Cancer Research

PHLDA1 Expression Marks the Putative Epithelial Stem Cells and Contributes to Intestinal Tumorigenesis

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Abstract

Studies employing mouse models have identified crypt base and position +4 cells as strong candidates for intestinal epithelial stem cells. Equivalent cell populations are thought to exist in the human intestine; however robust and specific protein markers are lacking. Here, we show that in the human small and large intestine, PHLDA1 is expressed in discrete crypt base and some position +4 cells. In small adenomas, PHLDA1 was expressed in a subset of undifferentiated and predominantly Ki-67–negative neoplastic cells, suggesting that a basic hierarchy of differentiation is retained in early tumorigenesis. In large adenomas, carcinomas, and metastases PHLDA1 expression became widespread, with increased expression and nuclear localization at invasive margins. siRNA-mediated suppression of PHLDA1 in colon cancer cells inhibited migration and anchorage-independent growth *in vitro* and tumor growth *in vivo*. The integrins *ITGA2* and *ITGA6* were downregulated in response to PHLDA1 suppression, and accordingly cell adhesion to laminin and collagen was significantly reduced. We conclude that PHLDA1 is a putative epithelial stem cell marker in the human small and large intestine and contributes to migration and proliferation in colon cancer cells. *Cancer Res; 71(10); 3709–19.* ©*2011 AACR.*

Introduction

Although it is generally accepted that intestinal epithelial stem cells (ISC) reside at the crypt base, their identity is subject to debate (1). In mice, 2 candidates for ISCs have been identified through Cre-mediated lineage tracing: Lgr5 (leucine-rich repeat containing G-protein coupled receptor 5) expressing crypt base cells which are interspersed between Paneth cells in the small intestine and goblet cells in the large intestine (2), and Bmi1 (Bmi1 polycomb ring finger oncogene) expressing cells at "position +4" relative to the crypt bottom in the proximal small intestine (3). In the small intestine, the Lgr5+ cells are also referred to as crypt base columnar cells (CBCC; ref. 4). Lgr5+ crypt base cells are the likely cells of origin of intestinal neoplasia (5). Whether CBCCs and position +4 cells in the small intestine represent different, overlapping, or identical populations of ISCs remains unresolved.

Additional candidate markers for the putative crypt base ISCs in mice have been identified through gene expression profiling of Lgr5+ cells, with validation for *Ascl2* (Achaete scute complex homolog 2) in the small and large intestine and *Olfm4* (olfactomedin-4) in the small intestine (6). Similarly Msi1 (Musashi-1) and Prom1 (Prominin-1/CD133) are expressed in crypt base cells, but these markers lack specificity with expression extending to transit amplifying cells (7, 8). Despite these substantive advances in mice, limited progress has been made regarding the assessment of candidate ISC markers in humans. Conflicting data exist on *BMI1* mRNA and protein expression in human small and large intestine (9–11), and systematic assessment of LGR5, ASCL2, and OLFM4 is hindered by a lack of appropriate antibodies and has been limited to surrogate *in situ* hybridization studies. *OLFM4* is perhaps the best studied candidate in humans, showing mRNA expression at the crypt base throughout the intestinal tract (12). *OLFM4* appears to be highly expressed in a subset of colorectal carcinoma cells, although data is limited to a small number of tumors (12).

We and others have previously identified overexpression of *PHLDA1* (pleckstrin homology-like domain family A member 1)

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in intestinal tumors of both humans and mice (13–15), and the gene has been shown to be coexpressed with *Lgr5* in murine crypt base cells (6). *PHLDA1* encodes a 401–amino acid protein that comprises a central pleckstrin homology domain common to proteins involved in intracellular signaling or as constituents of the cytoskeleton (16–18), a central polyglutamine tract, and 2 C-terminal regions rich in proline–glutamine and proline–histidine repeats. The gene is expressed in a wide range of normal and cancer tissues (19, 20). PHLDA1 function varies with cell type and context, with several studies reporting a proapoptotic (20–23) or antiproliferative role (24). *PHLDA1* expression is induced by external stresses such as heat shock (22, 23), and may be modulated by IGF-I (insulin-like growth factor I; ref. 25) and ERK (extracellular-regulated kinase) pathways (24).

Here we characterized the expression of PHLDA1 protein in the human small and large intestine, and compared this with other proposed markers of ISCs. We further determined the pattern of PHLDA1 expression in intestinal adenomas and carcinomas from all sites and stages, and explored PHLDA1 function in human colon cancer cell lines.

Materials and Methods

Patient specimens and cell lines

Archival and fresh-frozen intestinal tumor and adjacent normal tissues were retrieved from patients treated at the Royal Melbourne and Western Hospitals in Melbourne, Australia. Sample characteristics are summarized in Supplementary methods. This study was approved by the hospitals' ethics committee and all patients gave informed consent.

HCT116 (ATCC CCL-247) and SW480 (ATCC CCL-228) colon cancer cell lines were authenticated in 2010 by short tandem repeat (STR) analysis. Cells were cultured with Dulbecco's modified Eagle's medium (DMEM) and 10% FBS at 37°C and 5% CO_2.

In vitro experiments and functional assays

IHC, *in situ* hybridization, immunoblotting, and laser capture microdissection were performed using standard methods (Supplementary Methods). Colon cancer cell lines were analyzed for apoptosis (Annexin V/7-AAD), anchorage-dependent growth (MTT conversion assay), anchorage-independent growth (soft agar colony formation), cell migration (scratch and chamber migration assays), cell-matrix adhesion (collagen, laminin), response to Wnt pathway stimulation (Wnt3a-conditioned medium) and *in vivo* growth as xenografts as described in Supplementary Methods.

Transient transfections were conducted using lipofectamine 2000 (Invitrogen) with the following siRNAs: siPHLDA1_2 (Hs_PHLDA1_2, target sequence 5′AGGAGCGATGATGTACTGTAA3′, Qiagen), siPHLDA1_5 (Hs_PHLDA1_5, target sequence 5′CTAATCCGTAGTAATTCCTAA3′, Qiagen), control-siRNA (siNEG; AllStars Neg Control, Qiagen), β-catenin-siRNA pools (L-003482-00-0005, Dharmacon), and control-siRNA pools (D-001810–20, Dharmacon).

Total RNA was prepared using the RNeasy Mini Kit (Qiagen), and reverse transcribed using the SuperScript III First-Strand Synthesis Kit (Invitrogen). Quantitative real-time PCR was performed on a 7500 Fast Real-time PCR System (Applied Biosystems) using TaqMan assays (Applied Biosystems) against *PHLDA1* (Hs00378285_g1), *LGR5* (Hs00173664_m1), and the housekeeping gene *HPRT* (hypoxanthine phosphoribosyltransferase 1; HuHPRT_0604009) and SYBR green assays (Qiagen) against *MYC* (QT00035406) and *β-catenin* (QT00077882). Expression of 84 extracellular matrix (ECM) and cell adhesion genes was assayed using the Human Extracellular Matrix and Adhesion Molecules RT^2 Profiler PCR Array (SABiosciences).

