Hypoxia-Regulated Delta-like 1 Homologue Enhances Cancer Cell Stemness and Tumorigenicity

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Abstract

Reduced oxygenation, or hypoxia, inhibits differentiation and facilitates stem cell maintenance. Hypoxia commonly occurs in solid tumors and promotes malignant progression. Hypoxic tumors are aggressive and exhibit stem cell-like characteristics. It remains unclear, however, whether and how hypoxia regulates cancer stem cell differentiation and maintains cancer cell stemness. Here, we show that hypoxia increases the expression of the stem cell gene DLK1, or delta-like 1 homologue (Drosophila), in neuronal tumor cells. Inhibition of DLK1 enhances spontaneous differentiation, decreases clonogenicity, and reduces in vivo tumor growth. Overexpression of DLK1 inhibits differentiation and enhances tumorigenic potentials. We further show that the DLK1 cytoplasmic domain, especially Tyrosine339 and Serine355, is required for maintaining both clonogenicity and tumorigenicity. Because elevated DLK1 expression is found in many tumor types, our observations suggest that hypoxia and DLK1 may constitute an important stem cell pathway for the regulation of cancer stem cell-like functionality and tumorigenicity. [Cancer Res 2009;69(24):9271–80]

Introduction

How a tumor is initiated, sustains its growth, and progresses toward malignancy remains an ardently pursued topic. The cancer stem cell model posits that tumor growth is sustained by a small population of cancer cells that, like normal stem cells, are capable of self-renewal and differentiation (1). In contrast, the stochastic or clonal evolution model states that most of the tumor cells within a growing tumor mass are capable of self-renewal and that heterogeneity results from interclonal variations (2). For the most part, the majority of tumors, especially solid tumors, may be more appropriately explained by a combination of these two prevailing models (2). In either case, the tumor microenvironment may have profound impact on how cancer stem cells are maintained or how subclones with growth and survival advantages evolve and are selected. Currently, little is known about the role of the tumor microenvironment on the maintenance of stem cell characteristics, the cell-fate decision, and tumorigenic potential of poorly differentiated cancer cells.

Materials and Methods

Cell culture and hypoxia. SK-N-BE(2)C [BE(2)C], SK-N-ER (ER), and SH-SY5Y (SY5Y) cells were maintained in MEM and F12 (1:1), and IMR32 cells, in MEM, supplemented with 10% fetal bovine serum, 1 mmol/L sodium pyruvate, and 25 mmol/L HEPES at pH 7.4. Retinoic acid was used to mimic hypoxia effects at 21% O2 (11, 12). Culture media were replaced every other day inside the hypoxia chamber. To facilitate sphere formation, tissue culture dishes were coated with poly(hydroxyethylmethacrylate) (polyHEMA; Sigma-Aldrich) as described in Supplementary Materials and Methods. Cells were dissociated by repeated pipetting or with 0.05% trypsin to obtain single-cell suspension for passages or for cell counting.

Plasmids. The retroviral DLK-FL (full-length DLK1) and DLK-ΔCyto (without the cytoplasmic domain) constructs containing the GFP gene were provided by Dr. R. Bhatia (City of Hope National Medical Center, Duarte, CA; ref. 19). The mutations in the DLK1 cytoplasmic domain, Y339F, S355A, and Y339F/S355A, were created using the QuikChange site-directed mutagenesis kit (Stratagene). The constitutively active HIF-1α mutant (P402G/P564A) was described previously (20). The constitutively active HIF-2α mutant

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi:10.1158/0008-5472.CAN-09-1605
(Pro31A) was obtained from Dr. F. Lee (University of Pennsylvania School of Medicine, Philadelphia, PA; ref. 21) and was subcloned into the pLZRS retroviral vector.

