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Depletion of mitochondrial fission factor DRP1 causes increased apoptosis in human colon cancer cells

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ABSTRACT

Mitochondria play a critical role in regulation of apoptosis, a form of programmed cell death, by releasing apoptogenic factors including cytochrome *c*. Growing evidence suggests that dynamic changes in mitochondrial morphology are involved in cellular apoptotic response. However, whether DRP1-mediated mitochondrial fission is required for induction of apoptosis remains speculative. Here, we show that siRNA-mediated DRP1 knockdown promoted accumulation of elongated mitochondria in HCT116 and SW480 human colon cancer cells. Surprisingly, DRP1 down-regulation led to decreased proliferation and increased apoptosis of these cells. A higher rate of cytochrome *c* release and reductions in mitochondrial membrane potential were also revealed in DRP1-depleted cells. Taken together, our present findings suggest that mitochondrial fission factor DRP1 inhibits colon cancer cell apoptosis through the regulation of cytochrome *c* release and mitochondrial membrane integrity.

1. Introduction

Apoptosis, programmed cell death, plays essential roles in development, morphogenesis, differentiation, and homeostasis of multicellular organisms. Thus, dysregulation of apoptosis can be involved in pathogenesis of various human diseases such as autoimmune diseases, AIDS, neurodegenerative diseases, and cancer [1]. Apoptotic cell death is characterized by DNA fragmentation, nuclear condensation, cell shrinkage, and membrane blebbing; these features are orchestrated by activation of cysteine proteases, the caspases [2,3]. Two apoptotic pathways: the extrinsic pathway and the intrinsic pathway that eventually converge by caspase activation have been well characterized [4]. Mitochondria, highly dynamic cellular organelles, activate the intrinsic apoptotic cascade by releasing proapoptotic protein cytochrome *c* from the space between inner and outer mitochondrial membranes into the cytosol which triggers caspase activation.

Mitochondria continually change their morphology to maintain integrity, which is controlled by regulators of mitochondrial dynamics: fission factors mainly composed of DRP1 and Fis1, as well as fusion factors including OPA1 and Mfn1/2 [5]. Recent work suggests that these mitochondrial-shaping proteins are involved in cellular apoptosis [6–8]. The observation that OPA1-depleted HeLa cells containing excess fragmentated mitochondria display enhanced cytochrome *c* release into the cytosol [9,10], suggests that mitochondrial fission has an important role in cytochrome *c* release during apoptosis. In contrast, another report suggests that mitochondrial fission is not required for apoptosis. Indeed, inhibition of mitochondrial fission induced by depletion of DRP1 has no apparent effects on the apoptotic response of HeLa cells, although it causes partial reductions in cytochrome *c* release [11]. Therefore, further studies are awaited to elucidate the mechanistic link between mitochondrial shape and apoptosis, which will help to develop new therapies targeting mitochondrial dynamics for diseases caused by dysregulation of apoptosis including cancer.

In this study, we address the function of mitochondrial fission factor DRP1 in human colon cancer cells using RNA interference technology to down regulate DRP1 expression. Surprisingly, our findings showed that inhibition of mitochondrial fission factor resulted in enhanced cytochrome *c* release and apoptosis in colon cancer cells.

2. Materials and methods

2.1. Cell culture

Human colon cancer cells, HCT116 and SW480 were obtained from ATCC. HCT116 cells were cultured in McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS) under 5% CO_2 at 37 °C. SW480 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS under 5% CO_2 at 37 °C.

2.2. Knockdown of DRP1

To reduce expression levels of DRP1 in human cells, small interfering RNA (siRNA) targeting DRP1 sequence (siDRP1#1_5′-AAGCA-

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GAAGAATGGGGTAAAT-3′ [12] and siDRP1#2_5′-GGAGCCAGCTAGATATTAA-3′) was chemically synthesized in QIAGEN and Dharmacon. AllStars nonsilencing siRNA (QIAGEN) having no homology to any known mammalian gene was used as a negative control. Annealed siRNA was transfected into HCT116 and SW480 cells using RNAiMAX reagent (Invitrogen). After 72 h, DRP1 expression was analyzed by immunoblotting using antibodies against DRP1.

