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Knockdown of TWIST1 enhances arsenic trioxide- and ionizing radiation-induced cell death in lung cancer cells by promoting mitochondrial dysfunction

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ABSTRACT

TWIST1 is implicated in the process of epithelial mesenchymal transition, metastasis, stemness, and drug resistance in cancer cells, and therefore is a potential target for cancer therapy. In the present study, we found that knockdown of TWIST1 by small interfering RNA (siRNA) enhanced arsenic trioxide (ATO)- and ionizing radiation (IR)-induced cell death in non-small-cell lung cancer cells. Interestingly, intracellular reactive oxygen species levels were increased in cells treated with TWIST1 siRNA and further increased by co-treatment with ATO or IR. Pretreatment of lung cancer cells with the antioxidant N-acetyl-cysteine markedly suppressed the cell death induced by combined treatment with TWIST1 siRNA and ATO or IR. Moreover, treatment of cells with TWIST1 siRNA induced mitochondrial membrane depolarization and significantly increased mitochondrial fragmentation (fission) and upregulated the fission-related proteins FIS1 and DRP1. Collectively, our results demonstrate that siRNA-mediated TWIST1 knockdown induces mitochondrial dysfunction and enhances IR- and ATO-induced cell death in lung cancer cells.

1. Introduction

The term reactive oxygen species (ROS) collectively describes a broad class of O_2-derived free radicals as well as O_2-derived non-radical species, such as hydrogen peroxide [1]. Elevated levels of ROS can cause irreversible cellular injury and dysfunction through direct effects on bio-macromolecules, including nucleic acids, proteins, and lipids. High concentrations of ROS alter the balance of protective versus apoptotic systems. A number of anticancer agents currently used for cancer treatment have been shown to increase cellular ROS generation [2,3]. ROS levels above a critical homeostatic threshold that is incompatible with growth or survival of tumor cells, but tolerated by normal cells, leads to activation of apoptotic pathways [4]. Among therapeutic agents that act via this mechanism are arsenic trioxide (ATO), cisplatin, and irradiation (IR) [5–7].

The basic helix-loop-helix transcription factor TWIST1, a member of the TWIST family that also includes TWIST2/Dermo1, is one of the master regulators of the process of epithelial to mesenchymal transition [8]. Although TWIST1 is silent in most healthy adult tissue, it is frequently upregulated in a panel of carcinomas, including breast, prostate and hepatocellular carcinomas, where it is found to be a poor prognostic marker [9,10]. Thus, TWIST1 has been suggested to enhance the invasive and metastatic properties of cancer cells. TWIST1 also protects cancer cells from apoptotic cell death and is responsible for the stemness of cancer cells and the emergence of drug resistance [11].

The development of treatment strategies based on targeting TWIST holds great promise in cancer therapeutics. Recently, inactivation of TWIST1 by small interfering RNA (siRNA) was found to induce cellular senescence and growth arrest, and increase drug sensitivity. However, the underlying mechanisms have remained unclear. In this study, we demonstrate that siRNA-mediated knockdown of TWIST1 induces mitochondria fragmentation and thereby increases intracellular ROS levels in non-small-cell lung cancer

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(NSCLC) cells. Knockdown of TWIST1 acted through increased generation of ROS to markedly enhance ATO- and IR-induced cell death. These results strongly suggest that TWIST1 is involved in regulating ROS and could be a therapeutic target for enhancing the anti-cancer activity of ROS-inducing agents.

2. Materials and methods

2.1. Cell culture and reagents

H1299 and H460 human NSCLC cells, purchased from the American Type Culture Collection (Manassas, VA, USA), were grown in the recommended growth medium (Invitrogen, Carlsbad, CA, USA). Antibodies against DRP1, FIS1, MFN1, OPA1, and β-actin were acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA). ATO and N-acetyl-L-cysteine (NAC) were purchased from Sigma–Aldrich (St. Louis, MO, USA). siRNAs targeting TWIST1 and negative control (scrambled) siRNAs were from Santa Cruz Biotechnology.

