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Mitochondrial reactive oxygen species originating from Romo1 exert an important role in normal cell cycle progression by regulating $p27^{Kip1}$ expression

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Abstract

Reactive oxygen species (ROS) steady-state levels are required for entry into the S phase of the cell cycle in normal cells, as well as in tumour cells. However, the contribution of mitochondrial ROS to normal cell proliferation has not been well investigated thus far. A previous report showed that Romo1 was responsible for the high ROS levels in tumour cells. Here, we show that endogenous ROS generated by Romo1 are indispensable for cell cycle transition from G1 to S phase in normal WI-38 human lung fibroblasts. The ROS level in these cells was down-regulated by *Romo1* knockdown, resulting in cell cycle arrest in the G1 phase. This arrest was associated with an increase in the level of $p27^{Kip1}$. These results demonstrate that mitochondrial ROS generated by Romo1 expression is required for normal cell proliferation and it is suggested that Romo1 plays an important role in redox signalling during normal cell proliferation.

Keywords: *Reactive oxygen species, Romo1, $p27^{Kip1}$, Cell cycle arrest, mitochondria, redox signalling*

Abbreviations: *ROS, reactive oxygen species; Romo1, reactive oxygen species modulator 1; siRNA, small interfering RNA; PDL, population doublings; NAC, N-acetyl cysteine; H2O2, hydrogen peroxide; PBS, phosphate-buffered saline; MAPK, Mitogen-activated protein kinase; Erk, extracellular-signal-regulated kinase; MnSOD, manganese superoxide dismutase; FITC, fluorescein isothiocyanate; FBS, foetal bovine serum; RT-PCR, reverse transcription-polymerase chain reaction; BrdU, 5-bromo-2-deoxyuridine; MOI, multiplicity of infection*

Introduction

Reactive oxygen species (ROS) play important roles in various biological processes, including cell proliferation, transformation, cell death and senescence [1,2]. The main endogenous source of ROS generation is the mitochondrial electron-transport chain. Other sources of ROS include NADPH oxidase and xanthine oxidase, which play important roles in redox signalling [3]. ROS are continuously produced inside cells and are eliminated through antioxidant systems to maintain redox homeostasis. If this balance is disturbed, the increased ROS levels induce oxidative damage, resulting in a variety of pathological disorders. Excessive ROS production stimulates growth

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arrest or cell death. However, low ROS levels play an important role in cell proliferation and survival [4,5]. An appropriate ROS level is required for cell growth and survival. The physiological concentration of hydrogen peroxide (H_2O_2) ranges from 0.001–0.7 μM [6].

Treatment of fibroblasts, myoblasts, epithelial cells and endothelial cells with a low concentration of H_2O_2 (1–10 μM) induces cell proliferation [6]. Many factors are involved in signalling pathways that respond to ROS, such as mitogen-activated protein kinases (MAPKs), phosphoinositide 3-kinase (PI3K)/Akt, NF-kB, p53 and heat shock factor-1 [7]. Of these factors, extracellular-signal-regulated kinase (Erk) is reported to mediate H_2O_2-stimulated cell proliferation [8,9]. The intracellular ROS level is reduced by treating the cells with antioxidants and cell growth is inhibited by preventing activation of Erk and inhibiting the expression of cyclin D1 [8,10].

$p27^{Kip1}$ is another important mediator in antioxidant-mediated cell cycle arrest. An optimum level of ROS inside the cell is necessary for cell proliferation and cell cycle progression from G1 to S phase through the regulation of $p27^{Kip1}$ levels [11]. N-acetyl cysteine (NAC) treatment stimulates down-regulation of the cyclin D1 protein and induction of the $p27^{Kip1}$ protein; the expression levels of these proteins are recovered after removal of NAC from the system [12]. Exogenous treatment of cells with H_2O_2 stimulates cell proliferation through activation of Erk and Cdk2, as well as through reduction of $p27^{Kip1}$ levels [13]. However, H_2O_2 exerts no effect on $p21^{WAF1/CIP1}$ expression. In this report, decreased ROS levels caused by Bcl-2 expression enhanced $p27^{Kip1}$ expression and caused cell cycle arrest in the G1 phase.

Romo1 was first identified in the context of enhanced ROS production in the mitochondrion [14]. The expression of Romo1 was increased in senescent cells and blocking of Romo1 expression delayed replicative senescence of normal fibroblasts [15]. Recently, Romo1 was reported to be responsible for endogenous ROS production in various tumour cell lines and Romo1-derived ROS were shown to be necessary for tumour cell proliferation [10]. Down-regulation of ROS levels induced by *Romo1* knockdown resulted in G1 arrest in H1299 lung carcinoma cells [10]. Although we already showed that *Romo1* knockdown inhibited the growth of IMR-90 lung fibroblasts [10], we wanted to understand how *Romo1* knockdown-induced reductions in ROS levels inhibited the cell cycle progression of normal fibroblasts. In the present study, we show that Romo1-derived ROS levels are involved in the control of cell cycle progression through the regulation of $p27^{Kip1}$ expression in normal fibroblasts.

