Title: PDLIM1 stabilizes the E-cadherin/β-catenin complex to prevent epithelial-mesenchymal transition and metastatic potential of colorectal cancer cells

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Running title:
Loss of PDLIM1 induces EMT and promotes CRC metastasis

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The authors disclose no potential conflicts of interest.
Metastasis is a major cause of death in patients with colorectal cancer (CRC), and increasing evidence supports the contribution of the epithelial-mesenchymal transition (EMT) to cancer progression. The dissociation of the E-cadherin/β-catenin adhesion complex represents a key step in EMT and promotes cancer invasion and metastasis, but the upstream signaling pathways regulating this interaction are poorly understood. Here, we show that PDLIM1, a member of the PDZ and LIM protein family, was downregulated in highly metastatic CRC cells and liver metastases from CRC patients. We found that loss of PDLIM1 promoted the expression of EMT markers and increased the invasive and migratory properties of multiple CRC cell lines. Furthermore, PDLIM1 knockdown increased colon-derived liver metastasis in an orthotopic CRC model and promoted distant metastatic colonization in an experimental lung metastasis model. Mechanistic investigations revealed that PDLIM1 interacted with and stabilized the E-cadherin/β-catenin complex, thereby inhibiting the transcriptional activity of β-catenin and preventing EMT. Accordingly, PDLIM1 overexpression attenuated EMT of CRC cells. Moreover, the downregulation of PDLIM1 in CRC samples correlated with reduced E-cadherin and membrane β-catenin levels, and was associated with shorter overall survival. In conclusion, our study demonstrates that PDLIM1 suppresses EMT and metastatic potential of CRC cells by stabilizing β-catenin at cell-cell junctions, and its loss in metastatic tissues may represent a potential prognostic marker of aggressive disease.
Introduction

Colorectal cancer (CRC) is the third most common cancer in men and the second most common cancer in women worldwide, with an estimated incidence of more than 1.3 million in 2012, and mortality of 700,000 (1). Despite various advances in the diagnosis and treatment of CRC over the past decades, the overall prognosis for CRC patients remains poor (2). Tumor metastasis is a significant factor in the management of CRC and is a major obstacle to successful treatment. Although several therapeutic strategies including targeted drugs have been developed, the management of metastatic CRC remains problematic due to distinct molecular alterations in the tumor (3). Thus, a better understanding of the molecular mechanism underlying CRC metastasis may lead to new therapeutic strategies.

The epithelial-mesenchymal transition (EMT) is a biological process involving multiple molecular events by which the polarized epithelial cells acquire mesenchymal cell phenotypes (4). In CRCs, loss of E-cadherin has been characterized as a unique trait of EMT cells at the invasive front and is usually concomitant with deregulation of Wnt signaling pathway (4). β-catenin, a key component of the canonical Wnt signaling pathway, plays a crucial role in the negative regulation of E-cadherin and the induction of EMT (5). Under normal circumstances, most β-catenin and E-cadherin is present as an E-cadherin/catenin adhesion complex located to cell-cell adherent junctions at the membrane. Signaling pathways like TGF-β and EGF may activate EMT by causing disassociation of this complex to release β-catenin, which is pre-requisite for its nuclear translocation and subsequent posttranscriptional regulations (6,7). Thus, the E-cadherin/β-catenin complex provides a major strength for cell-cell association. However, the molecular mechanisms that act upstream of these factors are still not well understood. Thus, establishing the signaling
networks associated with these factors may elucidate the molecular mechanisms underlying
tumor invasion and metastasis.

PDLIM1 (also known as CLP36, Elfin or CLIM1) is a cytoskeletal protein that associates
with actin filaments and stress fibers (8). The PDZ and LIM protein family comprises
proteins containing a C-terminal PDZ and one or more N-terminal LIM domains (9). These
proteins are capable of recruiting signal proteins to actin and organizing signaling complexes
at the cytoplasmic or cellular membranes (10-12). To date, PDLIM2 and PDLIM4, which
belong to the same family as PDLIM1, have been implicated as candidate tumor suppressors
in several tumors (12,13). As an actin-binding protein, PDLIM1 is diffusely expressed in
heart, muscle, and gastrointestinal tract tissues and acts as a regulator of cell-cell adhesion
and focal adhesion (10). However, the functional significance of the PDLIM1 in cancers
remains to be determined.

In this study, we show that PDLIM1 is downregulated in CRC tissues, particular in CRC liver
metastasis. Furthermore, we show that loss of PDLIM1 results in disassociation of the E-
cadherin/β-catenin complex, leading to the nuclear translocation of β-catenin and the
repression of E-cadherin, resulting in enhanced invasive and metastatic potential of CRC.
The enhanced expression of PDLIM1 attenuates EMT induction in CRC cells. In addition, we
find a positive correlation between PDLIM1, E-cadherin, and membrane β-catenin expression
in CRC samples and their associations with prognosis. Taken together, our data indicate that
loss of PDLIM1 in CRC is an inducer of EMT and predicts poor outcomes in CRC patients,
implicating PDLIM1 as both a potential therapeutic target and a predictor of survival in CRC.

