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Parkin induces G2/M cell cycle arrest in TNF-α-treated HeLa cells

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ABSTRACT

Parkin is a known tumor suppressor. However, the mechanism by which parkin acts as a tumor suppressor remains to be fully elucidated. Previously, we reported that parkin expression induces caspase-dependent apoptotic cell death in TNF-α-treated HeLa cells. However, at that time, we did not consider the involvement of parkin in cell cycle control. In the current study, we investigated whether parkin is involved in cell cycle regulation and suppression of cancer cell growth. In our cell cycle analyses, parkin expression induced G2/M cell cycle arrest in TNF-α-treated HeLa cells. To elucidate the mechanism(s) by which parkin induces this G2/M arrest, we analyzed cell cycle regulatory molecules involved in the G2/M transition. Parkin expression induced CDC2 phosphorylation which is known to inhibit CDC2 activity and cause G2/M arrest. Cyclin B1, which is degraded during the mitotic transition, accumulated in response to parkin expression, thereby indicating parkin-induced G2/M arrest. Next, we established that Myt1, which is known to phosphorylate and inhibit CDC2, increased following parkin expression. In addition, we found that parkin also induces increased Myt1 expression, G2/M arrest, and reduced cell viability in TNF-α-treated HCT15 cells. Furthermore, knockdown of parkin expression by parkin-specific siRNA decreased Myt1 expression and phosphorylation of CDC2 and resulted in recovered cell viability. These results suggest that parkin acts as a crucial molecule causing cell cycle arrest in G2/M, thereby suppressing tumor cell growth.

1. Introduction

The parkin gene (*PARK2*) was first described in Parkinson's disease (PD) as a hereditary cause of familial PD [1]. *PARK2* is located on chromosome 6q25.2–6q27, a known and common fragile site [2]. Numerous studies have reported lack of parkin expression or mutations in *PARK2* in a variety of cancer types [2–6]. Hypermethylation of the parkin promoter region results in diminished expression of parkin in acute lymphoblastic leukemia, chronic myeloid leukemia, and colorectal cancers [4,7]. In pancreatic cancers, downregulation and copy number loss of the parkin gene have been observed [8]. Moreover, parkin expression has been shown to be inversely correlated with p53-linked brain tumor grade [9]. Many reports have suggested that parkin overexpression results in inhibition of cancer cell growth [4–6]. In the breast cancer cell line MCF7, parkin stabilizes microtubules, increases sensitivity to anti-cancer agents, and induces growth arrest [10]. There is evidence that parkin can also affect energy metabolism, namely the Warburg effect, leading to suppression of tumorigenesis in lung cancer and colon cancer cells [11]. In addition, the functional interplay between parkin and p53, a well-known tumor suppressor, has been reported. According to these reports, expression of parkin down-regulates p53, and p53 can, in turn, induce parkin expression [11,12]. Collectively, these reports highlight the possibility that parkin acts as a tumor suppressor. However, the mechanism by which parkin may act as a tumor suppressor remains to be fully elucidated.

The cell cycle is a tightly regulated process crucial to the survival of a cell. Cell cycle checkpoints exist to monitor and regulate cell cycle progression [13]. Cell cycle progression is prevented at specific points until the processes necessary for each phase are achieved and any DNA damage is properly repaired. There are two main cell cycle checkpoints, the G1/S checkpoint and the G2/M checkpoint, regulated by a variety of molecules. Among these molecules, the cell division cycle 2 (CDC2) protein is key in the