2.2. Transfections and treatments

Cells in 1 ml of serum-free medium were transfected with control siRNA or TWIST siRNA (100 nM) for 4 h at 37 °C in a CO_2 incubator using Lipofectamine (Invitrogen), as described by the manufacturer. After replacing media with fresh media containing 10% fetal bovine serum, cells were treated with ATO (1.5–2 μM) or IR (5 Gy) for 36 h and then analyzed as described below. Where used, NAC was applied 30 min before treatment with ATO or IR.

2.3. Reverse transcription-polymerase chain reaction

Total RNA was isolated using an Easy-Spin kit (Intron, Korea). An aliquot of total RNA (2 μg) was transcribed into cDNA using an RT^2 First Strand kit (Qiagen, Valencia, CA, USA). cDNA (2–5 μl) was amplified with a KAPA SYBR FAST qPCR kit (Kapa Biosystems, Woburn, MA, USA) using TWIST1 primer pairs from Origene Technologies (Rockville, MD, USA). PCR thermocycling conditions were as follows: 95 °C for 3 min (initial denaturation) followed by 40 cycles of 95 °C for 3 s (denaturation) and 60 °C for 30 s (annealing and extension).

2.4. Measurement of cell viability

Cell viability was determined by measuring the mitochondrial conversion of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) to a colored product. Cells were treated with

