Curcumin Promotes Autophagic Survival of a Subset of Colon Cancer Stem Cells, Which Are Ablated by DCLK1-siRNA

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Abstract
Curcumin is known to induce apoptosis of cancer cells by different mechanisms, but its effects on cancer stem cells (CSC) have been less investigated. Here, we report that curcumin promotes the survival of DCLK1-positive colon CSCs, potentially confounding application of its anticancer properties. At optimal concentrations, curcumin greatly reduced expression levels of stem cell markers (DCLK1/CD44/ALDH1A1/Lgr5/Nanog) in three-dimensional spheroid cultures and tumor xenografts derived from colon cancer cells. However, curcumin unexpectedly induced proliferation and autophagic survival of a subset of DCLK1-positive CSCs. Spheroid cultures were disintegrated by curcumin in vitro but regrew within 30 to 40 days of treatment, suggesting a survival benefit from autophagy, permitting long-term persistence of colorectal cancer. Notably, RNA interference-mediated silencing of DCLK1 triggered apoptotic cell death of colon cancer cells in vitro and in vivo, and abolished colorectal cancer survival in response to curcumin; combination of DCLK1-siRNA and curcumin dramatically reversed CSC phenotype, contributing to attenuation of the growth of spheroid cultures and tumor xenografts. Taken together, our findings confirm a role of DCLK1 in colon CSCs and highlight DCLK1 as a target to enhance antitumor properties of curcumin. Cancer Res; 1–12. ©2014 AACR.

Introduction
Colorectal cancer incidence remains very high in the United States and Western world (1), and even though several antibodies/molecules have been developed for treating colorectal cancers, surgical removal and chemo/radiotherapy remains standard of care. Colon cancer stem cells (CSC) are believed to be resistant to chemo/radiotherapy, and a major cause of relapse (2). Several colorectal cancer stem cell markers, with extracellular domains, have been identified, including CD44 (3), Lgr5 (4, 5), and DCLK1 (6, 7). We recently reported that immortalized embryonic epithelial cells, induced to overexpress progastrin (HEKmGAS), developed tumorigenic/metastatic potential (8). Transformed HEKmGAS CSCs coexpressed stem cell markers DCLK1/CD44, while nontumorigenic (HEKC) cells did not (8). Normal stem cells (NSC) from colonic crypts, positive for DCLK1/Lgr5, also do not coexpress CD44 (9). Here, we confirmed colorectal cancer cells (HCT-116, DLD-1, and HT-29) coexpress stem cell markers DCLK1/CD44, similar to tumorigenic HEKmGAS cells (8).

Curcumin (active ingredient in turmeric powder), isolated from Curcuma longa (10), is an important ingredient of Asian foods. Curcumin has potent anti-inflammatory, antibacterial, and anticancer effects (10), but lacks solubility in aqueous solutions; bioavailable formulations have been developed (11) and are being examined in phase I/II clinical trials (Dr. L. Helson, personal communication; ref. 12). Curcumin is nontoxic at high doses (12 g/d) and targets multiple oncogenic pathways (10, 12–14). However, proapoptotic effects of curcumin are attenuated by autocrine growth factors (14). Thus, subpopulations of cancer cells may escape inhibitory effects of curcumin; this concept was extended to CSCs in the current studies.

Curcumin induces caspase-3–dependent and -independent apoptosis due to autophagy (13, 15). Autophagy represents a double-edged sword that causes either cell death or survival of cells (16). Majority of the studies suggest that curcumin-induced autophagy is a prodeath signal (13, 17, 18). However, curcumin-induced autophagy also allows tumor-initiating cells to survive and either differentiate (19), or become senescent/quiescent (20, 21). Possible autophagic effects of curcumin on CSCs remain unknown. Inhibitory effects of curcumin have been reported on CSCs (22–25). However, response of CSCs, positive for DCLK1/CD44/Lgr5, to curcumin remains unknown. Here, we examined inhibitory effects of curcumin against colon cancer cells in vitro and in vivo, in relation to effects on apoptosis/autophagy/proliferation of DCLK1/CD44/Lgr5+ stem cells. Spheroidal regrowth assay was used to examine resistance of CSCs to curcumin. Our results suggest the novel possibility that DCLK1+ cells survive curcumin-induced autophagy.

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Because a subpopulation of DCLK1+ cells survived inhibitory effects of curcumin, we examined inhibitory efficacy of DCLK1-siRNA±curcumin, against growth of HCT-116 cells in vitro and in vivo. Our studies demonstrate that DCLK1-siRNA induces apoptosis of colon cancer cells/tumors in the absence of autophagy; combination of curcumin±DCLK1-siRNA induced massive apoptotic/autophagic cell death, resulting in almost complete loss of stem cell populations expressing DCLK1/CD44/Lgr5.