The lentiviral short hairpin RNA (shRNA) constructs (shDLK-2H and shDLK-4H) were cloned into CS-CDF-EG-PRE-K1f (22), and the siRNA oligonucleotides (siDLK-05 and siFLK-07) were from Dharmacon. Detailed cloning information and nucleotide sequences are shown in Supplementary Materials and Methods.

**Real-time reverse transcription-PCR.** First-strand cDNA was synthesized from total RNA. Real-time PCR was performed on StepOne Plus (Applied Biosystems) using Power SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer’s recommended protocol. The primer sequences can be found in Supplementary Materials and Methods.

**Chromosome immunoprecipitation.** BE(2)C cells were incubated at 1% O2 for 16 to 18 h and were used for chromosome immunoprecipitation (ChiP) according to our previously published protocol (20). The detailed procedure and ChiP primer sequences can be found in Supplementary Materials and Methods.

**Northern blot.** Total RNA was fractionated in 1% agarose gel and hybridized at 65°C overnight in Church’s Buffer with an [α-32P]dCTP-labeled DLK1/pref-1 cDNA probe prepared from pCMV-Sport6.1-pref-1 (IMAGE 639667). The radioactive blots were visualized on a Storm 860 Phosphor-Imager (GE Healthcare).

**Western blot.** Western blot was done as described previously (11) with the following antibodies: polyclonal rabbit anti-DLK1 (1:3,000; Chemicon International); anti-Sox2 (1:1,000; Chemicon International); c-kit (1:500; Zymed Laboratories); CD-133 (1:100; Abgent); Notch1 (1:2,000), HIF-1α (1:2,000), and HIF-2α (1:1,000; Novus Biologicals); proliferating cell nuclear antigen (PCNA; 1:500; Santa Cruz Biotechnology); and phospho–extracellular signal-regulated kinase (ERK) and total ERK (1:1,000; Cell Signaling), or β-actin (1:20,000; Sigma-Aldrich).

**Cell proliferation assay (MTS).** BE(2)C cells were transduced with lentivirus for 48 h and then reseeded at a density of 2 × 10^4 cells per well in 96-well plates. Cell growth was then analyzed every day using the MTS assay (Promega).

**Immunofluorescence.** Cells were fixed for 10 min with −20°C methanol and then incubated with mouse anti-DLK1 (1:100; R&D Systems) or rabbit anti–β-tubulin III (1:100; Sigma-Aldrich), followed by incubation with Alexa 594-conjugated donkey-anti-mouse (1:500; Invitrogen) or Alexa 488-conjugated goat-anti-rabbit (1:500; Invitrogen). Hoechst 33342 (2 μg/mL) was used for nuclear staining.

**Clonogenic assays.** NB cells were plated at 300 to 500 cells per well in six-well plates for 10 to 14 d, and colonies were stained with crystal violet. Plating efficiency = number of colonies (≥50 cells per colony) per input cells × 100%.

**Tumor xenografts.** NB cells were harvested by trypsinization, resuspended in cold PBS, then mixed with an equal volume of Matrigel (BD Bioscience), and then injected (100 μL/site) s.c. on both sides of the anterior backs of a group of five Nu/J mice (6-wk-old males; Jackson Laboratory). Tumor growth was monitored twice weekly. Tumor take was recorded when a palpable tumor was detected. Tumor size was measured using a precision caliper as the longest surface length (mm; LW) and width (W). Tumor volume (1/2 mm3) = LW^2/2 (23). All protocols were reviewed and approved by the Yale Institutional Animal Care and Use Committee.

**Statistics.** The statistical difference between two groups was analyzed by the two-tailed, unpaired Student’s t test using Prism 3.0 (GraphPad Software, Inc.). All results are expressed as mean ± SEM. For statistical analysis of tumor growth, the general statistical model used is:

\[
\log(\text{volume}) = \alpha + \beta\log(t)
\]

where \(\alpha\) and \(\beta\) are constants, and \(t\) is the elapsed time. Large values of \(\alpha\) correspond to uniformly larger tumor sizes across all dates. The slope \(\beta\) measures the rate of growth on this log scale. Statistical analysis was performed using linear regression in time.