Materials and methods

Cell culture and reagents

Normal WI-38 human lung fibroblasts and large SV40 T antigen-mediated immortalized WI-38 cells (WI-38 VA13) were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in Eagle's minimal essential media (EMEM) containing 10% heat-inactivated foetal bovine serum (FBS, Invitrogen), sodium bicarbonate (2 mg/ml; Sigma-Aldrich, St. Louis, MO), penicillin (100 U/ml) and streptomycin (100 μg/ml; Invitrogen). N-acetyl cysteine (NAC) and H_2O_2 were obtained from Sigma-Aldrich. MitoSOX and Mito-Tracker was obtained from Molecular Probes (Eugene, OR). Lipofectamine™ was purchased from Invitrogen.

Measurement of cell growth curve

Double-stranded small interfering RNA (siRNA) oligonucleotide targeting *Romo1* [16] was synthesized by Bioneer (Taejon, Republic of Korea). The cells (5×10^4) were seeded into 6-well plates and then transfected with *Romo1* siRNA using Lipofectamine™. They were then stained with trypan blue (Invitrogen) and counted using a haematocytometer.

ROS assay

Intracellular ROS levels were measured by a fluorescence microscope (Olympus LX71 microscope) or a FACScan flow cytometer (Becton Dickson, San Jose, CA), using MitoSOX (5 μM), as previously described [15].

Adenovirus-mediated gene expression

Adenovirus-expressing human manganese superoxide dismutase (Mn-SOD) [17] was infected into WI-38 VA13 cells 24 h after cell seeding. Adenovirus was infected at 50 multiplicity of infection (MOI) for 6 h at 37°C in EMEM medium without FBS. After infection, EMEM medium containing 20% heat-inactivated FBS was added and cells were incubated for 24 h.

Western blot analysis

The protein concentration was measured using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Equal amounts of protein (20 μg/ml) were separated by 10 ~ 12% SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) and electrophoretically transferred onto a nitrocellulose membrane. Western blotting was performed using anti-$p27^{Kip1}$ (BD Transduction Lab, San Jose, CA), anti-$p21^{WAF1/CIP1}$ (Santa Cruz Biotechnology, CA), anti-Cyclin D1 (Santa Cruz Biotechnology), anti-phospho Erk (Cell Signaling Technology, Danvers, MA), anti-Erk (Cell

Signaling Technology), anti-phospho Akt (Cell Signaling Technology), anti-Akt (Cell Signaling Technology), anti-Bcl-2 (Cell Signaling Technology) and anti-β-actin (Sigma-Aldrich) antibodies. Bands were visualized by chemiluminescence using the ECL Western blot detection kit (Amersham, Pharmacia Biotech, UK).

Cell cycle analysis

After harvesting, the cells were fixed with 70% ethanol for 24 h at 20°C and then resuspended in phosphate-buffered saline (PBS). Collected cells were stained with 50 μg/ml propidium iodide (Sigma-Aldrich) in PBS containing 20 μg/ml RNase A, incubated at 37°C for 30 min and then analysed using a FACScan flow cytometer (Becton Dickson).

BrdU uptake analysis

BrdU incorporation analysis was performed using a FITC BrdU flow kit (BD Pharmingen^{TM}). Cells were treated with 10 μM BrdU for 1 h. They were harvested and fixed using 100 μl of BD Cytofix/Cytoperm buffer per tube for 30 min, washed with BD Perm/Wash buffer and centrifuged for 5 min at 200–300 g, incubated with DNase (300 μg/ml) for 1 h at 37°C and washed with BD Perm/Wash buffer. After the wash buffer was removed, 50 μl of BD Perm/Wash Buffer containing diluted fluorescent anti-BrdU (1:50) was added directly to the cell pellet. The cells were then incubated for 20 min at room temperature in the dark. For staining of total DNA, the cells were treated with 7-amino-actinomycin D (7-AAD) solution. One millilitre of staining buffer (1 × DPBS + 3% FBS + 0.09% sodium azide) was added to each tube to resuspend the cells. Cell cycle analysis was carried out on a Becton Dickinson FACScan, using Cell Quest software.

Statistical analysis

Each assay was performed in triplicate and was independently repeated at least three times. Statistical analysis was conducted using the Student's ANOVA (analysis of variance). Statistical significance was defined as $p < 0.05$. Means, SEs and ps were calculated using GraphPad PRISM version 4.02 for Windows (GraphPad Software, San Diego, CA).

Results

Romo1-induced ROS are essential for normal cell growth

A previous study reported that *Romo1* knockdown decreased ROS levels in tumour cells, as well as in normal IMR-90 human lung fibroblasts and that Romo1-derived ROS were indispensable for tumour cell proliferation [10]. In the present study, we tried to determine how Romo1-derived ROS were involved in normal cell proliferation. First, we examined the changes in ROS levels induced by *Romo1* knockdown in normal WI-38 lung fibroblasts. Cells were transfected with *Romo1* siRNA and ROS levels in the mitochondria were measured using MitoSOX, a red redox dye sensitive for mitochondrial superoxide.

