Inhibition of c-Yes Induces Differentiation of HT-29 Human Colon Cancer Stem Cells through Midbody Elongation

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Recent research suggests that a small group of cells, named cancer stem cells (CSCs), is responsible for initiating tumor formation, recurrence, and metastasis. c-Yes, a proto-oncogene that is a subfamily of Src family kinase, is often activated in human colon cancer; this implicates c-Yes in the onset and progression of the disease. The objective of this study was to investigate the correlation between c-Yes and CSCs. We performed a sphere formation assay and reverse transcription-polymerase chain reaction for studying the differentiation of HT-29 human colon CSCs. To demonstrate the specific role of c-Yes in CSCs, we performed live cell microscopy and a cell cycle assay. These study shows, for the first time, that c-Yes is enriched in CD133⁺ CSCs, compared to their CD133⁻ counterparts, and that c-Yes depletion in CD133⁺ cells induces cell differentiation. Moreover, c-Yes depletion was found to elongate the midbody and increase the proliferation doubling time. This also suggested that the misregulation of microtubules during chromosomal separation causes aneuploidy. Our results suggest that c-Yes may play a crucial role in initiating, maintaining, and driving the tumorigenic property of colon cancer.

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INTRODUCTION

Cancer stem cells (CSCs), which constitute a small proportion of the total cell population in a tumor, possess greater ability to maintain and initiate tumor formation and promote metastasis than other tumor cells. They share many properties with normal stem cells, such as a long lifespan, pluripotency, and self-renewal ability [1]. CSCs have unique phenotypes and functional characteristics that promote and sustain tumors. CD133 is a unique marker expressed in the brain, colon, and pancreatic tumors [2]. Multiple studies have shown that the percentage of CD133⁺ cells correlates with tumor aggressiveness, metastases, and resistance to chemotherapy and radiotherapy [3-5]. Because CSCs are highly resistant to standard anti-cancer therapy, targeting these cells may halt the cancer at the source. c-Yes, a member of the Src tyrosine kinase family, is a potentially important

modulator of colon CSCs, as the CD133 marker is phosphorylated by Src family kinases [6].

The proto-oncogene c-Yes is an important non-receptor tyrosine kinase involved in various signaling pathways. Src family kinases play important roles in physiological and pathological processes such as cell progression, proliferation, and migration [7]. The frequent activation of c-Yes in human cancers, coupled with their viral homologs, suggests that they contribute to the onset or progression of malignant phenotypes [7]. The activation and function of Src family kinases have been studied more extensively in colon cancer than in any other cancer; however, the specific function of c-Yes in colon CSCs remains widely unknown.

MATERIALS AND METHODS

Cell culture

The human colorectal adenocarcinoma cell line HT-29 was purchased from the Korean Cell Bank (Seoul, Korea). These cells were cultured in Roswell Park Memorial Institute medium (RPMI-1640; GIBCO, Carlsbad, CA, USA) containing 2.05 mM glucose and 25 mM HEPES and supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The

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cells were incubated at 37°C with 5% CO₂. When the cells reached 80% confluence, they were detached using 0.05% trypsin and 0.53 mM ethylene diaminetetraacetic acid and then seeded into fresh flasks.

Fluorescence-activated cell sorting

HT-29 cells were detached from the flasks by using trypsin/EDTA and prepared for use in the BD FACSARIA system (Franklin Lakes, NJ, USA), according to the manufacturer's protocol. Cells were labeled with a primary CD133/1PE antibody (Miltenyi Biotec Australia Pty. Ltd., North Ryde, NSW, Australia) and sorted using non-labeled cells as controls. Data were analyzed using the software provided with the FACSARIA system.

Flow cytometry

Cells were labeled with a primary CD133/1-PE antibody (Miltenyi Biotec), according to the manufacturer's instructions. The purity of the sorted cells was evaluated by flow cytometry and Western blotting. Flow cytometry was performed using a BD FACS Calibur system. Data were analyzed using the software provided with the FACS Calibur system.

c-Yes transfection and selection

FACS-sorted CD133⁺ and CD133⁻ HT-29 cells were transfected with a c-Yes short hairpin RNA (shRNA) plasmid (Santa Cruz Biotechnology, Santa Cruz, CA, USA) by using shRNA plasmid transfection reagent (Santa Cruz Biotechnology). Cells were seeded in antibiotic-free medium in a 6-well plate. The cells were grown to 70% confluence, and then transfected at a concentration of 1 ng/μL. The transfection was performed according to the manufacturer's protocol. After 48 h of incubation with shRNA, the cells were selected with puromycin (5 μg/μL) for 5 days.

