Original Article

Doublecortin-like kinase 1 exhibits cancer stem cell-like characteristics in a human colon cancer cell line

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Objective: Colon cancer stem cells (CSCs) are implicated in colorectal cancer carcinogenesis, metastasis, and therapeutic resistance. The identification of these cells could help to develop novel therapeutic strategies. Doublecortin-like kinase 1 (DCLK1) has been viewed as a marker for gastrointestinal stem cells that fuel the self-renewal process, however others view them as a marker of Tuft cells or as an enteroendocrine subtype. The purpose of this study was to use a colon cancer cell line to identify and characterize the stem-like characteristics of the DCLK1+ cell population.

Methods: To enrich stem-like cells, HCT116 cells (derived from colon adenocarcinomas) were cultured using serum-free media to form spheres under both normal oxygen and hypoxia condition. DCLK1 transcript expression in the adherent parental cells and spheroids was quantified using quantitative real time reverse transcription- polymerase chain reaction [(q)RT-PCR]. DCLK1 protein expression was determined using flow cytometry. Self-renewal capability from adherent parental cells and spheroids was determined using extreme limiting dilution analysis (ELDA).

Results: Under both normal oxygen and hypoxia condition, the adherent parental cells were composed of cells that express low levels of DCLK1. However, spheroids exhibited an increased frequency of cells expressing DCLK1 on both mRNA and protein levels. Cells derived from spheroids also possess stronger self-renewal capability.

Conclusions: The higher fraction of DCLK1+ cells exhibited by spheroids and hypoxia reflects the stem-like characteristics of these cells. DCLK1 may represent an ideal marker to study and develop effective strategies to overcome chemo-resistance and relapse of colon cancer.

Key Words: Doublecortin-like kinase 1 (DCLK1); colorectal cancer; cancer stem cells; stem cell marker; spheroids

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Introduction

Colorectal cancer is the third most common cancer diagnosed and the third leading cause of cancer-related deaths in both men and women in the United States (1). In the last 20 years, progress in the treatment of colon cancer has improved the quality of life of patients through the use of innovative minimally invasive surgical techniques and multimodality treatments (2). However, the survival of patients with advanced disease has not improved (3). In fact, up to 50% of patients will relapse after surgical resection and ultimately die of metastatic disease (4-7). Although the reason for this is not fully understood, the presence of chemotherapy-resistant cancer stem cells (CSCs) is thought to be one the primary causes for tumor recurrence (8). This small subpopulation of cells has been identified in several solid tumors, including colon cancer. Recent evidence shows that CSCs possess certain characteristics that give them the ability to maintain the tumor population, metastasize, and be resistant to chemoradiotherapy. Thus, the CSC hypothesis has been proposed as a hierarchical model of

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tumor origin.

Putative CSC populations have been identified in several solid malignancies based on the up-regulation of specific surface markers when cultured under non-adherent conditions in serum-free media. The growth of these spherical colonies is also considered to reflect other stem cell-like features, such as the self-renewal ability (9). Interestingly, investigators have shown that restricted oxygen conditions can increase the CSC fraction and promote acquisition of a stem-like state (10). Recently, doublecortin-like kinase 1 [DCLK1, also known as KIAA0369 (11) and DCAMKL1 (12)], a microtubule-associated kinase expressed in postmitotic neurons, has been identified as a putative intestinal and colon stem cell surface marker (13-16). DCLK1 has been found to be associated with colon cancer (17) as well as other types of adenocarcinoma including esophageal (18) and pancreatic (19). The DCLK1 gene encodes different isoforms through alternative splicing mechanism and alternative promoters (11). DCLK1 functions in the regulation of microtubule polymerization through its doublecortin domain (20,21), and it serves as a serine-threonine protein kinase through its c-terminal protein kinase domain (22). It is involved in normal nervous system development (11,23,24), and it is correlated with general cognition and verbal memory function (25).

