

Pleckstrin homology-like domain, family A, member 1 (*PHLDA1*) and cancer (Review)

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Abstract. Pleckstrin homology-like domain, family A, member 1 (*PHLDA1*) encodes a member of an evolutionarily conserved pleckstrin homology-related domain protein family. It was first identified as a potential transcription factor required for Fas expression and activation-induced apoptosis in mouse T cell hybridomas. The exact molecular and biological functions of *PHLDA1* remain to be elucidated. However, its expression is induced by a variety of external stimuli and there is evidence that it may function as a transcriptional activator that acts as a mediator of apoptosis, proliferation, differentiation and cell migration dependent on the cellular type and context. Recently, *PHLDA1* has received attention due to its association with cancer. In the present review, the current knowledge of *PHLDA1* protein structure, expression regulation and function is summarized. In addition, the current data in the literature is reviewed with regards to the role of *PHLDA1* in cancer pathogenesis.

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1. Introduction

Pleckstrin homology-like domain, family A, member 1 (*PHLDA1*; also known as PHRIIP, TDAG51, DT1P1B11 and MGC131738) was first identified as a potential transcription factor required for Fas expression and activation-induced apoptosis in mouse T cell hybridomas (1). It was later mapped to human chromosome 12q15 by radiation hybrid mapping (2). The *PHLDA* family is evolutionarily conserved and comprises three members that are located on different chromosomes: *PHLDA1* (12q21.2), *PHLDA2* (11p15.4) and *PHLDA3* (1q32.1). All three share similar gene organization [i.e., two exons (one coding exon) and a small intron] and encode proteins with a PHL domain (3). During the last decade, *PHLDA1* has received increasing attention, although its functional role remains to be elucidated.

PHLDA1 encodes a protein that is 401 amino acids (aa) in length. This protein is rich in polyglutamine (QQ), proline-glutamine (PQ) and proline-histidine tracts with a PHL domain (1-3) (Fig. 1). Sequencing comparisons have shown that mouse, human and rat *PHLDA1* cDNA and protein exhibit high levels of sequence identity (2,4).

PHL domains are evolutionarily conserved and are found in proteins from prokaryotes and eukaryotes (5,6). These domains are composed of 100-120 aa residues, and their properties include binding to phosphatidylinositol lipids (5). Numerous proteins containing PHL domains (such as insulin receptor substrate proteins) have been found to interact with membrane components, eliciting a variety of cellular responses and participating in cell signaling transduction, vesicular trafficking and cytoskeletal rearrangement (7,8). QQ and PQ stretches are well-conserved motifs in eukaryotic proteins, and they are present predominantly in transcription factors that are involved in transcriptional activation and protein-protein interactions (9,10). In addition, a number of variations in

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Abbreviations: aa, amino acid; AICD, activation-induced cell death; BCC, basal cell carcinoma; EMT, epithelial-mesenchymal transition; ER, endoplasmic reticulum; FGF, fibroblast growth factor; IGF-1, insulin-like growth factor 1; NF- κ B, nuclear factor- κ B; OSCC, oral squamous cell carcinoma; PH, proline-histidine; PHL, pleckstrin homology-like; *PHLDA1*, pleckstrin homology-like domain, family A, member 1; PKC, protein kinase C; PQ, proline-glutamine; QQ, polyglutamine

Key words: *PHLDA1*, pleckstrin homology-like, cancer, tumor suppressor gene, oncogene, downregulation, stem cell

tandem repeats of the trinucleotide sequence CAG, which encodes homopolymeric stretches of QQ, have been associated with several inherited neurodegenerative diseases (10,11). Due to the presence of QQ stretches that function as transcriptional activation domains, PHLDA1 could act as a transcription factor required for Fas expression (1).