Statistical analysis

Statistical analyses were performed using the Student's *t* test for continuous variables. The Compare Groups of Growth Curves (CGGC; http://bioinf.wehi.edu.au/software/compareCurves) permutation test was used to compare groups of curves for the MTT cell proliferation, scratch, and cell line xenograft assays. All analyses were 2-sided and regarded as significant for $P < 0.05$.

Results

PHLDA1 is expressed in undifferentiated crypt base cells of the human intestine

Cytoplasmic PHLDA1 staining was detected by IHC in discrete cells at the crypt base along the entire human adult intestine (Fig. 1A). In the small intestine PHLDA1 staining cells were slender CBCCs interspersed between Paneth cells in all crypts, and cells immediately above the Paneth cells (position +4) in some crypts. Similar expression was seen for the proximal colon in regions where Paneth cells were present. In the distal colon and rectum, PHLDA1 was expressed in crypt base cells interspersed between goblet cells. These localizations were confirmed by double staining for PHLDA1 and the Paneth cell-specific marker α-defensin in the small intestine, and PHLDA1 and goblet cell-specific mucin in the large intestine (Supplementary Fig. 1). The location and morphology of PHLDA1 expressing cells in the human intestine closely resembled that of *Lgr5* expressing crypt base cells in mice as previously shown by Barker and colleagues (2). Accordingly, *in situ* hybridization for *LGR5* mRNA in humans demonstrated expression limited to the crypt base (Fig. 1B), and both *PHLDA1* and *LGR5* were shown to be largely restricted to the crypt base in laser capture microdissected samples from the human small and large intestine (Supplementary Fig. 2). In *Lgr5-EGFP-IRES-creERT2* knock-in mice (2), coexpression of Phlda1 and Lgr5-EGFP in CBCCs was further confirmed by IHC in serial sections (Fig. 1C). However, a decreasing gradient of Phlda1 protein was noted above CBCCs in murine tissue.

PHLDA1 expression was compared with putative ISC markers other than LGR5 in the human small and large intestine (Fig. 1B). MSI1 protein was expressed more widely in the crypt epithelium, including crypt base, position +4, and transit amplifying cells. BMI1 protein was detected in single cells, predominantly at the crypt base and mid crypt, occasional position +4 cells and in cells higher up the crypt-villus axis.

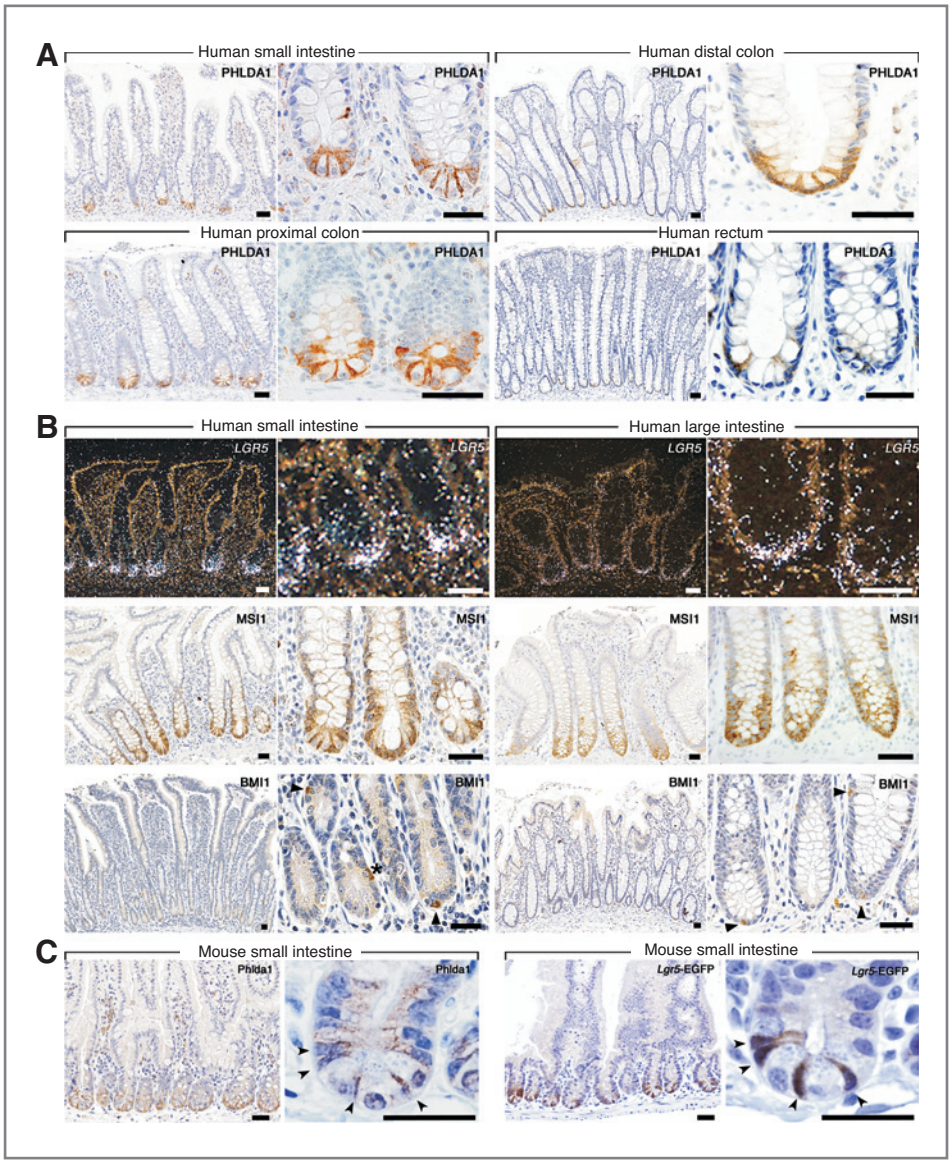

Figure 1. PHLDA1 marks undifferentiated crypt base cells in human small and large intestine: comparison with putative epithelial stem cell markers. A, PHLDA1 protein expression by IHC in CBCCs and some position +4 cells in small intestine and parts of proximal colon. Paneth cells are identified by apical cytoplasmic granules. In distal colon and rectum, PHLDA1 was expressed in crypt base cells interspersed between goblet cells. There was weak PHLDA1 staining of cells in the lamina propria. B, expression of other putative epithelial stem cell markers in human small and large intestine. *LGR5* mRNA expression by *in situ* hybridization resembled PHLDA1 protein expression in small and large intestine. MSI1 protein expression by IHC was seen in the crypt base and transit amplifying compartment in small and large intestine. BMI1 protein expression by IHC was seen in single cells (arrowheads) predominantly at the crypt base, including occasional position +4 cells (asterisk), in small and large intestine. Scale bars, 50 μm. C, Phlda1 and *Lgr5*-EGFP expression by IHC in small intestine from *Lgr5-EGFP-IRES-creERT2* knock-in mice. Strong staining for Phlda1 and *Lgr5*-EGFP was seen in slender CBCCs. Costaining for Phlda1 and *Lgr5*-EGFP (arrowheads) was demonstrated in 4 μm serial sections (right side). Scale bars, 25 μm.