**Results**

**Hypoxia increases DLK1 expression.** DLK1 was strongly increased at both mRNA and protein levels (Supplementary Fig. S1A and Fig. 1A) either at 1% O2 (hypoxia) or in the presence of deferoxamine, a hypoxia-mimicking compound in a panel of NB cell lines with MYCN amplification [BE(2)C and IMR32] or without MYCN amplification (ER and SY5Y). DLK1 expression can also be induced under anoxia (Supplementary Fig. S1B). We chose 1% O2 because it is close to the PO2 threshold (5–10 mmHg or 0.7–1.3% O2) widely used in clinical studies to define tumor hypoxia in vivo (3). We further found that hypoxia also increased DLK1 expression in glioblastoma cells as well as normal neuronal progenitor cells (Supplementary Fig. S1B and C), suggesting a common regulatory mechanism for DLK1 expression by hypoxia in neuronal progenitor cells and neuronal tumors. DLK1 is predominantly expressed as a full-length protein with a relative motility of around 50 kDa (Fig. 1A, left). An ∼45 kDa form of DLK1 was found in ER cells (Fig. 1A, lanes 5–6), likely due to the enzymatic cleavage of its extracellular domains (24).

Hypoxia induced the expression of HIF-1α protein in all the NB cell lines (Fig. 1A, left). Other hypoxia-inducible genes, such as vascular endothelial growth factor (VEGF) and glucose transporter 1 (GLUT1), were also induced by hypoxia (Supplementary Fig. S2A and B). However, only BE(2)C and SY5Y cells expressed HIF-2α (Supplementary Fig. S2C and D), and the HIF-2α expression was independent of the MYCN status. It is likely that a combination of HIF-α expression and the MYCN status could potentially explain different levels of DLK1 expression in NB cells.

Importantly, HIF-1α or HIF-2α alone is sufficient to enhance DLK1 expression (Fig. 1B, left), whereas deletion of both HIF-1α and HIF-2α is needed to abolish hypoxia-dependent increase of DLK1 expression (Fig. 1B, right). The proximal 5′ promoter/enhancer region of the DLK1 gene contains three putative hypoxia responsive elements (HREs) with the conserved motif of 5′-ACGTG-3′ (25) at −758, −402, and −248 bp, respectively. To ascertain HIF binding by ChiP assays, we designed two sets of primers flanking the HRE at −758 bp (DLK1-3D) and −248 bp (DLK1-4P), respectively. Both HIF-1α and HIF-2α were coimmunoprecipitated with this proximal DLK1 promoter/enhancer region in hypoxia-treated cells as determined by ampicloons with the predicted sizes (Fig. 1C, left) and by qRT-PCR (Fig. 1C, right). No promoter binding was found using the control IgG. Neither anti–HIF-1α nor anti–HIF-2α antibody precipitated the RAD51 promoter, a HIF-independent gene (26), further demonstrating the specificity of HIFs toward the DLK1 promoter. Consistent with the literature (7, 27), HIF-1α appeared to bind the VEGFA promoter more efficiently than HIF-2α did. These data clearly show that hypoxia increases DLK1 transcription via the HIF-dependent mechanism.

**DLK1 facilitates the maintenance of an undifferentiated NB phenotype.** We focused on BE(2)C cells because they have a high level of DLK1 expression (Fig. 1A) and possess cancer stem cell characteristics (28, 29). We found that DLK1 mRNA (Fig. 1D, lane 3) and protein (Fig. 1D, lane 7) were strongly decreased upon RA-induced differentiation. Interestingly, DLK1 expression was maintained by hypoxia (1% O2) even in the presence of RA (Fig. 1D, lanes 4 and 8). This result was consistent with the observation that hypoxia decreased RA-induced neurite growth, a hallmark of neuronal differentiation (Supplementary Fig. S3). In addition to RA, BrdUrd, an inducer of non-neuronal differentiation (28, 29), also dramatically decreased DLK1 expression as

