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Oncogenic role of *PDK4* in human colon cancer cells

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Background: Cancer cells maintain high rates of glycolysis. Pyruvate dehydrogenase kinases (PDK) contribute to this phenomenon, which favours apoptosis resistance and cellular transformation. We previously reported upregulation of *PDK4* in normal mucosa of colorectal cancer (CRC) patients compared with controls and in preneoplastic intestine of our mouse model. Decreased methylation of four consecutive *PDK4* CpGs was observed in normal mucosa of patients. Although other members of the PDK family have been investigated for transformation potential, *PDK4* has not been extensively studied.

Methods: *PDK4* methylation in blood of CRC patients and controls was evaluated by pyrosequencing. *PDK4* expression in human colon carcinoma cells was down-regulated by RNAi. Cellular migration and invasion, apoptosis and qRT-PCR of key genes were assessed.

Results: Pyrosequencing revealed decreased methylation of the same four consecutive CpGs in the blood of patients compared with controls. Cellular migration and invasion were reduced and apoptosis was increased following transient or stable inhibition of *PDK4*. Expression of vimentin, *HIF-1* and *VEGFA* was reduced.

Conclusions: These studies demonstrate the involvement of *PDK4* in transformation. Methylation assessment of *PDK4* in the blood may be useful for non-invasive CRC detection. *PDK4* should be considered as a target for development of anticancer strategies and therapies

Glycolysis is a cytoplasmic anaerobic pathway that uses glucose for energy. In the presence of oxygen, pyruvate enters the mitochondrial tricarboxylic acid (TCA) cycle and generates ATP through oxidative phosphorylation. Warburg *et al*, 1924 observed that tumours exhibit high rates of glycolysis and can generate a higher proportion of cellular ATP than that produced from oxidative phosphorylation (Warburg, 1956). Many oncogenes and tumour suppressors exert their effects through regulation of glycolysis (Hsu and Sabatini, 2008; Dang, 2012).

Pyruvate enters the TCA cycle through pyruvate dehydrogenase (PDH). PDH kinase (PDK) inhibits PDH activity and promotes the switch from mitochondrial oxidation to cytoplasmic glycolysis.

Dichloroacetate (DCA), a PDK inhibitor, shifts metabolism in the reverse direction. It induces apoptosis, inhibits tumour growth (Bonnet *et al*, 2007) and reduces expression of *HIF1A*, a master gene that controls the hypoxic response (Kumar *et al*, 2012).

PDKs form a family of four kinases in humans (*PDK1–PDK4*; Jeong *et al*, 2012). Knockdown of *PDK1* restores PDH activity, reverts the Warburg metabolic phenotype and decreases *HIF1A* expression, invasiveness and tumour growth (McFate *et al*, 2008). Inhibition of *PDK2* by small interfering (siRNA) increases apoptosis of cancer cells (Bonnet *et al*, 2007). *PDK3* expression is markedly increased in colon cancer and negatively associated with disease-free survival (Lu *et al*, 2011). *PDK4* is predominantly

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expressed in the muscle and affects the metabolic fate of glucose during exercise (Pilegaard and Neufer, 2004), but its role in oncogenesis has not been well studied.

We previously observed increased *PDK4* expression in normal colonic mucosa of colorectal cancer (CRC) patients compared with normal mucosa of controls (Leclerc *et al*, 2013). We also found decreased methylation of four consecutive CpG dinucleotides in the 5'-region of *PDK4* in normal colon of patients compared with normal colon of controls (Leclerc *et al*, 2013). Similarly, in our mouse model of intestinal neoplasia generated by low dietary folate, *Pdk4* expression was upregulated by folate deficiency (Leclerc *et al*, 2013). Based on changes in expression or methylation of *PDK4* and other genes in the two species, we suggested that tumorigenesis could relate to activation of peroxisome proliferator-activated receptor- α (PPARA); *PDK4* is a target of PPARA.

Given the role of *PDK4* in glycolysis and our observations of decreased methylation and increased expression of *PDK4* in preneoplastic colon, we hypothesised that reducing *PDK4* expression may disfavour CRC development or progression. Here we show that inhibition of *PDK4* disturbs the properties of CRC cells in culture, including effects on migration, invasion, apoptosis and expression of critical genes in transformation. Furthermore, we observed *PDK4* methylation differences in peripheral blood, between patients and controls, extending our earlier observations in normal colon. These findings may contribute to the development of a non-invasive test for CRC detection.