In the colon, we have found that DCLK1 positive cells in tumors reside in a niche at the crypt base among the Paneth cells and above the crypt base in an area, which includes the +4 compartment, were quiescent stem cells are thought to reside (13,26). However, currently, there are opposing opinions on the exact identity and functional characteristics of DCLK1 positive cells. Some view them as gastrointestinal stem cells that fuel the self-renewal process (13), whereas others view them as tuft cells (27) or as an enteroendocrine subtype (26).

The purpose of this study was to build on our initial observations of DCLK1 + cells in colon cancer and to identify and characterize the stem-like characteristics of the DCLK1 + cell fraction.

Materials and methods

Cell cultures

Human colon cancer HCT116 cells were a gift from Dr. Prescott Deininger in Tulane Cancer Center. Cells were maintained in McCoy's 5A medium (Gibco, Grand Island, NY, cat # 16600) supplemented with 10% FBS in 37 ℃ incubator with 5% CO_2.

Spheroid formation

HCT116 cells were dissociated with 0.25% Trypsin-EDTA solution (Sigma-Aldrich, St. Louis, MO) until single cell suspension was obtained. Cells were re-suspended in the serum-free spheroid formation medium, which consists of 100 μg/mL human recombinant epidermal growth factor (EGF, Stemgent, St. Diego CA, USA), 100 μg/mL human recombinant basic fibroblast growth factor (bFGF, Biosource, Grand Island, NY), and 0.5× B27 supplement (Gibco, Grand Island, NY) in the McCoy's 5A medium. 2×10^4 cells were plated in the ultra-low attachment 6 well plate (Fisher Scientific) in each well. Fresh medium was added to the cells every 3 days and spheroids were maintained in cultures for 7 days. For the spheroids formed under hypoxia condition, 1% or 5% O_2 was applied.

RNA harvest

Total RNA from HCT116 adherent parental cells and spheroids was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

Reverse transcription polymerase chain reaction (RT-PCR)

cDNA was generated using the Reverse Transcription System (Promega, Madison, WI) according to the manufacturer's instruction. Briefly, 1 μg of RNA was reverse transcribed in 20 μL reaction. The first-stand cDNA amplification mixture was diluted to 100 μL with nuclease-free water.

Quantitative real time PCR [(q)RT-PCR]

Gene expression was determined on an iCycler iQ5 fluorescence thermocycler (Bio-Rad Laboratories, Hercules, CA) using SYBR GreenER™ qPCR SuperMix for iCycler® Instrument (Life Technologies, Grand Island, NY, cat #11761-100). (q)RT-PCR was carried out according to the manufacturer's instruction with some modifications. Briefly, 5 μL cDNA from RT-PCR was added to a 25 μL reaction. Primers for the human DCLK1 are: 5'-TGAAGGGTACGCTCCTCAGT-3' (forward) and 5'-GCTACACTCTGACCGCATGA-3' (reverse). Beta-actin was used as an internal control and the primers are: 5'-GGACTTCGAGCAAGAGATGG-3' (forward) and 5'-AGCACTGTGTTGGCGTACAG-3' (reverse).

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Fluorescence-activated cell sorting (FACS)

CD44 is a universal cell surface glycoprotein expressed in a large number of mammalian cell types. It was used as an internal control of DCLK1 in this experiment. Briefly, HCT116 parental cells and spheroid cells were dissociated into single cell with Accutase (Invitrogen, Carlsbad, CA). Cells were suspended in PBS with 10% FBS and incubated with DCAMKL-1 polyclonal rabbit anti-human antibody (Abcam, Cambridge, MA) in dark at room temperature for one hour. After primary DCAMKL-1 antibody incubation cells were washed and incubated with FITC-conjugated goat anti-rabbit secondary antibody (Abcam, Cambridge, MA) in dark at room temperature for 30 minutes. After washing, cells were sorted through a cell sorter (FACS Aria III, BD). The cell line was independently analyzed three times and the mean value ± standard deviation calculated.