Materials and methods
Cell lines

Human CRC cell lines SW480, HT29, DLD1, SW620, Lovo were purchased from the American Type Culture Collection. KM12C cell line was a kind gift from Professor Min Wu (University of North Dakota, USA). All cell lines were tested by STR analysis and used within 6 months. The last time of authentication is November, 2015. All cell lines were routinely maintained in Dulbecco's Modified Eagle Medium (Invitrogen) supplemented with 10% fetal bovine serum (Biowest), penicillin (10^7 U/L), and streptomycin (10 mg/L) at 37°C in a humidified chamber under 5% CO2.

Patients and follow-up

This study enrolled two independent cohorts of CRC patients at the Department of Gastrointestinal Surgery, West China Hospital. Ethics approval for these studies was obtained from the Biomedical Ethics Committee of West China Hospital. A total of 127 tumor tissues and paired adjacent normal tissues (Cohort 1) were collected from patients undergoing resection for CRC between 2008 and 2009. Cohort 2 was collected from patients who underwent resection for CRC liver metastasis with matched primary tumor tissue available from 2010 to 2013. Data including clinical information, pathological characteristics, and AJCC stages were collected (14). The follow-up data were acquired at 3-month intervals through outpatient visits, telephone calls, or office visits. The follow-up data were censored in December 2014. Detailed clinicopathological features are listed in Supplementary Table 1.

Bisulfite genomic DNA sequencing

Genomic DNA was prepared using TIANamp Genomic DNA kit (TIANGEN), followed by the treatment of sodium bisulfite using the EZ DNA Methylation-Gold Kit (Zymo Research). The products were amplified by PCR. Primer pairs used to recognize the bisulfite-modified
regions (-6 to -290) of the PDLIM1 promoter were: forward 5'-
GTAGAGTTGAGAGTTTTGG-3', reverse 5'
AATCTATCTAAATAATCATAACAC-3'.

**TOP/FOP Flash assay**

The TOP/FOP Flash assay was performed in accordance with the protocol provided by the manufacturer (Upstate, USA). To measure the transcriptional activity of β-catenin, SW480 cells were seeded in 24-well plates at $2 \times 10^4$ cells/well. The next day, cells were transfected with Top-flash plasmid (Upstate) plus pRL-CMV plasmid (Promega) or Fop-flash plasmid (Upstate) plus pRL-CMV plasmid. Two days later, luciferase activity was measured in cell extracts using a Dual Luciferase Kit (Promega). The transfection efficiency was normalized to the activity of pRL-CMV (Renilla luciferase).

**In vivo models**

Male BALB/c nu/nu mice (7-week-old) were raised in a specific pathogen-free condition. Animal care and experimental protocols were in accordance with guidelines provided by the Institutional Animal Care and Use Committee of Sichuan University. For the experimental mouse lung metastasis model, $1 \times 10^6$ cells were injected into nude mice through the tail vein. The orthotopic mouse model of CRC was generated as described previously (15). Briefly, mice were anesthetized and $2 \times 10^6$ cells were injected into the wall of cecum. Mice were inspected daily and the date of death for each mouse was documented. Tumor metastasis was monitored by magnetic resonance imaging (MRI) at week 4 after injection. Mice were killed 6 and 8 weeks after injection to examine the lung and liver metastasis of tumor cells, respectively. The tumor metastases were confirmed by H&E staining and quantified based on visual examination and manual counting of formalin-fixed lungs and livers.
Statistical analysis

Statistical analyses and graphics were undertaken using Prism version 6.00 (GraphPad Software, USA) and R software, version 3.1.2 (R Core Team, Austria). A one-way ANOVA and Pearson’s $\chi^2$ test were used for the univariate analyses where appropriate. The survival analysis was performed as previously reported (16). Kaplan-Meier method was used to calculate the survival rates. Each single variable was analyzed using the Cox proportional hazards model. A p value $<0.05$ was considered statistically significant.

Results

PDLIM1 is downregulated in human CRCs and correlates with liver metastasis.

To explore the role of PDLIM1 in the progression of CRC, the expression of PDLIM1 was evaluated in tissue microarrays containing 127 samples of CRCs with patient-matched normal mucosal tissues. Immunohistochemical analyses demonstrated decreased expression of PDLIM1 in CRC samples compared with adjacent normal mucosal tissues (Fig. 1A-1B). In addition, PDLIM1 expression in six CRC tissues and paired normal mucosa was detected by Western blot analysis. Despite interindividual variations, PDLIM1 was found to be significantly downregulated in CRC tissues (Fig. 1C). Furthermore, the downregulated expression of PDLIM1 in CRC was verified by gene expression analysis based on three Oncomine datasets (Fig. 1D). To explore the role of PDLIM1 in CRC progression, six human CRC cell lines, KM12C, SW480, HT29, DLD1, SW620 and Lovo, were employed to evaluate PDLIM1 expression. The protein expression of PDLIM1 was significantly reduced in the two highly metastatic cell lines (SW620 and Lovo) compared with the weakly metastatic CRC cell lines (KM12C, SW480, HT29, DLD1) (Fig. 1E). To further investigate the correlation between PDLIM1 and CRC metastasis, CRC liver metastases with the
corresponding patient-matched primary tumors were obtained from 32 patients who underwent resection of the affected liver tissues. The PDLIM1 protein levels were found to be significantly reduced in CRC liver metastasis compared with primary CRC tissues (Fig. 1F-1G). Together, these results indicate that PDLIM1 is frequently downregulated in human CRCs, particular in metastatic lesions, suggesting that loss of PDLIM1 may play a potential role in CRC metastasis.