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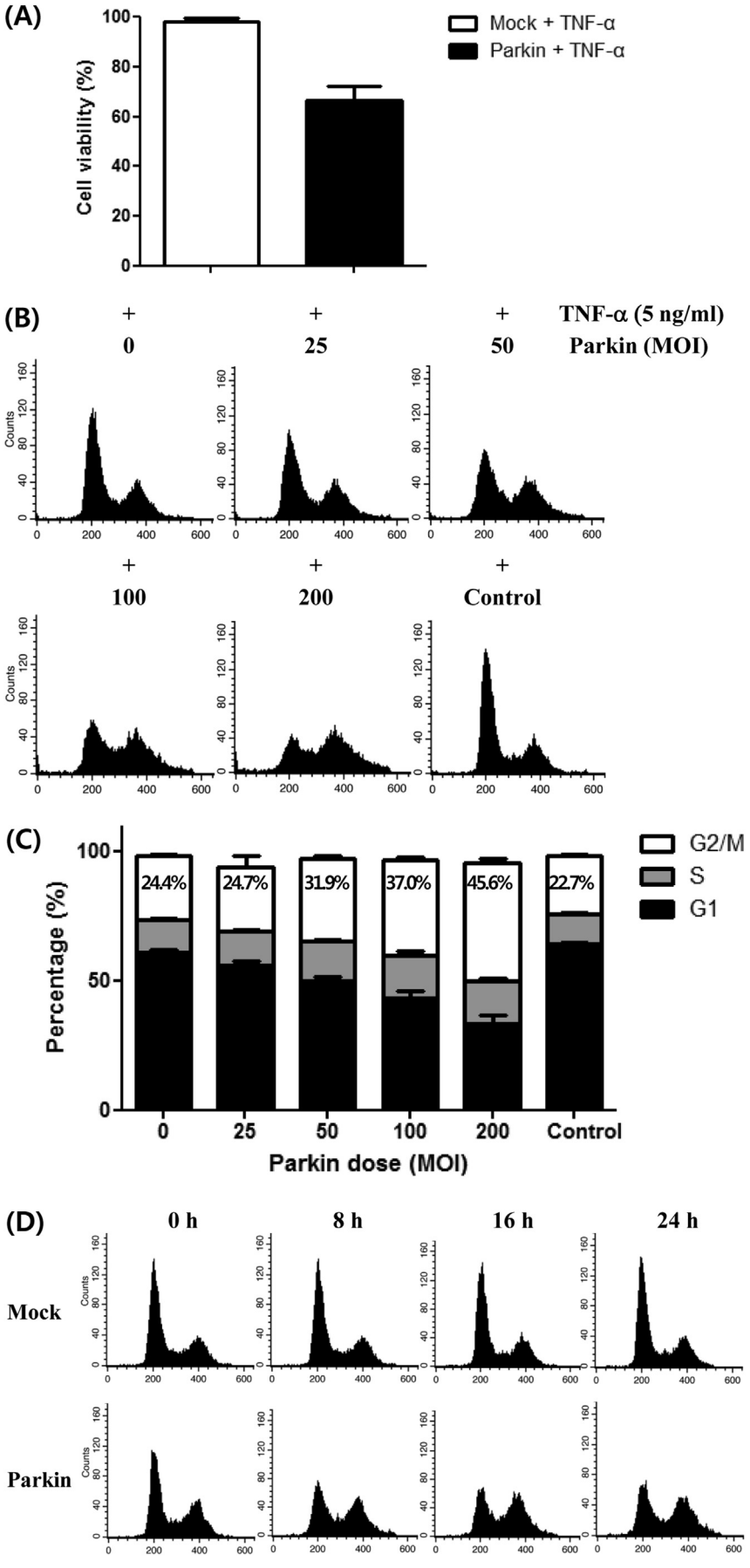
(A)
Mock + TNF-α
Parkin + TNF-α
Cell viability (%)
100
80
60
40
20
0
(B)
+ + + TNF-α (5 ng/ml)
0 25 50 Parkin (MOI)
+ + +
100 200 Control
Counts
(C)
G2/M
S
G1
Percentage (%)
24.4%
24.7%
31.9%
37.0%
45.6%
22.7%
0 25 50 100 200 Control
Parkin dose (MOI)
(D)
0 h
8 h
16 h
24 h
Mock
Parkin
Counts

regulation of the G2/M cell cycle transition. Entry into mitosis is regulated by activation of CDC2 which triggers reorganization of the nucleus, chromosome condensation, and formation of the mitotic spindle via the phosphorylation of various mitotic substrates [14]. The activity of CDC2 is controlled by cyclin binding and CDC2 phosphorylation [15]. The critical regulatory step in activating CDC2 during progression into mitosis appears to be dephosphorylation of CDC2 at Tyr15 and Thr14. Phosphorylation of the Tyr15 and Thr14 residues, which lie within the ATP-binding region of CDC2, inactivates CDC2, resulting in a block in the transition from G2 to the mitotic phase [15]. Several upstream molecules control CDC2 phosphorylation; phosphorylation of CDC2 at Tyr15 and Thr14 can be performed by Myt1 [16]. In addition, the cell division cycle 25C (CDC25C) protein is the phosphatase responsible for dephosphorylating and activating CDC2 [14]. Cyclin B is a G2 phase-associated cyclin essential for activation of CDC2 and for transition of cells to mitosis. The level of cyclin B is highest during the G2 phase, but is rapidly degraded by anaphase promoting complex-cell division cycle 20 (APC-CDC20) protein after entry into mitosis [17]. Therefore, if the entry of cells into mitosis is inhibited, the level of cyclin B remains elevated [18].