Extreme limiting dilution analysis (ELDA)

ELDA was performed according to Hu and Smyth's approach (28). In brief, HCT116 cells from passage and from spheroids were dissociated into single cell suspension. Cells of 1, 100, 1,000, and 10,000 in 100 µL per well were plated in ultra-low attachment 96 well plate (Fisher Scientific, Corning 3474) with 24 wells for each individual concentration and maintained in the serum-free spheroid formation medium for 5 days. Spheroids were fixed with 4% formalin and number of spheroids was counted under light microscope.

Statistical analysis

All data are shown as mean ± SEM. Statistical analysis between the DCLK1 mRNA and antigen expression in parent and the HCT116 spheroids was performed using the Student's *t*-test. The same analysis was applied to analyze the correlation between cell self-renewal capability and cell derivation. P<0.05 was considered to be statistically significant.

Results

DCLK1 mRNA is expressed in HCT116 cells

It has been reported that DCLK1 is expressed in both human normal (26) and colorectal cancer tissue (17). In order to determine whether DCLK1 is expressed in HCT116 cells and its relative expression level compared to beta-actin, a pair of primers that can recognize DCLK1 isoform_1(DCLK-long-B), isoform_2(DCLK-Short-B) and isoform_3(DCLK-Short-A) were used in the (q)RT-PCR experiment. The expression of DCLK1 isoform_4(CARP) was not included. Results indicated that the primers specifically identified DCLK1 isoform_1, 2 and 3 expression in the HCT 116 cells (*Figure 1A*) and their expression level was very low compared to beta actin, which is $1.92\times10^{-6}\pm 0.96\times10^{-6}$.

Expression of DCLK1 is up-regulated in HCT116 spheroids both transcriptionally and translationally under normal O_2 condition

Cancer stem cells (CSCs) consist of a higher percentage in spheroids (29-31). In order to evaluate DCLK1 expression in the CSCs, spheroids were formed from HCT116 cells under normal O_2 condition in serum-free condition. (q)RT-PCR was applied to monitor DCLK1 mRNA expression. Compared to the adherent parental cells, DCLK1 mRNA expression was significantly increased in the spheroids (3.86±1.16 fold; P=0.0026, *Figure 1B*), which indicates that DCLK1 mRNA is up-regulated in the CSCs. Since DCLK1 is a transmembrane protein (32) and it is expressed on the cell surface (14), FACS was carried out to determine whether DCLK1 is up-regulated on the protein level in spheroids. Results demonstrated that DCLK1 expression in the spheroids keeps the same up-regulation trend as that at the transcriptional level (*Figure 1B,C*). DCLK1 positive cells in the parental cells consist of 0.5±0.1%, whereas in the spheroid cells, DCLK1 positive cells consisted of 1.4±0.3%. That is 3.1-fold increase and it is statistically significant (P=0.015), which indicates that DCLK1 protein is up-regulated in the CSCs. So in summary, the expression of DCLK1 is increased in HCT116 cell spheroids both transcriptionally and translationally, which indicates that DCLK1 expression in CSCs is up-regulated on both transcription and translation level.

Expression of DCLK1 is up-regulated in HCT116 spheroids both transcriptionally and translationally under hypoxia condition

Hypoxia is a common feature of solid tumors under normal biological condition or during anti-angiogenesis, irradiation and chemotherapy (33). In order to determine whether hypoxia affects expression of DCLK1 in CSCs, HCT116 spheroids were cultured under hypoxia condition

