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Phosphatase POPX2 exhibits dual regulatory functions in cancer metastasis

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KEYWORDS

SILAC mass spectrometry, secretome, tumor angiogenesis, exosome, cytokine, POPX2
phosphatase

ABSTRACT

Cancer metastasis is a complex mechanism involving multiple processes. Earlier, our integrative proteome, transcriptome and phosphoproteome study reported that the levels of serine/threonine phosphatase POPX2 were positively correlated with cancer cell motility through modulating MAPK signaling. Surprisingly, here we found that POPX2 knockdown cells induced more numerous and larger tumor nodules in lungs in longer term animal studies. Interestingly, our analysis of DNA microarray data from cancer patient samples that are available in public databases shows that low POPX2 expression is linked to distant metastasis and poor survival rate. These observations suggest that lower levels of POPX2 may favor tumor progression in later stages of metastasis. We hypothesize that POPX2 may do so by modulation of angiogenesis. Secretome analysis of POPX2-knockdown MDA-MB-231 cells using LC-MS/MS based SILAC quantitative proteomics and cytokine array show that silencing of POPX2 leads to increased secretion of exosomes, which may in turn induce multiple pro-angiogenic cytokines. This study, combined with our earlier findings, suggests that a single ubiquitously expressed phosphatase POPX2 influences cancer metastasis via modulating multiple biological processes including MAPK signaling and exosome-cytokine secretion.

INTRODUCTION

Metastasis is the dissemination of tumor cells from their original sites to a new microenvironment.¹ It is a complex mechanism, which generally involves the following processes: local invasion, epithelial-mesenchymal transition (EMT), intravasation, circulation, arrest and extravasation, proliferation and angiogenesis.² Diverse cellular signaling networks and proteins are reported to regulate the various processes implicated in cancer metastasis. In addition, secreted proteins of the cancer cells are also found to regulate cell motility, invasion, cell matrix remodeling and angiogenesis.³ Secretomics has thus been proposed to be a promising strategy for cancer biomarker discovery and investigation.^{4, 5} Angiogenesis is not only affected by the well-reported pro- or anti-angiogenic macro-factors in the secretome, but also by other micro-effectors, such as exosomes.^{6, 7} In this study, we investigate the roles of POPX2 phosphatase in the regulation of tumor angiogenesis, in particular its influence on the cancer cell secretome.

POPX2 is a PP2C serine/threonine phosphatase. Its expression is ubiquitous and can be found in most human tissues. Thus far, we and others have identified three POPX2 substrates; they are p21-activated kinase (PAK), calcium-calmodulin kinase (CaMK) and the KIF3A kinesin motor protein.⁸⁻¹⁰ We have also reported that the activities of mitogen activated kinases (MAPK1/3) and glycogen synthase kinase 3 (GSK3 α/β) are regulated by POPX2 although they are not direct substrates of POPX2 through our proteome and phosphoproteome analysis.¹¹⁻¹³ POPX2 has been reported to interact with the formin protein, mDia1, and regulate transcription mediated by the serum response factor (SRF).¹⁴ Since MAPK1/3 and GSK3 can also phosphorylate and regulate transcription factors, it is conceivable that the activity of POPX2 may influence other

transcription factors through MAPK1/3 and GSK3.¹³ Our earlier study showed that high POPX2 level is positively correlated to the invasiveness of breast cancer cells.^{11, 13} POPX2 overexpressing cells exhibit higher cell motility and invasive property. POPX2 knockdown impairs initial lung colonization in the metastatic process but has no significant effect on longer-term colonization,¹¹ suggesting a different requirement for POPX2 function in later stages of tumor progression.

Many of the proteins which are functionally linked to POPX2, such as PAK,¹⁵⁻¹⁷ CaMK,^{18, 19} MAPK1/3^{20, 21} and GSK3 β ,²² have been reported to participate in regulating tumor progression, in particular tumor angiogenesis. Interestingly, we observed bigger and more surface nodules in the lungs of mice injected with POPX2 knockdown cells, which led us to further investigate possible involvement of POPX2 in angiogenesis. We found that silencing POPX2 resulted in increased secretion of exosomes, angiogenic factors and cytokines leading to enhanced angiogenesis thus favoring tumor growth. Based on our current and previous findings, we propose that POPX2 can exert dual functions in regulating cancer metastasis. POPX2 can exhibit either inhibition or promotion of metastasis depending on the stages of tumor development.

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MATERIALS AND METHODS

Animal lung metastasis assay

All animal work was done in accordance with protocols approved by the Institutional Animal Care and Use Committee. Female Balb/c nude mice at 4-6 weeks old were used for xenografting studies. For lung metastasis formation, 1×10^6 viable cells stably expressing control or POPX2 shRNA were washed and harvested in HBSS and subsequently injected into the lateral tail vein in a volume of 0.1 ml. Animals were monitored for metastasis-associated morbidity. Whole lungs were harvested and surface tumor foci were counted. All animal experiments were done according to the National Advisory Committee for Laboratory Animal Research (NACLAR) Guidelines.

Tube formation assay

The *in vitro* tube formation assay was optimized based on reported methods.^{23, 24} A 6-well plate was coated with 100 μ l matrigel per well. Similar numbers of HUVECs (human umbilical vein endothelial cells) were plated on the matrigel-coated wells and incubated in EGM-2 (complete endothelial cell medium) for 40 min for attachment. The HUVECs were then incubated in the respective conditioned media for 4 h to allow tube formation. The tubular structures were captured after 4 h using an Axio Observer microscope (Carl Zeiss, Germany) and analyzed by ImageJ software. Ten different fields per condition were captured and quantified. Measurements were expressed as the total tube length and the number of branch points.

CAM (chick chorioallantoic membrane) assay

Details of CAM assay have been described previously.⁶ Briefly, shells of 4-day old fertilized eggs (Chew's Agriculture, Singapore) were sawn equatorially with a cutoff wheel, and the contents were gently transferred into sterilized 80 mm² plastic weighing boats and incubated *ex vivo* in an egg incubator (Brinsea, North Somerset, UK) at 37.7 °C and 76 % humidity under a home-made plastic shield to prevent the albumen from drying out. For each onplant, two nylon mesh layers were embedded into 2 mg/ml collagen mixed with conditioned media (CM). Collagen onplants were placed randomly on CAMs. Distinct blood vessels appearing in the onplants above the plane of the lower mesh were counted.

