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Profilin-1 is dysregulated in endometroid (type I) endometrial cancer promoting cell proliferation and inhibiting pro-inflammatory cytokine production

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The Editor, *Biochemical and Biophysical Research Communications*,

We respectfully submit our manuscript entitled '**Profilin 1 is dysregulated in endometroid (type I) endometrial cancer promoting cell proliferation and inhibiting pro-inflammatory cytokine production**' by George, Winship, Sorby, Dimitriadis and Menkhorst for consideration for publication in *Biochemical and Biophysical Research Communications*.

Endometrial cancer is the most common female gynaecological cancer worldwide - alarmingly, the incidence and mortality of endometrial cancer is increasing, particularly in younger women of reproductive age. Unfortunately, there are limited treatment options for EC, particularly for recurrent or metastatic disease. Profilin 1 regulates tumorigenesis in numerous cancers but the role of profilin 1 has not been investigated in endometrial cancer.

Profilin 1 immunostaining was significantly reduced in the endometrial epithelial cancer cell compartment of grade II and III endometrial cancer compared to grade I endometrial cancer and normally cycling endometrium. Silencing profilin 1 *in vitro* increased endometrial epithelial cancer cell line (AN3CA) adhesion and proliferation. Profilin 1 immunostaining was strongly observed in infiltrating immune cells of the stromal compartment in endometrial cancer. Profilin 1 inhibited THP1 macrophage pro-inflammatory cytokine expression *in vitro*. Profilin 1 may play a role in the tumorigenesis of endometrial cancer due to increased endometrial epithelial cancer cell proliferation coupled with a pro-tolerance tumor microenvironment.

This an original study presenting novel, unpublished work. The material submitted in this manuscript has not been previously reported and is not under consideration for publication elsewhere. We will not submit this manuscript to another journal until a decision has been reached by *Biochemical and Biophysical Research Communications* as to its suitability for publication. All the authors concur with the submission. The authors have no conflicting financial or other interests.

Kind Regards,

Ellen Menkhorst

Blot #1

PFN1 and GAPDH for Figure 2B

Blot #2

Highlights

- PFN1 production is dysregulated in Type 1 EC.
- PFN1 protein is lost in Type 1 grade II and III endometrial epithelial cancer cells.
- Silencing PFN1 promoted AN3CA adhesion and proliferation.
- PFN1 is strongly expressed in the stromal compartment of EC.
- PFN1 down-regulated *TNF α* and *IL1 β* mRNA expression in THP1 cells

Profilin-1 is dysregulated in endometrioid (type I) endometrial cancer promoting cell proliferation and inhibiting pro-inflammatory cytokine production.

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Abstract

Endometrial cancer (EC) is the most common gynaecological malignancy. Alarming its incidence and mortality rate is increasing particularly in younger women of reproductive age. Despite this, there are limited treatment options for EC. Profilin-1 (PFN1) regulates tumorigenesis in numerous cancers, but the role of PFN1 in EC has not been investigated. We hypothesized that PFN1 would have altered expression in EC and contribute to the development of EC.

We quantified PFN1 in type 1 EC and benign/normal endometrium by RT-qPCR and IHC. The effect of silencing PFN1 on cell adhesion and proliferation was investigated using 2 EC cell lines (HEC1A and AN3CA). The effect of recombinant PFN1 (100 μ M) on pro-inflammatory cytokine gene expression was investigated using THP1 monocyte cell line.

PFN1 immunolocalized to glandular epithelial cells, vascular endothelial cells and leukocytes in the stromal compartment of normal endometrium and EC. PFN1 immunostaining intensity was significantly elevated in grade (G)I EC compared to normal endometrium, GI-II and GIII EC. In endometrial epithelial cancer cells alone, PFN1 immunostaining intensity was significantly reduced in GII and III EC compared to normal endometrium and GI EC. The stromal compartment of EC had strong PFN1 expression compared to benign and normal endometrium. Silencing PFN1 in the AN3CA endometrial epithelial cancer cell line significantly enhanced cell adhesion and proliferation. PFN1 treatment significantly down-regulated *TNF α* and *IL1 β* mRNA expression by THP1 cells.