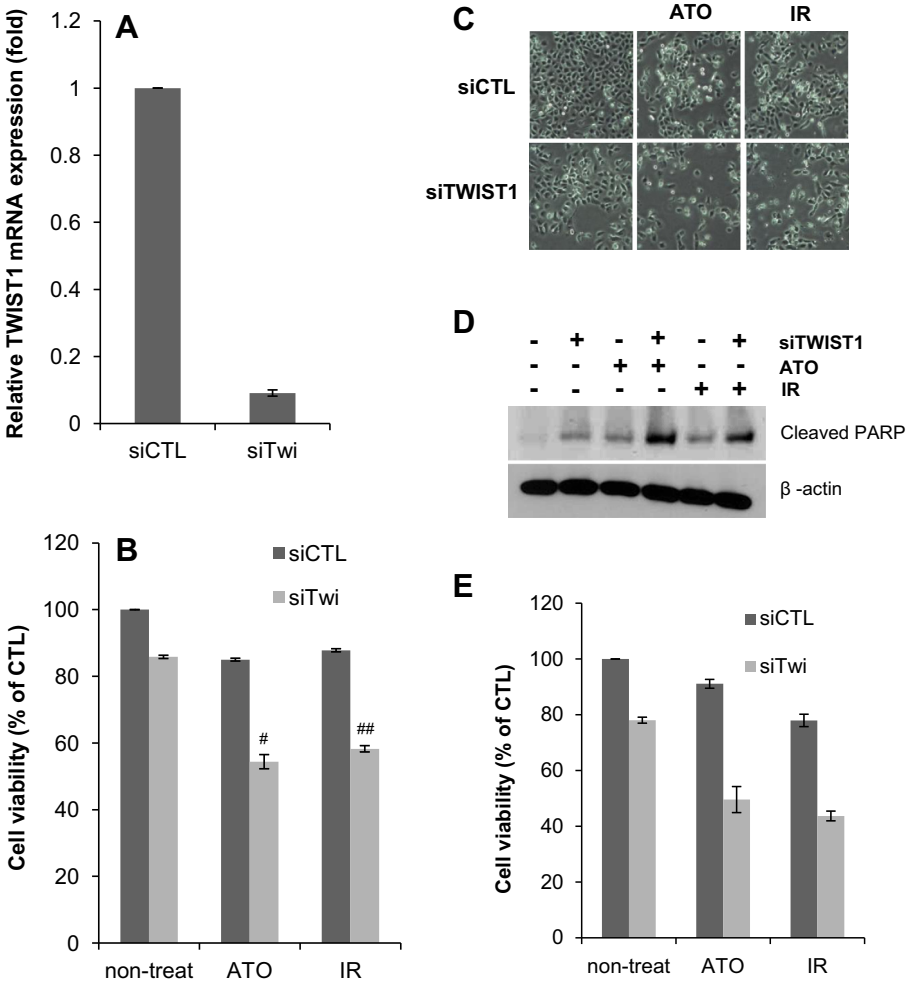

Fig. 1. Anticancer effects of combined treatment with TWIST1 siRNA and ATO or IR in NSCLC cells. Co-treatment with TWIST1 siRNA and ATO or IR exerts synergistic effects on cytotoxicity and PARP cleavage. (A) qRT-PCR. Cells were transfected with 100 μM negative control siRNA (siCTL) or TWIST1 siRNA and then incubated for 36 h. Data were normalized to that of β-actin and the relative TWIST1 mRNA levels are shown as fold. (B and D) MTT assay, morphology, and Western blotting. H1299 cells were transfected with 100 μM TWIST1 siRNA and then treated with 1.5 μM ATO or 5 Gy IR for 36 h. (E) MTT assay. H460 cells were transfected with 100 μM TWIST1 siRNA and then treated with 2 μM ATO or 5 Gy IR for 36 h. Each value represents the mean ± S.D. of three independent experiments ($^{\#\#}P < 0.01$, $^{\#}P < 0.05$ vs. TWIST1 siRNA-treated groups).

