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RESEARCH PAPER

PHLDA1 (pleckstrin homology-like domain, family A, member 1) knockdown promotes migration and invasion of MCF10A breast epithelial cells

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ABSTRACT

PHLDA1 (pleckstrin homology-like domain, family A, member 1) is a multifunctional protein that plays distinct roles in several biological processes including cell death and therefore its altered expression has been identified in different types of cancer. Progressively loss of PHLDA1 was found in primary and metastatic melanoma while its overexpression was reported in intestinal and pancreatic tumors. Previous work from our group showed that negative expression of PHLDA1 protein was a strong predictor of poor prognosis for breast cancer disease. However, the function of PHLDA1 in mammary epithelial cells and the tumorigenic process of the breast is unclear. To dissect PHLDA1 role in human breast epithelial cells, we generated a clone of MCF10A cells with stable knockdown of PHLDA1 and performed functional studies. To achieve reduced PHLDA1 expression we used shRNA plasmid transfection and then changes in cell morphology and biological behavior were assessed. We found that PHLDA1 downregulation induced marked morphological alterations in MCF10A cells, such as changes in cell-to-cell adhesion pattern and cytoskeleton reorganization. Regarding cell behavior, MCF10A cells with reduced expression of PHLDA1 showed higher proliferative rate and migration ability in comparison with control cells. We also found that MCF10A cells with PHLDA1 knockdown acquired invasive properties, as evaluated by transwell Matrigel invasion assay and showed enhanced colony-forming ability and irregular growth in low attachment condition. Altogether, our results indicate that PHLDA1 downregulation in MCF10A cells leads to morphological changes and a more aggressive behavior.

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Introduction

Pleckstrin homology-like domain, family A member 1 (PHLDA1) gene encodes an evolutionarily conserved 401-amino acid proline-histidine rich protein. Its deregulation has been correlated with cancer progression in clinical samples and *in vitro* studies.[1] In breast cancer, growth-inhibitory effect of PHLDA1 was described for transformed HME16C breast cells,[2] triple-negative MDA-MB-231,[3] ER+ T47D,[4] and ErbB2-positive SKBR3 breast cancer cells.[5] In a previous work from our group with a series of 699 invasive breast cancer patients, negative expression of PHLDA1 protein was a strong predictor of poor prognosis for breast cancer with rates of 5-year overall survival of 52.7% for patients with PHLDA1 negative tumor samples against 74.8% for patients with positive PHLDA1 tumor samples. Multivariate analysis showed that PHLDA1 protein expression was an independent prognostic factor of overall survival of breast cancer patients even after adjusting for clinical stage and lymph nodal status.[6] Otherwise, PHLDA1 was reported as a follicular stem cell marker in a set of studies[7-10] and, adding controversy over PHLDA1 role in breast, previous report suggested that PHLDA1 upregulation is associated with cancer stem cell properties in ER+ MCF7 breast cancer cell line.[11] Thereby, the role of PHLDA1 in breast cancer remains to be clarified.

Breast cancer is essentially a genetic disease where tumorigenesis involves alterations in oncogenes, tumor-suppressor genes and DNA stability genes. It is estimated that 5 to 10% of all breast cancers are attributable to well-defined breast cancer susceptibility genes.[12,13] Notably, BRCA1 and BRCA2 are arguably the most well characterized genes in which germline mutations are responsible for the majority of hereditary breast cancers. Mutations in BRCA1/2 and other genes of low, middle or high penetrance are believed

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to account for 30% of familial breast cancer.[14,15] Apart from familial breast cancer, the remaining majority of breast cancer cases are considered sporadic, and molecular alterations contributing to the disease have not been fully identified yet.[16]

The development of breast cancer is commonly postulated to be a multi-step process that progressively evolves from non-diseased to preclinical cancer, then clinical cancer states and ultimately metastasis.[17-19] As a longitudinal observation of this process is not tangible, inferences are only elusive and do not rule out the possibility that normal cells give rise to ductal carcinoma *in situ* or invasive ductal carcinoma, for example. In this context, the use of *in vitro* models for breast cancer investigation has emerged, as they are systems that allow mimicking the *in vivo* situation in a controlled manner at the same time that provide the possibility of testing each genetic change individually. The human mammary epithelial cell line MCF10A is a reliable and widely used model for studying normal breast cell function. MCF10A cells are mammary epithelial cells derived from human fibrocystic mammary tissue of a 36-years-old woman who neither had cancer nor a family history of cancer.[20] Remarkably, MCF10A cell line was sub-derived from MCF10, which is the unique cell line that is diploid and contains only a reciprocal translocation between chromosomes 3 and 9.[21] Also, MCF10A is near-diploid and became spontaneously immortalized, without viral infection, cellular oncogene transfection or exposure to carcinogens or radiation, preserving a variety of cell characteristics that mimic normal mammary epithelial cells in culture.[19,20,22]

The central hypothesis of our study was that PHLDA1 has tumor suppressive properties in breast cancer. Despite PHLDA1 had been reported deregulated in breast cancer studies, it has not yet been determined whether these changes are responsible for the initiation and/or the progression of the disease, nor its functional role or significance in those processes. In this sense, we believe that PHLDA1 relation with mammary epithelial transformation and tumorigenesis can be better understood if its imbalance appears as an individual event in non-tumoral breast cells, helping to avoid possible biases from the deeply distinct molecular characteristics of each breast tumor cell lineage. In the current study, we aimed to further dissect the role of PHLDA1 in breast cells, performing functional studies in MCF10A cells stably transfected with PHLDA1 shRNA. Our data revealed that PHLDA1 downregulation increases cell proliferation, migration and invasiveness contributing to a more aggressive phenotype in MCF10A cells.