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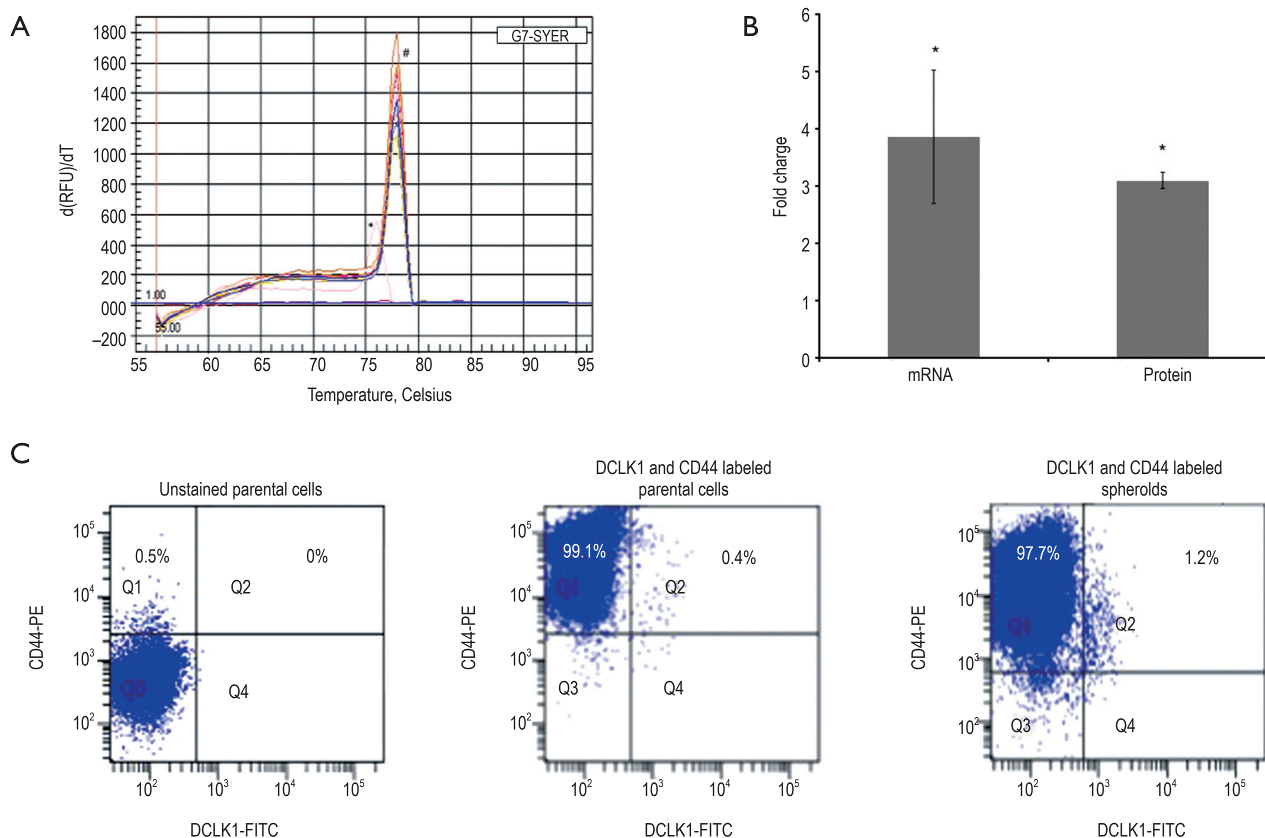

Figure 1 DCLK1 is up-regulated in the HCT116 spheroids under normal oxygen condition determined by (q)RT-PCR and FACS. A. Melt curve for DCLK1 examined by the designed primers. A pair of primers which can recognize DCLK1 isoform_1, 2, and 3 were designed. Genomic DNA from HCT116 cells (the only line with a smaller peak and shifted temperature, indicated by *) and cDNA from spheroids (lines with a similar sharp peak, indicated by #) were used as template in the (q)RT-PCR to test the specificity of the primers; B. DCLK1 expression in cells derived from spheroid formed under normal oxygen conditions was determined by (q)RT-PCR (Column labeled as mRNA) and FACS (Column labeled as Protein). For the (q)RT-PCR, DCLK1 expression was normalized to beta-actin before fold change calculation. DCLK1 fold change was calculated by comparing DCLK1 expression in the spheroids to the parental adhesive cells under the same culture condition. Data was expressed as mean±SEM from 5 [(q)RT-PCR]and 2 (FACS) independent experiments. For the statistical analysis, DCLK1 fold change in the spheroids was compared to adhesive parental cells, which was given as 1. *P<0.01 *vs*. adhesive parental cells; C. FACS data for determination of DCLK1 and CD44 expression in the adherent parental cells and spheroids under normal oxygen condition