**Downregulation of PDLIM1 in CRC cells involves DNA methylation**

To investigate the mechanism of PDLIM1 repression in CRC cells, we examined the mRNA level of six CRC cell lines. Consistently, a higher mRNA level of PDLIM1 was observed in highly metastatic CRC cell lines (SW620, Lovo) compared with primary CRC cell lines (KM12C, SW480, HT29, DLD1) (Fig. 2A). To date, PDLIM2, PDLIM3 and PDLIM4, which belong to the same family as PDLIM1, have been demonstrated to be down-regulated by promoter hypermethylation. In line with this, we evaluated the potential role of DNA methylation in PDLIM1 repression. A total of 44 CpG sites located between nucleotides -6 and -290 in the PDLIM1 promoter were examined using bisulfite genomic DNA sequencing. Dense cytosine methylation was observed in the CpG islands of the PDLIM1 promoter of highly metastatic CRC cells (SW620, Lovo) (Fig. 2B). Consistently, similar results were observed in methylation status of PDLIM1 promoter in clinical samples. Based on CRC tissues with patient-matched normal tissues, we found that the PDLIM1 promoter was methylated in CRCs tissues (Fig. 2C). This tendency was further validated in hypermethylated CRC liver metastatic tissues compared with primary foci (Fig. 2D). These results suggest that the PDLIM1 promoter was hypermethylated in CRCs with high metastatic potential. To further determine whether PDLIM1 promoter hypermethylation was responsible for PDLIM1 repression, the effect of 5-aza-dC (a DNA methyltransferase
inhibitor) on PDLIM1 expression was evaluated in CRC cells. Treatment with 5-aza-dC increased the mRNA level of PDLIM1 and restored the protein expression of PDLIM1 in CRC cells (Fig. 2E-2F). Consistently, cytosine methylation of the PDLIM1 promoter is largely reversed in response to 5-aza-dC treatment (Fig. 2B). Together, these data suggest that PDLIM1 repression in CRC is attributed to promoter methylation.

Loss of PDLIM1 promotes CRC cell invasion and metastasis in vitro and in vivo

To identify the role of PDLIM1 in CRC metastasis, we evaluated the invasive and metastatic potential in SW480 cells transfected with PDLIM1 shRNA transfected, and SW620 transfected with PDLIM1 cDNA. Successful shRNA-mediated knockdown of PDLIM1 in SW480 cells and cDNA-mediated overexpression of PDLIM1 in SW620 cells were confirmed using Western blot analysis (Fig. 3A). In the transwell migration and Matrigel invasion assays, PDLIM1 shRNA promoted the migration and invasion of SW480 cells, whereas the exogenous expression of PDLIM1 lead to a reduction in the migratory and invasive property of SW620 (Fig. 3B). In wound-healing migration assays, microscopic examination post-wounding revealed a significantly increased rate of wound closure in SW480-PDLIM1 shRNA-treated (hereafter referred to as SW480-shPDLIM1) cells compared with SW480 cells with control shRNA transfected (hereafter referred to as SW480-NC) (Supplementary Fig. S1). In addition, PDLIM1 cDNA-transfected SW620 cells (hereafter referred to as SW620-PDLIM1) had a significant delay in the wound closure rate compared with SW620 cells transfected with vector (hereafter referred to as SW620-Vector) (Supplementary Fig. S1).

To determine whether loss of PDLIM1 is involved in CRC metastasis in vivo, a lung metastasis model was generated by tail injection of two paired cell lines, SW480-
shPDLIM1/SW480-NC and SW620-PDLIM1/SW620-Vector. Each group was assigned with six mice. Representative MRI slides taken at four weeks post-injection are shown in Fig. 3C, confirming the occurrence of lung metastases. At six weeks post-injection, a higher number of lung metastatic nodules was observed in the SW480-shPDLIM1 group (n=6) compared with SW480-NC group (n=6) (Fig. 3D-3E). By contrast, SW620-PDLIM1 cells formed fewer lung metastatic nodules compared with SW620-Vector cells (Fig. 3D-3E). We next investigated the impact of PDLIM1 on CRC metastasis using an orthotopic mouse model of CRC (six mice for each group). Representative MRI slides taken at four weeks post-injection are shown in Fig. 3F. At eight weeks post-injection, the PDLIM1 knockdown in SW480 significantly increased the number of metastatic nodules in the liver, while overexpression of PDLIM1 decreased the metastatic capacity of SW620 (Fig. 3G-3I). Collectively, these data from both in vitro and in vivo experiments demonstrate that loss of PDLIM1 promotes both invasion and metastasis in CRC.