Results

PHLDA1 knockdown induces morphological changes in MCF10A cells

Morphological changes were observed in MCF10A cells with reduced expression of PHLDA1 compared with control cells. Plasmid vectors containing 2 different PHLDA1 specific shRNAs (named shPHLDA1 #1 and #2) were used to downregulate PHLDA1 in MCF10A cells. The knockdown efficiency was confirmed by Western blot analysis (Fig. 1A). Sub-confluent MCF10A cells grown in monolayer formed clusters that presented lamellipodia at the edges and showed cobblestone morphology with tight cell-to-cell contact upon confluence, which is characteristic of mammary epithelial cells (Fig. 1B). By contrast, MCF10A cells with reduced expression of PHLDA1 showed distinct nucleus/cytoplasm ratio, more pronounced lamellipodia formation at sub-confluence, and somewhat looser cell-to-cell contact in comparison with the control cells (Fig. 1B).

PHLDA1 knockdown increases proliferation and enhances mammosphere formation in MCF10A cells

We conducted CyQuant cell proliferation assay to examine whether reduced PHLDA1 expression could lead to changes in proliferation of MCF10A breast epithelial cells under attached conditions. We found that PHLDA1 downregulation in MCF10A cells significantly increased the proliferation rate compared with control cells for both knockdown sub-clones (shPHLDA1 #1 and #2) along 96 h (Fig. 2A). We further evaluated the effect of PHLDA1 down regulation on anchorage-independent growth conditions. After 9 d of culture, the majority of the cells died. However, a small fraction survived and formed mammospheres for both control and knockdown cells (Fig. 2B). Control MCF10A cells formed spheres with regular shape and exhibited low mammosphere-forming ability. In contrast, the number of spheroids for MCF10A cells with reduced expression of PHLDA1 was marginally increased, and these were more irregular compared with control cells. No differences in the size of the mammospheres were observed (Fig. 2B, b). The results showed that PHLDA1 knockdown increases proliferative behavior in attached conditions and change the growth pattern of mammospheres under anchorage-independent conditions corroborating with a negative regulation of chemotaxis for PHLDA1 function in breast cells.

PHLDA1 knockdown increases migration and invasiveness in MCF10A cells

The effect of PHLDA1 downregulation was further evaluated on cell motility, determined by wound-healing assay.