2. PHLDA1 expression and cell death

PHLDA1 mRNA and protein are expressed in several mammalian tissues, suggesting that it may have important cellular functions (1,4,12). In addition to being widely expressed in different tissues, PHLDA1 subcellular localization varies. PHLDA1 is found predominantly in the cytoplasm (13-16); however, perinuclear, nucleolar and nuclear localization have also been reported (12,13,16,17). PHLDA1 expression is induced by different stimuli, such as estrogens, growth factors, differentiation and endoplasmic reticulum (ER)-stress agents, and it is associated with different biological processes, such as cell apoptosis, cell proliferation and differentiation (Fig. 2).

Evasion of apoptosis or programmed cell death is a hallmark of cancer, and the development of resistance to apoptosis is associated with tumor pathogenesis and affects chemo- and radioresistance (18). Several lines of experimental evidence have implicated PHLDA1 in intrinsic and extrinsic apoptotic pathways; however, the mechanisms involved in its action as a proapoptotic or antiapoptotic agent remain to be elucidated.

The role of PHLDA1 in activation-induced cell death (AICD) was first reported by Park *et al* (1) in 1996, who used a T cell hybridoma model to determine that PHLDA1 expression is induced by T cell receptor activation in the presence of phorbol myristate acetate and ionomycin (1). The study also reported that PHLDA1 is required for Fas (CD95) expression, which has an important role in T cell receptor-induced apoptosis. These data point to an essential role for PHLDA1 in the induction of apoptosis in a T cell hybridoma model. However, the study by Rho *et al* (19) investigated the association between PHLDA1 and Fas expression *in vivo* and demonstrated that *PHLDA1*^{-/-} mice express normal levels of Fas and have normal T cell apoptosis, indicating that PHLDA1 is not essential for Fas expression. Furthermore, Oberg *et al* (20) demonstrated that in human T cells and pancreatic tumor cells, induction of PHLDA1 is inhibited by mitogen-activated protein kinase (MAPK) and protein kinase C (PKC) inhibitors, indicating that these two pathways may be involved in the regulation of PHLDA1 expression. However, no association was found between PHLDA1 expression and AICD or cell proliferation. By contrast, increased Fas expression was shown to be dependent on PKC activation (1,21), which induces Fas expression only in cells expressing wild-type PHLDA1 (21). Sequence analysis and experimental studies revealed that PHLDA1 contains putative casein kinase II and PKC phosphorylation sites (4,21). These data suggest that PHLDA1 transcriptional activation of the target gene FAS may be dependent on post-transcriptional modification upon PKC activation, which raises the possibility of transcriptional activity of PHLDA1 in a broader context.

In agreement with the proapoptotic role executed by PHLDA1 in rat neuronal cells, PHLDA1 mRNA and protein

were induced by fibroblast growth factor and phorbol dibutyrate, which is a differentiation agent that activates PKC (4). In addition, Gomes *et al* (4) reported that microinjection of differentiating H19-7 hippocampal cells with the neutralizing antibody anti-PHLDA1 increases cell survival. Furthermore, transient PHLDA1 expression decreases cell survival in differentiating and non-differentiating H19-7 cells, indicating that PHLDA1 acts as a mediator of differentiation-associated apoptosis in H19-7 cells (4). PHLDA1 expression is induced by the phorbol ester and ionomycin in different transformed T cell lines and was also found to interact with two mRNA-binding proteins, eIF3-p66 and PABP, in 293T cells. Additionally, PHLDA1 cotransfection with the luciferase gene leads to protein biosynthesis inhibition, a process that is important for the execution of apoptosis (13).

ER stress-inducing agents, such as homocysteine, tunicamycin and farnesol (FOH), have been reported to induce PHLDA1 expression in different cell types (12,22,23). Hossain *et al* (12) demonstrated that induction of PHLDA1 by homocysteine involves ER stress and is dependent on eIF2 α phosphorylation. In addition, transient overexpression of PHLDA1 leads to changes in cell morphology, decreases cell adhesion, and promotes detachment-mediated cell death in endothelial cells (12). To improve the understand how FOH induces apoptosis, Joo *et al* (22) performed cDNA microarray analysis and compared the gene expression profiles between vehicle-treated and FOH-treated H460 human lung adenocarcinoma cells. An induction of a number of genes associated with ER stress-response signaling, including PHLDA1, was observed. The study also reported that FOH-induced apoptosis in human lung carcinoma cells is coupled to the activation of an ER-stress response that includes activation of the sensors IRE1 and PERK, dependent on the activation of the MEK-ERK signaling pathway.