Loss of PDLIM1 induces EMT in CRC

Given the essential role of EMT in tumor metastasis, we evaluated the protein expression levels of EMT markers, E-cadherin, Vimentin, and a panel of EMT-associated transcription factors, including Snail, Slug, ZEB1, ZEB2, and TWIST in SW480, KM12C, SW620, and Lovo cells. Knockdown of PDLIM1 in SW480 and KM12C cells led to significantly lower levels of E-cadherin and higher levels of Vimentin and Snail (Fig. 4A). By contrast, the enhanced expression of PDLIM1 in SW620 and Lovo cells resulted in a concomitant reduction of Vimentin and Snail, accompanied by upregulation of E-cadherin (Fig. 4A). However, PDLIM1 showed no significant impact on the expression levels of other transcription factors, including Slug, ZEB1, ZEB2, and TWIST (Fig. 4A). Changes in EMT markers following stable downregulation or upregulation of PDLIM1 were further verified by
immunofluorescence analysis (Fig. 4B). Thus, these data suggest that loss of PDLIM1 may be an inducer of EMT in CRC cells.

On the basis of these findings, we next assessed a potential clinical relationship between PDLIM1 and EMT in human CRC tissues. Based on the immunohistochemical staining on TMAs composed of primary tumors and matched peritumoral tissues from 127 CRC patients, we found a similar pattern of distribution for PDLIM1, E-cadherin, and membrane β-catenin at the cell-cell junctions in normal mucosal tissues (Fig. 4D). Moreover, we found that CRC patients with low PDLIM1 expression tended to have lower E-cadherin and membrane β-catenin levels as well as higher Vimentin and Snail levels, suggesting that loss of PDLIM1 promoted the EMT process (Fig. 4E, Supplementary Fig. S2). Indeed, as shown in Fig. 4F, the protein expression of PDLIM1 is closely associated with E-cadherin ($R^2=0.1761$, $p<0.001$) and membrane β-catenin ($R^2=0.3274$, $p<0.001$), respectively. Representative images of four cases are shown to illustrate the correlation between these proteins in CRC tissues (Fig. 4G). Taken together, this specific expression pattern suggests a potential role of PDLIM1 in the EMT of CRC.

Loss of PDLIM1 activates β-catenin by disassociating E-cadherin/β-catenin complex

To explore the mechanism by which PDLIM1 regulates EMT in CRC, we evaluated whether the E-cadherin/β-catenin complex was affected by the manipulation of PDLIM1 expression. The cell lysate was immunoprecipitated by β-catenin and then immunoblotted with anti-E-cadherin antibodies to analyze E-cadherin/β-catenin complex formation. As shown in Fig. 5A, the abundance of β-catenin-associated E-cadherin was remarkably decreased following PDLIM1 knockdown in SW480 and KM12C cells, indicating that E-cadherin/β-catenin complex formation was reduced by knockdown of PDLIM1. Next, we tested whether
disassociation of the E-cadherin/β-catenin complex might contribute to the release of β-catenin. The immunoblot analysis showed that PDLIM1 knockdown in SW480 and KM12C cells resulted in downregulation of the epithelial marker E-cadherin (Fig. 4A). However, the level of β-catenin was not significantly altered (Fig. 5B). We separated cytosolic and nuclear fractions of cell lysates and found that PDLIM1 knockdown resulted in a decreased level of cytosolic β-catenin and an increased level of nuclear β-catenin, suggesting that loss of membrane-associated forms of β-catenin was due to redistribution to the nucleus (Fig. 5B).

Subcellular location of β-catenin was then evaluated using immunofluorescence analysis. Consistently, we found that β-catenin was located primarily in the plasma membrane in SW480-NC cells; however, PDLIM1 inhibition led to the nuclear accumulation of β-catenin (Fig. 5C). On the other hand, the expression of membrane β-catenin in SW620 cells significantly increased after enhanced expression of PDLIM1, while nuclear β-catenin was almost absent (Fig. 5C). Next, we evaluated the transcriptional activity of β-catenin-TCF by using TOP-Flash and FOP-Flash luciferase reporter. PDLIM1 knockdown increased the transcriptional activity of β-catenin-TCF in SW480, whereas enhanced expression of PDLIM1 decreased the transcriptional activity of β-catenin-TCF in SW620 (Fig. 5D). To investigate whether PDLIM1-regulated EMT involves β-catenin activation, we used siRNA to inhibit β-catenin expression. As shown in Fig. 5E, the knockdown of β-catenin results in significant reduction of both Snail and Vimentin levels. Moreover, the EMT induced by PDLIM1 downregulation was abolished on β-catenin knockdown (Fig. 5E), leading to the impairment of cell invasion and migration (Fig. 5F). Taken together, these results demonstrate that the loss of PDLIM1 leads to nuclear translocation of β-catenin from the E-cadherin/β-catenin complex, thereby resulting in the activation of Wnt signaling and induction of EMT.
To explore the mechanism by which PDLIM1 regulates the E-cadherin/β-catenin complex, we investigated whether PDLIM1 can interact with the E-cadherin/β-catenin complex. During growth in normal media when EMT was not observed, PDLIM1 interacts with β-catenin and E-cadherin respectively in SW480 and KM12C cells, indicating an interaction between PDLIM1 and E-cadherin/β-catenin complex in the absence of EMT in CRC cells (Fig. 6A-6B). Next, we mapped the binding domains of PDLIM1 in 293T cells transfected with PDLIM1 truncation mutants. Only N-terminal fragments containing PDZ domain, but not other regions of PDLIM1, interacted with the β-catenin/E-cadherin complex (Fig. 6C). Moreover, PDZ-containing fragments were able to inhibit the EMT process, as evidenced by induction of E-cadherin and repression of Snail (Fig. 6C), indicating that the PDZ domain of PDLIM1 forms the molecular basis of the interaction between PDLIM1 and the E-cadherin/β-catenin complex.