SILAC (Stable Isotope Labeling by Amino acids in Cell culture) and cell culture

Custom RPMI1640 medium lacking arginine and lysine, ¹³C₆-arginine (heavy R6) and ¹³C₆ ¹⁵N₂-lysine (heavy K8) were purchased from Cambridge Isotopes Laboratories, Inc. (Andover, MA, USA). ¹²C₆-arginine (light) and ¹²C₆-lysine (light) were purchased from Sigma-Aldrich. Dialyzed fetal bovine serum was purchased from Invitrogen. The control and POPX2 knockdown (X2-shRNA1/2) MDA-MB-231 stable cells used in this study were described previously.¹¹ The control cells were cultured in RPMI1640 medium containing heavy arginine and heavy lysine, along with 10 % (v/v) dialyzed fetal bovine serum and 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen, CA, USA). The X2-shRNA1 cells were maintained in RPMI1640 medium containing light arginine and light lysine, along with 10 % (v/v) dialyzed fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen, CA, USA). All cells were grown in 37 °C humidified incubator supplemented with 5 % CO₂. The cells used in the experiments were grown up to seven passages to ensure full incorporation of heavy and light amino acids. The complete incorporation of heavy K8 and R6 into cellular proteins were

confirmed by LC-MS/MS. Table S1 show complete SILAC labeling in control cells with K8 and R6 SILAC reagents in three randomly selected peptides with different abundances.

Conditioned media (CM) collection and concentration

When the cells were grown to 70 % confluence, all cells were washed with 1 × PBS for four times and serum free RPMI1640 medium once, followed by exposure to serum free medium for 6 h to remove serum proteins. Cells were then exposed to fresh serum free medium for another 24 h for secretome/CM collection. Protease inhibitor was added to the CM collected (400 ml each) to minimize protein degradation. The CM were centrifuged at 201 g for 10 min at 4 °C and filtered (0.22 µm pore size) to remove cell debris. The CM were then concentrated to 200-300 µl using 15 ml ultra filter with 10 kDa cut-off (Millipore) by centrifugation at 3220 g at 4°C. The concentrated CM were collected into a sterile 1.5 ml tube and stored at -80 °C until use.

SDS-PAGE

Conditioned media were dissolved in 10 % SDS, along with complete protease inhibitor cocktail (Roche) and phosSTOP phosphatase inhibitor cocktail (Roche) and dithiothreitol (Sigma). Protein concentration was determined by Bicinchoninic Acid (BCA) protein assay. Equal amounts of protein (50 µg each) from heavy and light samples were mixed at a 1:1 ratio and stored at -80 °C for the following experiments. Proteins (~ 50 µg) were separated on 4-12% NuPAGE gradient gel (Invitrogen). After SDS-PAGE, coomassie blue staining was adopted to visualize protein bands.

In-gel digestion

The whole lane was cut into 10 fractions for in-gel digestion. The gel pieces were washed with destaining solution several times until the color totally disappeared, followed by dehydration with 100 % acetonitrile and vacuum dried. Subsequently, proteins were reduced with 10 mM dithiothreitol at 56 °C for 1 h, followed by alkylation with 55 mM iodoacetamide at room temperature for 45 min. Gel pieces were dehydrated again with 100 % acetonitrile and vacuum dried. In-gel digestion was carried out by trypsin (Sigma-Aldrich) at 1:100 (m/m), and at 37 °C overnight. The supernatant was then transferred to a new 1.5 ml tube and the peptides were extracted with extraction buffer (50 % acetonitrile + 5 % acetic acid). Gel pieces were vortexed for 10 sec and sonicated for 30 min. The supernatant was collected into the same tube. The extraction steps were repeated several times for complete peptide extraction. Peptides were vacuum dried and stored at -80 °C until LC-MS/MS analysis.

LC-MS/MS analysis.

Three independent biological replicates (BR1, BR2 and BR3) were performed (Figure 3A). The tryptic peptides were resuspended in 0.1% formic acid and each fraction was injected twice for technical replicates (TR1 and TR2) in LC-MS/MS. The peptides were separated and analyzed on a Dionex Ultimate 3000 RSLCnano system coupled to a Q-Exactive (Thermo Fisher, MA) as previously described.²⁵ Approximately 2 µg of peptide from each fraction was injected into an Acclaim peptide trap column (Thermo Fisher, MA, USA) via the Dionex RSLCnano auto-sampler. Peptides were separated in a Dionex EASY-Spray 75 µm × 10 cm column packed with PepMap C18 3µm, 100 Å; (Thermo-Scientific, MA, USA) at 35°C. The flow rate was 300 nl min⁻¹. Mobile phase A (0.1% formic acid in 5% acetonitrile) and mobile phase B (0.1% formic

acid in 90% acetonitrile) were used to establish a 60 min gradient. Peptides were then analyzed on Q-Exactive with an EASY nanospray source (Thermo Fisher, MA) at an electrospray potential of 1.5 kV. A full MS scan (350-1600 m/z range) was acquired at a resolution of 70,000 at m/z 200 and a maximum ion accumulation time of 100 ms. Dynamic exclusion was set as 15 s. The resolution of the HCD spectra was set to 35,000 at m/z 200. The AGC settings of the full MS scan and the MS² scan were 3E6 and 2E5, respectively. The 10 most intense ions above the 2000 count threshold were selected for fragmentation in HCD with a maximum ion accumulation time of 120 msec. An isolation width of 2 was used for MS². Single and unassigned charged ions were excluded from MS/MS. For HCD, the normalized collision energy was set to 28%. The underfill ratio was defined as 0.2%.

Raw data files of the six replicates (three biological replicates × two injections) were processed and searched as six experiments using MaxQuant (v1.5.2.8)^{26, 27} and the Uniprot human protein database (downloaded on 15 July 2015, 90478 sequences, 35890546 residues) together with the common contaminant proteins. Standard search type with 2 multiplicity, 3 maximum labeled AAs and heavy labeled Lys8 and Arg6 were used for the SILAC quantitation. The database search was performed using the Andromeda search engine bundled with MaxQuant using the MaxQuant default parameters for Q-Exactive Orbitrap mass spectrometer. Briefly, the first and main searches peptide mass tolerance were 20 ppm and 4.5 ppm respectively while the MS/MS match tolerance was 20 ppm with FTMS de-isotoping enabled. The absence of two trypsin cleavage sites per protein was allowed. Carbamidomethylation (C) was set as a fixed modification. Oxidation (M), and deamidation (NQ) were set as variable modifications. The search was performed in the revert decoy mode with PSM FDR, protein FDR and site decoy fraction set to 0.01.

A total of 1560 proteins with at least two unique peptides were identified in the secretome by Andromeda in MaxQuant with FDR < 1% (Table S2). The scatter plots of inter-technical replicates and inter-biological replicates were used to evaluate the technical and biological reproducibility of the experiments respectively (Table S2). All peptide sequences were listed in Table S5.