This study demonstrated that whilst PFN1 production is retained in the stromal compartment of EC, PFN1 production is lost in endometrial epithelial cancer cells with increasing cancer grade. PFN1 may play a role in the tumorigenesis of EC. Loss of PFN1 in GII and GIII endometrial epithelial cancer cells associated with sustained PFN1 by infiltrating immune cells may promote EC tumorigenesis due to increased endometrial epithelial cancer cell proliferation coupled with a pro-tolerance tumor microenvironment.

45 **Keywords**

46 Profilin-1; AN3CA; THP1; Type 1 endometrial cancer;

47 **Abbreviations**

48 PFN1, profilin-1

49 EC, endometrial cancer

Introduction

Endometrial cancer (EC) is the most common female gynaecological cancer worldwide [1]. Of significant concern is the increasing incidence and mortality of EC, especially in reproductive age women [2], likely associated with increased life expectancy and obesity found in developed countries [3]. Therapeutic options beyond hysterectomy are limited for EC, and there are few treatments available for recurrent or metastatic disease [4].

The International Federation of Gynecology and Obstetrics guidelines are used to categorize EC. EC can be categorized into type 1 or type 2 based on histology. Over 80% of all EC is type 1 [5], a histologically endometrioid cancer associated with unopposed oestrogen, resulting in endometrial hyperplasia, which is characterized by excess proliferation of endometrial glands causing an increase in the glandular to stroma ratio [6]. Type 1 EC Tumor grade (GI-III) is defined by histology, metastatic behaviour and the degree to which the EC has invaded the uterine corpus and surrounding peritoneum [6]: i) Grade I (GI), well differentiated; ii) grade II (GII), moderately differentiated; and iii) grade III (GIII), poorly differentiated [6]. Type 1 EC is associated with gene mutations in *K-ras* and phosphatase and tensin homolog (PTEN) [4]. Type 2 EC are generally higher-grade, more aggressive adenocarcinomas which are non-endometrioid in histology [6]. Type 2 EC are not driven by oestrogen and are associated with gene mutations in tumor protein P53 (*p53*) [4]. However, treatments based on histological classifications can be ineffective, in part due to the highly variable gene mutations found in both EC types [7]. Personalized therapies based on molecular characterization of individual tumors may improve patient outcomes [8].

Profilin-1 (PFN1) is a small, 15kDa, multi-ligand protein expressed ubiquitously in mammalian cells [9] including endometrial epithelial cells [10, 11]. PFN1 was initially identified as an actin-binding protein and is now recognized as essential for cell survival due to its role in the regulation of the dynamic actin cytoskeleton. PFN1 is also released extracellularly although it doesn't have a secretion signal motif. PFN1 has been detected in dendritic-derived exosomes [12], conditioned media [11, 12] and serum [13].

Disruptions to the actin cytoskeleton is a hallmark of cancer cells [14] and PFN1 is dysregulated in multiple cancers [9], however PFN1 has cancer dependent functions in regulating metastatic ability [9]: in breast [14-17], hepatic [18] bladder [19] and pancreatic [20] cancers, down-regulation of PFN1 promotes metastatic potential, whereas in renal [21] and gastric [22] cancer metastatic disease, loss of PFN1 reduces metastatic potential.

The role of PFN1 in EC has not been investigated to date. PFN1 was identified as potential biomarker for Stage 1 EC by a proteomics screen [23], however this was not validated by another method. We hypothesized that PFN1 expression would be altered in human EC, similar to other epithelial malignancies and contribute to the development of EC. The aim of this study was to quantify PFN1 expression in type 1 human endometroid EC and determine the effect of down-regulated PFN1 on HEC1A and AN3CA (endometrial epithelial cancer cell lines) cell adhesion and proliferation. The effect of PFN1 on THP1 pro-inflammatory cytokine gene expression was investigated.

Materials and methods

Patient samples.

This study was approved by the Monash Health Human Research and Ethics Committee (approval no. 06014C) and the Victorian Cancer Biobank (Melbourne, Victoria, Australia; project no. 13018). Informed consent was obtained from each participant.

