

SCIENTIFIC REPORTS



OPEN

Role of Signal Regulatory Protein α in Arsenic Trioxide-induced Promyelocytic Leukemia Cell Apoptosis

Received: 08 December 2015

Accepted: 14 March 2016

Published: 24 March 2016

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Signal regulatory protein α (SIRP α) has been shown to operate as a negative regulator in cancer cell survival. The mechanism underneath such function, however, remains poorly defined. In the present study, we demonstrate that overexpression of SIRP α in acute promyelocytic leukemia (APL) cells results in apoptosis possibly via inhibiting the β -catenin signaling pathway and upregulating Foxo3a. Pharmacological activation of β -catenin signal pathway attenuates apoptosis caused by SIRP α . Interestingly, we also find that the pro-apoptotic effect of SIRP α plays an important role in arsenic trioxide (ATO)-induced apoptosis in APL cells. ATO treatment induces the SIRP α protein expression in APL cells and abrogation of SIRP α induction by lentivirus-mediated SIRP α shRNA significantly reduces the ATO-induced apoptosis. Mechanistic study further shows that induction of SIRP α protein in APL cells by ATO is mediated through suppression of c-Myc, resulting in reduction of three SIRP α -targeting microRNAs: miR-17, miR-20a and miR-106a. In summary, our results demonstrate that SIRP α inhibits tumor cell survival and significantly contributes to ATO-induced APL cell apoptosis.

SIRP α (also designated as CD172a, p84, SHPS-1) is a receptor-like membrane protein mainly present on mature myeloid leukocytes including neutrophils, monocytes, and macrophage^{1,2}. As an immunoglobulin superfamily member, SIRP α consists of three extracellular IgV-like loops and a cytoplasmic region with two immunoreceptor tyrosine-based inhibitory motifs (ITIMs). Previous studies have demonstrated that ligation of SIRP α by its ligand CD47, a ubiquitous cell membrane protein, leads to phosphorylation of its ITIMs, which in turn, recruits SH2 domain-containing protein tyrosine phosphatases SHP-1 or SHP-2 to initiate downstream inhibitory signal³. It has been shown that, through recruiting and activating SHP-1, SIRP α dephosphorylates Akt and GSK3 β , leading to the destabilization of β -catenin and the inactivation of Wnt/ β -catenin pathway. For example, Maekawa *et al.* reported that β -catenin was significantly induced by suppressing SIRP α level in K562 cells⁴. Qin *et al.* also reported that SIRP α significantly decreased the expression of β -catenin in hepatocellular carcinoma cell⁵. As canonical Wnt/ β -catenin signaling plays an important role in modulating proliferation and survival of tumor cells, particularly the leukemia cells^{6–9}, we speculate that SIRP α may promote acute promyelocytic leukemia (APL) cell apoptosis and suppress APL cell survival via inhibiting Wnt/ β -catenin signal pathway.

Arsenic trioxide (As₂O₃; ATO), a compound therapeutically used in Chinese traditional medicine, is effective in the treatment of patients with APL^{10–15}. Most APL cases are caused by the chromosomal translocation, resulting in the rearrangement of the promyelocytic leukemia (PML) gene and retinoic acid receptor (RAR α) gene and the production of PML-RAR α fusion protein¹⁴. PML-RAR α fusion protein is crucial for the pathogenesis of APL because it operates as a transcriptional silencer in the retinoic acid signaling pathway to block cell differentiation. While displaying a similar role of all-trans-retinoic acid (ATRA) in promoting the degradation of PML-RAR α fusion protein¹⁵, ATO is also a potent inducer of APL cell apoptosis^{16,17}. It has been reported that ATO induces

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cell apoptosis through producing reactive oxygen species to activate Chk2/p53 and p38 MAPK/p53 apoptotic pathways¹⁸ and inhibiting hTERT expression¹⁹. However, the mechanism underlying the ATO-induced APL cell apoptosis remains incompletely understood.