PHLDA1 is overexpressed in human intestinal tumors of all stages

We have previously shown by gene expression microarray analysis that *PHLDA1* is overexpressed in mouse and human intestinal tumors as compared with normal epithelium (15). Here, this was further validated by real-time reverse-transcriptase (RT)-PCR analysis in a series of normal colorectal tissues ($n = 20$), stage A to D colorectal cancers ($n = 20$ per stage) and HCT116 and SW480 colon cancer cell lines (Fig. 2A).

PHLDA1 IHC on small (<1 cm) colorectal adenomas revealed cytoplasmic staining in 100% (42 of 42) and nuclear staining in 19% (8 of 42) of cases (Supplementary Fig. 3). Notably, PHLDA1 expression was confined to discrete neoplastic cells of "undifferentiated" morphology interspersed with nonstaining neoplastic cells showing goblet cell differentiation, reminiscent of the differentiation hierarchy observed in normal crypts (Fig. 3A). Double staining of small adenomas with the active-proliferation marker Ki-67 showed a strong inverse association with PHLDA1 (Fig. 3C): for Ki-67 expressing cells from 5 small adenomas, 82.2% (411/500) were PHLDA1 negative and 17.8% (89 of 500) were PHLDA1 positive. This again mirrored the pattern found in normal crypts, where PHLDA1 staining was confined to the crypt base/stem cell compartment and Ki-67 staining to the mid crypt/transit amplifying cell compartment (Fig. 3C). In occasional small adenomas, a subset of the PHLDA1 expressing neoplastic cells displayed *de novo* expression of the Paneth cell marker α-defensin, irrespective of adenoma location in the intestinal tract (Fig. 3B).

In large adenomas and carcinomas, PHLDA1 staining became more widespread and included cells with goblet cell differentiation (Fig. 2B). Overall, 100% (19 of 19) of benign

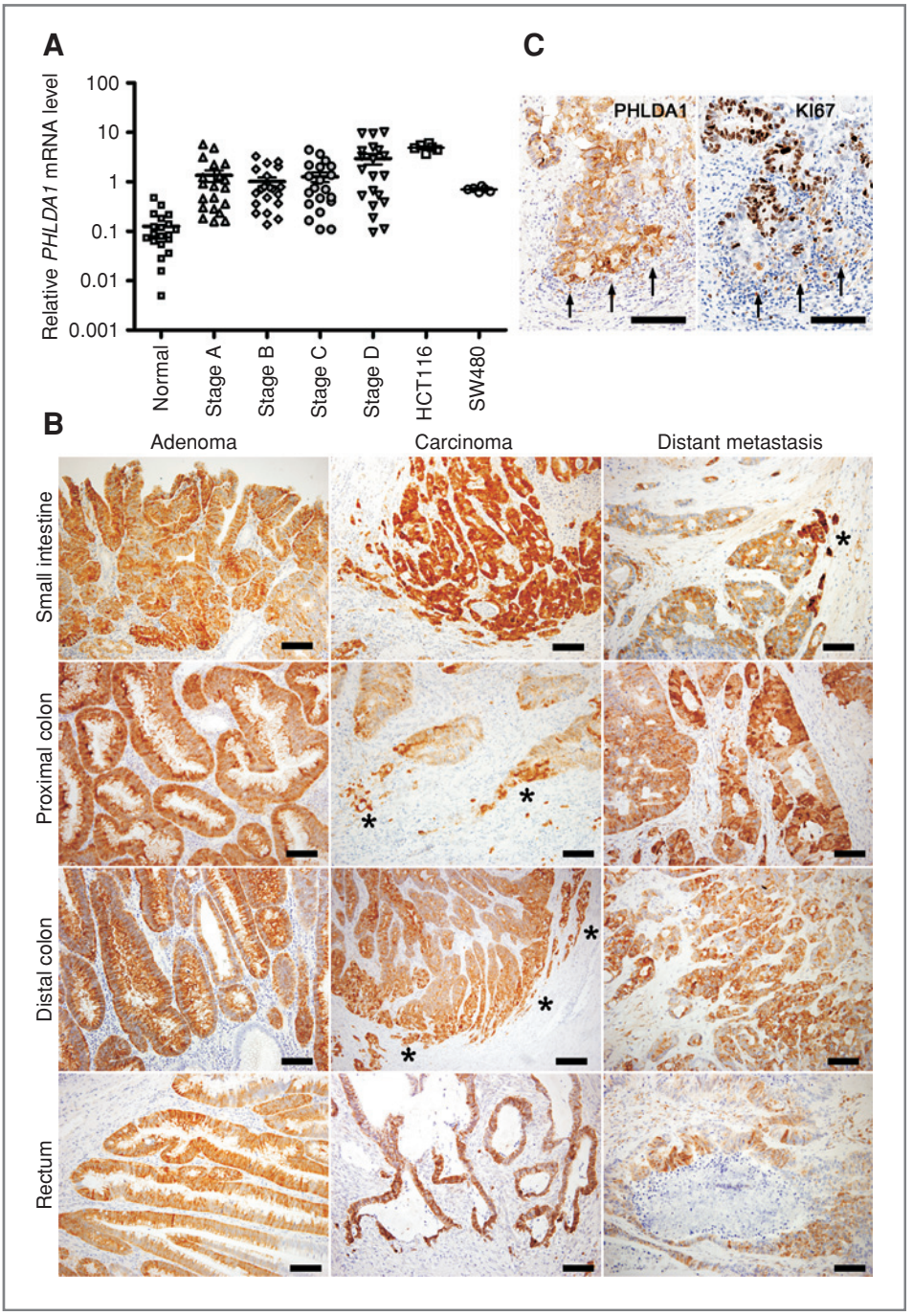

Figure 2. PHLDA1 is overexpressed in all stages of colorectal cancer. A, *PHLDA1* mRNA overexpression by real-time RT-PCR in stage A to D colorectal cancers ($n = 20$ per stage) and HCT116 and SW480 cells ($n = 6$ replicates) compared with normal mucosa ($n = 20$). Data are normalized to *HPRT* levels. B, representative images of PHLDA1 expression by IHC in adenomas, carcinomas, and distant metastases from small intestine, proximal colon, distal colon, and rectum. Asterisks mark areas with strong cytoplasmic and nuclear expression in tumor cells at the invasive front. C, PHLDA1 and Ki-67 IHC at the invasive front of a colorectal adenocarcinoma. PHLDA1 is strongly expressed at the leading edge with Ki-67 showing an opposite distribution (arrows). Scale bars, 100 μm.

lesions, 100% (20 of 20) of stage A, 95% (19 of 20) of stage B, 100% (20 of 20) of stage C, 87% (20 of 23) of stage D tumors, and 78% (7 of 9) of metastases showed cytoplasmic staining for PHLDA1 (Fig. 2B, Supplementary Fig. 3A). Cytoplasmic staining was of variable intensity in most cases, but tended to be stronger at invasive margins. Additional nuclear staining was seen in 21% (4 of 19) of large adenomas, 25% (5 of 20) of stage A, 40% (8 of 20) of stage B, 40% (8 of 20) of stage C, 52% (12 of 23) of stage D carcinomas, and 66% (6 of 9) of metastases (Supplementary Fig. 3B). In the majority of cases with nuclear staining this occurred predominantly in cells at invasive margins. Strong PHLDA1 staining at the invasive margin was inversely correlated with Ki-67 staining in serial sections, consistent with a switch from a proliferative to an invasive phenotype (Fig. 2C). There was no correlation between PHLDA1 staining and adenocarcinoma grade (data not shown).