2.3. Visualization of mitochondrial network

HCT116 and SW480 cells grown on glass coverslips were incubated in growth medium supplemented with 100nM MitoTracker Orange CMTMRos (Invitrogen) for 30 min under 5% CO_2 at 37 °C, washed in PBS, and fixed in 4% paraformaldehyde for 15 min at RT. After PBS washes, coverslips were treated with 1% Triton X-100 in PBS for 5 min and then mounted. Fluorescence images were acquired with a 63 × oil immersion objective on a LSM 510 META confocal microscope (Zeiss).

2.4. Cell fractionation and immunoblotting

HCT116 and SW480 cells were fractionated using a Qproteome Cell Compartment Kit (QIAGEN) according to the manufacturer's instructions. Whole cell lysates (WCL) were prepared from HCT116 and SW480 cells with protein extraction buffer (50 mM Tris–HCl pH 6.8, 2% SDS, 0.1 M DTT, 5 mM EDTA, 10% Glycerol, 0.1% BPB). Each cell fraction and WCL was separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) on 12 or 15% gels and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). PVDF membranes were incubated with antibodies to DRP1 (clone 8) (BD transduction laboratories), OPA1 (Abcam), Cleaved Caspase-3 (Asp175), CoxIV (Cell Signaling), GAPDH (B-2), and LDH (H-160) (Santa Cruz Biotechnology), followed by incubation with alkaline phosphatase-conjugated antibodies to mouse immunoglobulin G (IgG) or rabbit IgG (Santa Cruz Biotechnology). Blots were visualized with the BCIP/NBT system (Promega). The relative signal intensity of each protein was measured using NIH Image software.

2.5. Determining cell number

Cell number was quantified using a Scepter Handheld automated cell counter with 60 μm Scepter Sensors (Millipore), which employs the Colter principle of impedance-based particle detection. The concentration of viable cells was identified based on cell volume/size measurements using Scepter software Pro 2.0.

2.6. Apoptosis assay

To assess the apoptotic status of cells, HCT116 and SW480 cells were collected and stained with fluorescein isothiocyanate (FITC)-conjugated Annexin V and 7-aminoactinomycin D (7-AAD) in 1 × binding buffer (BD Pharmingen) for 15 min at RT and then analyzed using FACSCalibur flow cytometry (BD Biosciences).

2.7. DNA content analysis

DNA content was determined by flow cytometry after staining cells with Propidium Iodide (PI). In brief, HCT116 and SW480 cells were collected and fixed with 70% ethanol for 30 min at −20 °C. After washing with PBS containing 2% BSA, cells were then stained with PI/RNAse staining buffer (BD Pharmingen) for 15 min at RT and analyzed by flow cytometry.

2.8. Measurement of mitochondrial membrane potential

Mitochondrial membrane potential was analyzed using the dual emission fluorescent probe JC-1 (Invitrogen). In brief, collected cells were resuspended in growth medium containing 10 μg/ml JC-1 and cultured for 30 min under 5% CO_2 at 37 °C, then fluorescence changes were analyzed by flow cytometry.

3. Results

3.1. DRP1 knockdown induces mitochondrial elongation in HCT116 and SW480 cells

To investigate the function of DRP1 in colon cancer cells, we used two independent siRNAs (siDRP1#1 and siDRP1#2) designed specifically to block DRP1 expression. As shown in Fig. 1A, both siRNAs targeted to DRP1 but not nonsilencing control siRNA (siCtr) efficiently reduced DRP1 expression in HCT116 and SW480 cells. We first examined the effect of DRP1 depletion on mitochondrial morphology. We stained mitochondria of DRP1 siRNA-treated HCT116 and SW480 cells with the mitochondria-specific probe MitoTracker Orange CMTMRos. Visualization of mitochondria revealed that on DRP1 depletion, more than 85% of observed cells had elongated (highly connected) mitochondria, whereas we hardly detected mitochondrial elongation in siCtr-treated cells (Fig. 1B and C). Notably, many elongated mitochondrial tubules containing balloon-like structures were seen in SW480 cells transfected with DRP1 siRNAs (Fig. 1B) similar to the phenotype in DRP1-knockdowned HeLa cells [10]. Collectively, these results indicate that DRP1 is required for mitochondrial fission in colon cancer cells.