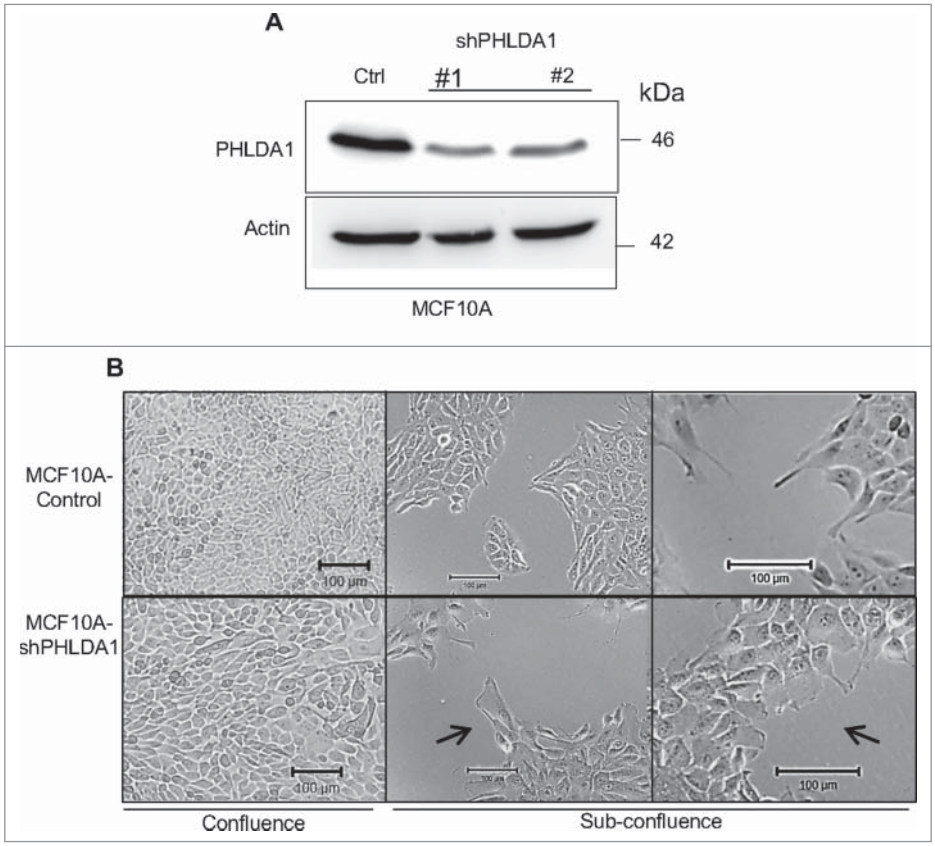

Figure 1. PHLDA1 protein expression and morphological features of MCF10A control and PHLDA1 knockdown cells. (A) Western blot analysis of PHLDA1 expression in MCF10A cells. First lane, MCF10A control cells (Ctrl); Lanes 2 and 3, MCF10A shPHLDA1 #1 and #2 sub-clones, respectively. (B) Phase-contrast images showing the morphological characteristics of MCF10A control and PHLDA1 knockdown cells grown in monolayer. Confluent and sub-confluent MCF10A control cells exhibiting typical morphology with lamellipodia often observed at the edges of clusters at sub-confluence. At confluence, MCF10A PHLDA1 knockdown cells assumed typical cobblestone morphology similar to control cells; at sub-confluence, cells exhibited lamellipodium more extensive than control cells (black arrows).

We found that PHLDA1 downregulation significantly increased cell migration (Fig. 3). Within 18 hours, the area of the wound was significantly recovered by the migrating of MCF10A cells with reduced expression of PHLDA1; by 24 hours, the wound area had been almost completely recovered (Fig. 3A, right). In marked contrast, the wound closure of MCF10A control cells exhibit comparatively less difference at the same imaged times (Fig. 3A, left), dramatically distinct from knockdown cells.

To independently investigate the role of PHLDA1 on migration and invasiveness capabilities of MCF10A cells we conducted the Transwell assay. As expected, MCF10A breast cells showed almost no invasive ability (Fig. 3B, left). Interestingly, downregulation of PHLDA1 induced an invasive behavior to these non-malignant and non-invasive cells, being able to degrade and invade through the Matrigel matrix (Fig. 3B, right), with an increment of 62 and 28 times to shPHLDA1 #1 and #2 subclones, respectively, in comparison to control cells ($p < 0.01$).

PHLDA1 downregulation enhances colony formation ability of MCF10A cells

The ability of cells to form colonies at low density was assessed. After 8 d of plating, PHLDA1 knockdown cells sub-clone #2 formed significantly more colonies than control cells in a clonogenic assay, $p < 0.05$ (Fig. 4). Similar results were found to shPHLDA1 #1 sub-clone, although not significantly (data no show). Moreover, colonies appearance differed markedly. Colonies of MCF10A cells with reduced PHLDA1 expression were weakly stained due to scattered morphology compared with control cells (Fig. 4B). MCF10A cells with reduced PHLDA1 expression exhibited a spindle-like morphology and lacked obvious cell-cell contacts when observed at high power magnification (Fig. 4C), confirming our observations of morphological changes assessed with phase-contrast morphology (Fig. 1B).

