Pancreatic Neuroendocrine Tumors and EMT Behavior Are Driven by the CSC Marker DCLK1

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Abstract

Doublecortin-like kinase 1 (DCLK1), a marker for intestinal and pancreatic cancer stem cells, is highly expressed in neuroblastomas. This study was conducted to assess DCLK1 expression levels in pancreatic neuroendocrine tumor (PNET) tissues and to explore the roles of this molecule in clinical tissue from multiple PNET patients, cells (BON1, QGP1, and CM) and tumor xenografts. Immunohistochemically, all PNET tissues highly and diffusely expressed DCLK1 as a full-length isoform, identical to that detected in primary liver NETs. A DCLK1-overexpressing PNET cell line (QGP1-DCLK1) exhibited epithelial–mesenchymal transition (EMT)-related gene signatures, and robust upregulation of Slug (SNAI2), N-Cadherin (CDH2), and Vimentin (VIM) was demonstrated here in human PNET tissue specimens and cells. DCLK1 characterized the PNET cell behavior, inducing p-FAK/SLUG-mediated EMT. These findings suggest the possibility of developing novel therapeutic strategies against PNETs by targeting DCLK1.

Implications: Evidence here reveals that human PNETs diffuse and robustly express the cancer stem cell marker DCLK1, which drives SLUG-mediated EMT, and suggests that NETs share biological features for druggable targets with other tumors, including neuroblastoma that also highly expresses DCLK1. Mol Cancer Res; 1–9. ©2017 AACR.

Introduction

Although neuroendocrine tumors (NET) are considered rare tumors, the number of patients with these tumors is gradually increasing worldwide (1–3). NETs, previously called “carcinoid or benign carcinoma” (4), grow slowly; however, they have metastatic potential and are categorized as malignant neoplasms (5). In addition, a study of 310 patients who underwent resection of pancreatic NETs (PNET) also showed high rates of lymph node metastasis (36.8%) and distant metastasis (20.3%) (6). Such high metastatic potential, despite slow tumor growth, is one of the most important features of NETs, including PNETs (7). Although molecular-targeted drugs have been used to treat NETs, such as everolimus and sunitinib (8, 9), the critical target molecules responsible for its aggressiveness have yet to be determined.

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were also used as pathologic controls, and two of the four LNET tissues were subjected to both Western blot analysis and immunohistochemistry for DCLK1. The patients’ tumors were surgically resected between 1997 and 2012 at Kurume University Hospital. Informed consent to participate in the study was obtained from all patients in accordance with the principles stated in the Declaration of Helsinki and the guidelines of the Ethical Committee of Kurume University (study registration no. 13149; Committee Chair, Kensei Nagata). The mean age of the patients with PNET was 56 years (range, 17–80 years). The diagnosis of NET was made by at least two pathologists independently according to the new World Health Organization guidelines for NET (24). The average diameter of the PNETs was 30.2 mm (range, 12–93 mm). Histologically, nine PNETs were graded as G1, and six were graded as G2. Three PNETs were endocrinologically functional. Distant metastasis or lymph node metastasis was detected in eight cases (Table 1).

**Cell lines and culture conditions**

The human PNET cell lines BON1 (25), QGP1, and CM were used in this study. BON1 was a kind gift from Professor B. Lankat-Buttgereit (Marburg, Germany). QGP1, a somatostatin-secreting cell line (26), was purchased from the Health Science Research Resource Bank (Japan Health Sciences Foundation). CM, an insulin-secreting line (27), was obtained from Professor Paolo Pozzilli (Rome, Italy). All cell lines were grown in DMEM (Wako) supplemented with 10% heat-inactivated (56°C, 30 minutes) FBS (Biowest), 100 units/mL penicillin, and 100 μg/mL streptomycin (Nacalai Tesque) in a humidified atmosphere containing 5% CO2 at 37°C.

