Title: Dysregulation of POPDC1 promotes breast cancer cell migration and proliferation

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Conflict of Interest

We declare that all co-authors of this manuscript have no conflict of interest.
Abstract
Breast cancer subtypes such as triple-negative that lack the expression of oestrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor 2 receptor (HER2), remain poorly clinically managed due to a lack of therapeutic targets. This necessitates identification and validation of novel targets. Suppression of Popeye domain-containing protein 1 (POPDC1) is known to promote tumourigenesis and correlate to poor clinical outcomes in various cancers, and also promotes cardiac and skeletal muscle pathologies. It remains to be established whether POPDC1 is dysregulated in breast cancer, and whether overcoming the dysregulation of POPDC1 could present a potential therapeutic strategy to inhibit breast tumourigenesis. We assessed the potential of POPDC1 as a novel target for inhibiting breast cancer cell migration and proliferation. POPDC1 was significantly suppressed with reduced cell membrane localisation in breast cancer cells. Furthermore, functional suppression of POPDC1 promoted breast cancer cell migration and proliferation, which were inhibited by POPDC1 overexpression. Finally, cAMP interacts with POPDC1 and upregulates its expression in breast cancer cells. These findings suggest that POPDC1 plays a role in breast tumourigenesis and represents a potential therapeutic target or biomarker in breast cancer medicine.

Keywords
POPDC1, BVES, breast cancer, cAMP, cell migration, cell proliferation

Highlights
- Expression and cell membrane localisation of POPDC1 is reduced in breast cancer cells
- Suppression of POPDC1 promotes breast cancer cell migration and proliferation
- Overexpression of POPDC1 inhibits breast cancer cell migration and proliferation
- cAMP interacts with, and upregulates POPDC1 in breast cancer
- Suppression of POPDC1 attenuates cAMP-mediated inhibition of breast cancer cell migration and proliferation
1. **Introduction**

Breast cancer is the second leading cause of cancer-related morbidity and mortality globally \[1\]. Despite major progress in the development of new breast cancer treatments and in the clinical management of the disease over the years, these have been met with major challenges including drug resistance \[2-5\]. Furthermore, breast cancer is a molecularly heterogeneous disease with subtypes that have proven challenging to target and which display varying levels of susceptibility to therapeutic agents \[4, 6, 7\]. This necessitates identification and validation of novel therapeutic targets that can be targeted to deliver maximum clinical efficacy with minimum side-effects especially in poorly managed breast cancer subtypes. Breast cancer can be classified on a molecular basis into 5 subtypes; luminal A, luminal B, HER2 positive, claudin-low and basal-like, according to the expression levels of oestrogen receptors (ER), progesterone receptors (PR) and human epidermal growth factor receptor 2 (HER2) in the cell. Luminal A tumours are ER\(^+\), PR\(^+/-\) and HER2\(^-\), luminal B tumours are ER\(^+\), PR\(^+/-\) and HER2\(^+\), HER2 positive tumours are ER\(^-\), PR\(^-\) and HER2\(^+\). Basal-like tumours and claudin-low tumours are subclasses of triple negative breast cancer and are ER\(^-\), PR\(^-\) and HER2\(^-\) \[8\]. Basal-like tumours are aggressive, have poor prognosis and are more challenging to therapeutically target due to a lack of hormone receptor expression. In contrast, luminal A tumours have a more positive prognosis, are less aggressive and easier to treat due to the expression of hormone receptors \[6, 8\].

Here we assess the potential of Popeye domain-containing protein 1 (POPDC1) (also known as blood vessel epicardial substance (BVES)), as a novel target for inhibiting cell migration and proliferation in breast cancer. We use a non-malignant breast cell line (MCF10A), a less aggressive luminal A breast cancer cell line (MCF7) and aggressive breast cancer cell lines MDA231 (also known as MDA-MB-231) which is triple-negative and the HER2+ cell line (SKBR3).

POPDC1 (also known as BVES), is a transmembrane protein encoded by the POPDC1 gene and is thought to be a tumour suppressor that is dysregulated to promote malignant cell behaviour. Loss of
POPDC1 expression has been correlated with enhanced cancer cell proliferation, migration, invasion, metastasis, drug resistance, and poor patient survival in various human cancers \cite{9-13}. Suppression of POPDC1 has further been shown to promote cell migration and invasion in hepatocellular carcinoma, and to promote tumourigenesis in colorectal cancer \cite{9, 11}. In addition, loss of POPDC1 has been shown to promote colorectal cancer tumourigenesis via activation of c-Myc regulated networks and activation of Wnt signalling \cite{12}. Although the exact functional mechanisms of POPDC1 are poorly understood, the known roles and correlations between POPDC1 with cancer and cardiovascular diseases have been recently reviewed \cite{14, 15}.