In the present study, we investigated the general pro-apoptotic effect of SIRP α on tumor cells, and as an extension, we studied the role of SIRP α in the ATO-induced apoptosis of APL cells. We found that expression of SIRP α resulted in apoptosis both of APL HL-60 cells and hepatocellular carcinoma Huh7 cells possibly by suppressing β -catenin signal pathway and upregulating Foxo3a. SIRP α -induced apoptosis could be reversed by pharmacological activation of β -catenin signal pathway. We also observed that ATO treatment induced SIRP α expression in APL cells in a time-dependent manner and abrogation of SIRP α induction prevented APL cell apoptosis and left the β -catenin signaling pathway unperturbed by the ATO treatment. The present study also further explored the miRNA-based mechanism that governs the induction of SIRP α by ATO in APL cells.

Results

SIRP α promoted the apoptosis and suppressed the proliferation of APL and hepatocellular carcinoma cells. Previous studies by us and others showed that APL HL-60 cells and hepatocellular carcinoma Huh7 cells lack SIRP α protein expression. To elucidate the effect of SIRP α , HL-60 and Huh7 cells were infected with either SIRP α -expressing lentivirus (LV-SIRP α) or control lentivirus (LV-CTL) and subjected to cell proliferation and apoptosis assay. The infection efficiency was approximately 90% with MOI 5 (supplementary Figure S1). As shown in Fig. 1a, HL-60 and Huh7 cells infected with LV-SIRP α both expressed significant amount of SIRP α protein at 48 h post-infection. MTT assay on day 3 demonstrated that the proliferation of cells infected with LV-SIRP α was significantly inhibited (Fig. 1b). Furthermore, we monitored the cell apoptosis on day 3 post-infection. The level of cleaved caspase-3, an activated form of caspase-3 was examined by western blotting using anti-caspase-3 antibody, while annexin V on cell surface was evaluated using annexin V-PI kit and flow cytometry. As shown in Fig. 1c, expression of SIRP α in HL-60 and Huh7 cells resulted in a drastic increase in the level of activated caspase-3, suggesting that the cells infected with LV-SIRP α underwent apoptosis. Consistent with this finding, HL-60 cells infected with LV-SIRP α displayed a significant increase in the percentage of annexin V-positive cells compared to cells infected with LV-CTL (Fig. 1d). Taken together, our results show that SIRP α expression inhibits the proliferation and promotes the apoptosis of tumor cells.

Downregulation of β -catenin by SIRP α contributes to tumor cell apoptosis. The involvement of Wnt/ β -catenin signal pathway in cell survival and various malignancies has been widely shown^{20,21}. To explore the mechanism underlying the inhibitory effect of SIRP α on cell survival, we examined whether SIRP α expression could possibly suppress β -catenin expression. For this experiment, we infected HL-60 cells and Huh7 cells with either LV-SIRP α or LV-CTL, and harvested the cells 3 days post-infection for western blotting analysis. As shown in the Fig. 2a, the level of β -catenin was significantly reduced in the HL-60 cells infected with LV-SIRP α but not LV-CTL. As the Wnt/ β -catenin signaling pathway can be regulated by Akt/GSK-3 β signaling²², we further tested whether SIRP α expression disrupted the Akt/GSK3 β pathway by measuring the levels of p-Akt, Akt, p-GSK3 β and GSK3 β in the HL-60 cells infected with LV-SIRP α . As shown in Fig. 2a, levels of p-Akt and p-GSK3 β were significantly reduced in HL-60 cells infected with LV-SIRP α , suggesting that SIRP α expression decreased the level of p-Akt and p-GSK3 β , leading to activation of GSK3 β and the degradation of β -catenin. Since Wnt/ β -catenin signaling has been shown to antagonize Foxo3a-mediated apoptosis^{23,24}, we also assessed whether SIRP α expression could lead to the increased expression of Foxo3a. As shown in Fig. 2a, expression of SIRP α alone significantly promoted the Foxo3a expression in HL-60 cells. Similar results were obtained in the hepatocellular carcinoma Huh7 cells (Fig. 2a). Taken together, these data suggest that SIRP α expression possibly suppress Wnt/ β -catenin signaling and promote Foxo3a expression in tumor cells.