Decreased ROS levels were observed in cells transfected with *Romo1* siRNA (Figure 1A). This decrease in ROS formation is quantified and illustrated in Figure 1B. The down-regulation of ROS by *Romo1* siRNA transfection was also examined in large, immortalized, SV40 T antigen-mediated WI-38 cells (WI-38 VA13) and similar results were obtained (Figure 1C and D). Intracellular ROS levels were also determined with flow cytometry using MitoSOX. WI-38 and W-38 VA13 cells were transfected with control siRNA-fluorescein isothiocyanate (FITC) or *Romo1* siRNA-FITC for 72 h and stained with MitoSOX for 30 min. ROS decrease was analysd by flow cytometry gating on 10 000 cells/sample (Figure 1E). *Romo1* knockdown by *Romo1* siRNA transfection was examined by reverse transcription-polymerase chain reaction (RT-PCR) in WI-38 and WI-38 VA13 cells (Figure 1F). To measure the transfection efficiency of *Romo1* siRNA by flow cytometry, *Romo1* siRNA-FITC was transfected into WI-38 and WI-38 VA13 cells for 72 h. The *Romo1* siRNA transfection efficiencies in these two cell lines were 98.34% and 86.94%, respectively (Figure 1F). To show that the *Romo1* siRNA-induced ROS decrease was not caused by some other oxidation process, we examined changes in fluorescence by looking at MnSOD, after which we quantitated the fluorescent signal. Adenoviral transfection efficiency was determined by examining MnSOD expression in 200 cells with a fluorescence microscope; transfection efficiency was above 80% (Figure 1G). The effect of MnSOD expression on the intracellular ROS level was measured by flow cytometry and MitoSOX staining, indicating that the mitochondrial ROS level was markedly decreased compared with control cells (Figure 1H). Immunofluorescence analysis also revealed that adenoviral expression of MnSOD (green) significantly decreased mitochondria ROS (Figure 1I). To identify the fluorescent signals shown in Figure 1, we stained the cells with MitoTracker. Figure 1J indicates that ROS stained with MitoSOX (red) was co-localized with MitoTracker (green). This indicates that the fluorescent signals shown in Figure 1 originated from the mitochondria.

Physiological ROS production is important for stimulating cell growth [4]. In order to determine if Romo1 expression is required for normal cell proliferation, *Romo1* siRNA was transfected into WI-38 cells and the cells were counted every day. Transfection of *Romo1* siRNA inhibited the growth of both WI-38 cells (Figure 2A) and WI-38 VA13 cells (Figure 2B). The percentage viabilities for the various

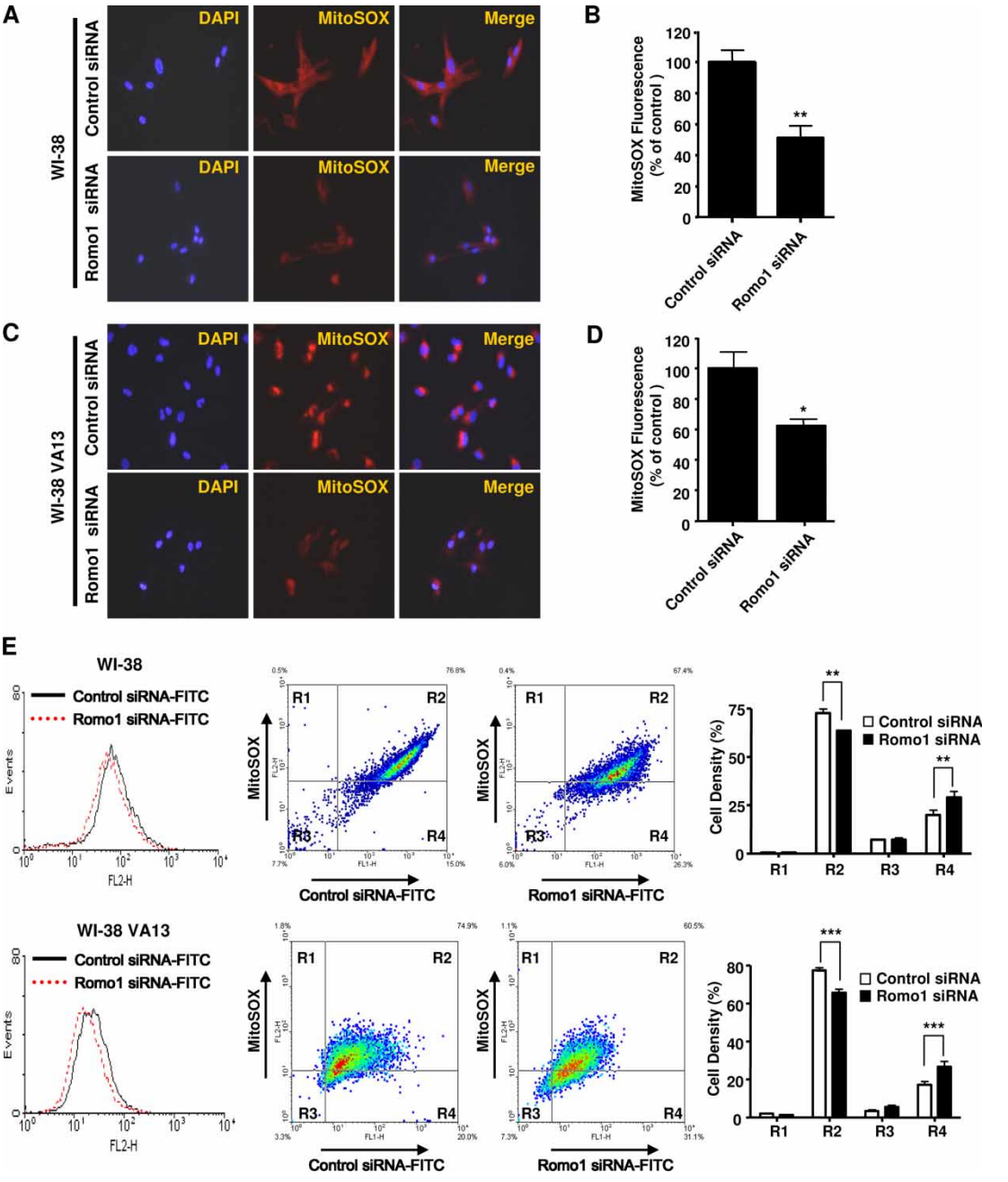

Figure 1 (*continued*).

groups within the WI-38 cell population were 72.6% (2 days), 55.2% (3 days) and 40.5% (4 days). The percentage viabilities of the various groups within the WI-38 VA13 cell population were 91.7% (2 days), 47.7% (3 days) and 28.5% (4 days). These results suggest that mitochondrial ROS originating from Romo1 is critical for normal cell growth.