The Victorian Cancer Biobank provided RNA from type 1 EC (n=9-10/grade) or benign post-menopausal endometrium (n=10). There was no difference in patient age between the three EC grades (median age of patients GI: 55.0 years, range 34-70; GII: 64 years, range 37-82; GIII: 66.5 years, range 42-82), while age was not available for patients donating benign endometrium. Samples were collected in Melbourne, Victoria, Australia between 2007 and 2014.

Cell lines

Cells were cultured in a 37°C humidified incubator containing 5% CO₂. GI-derived Ishikawa cells were provided by Dr Nishida (Tsukuba University, Tochigi, Japan) in 2014 and cultured in DMEM/F12 medium (Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc.). GII-derived HEC1A cells [authenticated by the Monash Health Translation Precinct (MHTP) Medical Genomics Facility in 2016] were cultured in McCoy's medium (Thermo Fisher Scientific, Inc.) supplemented with 10% FCS. GIII-derived AN3CA cells (authenticated by the MHTP Medical Genomics Facility in 2016) were cultured in DMEM/F12 medium supplemented with 10% FBS. THP-1 cells (human monocyte cell line non-adherent; authenticated by MHTP Medical Genomics Facility in 2016) were cultured in RPMI (Thermo Fisher Scientific, Inc) containing 10% FBS and 1% antibiotics (penicillin, streptomycin, amphotericin B; Gibco), before stimulation towards an adherent, macrophage phenotype by overnight treatment with phorbol 12-myristate 13-acetate (PMA; 12.5ng/ml).

RNA isolation, RT-PCR and qRT-PCR

Total RNA was isolated (TRI Reagent RNA Isolation system, Sigma #T9424) and DNase I treated (DNAfree™, Ambion) before reverse transcription (Superscript III First-Strand Synthesis System, Invitrogen) according to the manufacturer's protocol as previously described [24], except 0.5µl Superscript III was used for each reverse transcription reaction instead of 1µl.

PCR was performed as previously described [25] using GoTaq Green Master Mix (Promega) according to the manufacturer's instructions. Briefly, 1µg cDNA was combined with 2x Master Mix and 10µM primers (sequences shown in Table 1) and performed on a Veriti Thermal Cycler (Applied Biosystems). PCR products were visualized with GelRed Nucleic Acid Stain (Biotium) on a 1.6% agarose gel.

qPCR was performed as previously described [10]. Briefly, qPCR analyses were performed on the ABI 7500HT fast block real time PCR system (Applied Biosystems, Foster City, CA, USA) in triplicate in 384-well Micro Optical plates (Applied Biosystems) with the Power SYBR green master mix (Applied Biosystems) and 200 nM primers (sequences shown in Table 1).

The PCR and qPCR protocol was 95°C for 10 min and 40 cycles of 95°C for 15 s followed by 60°C for 1 min. qPCR relative expression levels were calculated by the comparative cycle threshold method ($\Delta\Delta C_t$), with 18S ribosomal RNA serving as the endogenous control for normalization.

Immunohistochemistry

An endometrial cancer mid-density tissue array slide (EMC1502; US Biomax, Inc) was used for PFN1 IHC as previously described for normal endometrial tissue [10]. Briefly, PFN1 (Santa Cruz Biotech #sc-137236; 1:400) or negative control antibody (Mouse IgG, Dako) was applied overnight at 4°C, before localization detected by Vectastain ABC Elite kit (Vector) and visualized using diaminobenzidine substrate (DAB, DakoCymation). As no negative control could be included on the array slide a positive and negative control section of proliferative phase endometrium was included in the run. CellSense software quantified DAB staining expressed as intensity per core. To give an intensity score for only the endometrial epithelial cell compartment for each core a blinded scorer semi-quantified epithelial cell staining (0, no staining, to 3, intense staining). Cores with less than ¼ tissue present or completely absent positive staining were excluded from analysis.

