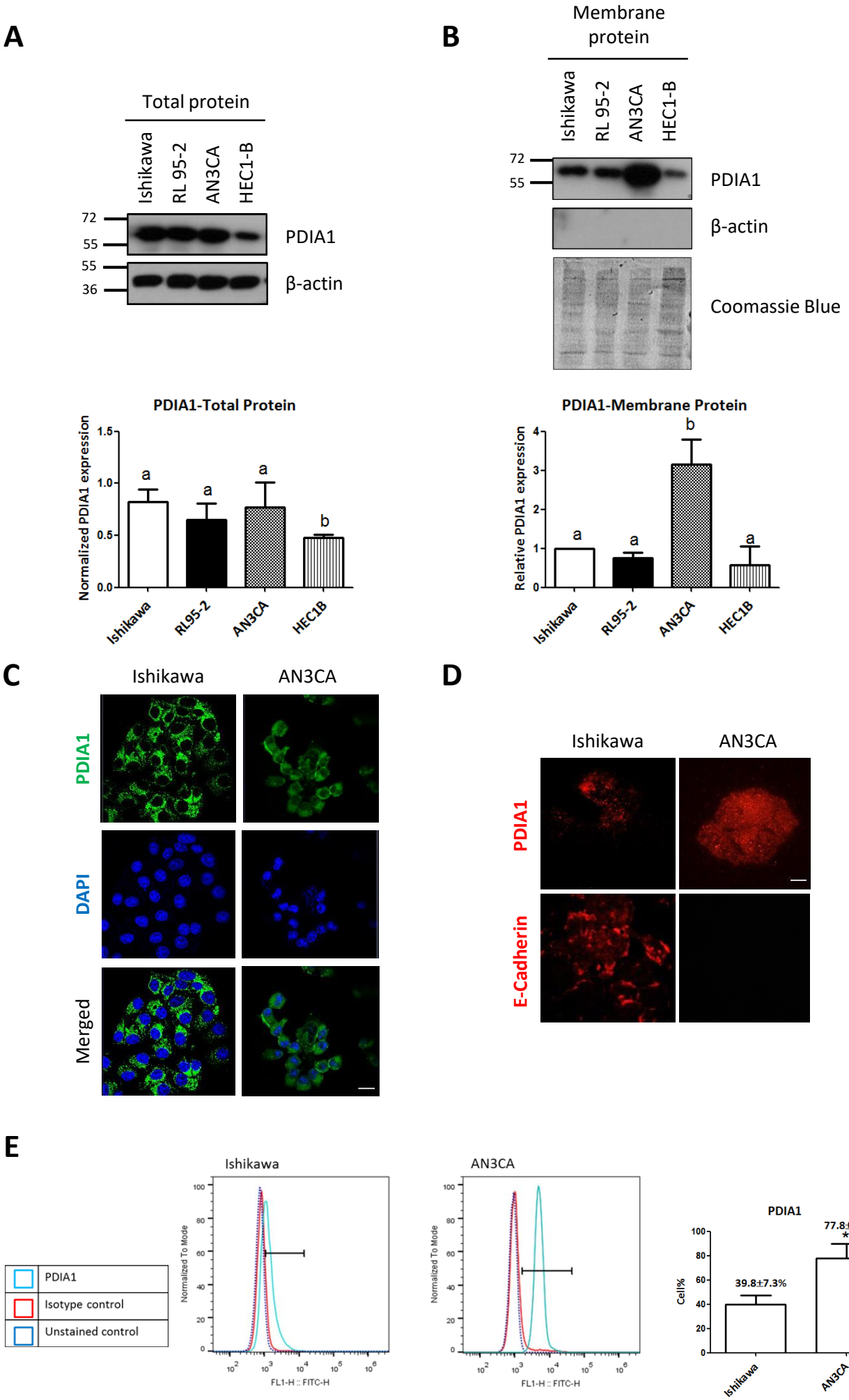
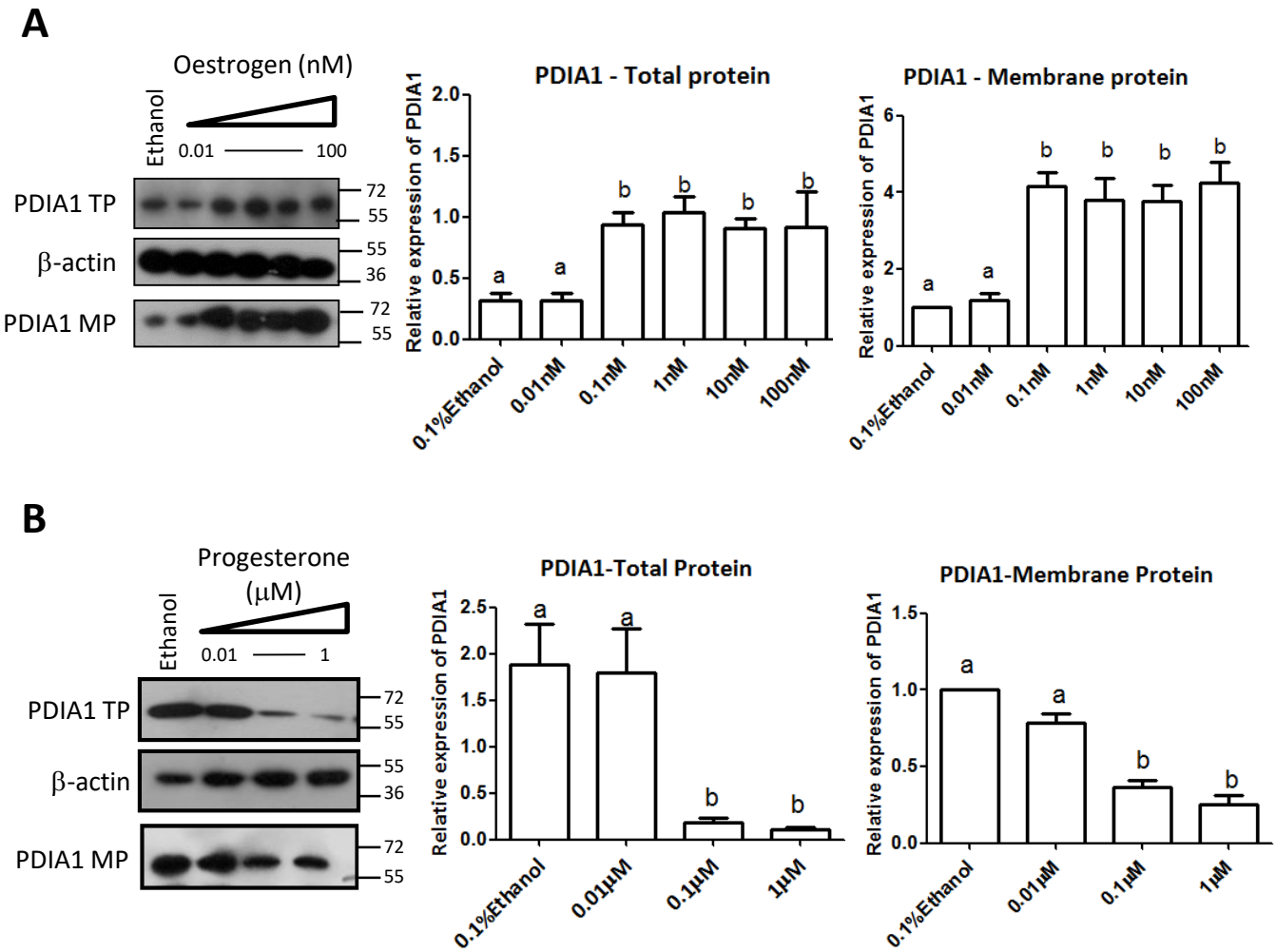