3.2. Knockdown of DRP1 results in decreased cell growth and increased apoptosis

We next examined proliferation of DRP1-depleted HCT116 and SW480 cells. As shown in Fig. 2, the cell number of siCtr-transfected control cells increased in a time-dependent manner, whereas treatment of DRP1 siRNAs significantly reduced proliferation of HCT116 and SW480 cells (Fig. 2).

To assess whether inhibition of DRP1 had an effect on apoptosis, we stained DRP1-knockdowned cells with fluorescently labeled Annexin V, an anticoagulant protein that has high affinity and selectivity for phosphatidylserine (PS), which is exteriorized during apoptosis, and 7-AAD, a membrane-impermeable dye, to monitor cell membrane integrity. Annexin V-positive/7-AAD-negative cells or double positive cells are defined as early apoptosis or late apoptosis, respectively. Flow cytometric analysis revealed that the percentage of total apoptotic cells (both early and late) was significantly higher in siDRP1-treated cells than that in siCtr-treated cells (Fig. 3A). We then analyzed caspase-3 processing by immunoblotting as a measure of activation of caspase-3, which is the central caspase responsible for the proteolytic cascade leading to cell death. We observed elevated caspase-3 cleavage on down-regulation of DRP1 (Fig. 3B), indicating that DRP1 depletion induces caspase-3 activation. Cell cycle analysis identified an increase in the number of sub-G0/G1 (<2 N DNA content) after DRP1 reduction (Fig. 3C). Accumulation of the sub-G0/G1 population is also characteristic of apoptotic cells and reflects fragmentation of chromosomal DNA in such cells. Taken together, these observations indicate that reductions in DRP1 enhance colon cancer cell apoptosis, suggesting that DRP1-mediated mitochondrial fission is important for survival of these cells.