PHLDA1 IHC on 2 adenomas, 5 carcinomas, and 1 distant metastasis from the small intestine showed a similar pattern to that observed for colorectal tumors (Fig. 2B).

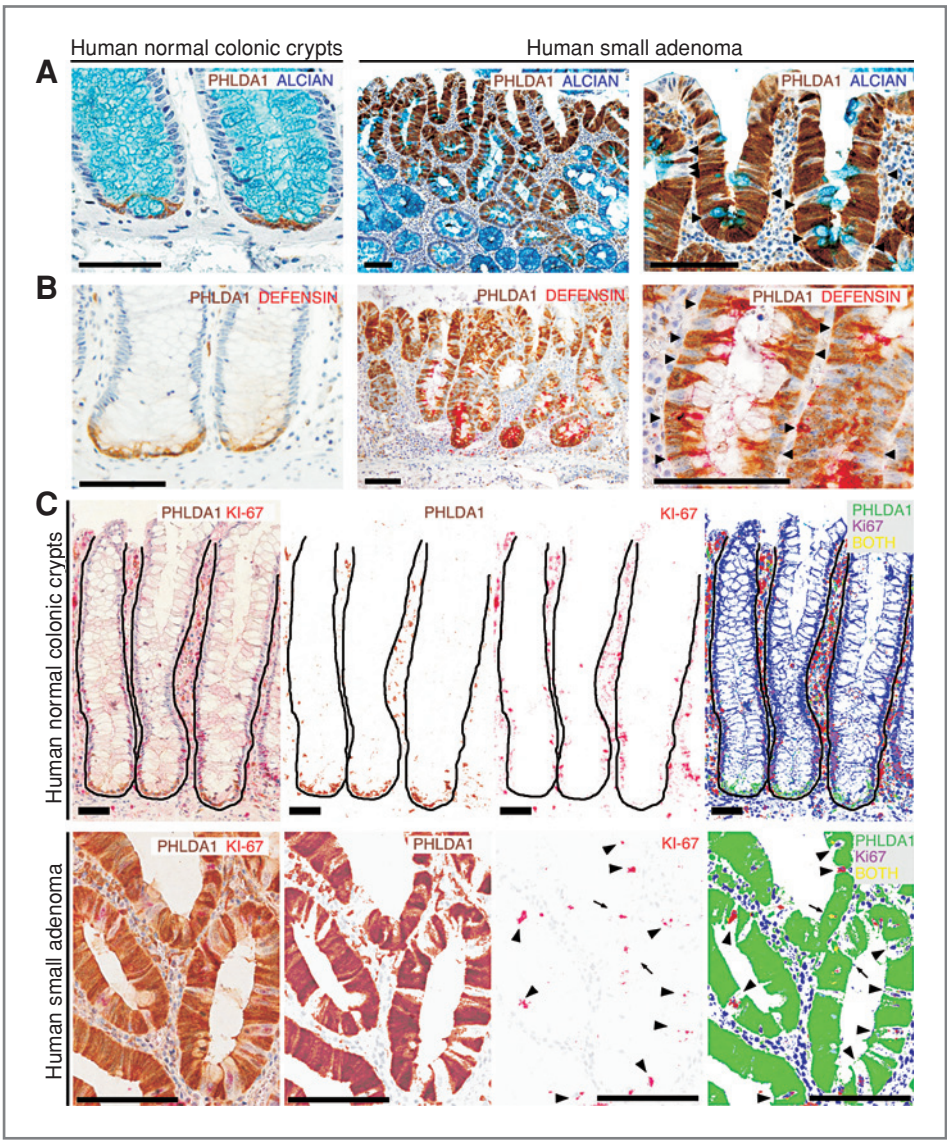

Figure 3. PHLDA1 staining in human normal large intestine and small (<1 cm) adenomas suggests maintenance of a stem cell/progenitor cell hierarchy of differentiation in early tumorigenesis. A, representative double staining of normal large intestine and a small (<1 cm) adenoma for PHLDA1 (DAB, brown) and goblet cell mucin (Alcian blue). In normal epithelium, PHLDA1-expressing cells were restricted to the crypt base and were situated between non-PHLDA1-expressing goblet cells. In the adenoma, PHLDA1 staining was confined to discrete neoplastic cells of "undifferentiated" morphology interspersed with PHLDA1-negative neoplastic goblet cells (arrowheads). B, double staining for PHLDA1 (DAB, brown) and the Paneth cell marker α-defensin (Fast Red, red). In normal epithelium, Paneth cells were absent and no α-defensin staining was detected. In occasional adenomas, a subset of PHLDA1 staining neoplastic cells showed *de novo* expression of α-defensin (arrowheads). C, double staining for PHLDA1 (DAB, brown) and Ki-67 (Fast Red, red) with color deconvolution and colocalization image analysis. In normal epithelium, PHLDA1 staining was confined to the crypt base/stem cell compartment and Ki-67 staining to the mid crypt/transit amplifying cell compartment. Staining for neither marker was seen in the upper crypt/differentiated cell compartment. In the adenoma, PHLDA1 and Ki-67 expression (arrowheads) was generally mutually exclusive, although occasional coexpression was seen (arrows). Colocalization color scheme: green/cyan, PHLDA1; red/purple, Ki-67; yellow, PHLDA1+Ki-67; and blue, haematoxylin. Scale bars, 50 μm.

PHLDA1 is not a direct target of the canonical Wnt signaling pathway

Overexpression of *PHLDA1* in intestinal tumors with demonstrated mutations activating the Wnt/β-catenin signaling pathway (13–15) suggests this gene as a potential direct downstream target of Wnt/β-catenin signaling. To test this hypothesis, we analyzed HCT116 and SW480 cells transfected with β-catenin-siRNA for changes in *PHLDA1* mRNA levels, using measures of *MYC*, a known direct target of Wnt/β-catenin signaling, as a positive control. Compared with control-siRNA (siNEG), both *β-catenin* and *MYC* expression were significantly downregulated in β-catenin-siRNA transfected cells ($P < 0.05$; Student's t test; Fig. 4A). In contrast, *PHLDA1* was upregulated in HCT116 cells and no significant change was detected in SW480 cells.