A
Total protein
Ishikawa
RL 95-2
AN3CA
HEC1-B
72
55
55
36
PDIA1
β-actin
B
Membrane protein
Ishikawa
RL 95-2
AN3CA
HEC1-B
72
55
PDIA1
β-actin
Coomassie Blue
PDIA1-Total Protein
Normalized PDIA1 expression
1.5
1.0
0.5
0.0
a
a
a
b
Ishikawa
RL95-2
AN3CA
HEC1B
PDIA1-Membrane Protein
Relative PDIA1 expression
4
3
2
1
0
a
a
b
a
Ishikawa
RL95-2
AN3CA
HEC1B
C
Ishikawa
AN3CA
PDIA1
DAPI
Merged
D
Ishikawa
AN3CA
PDIA1
E-Cadherin
E
Ishikawa
AN3CA
PDIA1
Isotype control
Unstained control
Normalized To Mode
FL1-H :: FITC-H
PDIA1
77.8±11.2%
*
39.8±7.3%
Cell%
Ishikawa
AN3CA

Figure 2


A
Oestrogen (nM)
Ethanol
0.01
100
PDIA1 TP
β-actin
PDIA1 MP
72
55
55
36
72
55
PDIA1 - Total protein
Relative expression of PDIA1
0.1%Ethanol
0.01nM
0.1nM
1nM
10nM
100nM
PDIA1 - Membrane protein
B
Progesterone (μM)
Ethanol
0.01
1
PDIA1-Total Protein
0.1%Ethanol
0.01μM
0.1μM
1μM
PDIA1-Membrane Protein

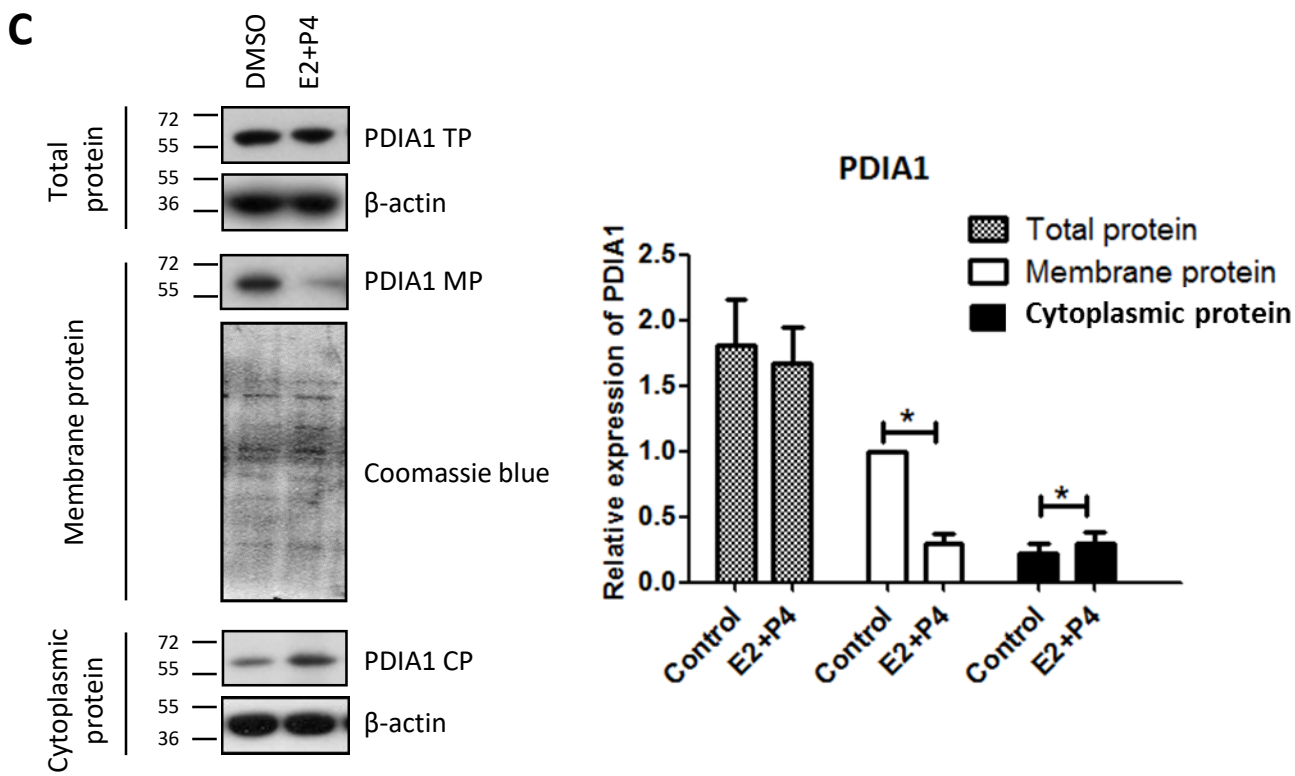
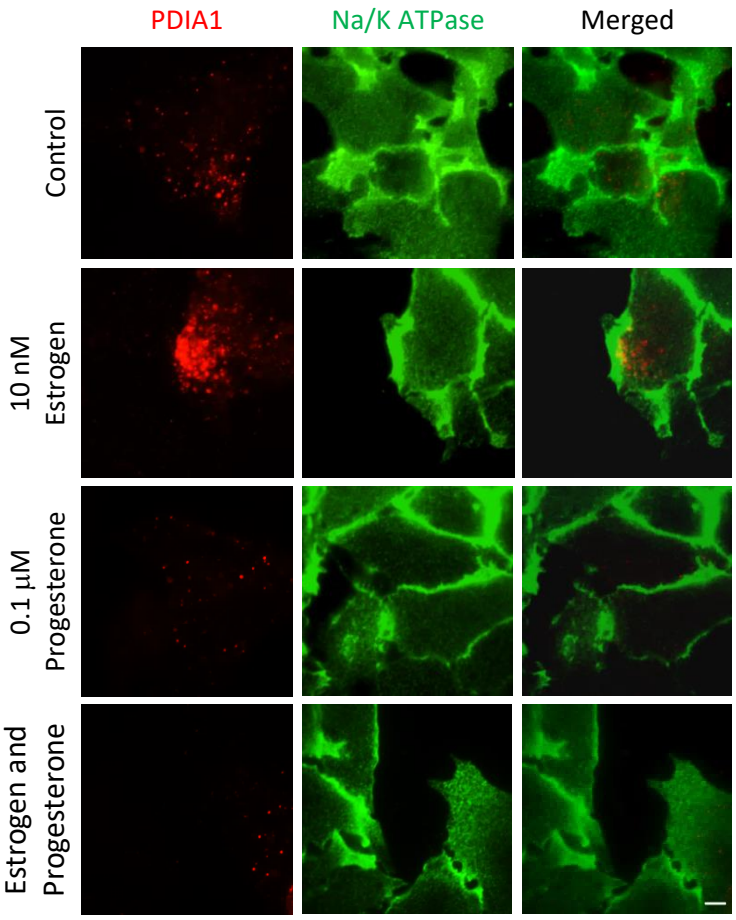