TNF-α, a potent pro-inflammatory cytokine, exerts a suppressive effect on tumors [19,20]. Lack of apoptosis due to unresponsiveness to pro-apoptotic stimuli causes uncontrolled cell proliferation, leading to cancer development [21]. Previously, we reported that expression of parkin in the HeLa cell line restored susceptibility to TNF-α-induced cell death [22]. This was mediated, in part, by activation of a caspase-dependent apoptotic pathway. However, the role of parkin in cell cycle regulation was not examined in our previous study. Recently, several reports have suggested that parkin is implicated in cell cycle control [5,23]. Parkin ubiquitinates cyclin D and cyclin E, resulting in proteasome-mediated degradation with subsequent cell cycle arrest in colon cancer, glioma and human embryonic kidney cell lines. Here, we investigated the involvement of parkin in cell cycle arrest and identified parkin-regulated cell cycle related molecules in TNF-α-treated HeLa cells. We report that parkin induces increased expression of Myt1 and phosphorylation of CDC2, thereby inducing G2/M cell cycle arrest.

2. Materials and methods

2.1. Cell line and cell culture

HeLa cells (ATCC, Manassa, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco) and streptomycin-penicillin (Gibco). HCT15 cells (ATCC) were cultured in RPMI-1640 supplemented with 10% FBS and streptomycin-penicillin. The cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2.

2.2. Parkin gene expression

A recombinant adenoviral vector containing the parkin gene (parkin virus) was produced as previously described [24]. Cells were plated in 6-well plates (2×10^5 per well) and 24 h later cells were infected with different concentrations of either parkin virus or a mock virus diluted in serum-free DMEM. 10% FBS-DMEM was added to each well 90 min post-infection. In experiments evaluating parkin virus dose-dependency, cells were infected with parkin virus at different multiplicities of infection (MOI) (0, 25, 50, 100, 200). To compensate for the effect of the viral vector, mock virus was added along with parkin virus to maintain a consistent MOI of 200.

2.3. Cell cycle analysis

Trypsinized cells were washed twice with PBS, fixed with 70% ethanol in PBS and incubated for 2 h at 4 °C. Fixed cells were stained with a solution containing RNase A (0.1 mg/ml) and propidium iodide (5 mg/ml) in PBS. After incubation for 40 min at 37 °C, the cell suspension was analyzed using FACS Calibur (BD Biosciences, Sparks, MD, USA).

2.4. MTT assay

HeLa cells (1.2×10^4 per well) were plated in 96-well plates and infected with mock or parkin virus at an MOI of 200 after 24 h. The cells were then treated with TNF-α (5 ng/ml) for 24 h. MTT assay was performed as previously described [25].

2.5. RT-PCR (reverse transcription–polymerase chain reaction)

Total RNA was extracted from cultured cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. cDNA was synthesized by reverse transcription and subjected to PCR as described previously [26]. The sequences of the PCR primers used are listed in Supplementary Table 1.

2.6. Western blot analysis

Cells were washed with PBS and then lysed at 4 °C with lysis buffer containing 1% Triton X-100, protease inhibitor cocktail (Sigma), phosphatase inhibitor cocktail (Roche, Mannheim, Germany) and PBS. Lysates were clarified and the supernatants subjected to Western blot as described previously [22].

2.7. Trypan blue dye exclusion assay

To count viable cells, the cells were trypsinized and trypan blue stain solution (10 μl of 0.4%) was mixed with 10 μl of the trypsinized cells. Non-stained cells were counted from this mixture using a hematocytometer.

3. Results

3.1. Expression of parkin induces G2/M arrest in TNF-α-treated HeLa cells

We previously reported that parkin overexpression decreased cell viability in TNF-α-treated HeLa cells via a caspase-dependent apoptotic cell death pathway [22]. However, the question of whether parkin influences cell proliferation, in addition to cell death, has not yet been investigated. In the current study, we investigated whether parkin exerts an effect on HeLa cell proliferation by influencing cell cycle progression. First, we reconfirmed that expression of parkin reduces cell viability in TNF-α-treated HeLa cells (Fig. 1A). Then, we investigated cell cycle status after parkin expression. HeLa cells were infected with varying doses of