(5% O_2 for mRNA harvest and 1% O_2 for FACS). (q)RT-PCR results showed that DCLK1 mRNA expression was significantly increased in the spheroids (3.4±1.08 fold; P=0.03, *Figure 2A*) compared to the parental cells, which indicates that DCLK1 mRNA is up-regulated in the CSCs under hypoxia condition. In order to let the cells grow under more extreme hypoxia condition, spheroids were cultured at 1% O_2 for the FACS. Results showed that DCLK1 positive cells account for 0.4±0.1% in the parental passage cells; while in the spheroids, DCLK1 positive cells account for 4.3±1.4% (*Figure 2A,B*). DCLK1 protein expression was increased significantly in the spheroids than the parental passage cells (10.8±0.3 fold; P=0.006), which indicates that DCLK1 protein is up-regulated in the CSCs under hypoxia condition. So in summary, under hypoxia condition, the expression of DCLK1 is increased in HCT116 cell spheroids both transcriptionally and translationally, which indicates that DCLK1 expression in CSCs is up-regulated on both transcription and translation level.

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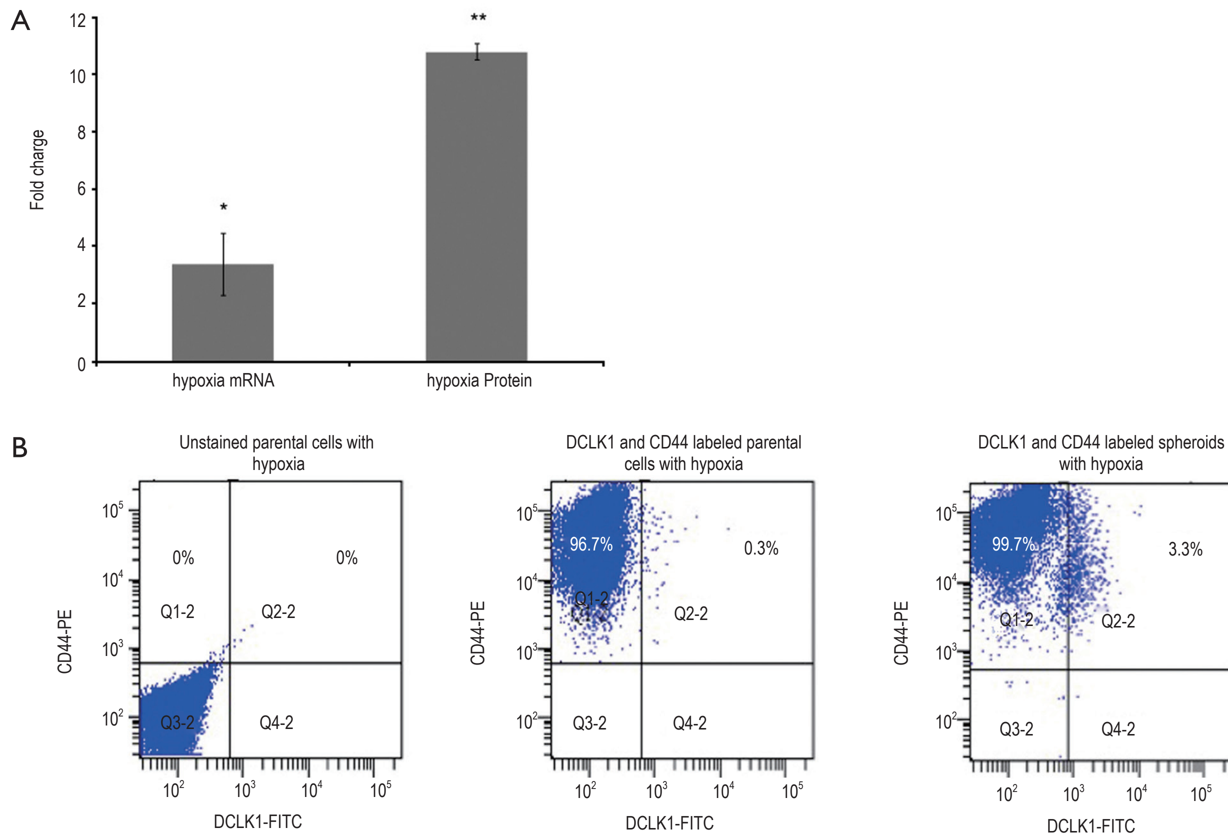

