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# The effects of nutrient depleted microenvironments and delta-like 1 homologue (DLK1) on apoptosis in neuroblastoma

Yuri Kim[§]

*Department of Nutritional Science and Food Management, Ewha Womans University, 11-1 Daehyun-dong, Seodaemun-gu, Seoul 120-750, Korea*

## Abstract

The tumor microenvironment, particularly sufficient nutrition and oxygen supply, is important for tumor cell survival. Nutrition deprivation causes cancer cell death. Since apoptosis is a major mechanism of neuronal loss, we explored neuronal apoptosis in various microenvironment conditions employing neuroblastoma (NB) cells. To investigate the effects of tumor malignancy and differentiation on apoptosis, the cells were exposed to poor microenvironments characterized as serum-free, low-glucose, and hypoxia. Incubation of the cells in serum-free and low-glucose environments significantly increased apoptosis in less malignant and more differentiated N-type IMR32 cells, whereas more malignant and less differentiated I-type BE(2)C cells were not affected by those treatments. In contrast, hypoxia (1% $O_2$) did not affect apoptosis despite cell malignancy. It is suggested that DLK1 constitutes an important stem cell pathway for regulating self-renewal, clonogenicity, and tumorigenicity. This raises questions about the role of DLK1 in the cellular resistance of cancer cells under poor microenvironments, which cancer cells normally encounter. In the present study, DLK1 overexpression resulted in marked protection from apoptosis induced by nutrient deprivation. This *in vitro* model demonstrated that increasing severity of nutrition deprivation and knock-down of DLK1 caused greater apoptotic death, which could be a useful strategy for targeted therapies in fighting NB as well as for evaluating how nutrient deprived cells respond to therapeutic manipulation.

**Key Words: Neuroblastoma, nutrient deprivation, apoptosis, DLK1**

## Introduction

Sufficient nutrients and oxygen are important to maintain tumor growth. Glucose is a major energy source in neural tissues, and the brain is dependent on glucose for its metabolic function. Since high glucose is required for tumor cells, brain tumors may be responsive to dietary and nutritional therapy. Nutrient depletion has been shown to cause cancer cell death [1,2]. Inadequate nutrient supply affects neuronal cell survival and causes damage to neuronal cells leading to apoptosis [3]. Apoptosis, programmed cell death, is a self-defense mechanism to remove potentially dangerous and damaged cells, or tumor cells. Apoptosis has morphologically distinct features such as nucleus and cytoplasm condensation as well as DNA fragmentation [4]. Apoptosis can be induced by various cytotoxic stimuli such as tumor necrosis factor α (TNFα), and nutrient deprivation including serum or glucose withdrawal [4-6]. It has been widely reported that nutrient deprivation causes tumor cell death through apoptotic pathways.

Neuroblastoma (NB) is the most common extracranial solid tumor in children [7]. NB originates from multipotent neural crest stem cells and is comprised of multiple cell phenotypes. NB tumors have high levels of cellular heterogeneity with cells at different stages of differentiation including neuroblasts, non-neuronal cells, and melanocytes [8]. There are three distinct cell types identified in NB cell lines and in primary NB tumors: the neuroblastic/neuroendocrine precursors (N-type), Schwannian/melanoblastic precursors, and non-neuronal cells (S-type), and cells with intermediate stem cells (I-type) [8-10]. Although the three cell types are multipotent and have the potential to differentiate further to neural crest cells, in particular, I-type stem cells can stably differentiate into either N- or S-type cells [8,11], indicating that I-type stem cells are precursors to both N- and S-type cells. Most importantly, the frequency of I-type cells is strongly increased in advanced NB. I-type cells exhibit the highest malignancy potential of all three types, whereas S-type cells are the least tumorigenic and N-type cells have an intermediate level of tumorigenicity [7]. These observations suggest that I-type cells represent the most immature and highly malignant NB cells. Although microenvironments in tumor have been widely documented, little is known about how different NB cell types respond to these conditions.