POPDC1 belongs to the \textit{POPDC} gene family which has 3 isoforms; \textit{POPDC1}, \textit{POPDC2} and \textit{POPDC3} which encode the POPDC1, POPDC2 and POPDC3 proteins respectively. POPDC proteins are transmembrane proteins normally tethered to the cell membrane as a dimer held together by a disulphide bond \cite{13, 16, 17}. They contain an extracellular amino terminus, three transmembrane domains and a cytoplasmic Popeye domain which binds cyclic nucleotides. The Popeye domain is evolutionarily conserved and has been shown to bind cAMP with high affinity. The binding of cyclic adenosine monophosphate (cAMP) to the Popeye domain is thought to induce structural changes in POPDC1 that affects protein function \cite{16, 18}. The signalling cascade downstream of POPDC1 has not yet been determined. Although the role of POPDC1 in breast cancer tumourigenesis remains to be established, POPDC1 presents a realistically druggable target for various reasons. Firstly, POPDC1 possesses a novel Popeye domain (PFAM: PF04831), that has not been identified in any other protein outside the POPDC protein family \cite{14, 18-20}. The Popeye domain has been linked to POPDC protein functions such as binding cAMP and maintenance of epithelial integrity \cite{15, 21}. For example, truncation of the protein following introduction of an early stop codon has been shown to prevent localisation of POPDC1 to the cell membrane and prevent POPDC1-mediated tight junction maintenance \cite{21}. Hence the Popeye domain can be targeted to potentially induce effects specific to POPDC signalling with less ubiquitous side-effects than targeting molecules such as cAMP. Secondly, the reduced
expression of POPDC1 consistently correlates to tumourigenesis in various cancers and to the 
promotion of cardiovascular and muscular pathologies \cite{14-16, 19}. POPDC1 can therefore potentially be 
targeted to stabilise the protein, prevent loss of function and withdrawal from the membrane to 
reduce pathological consequences.

Cyclic adenosine monophosphate (cAMP) is a second messenger molecule involved in signal 
transduction of for example, G-protein-coupled receptors. cAMP is synthesized when the enzyme 
adenyl cyclase catalyses the conversion of adenosine triphosphate (ATP) to cAMP. In breast cancer, 
elevation of intracellular cAMP concentrations has been shown to promote apoptosis and inhibit cell 
migration and invasion \cite{22, 23}. In addition, the elevation of intracellular cAMP concentrations has been 
shown to inhibit breast tumour growth in mouse xenografts \cite{24}. However, it remains to be established 
whether cAMP regulates POPDC1 in breast cancer, and whether POPDC1 is involved in cAMP-
mediated inhibition of cell migration, invasion and tumour growth.

We hypothesise that dysregulation of POPDC1 promotes malignant phenotypes in breast cancer and 
that restoration of POPDC1 can potentially inhibit cell migration and proliferation, and revert cells to 
a less malignant phenotype. To test this hypothesis we firstly, determined the expression levels of 
POPDC1 in breast cancer cells in comparison to normal breast cells. Secondly, we assessed the effects 
of loss and gain of POPDC1 functions on breast cancer cell migration and proliferation. Thirdly, we 
determined whether cAMP interacts with, and regulates the levels of POPDC1 in breast cancer cells. 
Finally, we assessed whether cAMP-mediated inhibition of cell migration and proliferation is 
potentially facilitated via POPDC1 signalling.

This paper demonstrates firstly the suppression and loss of cell membrane localisation of POPDC1 in 
breast cancer cells. Secondly, suppression of POPDC1 promotes cell migration and proliferation in 
breast cancer cells, which are significantly inhibited by the overexpression of POPDC1. Thirdly, cAMP 
interacts with and regulates POPDC1 expression in breast cancer cells and finally, cAMP-mediated
inhibition of breast cancer cell migration and proliferation is potentially mediated via POPDC1 signalling.

2. Materials and Methods

2.1. Cell culture

MCF10A cells (ATCC) were grown in mammary epithelial cell growth medium bullet kit (Lonza). MCF7 cells (ATCC), MDA231 cells (ATCC) and SKBR3 cells (ATCC) were grown in Dulbecco's Modified Eagle's medium (DMEM) (Sigma) supplemented with 10% foetal calf serum (FCS), 2 mM L-glutamine (PAA Laboratories) and 1% penicillin/streptomycin (penicillin 10,000 units/ml; streptomycin 10 mg/ml) (BioSera). Cells were grown at 37°C, 5% CO₂ in a sterile humidified incubator and passaged at ≥80% confluence. MCF10A is a normal breast cancer cell line and MCF7 is a luminal A breast cancer cell line classified as ER+, PR+/- and HER2-. MDA231 is a basal-like triple negative breast cancer cell line classified as ER-, PR- and HER2- and SKBR3 is a HER2+ cell line classified as ER-, PR- and HER2+.

2.2. Western blot

Cells were lysed in RIPA buffer containing 50 mM Tris-HCl at pH 8, 150 mM sodium chloride, 0.1% Triton-X 100, 0.1% sodium dodecyl sulphate, 0.5% sodium deoxycholate, 0.5% Nonidet P-40, 1% protease inhibitor (Sigma) and 0.1% phosphatase inhibitor. Lysates were subsequently homogenised through a 25 gauge needle. Gel electrophoresis was performed on a 10% agarose gel and proteins were transferred onto a nitrocellulose membrane (Biorad). Antibodies used for immunoblotting included rabbit anti-Popdc1 diluted 1:100 (Abcam), rabbit anti-phospho-CREB (Ser133) (Cell Signalling Technology) diluted 1:500, goat anti-rabbit conjugated with horse-radish peroxidase diluted 1:500 (Abcam) and mouse anti-β-actin-peroxidase diluted 1:10000 (Sigma). Pulldown assay membranes
were stained with anti-Popdc1 diluted 1:30 (Abcam), anti-β-actin-peroxidase diluted 1:5000 (Sigma) and goat anti-rabbit IgG conjugated with horse-radish peroxidase diluted 1:100 (Abcam). Protein bands were detected with an ECL chemiluminescence reaction kit (ThermoScientific).