Cell synchronization

For synchronization, HT-29 cells were incubated with nocodazole (5 mg/mL) for 18 h, and then transfected and selected using puromycin. Nocodazole treatment led to G2/M phase boundary arrest, which was then released by extensive washing with phosphate-buffered saline (PBS) and growth medium.

Reverse transcription-polymerase chain reaction

Total RNA was isolated using 1 mL of TRIzol (Invitrogen, Rockville, MD, USA), according to the manufacturer's protocol. Complementary DNA were synthesized with the Superscript III First Strand Synthesis system (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. Primers for polymerase chain reaction (PCR) were purchased from CosmoGeneTech (Seoul, Korea). The target regions were amplified, the

products were separated by electrophoresis on 2% agarose gels, and then detected with ethidium bromide staining and visualized using a Bio-Rad Molecular Imager GelDoc XR (Hercules, CA, USA).

Western blot analysis

HT-29 cells were lysed in RIPA PIC buffer and collected. The protein concentration in the cell lysate was assayed, and for each lysate sample, 10 μg of protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and then transferred to a nitrocellulose membrane. The membrane was blocked with 5% skim milk in Tris-buffered saline with Tween-20 (TBS-T). The primary antibody (mouse anti-c-Yes, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was diluted, as suggested by the manufacturer, and incubated with the membrane at 4°C overnight. The membrane was then washed with TBS-T 3 times for 10 min each. Following the washes, the membrane was incubated with a secondary antibody for 1 h. It was then washed with TBS-T 3 times for 10 min each. The proteins on the membrane were detected using an enhanced chemiluminescence system (Millipore, Billerica, MA, USA). Bands on the Western blot were scanned with the luminescent image analyzer (LAS-3000) and analyzed using Multi Gauge image analysis software (Fuji Film, Tokyo, Japan). Values are presented as mean ± standard error of the mean (SEM). Statistical significance was evaluated by ANOVA.

Cell viability assay

The transfected cells were seeded at a density of 3 × 10³ cells/well in 96-well plates, and allowed to attach for 24 h. Cell Counting Kit-8 (CCK-8; 10 μL) was added to each well, and the plate was incubated at 37°C for 1 h, and then cell proliferation and cytotoxicity were assessed by measuring the absorbance at 450 nm with a microplate reader (BioRad, Hercules, CA, USA).

Sphere formation assay

Sorted and c-Yes-depleted cells were seeded in 6-well plates at a density of 10³ cells/mL with serum-free RPMI, to promote sphere formation. The medium was supplemented with 10 ng/mL fibroblast growth factor, 10 ng/mL epidermal growth factor, and 2.75 ng/mL selenium (insulin-transferrin-selenium solution). Every week, fresh medium was added to the cells. On day 21, spheres larger than 60 μm were counted.

Live cell imaging

Cells were first placed on glass slides in a chamber and filmed for 3 h under constant conditions (5% CO₂, 37°C), and then observed with a confocal laser-scanning microscope (LSM 5 Exciter; Carl-Zeiss, Oberkochen, Germany). Images were recorded at a rate of 1 frame every 2 minutes and processed with the soft-

were provided with the system.

Propidium iodide-based cell cycle and aneuploidy assays

HT-29 cells were grown, washed thrice with PBS, and fixed with 70% ethanol overnight at 4°C. Cells were spun down and washed with PBS, and then treated with 100 g/mL RNase A and 100 µg/mL propidium iodide (PI) for 15 min. PI-labeled cells were measured using the FACS Calibur system and analyzed with the accompanying software.

Statistical analysis

All results are expressed as mean ± SEM. The statistical significance of differences in protein spot and band intensities between groups was determined by the Student's t-test by using SPSS statistical software, version 12 (SPSS Inc., Chicago, IL, USA).