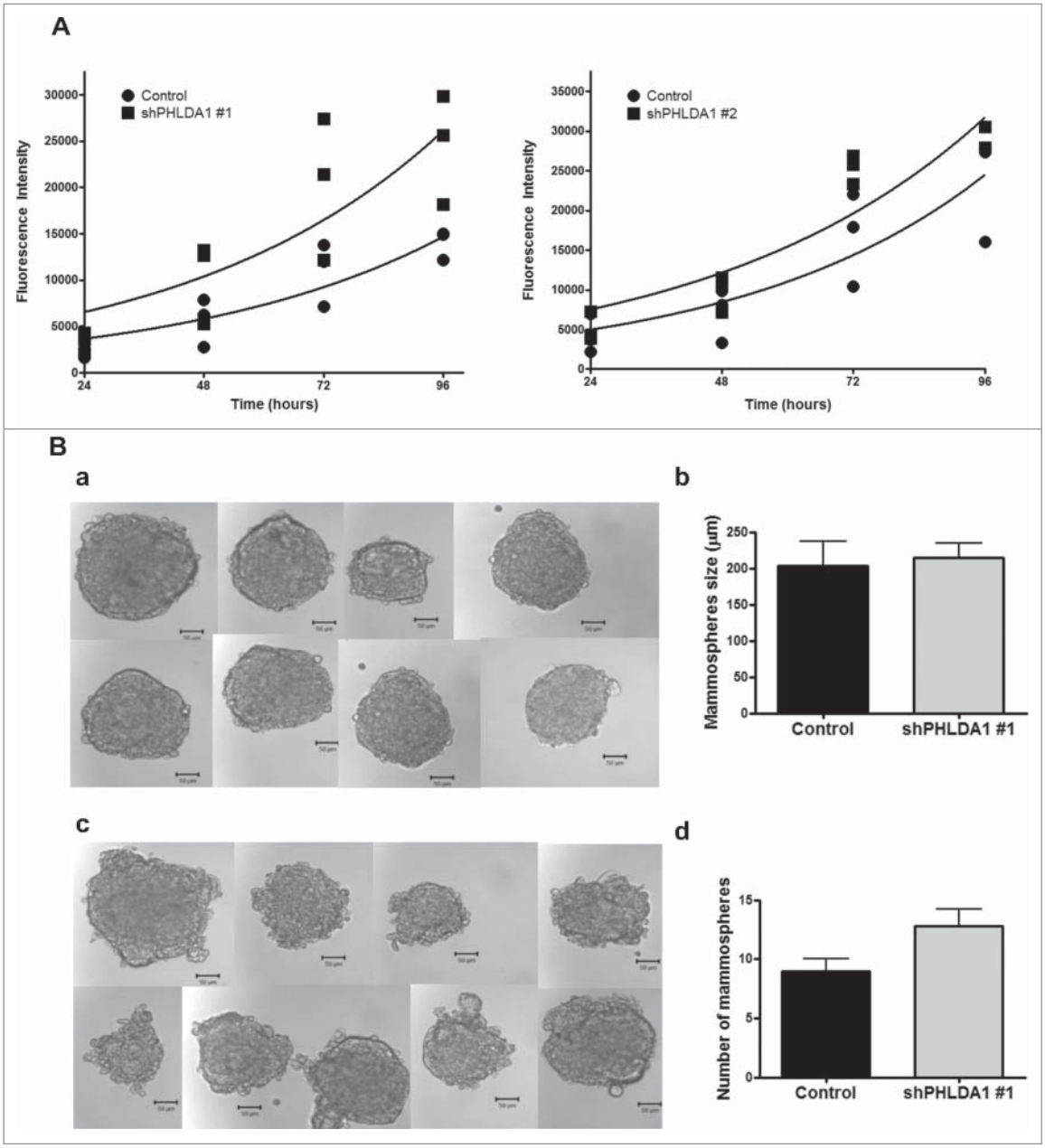

Figure 2. PHLDA1 knockdown increases proliferation and enhances mammosphere formation in MCF10A cells. (A) Increased proliferation rate in knockdown PHLDA1 MCF10A cells as compared with control cells. The cell proliferation rate was determined using a CyQUANT cell proliferation assay after 24, 48, 72 and 96 hours. Curve fit analysis was performed using GraphPad Prism software for non-linear regression. (Left) Control cells versus shPHLDA1 #1 sub-clone, $P < 0.01$. (Right) Control cells vs. shPHLDA1 #2 sub-clone, $P < 0.05$. (B) MCF10A control and PHLDA1 knockdown mammospheres characteristics. (a) MCF10A control and (c) PHLDA1 knockdown cells were seeded at single-cell density in low attachment plates and mammosphere were allowed to develop for 9 days, the mean size of mammospheres for each lineage was plotted (b) and the total number of mammospheres were counted (d) and plotted with mean ± s.e.m (*ns*, unpaired t test).

Down-regulation of PHLDA1 changes actin filaments distribution in MCF10A cells

Development of migratory and invasive properties involves a dramatic reorganization of the actin cytoskeleton and the concomitant formation of membrane protrusions required for invasive growth. Morphological changes in PHLDA1 knockdown cells such the presence of more lamellipodium in monolayer together with acquired invasive behavior and enhanced migration capacity, suggested a possible involvement of actin filaments reorganization. As assessed by immunofluorescence (Fig. 5A), cells with PHLDA1 downregulation (lower) showed highly actin-rich membrane projections