2.3. Immunocytochemistry

Sterile glass coverslips were pre-coated with 10 mg/ml poly-l-lysine (PLL) and dried under sterile conditions. 2 x 10^4 cells were seeded on sterile PLL-coated glass coverslips and incubated at 37°C, 5% CO_2 in a humidified incubator overnight. Cells were fixed onto coverslips with 4% paraformaldehyde for 20 minutes prior to 10 minute permeabilization with 0.5 % Triton X-100. Coverslips were rinsed three times in PBS prior to blocking, and after each step in the staining procedure. Non-specific binding sites were blocked with 5% BSA dissolved in PBS, for 30 minutes at room temperature. Cells were subsequently stained overnight at 4°C with CF-555 Phalloidin (Biotium) diluted 1:40 in PBS. Primary antibody staining with POPDC1 antibody (Sigma) (diluted 1:100) was subsequently performed with overnight incubation at 4°C. Secondary antibody staining was performed with Biotin-SP-conjugated goat anti-rabbit secondary antibody diluted 1:250 (Abcam) for 1 hour at room temperature. Tertiary antibody staining was subsequently performed with Streptavidin-flourescein diluted 1:150 (Abcam) for 30 minutes at room temperature. Counterstaining with 0.1 μg/ml DAPI dilactate nuclear probe (Sigma) was performed in the dark at room temperature for 10 minutes. Coverslips were mounted onto microscope slides with 1,4-diazabicyclo[2.2.2]octane (DABCO) mountant (Sigma), and stored in a dark cupboard. Confocal images were captured in single confocal plane with a Zeiss LSM700 confocal microscope at 63x magnification and processed using Zeiss Zen Black software. POPDC1 fluorescence intensity was measured with the ImageJ software. To quantitatively compare cell membrane expression of POPDC1 relative to overall expression in individual cells, fluorescence from the POPDC1 protein was measured per μm^2 in the membrane and
also across the whole cell. This was then presented as a comparative ratio of membrane fluorescence intensity/overall cell fluorescence intensity.

2.4. Protein suppression with siRNA

2 x 10^4 cells per/ml were grown in complete medium 24 hours prior to transient transfection with POPDC1 siRNA (Qiagen) or scrambled siRNA (Qiagen). Cells were incubated in serum-free medium 30 minutes prior to transfection. siRNA was pre-mixed with Lipofectamine 2000® (Invitrogen) in serum-free medium for 20 minutes and added to the cells. Complete medium was added to the cells 3 hours post-transfection to minimise toxicity to the cells. Boyden chamber assay, Alamar blue assay and Western blot analyses were performed 36 hours post-transfection. Cells were starved overnight prior to performing the Alamar blue assay.

2.5. Generation of stable cell lines

mPOPDC1 cDNA containing a G418 resistance cassette was kindly gifted by Professor Thomas Brand (Imperial College London). Plasmid DNA was transfected into MCF7, MDA231 and SKBR3 cells using Amaxa Nucleofector™ II (Lonza) to generate cell lines stably overexpressing POPDC1; MCF7 POP1++, MDA POP1++ and SKBR3 POP1++. Selection of stably transfected cells was performed with 1 mg/ml Geneticin sulphate (Biovision) (concentration verified using a kill curve) for 21 days. 0.5 mg/ml G418 was added to complete growth medium to maintain selection of MCF7 POP1++, MDA231 POP1++ and SKBR3 POP1++ cell lines thereafter.
2.6. **Boyden migration Assay**

Boyden chamber migration assays were performed to assess cell migration using 8 μm pore polycarbonate membranes (Neuro Probe Inc.). For experiments where the effects of POPDC1 knockdown and POPDC1 overexpression on cell migration were assessed, lower wells of the Boyden chamber were loaded with serum free medium lacking any migrant solution to ensure that the observed difference in migration was due to the effects of the difference in POPDC1 expression levels. For experiments where the effects of cAMP on cell migration were measured, lower wells were loaded with 60 μM Sp-8-Br-cAMPS (in serum free medium) for the test samples and simply serum free medium for the control samples. Cells were suspended in serum free medium to a density of 2 x 10^5 cells/ml prior to loading into upper wells of the chamber. After a 3 hour incubation at 37°C and 5% CO₂ in a sterile humidified incubator, non-migrated cells were scraped from the membrane surface and migrated cells were stained with a Diff Quik staining kit (Medion Diagnostix AG). Stained cells were subsequently counted using a light microscope and representative images were captured using Volocity software at 100 x magnification.