Carlisle *et al* (23) found that treatment of HK-2 cells (an immortalized proximal tubule epithelial cell line) with the ER-stress agents thapsigargin and cyclosporine A increases the cytosolic Ca²⁺ concentration and induces PHLDA1 expression, leading to epithelial-mesenchymal transition (EMT). In addition, overexpression of PHLDA1 in HK-2 cells leads to shape changes, β -catenin disruption and increased apoptosis, indicating that PHLDA1 may cause dissolution of adherens junctions, β -catenin nuclear localization and EMT induction (23). EMT is an important biological process that involves downregulation of epithelial markers and upregulation of mesenchymal markers, in which epithelial cells lose cell-cell adhesion and cell polarity, and gain the migratory and invasive properties that are characteristic of mesenchymal cells (24,25). As EMT has a key role in tumor progression and may influence therapy response, the potential role of PHLDA1 as an EMT regulator requires further investigation.

In support of the proapoptotic function exhibited by PHLDA1, Neef *et al* (14) reported that constitutive PHLDA1 expression in different melanoma-derived cell lines leads to reduced cell growth, reduced colony formation and increased basal apoptosis, which is associated with increased cleaved caspase 9 and cleaved poly adenosine diphosphate ribose polymerase. Upregulation of PHLDA1 has been identified in autophagy and apoptosis induced by rapamycin in T47D breast cancer cells (26). In addition, stress-induced heat-shock proteins (Hsp; including Hsp70, Hsp110 and Hsp40) bind

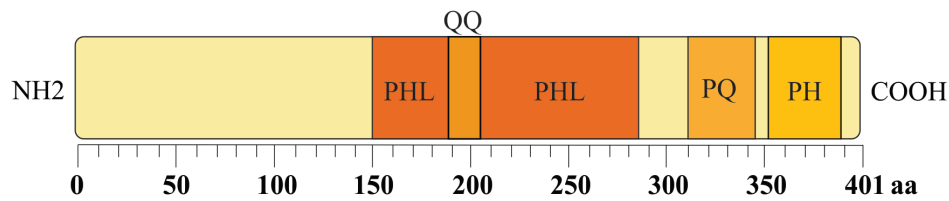


Figure 1. Schematic representation of the modular structure of the PHLDA1 protein. PHL, pleckstrin homology-like domain (aa 151-283); QQ, polyglutamine tract (aa 187-204); PQ, proline-glutamine tract (aa 311-346); PH, proline-histidine-rich tract (aa 352-389); aa, amino acid.

