Oncogenic role of PDK4 in human colon cancer cells

D Leclerc1, D N T Pham1, N Lévesque1, M Truongcao2, W D Foulkes3,4,5, C Sapienza2 and R Rozen*,1

1Department of Human Genetics and Pediatrics, The Research Institute of the McGill University Health Centre, McGill University, 1001 Decarie Boulevard, Room E.M0.3211, Montreal, QC H4A 3J1, Canada; 2Fels Institute for Cancer Research and Molecular Biology, Temple University School of Medicine, Philadelphia, PA, USA; 3Department of Medical Genetics, The Research Institute of the McGill University Health Centre, McGill University, Montreal, QC, Canada; 4Segal Cancer Centre, Lady Davis Institute, Jewish General Hospital, Montreal, QC, Canada and 5Program in Cancer Genetics, Department of Oncology and Human Genetics, McGill University, Montreal, QC, Canada

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...
expressed in the muscle and affects the metabolic fate of glucose during exercise (Pilegaard and Neuffer, 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 20 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 Gentra 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 (Laayette, CO, USA). The four target sequences were 5'-GAGCAUUUCUCGCGCUACA-3', 5'-CGACAAGAUAUGGCGUGUA-3', 5'-CAACGCCUGUAUGGAUA-3' and 5'-GACC GCCCUUCUAGAU-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 SMARTch-oice 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'-AACCAATTCCATCGTGA-3' and 5'-GATAATAAACCTTCGGGTG-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 ± 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 PDK4 oncogenicity in colon cancer
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 \( (P<0.05; \text{Figure 1B}) \) in patients, with a similar tendency for the two other CpGs. There was also a significant reduction in mean methylation of the four CpGs \( (P<0.05; \text{Figure 1B}) \).

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 \( (P\leq 0.52 \text{ for the four CpGs, independent } t\text{-tests}) \). The averaged values for patients were \( \sim 80\% \) of the averaged values for controls, for each CpG.

**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 \( (P<0.001; \text{Figure 2A, lower panel}) \); 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 \( (P<0.01, \text{Figure 3A}) \). Migration and invasion were significantly reduced, by \( \sim 80\% \) \( (P<0.001, \text{Figure 3B}) \), whereas viability was reduced by only 17% \( (\text{data not shown}) \). 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\textsubscript{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; \( P<5\times10^{-5} \) and \( 1\times10^{-4} \), 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% \( (P<5\times10^{-5} \) for both LV1 and LV3). Cell invasion was reduced by 48% and 31% compared with the negative control \( (P<5\times10^{-5} \text{ and } P<1\times10^{-4} \text{ for LV1 and LV3}) \).

**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 \( P<5\times10^{-4} \), respectively; Figure 4C). VEGFA expression was also decreased to 75 and 59% for LV1 and LV3 \( (P<5\times10^{-4} \text{ and } P<5\times10^{-5} \text{respectively}) \), compared with scrambled shRNA (Figure 4D).

![Figure 1](image1.png)

**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 ± 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.  

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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 murine 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 (Shahraz et al, 2010). It can also inhibit other enzymes. More specific PDK inhibitors

**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).

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would be beneficial for cancer therapy. Our results suggest that \textit{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 \textit{PDK4}, causing reduction of HIF1-\alpha levels and inhibition of invasion. Another group (Li et al, 2017) recently found that miR-182 is upregulated in lung tumours, with downregulation of \textit{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 \textit{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 \textit{PDK4} expression in LoVo is related to the advanced stage of the disease. Our results and other reports suggest a role for \textit{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 micrarrays (Grassian et al, 2011; Sun et al, 2014) and qRT-PCR (Blouin et al, 2011) which showed down-regulation of \textit{PDK4} when some cancer biopsies (including CRCs) were compared to their corresponding normal tissues.

Nevertheless, Kamarajugadda et al (2012) found overexpression of \textit{PDK4} in several human non-CRC cancer lines and we showed increased \textit{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 \textit{PDK4/Pdk4} was observed in both human and mouse normal colonic mucosa (Leclerc et al, 2013). The decreased methylation of \textit{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 \textit{PDK4} 5′-UTR (de la Rocha et al, 2016).

Barrès et al (2012, 2013) reported hypomethylation and increased expression of \textit{PDK4} in skeletal muscle, Bohl et al (2013) demonstrated higher expression of \textit{PDK4} in 5-aza-2′-deoxycydine- treated acute myeloid leukemia and loss of CpG methylation accompanied \textit{PDK4} upregulation during cardiomyocyte maturation (Kranzhofer et al, 2016). However, the impact of DNA methylation changes on \textit{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 \textit{PDK4} methylation in blood may have diagnostic utility. Knowledge of factors that influence \textit{PDK4} methylation would be very useful in this regard.

In summary, our results add yet another dimension to the multifaceted involvement of \textit{PDK4} in tumour progression. We suggest that \textit{PDK4} may be useful in both a therapeutic and diagnostic setting for CRC.
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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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