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[§] **Corresponding Author:** Yuri Kim, Tel. 82-2-3277-4485, Fax. 82-2-3277-2862, Email. yuri.kim@ewha.ac.kr
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DLK1 (Drosophila delta-like 1 homologue) is a type I transmembrane protein and secreted protein belonging to the epidermal growth factor (EGF)-like homeotic gene family. Preadipocyte factor 1 (pref-1), fetal antigen (FA1), pG2, and ZOG are other names for DLK1 [12]. DLK1 is expressed in animals from birds to mammals extensively in immature cells, and is down-regulated during fetal development, which implicates that DLK1 plays an important role in fetal development and growth. However, DLK1 is only detected in selected adult tissues and several tumor cells including NB [13,14], gliomas [15], small-cell lung carcinoma [14], and leukemia [16,17]. Previous studies have demonstrated that exogenous DLK1 increased NB cell clonogenic growth, which is a self-renewal characteristics of cancer stem cells [18], and regulated growth of pre-B cells and thymocytes [19,20]. Those results suggest that DLK1 performs an important role in stem/progenitor and cancer cell growth. However, the functional role of DLK1 in cellular resistant to poor microenvironments is still not well elucidated. The aim of this study was to evaluate the potential role of DLK1 in cancer cell survival and the malignancy of NB cells in nutrient deprivation-induced apoptosis.

## Materials and Methods

### *Cell culture*

The following two NB cell lines were used: SK-N-BE(2)C [BE(2)C] cells that were maintained in Minimum Essential Medium and F12 (1:1), and IMR32 cells that were kept in Minimum Essential Medium supplemented with 10% fetal bovine serum (v/v). The cells were cultured with 5% $CO_2$ and 95% atmospheric air at 37℃. The media were supplemented with 10% fetal bovine serum (v/v) (FBS) unless indicated otherwise. For all experiments, both BE(2)C and IMR32 cells were cultured to 60-80% confluence. Fresh medium was replaced every other day.

### *Induction of nutrient deprivation and hypoxia*

In order to induce serum depletion, the media were replaced with serum-free media. For glucose deprivation, after washing two times with PBS, the media were replaced with media containing 10% (v/v) FBS and 25 μM glucose, unless indicated otherwise. The cells were maintained in serum- or glucose-deprived media for 48 hr. For hypoxia (1% $O_2$) treatment, the cells were transferred to a hypoxia chamber (Invivo$_2$ 400, Ruskinn Technology) and then maintained for 48 hr.

### *Gene expression constructs*

DLK1-FL (full-length protein) expression and control vector constructs were kindly provided by Dr. R. Bhatia (City of Hope National Medical Center, Duarte, CA [21]). The DLK1-FL construct was cloned into a MIG-R1 retroviral vector containing the *GFP* gene under the control of an internal ribosome entry site (IRES). For viral infection to express DLK1-FL, IMR32 cells were infected at 50-60% confluence, which was performed as described previously [22]. The sequence was confirmed by DNA sequencing. The empty retroviral vector was used as a negative control. DLK1 expression was verified by Western blotting.

### *RNA interference*

Two siRNA oligonucleotides against human DLK1 were purchased from Dharmacon (Lafayette, CO). The sequences for the two siRNAs were as follows: DLK1 siRNA (05): 5'-GCACC UGCGUGGAUGAUGAUU-3', and DLK1 siRNA (07): 5'-GGA CGAUGGCCUCUAUGAAUU-3'. The sequence of the control siRNA was UAACAAUGAGAGCACGGCUUU. Transfection with the siRNAs was performed using DharmaFECT (Dharmacon, Lafayette, CO) according to the manufacturer's instructions.

### *Cell death and apoptosis analysis*

Apoptosis was analyzed by double staining with propidium iodide [PI; 3,8-diamino-5-(3-(diethyl-mehylamino)propyl)-6-phenyl phenanthridinium diiodide; Sigma, St. Louis, MO] and Hoechst 33342 (Hoechst; Sigma, St. Louis, MO) staining. After treatment of hypoxia and/or nutrient deprivation for 48 hours, the cells were washed with ice-cold PBS and fixed in paraformaldehyde solution. PI is widely used to evaluate neuronal membrane integrity and cell damage. 20 μl of 0.1 mM PI was added to the culture medium for 5 min at room temperature, leading to a final concentration of 2 μM PI. For Hoechst staining, the cells were stained with 2 μg/ml of Hoechst dye for 5 min at room temperature. PI uptake (red fluorescence) and Hoechst uptake (blue fluorescence) were observed, and photographed with a fluorescence microscope for image detection. Apoptotic cells were characterized by nuclear condensation/fragmentation revealed by nuclear staining with PI and Hoechst. Apoptosis was quantified by averaging cell counts in three to four randomly selected fields per dish. To evaluate the apoptotic cells, the ratio of both stained cells to total nuclei (%) was calculated.