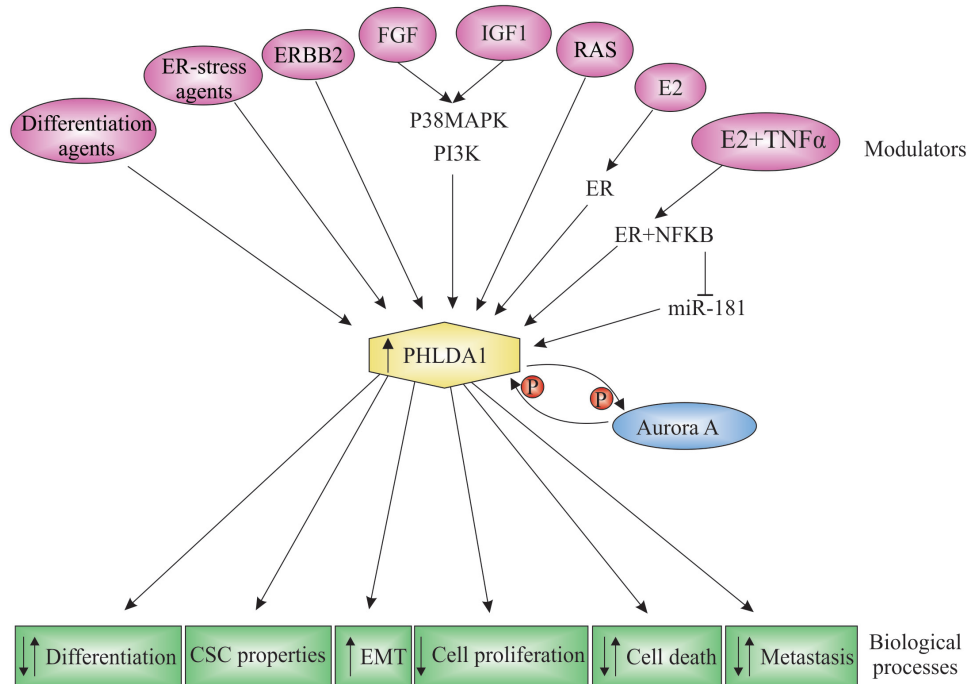


Figure 2. Modulators and biological functions of Pleckstrin homology-like domain, family A, member 1 (PHLDA1). PHLDA1 expression is modulated by a variety of external stimuli in different cell types. As a consequence, diverse biological processes are modulated. ER-stress agents, endoplasmic reticulum-stress agents; ERBB2, receptor tyrosine kinase 2; P, phosphorylation; FGF, fibroblast growth factor; IGF1, insulin-like growth factor 1; RAS, rat sarcoma virus oncogene; E2, 17 β -estradiol; ER, estrogen receptor; NF- κ B, nuclear factor of κ light polypeptide gene enhancer in B cells; p38 MAPK, p38 mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; TNF- α , tumor necrosis factor- α ; miR-181, microRNA 181.

directly to the pleckstrin domain of PHLDA1 and suppress its proapoptotic function in mouse embryonic fibroblast (MEF) cells (27). The promoter region of *PHLDA1* has heat-shock element binding sites, where heat-shock transcription factor 1 (HSF1) binds and modulates *PHLDA1* expression. In addition, HSF1 is able to directly bind to and activate *PHLDA1* in response to heat shock in male germ cells (27). Furthermore, during a time-course induction of cryptorchidism in rat testes, HSF1 and PHLDA1 were strongly expressed, suggesting that the HSF1/PHLDA1 pathways have an important role in the apoptosis of primary spermatocytes in cryptorchid testes (28).

In addition to its association with apoptosis induction, PHLDA1 has also been shown to act as an antiapoptotic agent. PHLDA1 mRNA and protein are induced by insulin-like growth factor 1 (IGF-1) through IGF-1R via the p38 MAPK pathway in NIH-3T3 (NWTb3) cells and via the phosphatidylinositol 3-kinase pathway in normal skin fibroblasts (29,30). Knockdown of *PHLDA1* expression by small interfering RNA (siRNA) abolishes the protective effects of IGF-1 against apoptosis induction in NIH-3T3 (NWTb3) cells in response to serum starvation (30). Toyoshima *et al* (29) also identified

that *PHLDA1* downregulation increases apoptosis, which is independent of IGF-1, suggesting that PHLDA1 may have antiapoptotic effects in NWTb3 cells.

PHLDA1 is also a direct target of the oncogenic fusion protein EWS/FLI1. Boro *et al* (31) demonstrated that EWS/FLI1 can bind to the promoter of *PHLDA1*, *in vivo* and *in vitro*, leading to its repression. In MEF cells derived from conditional EWS/FLI1 knockin embryos, EWS/FLI1 expression resulted in apoptosis and EWS/FLI1 was able to directly bind the caspase 3 promoter in CHP100 cells with a concomitant increase in endogenous caspase 3 expression (32). In addition, *PHLDA1* knockdown by siRNA in Ca9-22 cells increases activated caspase 3 expression, suggesting that PHLDA1 has a role as an apoptosis suppressor (33).