Materials and Methods

Materials and methods used in the current study are similar to that described previously (8). Reagents used are detailed in Supplementary Methods (A). HCT-116, DLD-1, and HT-29 colon cancer cells from American Type Culture Collection (ATCC) were maintained in Dulbecco’s Modified Eagle Medium as previously described (26). These cells were purchased in 1990s from ATCC and were authenticated by BioSynthesis DNA Identity Center in 2012.

Treatment of cells

Subconfluent cells in monolayer cultures were treated with either dimethyl sulfoxide (DMSO) or nontargeting siRNA (controls) or optimally effective concentrations of curcumin (25 μmol/L±DCLK1-siRNA (100 nmol/L), based on preliminary studies with increasing concentrations of these agents. After 24 to 48 hours of treatment, cells were processed for measuring viability/proliferation/apoptosis/autophagy by published methods (8, 17, 18), as detailed in Supplementary Methods (B).

Viability/proliferation. Briefly, viability/proliferation of cells was measured by Trypan blue exclusion test and MTT assay, respectively, as described previously (8).

Apoptosis/autophagy/proliferation. To assess the percentage of cells undergoing apoptosis/autophagy/proliferation, control and treated cells/spheroids, dissociated enzymatically and cytospun onto slides, were fixed and processed for staining with antibodies against apoptotic marker (activated caspase-3), autophagic marker (LC3A/B-I/II), and proliferation marker [proliferating cell nuclear antigen (PCNA)]. Live cells were also stained with Acridine Orange to visualize autophagic vesicles.

Colon cancer cells grown as primary/secondary spheroids

Colon cancer cells were grown as primary/secondary spheroids, as previously described by us (8). Primary spheroids were treated on day 6 after seeding the wells (at which time well-formed primary-spheroids were present), with optimally effective concentrations of curcumin±DCLK1-siRNA, as described in legend of Fig. 2A. For generating secondary spheroids, wells containing primary spheroids were enzymatically dissociated, and approximately 5,000 cells replated in low-attachment plates as described in Supplementary Methods (C).

Relapse experiment. For the relapse experiment (Fig. 2), control/treated primary spheroids were dissociated and replated as secondary spheroids and imaged daily with white light microscopy. In some experiments, spheroids were processed for paraffin embedding, followed by hematoxylin and eosin (H&E) staining/immunohistochemistry/immunofluorescence staining, as previously described (8). Control/treated spheroids were also processed for either Western blot analysis or cell viability, as described above.

FACsorted colon cancer cells and analysis for stem cell markers

Colon cancer cells were FACsorted and analyzed for stem cell markers, as described previously (8) and detailed in Supplementary Methods (D). Briefly, subconfluent cells were harvested and processed for labeling with fluorophore-tagged antibodies against DCLK1/Lgr5, FACsorted into distinct populations of positive/negative cells, cytospun and fixed on slides, and processed for immunofluorescence staining with anti-CD44 antibody and 4’,6-diamidino-2-phenylindole (DAPI). Images acquired with an epifluorescent microscope were analyzed using METAMORPH, v6.0 software (Molecular Devices).

Cells grown as subcutaneous xenografts in athymic (SCID/nude) mice

Cells were grown as subcutaneous xenografts in athymic (SCID/nude) mice as described previously (8) and detailed in Supplementary Methods (E). Briefly, 5 × 106 cells were inoculated subcutaneously in both flanks of female athymic mice. One week after injection, xenografts were visible on both sides. Mice, bearing tumors, were randomly divided into groups of 3 each. Mice were injected every 2 days with either 0.01% DMSO (Control), curcumin (3 mg/100 μL in 0.01%DMSO = 100 μmol/L), nontargeting siRNA (control; 100 (nmol/L)/100 μL PBS), DCLK1-siRNA (0.5 pmol/100 μL in PBS = 100 nmol/L), or curcumin (100 μmol/L) and DCLK1-siRNA (100 nmol/L). Tumor volume was measured every other day. Mice were sacrificed 3 weeks after initiating treatment, and tumors were removed and weighed and processed for Western blot/immunofluorescence analysis.

Western blot analysis of cells growing either as 2D cultures, 3D spheroids, or xenografts

Western blot analysis of cells growing either as two-dimensional (2D) cultures, three-dimensional (3D) spheroids, or xenografts was conducted as detailed in Supplementary Methods (F). Briefly, cells/tumors were processed for Western blot analysis as previously described (8). Blots were cut into horizontal strips containing target or loading control proteins and processed for detection of antigen–antibody complexes by chemiluminescence. Membrane strips containing target/loading control proteins were simultaneously exposed to autoradiographic films. In cases in which limited samples were analyzed for multiple proteins, loading control β-actin was measured in a corresponding sample containing equivalent protein. In a few cases, β-actin was stripped to measure target protein with equivalent protein with similar molecular mass within the same membrane. Relative band density on scanned autoradiograms was analyzed using Image program (rsbweb.nih.gov/ij/download) and expressed as a ratio of β-actin in corresponding samples.