**Immunohistochemistry and staining score**

Immunohistochemistry was performed as previously reported (23). The intensity of DCLK1 staining was scored on a scale of 0 to 3, in which 0 is negative staining, 1 is weakly positive staining, 2 is moderately positive staining, and 3 is strongly positive staining. The signal-positive area was scored on a scale of 0 to 2, in which 0 is positive staining in 0% to 20% of cells; 1 is positive staining in 21% to 60% of cells; and 2 is positive staining in 61% to 100% of cells. If the total score was greater than or equal to 2, it was judged as positive (Table 2). The antibodies used for immunohistochemistry and Western blotting are listed in Table 3.

**Western blotting**

Cells and homogenized liver NET tissues were lysed with RIPA buffer (Pierce) containing Protease Inhibitor Cocktail (Nacalai Tesque) and Halt Phosphatase Inhibitor Cocktail (Pierce). Protein concentrations were measured using the BCA Protein Assay Kit (Pierce). Samples containing 25 μg of protein were separated on 8% or 10% SDS-polyacrylamide gels and then transferred to equilibrated polyvinylidene difluoride membranes (Bio-Rad). After blocking with 5% nonfat milk, the membranes were incubated with diluted primary antibodies overnight at 4°C. The bound antibodies were detected with horseradish peroxidase-labeled secondary antibodies using ECL Plus Western Blotting Detection Reagents (Amersham Pharmacia Biotech). Positive signals from the target proteins were visualized using an image analyzer (LAS-4000; Fujifilm). Densitometric analysis of the signals was performed using Multi Gauge software, version 3.0 (Fujifilm).

**Overexpression of DCLK1**

A DCLK1-expressing plasmid (RC217050) and a control plasmid (RC208006) were obtained from OriGene Technologies, Inc. To generate stable transfectants, QGP1 cells and BON1 cells were transfected with the DCLK1 cDNA or the empty vector using TransIT-LT1 (Mirus) according to the manufacturer’s instructions. After transfection, DCLK1-overexpressing clones (QGP1-DCLK1 and BON1-DCLK1) were selected with 400 μg/mL G418 (Nacalai Tesque). Mock-transfected cells (QGP1-EV and BON1-EV) were similarly selected with G418 as control clones.

**Gene silencing of DCLK1 by siRNA**

DCLK1-targeting and nontargeting (NT) siRNAs were purchased from Thermo Fisher Scientific, Inc. Focal adhesion kinase (FAK)–targeting siRNA and paired NT siRNAs were obtained from Santa Cruz Biotechnology, Inc. The cells were transfected with these siRNAs using DharmaFECT (Dharmacon) according to the manufacturer’s protocol.

**Wound-healing assay**

Cells were seeded (6.0 × 10⁴) and grown in 35-mm dishes containing DMEM until confluence. A single scratch was made on the cell surface with a sterile 10-μL pipette tip. Perpendicular marks were made to standardize the viewing fields. Wound healing was monitored, and the distance between the edges of the scratched line was measured at 5 points under a microscope over 48 hours.

**cDNA microarray**

**Total RNA isolation.** Total RNA was isolated from QGP1-DCLK1 and QGP1-EV cells using TRIzol Reagent (Thermo Fisher Scientific) and purified using the SV Total RNA Isolation System (Promega Corporation) according to the manufacturer’s instructions. RNA samples were quantified using an ND-1000 spectrophotometer (NanoDrop Technologies), and RNA quality was confirmed with the Experion System (Bio-Rad).

**Gene expression microarrays.** cRNA was amplified, labeled, and hybridized to a 60K Agilent 60-mer oligomicroarray according to the manufacturer’s instructions. All hybridized microarray slides were scanned with an Agilent scanner. Relative hybridization intensities and background hybridization values were calculated using Agilent Feature Extraction Software (9.5.1.1).