Fig. 1. Effects of parkin expression on the cell cycle in TNF-α-treated HeLa cells. **(A)** HeLa cells were infected with either parkin virus or mock virus (200 MOI) for 24 h and then treated with TNF-α (5 ng/ml) for 24 h. Cell viability was measured by MTT assay. **(B)** HeLa cells were infected with the indicated concentrations (0, 25, 50, 100, 200 MOI) of parkin virus for 24 h and then treated with TNF-α (5 ng/ml) for 24 h. Uninfected cells were utilized as a control. Cells were stained with propidium iodide and cell cycle analyzed by flow cytometry. **(C)** The percentage of cells in each phase of the cell cycle was analyzed with CellQuestPro software. **(D)** HeLa cells were infected with either parkin virus or mock virus (200 MOI) for 24 h, treated with TNF-α (5 ng/ml) for each indicated time period and subjected to cell cycle analysis.

parkin virus (0, 25, 50, 100, 200 MOI) and then treated with TNF-α (5 ng/ml) for 24 h. Harvested cells were processed for analysis by flow cytometry. Our data revealed a dramatic parkin dose-dependent increase in the percentage of cells in the G2/M phase (Fig. 1B and C). To investigate when parkin induces G2/M arrest, HeLa cells were infected with parkin virus for 24 h and then cell cycle analysis was performed at the indicated time points after TNF-α treatment. In parkin virus-infected HeLa cells, the percentage of cells in the G2/M phase began to increase 8 h post-treatment with TNF-α (Fig. 1D). These results demonstrate that parkin expression induces cell cycle arrest in G2/M in TNF-α-treated HeLa cells.

3.2. Expression of parkin increases phosphorylation of CDC2 (Tyr15)

The transition from the G2 phase to the mitotic phase is regulated by CDC2, with dephosphorylation of CDC2 at Tyr15 being a critical step in CDC2 activation [15]. Therefore, we investigated whether parkin expression influences CDC2 phosphorylation at Tyr15 and found that parkin expression increased CDC2 phosphorylation in a parkin dose-dependent manner (Fig. 2A). Next, HeLa cells were infected with either parkin virus or mock virus (200 MOI) for 24 h and then treated with the indicated concentrations of TNF-α (0, 0.5, 1, 2, 5, 10 ng/ml) for an additional 24 h. CDC2 phosphorylation increased in a TNF-α dose-dependent manner in the parkin expressing cells compared to the mock virus infected cells (Fig. 2B). However, parkin expression did not affect CDC2 protein or mRNA levels (Fig. 2C and D). These results suggest that parkin expression in TNF-α-treated HeLa cells increases phosphorylation of CDC2 at Tyr15 and, consequently, inhibits CDC2 activity which results in cell cycle arrest in G2/M.

3.3. Cyclin B1 protein accumulates during parkin-induced G2/M arrest

Cyclin B1 is degraded in cells during exit from the mitotic phase [17]. Therefore, cell cycle arrest in G2/M results in cyclin B1 accumulation [18]. We found that cyclin B1 protein levels increased in a parkin dose-dependent manner (Fig. 2E), and that cyclin B1 levels also increased in a TNF-α dose-dependent manner in parkin-overexpressing cells (Fig. 2F). However, parkin expression did not affect Cyclin B1 mRNA levels (Fig. 2G). These results are concordant with a report that cyclin B1 accumulates during doxorubicin-induced G2/M arrest [18]. We reconfirmed this accumulation of cyclin B1 using doxorubicin treatment in HeLa cells (Supplementary Fig.1). These results demonstrate that parkin expression induces accumulation of cyclin B1, indirectly indicating that overexpression of parkin induces G2/M cell cycle arrest in TNF-α-treated HeLa cells.

3.4. Parkin induces an increase in Myt1 protein levels

Myt1 is a kinase responsible for inhibition of CDC2 activity by phosphorylating Tyr15 of CDC2. Consequently, we investigated whether Myt1 is involved in parkin-induced phosphorylation of