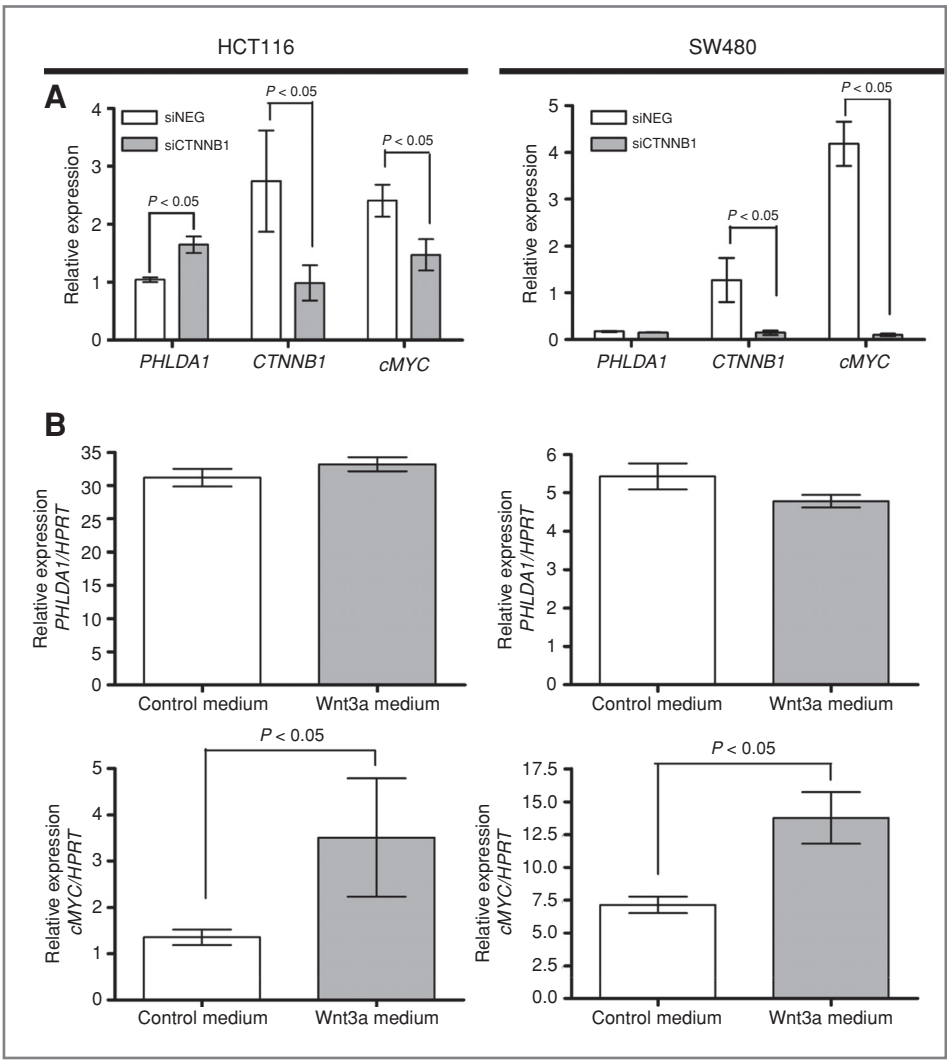

Figure 4. PHLDA1 is not a direct target of the canonical Wnt/β-catenin signaling pathway. A, HCT116 and SW480 cells transfected with β-catenin-siRNA (siCTNNB1) showed significant downregulation of *β-catenin* and the known direct Wnt/β-catenin pathway target *MYC* as compared with control-siRNA (siNEG) transfected cells. *PHLDA1* expression did not mirror these changes. B, HCT116 and SW480 cells cultured in the presence of Wnt3a-conditioned medium showed upregulation of *MYC* expression but no change in *PHLDA1* expression as compared with control medium. Data from 2 experiments; $n = 12$; means ± SEM.

Similar results were obtained for stimulation of HCT116 and SW480 cells with Wnt3a-conditioned medium. Compared to control medium, *MYC* expression showed significant upregulation after stimulation with Wnt3a ($P < 0.05$; Student's *t* test), whereas *PHLDA1* expression remained constant (Fig. 4B).

PHLDA1 suppression in colon cancer cells inhibits anchorage-independent growth *in vitro*

Several *in vitro* studies on nonintestinal cell lines have indicated a proapoptotic or antiproliferative role for PHLDA1 (20–24), whereas others have found no or opposing effects (25, 26). To evaluate these potential roles in colon cancer cell lines which express high levels of PHLDA1 protein, we analyzed HCT116 and SW480 cells for changes in apoptosis and cell proliferation following downregulation of endogenous PHLDA1 levels.

Cells were transiently transfected with 2 alternative PHLDA1-siRNAs or siNEG, and downregulation of *PHLDA1* mRNA and protein expression was confirmed to be greater than 70% using real-time RT-PCR and Western blotting (Fig. 5A). Early apoptosis and late apoptosis/necrosis were measured using flow cytometry and Annexin V/7-AAD staining. As HCT116 and SW480 cells show low basal levels of apoptosis, measurements were made for cells growing under standard conditions and following incubation with hydrogen peroxide, a potent inducer of programmed cell death (27). As anticipated, treatment with hydrogen peroxide resulted in a significant increase in the proportion of early apoptotic and late apoptotic/necrotic cells when compared with no treatment, but PHLDA1 suppression had no detectable influence under either experimental condition (Fig. 5B). Furthermore, we observed no difference in anchorage-dependent growth when siPHLDA1 and siNEG transfected cells were monitored over a 4 day period using MTT conversion assays (Fig. 5D).

However, when analyzed for anchorage-independent growth using colony formation assays in soft agar, PHLDA1 suppressed cells demonstrated clear inhibition of growth with significantly fewer colonies as compared with siNEG cells ($P < 0.002$ for all comparisons; Student's *t* test;