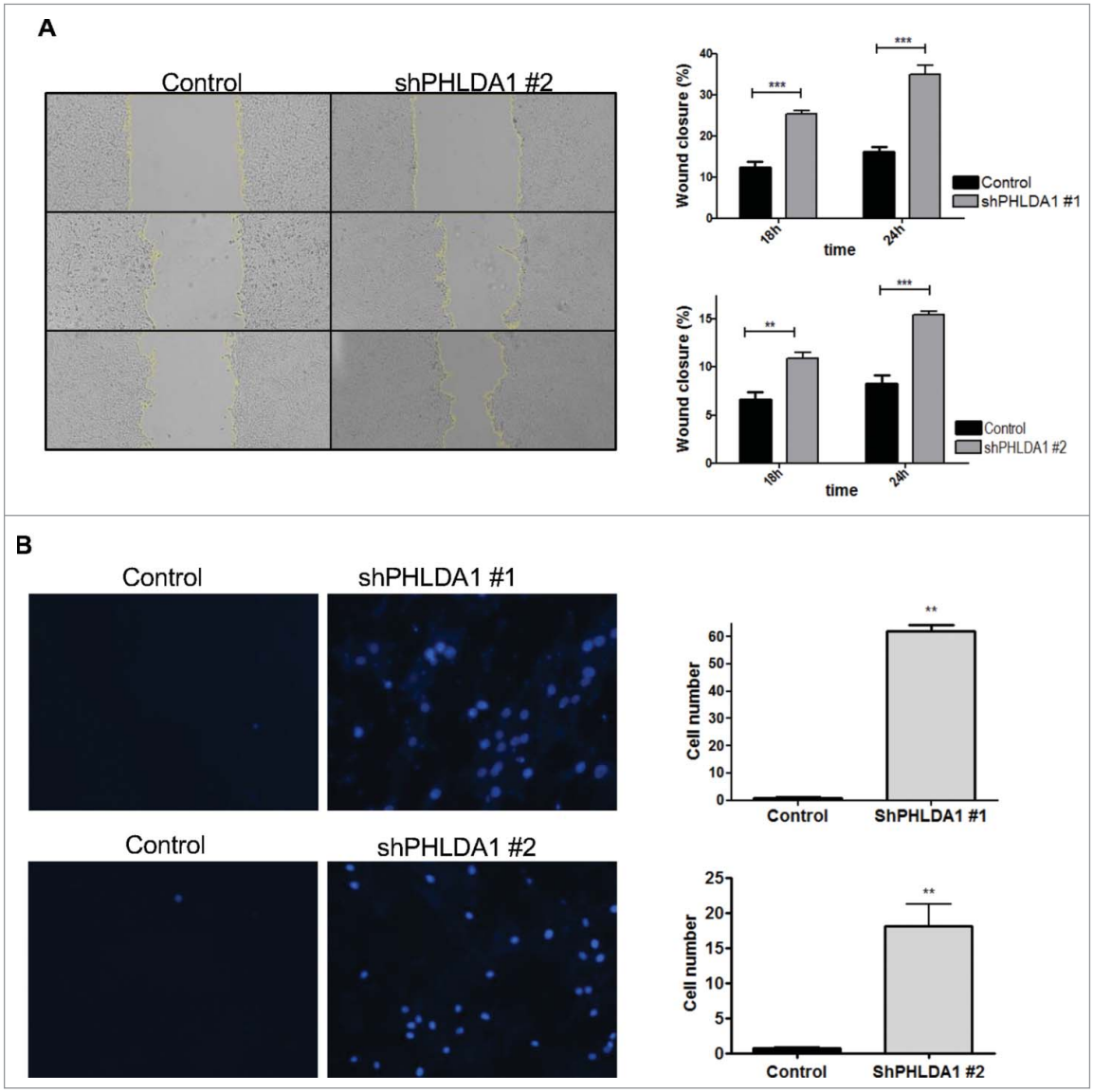

Figure 3. PHLDA1 knockdown enhances migration and promotes invasion of MCF10A cells. (A) Wound-healing assay shows that PHLDA1 knockdown significantly increases cell mobility. (Left) representative photomicrographs of cells. (Right) The bar graph shows the quantitative data for wound closure after 18 and 24 hours. Data represents mean ± s.e.m. $^{**}P < 0.01$; $^{***}p < 0.001$ by 2-way ANOVA test with Bonferroni correction. (B) PHLDA1 knockdown promotes invasion of MCF10A cells. (Upper) Representative photomicrographs of cells that have invaded, stained with DAPI. (Lower) Graph represents mean ± s.e.m. for the number of cells that have invaded/field in transwells from 3 independent experiments $^{**}P < 0.01$ (unpaired t test).

that were not observed in control cells (upper). Additionally, images taken after phalloidin staining allow seeing the scattered morphology and the loose cell-cell contact of shPHLDA1 cells when compared with control cells (Fig. 5B).

Discussion

Previously we have demonstrated that reduced expression of PHLDA1 is strongly associated with poor outcome in breast cancer patients.[6] In the present study, we demonstrate that PHLDA1 downregulation is related to the development of an aggressive phenotype in MCF10A cells, inducing morphological alterations, increasing proliferation rate, migration and clonogenic ability as well as inducing the acquisition of invasive behavior, in accordance with a putative tumor suppressor activity. Also, when stemness capacity was investigated with anchorage-independent mammosphere forming assay we found that MCF10A cells with reduced expression of PHLDA1 showed higher mammosphere-forming capacity in comparison to control cells.

Previous reports showed that PHLDA1 negatively regulates cell motility and proliferation in breast cancer