### *Western blotting*

The cell cultures were washed with cold PBS and lysates were prepared on ice in RIPA lysis buffer containing 50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1% Nonidet p-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and 1-2 mM PMSF. After protein concentrations were measured using BCA assay reagents (BioRad), 100 μg of whole cell lysate was used for 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the separated proteins were transferred to a PVDF membrane. The membrane was blocked in 5% fat-free milk in Tris-buffered saline (TBS) for 1 hr at room temperature

and was then incubated overnight at 4℃ with polyclonal antibody against DLK1 (1:3,000, Chemicon International, Temecula, CA) and anti β-actin (Sigma, St. Louis, MO). After washing three times with TBS, the membrane was further incubated with horse-radish peroxidase-conjugated anti-IgG antibody as a secondary antibody for 1 hr at room temperature to detect protein bands to which the primary antibodies were bound, using enhanced chemiluminescence (Pierce, Rockfold, IL).

*Statistical analysis*

The significant differences between different groups were analyzed using a one-way ANOVA analysis with Tukey's post-hoc test as a multiple comparison post test. When only two groups were statistically compared, unpaired Student's t-test was performed using Prizm 3.0 (GraphPad Software Inc. San Diego, CA, USA). All results are expressed as means ± SE. A significant difference between two groups was declared if $P < 0.05$.

## Results

*Advanced malignant cell type shows less apoptosis induced by serum deprivation*

Apoptosis assessment was performed based on nuclear morphology changes. BE(2)C and IMR32 cells were maintained in serum-starved media for 48 hr and then underwent double staining with PI and Hoechst. Apoptotic cells are positive for PI staining and display abnormal nuclear morphologies as revealed by Hoechst staining. The cells exhibited nuclear blebbing, indicating chromatin condensation characteristic of apoptosis (Fig. 1).

There are three phenotypes of the NB cell line, including N-type, S-type, and I-type. I type cells exhibit the highest tumorigenicity among the three types and are increased in advanced NB. To examine the association between cell malignancy and apoptosis induced by serum-deprivation, the more advanced I-type BE(2)C cells and the less malignant N-type IMR32 cells were treated with different concentrations of serum, and apoptosis was evaluated by double staining with PI and Hoechst. The percentage of apoptotic cells was not significantly different at any serum concentration dosage in more advanced and less differentiated I-type BE(2)C cells. However, the percentage of apoptotic cells was increased as serum concentration decreased in less malignant and more differentiated IMR32 cells (Fig. 1 and 2). These observations suggest that I-type cells, representing the most immature and highly malignant cells, were more resistant to apoptosis induced by serum deprivation compared to more mature and less malignant N-type cells. It is also implicated that NB malignancy may potentially be an important factor for apoptosis and resist chemotherapy that induces apoptosis.