<table>
<thead>
<tr>
<th>Table 1. Characteristics of patients and tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
</tr>
<tr>
<td>Age (range, years)</td>
</tr>
<tr>
<td>Tumor grade (G1/G2)</td>
</tr>
<tr>
<td>Long diameter of tumors (range, mm)</td>
</tr>
<tr>
<td>Functional/nonfunctional</td>
</tr>
<tr>
<td>Metastasis positive/negative</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 2. Evaluation criteria for immunostaining</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Score</strong></td>
</tr>
<tr>
<td><strong>Signal intensity</strong></td>
</tr>
<tr>
<td><strong>Positive area (%)</strong></td>
</tr>
</tbody>
</table>
Data analysis and filter criteria. Raw signal intensities and Flags for each probe were calculated from the hybridization intensities (gProcessedSignal) and spot information (gIsSaturated, etc.), according to the procedures recommended by Agilent. [Flag criteria in GeneSpring Software. Absent (A): "Feature is not positive and significant" and "Feature is not above background"; Marginal (M): "Feature is not Uniform," "Feature is Saturated," and "Feature is a population outlier"; Present (P): others.] The raw signal intensities of the samples were normalized using a quantile algorithm with the preprocessCore library package (28) in Bioconductor (29). We selected probes that called a “P” flag in two samples. To identify upregulated or downregulated genes, we calculated Z-scores (30) and ratios (non–log-scaled fold-change) from the normalized signal intensities of each probe to compare the control and experimental samples. Then, we established the following criteria for regulated genes: Z-score ≥ 2.0 and ratio ≥ 1.5-fold for upregulated genes and Z-score ≤ −2.0 and ratio ≤ 0.66 for downregulated genes.

Real-time qPCR analysis

RNA was extracted from cells using TRIzol, and 1 μg of total RNA was reverse transcribed using the TaqMan Reverse Transcription (RT) Reagent Kit (Applied Biosystems) according to the manufacturer’s instructions. qRT–PCR was performed on a 7500 Fast Real Time PCR System (Applied Biosystems) using TaqMan Universal PCR Master Mix (Applied Biosystems). The predesigned TaqMan probes for the genes of interest were as follows: DCLK1 (Assay ID, Hs00178027_m1), SLUG/SNAI2 (Hs00161904_m1), N-CADHERIN/CDH2 (Hs00362037_m1), E-CADHERIN/CDH1 (Hs01023894_m1), and VIMENTIN (Hs00958111_m1). Gene expression relative to GAPDH (Hs02758991_g1) was evaluated according to the ΔΔ-CT method using StepOne Software 2.0 (Applied Biosystems).

Sphere formation assay

QGP-DCLK1 and QGP-EV cells were seeded (at 1.2 × 10^4 per well) in ultralow attachment 6-well plates (Corning) and grown for up to 48 days. Then, the area of the cell spheres was measured using a microscope (BZ-X700) equipped with digital cell counting software (KEYENCE).

Xenograft model

QGP-DCLK1 and QGP-EV cells (2.5 × 10^6 per mouse) were subcutaneously injected into the back of 5-week-old male BALB/c athymic nude mice (n = 10 for each cell line; Clea Japan). Tumor size was measured weekly in two orthogonal directions using calipers, and tumor volume (mm^3) was estimated using the equation length × (width)^2 × 0.5. At 42 days after tumor cell inoculation, the mice were sacrificed, and the tumors were resected. The tumor tissues were subjected to hematoxylin and eosin (H&E) staining, immunohistochemistry, and Western blotting. The average number of mitotic figures (dividing tumor cell nuclei) per high-power field was quantified by counting the figures in six randomly selected fields. All

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Table 3. List of antibodies used in this study