Romo1 siRNA transfection induced G1 arrest by increasing p27^{Kip1} protein levels

Intracellular ROS play an important role in cell cycle progression and NAC-induced decreases in ROS levels cause cell cycle arrest in the G1 phase through p27^{Kip1} induction [8,12]. Therefore, we sought to determine if the down-regulation of ROS caused by *Romo1* knockdown induced cell cycle arrest. Cell cycle distribution was analysed by flow cytometry using propidium iodide staining. As shown in Figure 3A, progression of the cell cycle from the G1 to S phase was inhibited in cells transfected with *Romo1* siRNA. The proportion of cells in the G1 phase increased from 45.9% to 54.5%. G1 arrest was confirmed by a 5-bromo-2-deoxyuridine (BrdU) incorporation pulse chase experiment. Figure 3B showed that BrdU incorporation fell from 36.25% to 24.94% in the cells transfected with the *Romo1* siRNA. This clearly demonstrated that intracellular ROS levels, decreased by *Romo1* siRNA, retarded the

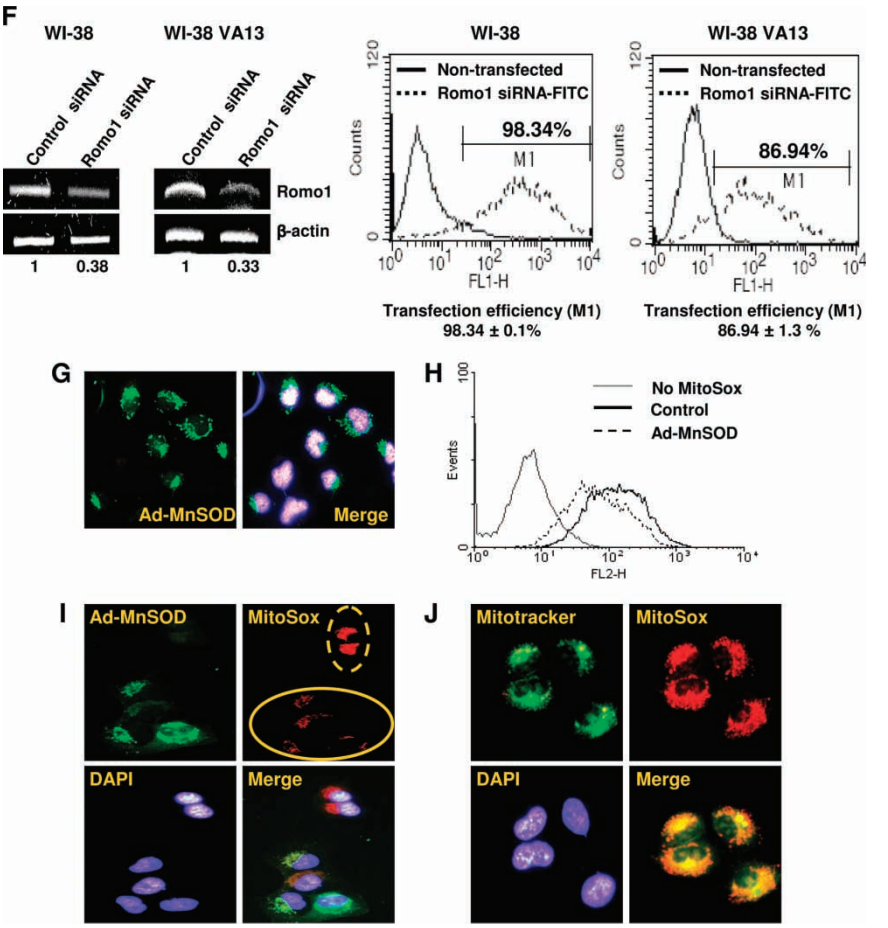

Figure 1. Down-regulation of endogenous ROS levels by Romo1 knockdown (A) WI-38 (PDL 34) cells were transfected with 100 nM of *Romo1* siRNA. After 72 h, cells were stained with MitoSOX and observed using fluorescence microscopy (Olympus LX71 microscope). (B) For quantification purposes, exposures were collected at equal times using the same plane of focus. MitoSOX fluorescence was analysed with MetaMorph software (Universal Imaging, Westchester, PA). (C) WI-38 VA13 cells were transfected with 100 nM Romo1 siRNA. After 72 h, cells were stained with MitoSOX and observed by fluorescence microscopy (Olympus LX71 microscope). (D) Mitochondrial ROS were quantified using MetaMorph software (Universal Imaging). (E) WI-38 (PDL 34) and WI-38 VA13 cells were transfected with *Romo1* siRNA-FITC for 72 h and stained with MitoSOX and ROS levels in transfected cells were measured by flow cytometry gating on 10 000 cells/sample. Density plot analysis of decreased ROS levels in 10 000 cells using FACS for FITC (FL1) and ROS intensity (FL2). (F) *Romo1* knockdown was analysed by RT-PCR. *Romo1* siRNA-FITC was transfected into both cells and transfection efficiency was analysed by flow cytometry gating on 10 000 cells/sample in three separate experiments. (G) Fluorescence microscopy images of cells expressing MnSOD. WI-38 VA13 cells were infected with adenoviral 50 MOI of MnSOD for 24 h. (H) WI-38 VA13 cells were infected with adenovirus expressing 50 MOI of MnSOD for 24 h. Decreased ROS levels were measured by flow cytometry. (I) Fluorescence microscopy images of MnSOD expression and ROS levels in WI-38 VA13 cells. Ad-MnSOD-infected cells were stained with an antibody to MnSOD (green) or MitoSOX (red). Straight lines show ad-MnSOD infected cells and dotted lines show non-infected cells. (J) Fluorescence microscopy images of cells stained with MitoTracker and MitoSOX. Error bar shows S.E. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