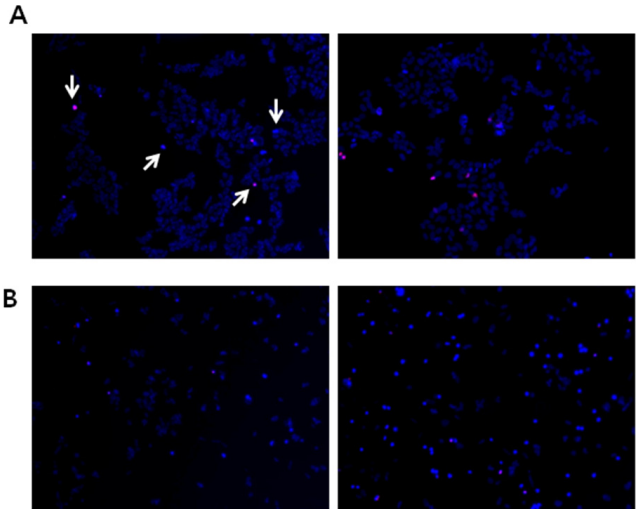


**Fig. 1.** Propidium iodide (PI) and Hoechst 33342 (Hoechst) staining demonstrate the appearance of nuclei with condensed chromatin in NB cells. BE(2)C cells (A) and IMR32 cells (B) were maintained with (left panels) or without (right panels) serum in media for 48 hr and then stained with PI (red) and Hoechst (blue) to visualize nuclear morphological changes (arrow). Both PI-and Hoechst-positive cells were counted as apoptotic cells. Images were detected by a fluorescence microscope. Magnification = x100.

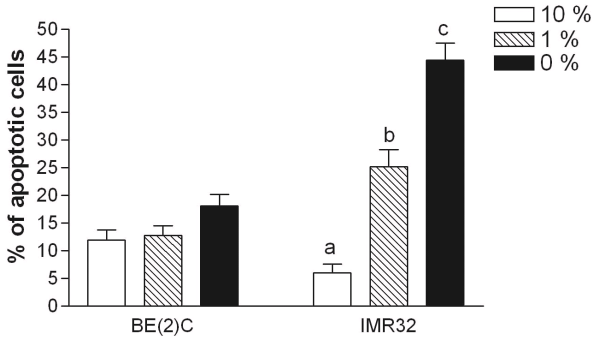


**Fig. 2.** More malignant I-type cells showed less apoptosis induced by serum deprivation. More advanced and less differentiated I-type BE(2)C cells and less advanced and more differentiated IMR32 cells were incubated at different concentrations of serum (10%, 1%, or 0%). After 48 hours, the cells were fixed and double stained with PI and Hoechst to evaluate apoptosis. Apoptotic cells with compromised membranes were positive for PI staining. Both PI- and Hoechst-positive cells were counted as apoptotic cells. A total of ≥ 300 cells were counted in 3-4 random fields. Bars are means ± SE. Different letters for given bars indicate the values are significantly different from each other ($P < 0.05$) (Tukey's Post Hoc test after one-way ANOVA).

*The effects of the tumor microenvironment (nutrient deprivation and hypoxia) on apoptosis*

In order to determine the effect of the microenvironment on apoptosis in NB, different malignancy state BE(2)C and IMR32 cells were incubated at various combinations of nutrient deprivation, including serum- and/or glucose-deprivation, as well as hypoxia. When BE(2)C cells were exposed to a combination of serum-free and low-glucose, apoptosis was increased significantly. However, the treatment of serum-free or low glucose alone was not sufficient to enhance apoptosis. On the