LC-MS/MS raw data of the six replicates (three biological replicates × two injections) and results for protein and peptide identification and quantification from MaxQuant were submitted to the ProteomeXchange Consortium²⁸ via the PRIDE data repository with the dataset identifier PXD003940. The submission details are:

Project Name: Phosphatase POPX2 exhibits dual regulatory functions in cancer metastasis

Project accession: PXD003940

Project DOI: Not applicable

Reviewer account details:

Username: reviewer43299@ebi.ac.uk

Password: sbeVCOgl

Cytokine protein array and MMP (Matrix Metalloproteinase) antibody array

RayBio Human Cytokine Array V (Catalog No.: AAH-CYT-5-2) was purchased from RayBiotech Inc., USA. The human MMP antibody array membrane (Catalog No.: ab134004) was purchased from Abcam, UK. To prepare the conditioned media, cells were plated in 100 mm tissue culture dishes and cultured in DMEM with 10 % fetal bovine serum until they reached 70 % to 80% confluence. The cells were washed twice with serum-free DMEM, and incubated for 24 hrs in serum-free DMEM. Assay for cytokine antibody arrays was carried out as per

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3 manufacturer's instructions. Briefly, cytokine array membranes were blocked with 5 %
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5 BSA/TBS (0.01 M Tris-Cl pH 7.6/0.15 M NaCl) for 1 h. Membranes were then incubated with 2
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7 ml of conditioned media prepared from different cell lines. After extensive washing with
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9 TBS/0.1 %v/v Tween 20 (three times, 5 min each) to remove unbound materials, the membranes
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11 were incubated in 1:250-diluted biotin-conjugated primary antibodies at room temperature for 1
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13 to 2 h and washed as described above before incubation in 1:1000-diluted horseradish
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15 peroxidase-conjugated streptavidin for 1h at room temperature. Unbound HRP-streptavidin was
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17 washed out with TBS/0.1 % Tween 20. Finally the signals were detected by ECL system. The
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19 MMP antibody array analysis was done as described in the manufacturer's manual and all the
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21 reagents were provided by the kit. Densitometric values of the spots were quantified using
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23 Protein Array Analyzer of ImageJ and listed in Table S4.
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34 **RNA extraction and Real-time quantitative PCR (qPCR) analysis**

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37 Total RNA of the cells were extracted using RNeasy Mini Kit (Qiagen), followed by synthesis of
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39 cDNA from 2 µg of total RNA using SuperScript® VILO™ cDNA Synthesis Kit (Life
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41 Technology). Real-time PCR was performed using SYBR® Green Real-time PCR Master Mixes
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43 and StepOnePlus™ Real-Time PCR Systems (Life Technology) according to the manufacturer's
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45 instruction. The relative gene expression levels were calculated using $2^{-\Delta\Delta C_T}$ method. The
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47 expression level of the target gene was normalized against GAPDH values. The data presented
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49 were expressed as fold changes with respect to their corresponding controls. The primers used
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51 were listed in Table S3.
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Cell adhesion assay

The 96-well dish was pre-coated with BSA (10 mg/ml), fibronectin (10 µg/ml) or collagen (100 µg/ml). Similar numbers (2×10^4) of MDA-MB-231 cells stably expressing either control shRNA or POPX2 shRNA1/2 were seeded in the coated 96-well dish and incubated for 30 min for attachment. After cell attachment, the 96-well dish was rinsed with $1 \times$ PBS to remove non-adhering cells. Cells were then fixed with 5 % glutaraldehyde/PBS at room temperature for 20 min before washing three times with distilled water, followed by staining with 0.1 % (w/v) crystal violet at room temperature for 1 h. The crystal violet was then solubilized using 10 % acetic acid and the absorbance was read by a spectrophotometer at 570 nm.

Immunoblotting, antibodies and plasmid constructs

Fifty µg protein from each cell lysate were subjected to reducing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 12 % resolving gels for 10-70 kDa proteins of interest or 10 % resolving gels for 20-100 kDa proteins of interest. SDS-PAGE was performed at 120 V at room temperature. Resolved proteins were subsequently transferred to nitrocellulose membranes (Bio-Rad, USA) at 80 V for 2 h at room temperature. The blot was blocked with 5 % (w/v) skimmed milk in 0.05% (v/v) Tween 20/Tris-buffered saline for 1h at room temperature, and probed with respective antibodies, followed by secondary antibodies dissolved in 5 % (w/v) skimmed milk. The blots were developed with either Novex[®] ECL Chemiluminescent Substrate Reagent Kit (Invitrogen) or Lumigen[®] (GE Healthcare). All western blot analyses were conducted with at least three independent experiments. The antibodies used in our work were anti-POPX2 (self-raised), anti-CD81 (Santa Cruz), anti-CD9 (Santa Cruz), anti-HSP70 (Santa Cruz), anti-HSP90 (Santa Cruz), anti-COL6A1 (Santa Cruz), anti-LAMA5 (Santa Cruz), anti-

actin (Millipore), anti-E-cadherin (BD transduction), anti-vimentin (BD transduction), anti- β -catenin (Sigma), anti-N-cadherin (BD transduction). The POPX2 siRNA sequence is 5'-ACCGCGCCUACUUUGCUGUGUUUGA-3', and siPOPX2-resistant construct (X2^r) was generated in pXJ40-GFP backbone.

Immunostaining

Similar numbers of cells were grown on coverslips in a 6-well dish until 90 % confluence before fixing with 5 % paraformaldehyde for 20 min at room temperature. The fixed cells were then permeabilized with 0.1 % TritonX-100/PBS for 10 min at room temperature, followed by blocking with 4 % BSA/PBS for at least 1 h at room temperature. Primary antibodies were 1:100 diluted in 1 % TritonX-100/PBS and incubated with the cells at 4 °C overnight. Secondary antibodies were also diluted 1:100 in 1 % TritonX-100/PBS and incubated with the cells at room temperature for 1h, followed by mounting with DAPI. Images were captured using an Axio Observer microscope (Carl Zeiss, Germany) and analyzed by ImageJ software.

Bioinformatics

Pathway analysis

The differentially expressed proteins were submitted to DAVID (Database for Annotation, Visualization and Integrated Discovery)²⁹ using their official_gene_symbol. The KEGG pathway annotations were downloaded and plotted. *P-value* for each KEGG pathway was - Log converted and plotted.