Transient transfection of cell/spheroids with double-stranded siRNA oligonucleotide

Transient transfection of cell/spheroids with double-stranded siRNA oligonucleotide is detailed in Supplementary Methods (G)
and was conducted as previously described (8). Transfected cells in 2D were propagated in normal growth medium containing 10% fetal calf serum, and growth was examined after 48 hours in an MTT assay. Transfected spheroids were maintained in spheroid medium as described in Supplementary Methods (C).

**Statistical analysis of data**

Data are presented as mean ± SEM of values obtained from four to six samples from two to three experiments/mice. To test for significant differences between means, nonparametric Mann–Whitney test was used using StatView 4.1 (Abacus Concepts, Inc.); \(P\) values were considered statistically significant if less than 0.05.

**Results**

**DCLK1+ colon CSCs coexpress CD44**

Monolayer cultures of colon cancer cell lines (HCT-116/DLD-1/HT-29) were analyzed by either immunofluorescence or FACSorting for expression levels of CSC markers, DCLK1/CD44/Lgr5, as previously described (8). On an average, 2% to 3% of cells expressed stem cell markers (data not shown). We recently reported that transformed/tumorigenic embryonic epithelial cells coexpressed stem cell markers DCLK1/CD44, unlike isogenic nontumorigenic cells (8). Human colon cancer cell lines were similarly positive for transformed phenotype (representative data from HCT-116 cells are presented in Supplementary Fig. S1i). Majority of FACSorted DCLK1+ cells (>80%) coexpressed CD44, whereas FACSorted Lgr5+ cells did not (Supplementary Fig. S1ii and S1iii). Surprisingly, a large number of CD44+ cells cosorted with Lgr5+ cells (Supplementary Fig. S1i and S1iii), suggesting that a subpopulation of Lgr5+ cells may be tightly adherent to CD44+ cells. Lineage-tracing studies in the future may allow us to determine whether the adherent CD44+ cells perhaps represent daughter progenitor cells, derived from Lgr5+ cells. CD44+ cells, which cosorted with Lgr5+ cells, did not coexpress DCLK1 (data not shown), unlike coexpression of CD44 by a majority of DCLK1+ cells (described above). CSCs, positive for either DCLK1 or Lgr5, were mostly present along outer edges of spheroids, derived from colon cancer cells (Supplementary Fig. S2A); CD44+ cells, on the other hand, were distributed throughout the spheroids, providing further evidence that cells positive for only CD44
may perhaps represent daughter progenitor cells. Coexpression of CD44 and DCLK1 was evident in cells along outer edges of spheroids, while coexpression of Lgr5 and CD44 was less frequent (Supplementary Fig. S2Bi and S2Bii).

Curcumin attenuates growth of HCT-116 cells/spheroids associated with loss of stem cell markers

Curcumin (25 μmol/L) was optimally effective in reducing growth of HCT-116 cells in 2D cultures by >50% (Fig. 1A), resulting in reduced expression of stem cell markers DCLK1/Lgr5/CD44 (Fig. 1B). Low doses of curcumin (10 μmol/L) did not significantly reduce number of spheroidal growths/well, but had morphologic effects (Fig. 1Ci and ii). Curcumin (25 μmol/L), reduced total number of tumorospheres by >60%/well, associated with disintegration of spheroids (Fig. 1Ci and ii), in a time-dependent manner (Fig. 1Ciii), along with caspase-3 activation (Fig. 1Di and ii).

Regrowth (relapse) of curcumin-treated HCT-116 spheroids

HCT-116 primary spheroids growing in 24-well plates were treated on day 6 with either control vehicle or 25 μmol/L of curcumin for 48 hours (Fig. 2Ai). Primary spheroids on day 8 were dissociated and replated as secondary spheroids. By day 4, cells from the control group started growing as secondary spheroids, whereas curcumin-treated cells did not (Fig. 2Ai). For approximately 28 days, secondary spheroids did not form from curcumin-treated samples, while control samples