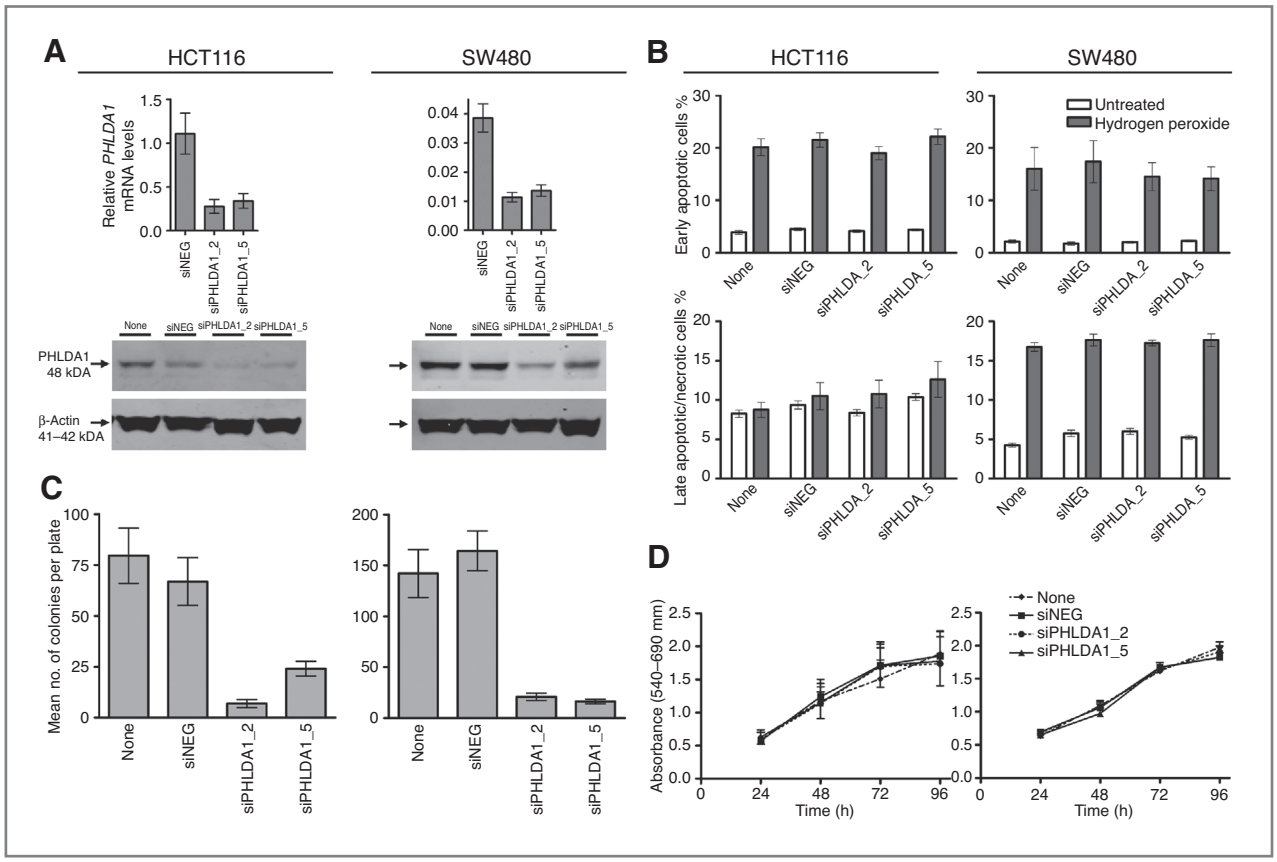

Figure 5. Suppression of PHLDA1 in colon cancer cells does not affect anchorage-dependent growth or apoptosis but inhibits anchorage-independent growth. A, confirmation of PHLDA1 suppression in HCT116 and SW480 cells transfected with PHLDA1-siRNAs by real-time RT-PCR and Western blotting. Real-time RT-PCR data are normalized to *HPRT* levels for 6 replicates; means ± SEM. B, Annexin V/7AAD staining showed similar proportions of cells in early or late apoptosis/necrosis for PHLDA1-siRNA and siNEG transfected cells under standard culture conditions or when treated with hydrogen peroxide to induce apoptosis. Data from 2 experiments; $n = 12$; means ± SEM. C, soft agar colony formation assays demonstrated significant reduction in anchorage-independent growth for PHLDA1-siRNA compared with siNEG transfected cells ($P < 0.002$). Data from 3 experiments; $n = 4$; means ± SEM. D, MTT assays showed similar rates of anchorage-dependent growth for PHLDA1-siRNA and siNEG transfected cells. Data from 2 experiments; $n = 16$; means ± SEM.

Fig. 5C). There was no apparent difference in colony formation between siNEG and untransfected cells indicating that the transfection process had little impact on this phenotype.

PHLDA1 suppression in colon cancer cells inhibits cell migration *in vitro*

Given the increased expression and nuclear localization of PHLDA1 at the invasive margin of human colorectal carcinomas, we assessed the effect of PHLDA1 suppression on colon cancer cell migration. In scratch assays, HCT116 and SW480 cells transiently transfected with siPHLDA1 showed significantly slower rates of wound closure when compared to siNEG cells ($P < 0.0001$ for all comparisons, CGGC; Fig. 6A). This was likely to reflect actual changes in migratory ability, given that anchorage-dependent growth was similar for siPHLDA1 and siNEG transfected cells. To confirm the role of PHLDA1 in cell migration, we performed chamber migration assays. Again siPHLDA1 transfected HCT116 or SW480 cells showed significantly reduced migration when compared with siNEG transfected cells ($P < 0.05$ for all comparisons, Student's t test; Fig. 6B). There was no apparent difference in migration between siNEG and untransfected cells for either assay.

PHLDA1 suppression results in downregulation of *ITGA2* and *ITGA6* and decreased cell-matrix adhesion

Given the apparent role of PHLDA1 in cell migration, we assessed the gene expression levels of 84 ECM and cell adhesion genes in siPHLDA1 and siNEG transfected HCT116 and SW480 cells using Human ECM and Adhesion Molecules RT^2 Profiler PCR Arrays. In both cell lines, 2 genes showed significant changes in expression, with downregulation of more than 2-fold in response to PHLDA1 suppression: *ITGA2 (Integrin alpha 2)* and *ITGA6* (*Integrin alpha 6*; $P < 0.05$, Student's t test; Fig. 6C).

ITGA2 and ITGA6 serve as integral membrane receptors that form focal adhesion contacts with various ECM-ligands including collagen (ITGA2) and laminin (ITGA2 and ITGA6; ref. 28). To determine the functional role of decreased *ITGA2* and *ITGA6* levels in PHLDA1-suppressed cells,