Secreted protein prediction

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3 Secreted proteins undergoing classical secretory pathway were predicted with SignalP³⁰ if the
4 protein received a signal peptide probability of ≥ 0.9 . Secreted proteins undergoing non-classical
5 secretory pathway were predicted with SecretomeP³¹ if the protein received a NN score ≥ 0.6 . It
6 is also possible that shed membrane receptors are released to the extracellular space. This class
7 of proteins was predicted with TMHMM.³² Proteins associated with exosome were obtained
8 from the ExoCarta database.³³ Proteins localized in the extracellular space (GO: 0005615) were
9 also retrieved from Gene Ontology.
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20 21 22 Cancer survival and metastasis analysis 23

24 Kaplan-Meier plotter^{34, 35} was employed to analyse the association between POPX2 (gene
25 symbol: PPM1F) expression and breast cancer survival rate using microarray data downloaded
26 from Gene Expression Omnibus (GEO), European Genome-phenome Archive (EGA) and The
27 Cancer Genome Atlas (TCGA) databases. The association between POPX2 expression and
28 relapse free survival (RFS) comprising 3557 patient samples, overall survival (OS) comprising
29 1117 patient samples, distance metastasis free survival (DMFS) comprising 1610 patient samples
30 were performed individually. All the other parameters were set as defaulted. Patient samples
31 were split into two groups based on various quantile expressions of the PPM1F: patients with
32 low PPM1F expression group (black) and patients with high PPM1F expression group (red). The
33 two patient cohorts were compared by Kaplan-Meier survival plot. The Hazard Ratio (HR) with
34 95 % confidence intervals and logrank P value were calculated. The mRNA expressions of
35 PPM1F at the primary site and metastasis site were compared in breast cancer, head and neck
36 cancer, kidney cancer, lung cancer, prostate cancer using Oncomine database.³⁶ For all these
37 analyses, patient samples were split into two groups: patient samples from the primary site and
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from the metastasis site with the specific number of patients indicated. The expression levels of PPM1F between these two groups were compared based on the mean of log2 median-centered expression ratio and *p-value* was calculated.

POPX2 expression analysis in different breast cancer sub-types

The mRNA expressions of PPM1F in a panel of basal or luminal breast cancer cell lines were extracted from Gene expression-based Outcome for Breast cancer Online (GOBO) analysis tool³⁷ and plotted. The mRNA expressions of PPM1F in the triple-negative (TNBC, also named as basal-like cells) and non-triple-negative (non-TNBC) breast cancer cells were compared in several breast cancer datasets (Tabchy Breast, Miyake Breast, Esseman Breast, Chin Breast, and Kao Breast) retrieved from Oncomine database.³⁶ For all these analyses, the cell lines were split into two groups, triple-negative (TNBC) and non-triple-negative (non-TNBC) breast cancer, with the specific number of samples indicated. The expression levels of PPM1F of these two groups were compared based on the mean of log2 median-centered expression ratio and *p-value* was calculated.

RESULTS

POPX2 knockdown enhanced tumor morbidity and metastasis

Our earlier studies have shown that POPX2 knockdown leads to reduced breast cancer cell motility possibly through modulating MAPK1/3 signaling.¹³ In addition, diminished initial attachment of POPX2 knockdown cells in the lung was observed.¹¹ These initial observations suggest that lower levels of POPX2 may effectively suppress tumor progression in early metastatic processes. Since not much is known about the roles of POPX2 during late stages of metastasis, we use a nude mouse model to examine the effects of POPX2 knockdown on long-term metastasis *in vivo*.

Stable cell lines of MDA-MB-231 breast cancer cells expressing control shRNA, POPX2-shRNA1 or POPX2-shRNA2 (Figure 1A, B) were injected into nude mice via their tail veins. The mice were monitored for metastasis-associated morbidity. Mice injected with both POPX2 knockdown cell lines exhibited parallel and dramatically decreased survival (Figure 1C), suggesting low POPX2 expression was associated with poor survival in mice. Moreover, the number of surface nodules in the lungs was markedly increased in mice injected with POPX2 knockdown cells compared to mice injected with control cells. The tumor nodules were also larger in sizes in mice injected with POPX2 knockdown cells (Figure 1D, E). Our data demonstrated that silencing POPX2 resulted in larger and more numerous tumors at the metastatic sites.

We also employed Kaplan-Meier survival analysis^{34, 35} to plot the breast cancer survival curves in relation to the gene expression of POPX2 (gene symbol, PPM1F) based on DNA microarray

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data from thousands of patient samples. We found significant correlations between low POPX2 expression and decreased probability of relapse free survival (RFS) and overall survival (OS). Low POPX2 expression was also associated with poor distant metastasis free survival (DMFS), although not significant enough (Figure S1A). These analyses indicate that low POPX2 expression may predict poor survival in patients with breast cancer, which is consistent with the results obtained from our mouse model (Figure 1C). In addition, POPX2 gene expression at primary tumor and metastasis tumor sites was also compared in multiple types of cancer using DNA microarray data downloaded from Oncomine.³⁶ We found lower POPX2 expression associated with metastasized tumors from breast, neck and head, kidney, lung, and prostate cancers (Figure S1B), which was consistent with the more severe metastasis found in the mice injected with POPX2 knockdown cells (Figure 1D, E).

Conditioned media from POPX2 knockdown cells contributed to angiogenesis

POPX2 substrates such as PAK,¹⁵⁻¹⁷ CaMK,^{18, 19} and proteins in the signaling pathways regulated by POPX2 such as MAPK1/3^{20, 21} and GSK3β²² have been reported to regulate tumor progression, particularly in tumor angiogenesis. We thus proceeded to analyze the roles of POPX2 in tumor angiogenesis using both *in vitro* and *in vivo* angiogenesis assays.

For the *in vitro* angiogenesis assay, human umbilical vein endothelial cells (HUVECs) were used as the angiogenesis model because they are capable of forming capillary-liked structures rapidly when cultured on matrigel in the presence of fetal bovine serum (FBS) and growth factors. The HUVEC tube formation system is thought to mimic the angiogenesis process.²³ Endothelial cells maintained in basal media, which are unable to form regular tubes, served as the negative control