To confirm that SIRP α expression promotes the expression of Foxo3a and cell apoptosis through suppressing the level of β -catenin, we tested whether the lithium chloride (LiCl) and SB-216763, two reagents widely used to repress the activity of GSK3 β and thus inhibit GSK3 β -mediated degradation of β -catenin²³, can abolish the inhibitory effect of SIRP α expression on β -catenin. As shown in the Fig. 2b, expression of SIRP α alone in the HL-60 cells suppressed β -catenin and promoted cell apoptosis as evidenced by enhanced Foxo3a expression and increased caspase-3 cleavage level. In contrast, treatment with LiCl or SB-216763 in LV-SIRP α -infected cells strongly rescued the β -catenin expression, hampered the increase of Foxo3a expression and blunted the Foxo3A-induced caspase 3 cleavage, suggesting the role of SIRP α in promoting cell apoptosis via reducing β -catenin and enhancing Foxo3a expression.

Involvement of SIRP α in ATO-induced APL cell apoptosis. To prove the pro-apoptotic effect of SIRP α on APL cells, we investigated the role of SIRP α in ATO-induced apoptosis of APL cells. We previously reported that APL cell lines, HL-60 and NB4, express no or little SIRP α protein despite harboring a significant amount of SIRP α mRNA²⁵. However, to our surprise, ATO can induce a *de novo* expression of SIRP α protein in both HL-60 and NB4 cells. As shown in the Fig. 3a, treatment of HL-60 and NB4 cells with ATO triggered a significant induction of SIRP α in a time-dependent manner. SIRP α protein was detectable within 8 h and reached peak level after 48 h of ATO treatment. Immunofluorescence analysis further showed that SIRP α protein induced by ATO treatment was correctly transported to the cell surface (Fig. 3b). Moreover, the induction of SIRP α in HL-60 and NB4 cells by ATO was positively correlated with the ATO-induced apoptosis. As shown in the Fig. 3c,d, ATO treatment led to an increase in cleaved caspase-3 level in a time-dependent manner. Treatment of APL cells with ATO was also found to induce a strong increase in the percentage of Annexin V-positive cells. These results are in agreement with previous reports that APL cells are susceptible to the apoptosis induced by ATO treatment²⁶. Interestingly, we found that, unlike APL cells, hepatocellular carcinoma Huh7 cells were not sensitive to ATO treatment and displayed no enhanced apoptosis induced by the same concentration of ATO