MATERIALS AND METHODS

Human subjects. Two groups of patients and controls were studied. Research was approved by the Temple University Office for Human Subjects Protections Institutional Review Board, protocol 11910 and the Research Ethics Office of the Jewish General Hospital, protocol 09-017.

For the first cohort (40 CRC patients and 40 controls), CRC patients samples came from the Temple/Fox Chase Cancer Center (FCCC) Biobank and controls were recruited from the Temple University Medical Center, as previously described (Leclerc *et al*, 2013). Average age of patients and controls was 59 and 58 years, respectively. Both groups comprise 19 men and 21 women each. The second cohort included 18 CRC patients and 29 controls. Patients were from the Temple/FCCC Biobank and controls were cancer-free persons having routine blood tests at the Jewish General Hospital, Montreal. Average age of patients and controls was 60 years. CRC patients included 8 men and 10 women; the controls had 15 men and 14 women.

Quantitative CpG methylation analysis. DNA was purified from blood using standard phenol–chloroform techniques or the Genra Puregene Blood Kit (Qiagen, Toronto, ON, Canada) and subsequently bisulfite-treated with the Qiagen EpiTect Bisulfite Kit (Qiagen). Bisulfite pyrosequencing was performed as before (Leclerc *et al*, 2013, 2013a). For description of oligonucleotides and representative pyrograms, please refer to Supplementary files of Leclerc *et al* (2013).

Cell culture. LoVo and DLD1 human colon carcinoma cells were kindly provided by François Houle and Jacques Huot (Université Laval, Quebec, Canada) or obtained from the American Type Culture Collection (Manassas, VA, USA), respectively. These cell lines were chosen, because they both perform particularly well in migration and invasion assays. Cells were maintained in a humidified incubator at 37 °C in 5% CO₂ and grown as monolayers in high-glucose Dulbecco's modified Eagle's medium with 5% fetal bovine serum, 5% bovine calf serum and 100 U ml⁻¹ penicillin and streptomycin. Culture materials were from GIBCO/BRL Life Technologies (Carlsbad, CA, USA).

siRNA transient transfection. ON-TARGETplus Human *PDK4* siRNA SMART pool was synthesised by Dharmacon (Lafayette, CO, USA). The four target sequences were 5'-GAGCAUUUCUC-GCGCUACA-3', 5'-CGACAAGAAUUGCCUGUGA-3', 5'-CAA-CGCCUGAUGGAUAA-3' and 5'-GACCGCCUCUUUAGU-UAA-3'. ON-TARGETplus Human GAPDH Control Pool and Non-targeting Pool (Dharmacon) were used as positive and negative controls, respectively. Double-stranded siRNA transient transfections were carried out on subconfluent (50–60%) LoVo or DLD1 cells seeded into six-well plates. Lipofectamine RNAiMAX (Life Technologies) transfection reagent was used as previously described (Pham *et al*, 2013). Effective transfection was confirmed by BLOCK-iT Alexa Fluor Red fluorescent Oligo (Life Technologies). Migration, invasion and viability assays were performed as before (Pham *et al*, 2013).

Lentiviral shRNA downregulation of *PDK4* expression. Seven constitutive promoters, driving the expression of TurboGFP and a non-targeting control shRNA, were tested using packaged, purified and concentrated high-titre lentiviral particles from the SMARTchoice Promoter Selection Plate (Dharmacon), at several multiplicities of infection. After transduction, wells were evaluated for TurboGFP reporter intensity using fluorescence microscopy. The most active promoter (mCMV) was chosen for expression of *PDK4* shRNA in LoVo cells. SMART vector 2.0mCMV Lentiviral shRNA Particles were synthesised by Dharmacon. The vector encompasses a puromycin resistance gene for selection and TurboGFP for identification of positive clones. Two different designs of SMART vector 2.0 mCMV particles (LV1 and LV3) targeting human *PDK4* were investigated. Target sequences for LV1 and LV3 were 5'-AACCAATTCACATCGTGTA-3' and 5'-GATAATAAACTTACCCTG-3', respectively. SMARTvector 2.0 mCMV Non-Targeting control particles (Dharmacon) were used as negative controls. Selection of cells stably expressing *PDK4* shRNA and control shRNA started 72 h post-transduction following the manufacturer's instructions. Briefly, growth medium was replaced with fresh medium containing 10 mg ml⁻¹ puromycin. This medium was replaced every 3 days and selection of stable transductants was completed in 4 weeks. Migration, invasion and viability assays were performed as above.