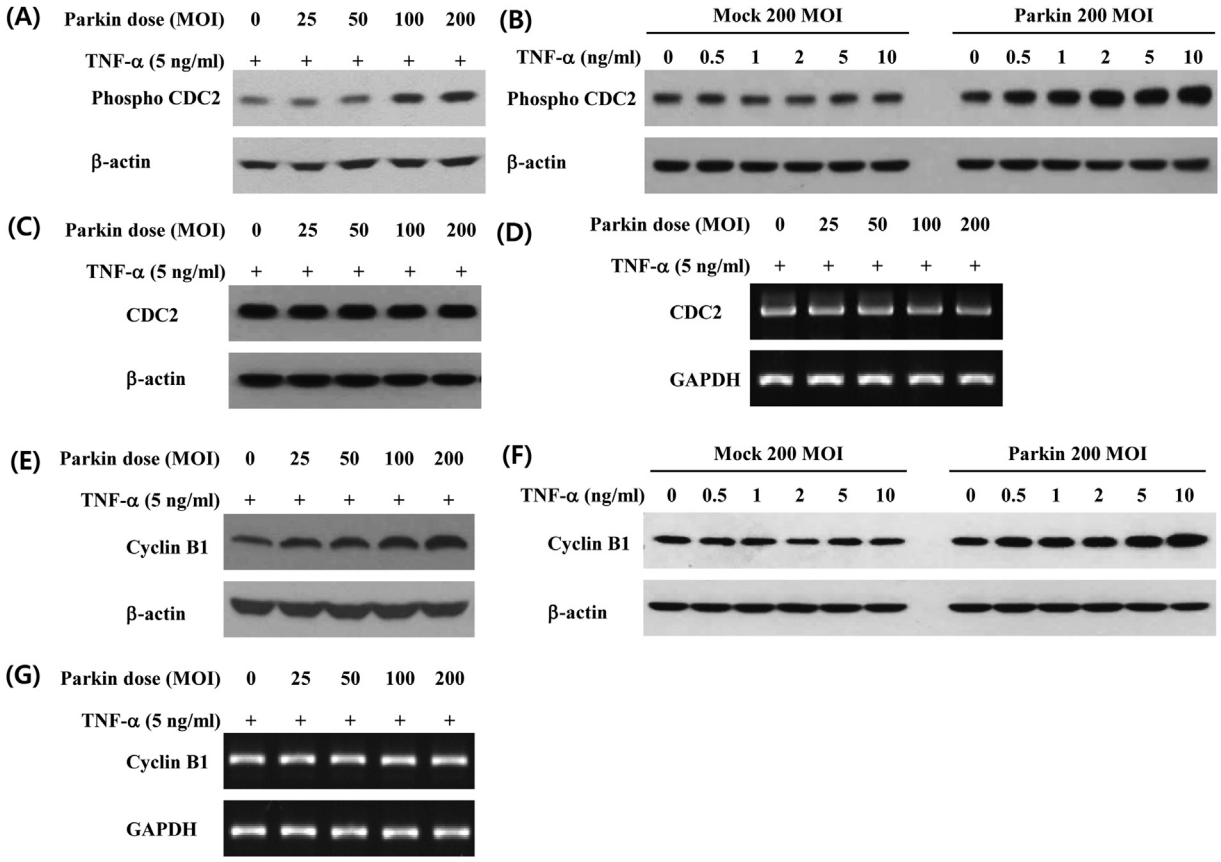

Fig. 2. Increases in phospho-CDC2 and accumulation of cyclin B1 following parkin expression in TNF-α-treated HeLa cells. **(A)** Phospho-CDC2 (Tyr15) Western blot. HeLa cells were infected with the indicated concentrations of parkin virus for 24 h and then treated with TNF-α (5 ng/ml) for 24 h. **(B)** Phospho-CDC2 (Tyr15) Western blot. HeLa cells were infected with either mock or parkin virus (200 MOI) for 24 h and then treated with the indicated concentration of TNF-α for 24 h **(C)** CDC2 Western blot. Cells were treated as in (A). **(D)** CDC2 RT-PCR analysis. Cells were treated as in (A). **(E)** Cyclin B1 Western blot. Cells were treated as in (A). **(F)** Cyclin B1 Western blot. Cells were treated as in (B). **(G)** Cyclin B1 RT-PCR analysis. Cells were treated as in (A).