C
DMSO
E2+P4
Total protein
Membrane protein
Cytoplasmic protein
PDIA1 TP
β-actin
PDIA1 MP
Coomassie blue
PDIA1 CP
72
55
55
36
PDIA1
Total protein
Membrane protein
Cytoplasmic protein
Relative expression of PDIA1
Control
E2+P4
*

Figure 2 conti …

D



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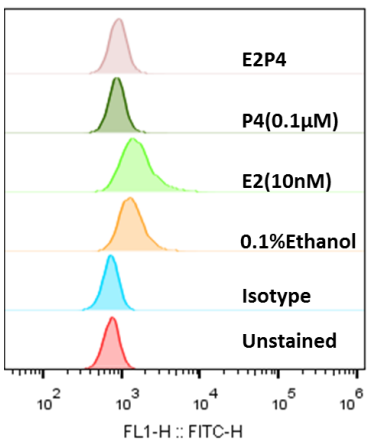

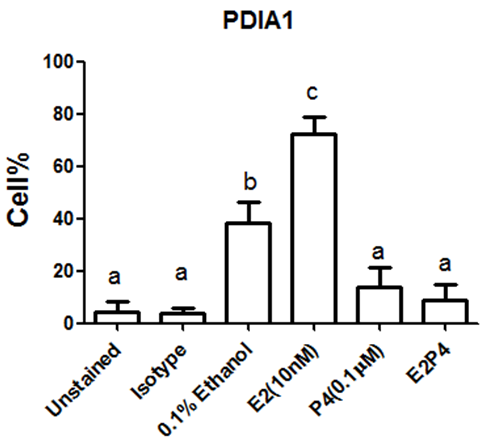

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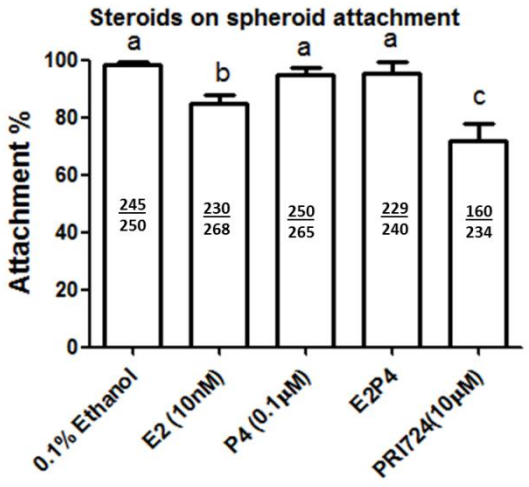