Western blotting

Endometrial cancer cell lysates from cells cultured under standard conditions (described above) were assayed for total protein and subjected to Western blotting for PFN1 as previously described [11], except 30µg total protein was loaded. Briefly, membranes were blocked using 0.1% Tween, 5% skim milk before primary antibody incubation (PFN1, overnight at 4°C, 1:1000, Santa Cruz Biotechnology sc-137236; GAPDH, 1hr at room temperature, 1:2000, CST #3683S) and PFN1 detected using ECL Plus Detection system (GE Healthcare).

siRNA transfection of PFN1

HEC1A and AN3CA's were transfected using RNAiMAX lipofectamine (Thermo Fisher Scientific, Inc) and ON-TARGETplus SMARTpool siRNA (FC: 1 μ M; PFN1 (5216): #L-102003-00-0010; scramble control: D-001810-10-05; Dharmacon) according to the manufacturer's instructions. xCELLigence assays and RT-qPCR for transfection efficiency were performed 72h after initial transfection.

xCELLigence real-time adhesion and proliferation assay

The real-time cell analyzer (RTCA) SP xCELLigence instrument (ACEA Biosciences; Agilent Technologies GmbH), was used as previously described [26]. Cells were seeded in E-plate 96 (ACEA Biosciences; Agilent Technologies GmbH) at ~10,000 cells/well in media supplemented with 5% FBS. Plates were monitored every 15min for a total of 72h.

PFN1 treatment for THP1 cytokine production

Adherent THP-1 macrophage cells were treated with recombinant human PFN1 (100 μ M; Abcam #ab87760) or vehicle control (PBS) for 24 and 48hr before RNA was extracted and RT-qPCR performed as described above.

Statistical Analysis

All statistical analyses were performed on raw data using GraphPad Prism 8.4.3 (GraphPad, SanDiego, CA, US). Data was tested for normality where possible (n>6) and paired t-tests, one-way ANOVA (followed by Tukey's multiple comparison test) and two-way-ANOVA (followed by Sidak's multiple comparison test) were used as appropriate: the test used is indicated in the figure legends. All data is presented as mean $\pm$ SEM. A p<0.05 was considered statistically significant.

Results

Endometrial epithelial PFN1 is down-regulated with increasing cancer grade.

There was no change in *PFN1* mRNA expression (Figure 1A) between benign endometrium or EC of any grade. PFN1 immunolocalized to endometrial epithelial, vascular endothelial and immune cells in normal

endometrium (Figure 1B-C) as previously described [10]. Strong PFN1 immunostaining was observed in glandular epithelial cells in normal endometrium and GI EC epithelial cancer cells but PFN1 immunostaining was reduced in epithelial cancer cells at GII and GIII (Figure 1C). Strong immunostaining of stromal compartment cells morphologically resembling immune cells was consistently observed in normal and cancerous (GI-III) endometrium although these cells were more common in EC (Figure 1C). PFN1 immunostaining intensity in whole tissue (quantified by CellSense software) was significantly increased in GI EC compared to normal, GI-II and GIII EC (Figure 1D; $F_{4,64}$ 5.580, $p<0.05$). PFN1 immunostaining of the endometrial epithelial cancer cellular compartment alone was significantly decreased in GII and GIII EC compared to normal endometrial tissue and GI EC (Figure 1E; $F_{4,74}$ 9.620; $p<0.05$).

Silencing PFN1 significantly increased AN3CA adhesion and proliferation

As the endometrial epithelial cell compartment showed significant loss of PFN1 with increasing cancer grade we determined the functional effect of silencing PFN1 on adhesion and proliferation in endometrial epithelial cancer cell lines. PFN1 mRNA and protein was highly detectable in Ishikawa (GI), HEC1A (GII) and AN3CA (GIII) cell lines and there was no difference in production between the three cell lines (Figure 2A&B). As loss of PFN1 was found in GII and GIII EC, we investigated the effect of silencing PFN1 in HEC1A (GII) and AN3CA (GIII) EC cell lines (Figure 2C-E). Silencing PFN1 increased AN3CA cell index (a measure of cell attachment) during the period of cell adhesion (up to 6h; 3-6h $p<0.05$; Figure 2C) and proliferation (up to 72h; 36-72h $p<0.05$; Figure 2D), but had no effect in HEC1A cells. Knockdown efficiency for each cell line is shown in Figure 2E.