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3 in our experiment. Whereas endothelial cells maintained in complete media supplemented with
4 FBS and growth factors, which are capable of forming regular tubular networks, were used as the
5 positive control (Figure 2A). To examine how POPX2 could affect angiogenesis, the conditioned
6 media collected from both the control and POPX2 knockdown MDA-MB-231 cells were used
7 for the *in vitro* angiogenesis assay. Total tube length and branch points were calculated. We
8 found that the tubular network was more regular when the endothelial cells were cultured in the
9 conditioned media obtained from POPX2 knockdown cells compared to those from control cells
10 (Figure 2A). Moreover, the total tube length and number of branch points were increased in
11 response to the conditioned media collected from POPX2 knockdown cells (Figure 2B, C).
12 When we introduced a siRNA-resistant POPX2 construct into POPX2-knockdown cells, we were
13 able to revert to the control phenotype in the tube formation assays. This observation suggests
14 that the enhancement in angiogenesis when POPX2 is silenced is specific and dependent on
15 POPX2 levels (Figure S2A-D). Our observations suggest that factors or proteins released by
16 POPX2 knockdown cells can induce angiogenesis *in vitro*.
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39 Next, we examined the effects of POPX2 on tumor angiogenesis using the well-established *in*
40 *vivo* CAM (chick chorioallantoic membrane) assay system.³⁸ Briefly, conditioned media
41 collected from control MDA-MB-231 cells and two independent lines of POPX2 knockdown
42 cells were separately mixed with collagen and randomly placed on CAM for 10 days to induce
43 angiogenesis. Representative images of control and POPX2 knockdown cells induced
44 angiogenesis are shown in Figure 2D. Onplant-induced new blood vessel outgrowth was counted
45 and plotted (Figure 2E). Conditioned media from POPX2 knockdown cells induced 1.8- to 2.4-
46 fold increase in angiogenesis compared to those from control (Figure 2E), suggesting that factors
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or proteins secreted from POPX2 knockdown cells induced angiogenesis *in vivo*, which is in agreement with our *in vitro* experiments (Figure 2A-C).

Quantitative mass-spectrometry analysis revealed secretomic alterations in POPX2 knockdown cells

In order to determine the effects of POPX2 on the secretome of breast cancer cells, we analyzed the secretome of POPX2 knockdown cells versus those from the control cells using LC-MS/MS-based SILAC quantitative proteomics. In this experiment, control cells were maintained in heavy medium supplemented with heavy arginine R6 and lysine K8, whereas the POPX2 knockdown MDA-MB-231 cells were grown in light medium supplemented with light arginine and lysine. Medium from multiple independently grown cells were conditioned for 24 h before harvested for secretome analysis. To reduce variation and increase reproducibility, three biological replicates along with two technical replicates were carried out (Figure 3A). Identified protein expression ratios were calculated as control vs POPX2 knockdown (heavy vs light). To evaluate the inter-technical and inter-biological reproducibilities, the SILAC ratio between technical replicates and biological replicates were scatter plotted and evaluated with linear regression analysis (Table S2). The R-square values varied from 0.92 to 0.99 in the technical replicates, indicating highly reproducible results in each LC-MS/MS injection. As expected, the biological replicates have larger variations with R-square values varied from 0.71 to 0.82. We performed the statistical analysis using all biological and technical replicates. P-value was calculated using Student's t-Test and a volcano plot was plotted to determine the cut off level of the data set (Figure S2E). Accordingly, a total of 609 proteins were enriched with statistical significance (p-value < 0.05) (Table S2_protein list). We further plot the ratios and designated the top 30% proteins as the

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3 differentially regulated hits. Therefore, proteins with ratios > 1.41 ($\text{Log}_2\text{Ratio} > 0.5$) were
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5 classified as down-regulated by POPX2 silencing, whereas proteins with ratios < 0.70
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7 ($\text{Log}_2\text{Ratio} < -0.5$) were classified as up-regulated by POPX2 silencing. Accordingly, a total of
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9 230 proteins were regarded as differentially-regulated hits in response to POPX2 silencing.
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15 Next, we validated the differentially-regulated hits (Figure S3). We noticed decreased levels of
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17 two ECM components in the secretome of POPX2 knockdown cells: (1) collagen (COL6A1), (2)
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19 laminin (LAMA5) (Figure S3C), which are the main components of the tumor microenvironment
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21 and important for tumor angiogenesis.³⁹ The up-regulation of matrix metalloproteinases (MMP3)
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23 and down-regulation of the matrix metalloproteinases inhibitor (TIMP2) were validated for both
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25 protein (Figure S3A-B and Table S4) and transcript levels (Figure S3D). The matrix
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27 metalloproteinases contribute to angiogenesis through modulating multiple signaling pathways.⁴⁰
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34 **Enrichment of predicted exosomes-associated proteins in the secretome of POPX2**

35 **knockdown cells**

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38 We found that not all the proteins identified in the secretome analysis are localized to the
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40 extracellular space. Contamination by non-secreted proteins during cell lysis or cell death is
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42 unavoidable even though the secretome sample preparation steps were done very carefully.
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44 Therefore, secreted protein prediction by different bioinformatics tools is necessary.⁴¹ Up to 99
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46 % of the total identified proteins are predicted to be secreted proteins (Figure 3B). The majority
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48 of the secreted proteins are from the highly evolved classical secretory pathway, which contains
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50 N-terminal signal peptides for directing them to the extracellular space.⁴¹ About 38% of proteins
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52 in our data set contain such signal peptides as analyzed by SignalP.³⁰ Proteins without the N-
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terminal signal peptide can be secreted via non-classical secretory pathways⁴²⁻⁴⁴ and are characterized using SecretomeP.³¹ About 31% of the total proteins are found to be secreted through the non-classical secretory pathways. TMHMM³² identified 17% of the proteins in our secretome as shed membrane receptors. In addition, Gene Ontology identified 31% of the proteins in the secretome as proteins localized to the extracellular space (GO: 0005615).

Apart from the different secretory pathways, proteins are also released through the exosomes. Links between the exosome and tumor angiogenesis have been uncovered recently as the genomic and proteomic materials buried in the exosomes are reported to be pro-angiogenic.^{45, 46} Interestingly, up to 91% of the proteins in our secretome data are found to be exosome associated proteins through the use of ExoCarta database³³ (Figure 3B). We found that the secreted levels of HSP70 and HSP90, the top 25 proteins that are often identified in the exosome,³³ were dramatically increased in the secretome of POPX2 knockdown cells (Table S2). In order to confirm the links between POPX2 and the regulation of exosome secretion, western blot analysis was performed to examine the levels of HSP70/90, and another two well-documented exosome markers, CD81 and CD9.^{45, 47} We found significant enrichment of exosomes in the secretome obtained from POPX2 knockdown cells (Figure 3C). This observation was consistent with the enhanced angiogenesis induced by conditioned media from POPX2 knockdown cells (Figure 2).