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Clone/type</th>
<th>Dilution</th>
<th>Antigen retrieval</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCLK1</td>
<td>EPR6085</td>
<td>1:700</td>
<td>H</td>
<td>Abcam</td>
</tr>
<tr>
<td>SYNAPTOPHYSIN</td>
<td>Z66</td>
<td>1:1</td>
<td>H</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>CHROMOGRANIN A</td>
<td>DAK-A3</td>
<td>1:400</td>
<td>H</td>
<td>DakoCytomation</td>
</tr>
<tr>
<td>CD56</td>
<td>1B6</td>
<td>1:200</td>
<td>H</td>
<td>Leica Microsystems</td>
</tr>
<tr>
<td>SLUG</td>
<td>(Polyclonal)</td>
<td>1:700</td>
<td>H</td>
<td>Abcam</td>
</tr>
<tr>
<td>Ki-67</td>
<td>MIB-1</td>
<td>1:200</td>
<td>H</td>
<td>DakoCytomation</td>
</tr>
</tbody>
</table>

Abbreviation: H, heat-activated.
animal experiments were conducted in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of the University of Kurume.

**Functional inhibition of DCLK1**

After functional inhibition of DCLK1 by using LRRK2-IN-1 (Calbiochem) and XMD 8-92 (Tocris Bioscience), the molecular changes in treated cells were assessed (22, 31, 32).

**Statistical analysis**

Statistical significance was assessed by the Mann–Whitney U test using StatView 5.0 J software (SAS Institute Inc.). *P* values less than 0.05 were considered statistically significant.

### Results

**DCLK1 is highly expressed in PNETs**

DCLK1 was diffusely expressed in the cytoplasm of tumor cells in PNET tissues (Fig. 1; Supplementary Fig. S1). All PNET

**Table 4. Immunoreactivity scores for DCLK1 and positivity for known neuroendocrine markers**

<table>
<thead>
<tr>
<th>Number</th>
<th>Age</th>
<th>Sex</th>
<th>Location</th>
<th>Grade</th>
<th>Size (mm)</th>
<th>Function</th>
<th>DCLK1</th>
<th>Cg A</th>
<th>CD56</th>
<th>SYP</th>
<th>MIB1</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>53</td>
<td>F</td>
<td>BT</td>
<td>G1</td>
<td>70 × 36</td>
<td>—</td>
<td>3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>&lt;2%</td>
</tr>
<tr>
<td>2</td>
<td>70</td>
<td>M</td>
<td>BT</td>
<td>G2</td>
<td>28 × 20</td>
<td>—</td>
<td>5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>&lt;2%</td>
</tr>
<tr>
<td>3</td>
<td>77</td>
<td>F</td>
<td>H</td>
<td>G1</td>
<td>14 × 12</td>
<td>—</td>
<td>3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>&lt;2%</td>
</tr>
<tr>
<td>4</td>
<td>51</td>
<td>F</td>
<td>T</td>
<td>G2</td>
<td>93 × 70</td>
<td>—</td>
<td>5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>5%</td>
</tr>
<tr>
<td>5</td>
<td>21</td>
<td>M</td>
<td>T</td>
<td>G2</td>
<td>32 × 22</td>
<td>—</td>
<td>5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>3%–5%</td>
</tr>
<tr>
<td>6</td>
<td>64</td>
<td>F</td>
<td>T</td>
<td>G2</td>
<td>20 × 15</td>
<td>+</td>
<td>4</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>10%</td>
</tr>
<tr>
<td>7</td>
<td>80</td>
<td>M</td>
<td>BT</td>
<td>G1</td>
<td>12 × 10</td>
<td>—</td>
<td>3</td>
<td>—</td>
<td>+</td>
<td>+</td>
<td>&lt;2%</td>
</tr>
<tr>
<td>8</td>
<td>90</td>
<td>F</td>
<td>H</td>
<td>G3</td>
<td>18 × 17</td>
<td>—</td>
<td>5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>&lt;2%</td>
</tr>
<tr>
<td>9</td>
<td>48</td>
<td>M</td>
<td>BT</td>
<td>G2</td>
<td>32 × 22</td>
<td>—</td>
<td>5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>8%–10%</td>
</tr>
<tr>
<td>10</td>
<td>65</td>
<td>M</td>
<td>H</td>
<td>G1</td>
<td>25 × 25</td>
<td>—</td>
<td>5</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>&lt;2%</td>
</tr>
<tr>
<td>11</td>
<td>52</td>
<td>F</td>
<td>B</td>
<td>G2</td>
<td>25 × 35</td>
<td>—</td>
<td>5</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>3%–5%</td>
</tr>
<tr>
<td>12</td>
<td>17</td>
<td>M</td>
<td>B</td>
<td>G1</td>
<td>20 × 18</td>
<td>+</td>
<td>5</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>&lt;2%</td>
</tr>
<tr>
<td>13</td>
<td>56</td>
<td>M</td>
<td>B</td>
<td>G1</td>
<td>32 × 38</td>
<td>—</td>
<td>3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>&lt;2%</td>
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<tr>
<td>14</td>
<td>57</td>
<td>M</td>
<td>T</td>
<td>G1</td>
<td>14 × 12</td>
<td>+</td>
<td>5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>3%–5%</td>
</tr>
<tr>
<td>15</td>
<td>71</td>
<td>F</td>
<td>B</td>
<td>G3</td>
<td>29 × 24</td>
<td>—</td>
<td>5</td>
<td>—</td>
<td>-</td>
<td>+</td>
<td>&lt;2%</td>
</tr>
</tbody>
</table>