2.7. **AlamarBlue® cell proliferation assay**

2 x 10^3 cells/well were seeded in 96 well plates and were serum starved overnight prior to drug treatment. AlamarBlue® dye (Thermoscientific) was added to each well at a final concentration of 10% (v/v) and cells were incubated for 4 hours at 37°C and 5% CO₂ in a sterile humidified incubator. Fluorimetric quantitation of the reduced AlamarBlue® dye was measured with a Biotek Synergy™ HT multi-detection microplate reader and Gen5 software at 530 nm excitation and 590 nm emission wavelengths.
2.8. Pulldown assay

Cells were lysed in NP-40 lysis buffer containing 50 mM Tris at pH 7.5, 150 mM sodium chloride, 1% Nonidet P-40, 5% glycerol, 1% protease inhibitor and 0.1% phosphatase inhibitor. Lysates were subsequently homogenisation through a 25 gauge needle. Adenosine 3’,5’-cyclic monophosphate (cAMP) agarose beads (Sigma) were mixed with phosphate buffered saline (PBS) at pH 7.4 to form a 50% slurry. 400 μl cAMP agarose bead slurry was centrifuged in a Pierce® spin cup (Thermo Scientific) at 1000 x g for 1 minute. 150 μl lysate was diluted with 150 μl PBS at pH 7.4 and gently mixed with cAMP agarose beads in a spin cup and incubated for 6 hours at 4°C with gentle agitation. The mixture was subsequently centrifuged at 1000 x g for 1 minute to filter out the liquid phase of the lysate. The beads were washed with 150 μl PBS at pH 7.4 and subjected to centrifugation at 1000 x g for 1 minute to wash off unbound elements from the beads. Four additional washes were subsequently performed. To elute specifically bound proteins, beads were gently mixed with 150 μl elution buffer (0.1 M glycine at pH 2.5) and centrifuged at 1000 x g for 1 minute. Four elution steps were performed separately. 5 μl neutralisation buffer (1 M Tris at pH 8.0) was added to each eluted fraction for pH neutralization. Western blot analysis was performed on the lysate, washed and eluted fractions as previously described.

2.9. cAMP treatment

Cells were grown to 70% confluence in 9 cm or 6 cm tissue culture dishes prior to Sp-8-Br-cAMPS (Biolog) treatment. Optimal drug concentration selection was performed with a range of Sp-8-Br-cAMPS concentrations; 20 μM, 40 μM and 60 μM diluted in serum free DMEM. Cells were incubated at 37°C and 5% CO₂ in a sterile humidified incubator for 1 hour. Time course analysis of the effects Sp-8-Br-cAMPS on POPDC1 expression was performed with 60 μM Sp-8-Br-cAMPS diluted in serum free DMEM, over 1 hour, 3 hours and 6 hours incubation periods at 37°C and 5% CO₂ in a sterile humidified incubator. Control samples were treated with serum free medium. To assess whether
upregulation of POPDC1 by cAMP pre-treatment affected cell proliferation, cells were treated with 60 µM Sp-8-Br-cAMPS for 1 hour, prior to replacement of the treatment solution with a fresh 60 µM Sp-8-Br-cAMPS solution and incubation for 24 hours. This 2 phase treatment approach initially upregulates POPDC1 (1st Sp-8-Br-cAMPS application), and then assesses the functional impact of the high intracellular cAMP levels provided by the second Sp-8-Br-cAMPS application on the upregulated POPDC1 levels. Cell lysis and Western blot analysis were performed as described above.

2.10. Statistical analysis

Unpaired 2-tailed Student’s t-test was performed on datasets with two comparison groups and one-way ANOVA with Dunnnett’s post hoc tests were performed on comparisons of more than two groups of data. A p-value ≤ 0.05 was considered statistically significant. Statistical significance was classified as follows: * = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001.
3. Results

3.1. The expression of POPDC1 is suppressed in breast cancer cell lines in comparison to non-malignant cells

![Figure 1: POPDC1 is suppressed in breast cancer cells.](image)

Western blot analysis of POPDC1 expression in breast cell lines. POPDC1 is expressed at lower levels in malignant MDA231 and SKBR3 cells in comparison to non-malignant MCF10A breast cells (n=3). Graph represents densitometric quantification of protein bands. Protein bands were normalised by calculating the POPDC1/β-actin ratio. Normalised ratios for each cell line were expressed as a ratio relative to MCF10A to show protein fold differences for each cell line relative to the MCF10A cells. Comparisons of protein fold ratios were conducted using ANOVA with Dunnett’s post-hoc test * = p ≤ 0.05.

Suppression of POPDC1 at protein and mRNA level has been observed in various cancers \cite{11-13, 25}. We assessed if POPDC1 was suppressed in breast cancer cells in comparison to non-malignant breast cells. Interestingly, POPDC1 was significantly suppressed in the more aggressive breast cancer subtypes; MDA231 triple-negative cells and SKBR3 HER2+ cells, in comparison to non-malignant MCF10A cells (Fig. 1A).
3.2. Cell membrane localisation of POPDC1 is reduced in breast cancer cell lines