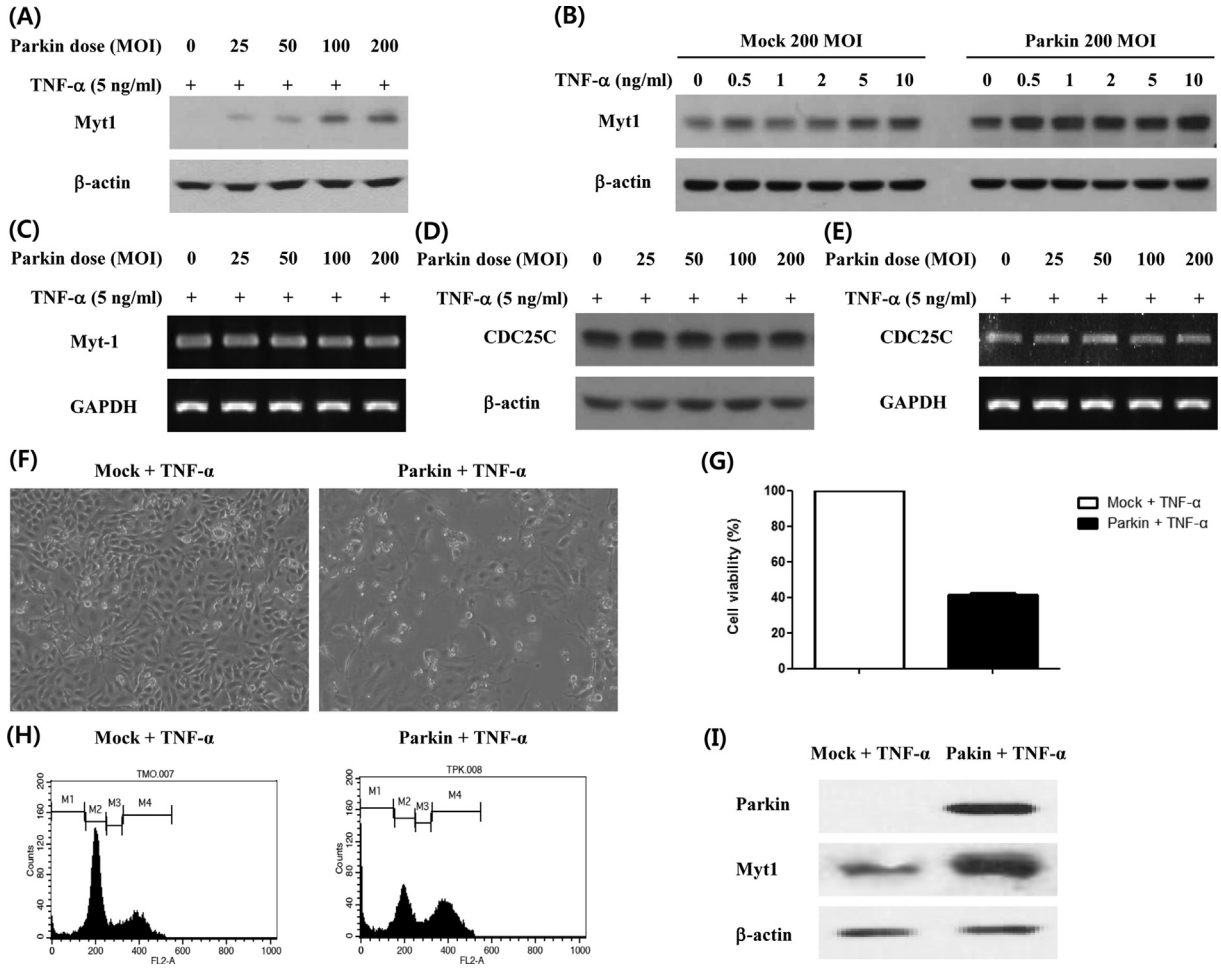

Fig. 3. Increased Myt1 protein levels following parkin expression in TNF-α-treated HeLa and HCT15 cells. **(A)** Myt 1 Western blot. HeLa cells were infected with the indicated concentrations (0, 25, 50, 100, 200 MOI) of parkin virus for 24 h and then treated with TNF-α (5 ng/ml) for 24 h. **(B)** Myt 1 Western blot. HeLa cells were infected with either mock or parkin virus (200 MOI) for 24 h and then treated with the indicated concentrations of TNF-α for 24 h. **(C)** Myt 1 RT-PCR analysis. Cells were treated as in (A). **(D)** CDC25C Western blot. Cells were treated as in (A). **(E)** CDC25C RT-PCR analysis. Cells were treated as in (A). HCT15 cells were infected with either mock or parkin virus (200 MOI) for 24 h and treated with TNF-α (5 ng/ml) for 48 h. Then, **(F)** images were captured using an inverted microscope, **(G)** cell viability was measured, **(H)** cell cycle analysis was performed, and **(I)** Myt1 was detected by Western blot.

CDC2. We found that Myt1 protein levels increased in both parkin (Fig. 3A) and TNF-α dose-dependent manner (Fig. 3B). However, Myt1 mRNA was not influenced by parkin expression (Fig. 3C). We next investigated whether the levels of CDC25C, a protein phosphatase associated with dephosphorylation of CDC2 at Tyr15, are affected by overexpression of parkin. We found that the levels of CDC25C protein and mRNA remained constant (Fig. 3D and E). These results suggest that the parkin-induced increase in phosphorylation of CDC2 results from increased levels of Myt1. In addition, we found that parkin expression also induced decrease of cell viability, cell cycle arrest in G2/M, and increase of Myt1 protein level in TNF-α-treated HCT15, a human colorectal adenocarcinoma cell line (Fig. 3F–I). These results imply that role of parkin in Myt1 protein expression, G2/M arrest, and reduced cell viability is not restricted to one specific cell line.