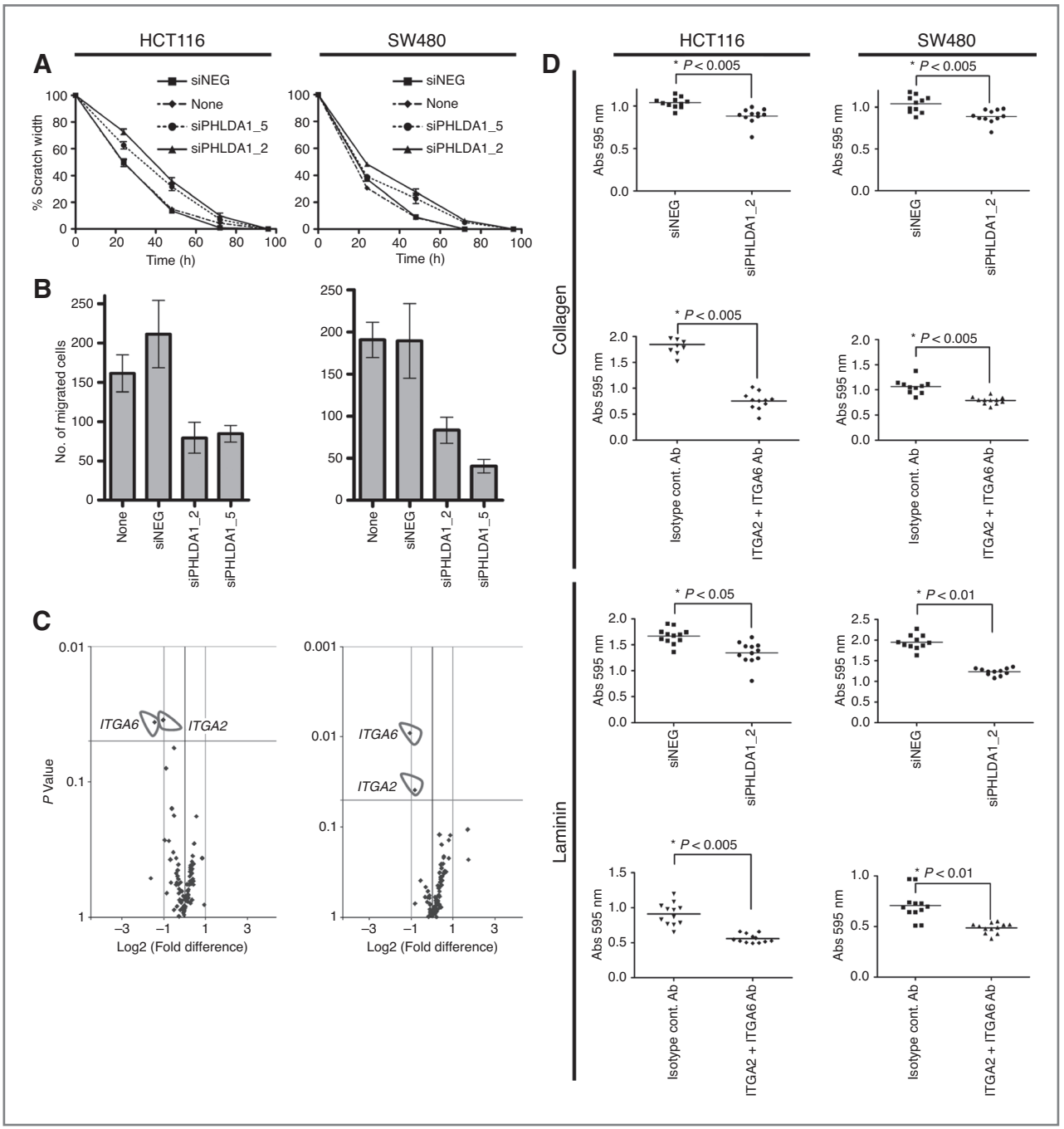

Figure 6. PHLDA1 suppression inhibits migration of colon cancer cells. A, scratch assays for HCT116 and SW480 cells transfected with PHLDA1-siRNA showed significantly reduced wound closure compared with control-siRNA (siNEG) transfected cells ($P < 0.0001$). Data from three experiments; $n = 14$; means $\pm$ SEM. B, chamber migration assays also demonstrated a reduction in migration ($P < 0.05$). Data from 2 experiments; $n = 8$; means $\pm$ SEM. C, relative gene expression levels for 84 ECM and cell adhesion genes for siPHLDA1_2 transfected as compared with siNEG transfected cells. *ITGA2* and *ITGA6* showed significant downregulation in both cell lines (>2-fold, $P < 0.05$). Data from 4 experiments; $n = 8$. D, cell-matrix adhesion assays ($n = 12$) demonstrated significantly reduced adhesion to collagen ($P < 0.005$) and laminin ($P < 0.05$), an effect phenocopied by functional-blocking of ITGA2 and ITGA6 with monoclonal antibodies ($P < 0.005$ and $P < 0.05$, respectively).

adhesion to collagen and laminin was assessed for siPHLDA1 and siNEG transfected cells. As anticipated, suppression of PHLDA1 was associated with a significantly reduced ability of HCT116 and SW480 cells to adhere to either ligand ($P < 0.01$, Student's t test; Fig. 6D). This effect was phenocopied by functional-blocking of ITGA2 and ITGA6 with monoclonal antibodies as compared with blocking with isotype control antibody (Fig. 6D).

PHLDA1 suppression in colon cancer cells inhibits tumor growth *in vivo*

To evaluate whether PHLDA1 suppression inhibited colon cancer cell growth *in vivo*, we analyzed xenografts generated by injecting HCT116 cells transfected with siPHLDA1_2 or siNEG into the rear flanks of BALB/c athymic nude mice. Xenograft growth was monitored for 18 days and tumor size measured on consecutive days (Fig. 7). Knockdown of PHLDA1 expression was confirmed by real-time RT-PCR prior to injection, and by IHC on a subset of tumors harvested on day 3 and 5 after xenograft. Measurements throughout the 18 day period demonstrated a significant reduction of growth for the siPHLDA1-HCT116 tumors as compared with siNEG-HCT116 tumors ($P < 0.0003$, CGGC): on day 18 postinjection the mean tumor volume was 145 and 251 mm^3 for siPHLDA1_2 and siNEG tumors, respectively. There were no apparent histological differences between siPHLDA1 knockdown and control tumors.

Discussion

We have identified PHLDA1 as a putative marker of epithelial stem cells in the human adult small and large intestine. In the small intestine, PHLDA1 protein was expressed in the morphologically distinct CBCCs and some position +4 cells. In the colon and rectum, PHLDA1 protein was expressed in undifferentiated columnar cells limited to the crypt base. This distribution of PHLDA1 expressing cells in the human intestine closely resembles that of Lgr5 in the mouse intestine, previously shown to mark intestinal stem cells through Cre-mediated lineage tracing (2). Accordingly, we confirmed by *in situ* hybridization on tissue sections and by real-time RT-PCR on microdissected crypts that *LGR5* expression is limited to the crypt base in the human intestine, in a similar distribution to *PHLDA1*. In transgenic mice, we further demonstrated coexpression of Phlda1 and Lgr5-EGFP in CBCCs, however a decreasing gradient of Phlda1 protein was evident above CBCCs in murine tissue. Consistent with this, Van der Flier and colleagues have previously demonstrated coexpression of *Phlda1* and *Lgr5* in flow cytometry isolated *Lgr5*-EGFP expressing crypt base cells from the mouse intestine (6).

The restricted crypt base expression of PHLDA1 protein in human intestine was illustrated by comparison with MSI1, a crypt base marker whose expression extends to transit amplifying cells. Similarly, PHLDA1 showed more restricted expression as compared with the published *in situ* hybridization results for *OLFM4*, a suggested marker of ISCs in the human (12). Although both PHLDA1 and BMI1 protein were expressed in a proportion of +4 cells, our data did not conclusively demonstrate an overlap between these 2 populations.

PHLDA1 was consistently expressed in human intestinal adenomas and the majority of carcinomas. In early adenomas (<1 cm) PHLDA1 staining was predominantly cytoplasmic and confined to neoplastic cells of undifferentiated morphology interspersed with neoplastic PHLDA1-negative cells showing goblet cell differentiation. Expression of