Figure 2: POPDC1 membrane localisation is reduced in cancer cells. (A) Cell membrane expression of POPDC1 is reduced in breast cancer cell lines. Immunocytochemical analysis of cell membrane expression of POPDC1 in breast cancer cells. Cells were counterstained with CF-555 Phalloidin (red) for visualisation of filamentous actin on the cytoskeleton and nuclear probe DAPI (blue). In MCF10A non-malignant cells, regions of high POPDC1 (green) localisation on the plasma membrane (white arrows) are observed. Plasma membrane expression of POPDC1 is reduced in MDA231 and SKBR3 cells. (White arrows indicate POPDC1 located in other cellular locations in these images). Scale bars = 20 µm (B) Quantitative analysis of the membrane expression of POPDC1 in different cell lines. Membrane POPDC1 fluorescence intensity per µm² was measured for individual cells along with fluorescence intensity per µm² across the whole cell. This was then converted to a ratio of fluorescence intensity in the membrane/fluorescence intensity across the entire cell. Membrane expression of POPDC1 is significantly reduced in MDA231 and SKBR3 cells compared to non-malignant MCF10A cells (n=4).
Cell membrane localisation of POPDC1 is known to be observed in various epithelial cells\textsuperscript{[26]}, so we next assessed if POPDC1 localisation is dysregulated in malignant MCF7 and MDA231 breast cells in comparison to non-malignant MCF10A breast cells (Fig. 3). POPDC1 (green) co-localised with Phalloidin (red) and is localised to the cell membrane of MCF10A non-malignant cells suggesting that POPDC1 is expressed primarily on the cell membrane of MCF10A cells (Fig. 2). This is consistent with its junctional function, and role in maintaining epithelial barrier integrity. Cell membrane expression of POPDC1 is significantly reduced in MDA231 cells and SKBR3 cells suggesting that the membrane localisation of POPDC1 is reduced in triple negative and HER2+ breast cancer cells. Loss of POPDC1 expression on the cell membrane could contribute to enhanced migration and malignant development by loss of the POPDC1 role in the maintenance of epithelial integrity, loss of tight junction maintenance and general molecular instability.
3.3. Suppression of POPDC1 promotes cell migration and proliferation in breast cancer cells

(A) Western blot analysis of POPDC1 expression following transfection with POPDC1 siRNA confirmed POPDC1 suppression in MCF7 (n=3), MDA231 (n=3) and SKBR3 (n=3) cells. Graphs below image panels represent densitometric quantification of protein bands. Protein bands were normalised by calculating the POPDC1/β-actin ratio. Normalised ratios were subsequently expressed as a ratio relative to control bands (no siRNA) to determine protein fold changes. (B) Suppression of POPDC1 with POPDC1 siRNA significantly promoted cell migration in MCF7 (n=5), MDA231 cells (n=5) and SKBR3 cells (n=5). Boyden chamber assay was performed over a 3 hour incubation period to allow cell migration across the polycarbonate membrane. Scale bars = 250 μm (C) Suppression of POPDC1 with POPDC1 siRNA significantly promoted cell proliferation in MDA231 cells (n=5) and SKBR3 cells (n=5), but not in MCF7 cells (n=4). Cells were transfected for 36 hours prior to overnight starvation in serum free medium and subsequent incubation in 10% AlamarBlue® dye for 4 hours. Comparisons of protein fold change ratios, mean % migration and mean % proliferation were conducted using ANOVA with Dunnett’s post-hoc test. Mean values presented ± SEM. ** = p ≤ 0.01, *** = p ≤ 0.001
Suppression of POPDC1 has been shown to promote cell migration, proliferation and invasion in various human cancer cells and tumour multiplicity in mice \cite{11, 12, 25}. To determine if suppression of POPDC1 affects breast cancer cell migration and proliferation, POPDC1 was suppressed with POPDC1 siRNA in MCF7, MDA231 and SKBR3 cells (Fig. 3A). A significant increase in cell migration was observed following suppression of POPDC1 in MCF7, MDA231 and SKBR3 cells (Fig. 3B). Furthermore, suppression of POPDC1 significantly promoted cell proliferation in the more aggressive breast cancer cell lines MDA231 and SKBR3 (Fig. 3C). This dataset suggests that suppression of POPDC1 promotes cell migration and proliferation in breast cancer cells and further supports the hypothesis that dysregulation of POPDC1 promotes a more malignant phenotype in breast cancer.