Real-time RT-PCR. Total cellular RNA was extracted using the RNeasy Mini kit (Qiagen). Concentration and integrity of RNA were determined as before (Leclerc *et al*, 2013a). cDNA synthesis and quantitative real-time RT-PCR were performed as previously described (Pham *et al*, 2013). Samples were run in triplicate and mRNA levels were expressed as a ratio relative to *TUBULIN* expression. Supplementary Table S1 describes oligonucleotide primers.

Western blotting. Cells were homogenised at 4 °C as described previously (Leclerc *et al*, 2013). Protein concentration was measured by the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA). Samples were run on SDS-polyacrylamide gels and western blotting was performed as previously reported (Leclerc *et al*, 2013). Experiments were performed twice.

Statistical analysis. Quantitative data are presented as average value of replicates \pm s.e.m. Levene's test assessed equality of variance in different samples and differences between control and treated cells were determined by independent *t*-test. Analyses were performed using SPSS for WINDOWS version 22.0 (IBM, New York, NY, USA). *P* < 0.05 was considered significant.

RESULTS

Decreased DNA methylation of *PDK4* in peripheral blood of CRC patients. We previously observed reduced methylation for

four consecutive CpGs in a 5'-potential regulatory region of *PDK4*, in normal colonic mucosa of CRC subjects compared with normal mucosa of controls (Leclerc *et al.*, 2013). We hypothesised that this methylation change may be systemic, in which case analysis of *PDK4* methylation in peripheral blood might be useful as a non-invasive CRC marker.

We analysed two cohorts of patients and controls (40 and 18 patients compared with 40 and 29 controls, respectively). The first cohort showed decreased methylation ($P < 0.01$) for all four CpGs and for mean methylation of these four CpGs (Figure 1A). In the second cohort, we observed significant methylation decreases for two of the four CpGs ($P < 0.05$; Figure 1B) in patients, with a similar tendency for the two other CpGs. There was also a significant reduction in mean methylation of the four CpGs ($P < 0.05$; Figure 1B).

Although there was some variation in the *PDK4* absolute methylation values between the two cohorts, the differences were small. More importantly, the methylation decreases between controls and patients in each cohort were very similar, that is, the magnitude of the decrease was not significantly different between the two cohorts ($P \leq 0.52$ for the four CpGs, independent *t*-tests). The averaged values for patients were $\sim 80\%$ of the averaged values for controls, for each CpG.

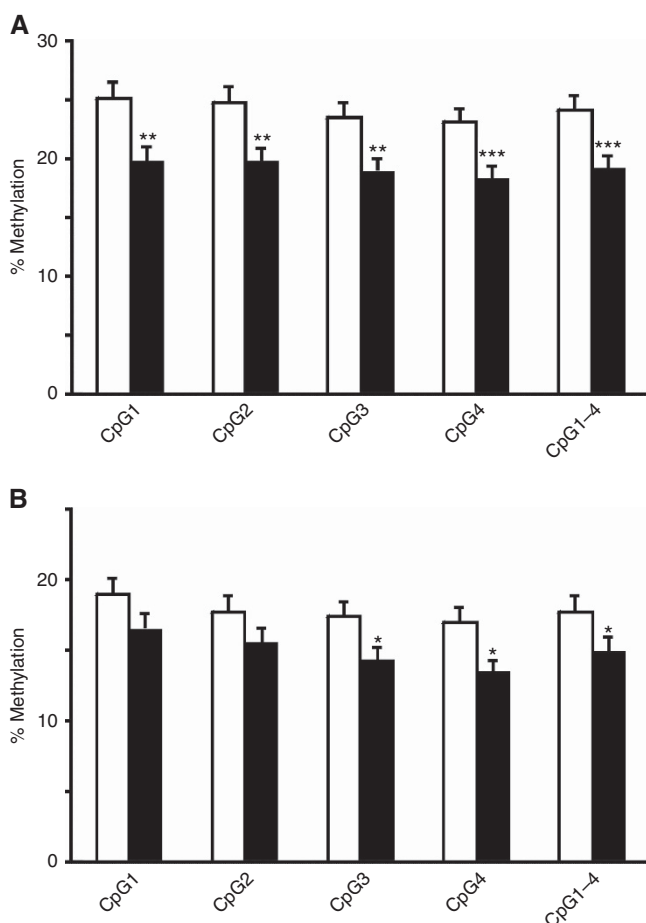


Figure 1. DNA methylation of *PDK4* in peripheral blood. (A) A cohort of 80 individuals was analysed for four CpGs individually and for average methylation of all four CpGs. Controls (40 individuals), white bars; CRC patients (40 subjects), black bars. Values are means \pm s.e.m. (B) Data for a second cohort (29 controls and 18 patients) are presented as in A. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.005$; independent *t*-tests.