transition from G1 to S. This is consistent with a previous report, in which *Romo1* siRNA transfection induced cell cycle arrest in the G1 phase of H1299 lung carcinoma cells [10].

We then examined the expression of Cyclin D1, Erk, Akt, p21$^{WAF1/CIP1}$, Bcl-2 and p27^{Kip1} using Western blot analysis. Down-regulation of Cyclin D1 expression was observed in WI-38 and WI-38 VA13 cells transfected with *Romo1* siRNA (Figure 4A and B). Erk activation was also inhibited in cells transfected with *Romo1* siRNA (Figure 4A and B). These results are consistent with a previous report [10]. We also studied the expression levels of p21$^{WAF1/CIP1}$ and p27^{Kip1}. Figure 4 shows that *Romo1* siRNA transfection increased p27^{Kip1} expression in WI-38, WI-38 VA13 and IMR-90 cells. However, there was no change in p21$^{WAF1/CIP1}$ expression, indicating that p27^{Kip1} was a major mediator in the G1 arrest induced by *Romo1* siRNA-triggered decreases in ROS.

Exposure to H_2O_2-induced cell growth recovery in Romo1 siRNA-transfected cells

The physiological level of H_2O_2 has been suggested to stimulate cell proliferation by regulating specific genes [6]. To determine if exogenous H_2O_2 treatment allowed for the recovery of cell proliferation in WI-38 VA13 cells transfected with *Romo1* siRNA, we treated

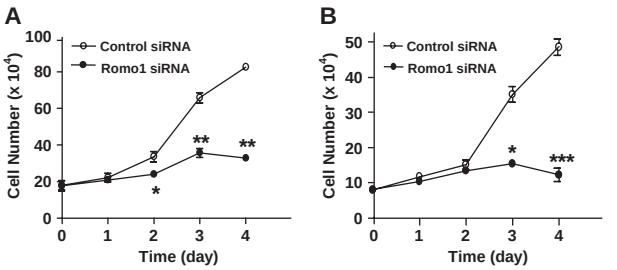

Figure 2. Growth inhibition after *Romo1* knockdown. (A) WI-38 (PDL 34) cells were transfected with 100 nM of *Romo1* siRNA. Cells were harvested every day and counted by staining with trypan blue. (B) WI-38 VA13 cells were transfected with *Romo1* siRNA and counted by staining with trypan blue. Error bar shows SE. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ vs control siRNA.

cells with various doses of H_2O_2. Figure 5A shows that cell growth was inhibited by *Romo1* siRNA transfection and that a low dose of H_2O_2 (3–6 μM) partially recovered the growth rate of cells transfected with *Romo1* siRNA. In contrast, cells transfected with control siRNA showed cell growth inhibition. Next, we examined $p27^{Kip1}$ protein levels by performing Western blot analysis. Interestingly, $p27^{Kip1}$ expression was down-regulated in cells exposed to low doses of H_2O_2 (3–6 μM), but was increased in cells treated with higher doses of H_2O_2 (Figure 5B). Since NAC treatment stimulates $p27^{Kip1}$ expression [12], this induction was also examined in both cell lines. As shown in Figure 5C, NAC treatment induced $p27^{Kip1}$ expression in both cell lines. These findings indicate both that intracellular ROS derived from the mitochondria participate in normal cell proliferation by regulating $p27^{Kip1}$ expression and that Romo1 is an important modulator in ROS production for normal cell cycle progression. To see that the direct link between Romo1 expression, change in ROS levels and the cell cycle change, a BrdU incorporation pulse chase experiment was carried out in the cells treated with H_2O_2. WI-38 VA13 cells were transfected with 100 nM Romo1 siRNA and treated with H_2O_2 (6 μM) for 1 h. After culturing for 24 h, a BrdU incorporation pulse chase experiment was performed.