Cytokine protein array analysis uncovers a subset of pro-angiogenic factors induced by POPX2 knockdown

It has been reported that tumor exosome is able to promote angiogenic progression by inducing cytokine signaling.^{6, 48, 49} Since we observed differential levels of exosomes, we next investigated

whether lower levels of POPX2 could modulate the cytokines profiles which in turn contribute towards increased angiogenesis. A cytokine protein array-based secretome analysis was used to examine cytokine changes in response to POPX2 knockdown. Secretome collected from both control and POPX2 knockdown cells were incubated with an antibody array membrane printed with 80 distinct cytokine specific antibodies in biological triplicates (Figure 4A, B). The levels of the various cytokines were firstly normalized with the internal positive control, and the fold increase of each cytokine was calculated as POPX2 knockdown vs control (Table S4). Cytokines with fold increase of more than two were selected for further investigation (Figure 4C). The levels of three quarters of the 80 distinct cytokines remained unchanged (Table S4 and Figure S3E_EGF validation). Of the 16 up-regulated cytokines, fibroblast growth factor (FGF-7), platelet-derived growth factor (PDGF-BB), hepatocyte growth factor (HGF) were significantly increased in the conditioned media derived from POPX2 knockdown cells (Figure 4A-C). The transcript levels of these cytokines were also significantly increased (Figure S3E). FGF has been identified as a pro-angiogenic factor. It has been reported that inhibition of FGF abrogates tumor angiogenesis through suppressing tumor growth and decreasing blood vessel density.⁵⁰ PDGF has been reported to directly stimulate angiogenesis *in vitro* by inducing endothelial cell growth and tube formation.⁵¹ Up-regulation of these well-known pro-angiogenic cytokines (Figure 4) is consistent with the increased angiogenesis (Figure 2) and tumor growth (Figure 1) observed in response to silencing POPX2. Further study of the pro-angiogenic factors secreted in response to POPX2 depletion using the KEGG pathway analysis revealed that POPX2 knockdown stimulated changes in pathways related to "cytokine-cytokine receptor interaction", "chemokine signaling", "melanoma", "pathway in cancer", "MAPK signaling", "renal cell carcinoma", and

"regulation of actin cytoskeleton" ($p\text{-value} < 0.05$) (Figure 4D). All data obtained are consistent with previously reported signaling pathways that POPX2 was found to be involved.¹³

Pathway analysis implicated the involvement of POPX2 in breast cancer cell-ECM and cell-cell interactions

In addition to analyzing the effects of POPX2 knockdown on cytokine secretion, we also performed pathway analysis on the secretome to obtain a comprehensive understanding of POPX2 related pathways. Two important cancer metastasis and angiogenesis related signaling pathways are over-represented. They are "focal adhesion" and "ECM-receptor interaction" ($p\text{-value} < 0.05$) (Figure 5A). We have previously reported that POPX2 positively regulates focal adhesion and actin cytoskeleton¹³. However, the impact of POPX2 in cell-ECM and cell-cell interactions has not been investigated. Hence, we further explored the effects of silencing POPX2 on cell-ECM and cell-cell interactions.

Based on the known roles of POPX2 in tumor metastasis and the overrepresented signaling pathways related to "cell-ECM interaction", we hypothesize that POPX2 could be involved in the regulation of cell attachment to the extracellular matrix. We next studied the cell attachment property of POPX2 knockdown MDA-MB-231 cells. We observed that POPX2 knockdown led to defective attachment onto both fibronectin and collagen coated surfaces (Figure 5B). This is consistent with our previous observation that POPX2 knockdown MCF7 breast cancer cells are defective in attachment onto collagen-coated surfaces.¹¹ However, the effect was less obvious for POPX2 knockdown MCF7 cells on fibronectin-coated surfaces.

Several cytokines, such as FGF and HGF, which are up-regulated in the cytokine array (Figure 4), have been shown to trigger EMT.⁵² The EMT process is essential for cancer metastasis and angiogenesis.⁵³ A number of EMT markers have been documented.⁵² Among which, E-cadherin and β -catenin are classified as epithelial markers, whereas vimentin and N-cadherin are known as mesenchymal markers. The loss of E-cadherin and β -catenin expression and the gain of vimentin and N-cadherin expression are hallmarks of EMT.⁵³ In our current study, the levels of the various EMT markers were examined in both MCF7 (epithelial cell type) and MDA-MB-231 (mesenchymal cell type) cells with or without POPX2 knockdown. E-cadherin and β -catenin distribution at the cell-cell junctions was significantly reduced when observed using immunostaining in POPX2-knockdown MCF7 cells (Figure 6A). On the contrary, increased N-cadherin levels were observed in POPX2-knockdown MCF7 cells from both immunostaining and immunoblotting experiments (Figure 6A, C). Vimentin was not expressed in MCF7 (Figure 6C). Reduced β -catenin distribution at the cell-cell junctions was also observed in POPX2-knockdown MDA-MB-231 cells (Figure 6B). Increased vimentin and N-cadherin levels were observed in POPX2-knockdown MDA-MB-231 cells from both immunostaining and immunoblotting experiments (Figure 6B, C). E-cadherin was not expressed in MDA-MB-231 (Figure 6C). However, the levels of β -catenin, remained unchanged with or without POPX2 knockdown (Figure 6C). These observations suggest that POPX2 knockdown might promote EMT in breast cancer cells.

DISCUSSION

A global view of the POPX2 phosphatase in tumor progression

In our earlier study, high levels of POPX2 are found to correlate with breast cancer cell invasiveness and lung colonization in early stages of metastasis.¹¹ However, high levels of POPX2 do not seem to support long-term tumor growth in the newly-formed metastatic microenvironment.¹¹ These observations led us to hypothesize that POPX2 may play distinct roles at different stages of tumor progression. Although POPX2 knockdown MDA-MB-231 cells showed poor initial attachment to the lungs, larger and more numbers of tumor nodules were observed in the lungs of nude mice in longer-term study (Figure 1). The long-term survival of the mice injected with POXP2-knockdown MDA-MB-231 cells was also reduced compared with control (Figure 1). One possible contributing factor could be the enhanced angiogenic potential of the POPX2 knockdown cells, which is consistent with our *in vitro* and *in vivo* angiogenesis assays (Figure 2). These observations suggest that lower levels of POPX2 favor tumor angiogenesis and tumor expansion in the new micro-environment of the metastatic cancer cells.

Tumor metastasis is a complex mechanism involving multiple biological processes. It is not solely dependent on cancer cell invasiveness and angiogenesis.⁵⁴ Thus, we further explore the roles of POPX2 in other tumor metastatic processes. Applying KEGG pathway analysis on the secretome, we noticed the overrepresented “ECM-receptor interaction” pathway (Figure 5A) and identified changes in ECM proteins secreted by POPX2 knockdown cells, such as laminin and collagen (Figure S3C). Altered levels of ECM proteins may impact on angiogenesis and vascular development in the longer term.⁵⁵ On the other hand, ECM expressions may also affect efficient cell-matrix interaction, which we have confirmed using cell attachment assays to evaluate initial

attachment of cells to the matrix (Figure 5B). POPX2 knockdown cells do not attach well on fibronectin and collagen coated surface when compared to control cells.