***PDK4* siRNA knockdown decreases migration, invasion and resistance to apoptosis in LoVo and DLD1 cells.** Experiments in LoVo cells demonstrated a dramatic decrease (96%; $P < 0.0005$) in *GAPDH* mRNA, our positive control, when a *GAPDH*-siRNA was used (Figure 2A, upper panel). These same conditions showed a significant decrease in *PDK4* mRNA after *PDK4* siRNA transfection ($P < 0.001$; Figure 2A, lower panel); *PDK4* expression was $\sim 40\%$ of that in mock-transfected cells. There were no significant expression changes after transfection with scrambled siRNAs.

To assess transformation potential, we examined migration and invasion in LoVo. Cells were significantly less motile (Figure 2B, top panel) after transfection with *PDK4* siRNAs (reduction of 71%; $P < 0.0005$) and significantly less invasive (Figure 2B, bottom panel; reduction of 60%; $P < 0.0005$) than cells transfected with scrambled siRNAs. Although viability was reduced by *PDK4* siRNAs, the decrease was small (34%, data not shown); this indicates that the decreased migration and invasion did not result primarily from cell death. Expression of vimentin (a marker of invasiveness, migration and poor prognosis; Lazarova and Bordonaro, 2016) was decreased by 40% by *PDK4* siRNA, compared with scrambled siRNA (Figure 2C). A similar decrease (46%) was observed with DCA (Figure 2C). As resistance to apoptosis is a hallmark of cancer cells, we assessed PARP cleavage, a common apoptosis marker. Figure 2D shows that PARP cleavage was undetectable in mock transfectants and cells treated with scrambled siRNA, whereas significant PARP cleavage was observed with *PDK4* siRNA. DCA and etoposide were used as positive controls (Bonnet *et al.*, 2007; Zhao *et al.*, 2013).

To validate the above results, we repeated these experiments in DLD1 cells. DLD1 expresses *PDK4* at much lower levels than LoVo ($\sim 12\%$ of LoVo, data not shown). Nonetheless, we demonstrated the same outcomes (Figure 3). siRNA significantly inhibited *PDK4* expression, compared with mock-transfected or scrambled siRNA-transfected cells ($P < 0.01$, Figure 3A). Migration and invasion were significantly reduced, by $\sim 80\%$ ($P < 0.001$, Figure 3B), whereas viability was reduced by only 17% (data not shown). Vimentin expression was significantly reduced (26% decrease, Figure 3C) and PARP cleavage was observed only in *PDK4*-siRNA-transfected cells, not in mock- or scrambled siRNA-transfected cells (Figure 3D).

***PDK4* shRNA knockdown decreases migration and invasion in LoVo cells.**

The mCMV promoter showed the highest expression in LoVo transductants (Supplementary Figure S1) and was therefore used to generate stable shRNA_{*PDK4*} transductants. After selection, we assessed two independent pools of transductants (LV1 and LV3) for *PDK4* mRNA. *PDK4* expression was significantly lower in LV1 and LV3 transductants than in shRNA-scrambled cells (35% and 38% of levels in scrambled control; $P < 5 \times 10^{-5}$ and 1×10^{-4} , respectively; Figure 4A). LV1 and LV3 cells were significantly less motile and invasive than shRNA-scrambled control cells (Figure 4B). Migration was reduced by 37 and 44% ($P < 5 \times 10^{-5}$ for both LV1 and LV3). Cell invasion was reduced by 48% and 31% compared with the negative control ($P < 5 \times 10^{-5}$ and $P < 1 \times 10^{-4}$ for LV1 and LV3, respectively).

***PDK4* shRNA knockdown decreases expression of HIF1A and VEGFA.**

As inhibition of expression of other PDK family members affects the HIF-1 pathway (McFate *et al.*, 2008; Sutendra *et al.*, 2013), we examined the impact of changes in *PDK4* expression on *HIF1A* and its target *VEGFA*.

HIF1A expression was significantly decreased for LV1 and LV3 transductants (72 and 68% of scrambled- shRNA levels, $P < 0.01$ and $P < 5 \times 10^{-4}$, respectively; Figure 4C). *VEGFA* expression was also decreased to 75 and 59% for LV1 and LV3 ($P < 5 \times 10^{-4}$ and $P < 5 \times 10^{-5}$ respectively), compared with scrambled shRNA (Figure 4D).