3. Role of PHLDA1 in cancer

Several lines of evidence suggest that PHLDA1 has an important role in cancer. Although the exact molecular and cellular functions of PHLDA1 have not been fully elucidated, evidence from experimental studies show that it is a potential

Table I. Aberrant expression of *PHLDA1* in cancer.

Type of cancer	Expression	Function	(Refs.)
Melanoma	Strongly expressed in benign melanocytic nevi and progressively reduced in primary and metastatic melanomas	Expression in melanoma cell lines is associated with reduced cell growth and colony formation, and increased basal apoptosis	(14)
Breast	Reduced expression, a strong predictor of poor prognosis for breast cancer patients		(15)
	Reduced expression associated with estrogen receptor expression in breast tumors	Regulatory feedback loop between Aurora-A and <i>PHLDA1</i>	(35)
	Elevated expression associated with a higher risk of distant metastasis in estrogen receptor-positive breast cancer patients		
		<i>PHLDA1</i> regulated by E2 and TNF- α at the transcriptional and post-transcriptional level in breast cancer cells	(40) (41)
		Silencing affects mammosphere formation and growth	(41)
		Reduced expression during ras-mediated cellular transformation induces proliferation under anchorage-independent conditions and increases ERK activation	(42)
	Upregulation found in autophagy and apoptosis induced by rapamycin in breast cancer cells	(26)	
	Expression reduced in ERBB2-negative tumor samples and ERBB2-negative breast cancer cell lines	Overexpression inhibits cell proliferation, colony-forming ability, and migration in SKBR3 cells	(36)
Oral	Reduced expression is associated with good prognosis in oral cancer	Silencing leads to apoptosis induction	(37) (33)
Gastric	Reduced expression associated with shorter overall survival in gastric cancer patients		(16)
Basal cell carcinoma	Reduced expression in basal cell carcinoma		(44)
Colorectal	Overexpressed in colorectal cancer	Silencing in colon cancer cells inhibits anchorage-independent cell growth and reduces cell migration	(38) (17)
Intestinal	Overexpressed at all stages of intestinal cancer development		(17)
Osteosarcoma		High expression associated high metastatic potential in osteosarcoma cells, and silencing delays overall survival in mouse xenograft models	(39)

PHLDA1, pleckstrin homology-like domain, family A, member 1; TNF- α , tumor necrosis factor- α ; ERK, extracellular-signal-regulated kinase; ERBB2, Erb-B2 receptor tyrosine kinase 2.

transcriptional activator that acts as a modulator of apoptosis and cell proliferation, two biological processes that impact cell survival and are considered hallmarks of cancer (34). In addition, altered expression of *PHLDA1* has been described in different types of tumor, such as melanoma, breast and intestinal and gastric tumors (Table I).

Reduced *PHLDA1* expression has been described in melanoma (14), breast carcinoma (15,35,36), oral carcinoma (37) and gastric adenocarcinoma (16). This indicates that *PHLDA1* may act as a tumor suppressor gene. However, *PHLDA1* was reported to be upregulated in colon cancer and in human

intestinal adenoma and carcinoma, and *PHLDA1* knockdown in colon cancer cells inhibits anchorage-independent cell growth and reduces cell migration (17,38). Furthermore, high expression of *PHLDA1* is associated with high metastatic potential in osteosarcoma cells and reduced *PHLDA1* expression delays overall survival metastasis progression in mouse xenograft models (39). These findings suggest that *PHLDA1* has tissue-specific functions and may have an oncogenic role in other cancer types.

In melanoma, downregulation of *PHLDA1* expression was found in metastatic lesions but not in primary tumors,

suggesting that loss of PHLDA1 expression may contribute to the progression of malignant melanoma (14). The effects of PHLDA1 expression on the biological characteristics of different melanoma-derived cell lines were also evaluated. PHLDA1 expression leads to reduced cell growth and colony formation, increased basal apoptosis, and increased sensitivity to apoptosis induced by chemotherapeutic agents (14).