![Figure 2. A and B, regrowth/relapse and reexpression of stem cell markers in curcumin-treated HCT-116 spheroids. Equal number of HCT-116 cells, seeded in 24 well plates, were treated on day 6 with or without curcumin (25 μmol/L; single arrow). Ai, after 48 hours, primary spheroids/cells were harvested and plated as secondary spheroids/cells (two arrows). Representative images of secondary spheroids/cells imaged until day 45. Aii, cell viability (%) of secondary spheroids/cells; data, mean ± SEM of 8 wells/two experiments. *P < 0.05 versus control values; †P < 0.05 versus curcumin values. Bi and ii, representative Western blot data from secondary spheroids/cells in duplicate wells derived from control/curcumin–treated samples. C, treatment of HCT-116 spheroids with DCLK1-siRNA. Representative Western blot data (i) and images (ii) of HCT-116 primary spheroids treated with control or DCLK1-siRNA (100 nmol/L) for 48 hours. Representative H&E staining of spheroid sections shown in the last panels.](https://cancerres.aacrjournals.org/content/74/6/1762/F2)
developed dense secondary spheroids. By days 28 to 30, small spheroidal structures appeared in wells containing curcumin-treated samples (Fig. 2Ai). By day 45, curcumin-treated spheroidal cells had regrown as secondary spheroids, suggesting that a subset of stem cells survived curcumin. Secondary cells/spheroids were isolated as single cells at indicated days and analyzed for cell viability (Fig. 2Aii). Surprisingly, approximate-ly 25% of curcumin-treated cells were viable on day 4 after replating (Fig. 2Aii), increasing dramatically by days 30 to 45, matching regrowth of treated-cells as secondary spheroids (Fig. 2Ai and ii). Relative levels of Lgr5 remained stable in secondary spheroids from control wells, while relative levels of DCLK1 increased 2- to 3-fold by days 30 to 45 (Fig. 2Bi and ii). Curcumin treatment of primary spheroids resulted in almost complete attenuation of Lgr5, but low levels of DCLK1 expression in HCT-116 spheroids (Fig. 2Ci), disintegrating HCT-116 spheroids (Fig. 2Bai and ii). Relative levels of Lgr5/DCLK1 were increased in curcumin-treated samples (Fig. 2Ai). By day 45, curcumin-treated spheroids were isolated as single cells at indicated days and analyzed for cell viability (Fig. 2Aii). Surprisingly, approximate-ly 25% of curcumin-treated cells were viable on day 4 after replating (Fig. 2Aii), increasing dramatically by days 30 to 45, matching regrowth of treated-cells as secondary spheroids (Fig. 2Ai and ii). Relative levels of Lgr5/DCLK1 were increased in curcumin-treated spheroids; by day 45, levels had increased 2- to 4-fold (Fig. 2Bii). Because curcumin attenuated Lgr5 expression but not DCLK1, we used DCLK1-siRNA for targeting DCLK1+CSCs.

**DCLK1-siRNA targets DCLK1 expression and induces disintegration of HCT-116 spheroids**

HCT-116 spheroids were treated with either control or DCLK1-siRNA. DCLK1-siRNA (100 nmol/L) attenuated DCLK1 expression in HCT-116 spheroids (Fig. 2Ci), disintegrating spheroids within 48 hours (Fig. 2Cii); lower concentrations were less effective (data not shown). Control-siRNA had no effects. Surprisingly DCLK1-siRNA was more effective than curcumin, while combination of DCLK1-siRNA+curcumin was significantly more effective than either agent alone, in both 2D (Fig. 3Ai) and 3D (Fig. 3Aii and iii).

**Inhibitory effects of curcumin±DCLK1-siRNA against growth of HCT-116 xenografts in vivo**

Mice inoculated subcutaneously with 5 x 10^6 HCT-116 cells had palpable tumors by day 7, and were injected on ventral side of tumors with either control or DCLK1-siRNA±curcumin.

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**Figure 3.** A–C, inhibitory effects of curcumin (25 μmol/L)± DCLK1-siRNA (100 nmol/L) on growth of HCT-116 cells, growing either as 2D (Ai)/3D (Aii, iii) in vitro or as xenografts in vivo (B and C). Ai–Aiii, cells/spheroids treated for 48 hours as shown. Controls treated with 0.01% DMSO or control-siRNA (100 nmol/L). Growth of cells/spheroids (absorbance at 560 nm) was analyzed as described in Supplementary Methods (C). Data, mean ± SEM of 8 to 12 wells/two experiments for 2D cells (Ai) and 3D-spheroids (Aii). Representative images of spheroids are presented in Aiii. B and C, athymic mice (3/group) inoculated bilaterally with 5 x 10^6 HCT-116 cells and treated with curcumin ± DCLK1-siRNA [as described in Supplementary Methods (E)]. B, representative images of tumor-bearing mice at day 28. Ci, tumor weights (g) at time of sacrifice. Data in Ci/ii, mean ± SEM of six tumors/three mice.