PFN1 down-regulated THP1 production of TNF α and IL1 β

As PFN1 immunostaining intensity was strong in infiltrating immune cells in EC, we investigated whether PFN1 regulated THP1 cytokine production. Recombinant human PFN1 treatment for 48h significantly reduced THP1 expression of *TNF α* and *IL1 β* (Figure 3A&B; $p < 0.05$). No significant effect was seen for *IL12 α* .

Discussion

This is the first study to characterize and investigate the function of PFN1 in type 1 EC. We demonstrated that PFN1 production was decreased with increasing EC grade in endometrial epithelial cancer cells. *In vitro* experiments indicated that loss of PFN1 could increase EC adhesion and proliferation. We detected PFN1 immunostaining in EC infiltrating immune cells and showed that exogenous PFN1 down-regulated THP1 macrophage production of pro-inflammatory cytokines *TNF α* and *IL1 β* .

In a previous EC biomarker discovery report, proteomics identified increased PFN1 in Stage 1 EC compared to adjacent peri-cancerous endometrial tissue collected from the same woman [23]. Here we found an increase in PFN1 immunostaining in GI EC (whole tissue) compared to benign endometrium and GI-II and GIII EC, however for the first time we report that PFN1 immunostaining was decreased specifically in epithelial cancer cells from GII-GIII EC. Our immunostaining suggests increased PFN1 in GI EC is likely due to increased immune infiltration into the cancer lesion which begins in GI EC, whereas the reduction in PFN1 protein found in GII and GIII EC is likely due to loss of PFN1 in the epithelial cancer cells. Overall, our data demonstrates the importance in localizing PFN1 expression to individual cells during EC progression.

The mechanism leading to altered PFN1 production by GII and GIII endometrial epithelial cancer cells is unknown. Changes to actin networks can alter PFN1 synthesis [9]; given that disruptions to the actin cytoskeleton is a hallmark of cancer cells [14] it is likely that PFN1 down-regulation reflects the transformation of these cells to cancerous cells. Whether the down-regulation of PFN1 in EC is transcriptional or post-transcriptional cannot be determined from this study: gene expression levels did not change with increasing cancer grade, probably due to increasing PFN1 positive immune cell

infiltration. miR-182 is a tumor promotor in a number of cancers including EC [27] and breast where it promotes tumorigenesis by down-regulating PFN1 [28]. In other tissues PFN1 regulates multiple pathways which are dysregulated in endometrial cancer, including PI3K, PTEN and p53 [9, 29, 30]. Our observation that the effect of silencing PFN1 was cell line specific may be related to the mutational background of these cells: AN3CA have mutations in *PI3KR1* and *PTEN*, whereas HEC1A has mutations in *KRAS* and *PI3KCA* [31]. Future experiments could be directed towards identifying the mechanism by which PFN1 inhibits cell proliferation in EC cell lines with differing mutational backgrounds.

PFN1 has tissue specific pro- or anti-tumorigenic actions [9]. It was demonstrated here that in EC cells, PFN1 may be anti-tumorigenic via its actions to suppress proliferation. Lower PFN1 is also seen in breast cancer where loss of PFN1 has been shown *in vitro* to increase the metastatic potential of epithelial breast cancer cells through increased proliferation, less apoptosis and increased migration and invasion [15-17]. PFN1 also impairs proliferation in hepatic [18] and bladder [19] cancer cells. However, breast and bladder cancer xenograft models made with cell lines where PFN1 is silenced show impaired tumorigenesis [14] [19]. This highlights the importance of the tumor microenvironment in tumorigenesis.

EC immunostaining presented here suggests that immune cells infiltrating into the EC strongly express PFN1. We have previously shown that endometrial stromal cells do not produce detectable levels of PFN1 by immunostaining and that the only cells staining for PFN1 in the stroma are likely leukocytes [10, 11]. Whilst we did not perform IHC for immune cell markers in this study, previous research have shown that the EC microenvironment is characterized by infiltration of immune cells which are thought to influence EC progression and patient outcomes [32]. We found that PFN1 treatment down-regulated THP1 pro-inflammatory cytokine expression *in vitro*. Our observation that *TNF α* and *IL1 β* expression was not reduced until 48h after PFN1 treatment suggests that PFN1 did not directly regulate *TNF α* and *IL1 β* mRNA expression. We have previously demonstrated that *in vitro* PFN1 down-regulates the production of arachidonate 5-lipoxygenase (ALOX5) in THP1 macrophages and human endometrial stromal cells [10].