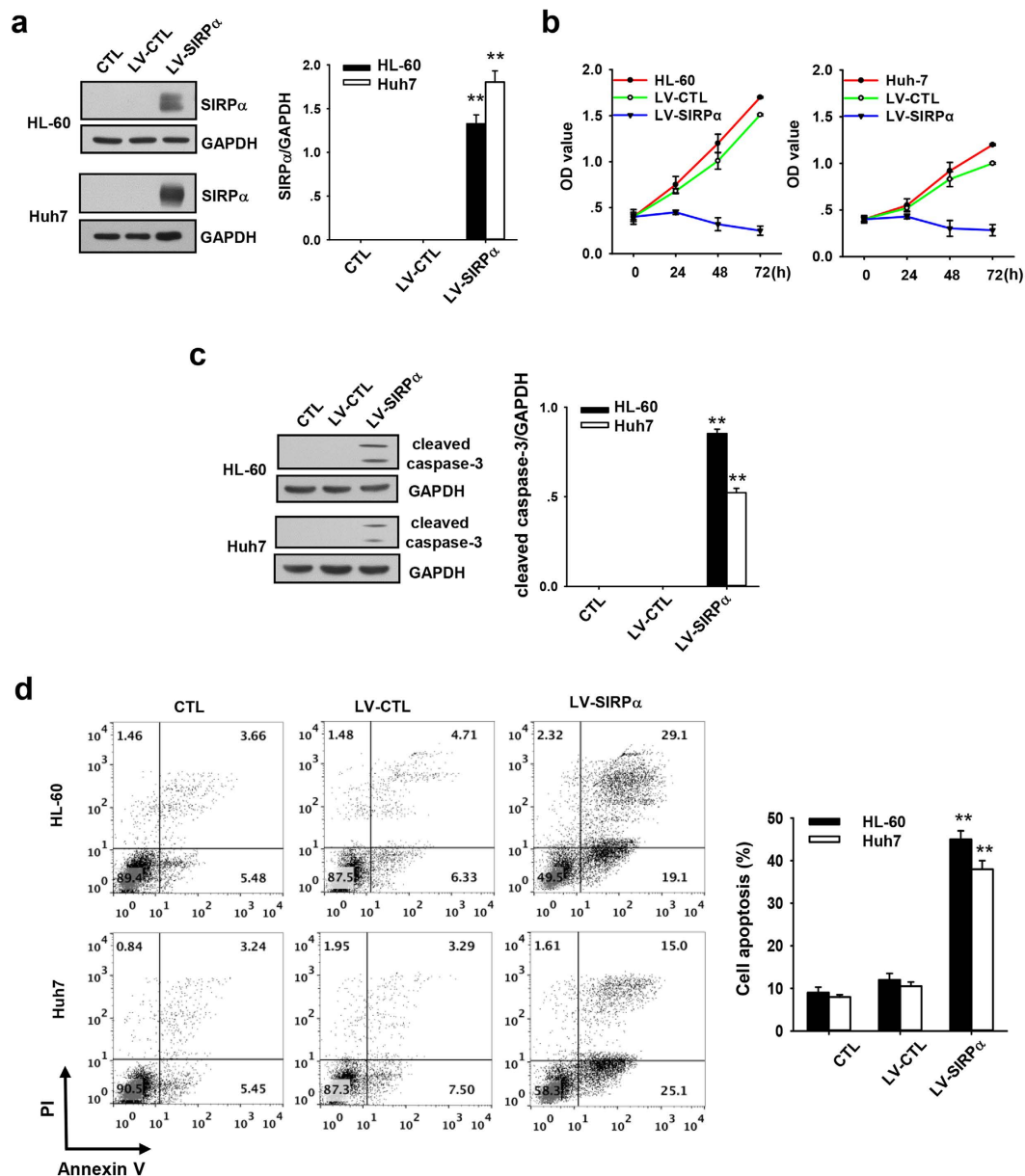


Figure 1. Forced SIRT α expression inhibited proliferation and caused apoptosis in APL and hepatocellular carcinoma cell line. (a) Western blotting of SIRT α level in HL-60 and Huh7 cells 48 h post-infection with SIRT α overexpression lentivirus, LV-SIRT α and control lentivirus, LV-CTL. (b) Growth curves of HL-60 and Huh7 cells infected with LV-SIRT α or LV-CTL. MTT assays were used to measure cell proliferation on days 1, 2, 3 post-infection. Absorbance was measured at 492 nm. (c) Western blotting of cleaved caspase-3 level in HL-60 and Huh7 cells infected with LV-SIRT α or LV-CTL on the third day post infection. (d) Flow cytometry analysis of apoptosis of HL-60 and Huh7 cells infected with LV-SIRT α or LV-CTL on the third day post infection with annexin V-PI staining: representative flow cytometer results (left panel) and quantitative analysis of apoptosis (right panel). Values were shown as the mean \pm SEM (n = 3). *P < 0.05. **P < 0.01.

within 48 h (Fig. 3c,d). Accordingly, no induction of SIRT α in Huh7 cells was observed in the process of ATO treatment (Fig. 3a,b). Taken together, these results suggest that ATO-induced apoptosis might be mediated by SIRT α expression.