1; P < 0.05 versus control values; 2; P < 0.05 versus curcumin values; 3; P < 0.05 versus DCLK1-siRNA values.
every second day for 3 weeks. Representative tumor-bearing mice, from the five treatment groups, are shown at day 28 in Fig. 3B. Tumor size (volume) continued to enlarge in mice in the order of control (DMSO) > control-siRNA > curcumin > DCLK1-siRNA (Fig. 3Ci). However, in curcumin+DCLK1-siRNA group, the preformed tumors actually began to shrink in size (Fig. 3Ci). After 3 weeks of treatment, mice were euthanized and tumor weights noted. Tumor weights in control versus treated mice followed a similar pattern described above for tumor size (Fig. 3Cii). Surprisingly, DCLK1-siRNA was more effective than curcumin against growth of HCT-116 cells/tumors (Fig. 3Ai and ii, B, and C), suggesting a functional role of DCLK1 in proliferative/tumorigenic potential of CSCs. The latter possibility was confirmed in the relapse experiment with spheroids. Primary spheroids treated with curcumin + DCLK1-siRNA did not re-form secondary spheroids even after 60 days of reploting (data not shown), and cell viability remained <5% after reploting (Fig. 2Aii).

**Inhibitory efficacy of curcumin on relative expression of stem cell markers/transcription factors in HCT-116 cells**

Autocrine progastrin exerts growth-promoting effects on colon cancer cells by activating NF-κB and upregulating relative expression of DCLK1/Lgr5/CD44 (9, 26–29). Control and curcumin-treated monolayer cultures (M), spheroids (S), and tumors (T) were harvested and analyzed by Western blot analysis for the above indicated proteins. Representative Western blot data are presented in Supplementary Fig. S3. Many blot data (Fig. S3Aii) are presented as a percentage change in the ratio of relative levels of target proteins/β-actin in control versus curcumin-treated cells (Supplementary Fig. S2B). Results confirmed that curcumin attenuates activation of β-catenin/NF-κBp65 and reduces relative expression of stem cell markers. Surprisingly, even though curcumin reduced expression of indicated proteins by >40% to 90% (Supplementary Fig. S3), inhibitory efficacy on growth of 2D cells/spheroids/tumors was <50% (Fig. 3A–C). Because curcumin induces autophagy (13, 15–20), which can lead to either cell survival or cell death (16), we next examined autophagy/apoptosis in response to curcumin+DCLK1-siRNA.

**Curcumin induces autophagy and apoptosis of colon cancer cells while DCLK1-siRNA only induces apoptosis**

Curcumin-treated HCT-116 cells were analyzed for relative levels of autophagic markers (LC3A/BI-II and Beclin-1) and activated caspase-3. Representative Western blot data are shown in Fig. 4Ai and ii. Data from several blots are presented as a percentage change in the ratio of relative levels of target proteins/β-actin in control versus curcumin-treated cells (Fig. 4Aiii and iv). Curcumin increased relative levels of LC3-I between 12 and 24 hours, which was processed to generate LC3-II (Fig. 4Aii and B), confirming previous reports (20). Beclin-1 (autophagy-latent gene, ATG6), required to initiate autophagosome formation (30), also increased in a time-dependent manner in response to curcumin (Fig. 4Aii and B). Acidic vesicular organelles (AVO) stain orange/red with acridine orange and specify autophagy (31). Formation of AVOs was confirmed in HCT-116 cells in response to curcumin in a dose- and time-dependent manner (Supplementary Fig. S4A–S4Aiv). Increased expression/formation of LC3-I/II in response to curcumin was confirmed by immunofluorescent staining of HCT-116 cells in culture (Fig. 4B). A significant percentage of LC3+ cells in curcumin-treated samples coexpressed activated caspase-3 (Fig. 4B, merged images), suggesting that curcumin-induced autophagy results in apoptotic death of many HCT-116 cells. DCLK1-siRNA significantly increased staining for activated caspase-3, but not LC3 (Fig. 4B). Combination of curcumin+DCLK1-siRNA significantly increased staining for LC3/activated caspase-3, suggesting that combined regimen may synergistically induce autophagy/apoptosis, wherein majority of autophagic cells go through apoptosis (Fig. 4B).

PCNA+ cells are mainly present at outer edges of control HCT-116 spheroids (Fig. 4C), similar to staining pattern of DCLK1/Lgr5 (Supplementary Fig. S2A and S2B). Curcumin-treated HCT-116 spheroids become positive for PCNA and LC3-I/II in an overlapping area of spheroids (Fig. 4C), suggesting that curcumin-induced autophagy is associated with both apoptosis (Fig. 4B) and proliferation (Fig. 4C).

To further examine cell death/cell survival role of autophagy, HCT-116 cells were treated with an inhibitor of autophagy (3-methylalanine, 3-MA; Supplementary Fig. S4B). Inhibitory effects of curcumin were partially reversed by 3-MA, but not to control levels (Supplementary Fig. S4B), suggesting that curcumin-induced autophagy results in both survival/apoptosis. 3-MA had insignificant effects on cells treated with DCLK1-siRNA or DCLK1-siRNA+curcumin (Supplementary Fig. S4B), confirming that autophagy in DCLK1-siRNA+curcumin–treated cells is mainly linked to apoptosis.