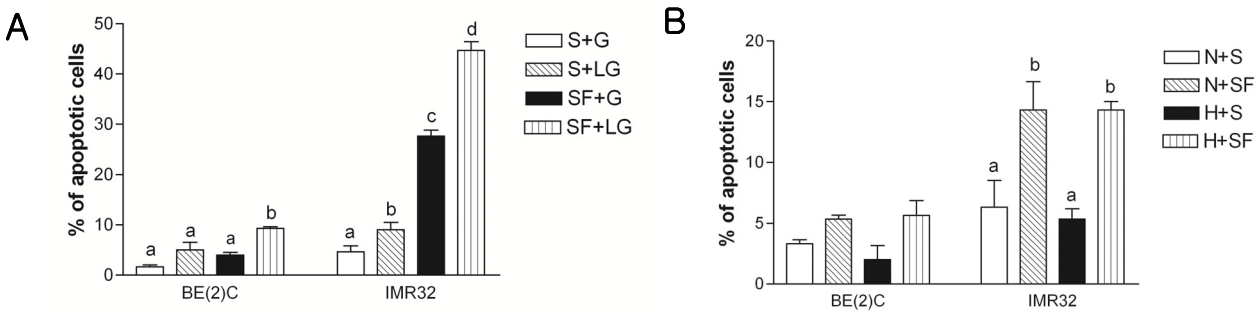


**Fig. 3. The effect of tumor microenvironment (nutrient deprivation and hypoxia) on apoptosis.** (A) BE(2)C and IMR32 cells were incubated at different combinations of nutrient deprivation (S+G, S+LG, SF+G, and SF+LG) for 48 hr. (B) BE(2)C and IMR32 cells were incubated under combination of hypoxia and serum deprivation (N+S, N+SF, H+S, and H+SF) for 48 hr. Determination of apotosis was performed as described in Fig. 2. A total of ≥ 300 cells were counted in 3-4 random fields. Bars are means ± SE. Different letters for given bars indicate the values are significant different from each other ($P < 0.05$) (Tukey's Post Hoc test after one-way ANOVA). S, 10% FBS (v,v); SF, serum-free; G, 25 mM of glucose; LG, low-glucose (25 μM); N, normoxia (21% $O_2$); H, hypoxia (1% $O_2$)

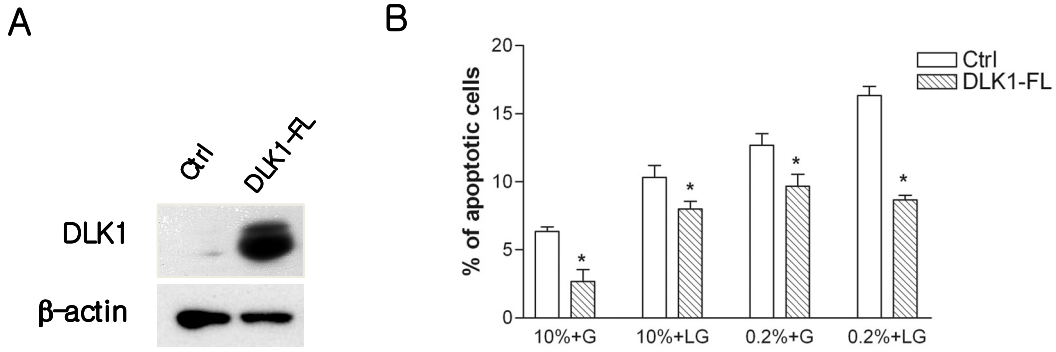


**Fig. 4. DLK1 ameliorated nutrient depletion-induced apoptosis.** (A) IMR32 cells were infected with full length DLK1 (DLK1-FL). Western blotting was performed to verify the over-expression of DLK1. β-actin was used as a loading control. (B) DLK1 over-expressed IMR32 cells were shifted to various conditions of media and maintained for 48h. The determination of apotosis was performed as described in Fig. 2. A total of ≥ 300 cells were counted in 3-4 random fields. Bars are means ± SE. * Statistically different, as compared with control (Student's t-test) ($P < 0.05$). 10%, 10% of serum (v,v); 0.2%, 0.2% of serum (v,v); G, 25 mM of glucose; LG, low-glucose (25 μM)

other hand, less malignant and more differentiated N-type IMR32 cells revealed a significant increase (2 fold) of apoptosis in low-glucose condition. Furthermore, serum-free only (7 fold) or the combination of serum-free and low-glucose (11 fold) resulted in dramatic increases in apoptosis in IMR32 cells (Fig. 3A).

Graeber *et al.* [23] demonstrated that hypoxia can select for apoptosis-resistant tumor cells. Hypoxic tumors with a low apoptotic index tend to be highly aggressive. Based on those observations, the effects of hypoxia on apoptosis of more advanced I-type BE(2)C cells and less aggressive N-type IMR32 cells were examined using double staining with PI and Hoechst. There was no increase of apoptosis in 1% $O_2$ for either cell line. However, more than a two-fold increase of apoptosis was examined under combined hypoxia and serum-free in IMR32, but not in BE(2)C cells (Fig. 3B). These data demonstrated that hypoxia had minor effects on the apoptosis of NB cells despite their different levels of malignancy or degrees of differentiation. Furthermore, more malignant and less differentiated tumor cells were more resistant to severe nutrient deprivation conditions than less malignant and more differentiated tumor cells.