Epithelial-mesenchymal transition is required for the initiation of cancer invasion. POPX2 may participate in the EMT process. Indeed, we saw the loss of E-cadherin and β -catenin expression and gain of vimentin and N-cadherin expression which are hallmarks of EMT⁵⁶ in POPX2 knockdown cells (Figure 6). These observations suggest that lower levels of POPX2 might favor EMT progression. Moreover, POPX2 knockdown cells exhibit lower GSK3 β kinase activity¹³ which in turn stimulates Snail transcription to repress E-cadherin expression and to promote EMT progression in breast tumor.⁵⁷

Based on all the observations, we are able to obtain for the first time a global view of the involvement of a phosphatase in tumor progression. More interestingly, our current findings combined with earlier reports suggest that although high levels of POPX2 may favor tumor cell motility and invasiveness at the earlier metastatic process,¹¹ once successfully attached to a new organ, in this case the lungs, low levels of POPX2 favor tumor cell maintenance and expansion at late metastatic stage (this study). It is possible that different levels of POPX2 activities are required at different stages of cancer metastasis: EMT, cell motility, invasion and angiogenesis. Hence the regulation of POPX2 is important in the control of cancer metastasis. Our study raises the possibility that cell motility may not be a good indicator for cancer metastasis and prognosis. Candidate protein cannot simply be classified as either tumor promoter or suppressor without drawing a global picture of its role throughout the different stages of metastasis. As such, targeted drug development should be more tumor-stage specific.

Low levels of POPX2 favor tumor angiogenesis through modulating exosome-cytokine signaling

Since low level of POPX2 elicited paracrine signaling, which induces tumor angiogenesis (Figure 2), we decided to investigate the secretome of POPX2-knockdown MDA-MB-231 cells. In this report, we adopted a SILAC based proteomics approach in combination with signal peptide analysis and predictions using different prediction programs and databases. From our secretome analysis, we found elevated levels of exosomes secreted by POPX2 knockdown cells (Figure 3B and C). Exosomes have been reported to induce angiogenesis.⁴⁵ Apart from increased exosome levels, we also found reduced level of ROBO4 (Table S2) which is potent negative regulator of angiogenesis.⁵⁸ The combined effects of increased exosome secretion and reduced levels of angiogenesis inhibitor may result in the enhancement of angiogenesis by the secretome of POPX2 knockdown cells.

It has been reported that tumor exosome can promote endothelial angiogenic responses by modulating the secretion of cytokines, thus contributing to tumor metastatic potential.^{6, 48, 49} Results from screening a cytokine array have revealed increased levels of angiogenic proteins, such as FGF and PDGF,^{50, 59} in the secretome of POPX2 knockdown cells (Figure 4). Interestingly, we did not observe many common angiogenic factors between our secretomic data and cytokine array screening. This could be due to the very low abundance of cytokines and the sensitivity of the methodologies used. Nevertheless, we used both methods to get complimentary datasets for a more complete coverage of the secretome and we did identify proteins that might be responsible for the observed increase in angiogenesis.

CONCLUSION

In conclusion, the phosphatase POPX2 shows dual roles in the regulation of metastasis by modulating multiple biological processes (Figure 7). Different levels of POPX2 activities are required at different stages of cancer metastasis. High levels of POPX2 are found to favor breast cancer cell motility and invasiveness at the early metastatic stage through modulating MAPK1/3 signaling¹³. Further analysis of POPX2 levels in the triple negative breast cancer (TNBC, also named as basal-like cells) cell lines (Figure S4A) and from microarray data available from multiple breast cancer datasets retrieved from Oncomine (Figure S4B), we are able to find the association of high POPX2 levels to higher invasiveness of the tumor (Figure S4). Consistently, the levels of POPX2 are higher in the more invasive triple-negative breast tumor cells than the less invasive non-triple negative cells. On the other hand, low levels of POPX2 favor exosome secretion which in turn modulate the cytokines profiles, thus contributing to tumor angiogenesis at later stages of metastasis. Hence it is important to adopt careful choices of biomarkers and strategies of therapeutic intervention to target different stages of cancer. What contributes to the switch of POPX2 signaling in the early and late stages still remains to be explored. Since tissue rigidity and topology varies widely, it is possible that tissue microenvironment differences between the primary and metastatic sites may fine tune POPX2-mediated signaling.

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3 **ASSOCIATED CONTENT**
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7 **Supporting Information**
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10 The Supporting Information is available free of charge on the ACS Publications website at
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17 Figure S1. Low expression of POPX2 is linked to poor survival rate and distant metastasis.
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19 Figure S2. Rescue of POPX2 knockdown-induced tube formation and Volcano Plot. Figure S3.
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21 Validation of secretome and cytokine array data. Figure S4. POPX2 is highly expressed in the
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23 more invasive triple-negative breast cancer cells (TNBC). Table S1. Complete SILAC labeling in
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25 control cells. Table S2. MaxQaunt identified and quantified protein groups. Table S3. qPCR
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27 primer list. Table S4. Quantification of densitometric values of spots for cytokine and MMP
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29 array. Table S5. Peptide list.
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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

CAM, chick chorioallantoic membrane; CaMKII, calmodulin-dependent protein kinase II; DAVID, Database for Annotation, Visualization and Integrated Discovery; DMFS, distance metastasis free survival; ECM, extracellular matrix; EMT, epithelial and mesenchymal transition; FGF, fibroblast growth factor; GOBO, Gene expression-based Outcome for Breast cancer Online; GSK3, glycogen synthase kinase 3; HUVEC, human umbilical vein endothelial cell; KEGG, Kyoto Encyclopedia of Gene and Genomes; MMP, matrix metalloproteinase; OS, overall survival; PAK, p21-activated kinase; PDGF, platelet-derived growth factor; RFS, relapse free survival; SILAC, Stable Isotope Labeling of Amino acid in Cell culture; TMHMM, TransMembrane prediction using Hidden Markov Models.

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FIGURE LEGENDS

Figure 1. POPX2 knockdown induces morbidity and increases surface nodules in experimental lung metastasis. MDA-MB-231 tumor cells with stable expression of either control shRNA or POPX2 shRNA1 and 2 were injected into the tail veins of nude mice. The knockdown efficiency of POPX2 was confirmed at both (A) protein (western analysis) and (B) transcript levels (qPCR). (C) Morbidity from experimental metastasis was assessed by calculating the survival rate as a function of time after experimental metastasis. (D) Representative images of endpoint assays conducted at 8 weeks after injection were shown. Scale bar: 5 mm. (E) The number of lung surface nodules was counted and plotted. Error bars represent standard deviations. The significance of difference was calculated by Student's t-test. * $P < 0.05$, **** $P \leq 0.0001$. Photographs courtesy of Hei Chan. Copyright 2016.