Figure 3

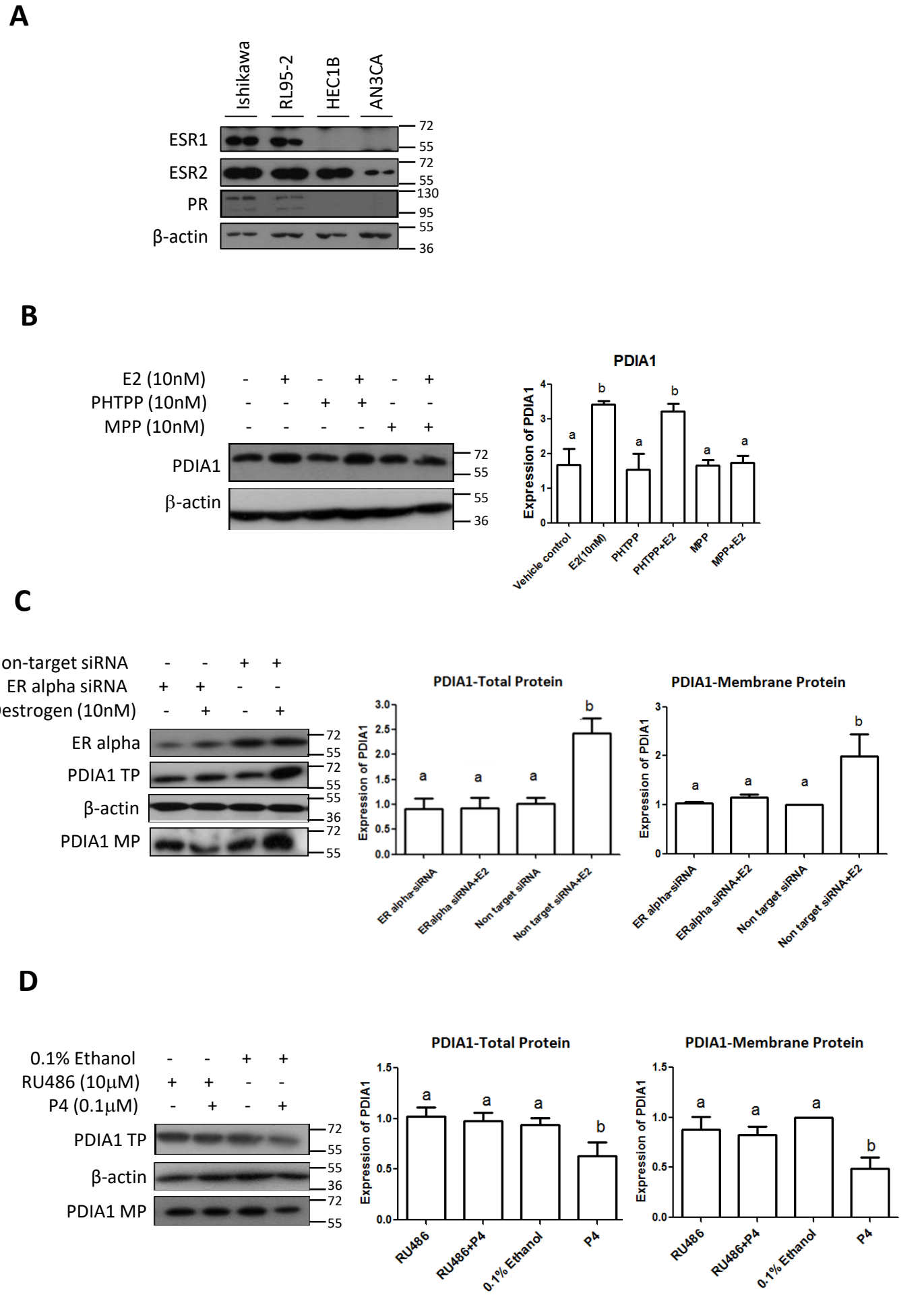

A
Ishikawa
RL95-2
HEC1B
AN3CA
ESR1
ESR2
PR
β-actin
72
55
72
55
130
95
55
36
B
E2 (10nM) - + - + - +
PHTPP (10nM) - - + + - -
MPP (10nM) - - - - + +
PDIA1
β-actin
72
55
55
36
PDIA1
Expression of PDIA1
Vehicle control
E2(10nM)
PHTPP
PHTPP+E2
MPP
MPP+E2
C
Non-target siRNA - - + +
ER alpha siRNA + + - -
Oestrogen (10nM) - + - +
ER alpha
PDIA1 TP
β-actin
PDIA1 MP
PDIA1-Total Protein
PDIA1-Membrane Protein
Expression of PDIA1
ER alpha-siRNA
ERalpha siRNA+E2
Non target siRNA
Non target siRNA+E2
D
0.1% Ethanol - - + +
RU486 (10μM) + + - -
P4 (0.1μM) - + - +
PDIA1 TP
β-actin
PDIA1 MP
PDIA1-Total Protein
PDIA1-Membrane Protein
Expression of PDIA1
RU486
RU486+P4
0.1% Ethanol
P4

Figure 4

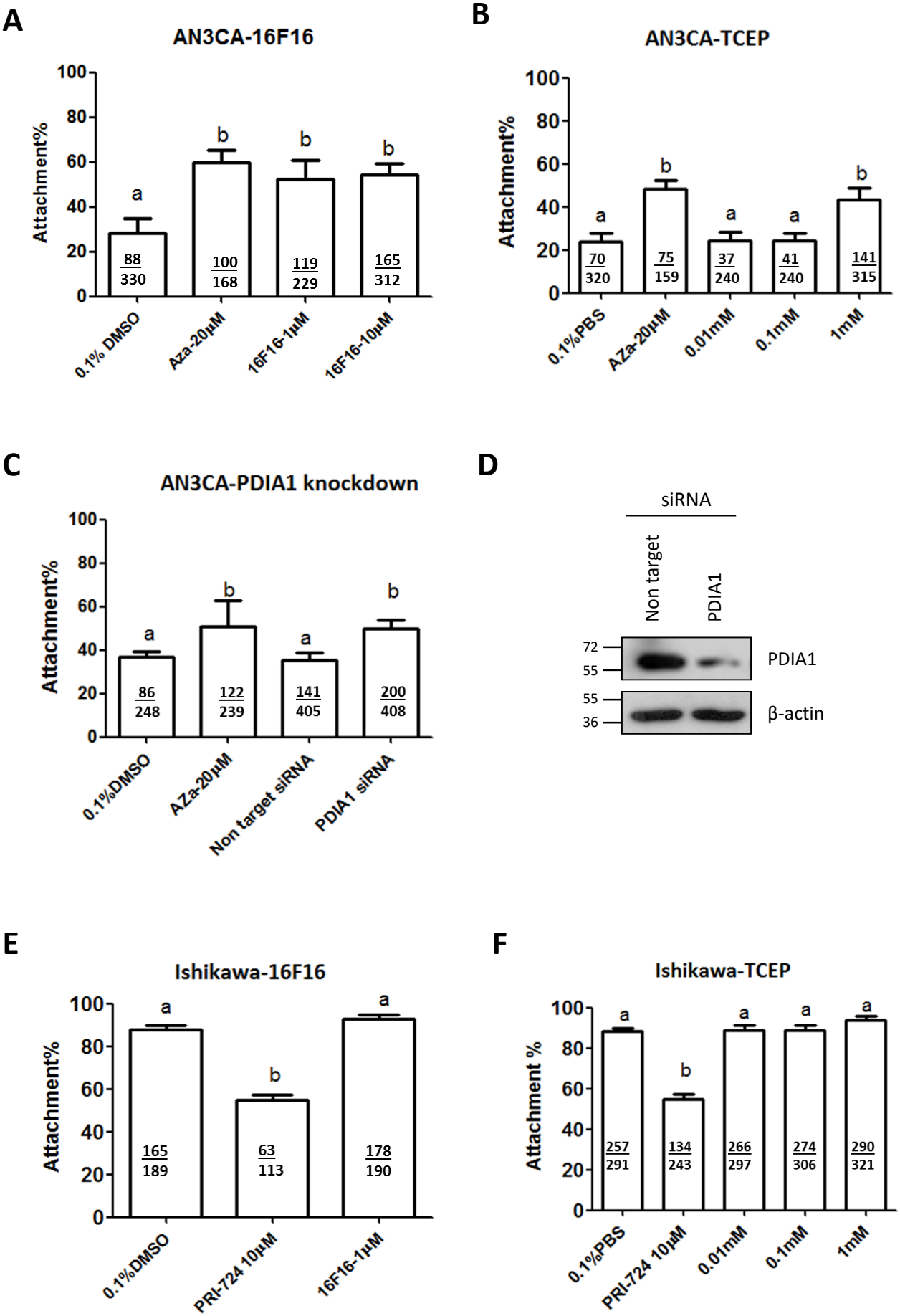
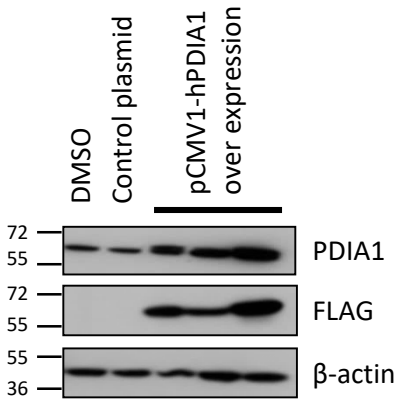