We next determined whether the induction of SIRT α by ATO treatment directly contributed to the cell apoptosis. In these experiments, we used a lentivirus-mediated SIRT α siRNA (SIRT α shRNA) to specifically abolish the induction of SIRT α protein in both HL-60 and NB4 cells by ATO. As shown in the Fig. 4a,b, SIRT α shRNA successfully decreased the induction of SIRT α protein in both HL-60 and NB4 cells by ATO treatment. More importantly, abrogation of ATO-induced SIRT α expression by SIRT α shRNA also blocked the ATO-mediated cell apoptosis, as shown by decreased caspase-3 cleavage (Fig. 4b,d). In agreement with this, Annexin V staining also showed that the percentage of Annexin V-positive cells in ATO-treated HL-60 and NB4 cells were decreased

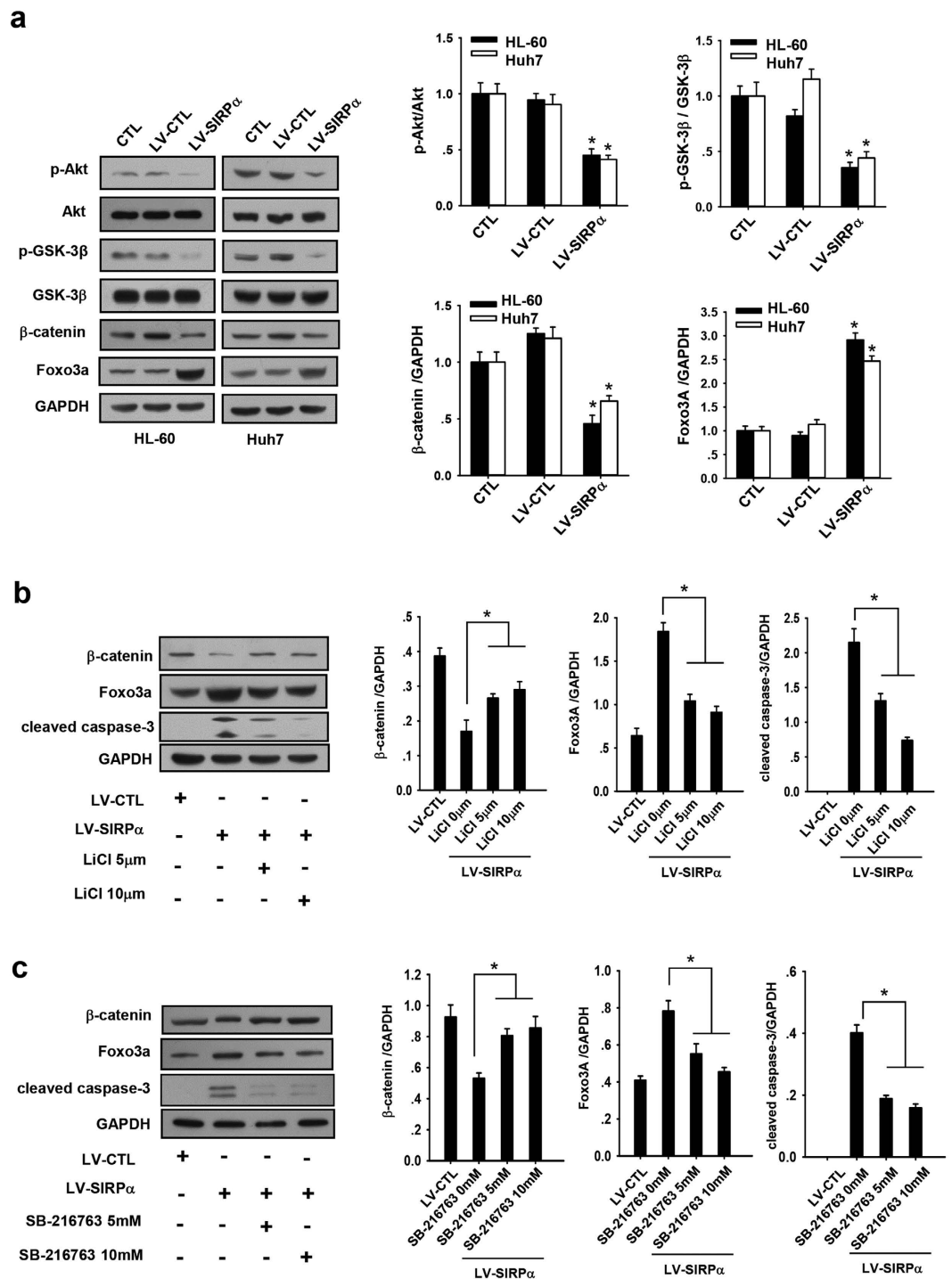


Figure 2. SIRP α contributed to Foxo3a expression and apoptosis possibly by inhibiting β -catenin. (a) Western blotting of p-Akt, Akt, p-GSK-3 β , GSK-3 β , β -catenin, Foxo3a and GAPDH in HL-60 and Huh7 cells infected with LV-SIRP α or LV-CTL on the third day post infection: representative Western blotting (left panels) and quantitative analysis (right panels). (b) Western blotting of β -catenin, Foxo3a, cleaved caspase-3 and GAPDH in LV-SIRP α -infected HL-60 cells co-incubated with 0, 5, or 10 μ M LiCl or 0, 5, or 10 mM SB-216763. 48 hours post-infection of lentivirus, cells were treated with LiCl, or SB-216763 for 4 h and then lysed for Western blotting analysis: representative Western blot (left panels) and quantitative analysis (right panels). Values were shown as the mean \pm SEM (n = 3). *P < 0.05. **P < 0.01.

after SIRP α was knocked down with SIRP α shRNA (Fig. 4e). These results collectively suggest that SIRP α possibly mediates ATO-induced apoptosis of APL cells.