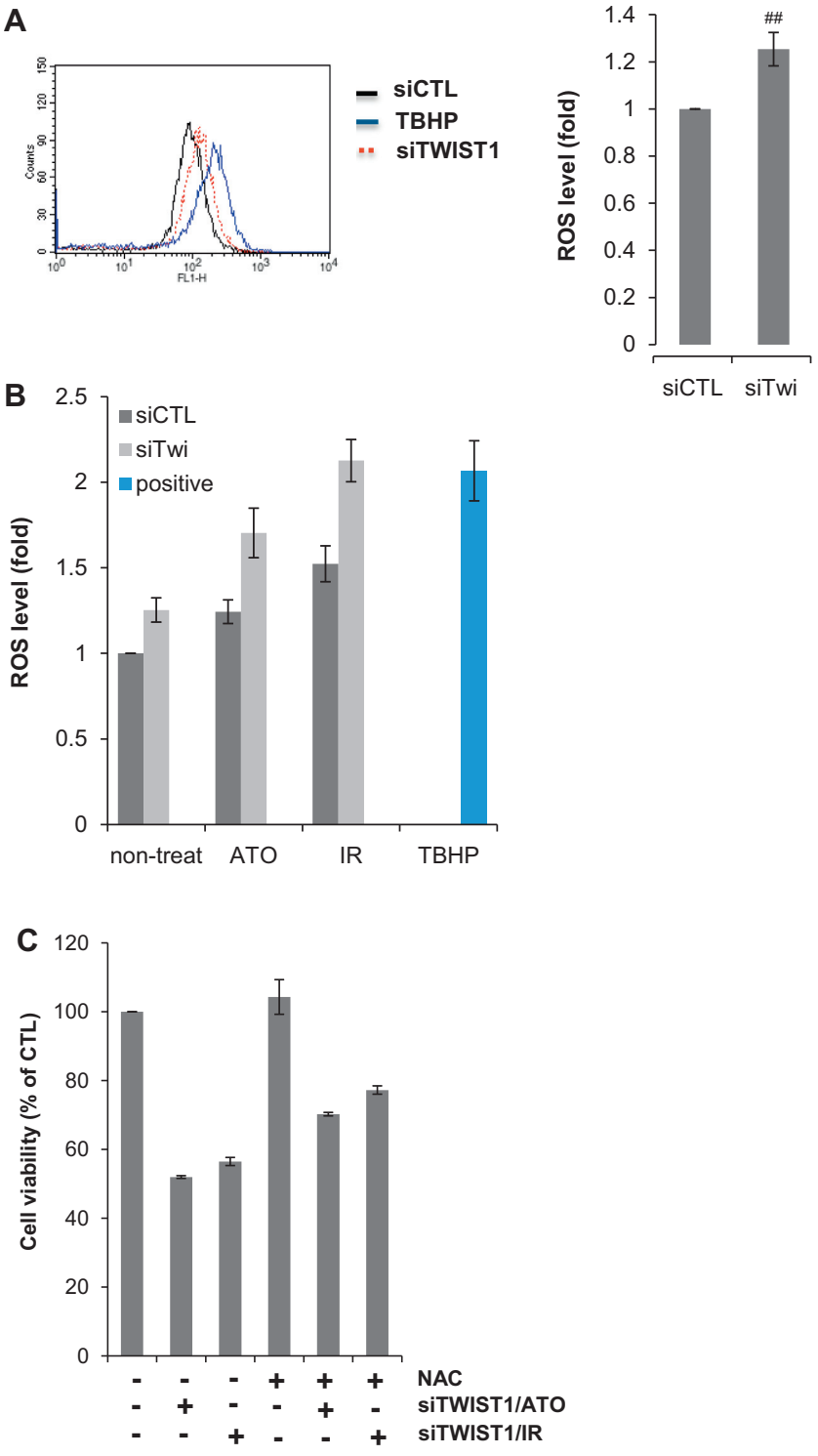

Fig. 2. Involvement of ROS in the effects of combined treatment with TWIST1 siRNA and ATO or IR in H1299 cells. Both TWIST1 siRNA alone and combined with ATO or IR induced the production of ROS. NAC partially blocked the cell death induced by combined treatment. (A and B) ROS generation measured by FACS analysis. Cells were treated as described in Fig. 1B. Representative results of three independent experiments are shown. Each value represents the mean ± S.D. ($^{\#\#}P < 0.01$ vs. control siRNA-treated groups). (C) MTT assay. Cells were transfected with 100 μM TWIST1 siRNA and then treated with 1.5 μM ATO or 5 Gy IR in the presence or absence of 10 mM NAC for 36 h.

drugs, as indicated, and the medium was exchanged with serum-free medium containing 1 mM MTT. After 1 h of incubation at 37 °C, cells were solubilized in dimethyl sulfoxide (DMSO). The amount of formazan, the converted form of MTT, was determined by measuring absorbance at 595 nm.

2.5. Measurement of ROS

ROS in cells were measured by fluorescence-activated cell sorting (FACS) analysis using a DCFDA Cellular Reactive Oxygen Species Detection Assay Kit (Abcam, Cambridge, MA, USA), as

described by the manufacturer. Tert-butyl hydrogen peroxide (50 μM) was used as a positive control.

2.6. Measurement of mitochondrial membrane potential

Loss of mitochondrial membrane potential (MMP) was measured using the MitoProbe JC-1 Assay kit (Molecular Probes, Invitrogen) as described by the manufacturer. Carbonyl cyanide 3-chlorophenylhydrazone (50 μM) was used as a positive control.

2.7. Confocal microscopy

Cells in 1 ml of serum-free medium were transfected with a mito-YFP (yellow fluorescent protein) expression plasmid (0.5 μg) and/or TWIST siRNA (100 nM). Thirty-six hours after transfection, changes in mitochondrial morphology were observed by confocal microscopy.

2.8. Western blotting

Cells were harvested and lysed in RIPA buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, and 0.1% SDS) supplemented with a protease inhibitor cocktail (Roche, Mannheim, Germany). Protein concentrations in lysates were determined by BCA assay. Equal amounts of protein (20–50 μg) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a nitrocellulose membrane. Membranes were blocked by incubating with 3% skim milk in Tris-buffered saline (TBS) for 1 h and then incubated overnight with the appropriate primary antibodies (diluted 1:1000). Membranes were then incubated with horseradish peroxidase-conjugated secondary antibody (1:3000) for 1 h. Immunoreactive proteins were visualized using enhanced chemiluminescence reagents (Amersham Biosciences, Little Chalfont, UK).