**NOTE:** H, B, and T in location indicate head, body, and tail of the pancreas, respectively.

**Figure 2.**

A, Overexpression of DCLK1 in QGP1 cells (QGP1-DCLK1 cells) shown by Western blotting. EV, empty vector-transfected QGP1 cells. B, An increase of polygonal and spindle-shape cells is observed in the QGP1-DCLK1 cells. C and D, In a wound-healing assay, significantly greater migration ability is observed in the QGP1-DCLK1 cells compared with that of the control cells. E, Confirmation of siRNA-mediated knockdown of DCLK1 expression in CM cells by Western blotting. siNT, non-targeted (NT) siRNA-treated CM cells. F and G, Wound healing is retarded in DCLK1-silenced CM cells (CM-siDCLK1). All data are expressed as mean ± SD. **P** < 0.01, Mann–Whitney U test.
Overexpression of DCLK1 induces EMT

Overexpression of full-length DCLK1 in QGP1-DCLK1 cells was confirmed by Western blotting (Fig. 2A). The cell shape changed from round to polygonal and spindle, that was morphologically reminiscent of EMT (Fig. 2B). In addition, the migration ability of these cells was clearly increased in the wound-healing assay (Fig. 2C and D). Conversely, the wound-healing speed of DCLK1-silenced CM cells was retarded (Fig. 2E–G). We then explored the genes involved in the DCLK1-induced EMT, focusing on EMT-associated transcription factors, including SNAI1 and SLUG, by cDNA microarray. The results showed that SLUG (SNAI2) expression was robustly upregulated in QGP1-DCLK1 cells, in concert with the remarkable upregulation in VIP (VIMENTIN) and TGFB1 (Transforming Growth Factor Beta 1; Supplementary Fig. S4). SLUG upregulation was reproduced in the qPCR analysis, which also showed upregulated expression of two other mesenchymal markers, VIMENTIN and N-CADHERIN (Fig. 3A). However, the expression of E-CADHERIN, an epithelial marker, was not always downregulated in these cells (Fig. 3A). These transcriptional changes were also confirmed at the protein level in not only QGP1-DCLK1 cells but also BON1-DCLK1 cells (Fig. 3B). Because cancer cells with EMT characteristics are prone to sphere formation, sphere-forming ability was semi-quantitatively evaluated with a digital microscope. As expected, QGP1-DCLK1 cells formed significantly more spheres than the control cells (Supplementary Fig. S5A and S5B). In addition, QGP1-DCLK1 cells showed slightly higher cell proliferation in vitro, but the difference did not reach a statistical significance.