Given the essential role of the E-cadherin/β-catenin complex in EMT induction, TGF-β1 was utilized as an EMT inducer to investigate the role of the interaction between PDLIM1 and this complex. Treating SW480 and KM12C cells with TGF-β1 for 48 hours resulted in EMT as evidenced by the nuclear translocation of β-catenin, decreased E-cadherin expression, and increased Vimentin and Snail levels (Fig. 6D-6E). Furthermore, the interaction between PDLIM1 and E-cadherin/β-catenin complex was decreased in response to TGF-β1 treatment (Fig. 6A-6B). However, enforced PDLIM1 expression in SW480 and KM12C cells significantly attenuated TGF-β1-induced EMT (Fig. 6D). Moreover, the overexpression of PDLIM1 was sufficient to abolish the increased cell invasion and migration induced by TGF-β1 (Fig. 6F). Similar results were also obtained when EGF was used as an EMT inducer.
(Supplementary Fig. S3). Taken together, these results suggest that PDLIM1 binds β-catenin and attenuates TGF-β1-induced EMT.

**Loss of PDLIM1 predicts poor survival in patients with CRC**

Detailed clinicopathological features are listed in Supplementary Table 1. Pearson χ² tests indicated that PDLIM1 was significantly related to microvascular invasion (P=0.002), tumor invasiveness (P=0.044), and lymph node involvement (P<0.001) (Supplementary Table 2). Using a median cutoff for PDLIM1 expression, we noted a trend toward better overall survival (OS) for patients with PDLIM1 high expression (Fig. 7A). Supplementary Table 3 shows the results of univariate analyses of 5-year OS and hazard ratios (HR), according to subgroups based on several prognostic variables. OS was significantly higher for CRC patients who had a high PDLIM1 expression (72% vs. 36%, p<0.001). Interestingly, patients with no detectable lymph node metastasis had better survival when PDLIM1 was highly expressed (76% in PDLIM1 high group vs. 33% in PDLIM1 low group, p<0.001) (Fig. 7B), as did those who had no distant metastasis (74% in PDLIM1 high group vs. 39% in PDLIM1 low group, p<0.001). These findings, in conjunction with our previous findings that PDLIM1 correlates with tumor invasiveness and metastasis, implicate PDLIM1 as a potential predictor of tumor micrometastasis in patients with no metastatic focus detectable in pathological specimens.

Additionally, we also observed significant OS differences for patients based on E-cadherin and membrane β-catenin expression (Fig. 7C-7D). When PDLIM1/E-cadherin and PDLIM1/β-catenin were analyzed using immunohistochemistry, the median density was determined and used as a cutoff in subsequent analyses. CRC Patients with low levels of PDLIM1, E-cadherin, and membrane β-catenin showed worse prognoses compared with
other groups (Fig. 7E-7F). Conversely, CRC patients who expressed high levels of PDLIM1, E-cadherin, and membrane β-catenin had best survival outcomes (Fig. 7E). These clinical data provide evidence that PDLIM1 in combination with E-cadherin and membrane β-catenin could be useful biomarkers in CRC to assess the invasive tumor biology and predict the prognosis of CRC patients.

Discussion

Tumor metastasis is a multistage process in which malignant cells disseminate from the site of origin and colonize distant organs or nodes. Although the general steps of this process may be the same, metastasis of different cancer types demonstrate heterogeneous biology and might be dependent on distinct sets of factors according to the tissue of origins and region of metastasis (17). Thus, the need to understand the biology of metastasis in different types of cancer becomes increasingly important. PDLIM1 is a cytoskeletal protein that is diffusely expressed in heart, muscle, and gastrointestinal tract tissues, while normal breast tissues show non-detectable or weak expression of PDLIM1 (18,19). Previous studies implicate PDLIM1 as a pro-metastasis factor in glioma and breast cancer (20,21). However, given the tissue specificity of PDLIM1, the role of PDLIM1 in different tumor types might vary accordingly. Based on the results derived from two cohorts of clinical samples, our study demonstrated that the expression of PDLIM1 was downregulated in CRC tissues compared with peritumoral tissues. More importantly, the samples from CRC liver metastases exhibited lower levels of PDLIM1 than patient-matched primary tumors. Based on a number of depletion and overexpression experiments in vitro, we revealed a negative role for PDLIM1 in regulating CRC cell invasion and migration. Moreover, we employed two independent in vivo models to elucidate the effect of PDLIM1 on the multistages of CRC metastasis. In line with the in vitro results, PDLIM1 knockdown increases colon-derived experimental liver
metastasis in the orthotopic CRC model as well as colonization in the distant metastatic site using the experimental lung metastasis model. Together, our study indicates a pivotal and negative role of PDLIM1 in CRC metastasis.