HCT-116 xenografts, harvested from control/treated mice, were processed for H&E/immunofluorescence (Fig. 5). The H&E sections from curcumin-treated tumors demonstrated unique hollow circular areas, surrounded by concentric layers of cells (Fig. 5Ai), not seen in other groups. As observed in 2D/3D cells in vitro (Fig. 4B and C), control/DCLK1-siRNA–treated tumor sections were largely negative for LC3 (Fig. 5Aii and iii). Curcumin-treated tumor sections demonstrated strong LC3 staining in concentric layers of cells surrounding the hollow areas, which seemed free of nucleated cells; combined treatment with curcumin+DCLK1-siRNA significantly augmented LC3 staining, but the hollow areas, surrounded by concentric layers of cells, were not evident any longer (Fig. 5Aii). PCNA+ cells were present along the edges of control tumors, but relatively absent in tumors treated with DCLK1-siRNA or curcumin+DCLK1-siRNA (Fig. 5Aii). Curcumin-treated tumor sections, on the other hand, demonstrated PCNA staining in concentric layers of cells surrounding the hollow areas (Fig. 5Aii), similar to PCNA staining pattern seen in curcumin-treated spheroids (Fig. 4C). Enhanced images from Fig. 5Aii are presented in Supplementary Fig. S5A to present the staining of LC3 and PCNA more clearly. A significant number of LC3-expressing cells coexpressed PCNA (Supplementary Fig. S5A, yellow color in merged images), while cells positive for LC3 and PCNA in control tumor sections were distinct and separate (Supplementary Fig. S5A). Percentage staining for LC3/PCNA per tumor section from 10 to 15
Figure 4. A–C, curcumin induces autophagy/apoptosis in HCT-116 cells/spheroids while DCLK1-siRNA induces apoptosis. A and ii, representative Western blot data from one of three experiments with curcumin-treated HCT-116 cells in 2D; LC3-I/II (Ai) and Beclin-1/activated caspase-3 (Aii) as shown. Aii–iv, % change in Western blot data from all three experiments in Ai–i. Ratio of target protein/β-actin at 0 hours assigned 0% value; ratios at increasing time points presented as % of 0-hour value. B, immunofluorescent staining for LC3-I/II and activated caspase-3 from representative control and activated caspase-3 treated cells, cultured on coverslips, from one of two experiments. Yellow color in merged images, costaining of LC3/activated caspase-3 (~7%), or DCLK1-siRNA increased several fold. Control tumor sections were positive for PCNA staining in 4% to 5% of the area, while curcumin-treated tumor sections demonstrated DCLK1 staining in concentric layers of cells surrounding the hollow areas (Fig. 6A and Supplementary Fig. S5Bi and S5Bii). Surprisingly, curcumin/curcumin + DCLK1-siRNA caused complete attenuation of transformed phenotype, with negligible coexpression of CDK1/CD44 and Lgr5/CD44 (Fig. 6A and Supplementary Fig. S5B), confirming transformed phenotype of CSCs. Treatment with curcumin/DCLK1-siRNA caused complete attenuation of transformed phenotype, with negligible coexpression of CD44 with DCLK1-Lgr5, while LC3 staining of tumor sections treated with curcumin/curcumin + DCLK1-siRNA increased 2-fold from control levels; DCLK1-siRNA + curcumin attenuated PCNA staining to <1% (Fig. 5Aiii). Activation of caspase-3 was minimal in control tumors (~1%) but significantly increased in tumors treated with either curcumin (~7%), DCLK1-siRNA (~6–7%), or DCLK1-siRNA + curcumin (~9%; Fig. 5Bi and ii). Importantly, none of the PCNA + cells were positive for activated caspase-3 (Fig. 5Bi), unlike costaining of LC3 with PCNA in curcumin-treated samples (Fig. 5Aii).
novel possibility that autophagic cells present among the concentric layers of cells, surrounding the hollow areas may represent a subpopulation of DCLK1\textsuperscript{+} cells, which retain the potential to proliferate (as suggested by PCNA-labeling of these cells; Fig. 5Aii and Supplementary Fig. S5A). Curcumin-treated tumor cells also continued to express CD44, unlike DCLK1-siRNA–treated tumors (Fig. 6A and Supplementary Fig. S5B). Curcumin significantly reduced expression of Lgr5, while DCLK1-siRNA was much less effective (Fig. 6A and Supplementary Fig. S5B). Curcumin+DCLK1-siRNA was most effective in attenuating DCLK1/Lgr5 staining along with significant loss in CD44 staining (Fig. 6A and B and Supplementary Fig. S5B). Tumors were also processed for Western blot analysis. Data from all tumors are presented as percentage change in the ratio of target protein/\textbeta-actin, wherein ratio for control samples was arbitrarily assigned 100\% value (Fig. 6B). Values from control groups (vehicle or control siRNA) were almost identical; therefore, a single bar for control values is shown in Fig. 6B. Curcumin significantly reduced relative levels of stem cell markers (DCLK1/Lgr5/ALDHA1) and pluripotent marker (Nanog), associated with a significant loss in levels of activated NF-\kappaBp65\textsuperscript{276} and total \textbeta-catenin, with a 3-fold increase in levels of activated caspase-3 and LC3-II (Fig. 6B; only changes in LC3-II are shown). DCLK1-siRNA attenuated relative levels of DCLK1/ALDHA1/Nanog, but had insignificant effects on Lgr5. DCLK1 had insignificant effects on activated NF-\kappaB, but significantly reduced total \textbeta-catenin, resulting in significantly increasing activated caspase-3 (apoptotic pathway), with no LC3II (autophagy). Curcumin+DCLK1-siRNA was most effective in attenuating...
relative levels of DCLK1/Lgr5/ALDHA1/Nanog/cellular β-catenin, resulting in a robust activation of both caspase-3 and LC3, suggesting that autophagic response to DCLK1-siRNA+curcumin likely leads to cell death rather than survival, unlike the response to curcumin alone.