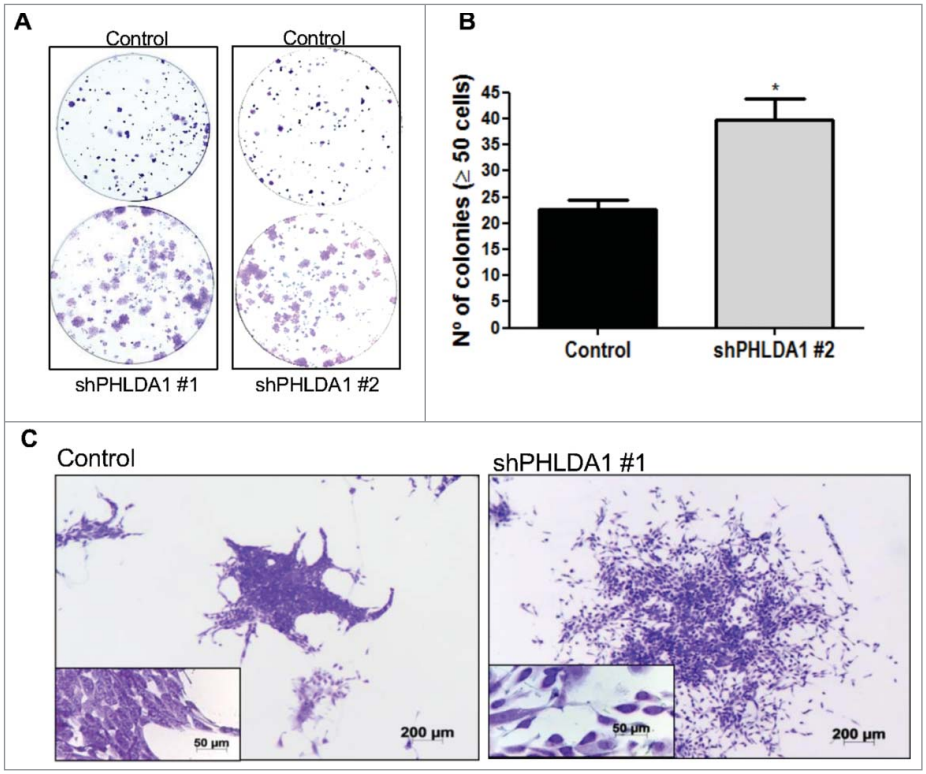

Figure 4. PHLDA1 knockdown enhances the ability of MCF10A cells to form colonies. (A) Representative images taken from 6 well plates showing colonies of MCF10A control cells, shPHLDA1 #1 and #2 cells. (B) Bar graph showing number of colonies after 8 d in culture. Data are expressed as mean ± s.e.m. $^{*}P < 0.05$ (unpaired t test). (C) Morphological differences between MCF10A control and PHLDA1 knockdown cells colonies stained with crystal violet. (Left) Representative photomicrograph of control cells. (Right) Representative photomicrograph of PHLDA1 knockdown cells.

cells under attached,[3,5] and unattached conditions.[2,3,5] Despite that, one study has found opposite results for growth under unattached conditions.[11] We, therefore, tested if these effects could be overcome by the PHLDA1 knockdown in non-malignant breast epithelial cells MCF10A in which cellular proliferative controls are intact.[20] Phase contrast microscopy observations of MCF10A cells with PHLDA1 knockdown showed distinct nuclei/cytoplasm ratio as they exhibited larger cytoplasm and cells at the edge of clusters had larger and more frequent lamellipodia protrusions than control cells. These projections are known to facilitate cell movement and act as sensory extensions of the cytoskeleton.[23] Also, actin-rich invadopodia exert a proteolytic function in ECM degradation, thus facilitating cell invasion.[24,25] In our clonogenic assay, morphological differences became more evident, scattered colonies and even spindle-shaped individual cells were observed for PHLDA1 knockdown cell line. These differences in morphology suggested a distinct pattern in actin distribution. Indeed, when we used immunofluorescence to assess actin organization, we observed actin-rich invadopodia in PHLDA1 knockdown cells, which play a proteolytic function in ECM degradation, thus facilitating cell invasion.[26] These findings are in accordance with the increased migratory activity and acquired invasive properties observed in MCF10A cells with PHLDA1 knockdown, phenomena that involve a dramatic reorganization of the actin cytoskeleton and the concomitant formation of membrane protrusions that are required for invasion.[26]

PHLDA1 is a pleckstrin homology-like domain protein.[27] Interestingly, pleckstrin homology (PH) domains are known to interact with phosphoinositides, a property shared by some PH-like domains (as reviewed by Scheffzek[28]). Phosphoinositides are known to play key roles in the regulation of the actin cytoskeleton and control membrane dynamics[29] and lamellipodia-dependent cell migration by inducing actin filament assembly at the plasma membrane and by regulating the direction of cell movement during chemotaxis.[30,31] However, a relationship between PHLDA1 and actin filament assembly at the plasma membrane has not been reported before, and this is also the first evidence that PHLDA1 knockdown can induce breast epithelial cell migration and invasiveness.

Johnson et al.[3] have investigated PHLDA1 and Aurora A kinase relation with invasiveness in MDA-