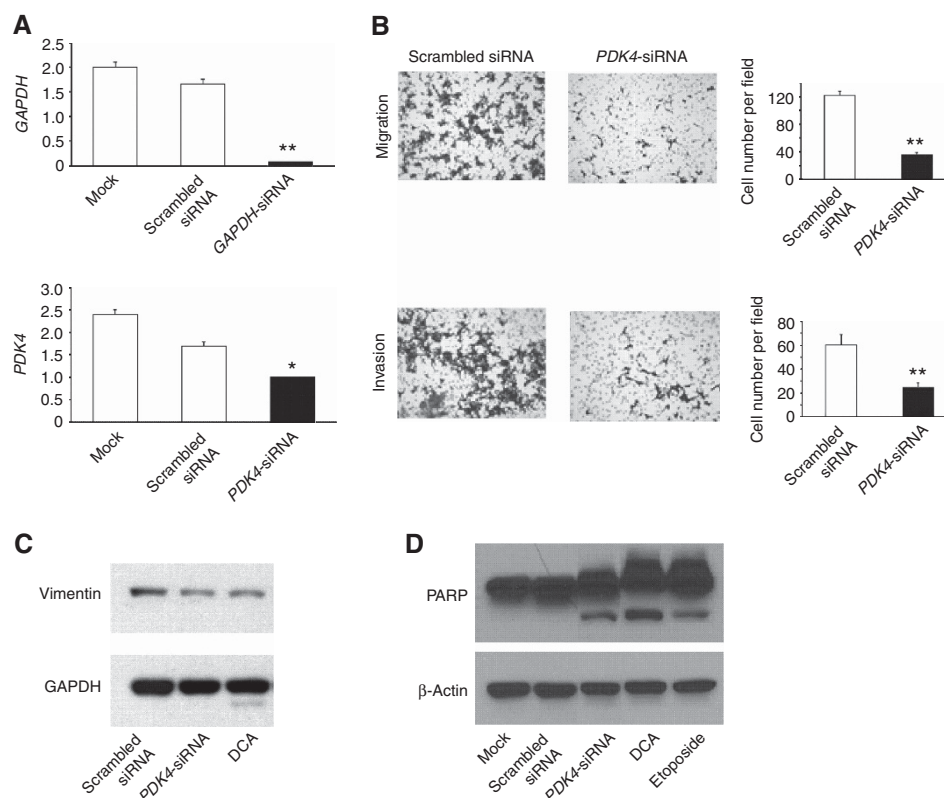


Figure 2. *PDK4* siRNA knockdown decreases migration, invasion and resistance to apoptosis in LoVo cells. At least three similar experiments were performed and representative results are shown. Data are expressed as means \pm s.e.m. **(A)** *GAPDH* or *PDK4* expression in mock-transfected cells or cells transfected with scrambled siRNA or specific siRNA against *GAPDH* (positive control) or *PDK4*. Expression is presented in arbitrary units with *TUBULIN* for normalisation. Bars represent means of triplicates. **(B)** Effect of scrambled siRNA- or *PDK4* siRNA transfection on migration and invasion. Bars represent the mean of stained cells in 15 fields. Average number of cells migrated/invaded was significantly lower with *PDK4* siRNA than scrambled siRNA. * $P < 0.001$ and ** $P < 0.0005$ (compared with mock- or scrambled siRNA-treated cells, independent t-test). **(C)** Vimentin was evaluated by Western blotting. DCA (positive control) was used at 50 mM. **(D)** Cleavage of PARP, assessed by western blotting. *PDK4* siRNA transfection resulted in PARP cleavage. DCA (50 mM) or etoposide (20 μ M) were used as positive controls. siRNA, small interfering RNA.

DISCUSSION

Cancer cells, unlike untransformed cells, rely on aerobic glycolysis for energy. PDH catalyses the oxidative decarboxylation of pyruvate in the TCA cycle. PDH is tightly regulated by PDKs, which prevent the entry of pyruvate into the TCA cycle and enhance glycolysis, a phenotype associated with apoptosis resistance (Plas and Thompson, 2002). PDK inhibition is accompanied by proapoptotic properties and antitumour activity (Olszewski *et al*, 2010).

Most studies on the PDK family have been performed on PDK1, PDK2 or PDK3 (Seyfried and Shelton, 2010; Lu *et al* 2011; Contractor and Harris, 2012). PDK2 and PDK4 are the most widely distributed PDK isoforms (Zhang *et al*, 2014), but PDK4 has not been extensively studied in transformation. To evaluate the role of *PDK4* in tumorigenesis, we used RNAi to attenuate *PDK4* expression in human CRC cells. Both siRNA and shRNA approaches decreased *PDK4* expression, which reduced the migratory and invasive properties of CRC cells.