DCLK1-overexpressing PNET cell xenograft tumors grow faster

QGP1-DCLK1 cells formed significantly larger xenograft tumors than QGP1-EV cells (Fig. 4A–C), suggesting that they had higher proliferative ability in vivo. Indeed, a significantly higher frequency of mitosis was observed in QGP1-DCLK1 tumor tissues stained with H&E than in QGP1-EV tumor tissues (Fig. 4D and E).

DCLK1 induces EMT-related characteristics, and tyrosine (Tyr)925-phosphorylated (p-) FAK is involved

Western blotting showed that the expression of the EMT regulator SLUG was increased in a representative QGP1-DCLK1 tumor tissue (Fig. 4F). Because QGP1-DCLK1 cells showed high...
migration potential (Fig. 2C and D), we focused on the expression levels of FAK and its downstream molecules, including PAXILLIN, ERK1/2, AKT, and CYCLIN D1. Notably, Tyr925-p-FAK, but not Tyr397-p-FAK, was highly expressed in the QGP1-DCLK1 tumor tissues, which was accompanied by increased expression of PAXILLIN, p-ERK1/2, p-AKT, and CYCLIN D1 (Fig. 4F). Then, we confirmed significant upregulation of both SLUG and Tyr925-p-FAK in all QGP1-DCLK1 tumors studied (n = 5 for each group) by immunohistochemistry (Fig. 4G with graphs for immunostaining scores).

Silencing DCLK1 by siRNA

Knockdown of DCLK1 with an siRNA restored the alterations in the above-mentioned EMT-associated proteins and CYCLIN D1 in QGP1-DCLK1 cells in vitro (Fig. 5A). This trend was also confirmed in CM cells, in which the expression of Tyr925-p-FAK and SLUG was constitutively high (Fig. 5B).

Tyr925-p-FAK induces EMT

To determine the hierarchal relationship between FAK and SLUG, FAK was silenced in QGP1-DCLK1 cells. In FAK-silenced QGP1-DCLK1 cells, SLUG was lost upon loss of FAK (Fig. 6A). This finding suggested that FAK was upstream of SLUG. Of note, in the next experiment using 1 μmol/L of LRRK2-IN-1, an inhibitor of DCLK1, it was suggested that loss of SLUG was through loss of Tyr925-p-FAK in QGP1-DCLK1 cells, whose expression level of total FAK was not altered (Fig. 6B). In addition, affecting DCLK1 activity by using another small-molecule XMD 8-92 resulted in decreased expressions of both Tyr925-p-FAK and SLUG in a
DCLK1 Deﬁnes PNET Behavior

The important findings of this study are as follows: (1) human PNET tissues and the PNET cell line CM robustly expressed DCLK1, (2) DCLK1-overexpressing PNET cells exhibited EMT characteristics, which were highlighted by SLUG upregulation, and (3) mechanistically, upregulated SLUG expression was under the control of Tyr\textsuperscript{925}-p-FAK.

Diffuse and robust expression of DCLK1 in human PNET tissues is a novel ﬁnding, when compared with DCLK1 expression patterns in a recent report on primary and metastatic pancreatic cancer tissues (20). In pancreatic cancer tissues, the CSC marker DCLK1 was often expressed in metastatic sites, including the liver and lung, whereas it was rarely expressed in primary tumor sites (20). Such ﬁndings are reasonable because the population of CSCs should be small; thus, DCLK1-positive cells would be rarely detectable in clinically resected primary cancer tissues, even though the tissues were derived from metastatic lesions. Thus, diffuse and robust expression of DCLK1 seems to be a unique characteristic of human PNET tissues; however, it may be a universal trait of NET because a similar expression pattern was also demonstrated in rectal NET tissues (23). Such diffuse, strong expression of DCLK1 in NETs may explain their clinical features, such as high metastatic potential despite their early developmental stage (7). The mechanism underlying robust DCLK1 expression remains to be elucidated. In the immunohistochemical analysis, DCLK1 staining scores did not predict patient prognosis. To validate this, additional studies including larger numbers of cases are needed. In contrast to the clear DCLK1 positivity in all tested PNET tissues, two of the three human PNET cell lines were negative for DCLK1. The reason for this was not clariﬁed in this study; however, full-length DCLK1-negative clones may have been incidentally selected in the establishment of the BON1 and QGP1 cell lines.