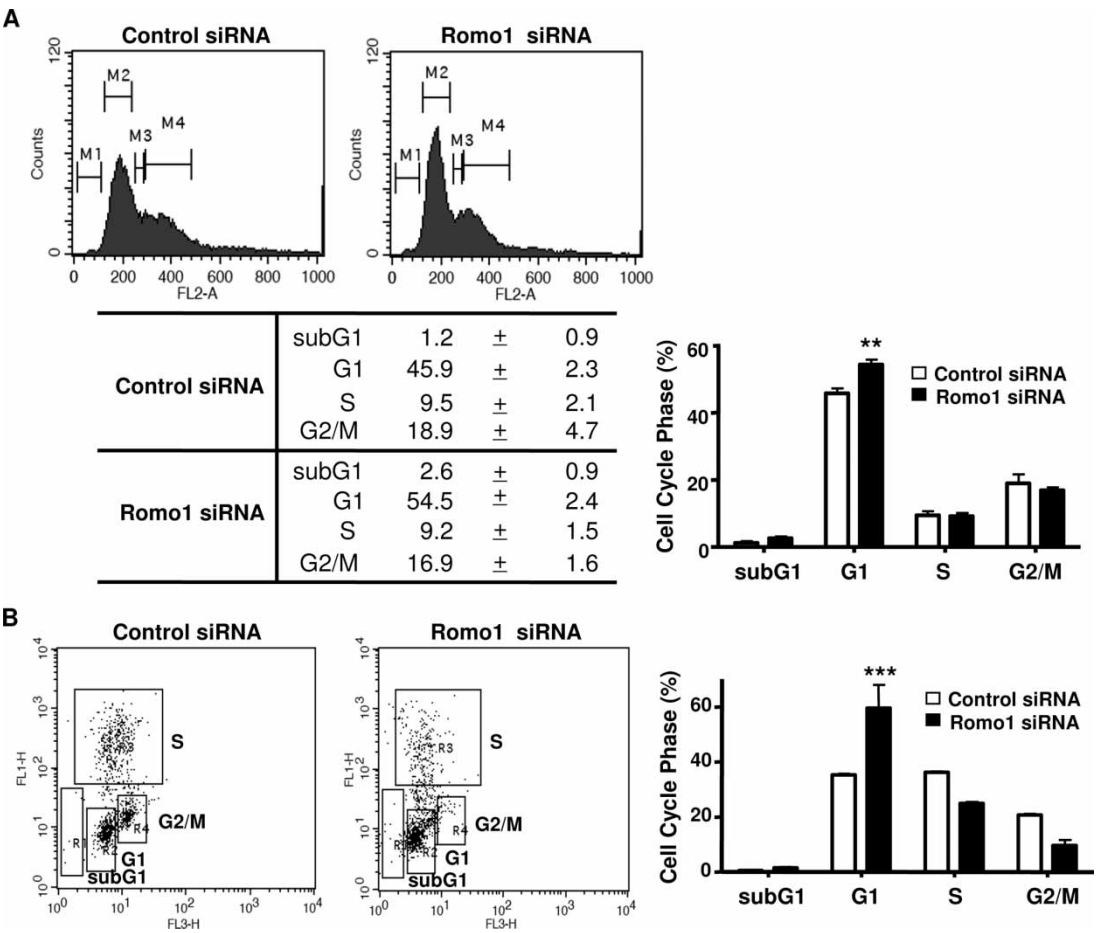

Figure 3. Cell cycle arrest in cells transfected with *Romo1* siRNA. (A) WI-38 VA13 cells were transfected with *Romo1* siRNA for 48 h. Cells were stained with propidium iodide containing 20 μg/ml RNase and analysed by flow cytometry. The cell cycle was analysed using Cell Quest software in three separate experiments. (B) WI-38 VA13 cells were transfected with *Romo1* siRNA for 48 h. Cells were treated with 10 μM BrdU for 1 h and analysed by FACS. Total DNA was stained with 7-AAD (FL3) and cells in the S phase were analysed by measuring the incorporation of BrdU (FL1). Dot plot shows each phase of the cell cycle total DNA content in three separate experiments. Error bar shows SE. ** $p<0.01$;*** $p<0.001$ vs control siRNA.