3.5. The parkin induced decrease in cell viability is rescued by parkin siRNA treatment

To confirm that parkin increases the levels of phosphorylated CDC2 and Myt1, we introduced a parkin siRNA into parkin-overexpressing cells. When parkin expression was decreased by the introduction of parkin siRNA, both Myt1 and phosphorylated CDC2, which had been increased by parkin overexpression, decreased (Fig. 4A). These results demonstrate that parkin induces an increase in Myt1 and phosphorylated CDC2 levels. Furthermore, the parkin-induced decrease in cell viability was partially recovered by introduction of parkin siRNA (Fig. 4B). From these results, we propose that parkin overexpression induces Myt1 expression and phosphorylation of CDC2, causing cell cycle arrest in G2/M and, consequently, resulting in decreased cell viability in TNF-α-treated HeLa cells.

4. Discussion

Currently, cumulative data suggest that parkin performs a tumor suppressive role. Lack or mutation of parkin has been observed in a variety of cancers [2,3], and reintroduction of parkin is known to suppress cancer cell viability [4,5]. However, the mechanism through which parkin acts as a tumor suppressor has yet to be fully revealed. In a previous study, we reported that parkin expression induced TNF-α-induced apoptotic cell death in HeLa cells which are naturally resistant to TNF-α-induced cell death [22]. In this study, we investigated the role of parkin in cancer cell proliferation. We found that i) parkin expression arrests cancer cells in G2/M, ii) this