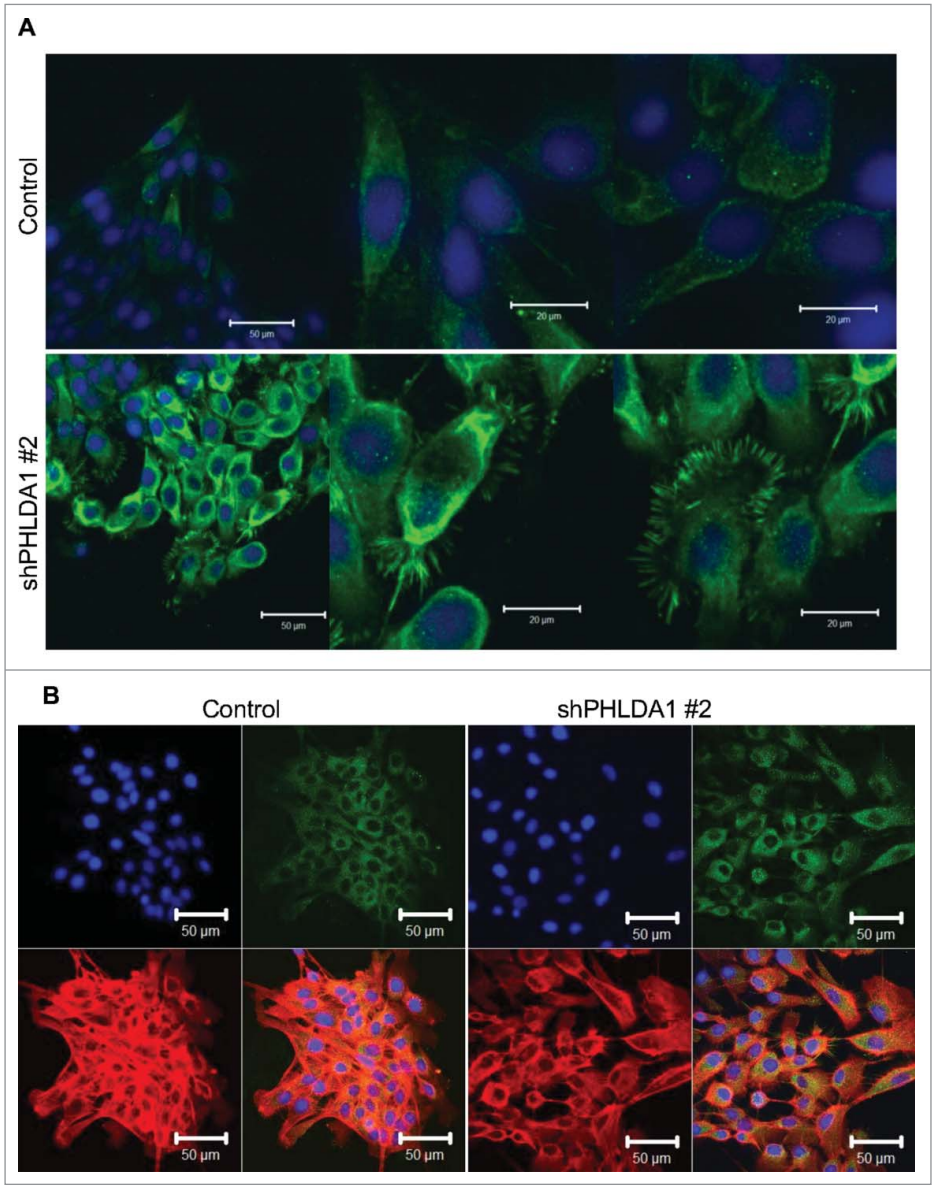

Figure 5. PHLDA1 downregulation induces morphological changes in MCF10A cells. Actin staining revealed morphological differences between Control and shPHLDA1 cells. (A) MCF10A PHLDA1 knockdown cells display highly actin-rich membrane projections. Representative photomicrographs of cells immunostained with actin primary antibody and Alexa-Fluor 488 secundary antibody (green) and nuclei counterstained with DAPI. (B) Contrast between the tight cell-cell contact of control cells with the loose cell-cell contact of shPHLDA1 cells. Representative photomicrographs of cells stained with actin antibody (green), rhodamin phalloidin (red) and nuclei counterstained with DAPI. Scale bar: 20 μm.

MB-231 breast adenocarcinoma cells. They found that knockdown of PHLDA1 enhanced invasion whereas its overexpression had the opposite effect. Indeed, when using Ser98 phosphorylation-resistant PHLDA1 mutant, cell motility was decreased even upon Aurora A overexpression (Aurora A cells were highly motile). The authors also report decreased proliferation rates after overexpression of PHLDA1. Consistent with these findings we found that PHLDA1 knockdown also increases cell proliferation in MCF10A cells, corroborating with the tumor suppressor role of PHLDA1. Li et al.[5] described similar effects on HER2 positive breast cancer cell line SKBR3 after overexpression of PHLDA1, where decreased motility and proliferation rates was seen. Under low attachment conditions, the authors report a similar inhibitory effect. In our study knockdown of PHLDA1 lead to a slight increase in the number of mammospheres formed under low attachment conditions in comparison to control MCF10A cells.

Although clinical and experimental studies are required to elucidate the molecular and cellular functions of PHLDA1, evidence reported in the literature indicate that PHLDA1 expression can be modulated by various stimuli, resulting in pleiotropic effects regulating different biologic

processes that might impact tumor initiation, progression, and therapeutic response. In a previous study, we raised the possibility of PHLDA1 being useful as a predictor of prognosis for breast cancer: its paucity was related to worse outcome. Here, we showed that PHLDA1 downregulation in non-malignant breast epithelial cells led to morphological and behavioral changes conferring a more aggressive phenotype. We show for the first time that PHLDA1 knockdown induces actin filaments reorganization and acquisition of migration and invasion capacities in MCF10A cells allowing us to speculate that PHLDA1 downregulation could be useful for early detection of breast cancer. Moreover, Li et al., showed that PHLDA1 plays a role in the negative feedback regulation of ErbB2 activity and its upregulation enhances sensitivity to lapatinib in SKBR3 cells, indicating that PHLDA1 could be a potential therapeutic response indicator. Based on that we could formulate the hypothesis that patients with different levels of expression of PHLDA1 may exhibit differences in drug response, helping to provide information for patients that may or may not be beneficial for one such treatment scheme.