**Cancer Res 2009; 69: (24). December 15, 2009 9272 www.aacrjournals.org**

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Figure 1. Hypoxia induces DLK1 expression. A, quantitative reverse transcription-PCR (left) for DLK1 mRNA. Total RNA was prepared from NB cells after 16 h of incubation at 1%, 21% O2, or in the presence of 100 μmol/L deferoxamine. Data were normalized to HPRT mRNA that did not change under hypoxia (columns, mean from ≥3 experiments; bars, SEM; *, P < 0.05). Western blot for DLK1 (right) with HIF-1α as a control for hypoxia and β-actin or PCNA as a loading control. N.D., not determined. B, Western blot for DLK1. BE(2)C cells were transduced with retrovirus containing either the constitutively active HIF-1α, constitutively active HIF-2α, or empty vector (ctrl, left) or with lentivirus expressing shHIF1α, shHIF2α, or simultaneously infected with both shHIF1α and shHIF2α virus (right). Cells were cultured for 16 h at 1% (hypoxia, H) or 21% O2 (N). C, ChIP using @HIF-1α or @HIF-2α antibodies. BE(2)C cells were incubated at 1% O2 for 16 h to induce the HIF-α subunits. Two different sets of primers (DLK1-3D and DLK1-4P) were used to detect the DLK1 promoter gene with RAD51 primers as negative control and VEGFA primers as positive control. Amplicons with the predicted sizes were validated using the end-point PCR for 28 cycles (left). Relative levels of promoter binding were analyzed by qRT-PCR (right). D, Northern (left) and Western blot (right) for DLK1 in BE(2)C cells cultured at 1% (+) or 21% (−) O2 with or without 1 μmol/L RA for 24 h.
shown by indirect immunofluorescence (Supplementary Fig. S4A). We further found (Fig. 2A) that DLK1 expression was highly correlated with the expression of other neuronal stem cell markers including c-kit, CD-133, and SOX2 (29, 30). These observations show that DLK1 is a bona fide stem/progenitor cell marker for immature NB cells.

To examine the role of DLK1 in NB cell differentiation, we designed two lentivirus-based shRNA interference constructs, shDLK-2H and shDLK-4H, which target two separate regions of DLK1 mRNA. Both shDLK constructs inhibited expression of endogenous DLK1 in BE(2)C cells (Fig. 2B), although shDLK-4H appeared to be more effective (up to 2-fold more efficient based

**Figure 2.** DLK1 maintains an undifferentiated NB phenotype. A, BE(2)C cells were differentiated with 1 μmol/L RA or 10 μmol/L BrdUrd for 5 d. DLK1, SOX2, c-kit, and CD-133 were detected by Western blot. B, BE(2)C cells were infected with lentivirus expressing shDLK-2H, shDLK-4H, or empty lentiviral vector (chr). Left: Infected cells were stained using antibodies against DLK1 (red) or neuron-specific β-tubulin III (green) with Hoechst 33342 as nuclear stain (blue). Magnification, ×200. Right, expression of DLK1 was analyzed by Western blot, and cells with β-tubulin III–positive neurites were enumerated from five to six random fields (columns, mean; bars, SEM; *, P < 0.0001 versus control). C, left, fluorescence-activated cell sorting (FACS)–selected BE(2)C cells expressing the full-length DLK1 (DLK-FL) or empty retroviral vector (chr) were cultured for 3 d with or without 1 nmol/L RA and then stained as in B. Magnification, ×200. Right, neurite-positive cells were counted from five to six random fields (columns, mean; bars, SEM; *, P = 0.0001 versus RA-treated control). D, Western blot for CD133 and Notch1 in ER cells expressing the full-length DLK1 with the empty retroviral vector as control (chr).
on qRT-PCR analysis) than shDLK-2H in decreasing DLK1 mRNA. Interestingly, BE(2)C cells with DLK1 knockdown spontaneously developed neuronal processes under normal culture conditions, as revealed by immunofluorescence of the neuron-specific β-tubulin III (Fig. 2B). Spontaneous differentiation was observed only in cells expressing shDLK-2H or shDLK-4H but not in control-infected cells (Supplementary Fig. S4B). We further confirmed this observation using two separate siRNA oligonucleotides (Supplementary Fig. S4C). We also found that expression of the sympathetic neuronal peptide neurotransmitter gene neuropeptide tyrosine (NPY; ref. 5) was increased in BE(2)C cells either treated with RA or transduced with shDLK-4H (Supplementary Fig. S5A and B). However, expression of HASH-1 (ASCL1), a gene involved in early sympathetic lineage commitment (5), was decreased during RA-induced differentiation (Supplementary Fig. S5A), but not upon transduction of the lentiviral shDLK-4H (Supplementary Fig. S5B), suggesting nonoverlapping pathways may be involved between RA- and shDLK-induced differentiation. These results clearly show that loss of DLK1 predisposes NB cells to spontaneous differentiation.