RESULTS

Isolation of CD133⁺ and CD133⁻ HT-29 cells

CD133⁺ and CD133⁻ HT-29 cells were sorted using the FACS Aria system (Fig. 1A), and their purity was checked on day 18. Sorting was performed using CD133-PE-antibody labeling. The time required for cell sorting and other experiments was 18 days. Therefore, CD133 expression was measured by flow cytometry to verify that no changes occurred in the cell population after FACS sorting. CD133⁺ cells were grown for 18 days, and then separated into 4 groups. Verification showed that the purity of the CD133⁺ cells and CD133⁻ cells was 90–95% and 84–90%, respectively (Fig. 1B). Western blot analysis was also performed to confirm cell purity (Fig. 1C).

c-Yes is required to maintain stem cell-like properties

Sorted and depleted HT-29 cells were plated in serum-free medium in low-attachment 6-well plates. Figure 2A, B, and C shows the morphological changes of cells that were sorted as either CD133⁺ or CD133⁻ and transfected with either c-Yes shRNA or scrambled shRNA. By week 3, CD133⁺ cells showed significant tumor sphere formation. Spheres larger than 400 µm were counted and quantified; the results are shown in Figure 2D, which focuses on the c-Yes-depleted, CD133⁻ cells. No significant difference in sphere formation was noted in c-Yes-depleted cells. This suggests that either the numbers of stem cell-like cells were significantly reduced in these cultures or that c-Yes depletion induced differentiation of the CSCs.

Depletion of c-Yes induces stem cell differentiation

Total RNA and protein were obtained from cells that were transfected with either c-Yes or scrambled shRNA. Figure 1D

shows that c-Yes was indeed depleted by the c-Yes-specific shRNA. Figure 2E shows the mRNA expression levels of stemness-related markers and differentiated HT-29 cells. The expression levels of *CD133*, *Nanog*, *Oct4*, and *CK-20* in CD133⁺ and CD133⁻ cells were compared. The relative expression of *CD133*, *Nanog*, and *Oct4* was upregulated in CD133⁺ cells, whereas *CK-20* expression was significantly upregulated in CD133⁻ cells. *CD133*, *Nanog*, and *Oct4* are specific markers of stem cells, whereas *CK-20* is a marker of differentiated colorectal cells.

Depletion of c-Yes induces elongation of the midbodies

Figure 3A shows the HT-29 cells transfected with the control shRNA. These cells grew in a colony with tight junctions. HT-29 cells also exhibited polyp-like growth *in vitro*, and grew on top of each other rather than in a single layer. Figure 3B shows HT-29 cells transfected with c-Yes shRNA, which induced c-Yes depletion. The morphology of these cells was dramatically different from that of the control-transfected cells. First, the cells grew in a non-colony-like single layer and showed outgrowths from the midbody, which has a morphology similar to that of cells that have undergone incomplete abscission. A morphological capture technique was conducted without cell cycle synchronization, which also showed that incomplete cell division is an accumulation process. To capture a single cell during abscission, live cell imaging was performed. After sorting, transfection, and cell cycle synchronization, the images of these cells were captured during the M phase, more specifically during abscission. The cells were captured for 3 h, and the division was filmed. Cells transfected with scrambled shRNA showed a connected, yet very short, midbody. After 90 min, the two cells separated (Fig. 3D). The c-Yes-depleted cells showed a thicker, more elongated midbody. After 90 min, the midbodies showed no change (Fig. 3E).

Depletion of c-Yes induces multinucleation and aneuploidy

HT-29 cells that were sorted and transfected with either c-Yes or scrambled shRNA were observed by both optical and confocal microscopy. Figure 4D and F show normal cell processes, whereas in Figure 4E and G, the bottom two panels show abnormal morphologies. Views obtained using the optical microscope show the cells during interphase. Cells depleted of c-Yes were multinucleated. The images captured using the confocal microscope show the cells during M phase. The cells transfected with the scrambled control shRNA showed proper cytokinesis, starting with a cleavage furrow and proceeding to cell division. The c-Yes-depleted cells showed two nucleus-like structures. To quantify the degree of aneuploidy, a PI assay was conducted using FACS to measure DNA quantity. Figure 4A and B show that the

percentage of aneuploidy doubled from approximately 7% to 15%. The arrow indicates the slight peak that represents 6n. The samples were quantified, and the results are shown in Figure 4C.

Depletion of c-Yes stunts the proliferation of HT-29 cells

The viability of cells that were transfected with either c-Yes shRNA or scrambled shRNA was analyzed. The optical density was measured at 24 h and 48 h (Fig. 3C). Initially, 3000 cells

were seeded, and the proliferation rate was measured using CCK-8. After 24 h, a difference in the proliferation rate was observed, which can also be interpreted as a difference in the cell division rate. The incomplete cell division caused by the depletion of c-Yes also affected the overall division rate and cell number; this difference was more pronounced after 48 h. Considering the doubling time of HT-29 cells, which is approximately 22 h, the doubling time of the c-Yes-depleted cells doubled from 24