A
AN3CA-16F16
Attachment%
100
80
60
40
20
0
a
b
b
b
88/330
100/168
119/229
165/312
0.1% DMSO
Aza-20μM
16F16-1μM
16F16-10μM
B
AN3CA-TCEP
Attachment%
a
b
a
a
b
70/320
75/159
37/240
41/240
141/315
0.1%PBS
AZa-20μM
0.01mM
0.1mM
1mM
C
AN3CA-PDIA1 knockdown
Attachment%
a
b
a
b
86/248
122/239
141/405
200/408
0.1%DMSO
AZa-20μM
Non target siRNA
PDIA1 siRNA
D
siRNA
Non target
PDIA1
72
55
55
36
PDIA1
β-actin
E
Ishikawa-16F16
Attachment%
a
b
a
165/189
63/113
178/190
0.1%DMSO
PRI-724 10μM
16F16-1μM
F
Ishikawa-TCEP
Attachment %
a
b
a
a
a
257/291
134/243
266/297
274/306
290/321
0.1%PBS
PRI-724 10μM
0.01mM
0.1mM
1mM

Figure 4 Conti …

G



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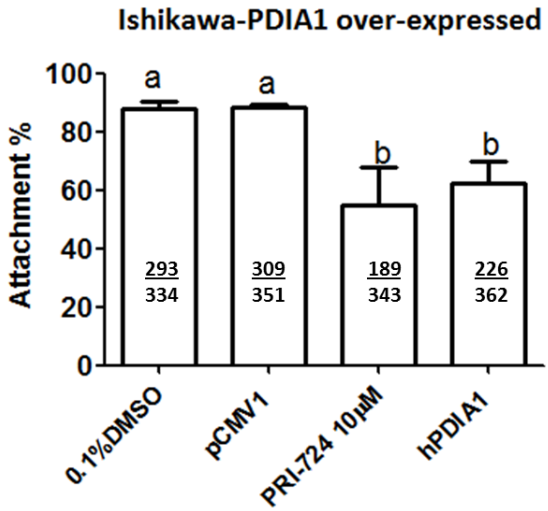

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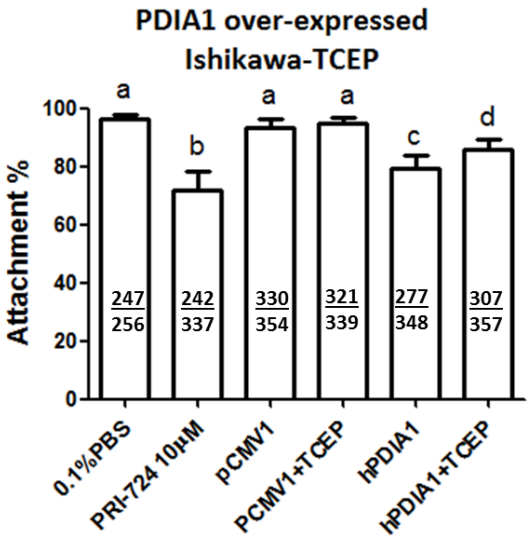