3. Results

3.1. siRNA-mediated TWIST1 knockdown enhances ATO- and IR-induced cell death

To investigate whether knockdown of TWIST1 sensitizes H1299 non-small-cell lung cancer cells to arsenic trioxide (ATO)- or ionizing radiation (IR)-induced cytotoxicity, we transfected cells with TWIST siRNA for 4 h, followed by exposure to 1.5 μM ATO or 5 Gy ionizing radiation (IR) for 36 h. The specificity of TWIST siRNA toward TWIST1 mRNA was confirmed using qRT-PCR (Fig. 1A). TWIST1 siRNA sensitized cells to the cytotoxic effects of ATO and IR (Fig. 1B and C) and enhanced poly-(ADP-ribose) polymerase (PARP) cleavage (Fig. 1D). A similar sensitizing effect of TWIST1 siRNA on ATO- and IR-induced cytotoxicity was obtained in the NSCLC cell lines, H460 (Fig. 1E). These results clearly indicate that knockdown of TWIST1 enhances the sensitivity of lung cancer cells to ATO and IR.

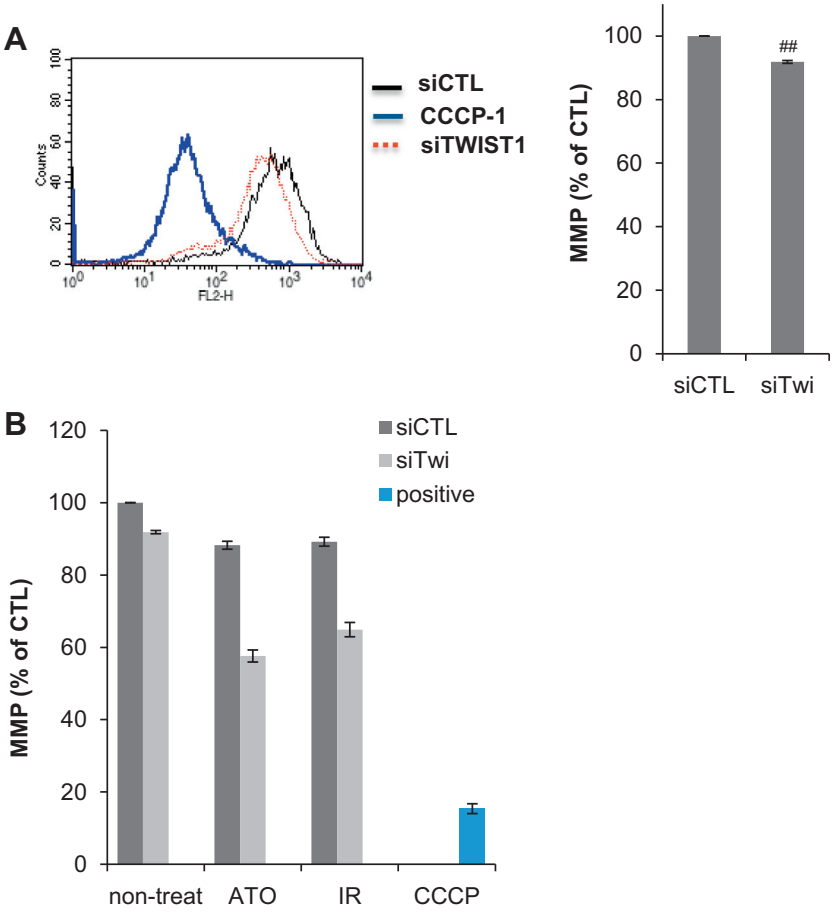

Fig. 3. Loss of MMP following combined treatment with TWIST1 siRNA and ATO or IR in H1299 cells. TWIST1 siRNA alone induced mitochondrial dysfunction and synergized with ATO and IR to further decrease MMP. MMP measured by FACS analysis. Representative results of three independent experiments are shown. Each value represents the mean ± S.D. ($^{\#\#}P < 0.01$ vs. control siRNA-treated groups).