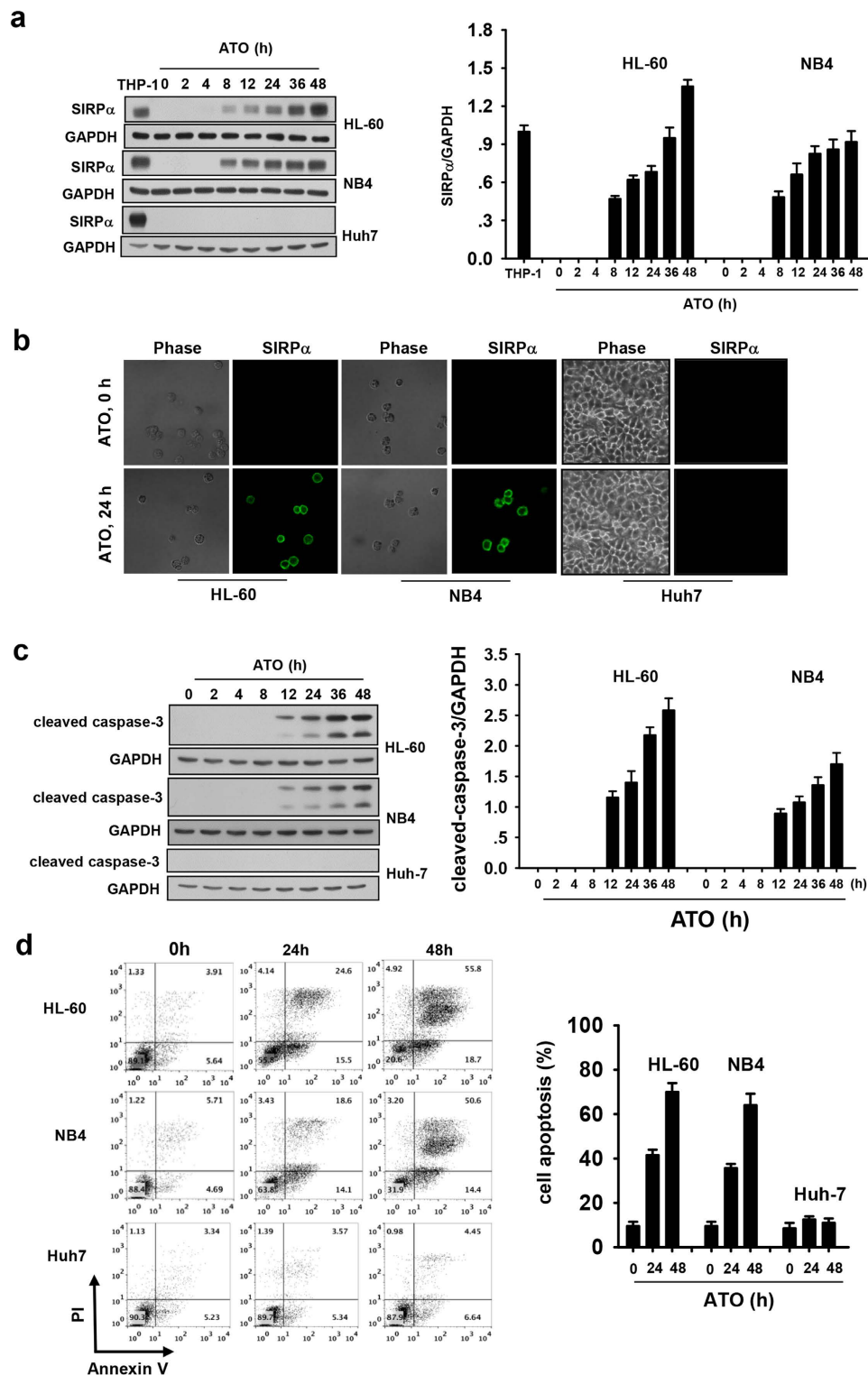


Figure 3. ATO induced expression of SIRP α protein and apoptosis in APL cell lines but not in hepatocellular carcinoma cell line. (a) Western blotting of SIRP α level in HL-60, NB4 and Huh7 cells treated with ATO for indicated time, the THP-1 whole cell lysate was used as a positive control: representative Western blotting (left panel) and quantitative analysis of SIRP α level (right panel). (b) Immunofluorescence analysis of SIRP α protein induced in HL-60, NB4 and Huh7 cells with ATO treatment for 24h. (c) Cleaved caspase-3 level in HL-60, NB4 and Huh7 cells treated with ATO at indicated time: representative Western blot (left panel) and quantitative analysis (right panel). (d) Flow cytometry analysis of ATO-treated HL-60, NB4 and Huh7 cells for indicated time with annexin V-PI staining: representative flow cytometer data (left panel) and quantitative analysis of apoptosis (right panel). The percentage of annexin V positive cells was calculated. Values were shown as the mean \pm SEM (n = 3). *P < 0.05. **P < 0.01.