Reduced expression of PHLDA1 was observed in 60.7% of oral squamous cell carcinomas (OSCC), particularly in well-differentiated tumors. Positive PHLDA1 immunostaining was associated with advanced clinical stages of the disease, suggesting that PHLDA1 has a functional role in oral tumorigenesis. Overall and disease-free survival rates were significantly improved in patients with tumors that were negative for PHLDA1, and a multivariate analysis suggested that PHLDA1 is an independent prognostic factor in OSCC patients (37).

Downregulation of PHLDA1 mRNA and protein expression is frequently observed in primary invasive breast tumors. In a large series of primary breast tumors, Nagai *et al* (15) reported that downregulation of the PHLDA1 protein is a strong predictor of poor prognosis for breast cancer patients. This suggests that reduced PHLDA1 expression contributes to breast cancer progression and may serve as a useful prognostic biomarker of disease outcome. The study also found that the subgroup of breast tumors that were negative for estrogen receptor and expressed PHLDA1 had a more favorable outcome. PHLDA1 downregulation has also been associated with estrogen receptor expression in breast tumors (35). In addition, high *PHLDA1* mRNA expression is associated with a high risk of distant metastasis in estrogen receptor-positive breast tumors. This suggests that in breast cancer cells, PHLDA1 may have different functions depending on the estrogen receptor status, which affects the clinical behavior of tumors.

In an investigation of the effects of E2 and the antiestrogen agent ICI 182780 on transcriptional regulation of *PHLDA1* in MCF7 breast cancer cells, Marchiori *et al* (40) demonstrated that PHLDA1 is upregulated by E2 via estrogen receptor in a time-dependent manner. More recently, PHLDA1 was shown to be regulated by crosstalk between ER and inflammatory nuclear factor- κ B (NF- κ B) (41). PHLDA1 is regulated by E2 and tumor necrosis factor- α in MCF7 breast cancer cells through estrogen receptor and NF- κ B activation. Kastrati *et al* (41) also found that estrogen receptor and the NF- κ B pathway activation upregulates PHLDA1 directly by transcriptional activation and by indirectly repressing the expression of miR-181a and b, which directly target PHLDA1.

Reduction of PHLDA1 expression in HME16C mammary epithelial cells during ras-mediated cellular transformation induces proliferation under anchorage-independent conditions and results in increased ERK activation, which suggests that PHLDA1 opposes ERK-mediated transformation in breast cancer cells (42). In a profiling study with SKBR3 breast cancer cells treated with lapatinib, Li *et al* (36) identified PHLDA1 as a novel downstream target in the Erb-B2 receptor tyrosine kinase 2 (ERBB2)/epidermal growth factor receptor pathway and demonstrated that PHLDA1 expression is reduced in ERBB2-negative breast tumors and breast cancer cell lines. In addition, overexpression of PHLDA1 was found

to inhibit proliferation, colony-formation ability and migration in SKBR3 breast cancer cells (36).

A regulatory loop between Aurora-A and PHLDA1 was also reported in breast cancer cells (35). In MDA-MB-231 breast cancer cells, Aurora-A predominantly phosphorylates PHLDA1, leading to its degradation. PHLDA1 overexpression results in decreased levels of Aurora-A, suggesting that PHLDA1 acts as a negative regulator of Aurora-A-mediated breast oncogenesis (35).

In an expression profiling study, Ohyama *et al* (43) determined that PHLDA1 is highly expressed and precisely localized in the bulge, which is the multipotent compartment of the hair follicle. This indicates that PHLDA1 may be an epithelial stem cell marker. The identification of PHLDA1 as a potential epithelial stem cell marker has attracted the attention of several investigators who are researching the possible role of PHLDA1 in the tumorigenic process; however, this area of investigation is in the early stages.

In an investigation of the expression patterns of PHLDA1 in 16 desmoplastic trichoepitheliomas and 14 morphoeic basal cell carcinomas (BCCs), the study by Sellheyer and Krahl (44) reported PHLDA1-positive immunostaining in >80% of the trichoepitheliomas and PHLDA1-negative immunostaining in all the BCCs examined, indicating that expression of the hair follicle bulge marker PHLDA1 can be used to differentiate between trichoepitheliomas and BCCs. Thus, PHLDA1 could be used as a biomarker in the differential diagnosis of basaloid tumors.