Figure 5

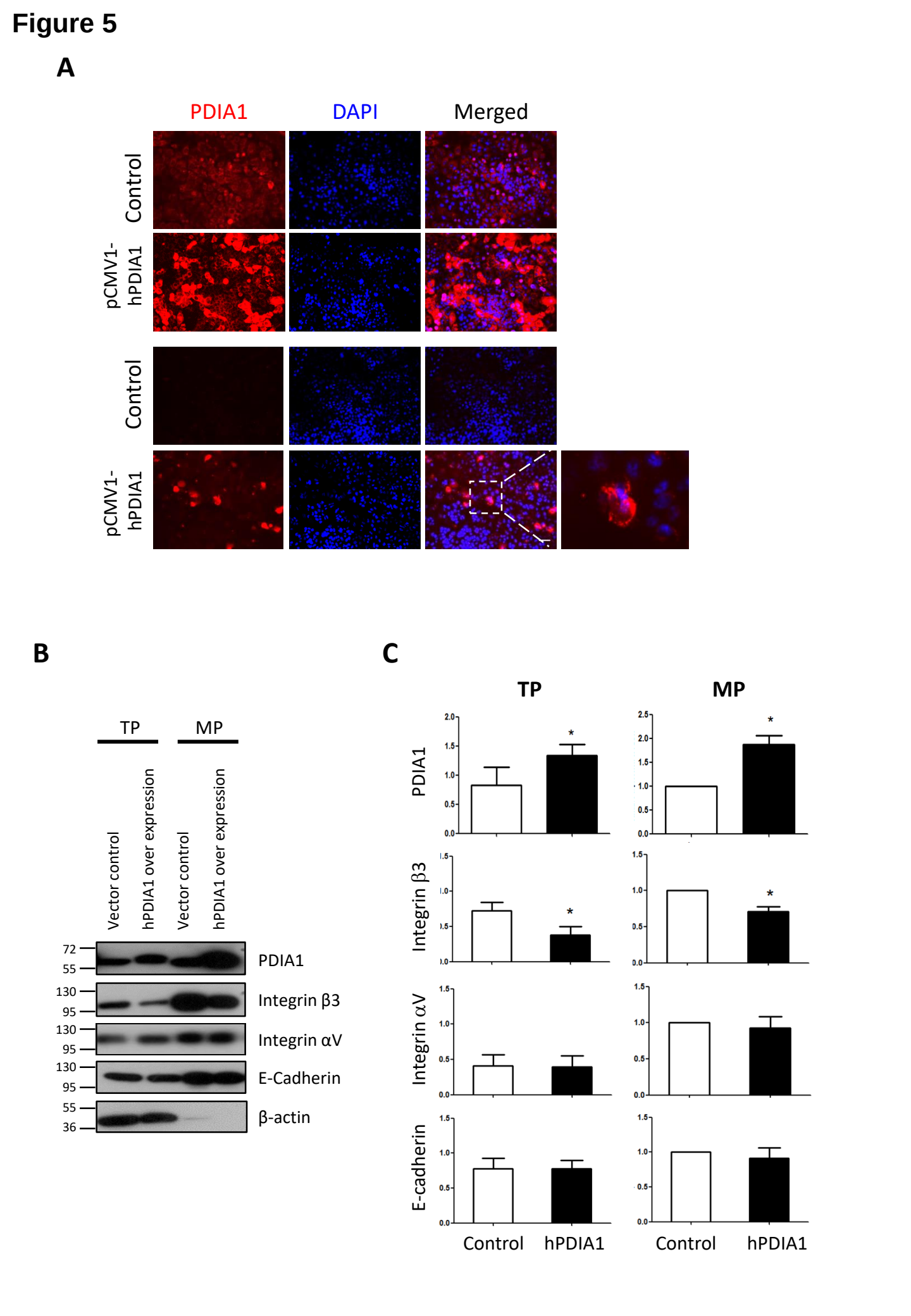
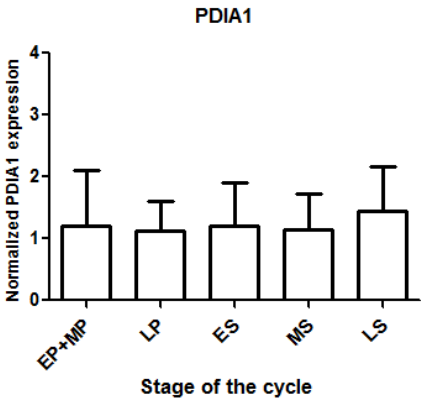
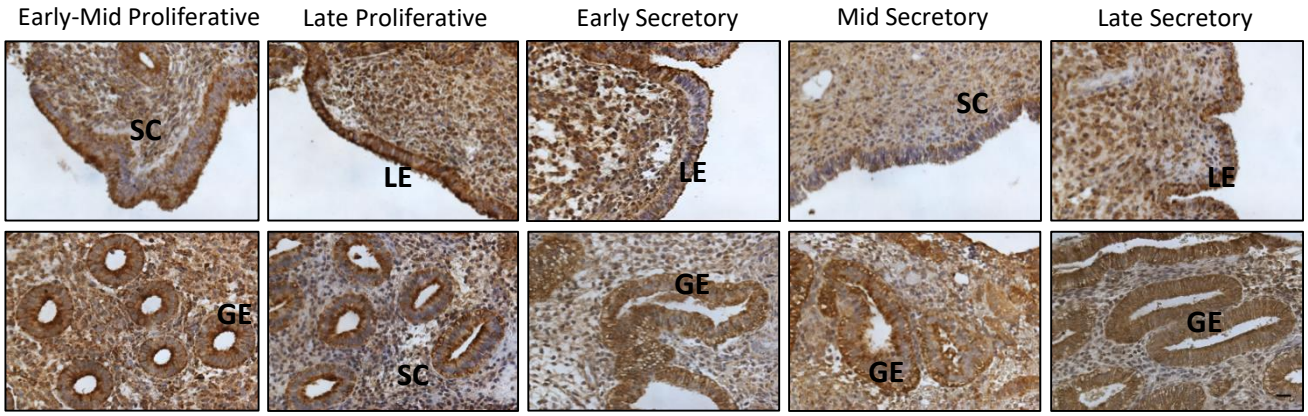

A
PDIA1
DAPI
Merged
Control
pCMV1-hPDIA1
Control
pCMV1-hPDIA1
B
TP
MP
Vector control
hPDIA1 over expression
Vector control
hPDIA1 over expression
72
55
PDIA1
130
95
Integrin β3
130
95
Integrin αV
130
95
E-Cadherin
55
36
β-actin
C
TP
MP
PDIA1
Integrin β3
Integrin αV
E-cadherin
Control
hPDIA1
Control
hPDIA1