Experimental and clinical studies have shown that EMT plays a gatekeeper role in the regulation of tumor invasion and metastasis (4). Recent microarray studies have investigated the molecular alterations associated with EMT in CRCs and found that PDLIM1 is downregulated in cells with an EMT phenotype (22,23). Here, we reported that CRC cells with low levels of PDLIM1 expressed high levels of Vimentin, Snail, and low levels of E-cadherin, indicating a potential correlation between PDLIM1 and EMT, which might account for the invasive and metastatic potential of these cells. Moreover, knockdown of PDLIM1 in CRC cells reduced E-cadherin expression whereas enhanced expression of PDLIM1 induced E-cadherin expression. As downregulation or loss of E-cadherin marks the initiation of EMT and the gain of invasive and metastatic potential of cancer cells, we postulated that PDLIM1-dependent regulation of E-cadherin expression was a potential mechanism that results in CRC metastasis.

The disruption of cell-cell adherens junctions by misregulation of E-cadherin is a key step in EMT that contributes to CRC cell invasion and metastasis (4). Normally, E-cadherin and β-catenin form a complex in the cell-cell junction area, which provides the basis for cell-cell association (7). In response to several signaling pathways such as TGF-β or EGF, the E-cadherin/β-catenin complex is disassociated to release β-catenin for subsequent posttranscriptional regulation (6). In line with this notion, we showed that PDLIM1 inhibition induced disassociation of the E-cadherin/β-catenin complex and nuclear translocation of membrane β-catenin, followed by the activation of β-catenin-TCF transcription, increasing
Snail expression and decreasing E-cadherin levels. Thus, the frequent downregulation of PDLIM1 in CRCs may account for one potential mechanism underlying the dysfunction of Wnt signaling pathway in these tissues. Using either TGF-β1 or EGF as the inducer of EMT (7,24), we found that enhanced PDLIM1 expression in CRC cells was capable of attenuating EMT. Therefore, we conclude that PDLIM1 downregulation promotes CRC metastasis by inducing EMT through a β-catenin-dependent pathway.

Another key finding in our study is the prognostic value of PDLIM1 protein expression in CRC patients. Based on a cohort of 127 patients, we demonstrated that low expression of PDLIM1 in CRC was an indicator of poor prognosis. Given the roles of PDLIM1 in CRC invasion and metastasis, these findings implicated PDLIM1 as a marker for tumor aggressiveness and a predictor for CRC patients' survival. Our TMA results also confirmed that low expressions of either E-cadherin or membrane β-catenin were indicators of poor prognosis for CRC patients, which is compatible with previous studies (22,25). Unlike tumors such as breast cancer where metastasis might take decades to acquire the function for colonization (17), malignant progression and most metastatic traits in CRC are acquired during progression to the invasive carcinoma stage in the primary site (26). Under this scenario, the molecular alterations of CRC cells in the primary site may carry the potential to determine whether metastatic progression may proceed or not, underscoring the feasibility of using one or a set of particular biomarkers to evaluate the risk of metastasis. Our study reveals the clinical relevance and prognostic significance of PDLIM1-dependent regulation of β-catenin and E-cadherin, indicating that the assessment of PDLIM1, in conjunction with E-cadherin and β-catenin, may provide a more detailed appraisal of metastasis risk for individual CRC patients, which may assist in the development of individualized therapeutic strategies.
In summary, our current findings demonstrate a role for PDLIM1 in the regulation of EMT via a β-catenin-dependent pathway. Loss of PDLIM1 in CRC is a potential indicator of aggressive CRC phenotypes and correlates with poor clinical outcome. Thus, PDLIM1 may be used as a biomarker for the individualized management of CRC patients.

References


FIGURE LEGEND

Figure 1. PDLIM1 is downregulated in human CRCs.

(A) Immunohistochemical staining of PDLIM1 in paraffin-embedded human CRC tissues. Scale bar, 200 μm (Left) 50 μm (Right) (B) The relative protein level of PDLIM1 is significantly lower in CRC tissues than adjacent normal tissue. Data represent the mean ± SD. (C) Western blot analysis of PDLIM1 expression in CRC tissues and patient-matched adjacent normal tissues. (D) The relative mRNA expression of PDLIM1 in three Oncomine datasets, GDS2947, GDS3756, and GDS2609. Data represent the mean ± SD. (E) Relative PDLIM1 protein levels in six CRC cell lines as determined by western blot analysis. (F) Immunohistochemical staining of PDLIM1 in CRC liver metastasis and patient-matched primary CRC tissues Scale bar, 50 μm. (G) The relative protein level of PDLIM1 is lower in CRC liver metastases than patient-matched primary CRC tissues. *** p<0.001.