The switch from oxidative to glycolytic metabolism is an active response to hypoxia and up-regulation of *HIF1A* represents a general mechanism underlying the Warburg effect (Semenza, 2009). Decreased expression of *HIF1A* reduces glucose transporters and glycolytic enzymes (Iyer *et al*, 1998; Luo *et al*, 2006) and, in contrast, overexpression promotes glycolysis (Luo *et al*, 2006). We observed that inhibition of *PDK4* decreases *HIF1A* expression in colon carcinoma cells; this finding is consistent with the

observation that *HIF1- α* correlates with *PDK4* expression (Lee *et al*, 2012). Solid tumours including colon carcinoma require neovascularisation for progression and metastasis. Regions of hypoxia are common in a tumour mass and the consequent expression of *HIF1A* results in constitutive activation of specific hypoxia-induced pathways, including *VEGFA* synthesis (Zhong *et al*, 1999). *HIF-1* is the main activator of VEGF (Pellizzaro *et al*, 2002), which increases progression and metastasis of colon cancer (Kondo *et al*, 2000); we found decreased expression of *VEGFA* after *PDK4* inhibition.

To explore another *HIF-1* target gene, we measured expression of vimentin, which is required for motility (Semenza, 2003). We found decreased expression concomitant with the attenuation of migration/invasion. Our results indicate that *PDK4* affects multiple steps in the complex process of invasion by promoting the ability of cells to reprogram key genes and to migrate. In addition, PDKs facilitate fatty acid oxidation for energy supply in cancer cells (Zhang *et al*, 2014). We previously reported increased expression of fatty acid oxidation genes and proteins in our mouse model of preneoplastic intestine (Leclerc *et al*, 2013a, 2014) with increased *Pdk4* expression.

Knockdown of *PDK4* also induced PARP cleavage, an observation consistent with another report (Bonnet *et al*, 2007), demonstrating that DCA induces apoptosis. DCA also decreased tumour growth in rats *in vivo* (Michelakis *et al*, 2008; Sun *et al*, 2010). However, this compound is toxic and can lead to liver toxicity and neoplasia, as well as skin cancer (Shahrzad *et al*, 2010). It can also inhibit other enzymes. More specific PDK inhibitors