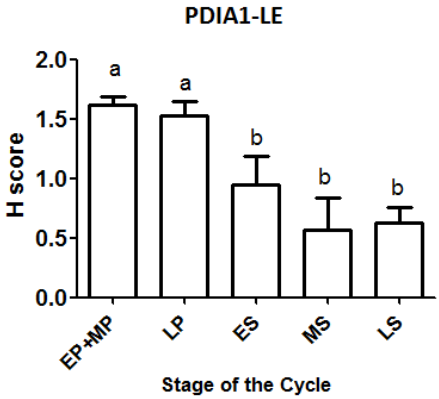
Figure 6

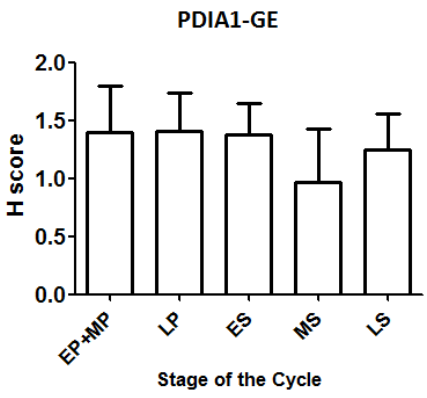
A


PDIA1
Normalized PDIA1 expression
0
1
2
3
4
EP+MP
LP
ES
MS
LS
Stage of the cycle

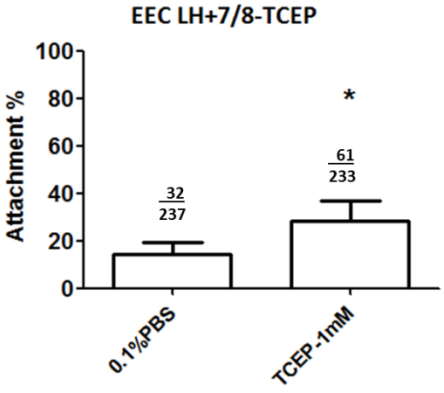
B


Early-Mid Proliferative
Late Proliferative
Early Secretory
Mid Secretory
Late Secretory
SC
LE
GE
SC
LE
GE
SC
LE
GE
GE
LE
GE


PDIA1-LE
H score
0.0
0.5
1.0
1.5
2.0
a
a
b
b
b
EP+MP
LP
ES
MS
LS
Stage of the Cycle

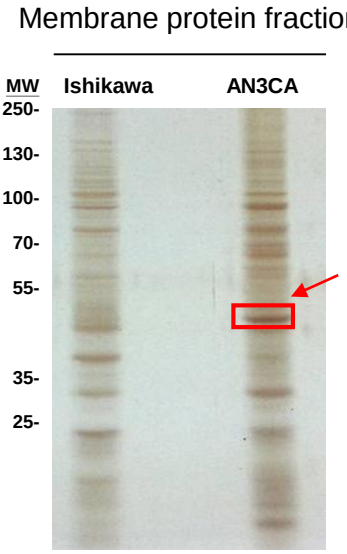

PDIA1-GE
H score
0.0
0.5
1.0
1.5
2.0
EP+MP
LP
ES
MS
LS
Stage of the Cycle

C


EEC LH+7/8-TCEP
Attachment %
0
20
40
60
80
100
32
237
61
233
*
0.1%PBS
TCEP-1mM

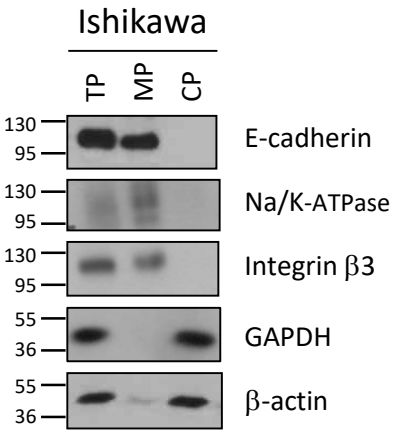
Supportive Information- Figure 1

A



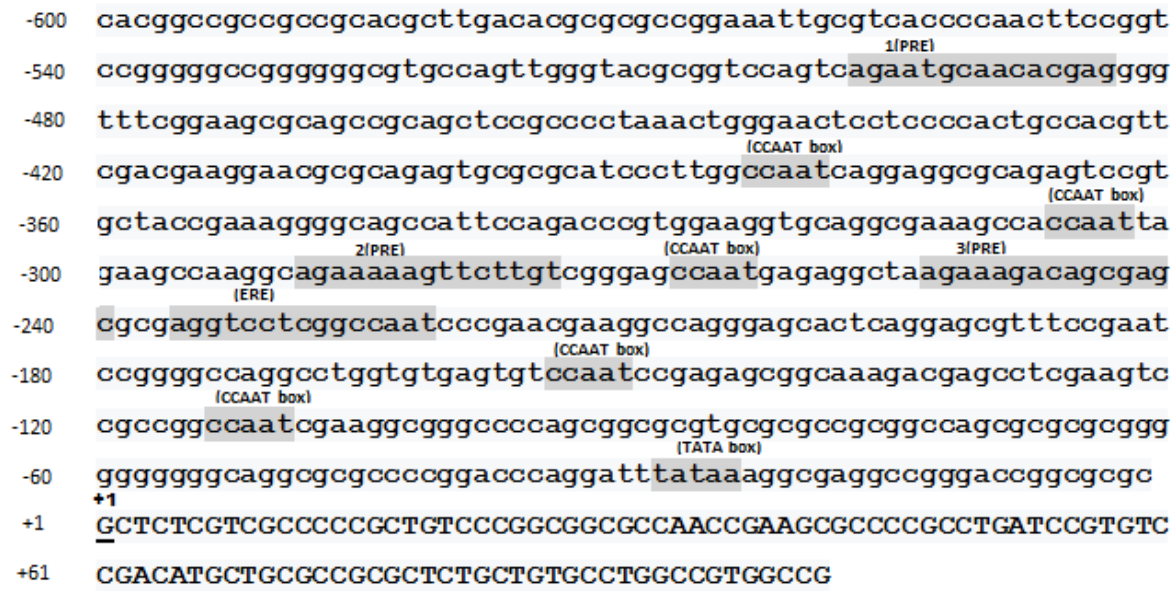
Protein Name	Accession number	Protein Score	Total ion score	Peptide	Ion score	Sequence	*m/z*
Human Protein Disulfide Isomerase, chain A [Homo sapiens]	gi159162689	287	279	GYPTIKFFR DAPEEEDHVLVLR DAPEEEDHVLVLRK	35 88 107	81-89 1-13 1-14	1128 1521 1649