Discussion

A novel finding of the current study is that a subset of DCLK1+ colon CSCs is resistant to inhibitory effects of curcumin, and that DCLK1-siRNA is more effective than curcumin in reducing tumor mass in vivo. Conventional anticancer therapies (radio/chemotherapy) primarily kill rapidly proliferating cancer cells that form bulk of the tumors, but are believed to spare relatively quiescent CSCs. Dietary agents, such as curcumin, are believed to suppress self-renewal of CSCs, thus, sensitizing drug-resistant tumors (2, 23). A curcumin analog, G0-Y030, inhibited tumorosphere formation from ALDHA1+/CD133+ colon CSCs (22). We used DCLK1/Lgr5/CD44 as markers of colon CSCs, as they mark both normal and cancer intestinal/pancreatic stem cells (4–6, 32, 33). The spheroid relapse assay provided the first evidence that a subset of DCLK1+ cells may be resistant to curcumin, becoming quiescent/dormant for a period of time before re-forming spheroids (Fig. 2A and B), which is a hallmark of stem cells. Resistance of a subset of esophageal squamous carcinoma cells to curcumin has been reported; but the authors concluded that curcumin eliminates CSCs as ALDHA1+/CD44+ CSCs were eliminated by curcumin (24). Another dietary agent was reported to significantly target DCLK1+ CSCs, via the Notch signaling pathway (34). Our findings, however, suggest that DCLK1+ CSCs are not eliminated by curcumin.

Multiple signaling pathways are inhibited by curcumin in epithelial cancers, resulting in apoptotic death (10).
Besides apoptosis, curcumin induces caspase-3–independent apoptosis (autophagy) in cancer cells (15). Autophagy culminates in either cell death or survival/quiescence/differentiation of tumor cells (15, 21, 18). Curcumin-induced autophagy in cancer cells is mainly reported to result in cell death (12, 18). Our results, however, suggest that while curcumin induces apoptotic/autophagic cell death of many cancer cells/CSCs, it seems to induce autophagic survival/quiescence of a subset of DCLK1⁺ CSCs (Figs. 2A and B, 5Aii, and 6A), representing a novel aspect of our findings.

Autophagy has been described as a prosurvival mechanism that acts as a cellular switch between apoptosis and quiescence/senescence (21). Specific pathways, activated in autophagic cells in response to curcumin, protect cells from cell death and allow cells to differentiate or become quiescent (19, 20). Curcumin induces differentiation of autophagic glioma-initiating/embryonic stem cells (19, 35). Similar to our findings, Mosieniak and colleagues (20) observed that a subpopulation of curcumin-treated autophagic colon cancer cells survived, becoming senescent/quiescent; the authors, however, did not examine possible regrowth of cells beyond 72 hours. Our results suggest that curcumin-induced quiescent colon cancer cells may represent DCLK1⁺ CSCs, which become dormant for a period of time, followed by reformation of spheroids.