*DLK1 regulates apoptosis induced by serum and/or glucose deprivation*

Investigators have reported that DLK1 participates in cell growth and proliferation in pre-B cells, thymocytes, and gliomas [24,25]. However, very little is known about the role of DLK1 in the apoptosis of tumor cells. To investigate the function of DLK1 in the apoptosis of NB, DLK1 was over-expressed using retroviral infection containing full-length DLK1 (DLK1-FL). The expression of DLK1 was highly amplified in highly malignant and less differentiated BE(2)C cells whereas its expression was quite low in less advanced and more differentiated IMR32 [18]. However, after infection of DLK1-FL, high expression of DLK1 in IMR32 cells was found. Over-expressed DLK1 expression was evaluated by Western blotting (Fig. 4A). The over-expression of DLK1-FL caused marked resistance against various nutrient deprivation condition-induced apoptosis. In particular, apoptosis was dramatically suppressed (43%) by overexpressed DLK1 under a combination of serum deprivation (0.2%) and low-glucose (25 μM) when compared to the control (10% serum and 25 mM of glucose) (Fig. 4B).

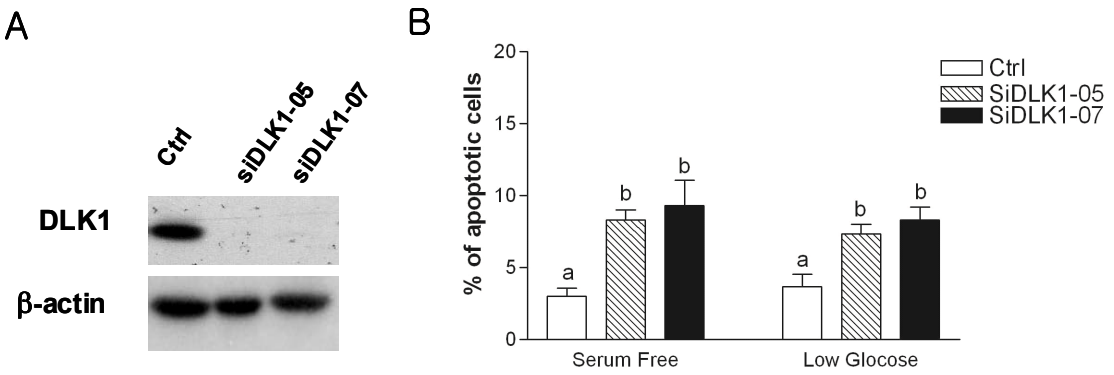


**Fig. 5.** DLK1 knock-down increased nutrient depletion-induced apoptosis. (A) BE(2)C cells were transfected with siDLK1 oligonucleotides (siDLK1-05 or siDLK1-07). Knockdown of DLK1 expression by siDLK1-05 or -07 was confirmed by Western blotting. β-actin was used as a loading control. (B) After transfection with siDLK1 oligonucletides, cells were incubated with nutrient depletion conditions, either serum-free or low-glucose (25 μM of glucose). The determination of apotosis was performed as described in Fig. 2. A total of ≥ 300 cells were counted in 3-4 random fields. Bars are the mean ± SE. Different letters for given bars indicate values are significantly different from each other ($P$< 0.05) (Tukey's Post Hoc test after one-way ANOVA)

To confirm the role of DLK1 in NB apoptosis, two different siDLK1 oligonucleotides were transfected individually into highly malignant BE(2)C cells, which have high DLK1 expression endogenously. Knock-down DLK1 expression was evaluated by Western blotting (Fig. 5A). Both serum and glucose depletion-induced apoptosis were significantly increased (relatively 2 fold) by DLK1 knock-down in this cell line (Fig. 5B). Collectively, these data suggest a critical role of DLK1 in the regulation of apoptosis in NB cells.