Aberrant PHLDA1 expression has been described in intestinal and colorectal cancers (17,38). Upregulation of *PHLDA1* mRNA expression was associated with the presence of anemia in patients with colon cancer (38), and increased nuclear expression of PHLDA1 was observed in the invasive margins of human colorectal carcinomas (17). siRNA knockdown of *PHLDA1* expression in HCT116 and SW480 colon cancer cells under anchorage-dependent growth leads to inhibition of cell growth and colony-formation ability, and *PHLDA1* downregulation also inhibits cell migration in these colon cancer cells (17). In addition, strong PHLDA1-positive immunostaining has been found in undifferentiated crypt base cells that exhibit a similar pattern of LGR5 expression. LGR5 is a putative epithelial stem cell marker for human small and large intestine (17). In agreement with this finding, Guezguez *et al* (45) determined that PHLDA1 is part of a primordial stem-like cell signature in normal human intestinal epithelial crypt cells treated with noggin, an antagonist to bone morphogenetic protein signaling.

In an evaluation of a large number of gastric carcinomas and normal gastric mucosa by immunohistochemistry, Zhao *et al* (16) observed that PHLDA1 is expressed diffusely in the cytoplasm and nucleus in crypt base cells. PHLDA1 expression appeared to mark putative epithelial stem cells in all the normal gastric mucosa analyzed. The study by Zhao *et al* (16) identified negative PHLDA1 expression in 57% of the gastric carcinomas and the most poorly differentiated cases were negative for PHLDA1. Strong nuclear staining was found in invasive margins. Furthermore, reduced PHLDA1 expression was associated with shorter overall survival in gastric cancer patients (16).

In breast cancer, PHLDA1 was identified among the gene set of oncogenic ras-pathway and interferon-response

signatures in selected small populations of cluster of differentiation (CD) 24^{-low}/CD44⁺ stem cells from several breast cancer cell lines (46). More recently, high PHLDA1 expression was shown to contribute to the survival and growth of breast cancer stem cells (41). Kastrati *et al* (41) also found upregulation of PHLDA1 mRNA and protein expression in a panel of estrogen receptor-positive breast cancer cells that were growing in mammosphere cultures compared to cells growing in adherent monolayer cultures. The study also observed that PHLDA1 knockdown affects mammosphere formation and growth, leading to a reduction in the number and size of the mammospheres.

4. Conclusions

Over the last few years, PHLDA1 has attracted increasing attention. However, the data reviewed herein show that the precise molecular and cellular functions of PHLDA1 remain to be fully elucidated. Although several lines of evidence have implicated PHLDA1 as a potential transcriptional activator that acts as a proapoptotic and antiproliferative factor, the exact mechanisms by which PHLDA1 mediates cell survival are still under investigation. PHLDA1 expression can be modulated by various stimuli, resulting in pleiotropic effects, with upregulation and downregulation of different biological processes, such as proliferation, apoptosis, migration and invasion, which are considered key hallmarks of carcinogenesis. The pleiotropic effects of PHLDA1 indicate tissue-specific activities that depend on the cellular context. PHLDA1 is expressed in different tissues with variable subcellular localization and function, and its expression pattern is altered in different types of tumors. Data gathered thus far indicate that PHLDA1 has an important role in the tumorigenic process; however, new results from clinical and experimental studies are necessary to improve the definition of its role as a tumor suppressor gene or oncogene and its contribution to tumor progression. Further, well-designed clinical and experimental studies, *in vitro* and *in vivo*, that use informative and testable animal models are required to improve the understanding of the PHLDA1 mechanisms of action in normal and cancer cells. In addition, the identification of PHLDA1 as a potential stem cell marker and evidence of its possible roles in the EMT indicate that there are new perspectives and research fields to be explored to evaluate the actual contributions of PHLDA1 to the tumorigenic process and its role not only as prognostic factor, but also as a potential therapeutic target.

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