Figure 1. Cancer stem cells. (A) Sorting of cancer stem cells. HT-29 cells were detached, washed, and labeled with an anti-CD133-PE antibody. Panel a shows the upper and lower gates, which include the populations of cancer stem cells and non-cancer stem cells, respectively (green: CD133⁺ cells, blue: CD133⁻ cells). (B) HT-29 cell populations at 18 days after sorting. The cells generally maintained their sorted states; 92% of the positive cells were maintained and 84% of the negative cells were maintained. (C) Immunoblotting of the sorted cells to confirm CD133 levels and to verify the differences in c-Yes levels. (D) Confirmation of c-Yes depletion in HT-29 cells transfected with either c-Yes short hairpin RNA (shRNA) or scrambled shRNA by Western blotting.

Figure 2. mRNA expression of stemness and differentiation markers. (A-D) Tumor spheres from CD133⁺ and CD133⁻ cells transfected with either c-Yes short hairpin (shRNA) or scrambled shRNA were grown in SFM medium supplemented with 10 ng/mL epidermal growth factor and 10 ng/mL fibroblast growth factor. After 3 weeks, spheres larger than 400 μ m in diameter were counted, and these data were graphed (D). When c-Yes was depleted, the original sphere-forming ability of CD133⁺ cells was diminished to levels even lower those of the CD133⁻ cells. (E) The expression levels of stemness and differentiation markers in CD133⁺ and CD133⁻ cells were measured by RT-PCR. FACS-sorted HT-29 cells were transfected with plasmids expressing c-Yes shRNA (Santa Cruz) or scrambled sequence (control). cDNA samples from these cells were then subjected to RT-PCR to detect *CD133*, *c-Yes*, *Nanog*, *Oct4*, *CK20*, and β -actin. *CD133*, *Nanog*, and *Oct4* are stemness markers, whereas *CK20* is a marker of differentiated colon cancer cells. mRNA: messenger RNA, SFM: serum-free medium, RT-PCR: reverse transcription-polymerase chain reaction.

h to approximately 48 h.

DISCUSSION

Accumulating evidence suggest the existence of a tumor-initiating

cell population known as CSCs [8-10]. The CSC model is one of the current hotspots in cancer research, for which a distinct population of cells with stem cell-like behavior has been identified [10,11]. Colon tumors contain cells with stem cell-like properties such as self-renewal and heterogeneous dif-

Figure 3. c-Yes depletion causes abscission failure. (A) HT-29 cells transfected with control short hairpin RNA (shRNA) or scrambled sequence. (B) HT-29 cells transfected with c-Yes shRNA to induce gene depletion. Arrows indicate midbody elongation and incomplete cytokinesis. (C) HT-29 cells were transfected with either c-Yes shRNA or the control scrambled shRNA. Cell proliferation was measured at 24 h and 48 h by using CCK-8. c-Yes-depleted cells showed a slower doubling rate. (D and E) Sorted, transfected, and synchronized HT-29 cells were examined using live cell microscopy for 3 h (up to 90 min are shown). Cells transfected with scrambled shRNA showed complete cell division by 90 min, whereas c-Yes-depleted cells showed unchanged elongation of midbodies at 90 min.

ferentiation. In colorectal cancer, CD133 is considered to be a CSC marker [12].

c-Yes encodes a 62-kDa tyrosine kinase protein that has ex-

tensive homology with *c-Src*. *c-Yes* expression has been detected in a variety of tissues, including epithelial, connective, vascular, smooth muscles, and neuronal tissues [13-15]. Although

Figure 4. *c-Yes* is involved in chromosomal control. (A and B) FACS PI DNA analysis was used to analyze the cell cycle in HT-29 cells transfected with either scrambled (A) or *c-Yes* (B) short hairpin RNA (shRNA). *c-Yes*-transfected cells showed an increase in the number of cells with abnormal content, especially in multiples of *n*, which indicates multinucleation (arrow at 6*n*). (C) The percentage of cells with aneuploidy was plotted (D-G). Multinuclear cell formation was observed in HT-29 cells depleted of *c-Yes* by optical microscopy (D and E) and confocal microscopy (F and G). PI: propidium iodide.