### 3.4. Overexpression of POPDC1 suppresses cell migration and proliferation in breast cancer cell lines

We next assessed if gain of POPDC1 function would inhibit cell migration and proliferation. Due to a lack of available POPDC1 activators or modulators, gain of function studies were conducted by overexpression of POPDC1 into MCF7, MDA231 and SKBR3 cells (Fig. 4A).
Figure 4: Overexpression of POPDC1 suppresses breast cancer cell migration and proliferation. (A) Western blot analysis of POPDC1 expression in wild type cells and cell lines stably transfected with POPDC1; MCF7 POP1++, MDA231 POP1++ and SKBR3 POP1++. Stably transfected cell lines expressed significantly higher levels of POPDC1 MCF7 (n=4), MDA231 (n=4) and SKBR3 (n=4). Graphs below Western blot band panels represent densitometric quantification of protein bands. Protein bands were normalised by calculating the POPDC1/β-actin ratio. Fold differences in protein expression were calculated by expressing normalised band values of POPDC1 overexpressing cell lines as a ratio to normalised band values of wild type cell lines. (B) Overexpression of POPDC1 significantly suppressed cell migration in MCF7 cells (n=4), MDA231 (n=5) and SKBR3 cells (n=5). Boyden chamber assay was performed over a 3 hour incubation period to allow cell migration across the polycarbonate membrane. Scale bars = 250 μm (C) Overexpression of POPDC1 significantly suppressed cell proliferation in MCF7 cells (n=4), MDA231 cells (n=5) and SKBR3 cells (n=5). Cells were starved in serum free medium overnight prior to incubation in 10% AlamarBlue® dye for 4 hours. Comparisons of protein fold change ratios, mean % migration and mean % proliferation were conducted using an unpaired t-test. Mean values presented ± SEM. ** = p ≤ 0.01, *** = p ≤ 0.001
Given that overexpression of POPDC1 has been shown to attenuate colorectal cancer tumour growth and metastasis in mice \[^{12}\] , we next asked if POPDC1 overexpression affects breast cancer cell migration and proliferation. Overexpression of POPDC1 significantly inhibited cell migration and proliferation in MCF7, MDA231 and SKBR3 cells (Fig. 4B and Fig. 4C); consistent with our theory that POPDC1 suppresses malignant cell behaviour.

3.5. cAMP interacts with and upregulates POPDC1 in breast cancer cells

The Popeye domain of POPDC1 has been shown to bind cAMP with high affinity using radioligand binding assays and affinity precipitation \[^{16}\]. The interaction between POPDC1 and cAMP has not been reported in breast cancer cells. To confirm if cAMP interacts with POPDC1 in breast cancer cells, we performed a pulldown assay with cAMP agarose beads. To confirm specificity of the assay, lysates were further probed for β-actin, which is known to not interact with cAMP.
Figure 5: cAMP interacts with POPDC1 and upregulates its expression in breast cancer cells. (A) Western blot analyses of cAMP agarose pulldown assays in MCF7, MDA231 and SKBR3 cells. POPDC1 was detected at high levels in the first eluted fraction (eluate 1) and at very low levels in the second eluted fraction (eluate 2) in MCF7 (n=3), MDA231 (n=3) and SKBR3 (n=3) cells confirming protein interaction between POPDC1 and cAMP. β-actin, the negative control, was detected in the unbound protein fraction but not in the eluted fractions, confirming specificity of the assay. (B) Western Blot analysis of the effects of 20 µM, 40 µM and 60 µM Sp-8-Br-cAMPS on the expression of POPDC1 and CREB following 1 hour treatment duration in MCF7 (n=3), MDA231 (n=3) and SKBR3 (n=3) cells. Graphs below Western blot band panels represent densitometric quantification of POPDC1 bands as a ratio to corresponding β-actin bands. Comparisons of normalised β-actin/POPDC1 ratios were conducted using ANOVA with Dunnett’s post-hoc test. Mean values presented ± SEM. ** = p ≤ 0.01, *** = p ≤ 0.001.

POPDC1 was detected in the first and second eluted fractions of cAMP agarose pulldown assays in MCF7, MDA231 and SKBR3 cells. The eluted fractions contain proteins that interact with cAMP. Presence of POPDC1 in the eluted fraction confirm that POPDC1 indeed interacts with cAMP in MCF7, MDA231 and SKBR3 cells, and is pulled out of the lysate using immobilised cAMP (Fig. 5A). Furthermore, the negative control β-actin was detected in the lysate fraction containing unbound proteins, but not in the eluted fractions confirming that the assay specifically eluted cAMP bound proteins. To assess the effects of cAMP on POPDC1 expression in breast cancer cells, we next asked if
upregulation of cAMP with the cAMP analogue Sp-8-Br-cAMPS can affect the levels of POPDC1. The effects of a range of Sp-8-Br-cAMPS concentrations were assessed over 1 hour treatment duration (Fig. 5B). Detection of POPDC1 was upregulated by 40 µM and 60 µM Sp-8-Br-cAMPS in MCF7 cells, significantly upregulated by 20 µM, 40 µM and 60 µM Sp-8-Br-cAMPS in MDA231 cells and by 40 µM and 60 µM Sp-8-Br-cAMPS in SKBR3 cells. To confirm activation of the cAMP pathway, we assessed if 20 µM, 40 µM and 60 µM Sp-8-Br-cAMPS affects the expression of phospho-CREB, another molecule that functions downstream of cAMP. Treatment with different Sp-8-Br-cAMPS concentrations increased the expression levels of phospho-CREB in MCF7, MDA231 and SKBR3 cells confirming activation of cAMP signalling (Fig. 5B). This dataset confirms that cAMP indeed interacts with POPDC1 and can increase its presence and stability in breast cancer cells.
3.6. cAMP inhibits cell migration and proliferation in breast cancer cells