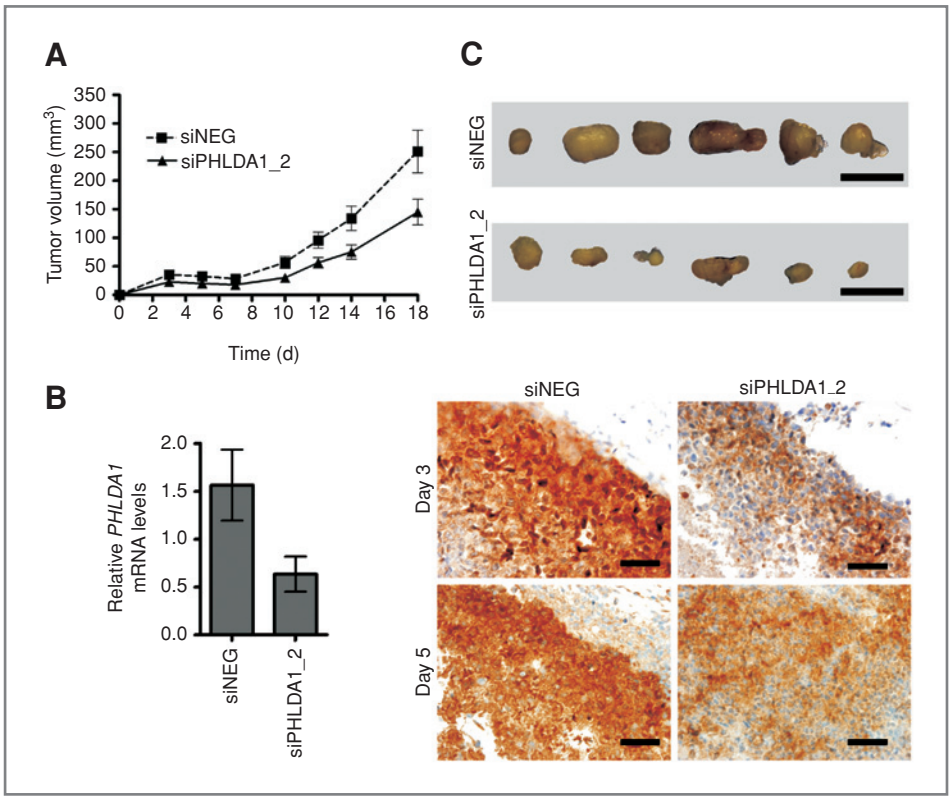

Figure 7. PHLDA1 suppression in colon cancer cells inhibits tumor growth in xenografts. A, HCT116 cells transfected with PHLDA1-siRNA (siPHLDA1_2) showed significantly reduced growth in athymic nude mice compared with control-siRNA (siNEG) transfected cells ($P < 0.0003$). Data from 2 xenograft experiments; $n = 18$; means ± SEM. B, confirmation of PHLDA1 knockdown by real-time RT-PCR before injection and by IHC on days 3 and 5 after xenograft. RT-PCR data are normalized to *HPRT* levels; means ± SEM. Scale bars, 50 μm. C, representative images of tumors harvested on day 18. Scale bar, 1 cm.

PHLDA1 in early adenomas was further inversely associated with the proliferation marker Ki-67. This staining pattern was reminiscent of that observed for normal intestinal crypts, where undifferentiated PHLDA1 expressing crypt base cells give rise to Ki-67 expressing transit amplifying cells and then to the differentiated cells of the intestine including goblet cells. PHLDA1 expression in early human adenomas also resembled that reported for murine adenomas produced by targeted transformation of Lgr5-EGFP expressing stem cells, in which Lgr5-EGFP staining is restricted to small populations of neoplastic cells with undifferentiated stem cell-like morphology (5). However, in occasional early human adenomas a small proportion of PHLDA1 expressing neoplastic cells showed *de novo* expression of the Paneth cell marker α-defensin, indicating that the apparent hierarchy of differentiation in early adenomas is somewhat aberrant. Induction of *de novo* expression of Paneth cell-specific genes in intestinal tumorigenesis has previously been described in mouse models following targeted *Apc* inactivation or gain-of-function β-catenin mutation, as well as in human intestinal tumors (29, 30).

In large adenomas and carcinomas, PHLDA1 cytoplasmic expression became more diffuse and included neoplastic cells with goblet cell differentiation, consistent with a more substantial deregulation of the differentiation hierarchy. With increasing carcinoma stage there was a slight decrease in cytoplasmic PHLDA1 staining, but an increase in cytoplasmic and nuclear staining at the invasive front. The switch to an invasive migratory phenotype at the invasive front was accompanied by a clear reduction in cell proliferation as indicated by decreased Ki-67 staining. To what extent the nuclear and cytoplasmic pools of PHLDA1 have different functions remains to be elucidated. Of interest, the polyglutamine tract in PHLDA1 is a feature common to several transcription factors, suggesting a possible role as a transcription factor or coactivator (31).

Previous studies demonstrating overexpression of *PHLDA1* in intestinal tumors carrying mutations leading to aberrant activation of Wnt/β-catenin signaling have suggested this gene as a potential direct downstream target of the pathway (13–15). Our results from manipulation of Wnt/β-catenin signaling levels in colon cancer cell lines do not support this hypothesis. *PHLDA1* showed upregulation in HCT116 cells and no change in SW480 cells in response to β-catenin suppression and no change in response to Wnt3a stimulation. However, while our data provide evidence against direct regulation of *PHLDA1* expression by the canonical Wnt/β-catenin pathway, they do not exclude the possibility that *PHLDA1* might be a downstream target of noncanonical Wnt signaling.

Some studies on nonintestinal cell types have identified a proapoptotic or antiproliferative role for PHLDA1 (20–24, 32), whereas others have found no or opposite effects (25, 26), suggesting cell type and/or context-dependence for PHLDA1 function. Consistent with context-dependence, downregulation of endogenous PHLDA1 in human colon cancer cells had no effect on anchorage-dependent growth or apoptosis, but did result in inhibition of anchorage-independent growth *in vitro* and xenograft growth *in vivo*. We further found evidence for a novel role for PHLDA1 in cell migration, initially suggested by the increased staining and nuclear relocalization of the protein at the invasive front of intestinal carcinomas. Accordingly, colon cancer cells showed significantly reduced migratory behavior in response to PHLDA1 suppression.

Investigation of the molecular mechanisms by which PHLDA1 may mediate its pro-migratory and context-dependent proproliferative function revealed a direct association between PHLDA1 suppression and downregulation of *ITGA2* and *ITGA6* expression. *ITGA2* and *ITGA6* are members of the integrin receptor family involved in regulation of cell-matrix adhesion and cellular signaling, and their expression is strongly implicated in tumor proliferation, migration, and invasion (33). For example, expression of the α6β4 receptor has been associated with increased tumor size and grade and decreased survival in breast cancer (34, 35). Similarly, upregulation of the α6β4 receptor has been reported for primary tumors of the human colon (36) and at the invasive front of colorectal cancers (37). Consistent with the functional role of *ITGA2* and *ITGA6*, adhesion to collagen and laminin was markedly decreased for PHLDA1 suppressed colon cancer cells, an effect phenocopied by functional-blocking of ITGA2 and ITGA6 with monoclonal antibodies.

In summary, our data indicate that PHLDA1 marks candidate epithelial stem cells in the human intestine. Small adenomas (<1 cm) show retention of some hierarchy of differentiation, and PHLDA1 overexpression through all stages of intestinal cancer development appears to contribute to tumorigenesis, with roles in cell migration and anchorage-independent proliferation. PHLDA1 function in intestinal tumors may be partly mediated by modulation of *ITGA2* and *ITGA6* expression levels. The availability of a protein marker of putative human ISCs will facilitate the study of stem cell biology and tumorigenesis.

Disclosure of Potential Conflicts of Interest

The authors disclose no potential conflicts of interest.

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