c-Src is expressed in the same tissues as c-Yes, their expression patterns are quite different. The difference in the expression patterns of these two proteins provides evidence that c-Yes performs functions unique from those of c-Src. Although c-Yes has not been studied as much as c-Src, their respective signaling pathways have been determined. c-Yes has been suggested to be involved in the control of cell proliferation, and recently, it was shown to be involved in differentiation and various signal transduction pathways. One of the key issues in stem cell biology is the mechanism that regulates pluripotency, a crucial feature of stem cells [16]. c-Yes is highly expressed in pre-replicative and undifferentiated cells; this suggests that c-Yes has possible roles in differentiation [17]. Indeed, subsequent research has shown that treatment of various embryonic stem cell lines with Src inhibitors induces their differentiation [16,17]. Blake et al. [18] showed that treatment with the Src inhibitor SU6656 induced the differentiation of murine embryonic stem (ES) cells, whereas Weinstein et al. [19,20] implicated Src in germ layer formation in *Xenopus laevis* embryos. Meyn et al. [16] showed that treatment with the Src inhibitor SU6656 induced the differentiation of murine ES cells, whereas Annerén et al. [17] showed that c-Yes is necessary to maintain mouse and human ES cells in undifferentiated states.

We used three different stemness-related genes, namely, *Nanog*, *Oct4*, and *CK-20*, whose expression levels are indicative of the differentiation status of CSCs [17]. *Nanog* and *Oct4* are nuclear transcription factors of stem cells, whereas *CK-20* is a marker of differentiated colorectal cells [10,17]. The expression of these genes, which reflects the CSC differentiation status, was assessed by RT-PCR in both c-Yes-depleted CD133⁺ and CD133⁻ HT-29 cells. c-Yes was depleted by transfecting a plasmid expressing a c-Yes-specific shRNA, and a shRNA with a scrambled sequence was used as a control. Depletion of c-Yes caused a decrease in the population of cells with stem-like properties, and induced their differentiation, compared to the cells that were transfected with a scrambled shRNA (Fig. 2). To confirm the RT-PCR data, we performed a sphere formation assay. Taken together, these results suggest that c-Yes activation is important for maintaining human colon CSCs in an undifferentiated state in order to maintain their self-renewal and stem cell-like properties. Future studies may validate the use of c-Yes as a candidate for differentiation therapy, because of its ability to induce CSC differentiation.

The last stage of mitosis is cytokinesis, when the two daughter cells split into two separate cells [21,22]. During cytokinesis, the two daughter cells are connected by a bundle of microtubules called the midbody. The midbody is known to harbor the proteins required for abscission, which is the final severing of the connected cells [21]. Previous studies have localized c-Yes

to the midbody, which indicated that it plays a role in cell cycle completion [14]. In the present study, we showed that c-Yes is required for abscission in HT-29 cells. c-Yes depletion caused abscission failure, as evidenced by an unusually elongated intracellular bridge.

According to Boivin et al. [6], the midbody is also the site where CD133 is located, clumped around this area. According to Ettinger et al. [23], CD133 is released from differentiated cells after abscission, whereas it is internalized in the midbody when the cells maintain their self-renewal properties. In addition, Sancier et al. [7], Park et al. [24], and Cartwright et al. [25] demonstrated an increase in the specific activity of c-Yes as cells exit mitosis. In their study, it was proposed that because of abnormal abscission, the division rate of these cells is significantly slower than that of undepleted cells. Indeed, our study shows that the proliferation of c-Yes-depleted cells was 1.5-times slower than that of control cells. Using live cell imaging, synchronized cells were captured during cytokinesis (Fig. 3).

Aneuploidy refers to cells in which chromosomal separation is aberrant, which results in abnormal chromosomal content. Aneuploidy is the most common characteristic of human tumors; therefore, we propose that aneuploidy contributes to and drives tumor development. Previous studies have shown that, for many colorectal cancer cell lines, the chromosomal content is constantly reshuffled. The distribution of chromosomal aneuploidy can be explained in part by the localization of c-Yes in the centrosomes [14]. The centrosomes are the organizing centers of the microtubules that are responsible for proper chromosomal division during the M phase. The localization of c-Yes in the centrosomes provided clues as to how c-Yes may be involved in the formation of aneuploidy or multinucleation. Figure 4 show that c-Yes-depleted cells induce multinucleation. According to Ratushny et al. [26], inhibition of Src resulted in the overexpression of the mitotic regulator Aurora kinase A, which induced tumor aneuploidy and chromosomal instability. This suggests that c-Yes functions to induce aneuploidy and to further promote tumorigenesis; however, further mechanistic studies are needed to confirm this hypothesis.

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Conflicts of Interest

The authors have no conflicts of interest.

Ethical Statement

There are no animal experiments carried out for this article.

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