Figure 2. Down-regulation of PDLIM1 in CRC cells involves DNA methylation.

(A) Relative mRNA levels of PDLIM1 in indicated cells were determined by real-time PCR. bars, SD (n = 4). (B) Cells were treated with 5-aza-dC (5μM) for 48 h. Methylation status of PDLIM1 promoter was determined by bisulfite genomic DNA sequencing as described in Methods. Each dot represents a CpG site; White dots represent unmethylated CpG dinucleotides whereas each black dots represents a methylated cytosine residue within the CpG islands. (C) Methylation statuses of PDLIM1 promoter in five pairs of CRCs tissues and patient-matched normal tissues were determined by bisulfite genomic DNA sequencing (D) Methylation statuses of PDLIM1 promoter in five pairs of CRCs tissues and patient-matched liver metastatic tissues were determined by bisulfite genomic DNA sequencing (E) mRNA levels of PDLIM1 were determined by real-time PCR. Changes in PDLIM1 mRNA levels following 5-aza-dC treatment were represented as fold induction relative to control cells.
bars, SD (n = 4) (F) Western blot analysis of PDLIM1 expression in indicated cells as treated in B

**Figure 3. Loss of PDLIM1 enhances invasive and metastatic potential of CRC cells.**

(A) PDLIM1 knockdown and overexpression as detected by Western blot analysis. (B) Representative data for transwell matrigel invasion and migration assay for indicated colorectal cancer cells. Magnification, ×200. Data represent the mean ± SD and are representative of three independent experiments. (C) Representative slides of lung tumor metastasis monitored by MRI at 4 weeks post-injection. (D) The number of lung metastasis nodules from experimental metastases generated by indicated cells 6 weeks after tail vein injection. Data represent the mean ± SD. (E) Representative images of lung metastasis from mice as in D. (F) Representative slides of liver tumor metastasis monitored by MRI at 4 weeks post-injection. (G) The number of liver metastasis nodules from the orthotopic CRC models generated by indicated cells 8 weeks post-injection. Data represent the mean ± SD. (H) Representative images of liver metastasis from mice as in G. (I) Kaplan-Meier curves of mice from G. *** p<0.001.

**Figure 4. Loss of PDLIM1 induces EMT in CRC.**

(A) EMT marker expression following stable downregulation of PDLIM1 expression as detected by Western blot analysis. (B) EMT marker expression following stable upregulation of PDLIM1 expression as detected by Western blot analysis. (C) PDLIM1, E-cadherin, Vimentin, and Snail expression in indicated cells as detected by immunofluorescence assay, Scale bar, 20 μm. (D) Representative images show normal colon epithelial cells with intact PDLIM1, E-cadherin, and β-catenin membrane expression. Scale bar, 20 μm (E) The association between expression of PDLIM1, E-cadherin, membrane β-catenin, Snail,
Vimentin in CRC tissues. **, p < 0.01; *** p < 0.001. (F) The correlation test of immunostaining intensity between PDLIM1 and membrane β-catenin or E-cadherin respectively. (G) Immunohistochemical analyses of PDLIM1, E-cadherin, β-catenin, Snail, and Vimentin expression in CRC tissues (scale bar, 50 mm).

Figure 5. Loss of PDLIM1 activates WNT-β-catenin signal pathway by disassociating E-cadherin/β-catenin complex

(A) Immunoprecipitation of β-catenin with E-cadherin in indicated CRC cells as detected by Western blot analysis. (B) β-catenin expression in whole cell lysates, cytoplasm, and nuclear fractions as detected by Western blot analysis. (C) Immunofluorescence analysis of β-catenin (green) in indicated CRC cells. Merged images represent overlays of β-catenin (green) and nuclear staining by DAPI (blue). (D) TOP-Flash/FOP-Flash assay depicting Wnt activity in indicated CRC cells. (E) Protein expression of PDLIM1, E-cadherin, β-catenin, Vimentin, and Snail in SW480 cells with indicated treatments as detected by Western blot analysis. (F) Matrigel invasion and transwell migration assay for SW480 cells as treated in E. Data represent the mean ± SD and are representative of three independent experiments. *** p<0.001.

Figure 6. PDLIM1 binds E-cadherin/β-catenin complex and attenuates EMT

(A) Immunoprecipitation of PDLIM1 with E-cadherin and β-catenin in SW480 and KM12C cells as detected by Western blot analysis. Cells were treated with 10 ng/mL TGF-β1 for 24 hours. (B) Immunoprecipitation of β-catenin with E-cadherin and PDLIM1 in SW480 and KM12C cells as detected by Western blot analysis. Cells were treated with 10 ng/mL TGF-β1 for 24 hours. (C) 293T cells transfected with myc-tagged full-length or PDLIM1 fragments were immunoprecipitated with an anti-myc antibody. β-catenin, E-cadherin, Snail and myc-
PDLIM1 (full length or fragments) levels were shown. (D) Protein expression of PDLIM1, E-cadherin, Vimentin, and Snail in SW480 and KM12C cells with indicated treatment. (E) Immunofluorescence analysis of β-catenin (green) in SW480 cells as treated in C. Merged images represent overlays of β-catenin (green) and nuclear staining by DAPI (blue). (F) Data of Matrigel invasion and transwell migration assay for SW480 cells as treated in D. Data represent the mean ± SD and are representative of three independent experiments. ***

p<0.001.