Apoptotic and autophagic cell death are not mutually exclusive but can induce cell death simultaneously and cooperatively (36), which may explain coexpression of apoptotic/autophagic markers within same cells in response to curcumin (Fig. 4B). Even though autophagy and apoptosis can occur in same cells, autophagy in response to curcumin is believed to be due to endoplasmic reticulum (ER) stress, independent of apoptosis (37). Autophagy either allows ER-stressed cells to survive or drives them toward apoptosis (37); it remains to be determined whether ER stress plays a role in survival of a subset of DCLK1⁺ colon cancer cells, in response to curcumin. Besides ER stress, curcumin also induces reactive oxygen species (ROS), which can result in autophagy (17). It is thus possible that a subset of DCLK1⁺ cells are resistant to curcumin-induced ROS and/or ER stress, and spared from autophagic cell death. A complex cross-talk between several signaling pathways is believed to dictate the outcome of autophagy in cancer cells, which can either survive or proceed to cell death, as recently reviewed (38). In future studies, we will examine the role of some of these pathways in allowing a subpopulation of DCLK1⁺ cells to go through autophagy, associated with survival, rather than apoptosis.

Results of our in vivo studies also suggest that long-term treatment of tumors with curcumin perhaps allows a subset of DCLK1⁺ CSCs to proliferate and maintain tumor mass, which may explain the disconnect between potent inhibitory effects of curcumin against multiple growth-promoting pathways (current studies and refs. 10, 13, 14), but less than optimal inhibitory effects against tumor growth (Fig. 3B and C). Previously also, curcumin was reported to increase a subset of colon cancer cells in S-phase (20); based on our results, we believe that the PCNA⁺ cells likely represent DCLK1⁺ CSCs, which survive curcumin. Results of our preliminary studies further suggest that DCLK1⁺ CSCs also survive other insults, such as radio/chemotherapy (unpublished data from our laboratory), strongly suggesting that DCLK1 expression in CSCs is perhaps linked to chemoresistance of CSCs, allowing cells to survive autophagy by as yet unknown mechanisms.

Because DCLK1⁺ cells seemed to be resistant to curcumin and other chemotherapeutic insults as well, we examined inhibitory effects of DCLK1-siRNA. Downregulation of DCLK1 expression significantly reduces growth of colon cancer cells in vitro and in vivo (7, 39), as confirmed by us (Fig. 3). A surprising and unexpected finding was that DCLK1-siRNA was more effective than curcumin in reducing size/weight of colon cancer tumors growing as subcutaneous xenografts (Fig. 3C). Combination of DCLK1-siRNA+curcumin was even more effective than either agent alone, and caused preformed tumors to lose tumor mass (Fig. 3C). Synergistic inhibitory effects of curcumin against cancer cells/CSCs from many different organs have been previously reported with chemotherapeutic agents (reviewed in ref. 23). Here, we demonstrate for the first time, synergistic inhibitory effects of curcumin with siRNA molecules against a stem cell marker, DCLK1. Although curcumin treatment resulted in both apoptotic cell death and autophagic survival, DCLK1-siRNA caused only apoptotic cell death of colon cancer cells (Figs. 4B and 5B). RNA interference against other CSC markers, such as CD44, also inhibit proliferation and induce apoptosis of colon cancer cells (3), suggesting that DCLK1 and CD44 may represent functional CSC markers, which play an important role in maintaining proliferative potential of cancer cells. Many reports strongly suggest that DCLK1 expression is critically required for maintaining tumorous growths in many different organs, including intestines and pancreas (6, 7, 33, 39, 40), as further confirmed in the current studies.

Combined regimen of curcumin+DCLK1-siRNA augmented both apoptotic/autophagic cell death pathways, with no sign of proliferation/survival of CSC populations. It is therefore speculated that addition of DCLK1-siRNA overcomes resistance of a subset of DCLK1⁺ cells to curcumin, resulting in possible elimination of CSCs, as suggested by the results of the relapse assay (Fig. 2Aii); however, it remains possible that a subset of quiescent CSCs, positive for other stem cell markers, such as Lgr5, remain dormant/undetectable. Our results suggest that while curcumin targets Lgr5⁺ CSCs, DCLK1-siRNA does not eliminate LGR5⁺ CSCs (Fig. 6). On the basis of our results, it is proposed that combination of curcumin+DCLK1-siRNA may therefore be effective toward eliminating CSCs positive for Lgr5/DCLK1/CD44. In a previous study, we had reported that addition of p38MAPK may overcome resistance of insulin–like growth factor-II (IGF-II)–expressing colon cancer cells to curcumin (14). On the basis of recent findings, as discussed above, it may be possible to overcome resistance of a subset of DCLK1⁺ CSCs against curcumin by adding inhibitors of ROS, and/or autophagy, to avoid possible deleterious effects of downregulating DCLK1 in normal intestinal stem cells and neuroprogenitor cells (6, 41, 42). Thus, in the future, it may be possible to avoid toxic effects of radio/chemotherapy by
using combinatorial strategies with nontoxic agents (such as curcumin and DCLK1-siRNA), which target CSCs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: C. Kantara, R. Ulrich, P. Singh
Development of methodology: C. Kantara, M. O'Connell, S. Sarkar, P. Singh
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Kantara, S. Moya
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Kantara, M. O'Connell, S. Sarkar, P. Singh

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