ALOX5 is a lipoxygenase enzyme which converts arachidonic acid to leukotrienes [33] and inhibition of ALOX5 down-regulates the expression of pro-inflammatory cytokines including TNF α [34], IL6 [34] and IL1 β [35]. Strong PFN1 staining of cells within the stromal compartment of EC therefore might reflect a pro-tolerance tumor microenvironment: strong PFN1 staining within the stromal compartment of renal and gastric cancer is also observed [21, 22].

In conclusion, we have identified that PFN1 is dysregulated in EC and that differential expression of PFN1 between endometrial epithelial cancer cells and infiltrating immune cells may play a key role in the tumorigenesis of EC. Loss of PFN1 in GII and GIII endometrial epithelial cancer cells associated with sustained expression of PFN1 by infiltrating immune cells may promote tumorigenesis due to increased EC cell proliferation coupled with a pro-tolerance tumor microenvironment. The role of PFN1 in EC should be confirmed using primary EC cells and *in vivo* mouse models.

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Table 1. Primer sequences.

Primer	Forward 5'-3'	Reverse 5'-3'
<i>18s</i>	GATCCATTGGAGGGCAAGTCT	CCAAGATCCAACTACGAGCTT
<i>IL1β</i>	CAGCCAATCTTCATTGCTCA	TCGGAGATTCGTAGCTGGAT
<i>IL12α</i>	AGAGTCCCGGGAAAGTCCT	TCCAGGAGGACCAGGGTAG
<i>PFN1</i>	GATGGGGAATTTAGCATGGA3	GAAGGGACAGACGAGGTCAG
<i>TNFα</i>	TCAGCTCTTCTCCTTCCTG	CAGCTTGAGGGTTTGCTACA

Figure 1. PFN1 production in endometrial cancer (EC). A. *PFN1* mRNA expression by benign post-menopausal endometrium (B) and EC from grades (G) I, II and III (n=9-10/group); B. Positive and negative control for PFN1 IHC. C. PFN1 immunostaining in normal (N) endometrium and EC. D. PFN1 immunostaining intensity in normal cycling endometrium (N) and GI, GI-II, GII and GIII EC (one-way ANOVA, n=6-18/group). E. PFN1 immunostaining in endometrial epithelial cells from N endometrium and EC (one-way ANOVA, n=7-20/group). Data presented as mean $\pm$ SEM; *, P<0.05, significant difference from normal cycling endometrium (N); ^, P<0.05, significant difference from GI endometrial cancer; e, epithelial cell; s, stroma; v, blood vessel; arrow, PFN1 positive cell in stroma, likely immune cell.

Figure 2. PFN1 silencing promoted AN3CA cell adhesion and proliferation. A. *PFN1* mRNA expression by EC cell lines Ishikawa (I), HEC1A (H), AN3CA (A). B. PFN1 protein production by EC cell lines. C. PFN1 silencing enhanced AN3CA adhesion (two-way ANOVA; n=5/group). D. PFN1 silencing enhanced AN3CA proliferation (two-way ANOVA; n=5/group). E. Confirmation of *PFN1* knockdown by qPCR (paired t-test; n=2-5/group). Data presented as mean $\pm$ SEM; *, P<0.05; Scr, scramble.

Figure 3. PFN1 treatment (100 μ M) impaired THP1 macrophage pro-inflammatory cytokine production. A. *TNF α* expression (paired t-test; n=6/group); B. *IL1 β* expression (paired t-test, n=5-6/group); C. *IL12 α* expression (paired t-test, n=3/group). Data presented as mean $\pm$ SEM; *, P<0.05; Con, control.

Figure

Figure 1

Figure 2

Figure 3

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