On the other hand, overexpression of DLK-FL significantly suppressed RA-induced neurite formation ($P < 0.0001$ versus RA-treated control; Fig. 2C) and resulted in increased levels of the stem cell markers CD133 and Notch1 (Fig. 2D), suggesting enhanced stem cell characteristics. Collectively, these data suggest a critical role of DLK1 in the maintenance of undifferentiated NB cell phenotype.

In addition, we found that DLK1 may have an impact on other cytoplasmic pathways actively involved in neural stem cells including epidermal growth factor receptor (EGFR), fibroblast growth factor receptor (FGFR), and Notch (31–36). Levels of EGFR, FGFR1, and Notch were strongly reduced (Supplementary Fig. S6A, C, and E) in cells treated with RA or BrdUrd, as well as DLK1 knockdown (Supplementary Fig. S6B, D, and F). On the other hand, ERK phosphorylation was strongly increased in BE(2)C cells treated by RA or BrdUrd (Supplementary Fig. S6G), as well as by shRNA (Supplementary Fig. S6H), which is consistent with increased ERK phosphorylation during differentiation of neuronal progenitor cells (37). Together, these observations show that multiple signaling pathways are potentially involved during shDLK-induced spontaneous differentiation of NB cells. However, it remains to be determined...
whether DLK1 directly regulates these different pathways. Furthermore, it is worth noting that elevated NPY expression (Supplementary Fig. S5B) and ERK phosphorylation (Supplementary Fig. S5C) still occurred in shDLK-transduced cells under hypoxia, suggesting that hypoxia is not able to completely block the shDLK-induced spontaneous differentiation.

**DLK1 regulates tumor sphere formation.** The ability to grow as nonadherent spheroids in the sphere medium has been widely used to assess cancer stem cell characteristics (30, 38). All four NB cell lines used in this study were able to form tumor spheres (Supplementary Fig. S7A), albeit to different degrees. DLK1 expression was strongly increased in tumor spheres compared with adherent cells maintained in the serum-containing medium (Fig. 3A, left). When plated in tissue culture dishes, BE(2)C cells formed tumor spheres gradually in the sphere medium (Supplementary Fig. S7B). Interestingly, DLK1 expression increased as the tumor spheres grew (Fig. 3A, middle), further suggesting a relationship between enhanced DLK1 expression and tumor sphere formation.