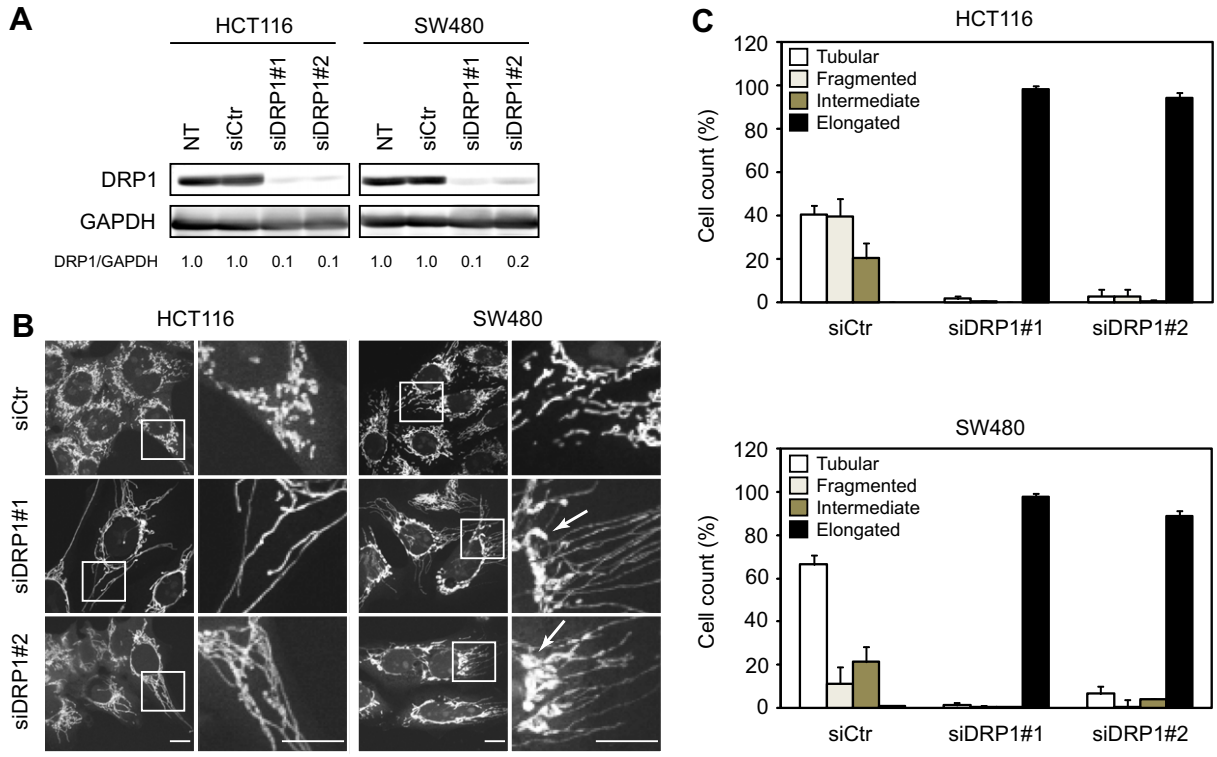

Fig. 1. Reduction of DRP1 expression by siRNA results in mitochondrial elongation. (A) Expression level of DRP1 protein. Whole cell lysates prepared from HCT116 and SW480 cells transfected with siRNAs targeted to DRP1 (siDRP1#1, siDRP1#2) or control siRNA (siCtr) for 72 h were subjected to immunoblotting for DRP1 and GAPDH. Densitometric measurements in the arbitrary units of DRP1 protein bands after normalization to GAPDH protein (DRP1/GAPDH) are indicated under each lane. (B) Visualization of mitochondria. Images of MitoTracker Orange CMTMRos-stained mitochondria in each cell were captured under confocal microscopy. Enlargement shows the area indicated by the box in the left panel. Arrows represent balloon-like structures. Scale bars: 10 μm. (C) Percentage of cell population with tubular, fragmented, intermediated, or highly interconnected (elongated) mitochondria. At least 100 cells in several fields were counted in three independent experiments. Error bars represent standard deviation of the mean (SDM). *NT*, no treatment with siRNA; *siCtr*, 10 nmol/L nonsilencing siRNA; siDRP1, 10 nmol/L siRNAs targeted against the DRP1 gene.

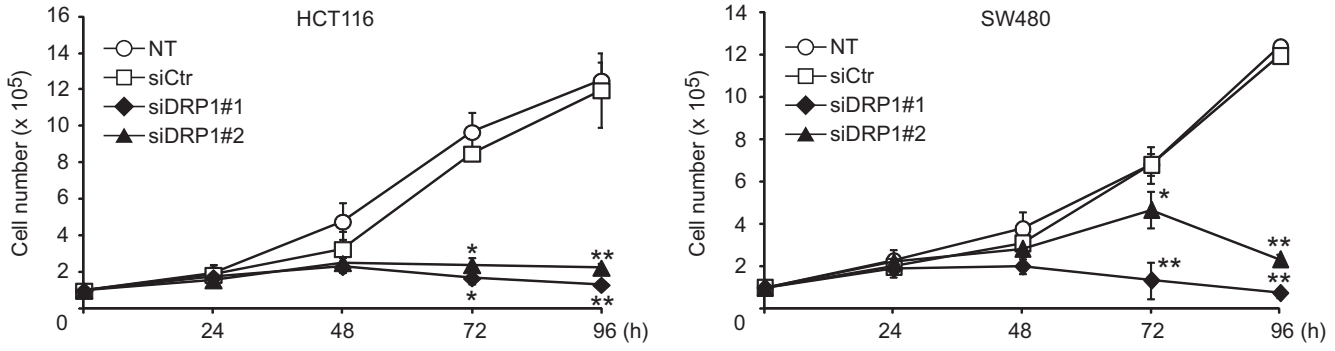

Fig. 2. DRP1 knockdown decreases colon cancer cell proliferation. Growth curves of HCT116 and SW480 cells transfected with DRP1 siRNAs for 96 h. *, **: significantly different from control siRNA (siCtr)-treated cells at $^{*}P < 0.05$, $^{**}P < 0.01$ by the unpaired Student's *t* test. Error bars represent SDM ($n = 3$). *NT*, no treatment with siRNA; *siCtr*, 10 nmol/L nonsilencing siRNA; siDRP1, 10 nmol/L siRNAs targeted against the DRP1 gene.