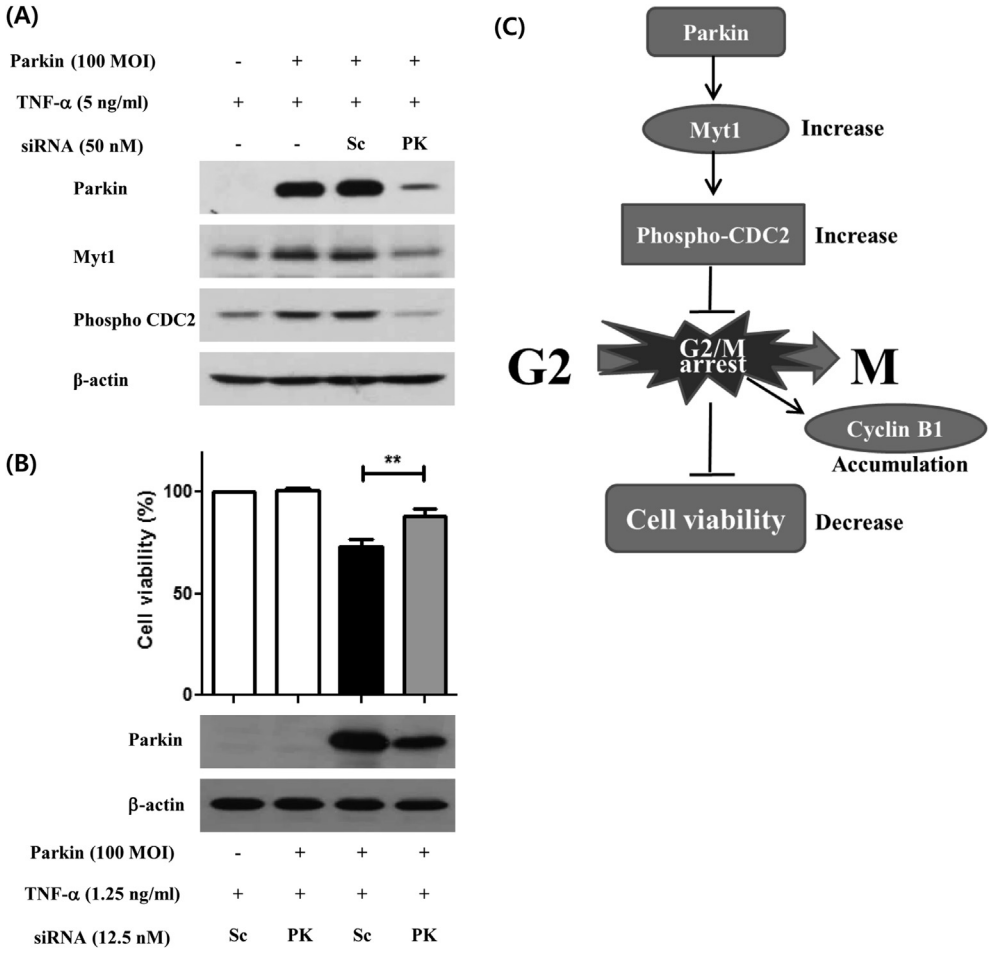

Fig. 4. Recovery of cell viability and alterations in cell cycle regulatory proteins resulting from siRNA knockdown of parkin. **(A)** Western blot analysis of parkin, phospho CDC-2 and Myt 1. HeLa cells were infected with either mock or parkin virus (100 MOI) or transfected with either scrambled siRNA (Sc-siRNA) or parkin siRNA (PK-siRNA). After 24 h, TNF-α (5 ng/ml) was added for 24 h **(B)** HeLa cells were infected with either mock or parkin virus (100 MOI) and transfected with either scrambled-siRNA (Sc-siRNA) or parkin-siRNA (PK-siRNA). After 24 h, TNF-α (1.25 ng/ml) was added for 24 h and cell viability was subsequently measured by trypan blue dye exclusion assay. Data are from three independent experiments. Values are shown as mean and standard error. *P*-values were determined using the Student's *t*-test. $^{**}P < 0.01$. **(C)** Schematic model showing the highlights of parkin-induced G2/M arrest in TNF-α-treated HeLa cells.

G2/M arrest seems to be due to phosphorylation-mediated inactivation of CDC2 and iii) the levels of the CDC2-regulating molecule Myt1 increased in response to parkin expression in TNF-α-treated HeLa cells.

CDC2 is an important molecule involved in regulating the G2/M cell cycle check point. Activation of CDC2 kinase activity is a key process in cell cycle progression, with dephosphorylation of CDC2 at Tyr15 being the critical regulatory step in CDC2 activation during progression into mitosis [15]. Therefore, we investigated the phosphorylation status of CDC2 after we observed G2/M arrest and found that phospho-CDC2 increased in both TNF-α and parkin dose-dependent manners. We propose that parkin expression resulted in inactivation of CDC2 by increasing phosphorylation of CDC2, and thus induced G2/M cell cycle arrest. CDC2 is responsible for the attachment of chromosomes to the mitotic spindle, spindle positioning, and spindle elongation; all of which are crucial events in mitosis [27–29]. Moreover, CDC2 activity is required for activation of anaphase promoting complex (APC^{cdc20}), an E3 ubiquitin ligase essential for initiation of anaphase [30]. Further studies will be needed to examine the role of parkin in additional events in the mitotic process.

Several molecules are known to be associated with the phosphorylation of CDC2, including CDC25C, a protein phosphatase responsible for dephosphorylation and activation of CDC2 [14]. In the current study, there was no significant change in CDC25C protein levels. However, the activity of CDC25C is regulated by phosphorylation at more than 12 different sites by a variety of kinases, such as CDC2/cyclin B and polo-like kinase [14]. Therefore, additional studies will be necessary to examine the phosphorylation status of CDC25C in parkin-overexpressing cells. Other protein kinases, such as Wee1 and Myt1, are also involved in phosphorylation of CDC2 [14,16]. In the current study, Myt1 levels increased in response to parkin expression. These results may explain the observed increase in CDC2 phosphorylation, as phosphorylation of Thr14 and Tyr15 can be performed by Myt1 [16].

TNF-α is known to exert a suppressive effect on tumors [20]. In particular, the accumulation of cells in G2/M in response to TNF-α treatment has been observed in a variety of cancer cells, including human ovary adenocarcinoma and rat mammary carcinosarcoma cells [31,32]. However, unresponsiveness to TNF-α stimulation has also been reported in various types of cancer cells [33]. In the current study, we found that parkin induces G2/M arrest in TNF-α-treated HeLa cells, suggesting that parkin restores TNF-α-responsiveness in HeLa cells and results in cancer cell growth inhibition. In conclusion, we report the novel finding that parkin induces G2/M arrest and inhibits proliferation of TNF-α-treated HeLa cells.

Although additional studies of the other possible tumor suppressive mechanisms of parkin are necessary, we expect this study will contribute to our understanding of the role of parkin as a tumor suppressor and provide a basis for future studies on the regulation of the cell cycle by parkin.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.05.101.

Transparency document

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