3.2. Knockdown of TWIST1 induces ROS production and further enhances ATO- and IR-induced ROS production

It has been reported that ATO and IR induce cell death in cancer cells by promoting the production of ROS [5,7]. Therefore, we examined ROS generation in response to TWIST1 siRNA alone and in combination with ATO or IR in lung cancer cells. Unexpectedly, we found that TWIST1 siRNA alone generated ROS (Fig. 2A) and synergized with ATO and IR to produce further increases in ROS (Fig. 2B). Next, we determined whether this elevated production of ROS is crucial for the combined effects of TWIST siRNA and ATO or IR on cell viability. We found that NAC, a free radical scavenger, markedly attenuated the cytotoxic effects of combined treatment. Our results imply that elevated ROS levels are necessary for the induction of cell death by combined treatment with TWIST1 siRNA and ATO or IR.

3.3. Knockdown of TWIST1 induces a loss of mitochondria membrane potential

We previously reported that suppression of TWIST1 leads to a marked increase in the cellular ADP/ATP ratio, indicating a decrease in the ATP content in cells [12]. Therefore, we assessed the changes in mitochondria membrane potential (MMP) after treatment of H1299 cells with TWIST1 siRNA alone or together with ATO or IR. As shown in Fig. 3A, TWIST1 siRNA alone significantly decreased MMP. In cells treated with TWIST1 siRNA together with ATO or IR, the loss of MMP was enhanced compared to that observed with ATO or IR treatment alone (Fig. 3B). These results show that silencing TWIST1 induces mitochondrial dysfunction, at least in lung cancer cells.

3.4. Knockdown of TWIST1 induces mitochondrial fragmentation and changes in fragmentation-related mitochondrial proteins

Mitochondrial fragmentation has been reported to be a prerequisite for ROS production [13]. Since knockdown of TWIST1 induced ROS production and a loss of MMP, we investigated mitochondrial fragmentation (fission) after treatment with TWIST1 siRNA using a plasmid expressing a YFP protein fused with a mitochondria tracker (mito-YFP) [14]. H1299 cells were co-transfected with mito-YFP and TWIST siRNA and incubated for 36 h, after which cell morphology was examined by confocal microscopy. As shown in Fig. 4A, TWIST1-knockdown cells exhibited a fragmented mitochondrial phenotype. Moreover, mitochondrial proteins involved in mediating mitochondrial fission and fusion were dynamically changed. Specifically, Western blot analyses revealed that expression levels of dynamin-related protein 1 (DRP1) and fission 1 (FIS1) were increased in TWIST1 siRNA-treated cells, whereas those of the fusion proteins mitofusin1 (MFN1) and optic atrophy protein 1 (OPA1) were decreased (Fig. 4B). These general increases in the expression of fission proteins and decreases in the expression of fusion proteins indicate that TWIST1 knockdown results in abnormal mitochondrial dynamics.