Figure 7. Loss of PDLIM1 predicts poor prognosis in patients with CRC.

Kaplan-Meier analyses of overall survival (OS) based on PDLIM1, E-cadherin and membrane β-catenin expression. (A) OS was significantly higher in the PDLIM1 high group compared with the PDLIM1 low group. (B) For patients with no lymph node metastasis detected, OS was significantly higher in the PDLIM1 high group compared with the PDLIM1 low group. (C) Patients in the E-cadherin high group have higher OS when compared with the E-cadherin low group. (D) Patients in the membrane β-catenin high group have higher OS when compared with the membrane β-catenin low group. (E) Prognostic values of PDLIM1 combined with E-cadherin expression. (F) Prognostic values of PDLIM1 combined with membrane β-catenin expression.
Figure 1. PDLIM1 is downregulated in CRC

A

Normal tissue

Colorectal cancer

Normal colon
Transition Cancerous Area ▲ colon

B

Relative PDLIM1 protein expression

***

N T N T N T
PDLIM1
β-actin

C

Sample 1 Sample 2 Sample 3
Sample 4 Sample 5 Sample 6
PDLIM1
β-actin

D

Relative mRNA level of PDLIM1

GDS2947

GDS93756

GDS2909

5000 Normal Adenoma
15000
25000

Relative mRNA level of PDLIM1
0 20000 40000 60000

Relative mRNA level of PDLIM1
0 10000 20000 30000

0 Normal CRC

F

Case 1
Case 2
Case 3
Case 4

Primary tumor

Liver metastases

mCRC

G

Relative PDLIM1 protein expression

***

0 50 100 150 200
Primary tumor Liver metastasis
Figure 2. Down-regulation of PDLIM1 in CRC cells involves DNA methylation

A

Relative mRNA levels of PDLIM1 (%)

Nonmetastatic
Metastatic

KM12C
SW480
HT29
DLD1
SW620
LoVo

B

CpG Islands

Clone -290

CpG Islands

Primary tumor

Liver metastasis

C

CpG islands

-290

Normal
CRC

D

E

Induction of PDLIM1 by 5-aza-dC (fold)

Nonmetastatic
Metastatic

KM12C
SW480
HT29
DLD1
SW620
LoVo

F

5-aza-dC

PDLIM1

β-actin

SW480
KM12C
HT29
SW620
LoVo
DLD1

Figure 3. Loss of PDLIM1 promotes CRC cell invasion and metastasis in vivo and in vitro.

A

B

C

D

E

F

G

H

I

Table and figures showing migration, invasion, and metastasis assays for SW480 and SW620 cell lines with PDLIM1 knockdown or overexpression. The graphs show quantitative analysis of cell migration, invasion, and lung metastasis. Cross-sections and microscopy images are included to demonstrate the cellular and tissue-level effects.
Figure 5. Loss of PDLIM1 activates WNT-β-catenin signal pathway by disassociating E-cadherin/β-catenin complex

A

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C

SW480-NC | SW480-shPDLIM1 | SW620-Vector | SW620-PDLIM1

D

![Luciferase Reporter Assays](FOP-Flash TOP-Flash)

E

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<td>β-actin</td>
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F

Relative cell number:

- Invasion
- Migration

Lines:

- SW480-NC
- SW480-siβ-catenin
- SW480-shPDLIM1
- SW480-shPDLIM1+siβ-catenin

Significance:

- ***
- **
- ns
Figure 6. PDLIM1 binds E-cadherin/β-catenin complex and attenuates EMT

(A) TGF-β1 (10 ng/mL) was added to SW480 and KM12C cells. Immunoprecipitation (IP) of PDLIM1 was performed and Western blot analysis was conducted for E-cadherin and β-catenin.

(B) Similar IP and Western blot analysis was conducted for KM12C cells.

(C) Schematic representation of PDLIM1 constructs: PDZ and LIM domains. Various truncated constructs were used for further analysis.

(D) Western blot analysis for PDLIM1, E-cadherin, Vimentin, Snail, and β-actin expression with the indicated constructs.

(E) Immunofluorescence images showing the localization of β-catenin and DAPI in SW480 cells with and without TGF-β1 treatment.

(F) Graphical representation of the relative cell number for invasion and migration assays with different treatments.
Figure 7. Loss of PDLIM1 predicts poor prognosis in patients with CRC
PDLIM1 stabilizes the E-cadherin/β-catenin complex to prevent epithelial-mesenchymal transition and metastatic potential of colorectal cancer cells

Hai-Ning Chen, Kefei Yuan, Na Xie, et al.

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