3.3. Depletion of DRP1 causes enhanced cytochrome c release and reduction of mitochondrial membrane integrity

In apoptotic stimulation, cytochrome *c*, a key regulator of mitochondria-mediated apoptosis, is released from mitochondria to the cytosol, which triggers apoptotic cell death through caspase activation. Therefore, we next examined cytochrome *c* release on down-regulation of DRP1. Immunoblot analysis of membranes (containing mitochondrial membranes) and cytosol fractions extracted from DRP1-knockdowned HCT116 and SW480 cells revealed elevated cytochrome *c* release to the cytosol on DRP1 depletion (Fig. 4A).

Mitochondrial membrane potential ($\Delta\psi m$) is known to change during apoptosis and may in fact be responsible for the release of cytochrome *c* [13,14]. Therefore, we investigated the effect of DRP1 depletion on $\Delta\psi m$ by flow cytometry using the cationic fluorescent probe JC-1. The high $\Delta\psi m$ of normal cells loaded with JC-1 allows for the formation of red-fluorescent J-aggregates. Upon loss of $\Delta\psi m$, these J-aggregates dissipate into monomers leading to a shift from red to green fluorescence [14]. As shown in Fig. 4B, DRP1-depleted HCT116 and SW480 cells displayed significantly reduced $\Delta\psi m$ expressed as the red:green ratio of fluorescence intensity, indicating attenuation of mitochondrial membrane potential on DRP1 depletion.