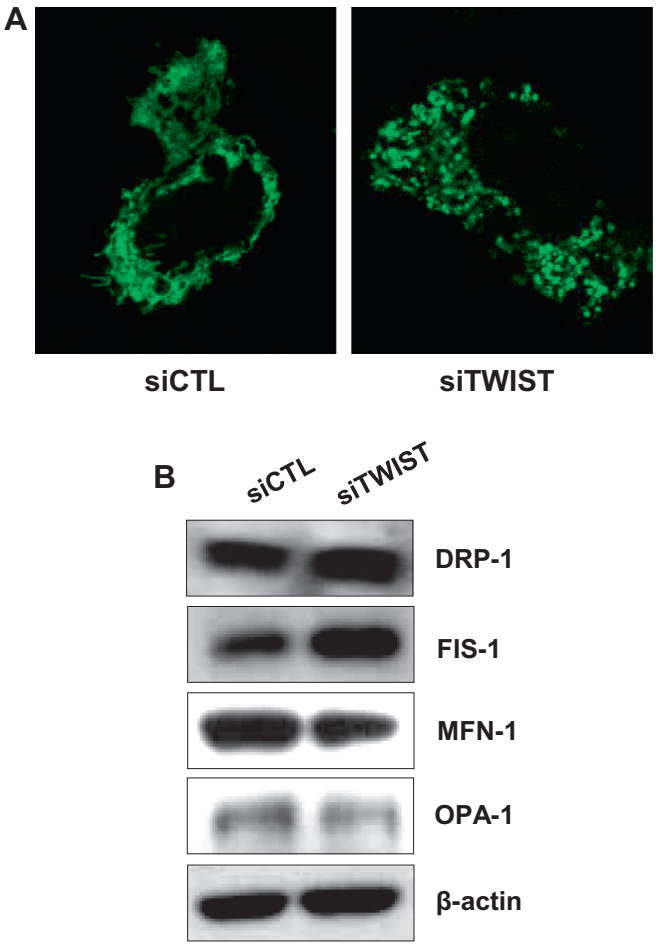

Fig. 4. Mitochondria fragmentation and changes in mitochondrial proteins induced by treatment with TWIST1 siRNA in H1299 cells. Treatment with TWIST1 siRNA induced mitochondrial fragmentation and increased DRP1 and FIS1 expression. Fluorescence micrographs of mitochondrial morphology and Western blotting. Cells were transfected with 100 μM negative control siRNA (siCTL) or TWIST1 siRNA and then incubated for 36 h. Data are representative of at least three independent experiments.

4. Discussion

TWIST1 is frequently overexpressed in cancer cells and is suggested to play an important role in cancer progression and resistance to chemotherapy [15]. In the present study, we demonstrated that knockdown of TWIST1 using siRNA enhanced the sensitivity of lung cancer cells to ATO and IR through enhanced ROS production and dissipation of MMP. Unexpectedly, we found that knockdown of TWIST1 induced mitochondrial fragmentation with a concomitant increase in the expression of fission-related proteins (DRP1 and FIS1) and a decrease in the expression of fusion-related proteins (MFN1 and OPA1) in lung cancer cells. Moreover, knockdown of TWIST1 decreased MMP and increased ROS production. These results are in line with previous reports that increased DRP1 expression and decreased MFN1/2 expression enhances ROS production in dexamethasone-treated hepatoma cells and radiation-treated human epithelial cells [16,17]. In this context, mitochondrial fragmentation induced by knockdown of TWIST1 increased ROS production in NSCLC cells, thereby sensitizing cells to ATO- and IR-induced cell death. Importantly, scavenging of ROS by NAC suppressed the cell death induced by combined treatment with TWIST1 siRNA and ATO or IR.

The mitochondrial network is continually remodeled through dynamic regulation of fusion and fission [18–21]. At the molecular level, fusion and fission are regulated by several proteins, including Drp1, Fis1, Mfn1/2, and Opa1 [22–27]. Diverse regulatory pathways affect mitochondrial dynamics via these proteins by changing their expression levels and/or post-translational modifications, such as phosphorylation and S-nitrosylation. Here, we showed that knockdown of TWIST1 differentially regulated these proteins, upregulating the fission proteins DRP1 and FIS1 and downregulating the fusion proteins MFN1 and OPA1. These findings imply that enhanced fission and impaired fusion induced by knockdown of

TWIST1 contribute to the mitochondrial fission process. Although it is clear that knockdown of TWIST1 regulates mitochondrial fusion and fusion proteins and contributes to mitochondria fragmentation, the mechanism by which TWIST1 knockdown regulates these processes is not yet known. Further study is needed to clearly define how TWIST1 regulates mitochondrial fusion and fusion proteins. In conclusion, targeting TWIST1 efficiently sensitizes NSCLC cells to ATO and IR. This effect is mediated by disrupting mitochondrial function, resulting in elevated production of ROS. Our findings provide a rational for combination treatment with TWIST1 siRNA and ATO or IR as a promising therapeutic approach to lung cancer therapy.

5. Conflict of interest

The authors declare no conflicts of interest.

Acknowledgement

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