To ascertain that increased DLK1 expression results from sphere formation and not just the effects of the sphere medium, we compared DLK1 expression under three different conditions: (a) a monolayer in tissue culture dishes with the regular growth medium (GM/TC), (b) as a mixture of adherent and nonadherent cells in tissue culture dishes with the sphere medium (SphM/TC), or (c) as completely nonadherent culture in polyHEMA-coated dishes with the sphere medium (SphM/pHEMA). As shown in Fig. 3A (right), the sphere medium generally enhanced DLK1 expression over the GM/TC control. More robust increases of DLK1 expression occurred under the SphM/pHEMA condition that forced formation of tumor spheres. We further determined whether hypoxia developed in the growing tumor spheres using the hypoxia marker pimonidazole hydrochloride (hypoxyprobe-1). Only \( \leq 16\% \) cells became hypoxic in the 6-day-old tumor spheres (Supplementary Fig. S7C), suggesting that the increased DLK1 expression in tumor spheres is unlikely to be driven by hypoxia. Because the “sphere culture” condition facilitates the expansion or enrichment of stem cells and/or progenitors, NB cells growing as spheroids likely possess enhanced stem...
cell characteristics. Therefore, increased DLK1 expression under the “sphere culture” condition may be associated with enhanced stem cell characteristics.

BE(2)C cells were only able to form loose cell aggregates (Supplementary Fig. S7A), which made it difficult to accurately measure the sizes or the numbers of spheres/aggregates. Therefore, we examined the role of DLK1 in regulating tumor sphere growth by enumerating total cell numbers in tumor spheres. We found that knocking down DLK1 expression with the shRNA constructs (Fig. 3B, left) or the siRNA oligonucleotides (Fig. 3B, right) resulted in a significant decrease in tumor sphere growth. The shDLK constructs also decreased cell growth when cells were maintained in the regular medium (Fig. 3C), consistent with its role in facilitating spontaneous differentiation. Conversely, expression of DLK-FL in SY5Y cells (DLK1-low) enhanced tumor sphere growth (Fig. 3D). However, overexpression of DLK-FL did not change the growth rate of SY5Y cells under the conventional culture condition (Supplementary Fig. S7D). Nonetheless, these results show that DLK1 plays an important role in regulating the ability of NB cells to grow as anchorage-independent tumor spheres, an important aspect of cancer stem cell characteristics.

**DLK1 promotes clonogenicity in vitro.** Clonogenicity measures the ability to form a colony from a single cell or the self-renewal potential. As shown in Fig. 4A, overexpression of DLK-FL in ER cells (DLK-low) significantly enhanced clonogenicity (% plating efficiency) under both normoxia and hypoxia, whereas shRNA-mediated knockdown of endogenous DLK1 in BE(2)C cells (DLK1-high) resulted in a dramatic decrease of clonogenicity (Fig. 4B). Similarly, transient transfection with the siRNA oligonucleotides also significantly decreased clonogenic growth of BE(2)C cells (Supplementary Fig. S8), albeit less potently than the lentiviral shRNAs. This difference is likely due to the short-term effects of oligonucleotides compared with the constitutively expressed shRNAs.

The DLK1 intracellular domain (∼50 amino acids) contains two putative phosphorylation sites Y339 and S355 that are highly conserved among mammals (Fig. 5A), suggesting that the DLK1 cytoplasmic domain may be required for its biological functions. To test this hypothesis, we created the following retroviral constructs:

![Figure 5. DLK1 cytoplasmic domain is required for clonogenic growth and tumor sphere formation. A, comparison of the DLK1 cytoplasmic domains among different mammalian species. Arrows, the putative phosphorylation sites [tyrosine (Y)-339 and serine (S)-355]. B, BE(2)C cells were FACS-sorted for DLK1 lacking cytoplasmic domain (DLK-ΔCyto; left), DLK1 with point mutation [Y339F (phenylalanine), S355A (alanine), or Y339F/S355A; right] or empty vector (ctrl). Clonogenic assays were performed as described in Fig. 4. *, P < 0.0002 versus control. C, FACS-sorted BE(2)C cells were cultured in the sphere medium for 4 d (Fig. 3B). *, P < 0.002 versus control.](cancerres.aacrjournals.org)
(a) DLK1 cytoplasmic domain deletion (DLK-ΔCyto), (b) full-length DLK1 with a single mutation, Y339-to-phenylalanine(F) or S355-to-alanine(A), and (c) full-length DLK1 with both mutations (Y339F/S355A). BE(2)C cells with stable expression of DLK-ΔCyto, Y339F, S355A, or Y339F/S355A exhibited significantly reduced clonogenicity under both normoxia and hypoxia (Fig. 5B), as well as decreased growth of tumor spheres (Fig. 5C). These results suggest that the DLK1 cytoplasmic domain may play an essential role in facilitating clonogenic growth and tumor sphere formation. However, DLK-ΔCyto and Y339F/S355A did not appear to have significant impact on either spontaneous differentiation or cell growth under conventional tissue culture conditions. It is likely that the two DLK1 mutants exert their dominant-negative effects only under certain stress conditions, such as in the tumor sphere media or under the clonogenic conditions.

**DLK1 promotes tumorigenicity in vivo.** Using the subcutaneous xenograft assay in athymic mice, we found that BE(2)C cells with shRNA-mediated downregulation of DLK1 expression exhibited longer tumor delay (Fig. 6A, left). Both shDLK-2H and shDLK-4H tumors were significantly smaller in volume during growth (P < 0.002 for each group versus control; Fig. 6A, middle) and by weight at the end of the experiment (P < 0.05 for each group versus control; Fig. 6A, right). Interestingly, overexpression of both DLK-ΔCyto and Y339F/S355A mutants in BE(2)C cells decreased overall tumor take rate and retarded tumor growth (Fig. 6B). Statistically, the Y339F/S355A tumors were smaller (P = 0.044) and grew more slowly (P = 0.022) than the vector control within the first 21 days of growth. Tumor growth rate appeared to be similar during the late stage of tumor growth. The mean growth rate of the DLK-ΔCyto tumors was nearly identical to that of the Y339F/S355A tumors. However, the differences between the DLK-ΔCyto and the control group were not statistically significant, likely due to the variability among individual tumor sizes. Nonetheless, these data suggest that the DLK-ΔCyto and Y339F/S355A mutants may possess dominant-negative functions.

On the other hand, overexpression of DLK-FL in ER cells (DLK1-low) accelerated tumor take or shortened tumor latency (Fig. 6C). Tumors developed from ER cells with DLK-FL were larger (P < 0.01) and grew faster (P < 0.04) than the control cells (Fig. 6C). However, the difference in the growth rate between the DLK-FL and the control group appears to narrow after day 16. Nevertheless, results from our additional xenograft experiments also showed that DLK-FL enhanced tumorigenicity, whereas DLK-ΔCyto and Y339F/S355A decreased tumorigenicity (Supplementary Fig. S9). These results indicate that DLK1 plays an important role in the regulation of tumorigenicity in vivo.

**Discussion**

Hypoxia is an important environmental factor that seems to favor undifferentiated stem or progenitor cells, including human embryonic stem cells, neuronal, and other mesenchymal progenitor cells (9, 10, 12, 20). Jögi and colleagues (5) have shown that hypoxic NS cells acquire an immature phenotype and that hypoxia-pretreated...
NB cells grow slightly faster in vivo. Recent genomics studies have further revealed that poorly differentiated human tumors display a gene expression signature similar to that found in normal embryonic stem cells (39) or lineage-committed progenitor cells (40). However, it remains largely unknown how stem cell genes regulate tumor progression, and how their expression is regulated by tumor microenvironment.

DLK1, a member of the notch/delta/serrate family, is preferentially expressed in immature cells with regenerative potentials (13, 15). DLK1 inhibits adipogenesis (24) and also seems to regulate the differentiation of hematopoietic stem cells (17, 19) and lymphoid progenitors (41, 42). Elevated expression of DLK1 is found in a variety of tumor cells, including NB (18), gliomas (16), small-cell lung carcinoma (43), and leukemia (17, 19). Other evidence suggests that DLK1 may inhibit tumor cell differentiation and increase proliferation (16, 19). Nonetheless, the role of DLK1 in tumor progression remains poorly understood.