Material and methods

Cell culture

MCF10A cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured at 37°C in an atmosphere of 5% CO2 and 95% air and passaged weekly in DMEM/F-12 supplemented with 5% horse serum, 50 ng/ml epidermal growth factor (EGF), 10 μg/ml insulin, 0.5 μg/ml hydrocortisone, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 0.1 μg/ml cholera toxin.

Cellular transfection

MCF10A cells were transfected with pRS vector empty or expressing shRNA targeting PHLDA1 mRNA (OriGene Technologies, Inc.) using Fugene HD reagent (Roche Applied Science). Polyclonal populations of transfected cells were selected with Puromycin 0.7 μg/ml. After selection, subclones were maintained with 0.5 μg/ml puromycin.

Migration assay

For migration assays, confluent monolayers of MCF10A control and shPHLDA1 stable transfected subclones were scratched with a sterile 20 μl pipette tip. The plates were then washed and incubated at 37°C in 5% Horse serum and DMEM-F12 supplemented medium. Images were taken after 18 and 24 hours.

Invasion assay

Invasion assays were performed using BD BioCoat growth factor reduced Matrigel invasion chambers following manufacturer's instructions (BD Biosciences). Briefly, control or shPHLDA1 cells (1×10^5) were introduced into the upper compartment. After 18 h cells were wiped off from the upper surface of each insert. The cells on the lower surface, which represented the cells that migrated and invade through control insert membrane, were fixed with methanol, stained with DAPI and counted by microscopic examination in 10 representative fields. Cell invasion data was expressed as the mean number of cells per field.

Cell proliferation assays

CyQUANT kit (Invitrogen) assay was performed following manufacturer's instructions. Briefly, cells in 5% horse serum and DMEM-F12 supplemented medium were seeded in triplicate in 96 multi-well plates (Corning) at a density of 1×10^4 cells/well. Following 24, 48, 72 and 96 h medium was removed from the plates and cells were incubated with 1x dye binding solution at 37°C for 1 h in the dark. The fluorescence was measured using the Fluostar Optima microplate reader (Fluostar Optima, BMG Labtech) with excitation maximum at 485 nm and emission maximum at 530 nm.

Anchorage independent growth assay

Cells were seeded at a single-cell density of 526 cells/cm^2 into wells of ultralow attachment 6-well-plate (Corning) with serum-free supplemented DMEM/F12 medium (Gibco) and 1 x B27 supplement (Gibco) and incubated in a humidified atmosphere with 5% CO2 at 37°C. Mammospheres were evaluated after 9 d. For secondary spheres, the cells were collected, enzymatically disaggregated with trypsin and single-cell suspensions were seeded at the same prior density and culture conditions as described above, cultivated for further 9 d. Images were taken and mammospheres were counted in each final day.

Clonogenic assay

Single cells were plated onto 6 well plates and were allowed to grow for 8 d. Next, the cells were fixed with ice-cold methanol, stained with crystal violet for 30min and the number of colonies with more than 50 cells in each plate was determined.

Western blot

Whole-cell lysates were prepared from cells cultured in monolayers. Thirty μg of lysates were analyzed on 10%

SDS-polyacrylamide gels followed by transfer at 2.5 V for 30min to nitrocellulose membranes (Pierce Biotechnology, Rockford, IL, USA). Protein concentration was measured using the Coomassie Plus Protein Assay Reagent™ (Pierce Biotechnology). Blots were blocked with 5% skim milk in Tris-buffered saline containing 0.1% of Tween 20 (TBS-T) for 1 h at room temperature and then incubated with primary antibody overnight at 4°C for all proteins but PHLDA1 which membrane was blocked overnight (4°C) and incubated with primary antibody for 1h at room temperature. Membranes were then washed and incubated with a peroxidase-conjugated secondary antibody for 1h. Antibodies were diluted in 5% skim milk in TBS-T. After washing the membranes, the signals of reactive proteins were developed using the Western Lightning® Plus-ECL enhanced chemiluminescence substrate (PerkinElmer) and visualized in the ImageQuant LAS 4000 (GE Healthcare Life Sciences).

Immunofluorescence

Cells were plated on 8-well chamber slides at a density of 1.5×10^4 cells per well. Once 40–60% of confluence was reached cells were immunostained using actin primary antibody followed by Alexa-488-labeled secondary antibodies. Nuclear staining was performed with Hoechst 33342 (Invitrogen) for 15 min. After washing with PBS, coverslips were mounted on microscope slides with PBS/Glycerol or PBS/Glycerol/rhodamine-phalloidin staining. Images were taken using Zeiss LSM Meta 510 scanning confocal microscope.

Statistical analysis

Statistical analyses were performed using t-student test or ANOVA as appropriate, with P-value of < 0.05 considered statistically significant. Data are expressed as mean ± SD. The data are representative of 3 separate experiments.

In all figures, asterisks denote significance levels as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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