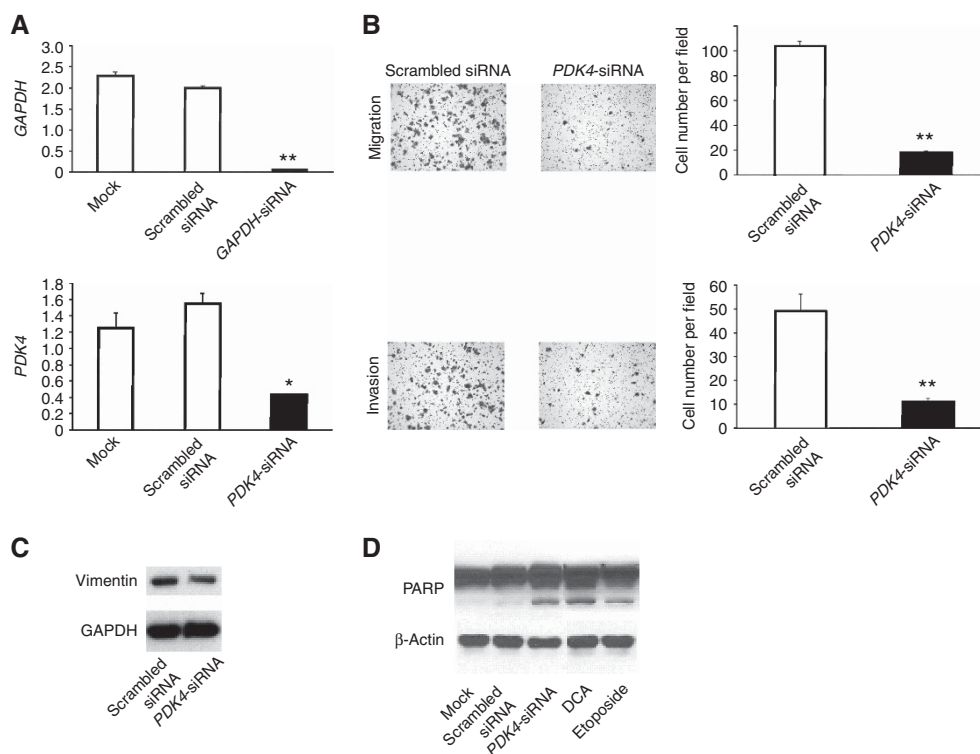


Figure 3. *PDK4* siRNA knockdown decreases migration, invasion and resistance to apoptosis in DLD1 cells. Representative results from two experiments are shown. Data are expressed as means \pm s.e.m. (A) *GAPDH* or *PDK4* expression after mock- or scrambled siRNA (white bars) or after specific siRNAs (black bars) against *GAPDH* (positive control) or *PDK4*. Expression is presented in arbitrary units with *TUBULIN* for normalisation. Bars represent mean of triplicates. (B) Effect of scrambled siRNA- or *PDK4* siRNA transfection on migration/invasion. Bars represent mean of stained cells in 15 fields. Number of cells migrated/invaded was significantly lower in *PDK4* siRNA transfectants than in scrambled siRNA. * $P < 0.01$ and ** $P < 0.001$ (different from mock- or scrambled siRNA-treated cells, independent t-test). (C) Vimentin was evaluated by western blotting. (D) Cleavage of PARP, assessed by western blotting. *PDK4* siRNAs transfection resulted in PARP cleavage. DCA (50 mM) or etoposide (20 μ M) were used as positive controls. siRNA, small interfering RNA.

would be beneficial for cancer therapy. Our results suggest that *PDK4* should be considered as a CRC target. In the course of our experimentation, Mazar *et al* (2016) reported that miR-211 acts in melanomas as a tumour suppressor that can negatively regulate *PDK4*, causing reduction of HIF1- α levels and inhibition of invasion. Another group (Li *et al*, 2017) recently found that miR-182 is upregulated in lung tumours, with downregulation of *PDK4*. To explain this puzzling observation, they suggested that promotion of lipogenesis (as opposed to lipid oxidation) may increase lung tumorigenesis and that dysregulation of *PDK4* can have opposite effects on tumorigenesis in different tissues.

Although our work was performed with two very different cell lines, it would be interesting to test other CRC cell lines in future studies. LoVo is derived from CRC metastatic cells, whereas DLD1 is from a primary CRC. It remains to be determined whether the higher *PDK4* expression in LoVo is related to the advanced stage of the disease. Our results and other reports suggest a role for *PDK4* and other *PDKs* in the initiation of tumorigenesis, but Grassian *et al* (2011) proposed that the consequence of a change in PDH flux can be different at later stages of tumour development. This concept could explain some findings from microarrays (Grassian *et al*, 2011; Sun *et al*, 2014) and qRT-PCR (Blouin *et al*, 2011) which showed down-regulation of *PDK4* when some cancer biopsies (including CRCs) were compared to their corresponding normal tissues.

Nevertheless, Kamarajugadda *et al* (2012) found overexpression of *PDK4* in several human non-CRC cancer lines and we showed increased *PDK4* expression in the normal mucosa of CRC patients (Leclerc *et al*, 2013). Similar observations were obtained in

preneoplastic intestine in our mouse model of intestinal neoplasia. Decreased methylation of *PDK4/Pdk4* was observed in both human and mouse normal colonic mucosa (Leclerc *et al*, 2013). The decreased methylation of *PDK4* in blood of CRC patients in this study is consistent with our methylation results in colonic mucosa and suggests the presence of a systemic effect on metabolism. This phenomenon may be related to a recently reported association between levels of specific fatty acids in peripheral blood and methylation of the *PDK4* 5'-UTR (de la Rocha *et al*, 2016).

Barrès *et al* (2012, 2013) reported hypomethylation and increased expression of *PDK4* in skeletal muscle, Bohl *et al* (2013) demonstrated higher expression of *PDK4* in 5-aza-2'-deoxycytidine-treated acute myeloid leukemia and loss of CpG methylation accompanied *PDK4* upregulation during cardiomyocyte maturation (Kranzhöfer *et al*, 2016). However, the impact of DNA methylation changes on *PDK4* expression in CRC cells requires further study (e.g., using 5-azacytidine).

Our observation of altered methylation in colon and blood of CRC patients may be useful for designing a non-invasive test for CRC detection. CRC detection strategies include colonoscopy, which is invasive and labor-intensive, analysis of stool, and studies of methylation of cell-free DNA in blood, a tedious procedure (Molnár *et al*, 2015). More direct analysis of *PDK4* methylation in blood may have diagnostic utility. Knowledge of factors that influence *PDK4* methylation would be very useful in this regard.