Comprehensive analysis of the DCLK1-overexpressing PNET cells showed that SLUG is a core factor involved in the DCLK1-induced EMT. This is consistent with previous ﬁndings demonstrating a similar type of EMT in colon and pancreatic cancer cells (20, 21). This similarity suggests that one of the crucial functions of DCLK1 is to induce EMT independent of cell type. Although the causative role of DCLK1 in SLUG-mediated EMT in PNET cells was not clariﬁed in this study, we showed that Tyr\textsuperscript{925}-p-FAK, but not Tyr\textsuperscript{397}-p-FAK, was involved in SLUG-mediated EMT in DCLK1-overexpressing PNET cells. Indeed, it is known that FAK phosphorylation at Tyr\textsuperscript{397} preferentially induces EMT via growth factor receptor–bound protein 2 and downstream activation of the RA$S$ and ERK cascades (36–39). In contrast, phosphorylation of FAK at Tyr\textsuperscript{925} promotes autophosphorylation of this site, relaying extended phosphorylation at other N-terminal phosphorylation sites, including Tyr\textsuperscript{397}, Tyr\textsuperscript{376}, and Tyr\textsuperscript{377} (37, 38), and contributing to the physiologic development of organs. In this context, we suggest that DCLK1 and Tyr\textsuperscript{925}-p-FAK-mediated EMT involving p-ERK1/2 and SLUG upregulation plays a critical role in determining tumor cell behavior in PNETs (40). Such activation of FAK in the DCLK1-overexpressing PNET cell line might also contribute to the increase in tumor size and cellular proliferation through upregulating the cell-cycle driver CYCLIN D1.

It is well known that SRC phosphorylates FAK at both Tyr\textsuperscript{397} and Tyr\textsuperscript{925} (37, 38), although it is not yet understood how these distinct phosphorylations are so tightly regulated. Of note, SRC is abundantly expressed in PNET and is involved in its cell adhesion and migration (41). Thus, we speculate that DCLK1 may interact with SRC to preferentially phosphorylate Tyr\textsuperscript{925} (Fig. 7). From a therapeutic point of view, abundant expression of FAK in PNET is

**Figure 5.**
DCLK1 knockdown downregulates Tyr\textsuperscript{925}-p-FAK and SLUG. Western blotting analysis showing that Tyr\textsuperscript{925}-p-FAK and SLUG protein expression is restored by 50 nmol/L DCLK1-speciﬁc siRNA (siDCLK1). A. In DCLK1-positive CM cells, DCLK1 silencing results in downregulation of SLUG and Tyr\textsuperscript{925}-p-FAK, and Tyr\textsuperscript{925}-p-FAK downregulation occurs earlier and is stronger (B).

**Figure 6.**
FAK activation is upstream of SLUG. Silencing of FAK by siRNA in QGP1-DCLK1 cells leads to a clear downregulation of SLUG (A), and this effect is mimicked by treatment with 1 μmol/L of LRRK2-IN-1 (LRRK2), a DCLK1 inhibitor (B), where loss of SLUG is through loss of Tyr\textsuperscript{925}-p-FAK in QGP1-DCLK1 cells. Note, total FAK expression is unaltered (B). C. Treatment with another small-molecule XMD8-92 results in decreased expressions of both Tyr\textsuperscript{925}-p-FAK and SLUG in a dosage-dependent manner, in not only QGP1-DCLK1 cells but also CM cells.
attractive (42). In a previous study, it was demonstrated that inactivation of FAK, coupled with inhibition of mTOR by everolimus, led to increased apoptosis of PNET cell lines by preventing feedback activation of AKT (42). Future investigations on how DCLK1 precisely regulates the site-specific phosphorylation of FAK by SRC may provide insights into more druggable targets in PNETs.

**References**

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