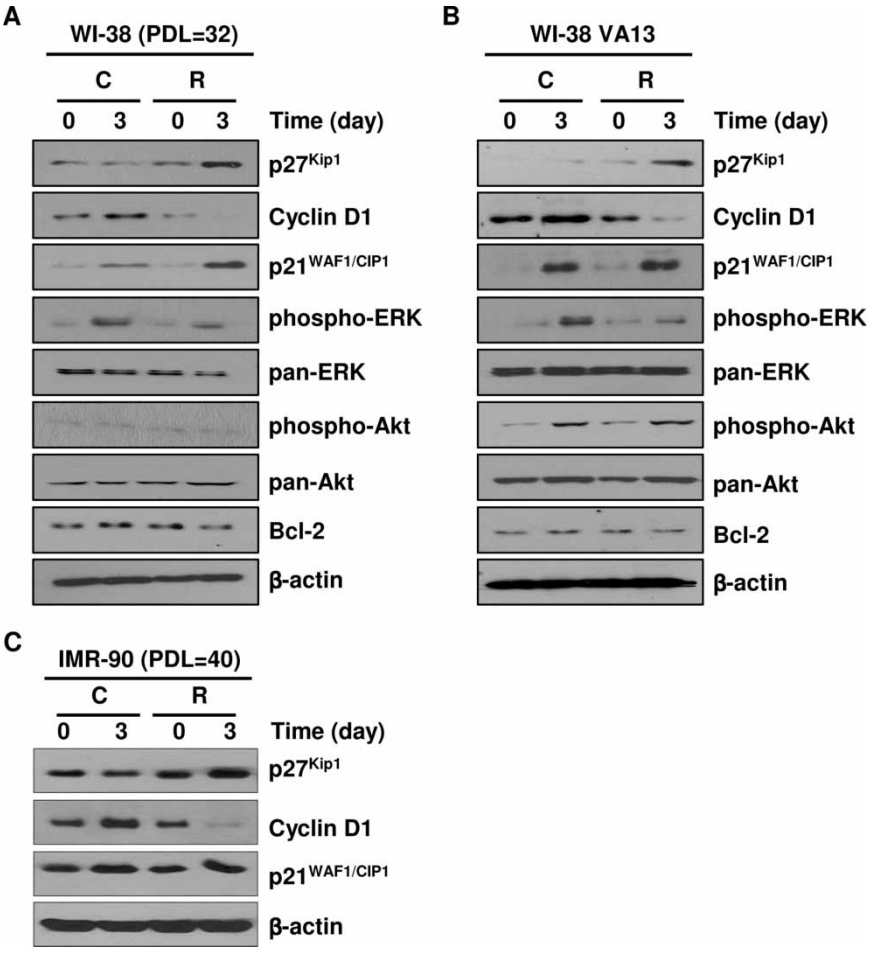

Figure 4. Western blot analysis using cells transfected with *Romo1* siRNA. WI-38 (PDL 32, A), WI-38 VA13 (B) and IMR-90 (PDL 40, C) cells were transfected with 100 nM *Romo1* siRNA for 72 h and Western blot analysis was conducted using the indicated antibodies. C, cells transfected with control siRNA; R, cells transfected with *Romo1* siRNA.

Figure 5D shows that BrdU incorporation fell from 32.58% to 20.48% by *Romo1* siRNA transfection and that a low dose of H_2O_2 (6 μM) partially recovered the transition rate from G1 to S of cells transfected with *Romo1* siRNA. This clearly demonstrated that intracellular ROS levels, decreased by *Romo1* siRNA, retarded the transition from G1 to S.

Discussion

Although excessive ROS production is harmful to cells, there have been many reports demonstrating that low levels of cellular ROS are essential for cell proliferation [4,5]. An antioxidant-induced reduction in endogenous ROS causes cell cycle arrest in the G1 phase, demonstrating that ROS steady-state levels are required for entry into the S phase [8]. These reports demonstrate that serum-induced cell growth is inhibited by a decrease in endogenous ROS. Many reports have also demonstrated that intracellular ROS are generated by growth factors and cytokines and that they are indispensable for cell proliferation [18,19]. ROS production required for redox signalling is mainly induced by NADPH oxidase and various growth factors and cytokines stimulate ROS generation by activating this enzyme [1]. However, it is not known if ROS derived from the mitochondria contribute to redox signalling.

In a previous report, we showed that endogenous ROS levels generated by Romo1 were required for tumour cell proliferation [10]. We also showed that *Romo1* knockdown inhibited the growth of various tumour cell lines, as well as normal IMR-90 fibroblasts. Tumour cell growth was inhibited by *Romo1* siRNA transfection and this inhibition was mediated by inhibition of Erk activation and down-regulation of Cyclin D1 expression. Mitochondrial ROS were shown to originate from Romo1 and to play an important role in tumour cell proliferation [10]. In the present study, we determined if a *Romo1* knockdown-induced decrease in endogenous ROS inhibited normal cell growth. We found that a decrease in ROS in lung fibroblast cell lines inhibited cell growth through the Erk pathway. Figure 5A depicts the