In summary, our results add yet another dimension to the multifaceted involvement of *PDKs* in tumour progression. We suggest that *PDK4* may be useful in both a therapeutic and diagnostic setting for CRC.

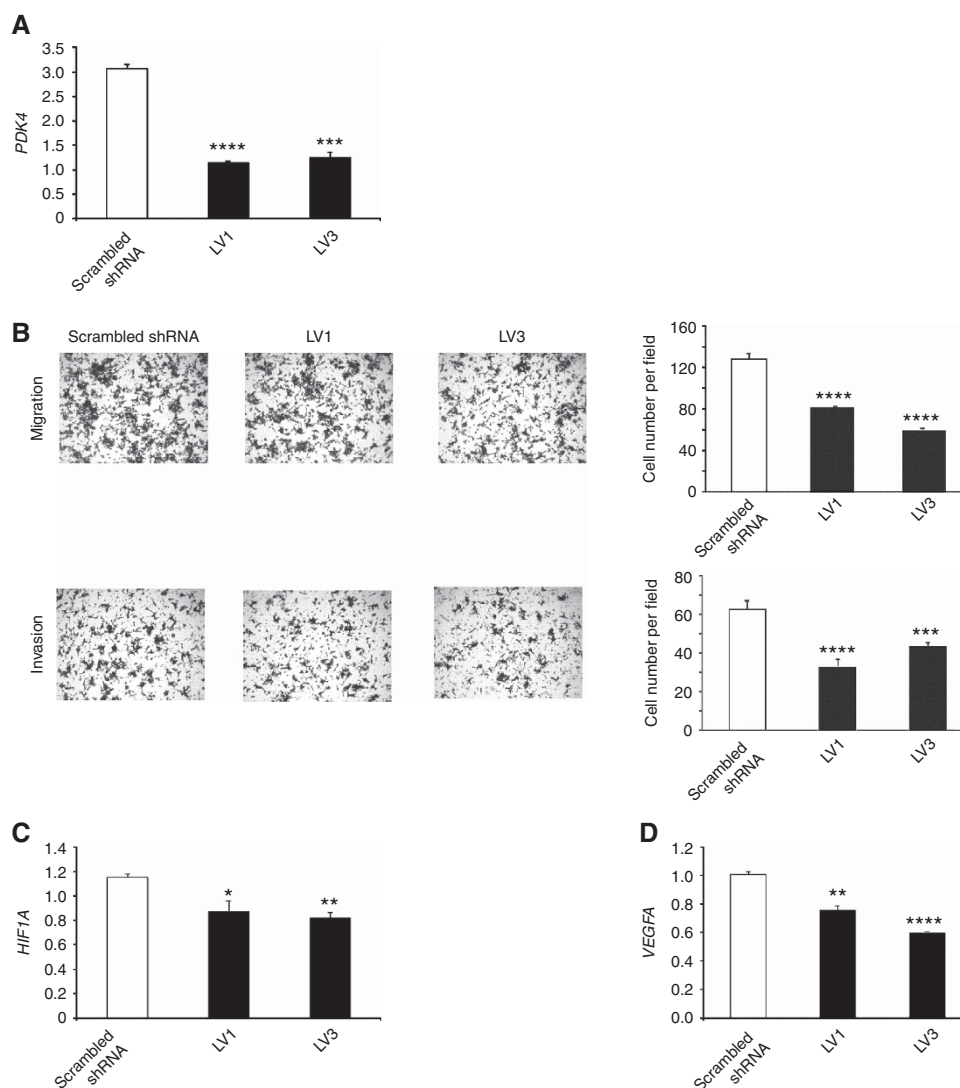


Figure 4. PDK4 shRNA knockdown in LoVo cells decreases migration, invasion and expression of *HIF1A* and *VEGFA* genes. **(A)** PDK4 knockdown by shRNA was effective in the two transductants, assessed by qRT-PCR with *TUBULIN* for normalisation. **(B)** Average number of cells migrated or invaded was significantly lower for cells expressing PDK4 shRNA. Three experiments were performed and representative results are shown. **(C)** Significantly decreased expression of *HIF1A* was observed compared with scrambled shRNA. **(D)** Similar decreases were observed for *VEGFA*. Results are expressed as means \pm s.e.m. Bars represent mean of triplicates for expression assays and mean of stained cells in 15 fields for migration/invasion assessments. Asterisks denote significant differences compared with scrambled shRNA. * $P < 0.01$, ** $P < 5 \times 10^{-4}$, *** $P < 1 \times 10^{-4}$ and **** $P < 5 \times 10^{-5}$ (independent t-tests).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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