Figure 6: Sp-8-Br-cAMPS inhibits cell migration and proliferation in breast cancer cell lines (A) Boyden chamber assay analysis of the effects of 60 µM Sp-8-Br-cAMPS on breast cancer cell migration in MCF7 (n=4), MDA231 (n=4) and SKBR3 (n=4) cells. Boyden chamber assay was performed over a 3 hour incubation period to allow cell migration across the polycarbonate membrane. Scale bars = 250 μm (B) Alamar Blue assay analysis of the effects of 60 µM Sp-8-Br-cAMPS on MCF7 (n=4), MDA231 (n=4) and SKBR3 (n=4) cell proliferation. Cells were starved in serum free medium overnight prior to drug treatment. (C) Alamar Blue assay analysis of the effects of a 2-phase treatment with 60 µM Sp-8-Br-cAMPS, on MCF7 (n=5), MDA231 (n=5) and SKBR3 (n=5) cell proliferation. Cells were starved in serum free medium overnight prior to drug treatment. Cells were subsequently treated with 60 µM Sp-8-Br-cAMPS for 1 hour prior to treatment replacement with a fresh 60 µM Sp-8-Br-cAMPS solution and a further 24 hour incubation. Alamar blue assays were performed by incubating cells in 10% AlamarBlue® dye (diluted in serum free medium) for 4 hours. Mean % migration and mean % proliferation values were compared using an unpaired t-test. Mean values presented ± SEM. ** = p ≤ 0.01, *** = p ≤ 0.001.
Increasing intracellular levels of cAMP has been shown to inhibit breast cancer cell migration and invasion \([22, 23]\). We asked if increasing intracellular levels of cAMP with 60 µM Sp-8-Br-cAMPS affects cell migration and proliferation in MCF7, MDA231 and SKBR3 cells. 60 µM Sp-8-Br-cAMPS significantly inhibited cell migration in MCF7, MDA231 and SKBR3 cells (Fig. 6A) and cell proliferation in SKBR3 cells (Fig. 6B). However, no effect was observed on MCF7 and MDA231 cell proliferation. We hypothesised that the initial treatment with Sp-8-Br-cAMPS served to upregulate the expression of POPDC1, but that insufficient concentrations then remained to actually act on the upregulated protein due to the gradual degradation of the analogue. Therefore, we applied a further 60 µM after an hour to see what functional effects were evident when POPDC1 expression was raised. Following this 2-phase treatment regimen, significant inhibition of cell proliferation was observed in MCF7, MDA231 and SKBR3 cells (Fig. 6C). Taken together this dataset suggests that increasing intracellular levels of cAMP inhibits cell migration and proliferation, and that high cellular POPDC1 expression interacts with cAMP to inhibit cell proliferation. It also suggests that cAMP control of cell migration and proliferation varies in sensitivity suggesting mechanistically diverse effects.

3.6. Suppression of POPDC1 attenuates cAMP-mediated cell migration and proliferation

Although the dataset so far suggests that cAMP-mediated inhibition of cell migration and proliferation are potentially facilitated via POPDC1 signalling, cAMP-mediated signals could be facilitated via other cAMP-binding molecules such as POPDC2 and POPDC3. It is therefore essential to determine whether cAMP-mediated suppression of breast cancer cell migration and proliferation is indeed dependent on cAMP-induced increase in POPDC1 protein levels. Hence we next asked if the POPDC1 suppression attenuates cAMP-mediated breast cancer cell migration and proliferation in these cell lines. To test this, the effects of 60 µM Sp-8-Br-cAMPS on breast cancer cell migration and proliferation were assessed following POPDC1 knockdown in MCF7, MDA231 and SKBR3 cells (Fig. 7).
Figure 7: Suppression of POPDC1 inhibits cAMP-mediated cell migration and proliferation in breast cancer cells. (A) Boyden Chamber assay analysis of the effects of 60 µM Sp-8-Br-cAMPS on MCF7 (n=4), MDA231 (n=4) and SKBR3 (n=4) breast cancer cell migration following POPDC1 suppression with siRNA. Boyden chamber assay was performed over a 3 hour incubation period to allow cell migration across the polycarbonate membrane. Scale bars = 250 μm (B) Alamar Blue assay analysis of the effects a 2-phase 60 µM Sp-8-Br-cAMPS treatment on the proliferation of MCF7 (n=5), MDA231 (n=5) and SKBR3 (n=5) cells following POPDC1 suppression with siRNA. Cells were transfected for 36 hours and starved overnight prior to drug treatment. Cells were treated with 60 µM Sp-8-Br-cAMPS treatment for 1 hour prior to treatment replacement with a fresh 60 µM Sp-8-Br-cAMPS treatment solution and 24 hours incubation. Alamar blue assay was performed by incubating cells in 10% AlamarBlue* dye for 4 hours. Mean % migration and mean % proliferation values were compared using an unpaired t-test. Mean values presented ± SEM. **= p ≤ 0.01, *** = p ≤ 0.001.

POPDC1 knockdown attenuated cAMP-induced cell migration and proliferation in MCF7, MDA231 and SKBR3 cells (Fig. 7A-B) reducing migration and proliferation levels to baseline. This confirms that cAMP-mediated inhibition of breast cancer cell migration and proliferation is depend on high cellular
4. Discussion

In this study, we have shown that POPDC1 is expressed in breast cells, but the expression is significantly suppressed in aggressive MDA231 and SKBR3 breast cancer cells, in comparison to non-malignant MCF10A breast cells (Fig. 1). This is consistent with suppression of POPDC1 protein and mRNA observed in various cancers including gastric cancer, colorectal cancer and hepatocellular carcinoma [11-13].