## Discussion

It has been reported that nutrient deprivation and/or energy restriction decrease tumor growth by reducing inflammation associated with ageing, elevating glucocorticoid hormone, and decreasing angiogenesis [26-28]. Furthermore, nutrient deprivation including a lack of serum or glucose is a potent inducer of apoptosis in cultures of many different cell types. The absence of serum in culture media inhibited cancer cell proliferation and increased apoptosis in K562 leukemia cells [29]. Since glucose is an exclusive energy source for the brain, a lack of glucose may be a critical influence on brain tumor cell metabolism and survival. In the present study, we employed human NB cell lines as an *in vitro* model system to examine the effects of the tumor cell microenvironment, including nutrient deprivation (serum or glucose depletion) and hypoxia, on apoptosis. In this study, in less malignant and more differentiated N-type IMR32 cells, apoptosis was affected by culture in serum-free conditions and further increased in low-glucose condition. This is consistent with a report demonstrating that other N-type cells, SK-N-SH-SY5Y (Sy5y), showed a marked decrease in cell number by glucose withdrawal for 72 hr [3].

Mukherjee *et al.* [28] reported that dietary restriction reduced tumor cell growth by reducing angiogenesis and by enhancing apoptosis in syngeneic CT-2A mouse brain cancer. Reduced glucose uptake in cancer cells by the glucose metabolic inhibitor, 2-deoxy-D-glucose, causes apoptosis in breast cancer cells [30]. Normal and transformed cancer cells respond to nutrition depletion in opposing manners. Whereas normal cells maintain their growth by regulating growth signals and nutrition support, transformed cancer cells lose responsiveness to most external growth signaling. During glucose deprivation, in normal cells, glucose uptake and glucose transporter expression is increased or modified to compensate. In contrast, when cancer cells are stressed by glucose-deprivation, stress related genes are upregulated leading to apoptosis [30]. The present study has revealed the response of cancer cells, particularly the status of malignancy, to nutrient depletion-induced apoptosis.

There was no increased apoptosis under hypoxia (1% $O_2$) despite different levels of malignancy or the degree of differentiation. These data are consistent with a study [31] demonstrating that NB cells treated under hypoxia (1% $O_2$) for 72 hr did not show any substantial changes in cell death, suggesting that hypoxia has minor effects on apoptosis in NB cells despite different levels of malignancy or degrees of differentiation.

Many genetic abnormalities have been identified and used clinically for prognosis of NB, such as MYCN amplification. MYCN amplification has been related to advance stage and adverse prognosis of NB [32,33]. Different malignant stages of IMR32 and BE(2)C cells showed MYCN-amplification (data not shown) [34]. However, despite the over-expression of MYCN in both cell lines, I-type BE(2)C cells were more resistant to stress-induced apoptosis than N-type IMR 32 cells in the present study. This result indicates that tumor cell malignancy and differentiation stage are more important than MYCN status to regulate tumor cell growth and apoptosis, suggesting an involvement of other genetic and/or epigenetic factors. It was previously demonstrated that DLK1 constituted an important role in tumorigenicity and stem cell-like functionality [18], which

supported the pro-survival function of this protein. It has also been reported that poorly differentiated and more malignant I-type BE(2)C cells expressed more DLK1 than more differentiated and less aggressive IMR32 cells [18], supporting the pro-survival role of DLK1 in malignancy and differentiation. The over-expression of this particular stem cell gene, DLK1, was able to ameliorate apoptosis in both low-serum (0.2%) and low-glucose (25 uM) conditions, having a potent effect on survival in IMR32 cells. Furthermore, knock-down of DLK1 enhanced apoptosis in a microenvironment characterized by a lack of serum and glucose. These data are consistent with a previous study where exogenous DLK1 inhibited an increase of apoptosis induced by $AS_2O_3$ in K562 leukemia cells [35]. Taken together, the present results demonstrate that an elevated DLK1 level provided a survival advantage for NB cells in a poor microenvironment characterized by serum and glucose deprivation. For future research, it is necessary to determine the stress-related signal mechanism for triggering apoptosis by nutrient deprivation. The findings from this study raise the interesting potential for further mechanistic studies to investigate DLK1-involved signal pathways ameliorating apoptosis in NB.

The prevention and treatment of NB remains a major challenge. Therefore, the development of a potential new preventive and therapeutic strategy is desirable. The implementation of nutrient deprivation in cancer prevention as well as therapeutic strategies, could be effective without having adverse side effects. Furthermore, DLK1 could provide a novel therapeutic strategy for regulating apoptosis in NB cells.

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