Figure 2 DCLK1 is up-regulated in the HCT116 spheroids under hypoxia condition determined by (q)RT-PCR and FACS. A. Fold change of DCLK1 expression in spheroids compared to parental adhesive cells cultured under hypoxia condition. Results from (q)RT-PCR was labeled as Hypoxia mRNA (5% O_2). Results from FACS were labeled as Hypoxia protein (1% O_2). For the (q)RT-PCR, DCLK1 expression was normalized to beta-actin before fold change calculation. DCLK1 fold change was calculated by comparing DCLK1 expression in the spheroids to the parental adhesive cells under the same culture condition. Data was expressed as mean±SEM from 2 independent experiments for both (q)RT-PCR and FACS . For the statistical analysis, DCLK1 fold change in the spheroids was compared to adhesive parental cells, which was given as 1. *P<0.05 *vs*. the adherent parental cells, **P<0.01 *vs*. the adherent parental cells; B. FACS data for determination of DCLK1 and CD44 expression in the adherent parental cells and spheroids under hypoxia condition

Cells derived from spheroids possess stronger spheroids formation capability

In the CSCs hypothesis, it is believed that CSCs possess similar biological properties as normal stem cells, such as its capability in indefinite self-replication. Through aberrant differentiation, CSCs can generate other cancer cells (34). In order to investigate whether CSCs possess stronger spheroids formation capability, cells from parental HCT116 cells or spheroids were plated at different concentration for spheroid formation according to the ELDA approach. Results demonstrated that cells from spheroids possess stronger spheroid formation capability compared to the parental cells, and this capability is dose dependent (*Figure 3*). Cells from spheroids at 100 and 1,000 cells/well possess significant stronger spheroids formation capability (P=8.357e-05 and 1.324e-06, respectively). Whereas for the concentration of 1 and 10 cell(s)/well, there is no significant difference was found between the cells derived from either parental or spheroids. This data indicates that CSCs possess the capability of giving rise to other cancer cells, and this capability is significantly stronger than parental cells. Up-regulation of DCLK1 in the CSCs may be correlated with the stronger cell proliferation capability.

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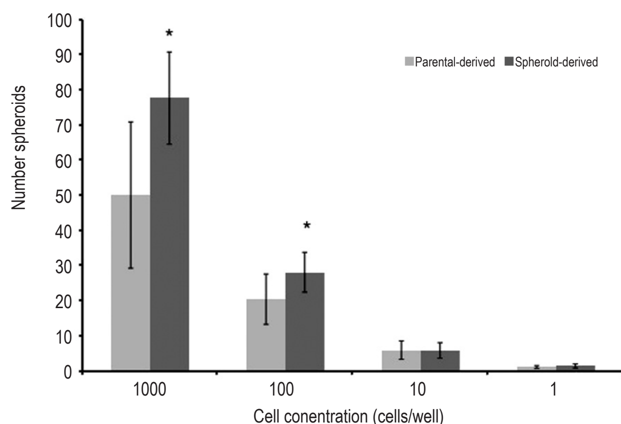

Figure 3 Cells derived from spheroids possess stronger self-renewal capability. Extreme limiting dilution analysis was used to measure self-renewal capability of cells derived from parental adhesive cells and spheroids under normal oxygen condition. Each cell concentration has 24 replicates in 96-well plate. Progeny spheroid number formed from spheroids was compared to that from parental adhesive cells. *P<0.001 *vs*. cells derived from the adherent parental

Discussion

In this report, we present evidence that one of the best-known human cancer cell lines derived from colorectal adenocarcinomas contains a subpopulation of DCLK1 + cells with stem-like properties as demonstrated by their up-regulation when cultivated under stem-cell-selective conditions. Under these conditions, we also demonstrated that DCLK1 + cells exhibited other key biological properties of CSCs, including self-renewal and proliferation.