This study has further demonstrated differential POPDC1 localisation in breast cancer cells in comparison to non-malignant breast cells. Cell membrane localisation of POPDC1 is reduced in MDA231 and SKBR3 cells in comparison to high localisation of POPDC1 to the cell membrane in normal MCF10A breast cells (Fig. 2). We hypothesise that suppression of POPDC1 expression in breast cancer cells prevents the POPDC1 functioning at the cell membrane. Suppression of POPDC1 on the cell membrane has been shown to impair POPDC1-mediated tight junction maintenance in epithelial cells [21]. We postulate that the absence of functional POPDC1 dimers on breast cancer cell membranes potentially leads to a reduction in POPDC1-mediated tight junction maintenance thereby promoting cell migration.

We have further demonstrated the functional relevance of POPDC1 dysregulation in breast cancer. Suppression of POPDC1 significantly promotes a malignant phenotype in breast cancer by promoting cell migration in MCF7, MDA231 and SKBR3 cells, and cell proliferation in the more aggressive MDA231 and SKBR3 cells (Fig. 3B and Fig. 3C). Additionally, the overexpression of POPDC1 reverts cells to a less malignant phenotype by significantly inhibiting cell migration and proliferation in MCF7, MDA231 and SKBR3 breast cancer cells. Taken together with data from other studies which show that...
the suppression of POPDC1 promotes cell migration and invasion in glioblastoma, hepatocellular carcinoma, gastric cancer and colorectal cancer \[12, 13, 25, 27\], these data provide strong evidence to support the hypothesis that the dysregulation of POPDC1 promotes malignant phenotypes in breast cancer. Loss of POPDC1 expression has been shown to not only promote malignant behaviour in cancer, but also promotes cardiac and skeletal muscle pathologies, which are inhibited or partially reversed by gain of POPDC1 function \[14, 16, 19\]. These suggest that POPDC1 could potentially be therapeutically targeted in a similar manner to treat various diseases. Therapeutic targeting to prevent POPDC1 dysregulation or to promote gain of POPDC1 function can potentially be achieved in various ways. Firstly, gain of function could be achieved with small molecules that bind to the Popeye domain to induce protein stabilisation. Secondly, truncation of the protein has been shown to prevent POPDC1-mediated tight junction maintenance by preventing localisation of POPDC1 to the cell membrane \[21\]. Loss of POPDC1 function can therefore potentially be prevented by targeting mutations that prevent protein localisation to the cell membrane or stabilising the protein in the membrane.

This study has further shown that cAMP interacts with POPDC1 and upregulates its expression in MCF7, MDA231 and SKBR3 breast cancer cells (Fig. 5A and Fig. 5B). Furthermore cAMP significantly inhibited cell migration in MCF7, MDA231 and SKBR3 cells (Fig. 6 A), consistent with data from other studies showing that cAMP inhibits cell migration and invasion in breast cancer cells \[22, 23\]. However, significant inhibition of cell proliferation in MCF7 and MDA231 cells was only observed following a 2 phase treatment regimen with 60 µM Sp-8-Br-cAMPS (Fig. 6C). The initial Sp-8-Br-cAMPS treatment served to upregulate the expression of POPDC1, with the subsequent treatment permitting the functional consequences of Sp-8-Br-cAMPS binding to elevated POPDC1 expression to be measured. This suggests cAMP-mediated cell proliferation is facilitated via a mechanism that at least in part, involves the upregulation of POPDC1. The hypothesis that cAMP-mediates inhibition of breast cancer
Cell migration and proliferation via POPDC1 signalling is further strengthened by the attenuation of cAMP-mediated cell migration and proliferation in MCF7, MDA231 and SKBR3 cells following POPDC1 knockdown (Fig. 7A-B) which suggests that these effects are dependent upon a cAMP-induced increase in POPDC1 levels. Furthermore, it suggests that migration does not require the upregulation of POPDC1 as it was inhibited by a single treatment. This is consistent with a mechanism that involves direct action of Sp-8-Br-cAMPS in stabilising the existing POPDC1 protein being sufficient to reduce migration. It further suggests a more complex and less direct mechanism involved in cAMP potentially exerting control of proliferation via POPDC1. This firstly requires upregulation of the protein, and subsequent interaction with cAMP to influence proliferation perhaps via a downstream moiety. This molecular diversity is a novel and interesting finding that may further extend the therapeutic potential of POPDC1 as a target.

In conclusion, POPDC1 represents a druggable target that can potentially be manipulated to inhibit breast cancer cell migration and proliferation. In particular, this seems possible by binding of the cAMP-binding Popeye domain, which seems to establish the protein’s function in the plasma membrane. As a unique molecular domain, molecules that can interact in a cAMP-mimicking manner seem like exciting candidates for investigation going forward. However, mechanisms by which POPDC1 regulates cell migration and proliferation in breast cancer need to be clearly elucidated in order to permit development of directed therapeutics to take advantage of POPDC1 and its diverse molecular role.

5. **Author contribution statement**

S.J.T contributed to conceptualisation of the study, study design, manuscript editing, resources and supervised the project. J.N.A contributed to conceptualisation of the study, study design, conducted experiments and manuscript writing and editing.
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