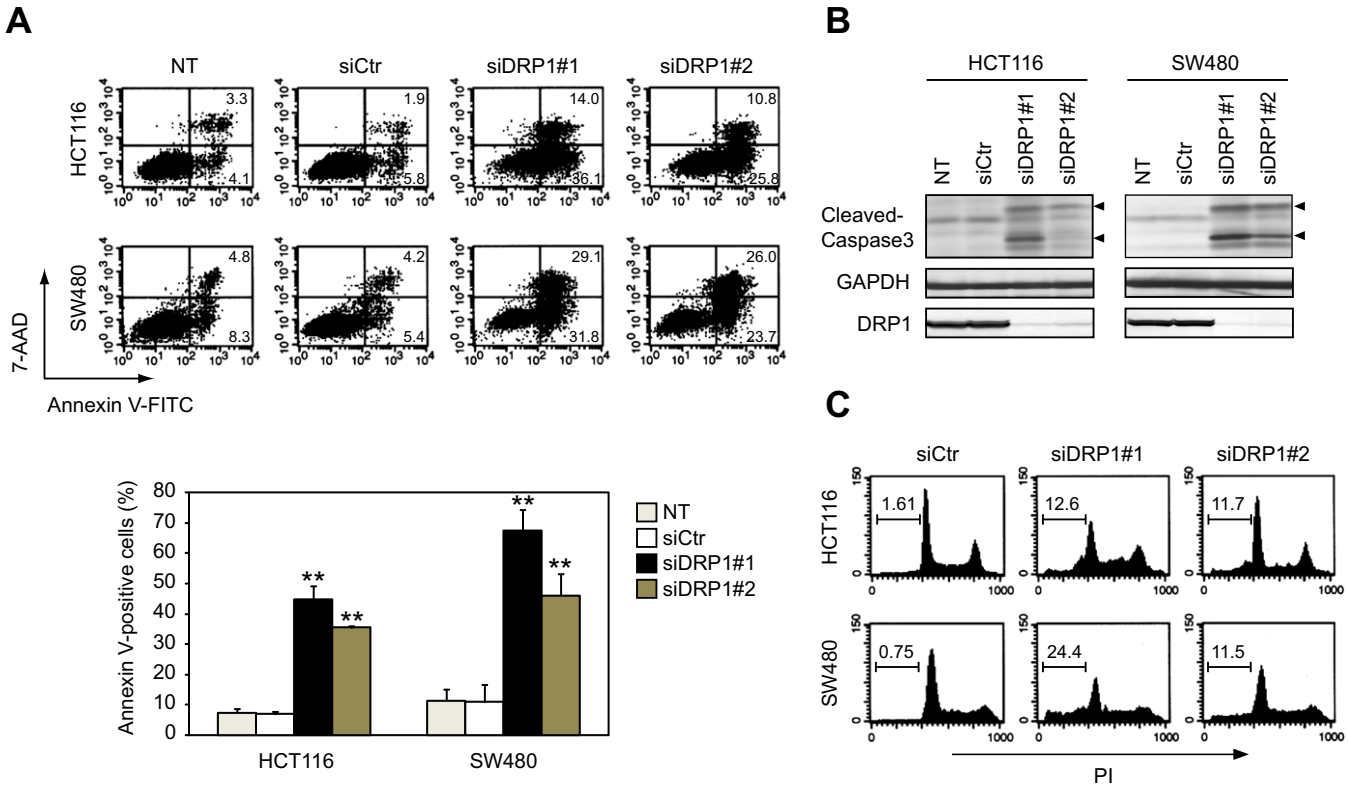

Fig. 3. DRP1 knockdown triggers apoptosis in colon cancer cells. (A) Annexin V-FITC and 7-AAD double staining and following flow cytometric analysis of HCT116 and SW480 cells transfected with DRP1 siRNAs for 72 or 96 h. (Upper) Representative dot plots are displayed. The lower right quadrant shows early apoptotic cells and the upper right quadrant shows late apoptotic cells. Numbers indicate the percentage of early or late apoptotic cells. (Lower) Percentage of Annexin V-positive apoptotic cells determined by flow cytometry is shown. **: significantly different from control siRNA (siCtr)-treated cells at $^{**}P < 0.01$ by the unpaired Student's *t* test. Error bars represent SDM ($n = 3$). (B) Caspase-3 activation was examined by immunoblotting for cleaved caspase-3 in HCT116 and SW480 cells transfected with DRP1 siRNAs for 72 or 96 h. Arrowheads indicate the position of cleaved caspase-3. The figure shown is representative of three independent experiments. (C) DNA content was assessed by flow cytometry of PI-stained HCT116 and SW480 cells transfected with DRP1 siRNAs for 72 or 84 h. Numbers indicate the percentage of the sub-G0/G1 cell population. *NT*, no treatment with siRNA; *siCtr*, 10 nmol/L nonsilencing siRNA; siDRP1, 10 nmol/L siRNAs targeted against the DRP1 gene.