Serum-free culture conditions supplemented with growth factors have been successfully applied to cultivate cells with stem-like properties from several malignancies (31,35-37). In this study, we applied a serum-free culture condition to cultivate CSCs from a colon cancer cell line. Tumor sphere formation was observed within 8 weeks, could be sustained in culture for more than 5 months, and retained the expression of well-known cell surface markers, such as CD44. As controls, adherent primary cultures were also generated under traditional culture conditions using a serum-containing medium. Interestingly, DCLK1 + cells were found to comprise only a small fraction of the total tumor population in our adherent primary cultures of HCT116 human colon cancer cells. However, consistent with stem like properties, the percentage of DCLK1 + cells significantly increased in the tumor sphere-forming colonies. Considering the level of DCLK1 expression observed in our colon cancer cells and the belief that CSCs are only a small sub-population of tumor specimens, our results suggest that DCLK1 may be a marker expressed on functional CSCs.

High level of DCLK1 expression has been observed in other gastrointestinal tumors/cancers as well. Vega and colleagues (18) reported that DCLK1 increased significantly in both Barrett's esophagus with/without dysplasia and esophageal adenocarcinoma when compared to the normal esophageal mucosa. They also observed DCLK1 was progressively increased during the progression from Barrett's esophagus with dysplasia to esophageal adenocarcinoma. It was also reported that in the Hepatitis C virus-induced liver carcinogenesis, DCLK1 was highly expressed and DCAMKL-1-positive cells isolated from hepatoma cell lines possess the capability to form spheroids (38). Sureban and colleagues found that DCLK1 was increased in both mouse pancreatic cancer model and human pancreatic adenocarcinoma (19). Taken together, these observations suggest that the up-regulation of DCLK1 plays a critical role in the development of carcinogenesis.

Whether DCLK1 can be used as an additional marker to further define the CSC population in colorectal cancer remains a topic of debate. May and colleagues (13,14) identified DCLK1 as a novel gastrointestinal stem cell and adenoma stem cell marker following radiation injury and in adenomatous polyposis coli (APC)/multiple intestinal neoplasia mice. They observed that DCLK1 positive cells are located at the +4 position, just above Paneth cells, and co-localized with some musashi-1 (MSI-1), a well-accepted stem cell marker. They further identified that DCLK1 was a quiescent intestinal stem cell marker, and it can be used to distinguish quiescent stem cells from the cycling stem/progenitors. However, Gerbe and colleagues (39) proposed that DCLK1 is not a marker for intestinal epithelial stem cells, but a highly specific and robust marker for post-mitotic, differentiated, tuft cells, which is a minority cell lineage of the intestinal epithelium. Using DCLK1 immunohistochemistry staining in normal colorectal biopsies from 14 patients, our lab identified that expression of DCLK1 was not confined to the stem cell compartment, with 70% of DCLK-1 positive cells located in the lower third of the crypt, 26% in the middle third and 4% in the upper third. We also identified that DCLK1 only co-localized with leucine-rich-repeat-containing G-protein-

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coupled receptor 5 (Lgr5), an intestinal stem cell marker, at the crypt base, but co-stained with chromogranin-A (CgA), an enteroendocrine cell marker, throughout the crypt. So we believe that DCLK1 marks a subset of colorectal stem cells, as well as a subset of enteroendocrine cells (26). The most recent publication from Nakanishi and colleagues reported that DCLK1 is only expressed in the tumor stem cells but not in the normal stem cells, so it is a unique intestine tumor stem cell marker (15).