Figure 2. Conditioned media from POPX2 knockdown cells contribute to angiogenesis both *in vitro* and *in vivo*. (A) Similar numbers of HUVECs (human umbilical vein endothelial cells) were incubated in EBM (endothelial cells basal medium), EGM (EBM + 10 % FBS + essential endothelial cells growth factors), control-CM, and X2-shRNA1/2-CM (conditioned media obtained from control and two independent POPX2-knockdown MDA-MB-231 cell lines, respectively). Images of tube formation on matrigel was captured 4 h after incubation. Representative images of independent biological triplicate were shown. Scale bar: 200 μm . (B) Total tube length and (C) the number of branch points were quantified from at least 10 random fields of view for each sample. Results presented were the average from three independent biological experiments. Error bars represent standard deviation. Student's t-test was used to compare the difference between groups. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$. (D) Collagen

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onplants, with conditioned media from control and two independent POPX2 knockdown cell lines, were supported by two layers of nylon mesh and randomly placed on the chick chorioallantoic membrane (CAM). Images were captured using a dissecting microscope. Scale bar, 1 mm. (E) Angiogenesis activities were analyzed by counting the number of vessel branch points. The angiogenic index was calculated as the percentage of newly formed blood vessel branch points induced by the POPX2 knockdown condition media relative to those by the control. The error bars represent standard deviations. Photographs courtesy of Hei Chan. Copyright 2016.

Figure 3. Work-flow and secreted proteins prediction. (A) Conditioned media harvested from both control and POPX2 knockdown cells were used for secretome analysis. SILAC-based quantitative proteomics approach was adopted. (B) The sub-cellular localizations of the identified proteins were predicted using multiple bioinformatics tools. Proteins secreted using the classical secretory pathway was predicted with SignalP. Secreted proteins from non-classical secretory pathway were predicted with SecretomeP. Shed membrane receptors that were released to the extracellular space were predicted with TMHMM. Proteins associated with exosomes were obtained from ExoCarta database. Proteins localized to the extracellular space were also retrieved from Gene Ontology. (C) Both the conditioned media and cell lysates were harvested for western blot analysis. Exosome secretion was investigated using specific antibodies against CD81, CD9, HSP70 and HSP90. Ponceau S stain of the whole membrane was used to indicate equal loading. Results shown are representative of three independent biological experiments.

Figure 4. Increased angiogenetic cytokines were found in conditioned media from POPX2 knockdown cells. (A) A human cytokine antibody array was used to analyze the secretion of 80 cytokines found in the conditioned media from control or POPX2 knockdown cells. Significantly increased cytokines were highlighted with green rectangle (control) and red rectangle (POPX2 knockdown). (B) Profile of different cytokines on the array. (C) The dot intensities of different cytokines were quantified by densitometry using ImageJ software and normalized against the intensity of internal positive controls for comparison. The fold change of cytokines in conditioned media from POPX2 knockdown cells vs control cells was determined. Cytokines with fold change ≥ 2 were shown. Three independent experiments were performed. Error bars represent standard deviations. (D) The KEGG pathway annotations were downloaded and plotted. *P-value* for each KEGG pathway was - Log converted.

Figure 5. KEGG pathway analysis implicated the involvement of POPX2 in breast cancer cell-ECM interactions. (A) The differentially regulated hits obtained from the secretome were submitted to the Database for Annotation, Visualization and Integrated Discovery (DAVID) using their official_gene_symbol. The KEGG pathway annotations were downloaded and plotted. *P-value* for each KEGG pathway was - Log converted. (B) Control and two independent lines of POPX2 knockdown cells were plated on bovine serum albumin (BSA), fibronectin and collagen coated 96-well plate. The cells were allowed to attach for 30 min before fixation. Attached cells were stained with crystal violet and quantified by measurement of the absorbance at wavelength 570 nm. The results were obtained from three independent experiments. Error bars represent standard deviations. The significance of difference was calculated by Student's t-test. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

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Figure 6. Examination of the effects of silencing POPX2 on the EMT process. (A) Immunostaining with antibodies against various EMT markers, including epithelial markers (E-cadherin, β -catenin) and mesenchymal markers (vimentin, N-cadherin), were carried out in both MCF7 and (B) MDA-MB-231 with or without POPX2 knockdown. The experiments were conducted in triplicates. Representative images were shown. Scare bar: 30 μ m. (C) Cell lysates obtained from both MCF7 and MDA-MB-231 with or without POPX2 knockdown were subjected to western blot analysis to determine the levels of the various EMT markers using their respective specific antibodies. Actin was used as a loading control. Results shown are representative of three independent biological experiments.

Figure 7. POPX2 phosphatase shows dual effects on cancer metastasis by modulating multiple biological processes. At early stages of metastasis, high levels of POPX2 enhance MAPK signaling and contribute to increased cell motility. At late stages of metastasis, low levels of POPX2 favor exosome-cytokine signaling and contribute to angiogenesis. Since POPX2 shows dual functions toward cancer progression, the balance of POPX2 levels and activities are important at different stages of metastasis.

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C

RayBio® Human Cytokine Antibody Array 5 Map

B

	A	B	C	D	E	F	G	H	I	J	K
1	Pos	Pos	Pos	Pos	Neg	Neg	ENA-78	GCSF	GM-CSF	GRO	GRO-α
2	I-309	IL-1α	IL-1 β	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7	IL-8	IL-10
3	IL-12	IL-13	IL-15	IFN- γ	MCP-1	MCP-2	MCP-3	MCSF	MDC	MIG	MIP-1β
4	MIP-1δ	RANTES	SCF	SDF-1	TARC	TGF-β1	TNF-α	TNF-β	EGF	IGF-I	Angiogenin
5	Oncostatin M	Thrombopoietin	VEGF	PDGF-BB	Leptin	BDNF	BLC	Ck β 8-1	Eotaxin	Eotaxin2	Eotaxin3
6	FGF-4	FGF-6	FGF-7	FGF-9	Flt-3 Ligand	Fractalkine	GCP-2	GDNF	HGF	IGFBP-1	IGFBP-2
7	IGFBP-3	IGFBP-4	IL-16	IP-10	LIF	LIGHT	MCP-4	MIF	MIP-3α	NAP-2	NT-3
8	NT-4	Osteopontin	Osteoprotegerin	PARC	PIGF	TGF- β2	TGF- β3	TIMP-1	TIMP-2	Pos	Pos

Figure 4

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