In this report, we have shown that hypoxia increases DLK1 expression via the HIF-dependent mechanism. DLK1 knockdown results in enhanced spontaneous differentiation of NB cells, reduced tumor sphere growth, decreased clonogenicity in vitro, and decreased tumorigenicity in vivo. On the other hand, overexpression of DLK1 inhibits differentiation and promotes tumorigenicity. Our data have further revealed an important role of the DLK1 cytoplasmic domain, especially the conserved putative phosphorylation sites Y339 and S355. Consistent with our findings, Li and colleagues (19) have found that the DLK1 cytoplasmic domain is required for blocking RA-induced differentiation of human promyelocytic HL-60 cells. Our observations have provided strong evidence establishing DLK1 as a new class of stem cell genes that play a significant role in the regulation of cancer stem cell characteristics and tumorigenicity.

Our data suggest that DLK1 can exert potential impact on several membrane-associated signaling pathways including EGFR, FGFR, and Notch that facilitate maintenance or self-renewal of neural stem/progenitor cells (31–36), as well as ERK phosphorylation (37). Obviously, the mechanisms of DLK1 function likely involve multiple pathways. We cannot rule out the possibility that changes in these signaling pathways result from shDLK-induced spontaneous differentiation, rather than directly from the loss of interaction with DLK1. The exact mechanisms of DLK1 function warrant further investigation.

Recent studies have shown that a small population of immature NB tumor cells is localized in a perivascular space in vivo and shows strong immunohistochemical staining for HIF-2α (7, 44). These HIF-2α+ cells also seem to be positive for MYCN amplification (44). However, we have found that HIF-2α is differentially expressed in NB cell lines, although HIF-1α is ubiquitously expressed and does not correlate with MYCN amplification. It is possible that HIF-1α and HIF-2α are regulated by different mechanisms, especially under in vivo conditions. Consistent with this notion, our previous study (11) has found that HIF-2α is expressed only in the differentiated adipocytes, but not in the progenitor cells, and is stabilized at 21% O2. It seems that elevated HIF-2α expression is preferentially associated with a stem cell–like population from NB tumors (7, 44) or gliomas (8). Since our data have shown that both HIF-1α and HIF-2α are capable of enhancing DLK1 transcription, it will be of great interest to determine whether elevated HIF-2α expression, especially in such perivascular niches, has an effect on DLK1 expression in vivo. Our observations, together with others, support a new paradigm that hypoxia signaling promotes tumor progression by upregulation of stem cell genes including DLK1 that facilitate the maintenance or selection of cancer cells with stem cell characteristics.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 4/30/09; revised 9/10/09; accepted 10/6/09; published OnlineFirst 11/24/09.

Grant support: NIH grant R01CA125021 (Y. Yun); Y. Kim is supported in part by an institutional postdoctoral training grant (5-T32-CA009295) from the NIH and the Anna Fuller Fund Fellowship from Yale University School of Medicine. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. Nai-Kong V. Cheung of Memorial Sloan-Kettering Cancer Center for SK-N-ER cells, Dr. Robert Ross of Fordham University for BE(2)C cells, Dr. A.N. Van den Pol of Yale University for the human astrocytic progenitors from adult brain and embryonic tissues, Dr. Ravi Bhatia of City of Hope National Medical Center for full-length DLK1 and DLK1-ΔCyto, Dr. Frank Lee of the University of Pennsylvania for HIF-2α/P331A, Dr. Kazufumi Katayama of the Tokyo Metropolitan Institute of Medical Science for the lentiviral shRNA vectors, members of the Yun Laboratory, especially Dr. Yongming Ren, for constructive suggestions and technical assistance, and Lisa Cabral for her excellent editorial assistance.

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