Using DCLK1 as a target for tumor/cancer therapy has gained promising progress in research. Sureban and colleagues (40,41) reported that growth of HCT116 xenografts in nude mice was arrested after DCLK1 knockdown by siRNA. Verissimo and colleagues observed that apoptosis was induced in neuroblastoma cells after DCLK1 knockdown (42). Nakanishi and colleagues investigated whether targeting DCLK1 marked tumor stem cells can be an effective approach to treat tumor. They found that diphtheria toxin only cause apoptosis in the DCLK1 positive tumor stem cells, but not in the DCLK1 negative cells, and interruption of the tumor progeny supply by DCLK1-positive TSCs can cause tumor regression during turnover of polyps (15). So targeting DCLK1, either directly or indirectly, might be an effective approach for the treatment of tumors/cancers.

Mechanism of how DCLK1 affect tumor genesis still remains unclear. Sureban and colleagues (19,40,41) reported that in the HCT116 xenografts or pancreatic cancer cells after DCLK1 siRNA treatment, the antitumor microRNA let-7a and miR-144 were up-regulated, and their downstream proto-oncogene c-Myc and Notch-1 were down-regulated, respectively. What's more, they also observed that the epithelial mesenchymal transition (EMT) inhibitor miR-200a was up-regulated and the EMT-associated transcription factors ZEB1, ZEB2, Snail and Slug were down-regulated. So they believe that the direct regulatory links between DCAMKL-1, microRNAs, and EMT might enable DCLK1 to become a novel target for tumor/cancer therapy.

Oxygen tension is tightly regulated under homeostasis condition. Disorder of oxygenation levels can result in severe diseases. About 70 years ago, Warburg had realized that low oxygenation levels (now termed as hypoxia) is the prime cause of cancer (43,44). Hypoxia is a common fundamental feature of solid tumors under normal biological condition or during anti-angiogenesis, irradiation and chemotherapy (33). Hypoxia affects tumor progression directly or in-directly, and many molecular pathways and genes are involved, including hypoxia induced factor-1 (HIF-1), VEGF, etc. It has been identified that low oxygen tension associated with maintenance of an undifferentiated cell state (45,46). The response of stem cells (SC) to hypoxia is one of the main mechanisms of an organism's adaptation to changing terms of external and internal environment. Hypoxia is likely to be a functional component of a normal stem cell niche. Interestingly, hypoxia promotes the self-renewal of embryonic stem (ES) cells and has been shown to regulate cancer stem cells and promotes their self-renewal capability (45,47,48). Indeed, investigators have shown that restricted oxygen conditions increase the CSC fraction and promote acquisition of a stem-like state. Lin and colleagues (10) observed that CD133 + enriched colorectal CSCs can survive under hypoxia and serum depletion condition. Long-term hypoxia condition significantly enhanced HCT116 xenografts growth rate, increased HIF-1 expression, and increased microvessel density in the tumor tissue (49). Our results demonstrated that under hypoxia condition, DCLK1 expression was significantly increased in the HCT116 spheroids transcriptionally and translationally. To our knowledge, this is the first time that DCLK1 expression was investigated under hypoxia condition in the colorectal cancer cells. Whether the increase of DCLK1 is correlated with hypoxia-induced tumor angiogenesis, increasing mutation rate, metastasis and resistance to radiation and chemotherapy needs further investigation.

Two important characteristics of stem cells are its self-replication and asymmetric cell division capability. CSCs are hypothesized to possess those stem-like properties and that's why radio-chemoresistance exists. In our experiment, we observed that cells generated from HCT116 spheroids possess significantly stronger self-renewal capability compared to the parental adhesion HCT116 cells, and it is cell dose-dependent. This may be due to the higher percentage of CSCs in the spheroids. So our results indicate that CSCs are capable for self-renewal. To target the CSCs for tumor therapy will be an effective approach for tumor therapy.

In summary, we demonstrated that DCLK1 expression was up-regulated in the HCT116 spheroids under both normal and hypoxia condition. Cells from spheroids are more capable to produce progenies. Our data also indicates that DCLK1 is up-regulated in the CSCs, and this may be correlated with their stronger self-renewal capability. DCLK1 may become a novel target for colorectal cancer therapy. Further studies from patients' specimens are needed for a continued clarification of the role of these

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cells in therapy response and therefore to contribute for the establishment of novel therapeutic strategies.

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