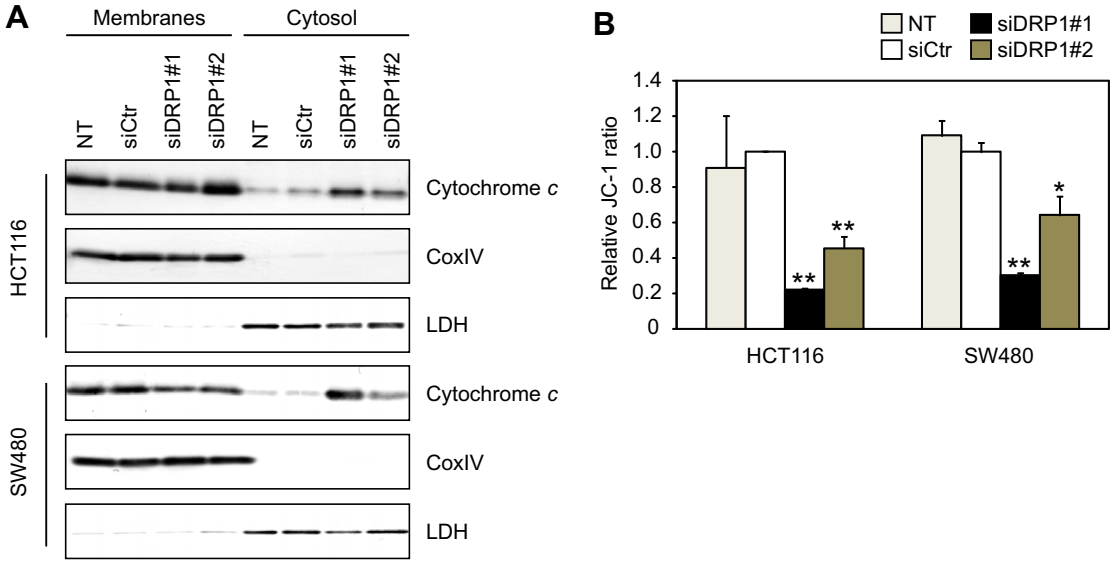

Fig. 4. Loss of DRP1 increases cytochrome *c* release and decreases mitochondrial membrane potential in colon cancer cells. (A) The distribution of cytochrome *c* was examined with HCT116 and SW480 cells transfected with DRP1 siRNAs for 72 or 96 h, respectively. Fractionation quality was verified by the distribution of specific subcellular markers: CoxIV for membranes and LDH for cytosol. The figure shown is representative of three independent experiments. (B) Mitochondrial membrane potential ($\Delta\psi m$) was assessed using $\Delta\psi m$-sensitive JC-1 fluorescence dye-stained HCT116 and SW480 cells transfected with DRP1 siRNAs for 72 h. The mean ratio of red:green fluorescence intensity obtained by control siRNA (siCtr) treated cells was defined as 1. *, **: significantly different from siCtr-treated cells at $^{*}P < 0.05$, $^{**}P < 0.01$ by the unpaired Student's *t* test. Error bars represent SDM ($n = 3$). *NT*, no treatment with siRNA; *siCtr*, 10 nmol/L nonsilencing siRNA; siDRP1, 10 nmol/L siRNAs targeted against the DRP1 gene.

4. Discussion

In the present study, we first described a role for DRP1 in regulation of apoptosis in colon cancer cells. Our results show that down-regulation of DRP1 results in elongation of mitochondria and causes enhanced apoptosis in colon cancer, indicating that a mitochondrial fission factor participates in inhibition of colon cancer cell apoptosis.

The majority of cytochrome *c* protein is sequestered in the narrow necks of mitochondrial inner membrane cristae folds [15]. It has been reported that OPA1 maintains cristae structure in a mitochondrial fusion-independent manner [16,17]. Upon apoptosis, OPA1-organized cristae are destructured to facilitate cytochrome *c* release from the intermembrane space to the cytosol [16,17]. Indeed, depletion of OPA1 causes perturbation of mitochondrial inner membrane structure and integrity and enhances cytochrome *c* release during apoptosis [9]. Because DRP1-depleted colon cancer cells display disruption of mitochondrial membrane integrity and enhanced cytochrome *c* release to the cytosol (Fig. 4), resembling the phenotype of OPA1-depleted cells, dysregulation of OPA1-mediated maintenance of cristae structure is likely to be involved in the acceleration of cytochrome *c* release on DRP1 depletion. Interestingly, depletion of DRP1 altered the expression pattern of OPA1 isoforms involving enhanced expression of short form OPA1 in HCT116 and SW480 cells (Supplementary Fig. S1). Although it remains to be elucidated whether this aberrant OPA1 expression pattern results in cristae disorganization, the selective loss of long isoforms and accumulation of short OPA1 leads to apoptosis mediating the aberrant cristae morphogenesis is reported [18]. DRP1 may be involved in OPA1-dependent cristae organization to control colon cancer cell apoptosis.

Recent reports suggest that DRP1 is required for HeLa cervical cancer cell proliferation [19,20]. Indeed, prolonged DRP1 depletion caused decreased cell growth, which is thought to be caused by mitochondrial dysfunction including a drop in cellular ATP levels rather than inducing apoptosis [19,20]. Although we have no evidence to explain the discrepancy between these previous results [19,20] and our results obtained from DRP1-depleted HCT116 and SW480 colon cancer cells, it is possible that differences in the nuclear genetic background among these cells contribute differential susceptibility to apoptosis on DRP1 depletion.

Our finding that depletion of DRP1 commits colon cancer cells to apoptosis without any other stimulus suggests that DRP1 may serve as a new therapeutic target for colon cancer. Importantly, DRP1 deficiency had no effects on proliferation or viability of normal cells such as MEF or ES cells [21], suggesting that DRP1 inhibition may cause apoptosis specifically in colon cancer cells without affecting normal cell growth. Thus, mdivi-1, a chemical inhibitor of DRP1-mediated mitochondrial fission [22], may provide effective colon cancer chemotherapy.

In summary, our results provide, for the first time, evidence that mitochondrial fission factor DRP1 is involved in inhibition of colon cancer cell apoptosis, which gives new insight into the function of mitochondrial dynamics in apoptosis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.03.118.

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