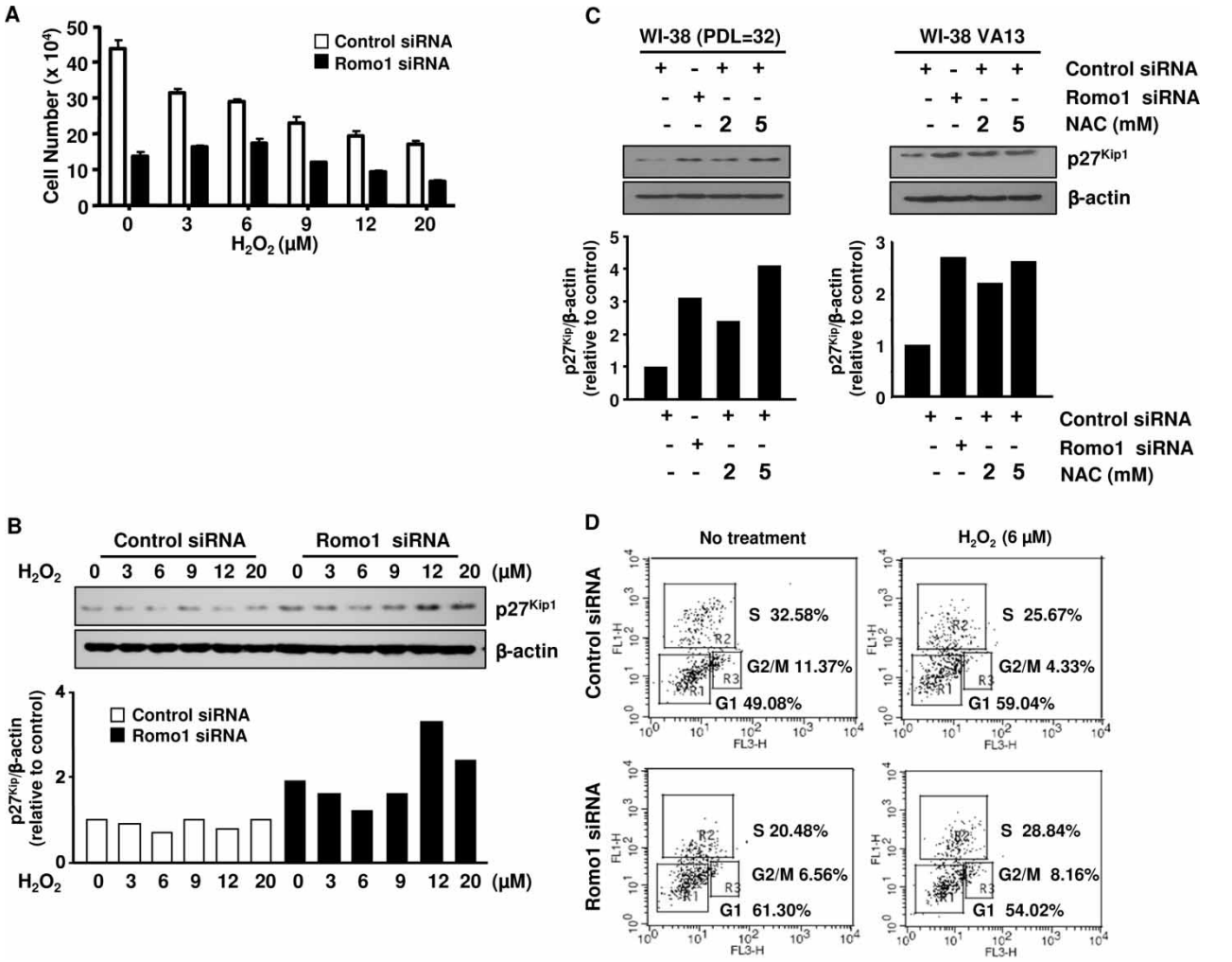

Figure 5. Cell growth and $p27^{Kip1}$ expression in cells treated with low-dose H_2O_2. (A) WI-38 VA13 cells were transfected with 100 nM *Romo1* siRNA for 48 h and treated with H_2O_2 (0, 3, 6, 9, 12 or 20 μM) for 1 h. After culturing for 24 h, the cells were counted. (B) After transfection with *Romo1* siRNA, the cells were exposed to H_2O_2 (0, 3 6, 9, 12 or 20 μM) for 30 min and Western blot analysis was performed using an antibody against $p27^{Kip1}$. (C) Cells were treated with 2 or 5 mM NAC for 24 h and Western blotting was performed using an antibody against $p27^{Kip1}$. Densitometric analysis of $p27^{Kip1}$ expression is provided in the lower panels, expressed relative to controls. (D) The cell cycle changes in cells treated with low-dose H_2O_2. After transfection with *Romo1* siRNA, the cells were exposed to H_2O_2 (6 μM) for 1 h, After culturing for 24 h, cells were treated with 10 μM BrdU for 1 h and analysed by FACS. Total DNA was stained with 7-AAD (FL3) and cells in the S phase were analysed by measuring the incorporation of BrdU (FL1). Dot plot shows each phase of the cell cycle total DNA content.

complete growth curve in *Romo1* siRNA-transfected cells under various H_2O_2 conditions. In an additional experiment, cells were treated with various concentrations of H_2O_2 for 1 h every day and then counted. A complete growth curve was also charted for 3 days under various H_2O_2 conditions. There was no H_2O_2 effect on cell proliferation (data not shown). This suggests that the cell growth effects of Romo1 knockdown can be partially countered by H_2O_2 treatment at 1 day. The fact that 3 days of H_2O_2 treatment could not recover cell growth can be explained two ways. First, adjustment of the redox imbalance resulting from intracellular Romo1 knockdown is difficult to accomplish with exogenous H_2O_2 treatment. Additional signalling pathways may exist in relation to endogenous Romo1-induced cell growth and this experiment needs to be clarified in the future. Romo1 is an important modulator of ROS production in normal cell cycle progression and Romo1-derived intracellular ROS participates in normal cell proliferation by regulating $p27^{Kip1}$ expression.

Even though the exact mechanism by which Romo1 increases intracellular ROS in the mitochondria of normal and tumour cells has been not identified, Romo1 appears to modulate the release of ROS from the mitochondria into the cytoplasm. In a previous report, we showed that the increased ROS formation caused by Romo1 over-expression induced nuclear damage, indicating that ROS produced in the mitochondria were translocated into the nucleus [15]. We also showed that inhibitors of complex III in the

mitochondrial respiratory chain inhibited the Romo1-mediated increase of ROS levels, indicating that Romo1-derived ROS originated from complex III of the respiratory chain. Complex III is a major source of ROS released into the cytosol and the majority of ROS in the mitochondria are produced at complexes I and III [20]. Therefore, it is possible that Romo1 is located in the mitochondrial membrane and that Romo1 exerts a role as a modulator to supply ROS required for redox signalling in cell proliferation. Further studies will be needed to clarify the exact mechanism by which Romo1 releases ROS into the cytoplasm. Although little Romo1 information has been published thus far, the present study suggests that Romo1 is a major molecular contributor in the maintenance of cellular ROS levels. Interestingly, ROS derived from the mitochondria play a major role in normal cell proliferation. However, further research, preferably using *in vivo* systems, is required to understand how Romo1 generates ROS in the mitochondria and functions in cell proliferation.

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