B



Supportive Information- Figure 2

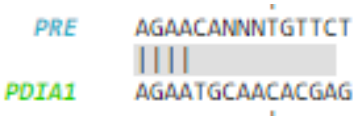
A



B

C

Site 1



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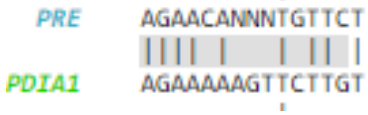

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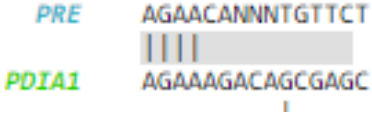
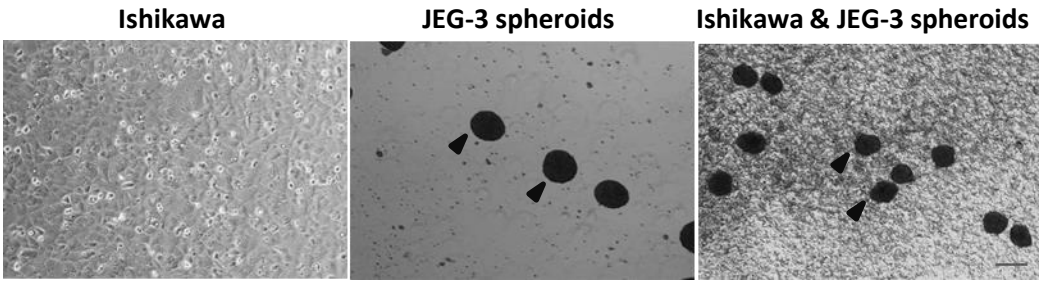
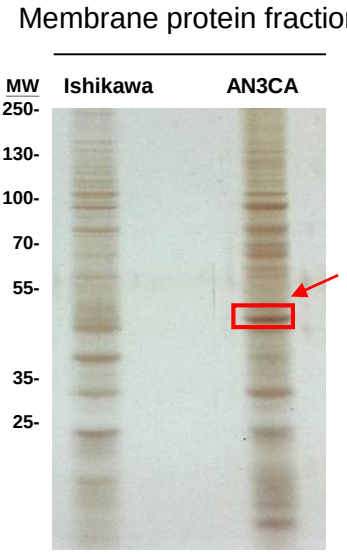


Supportive Information- Figure 3



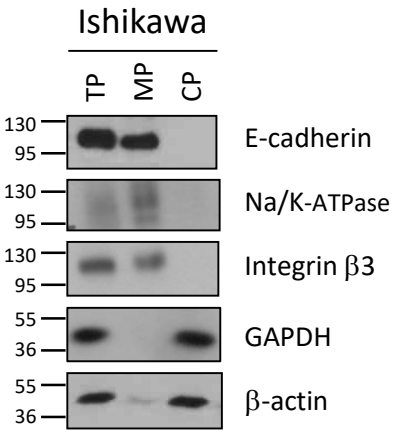
Supportive Information- Figure 1

A



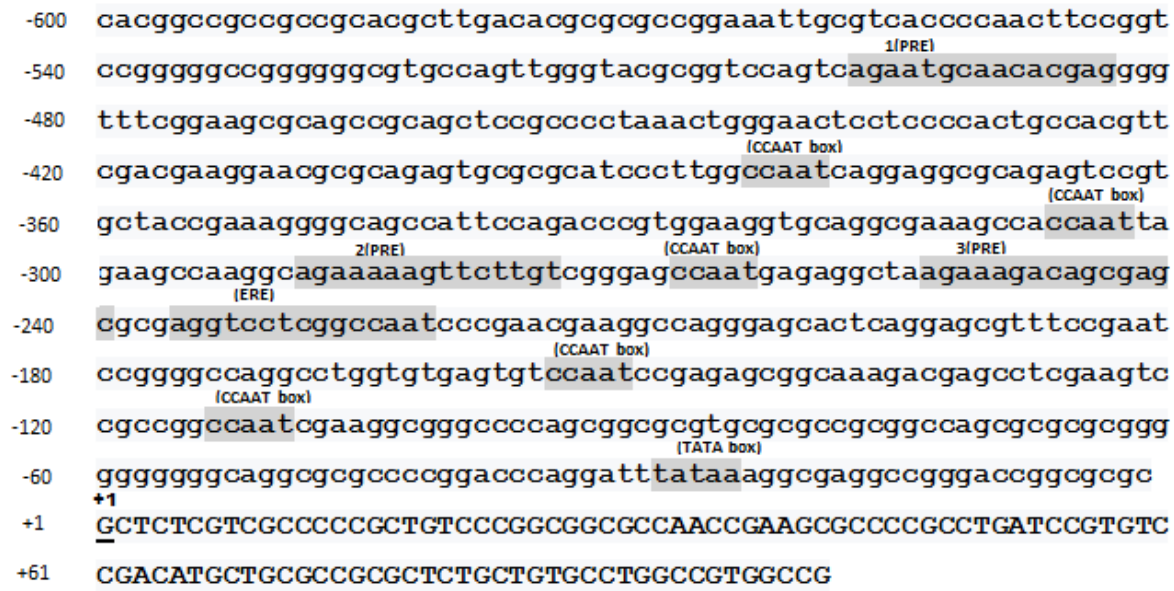
Protein Name	Accession number	Protein Score	Total ion score	Peptide	Ion score	Sequence	*m/z*
Human Protein Disulfide Isomerase, chain A [Homo sapiens]	gi159162689	287	279	GYPTIKFFR DAPEEEDHVLVLR DAPEEEDHVLVLRK	35 88 107	81-89 1-13 1-14	1128 1521 1649

B



Supportive Information- Figure 2

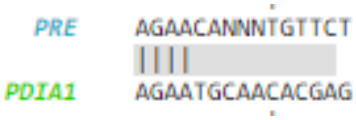
A



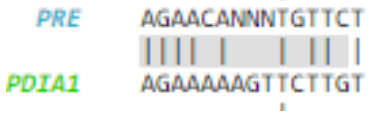
B

C

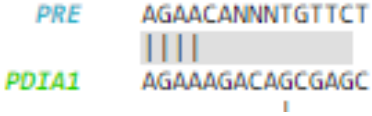
Site 1



Site 2



Site 3



Supportive Information- Figure 3