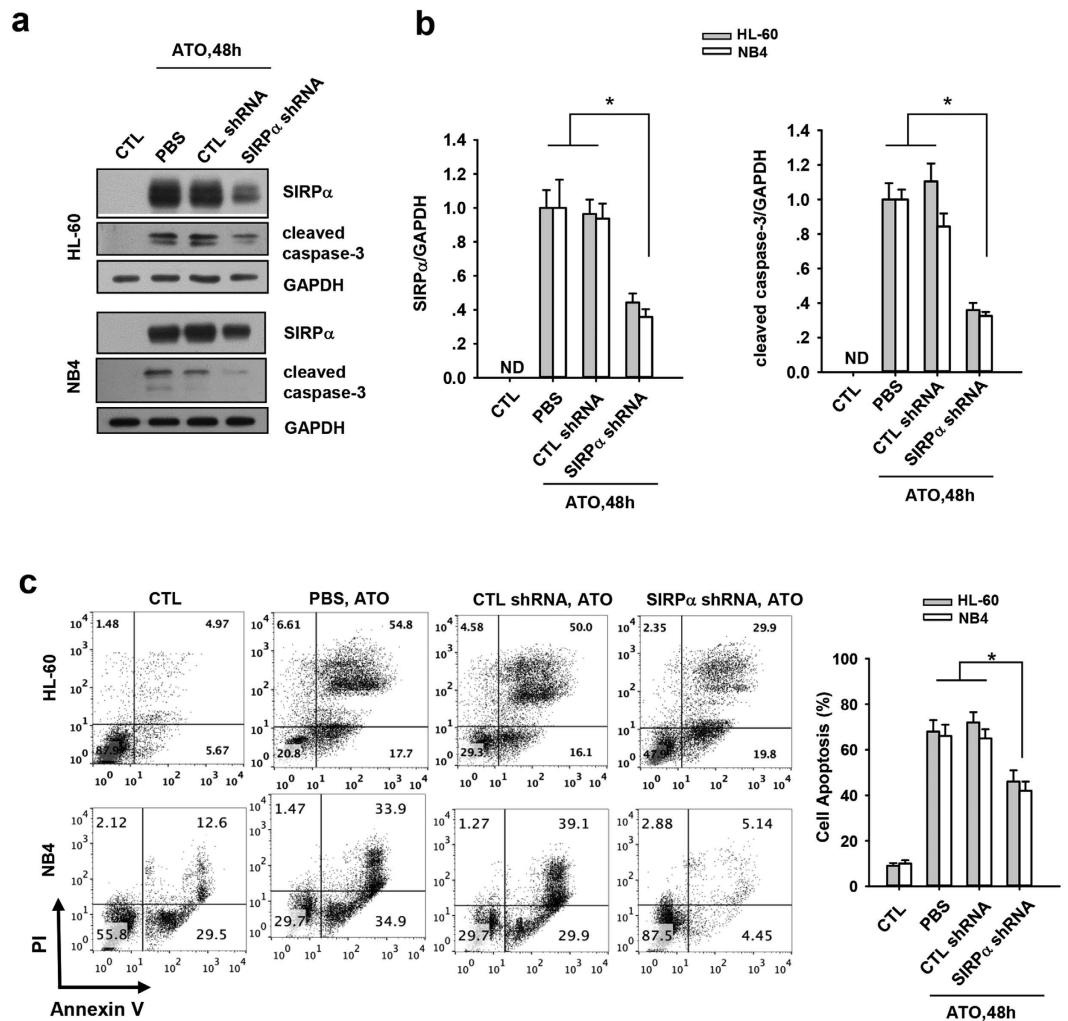


Figure 4. Block of SIRP α induction attenuated ATO-induced apoptosis of APL cell lines. SIRP α and cleaved caspase-3 protein level in SIRP α shRNA lentivirus-infected HL-60 or NB4 cells treated with ATO for indicated time: representative Western blots (a) and quantitative analysis of Western blot (b). Cells treated without lentivirus (PBS) or the cells infected with CTL shRNA lentivirus were used as controls. (c) Flow cytometry analysis of annexin V-PI staining in SIRP α shRNA lentivirus-infected HL-60 or NB4 cells in the presence of ATO for indicated time. Left panel, representative flow cytometer data. Right panel, quantitative analysis of apoptosis. Values were shown as the mean \pm SEM (n = 3). *P < 0.05. **P < 0.01.

To test whether the contribution of SIRP α on ATO-induced apoptosis is possibly through inhibiting β -catenin signal pathway, we studied the effect of SIRP α on β -catenin levels in both HL-60 and NB4 cells that were treated with ATO. As expected, treatment of HL-60 and NB4 cells with ATO alone resulted in a drastic suppression of β -catenin, as assessed by western blotting (Fig. 5a,b). Consistent with the suppression of β -catenin, the phosphorylation of Akt and GSK-3 β was reduced but the expression of Foxo3a significantly increased by ATO treatment (Fig. 5a,b). To confirm that SIRP α induction is required for the suppression of β -catenin and upregulation of Foxo3a in HL-60 and NB4 cells by ATO, we knocked down SIRP α in both HL-60 and NB4 cells using SIRP α shRNA lentivirus and then evaluated the expression of β -catenin, as well as the expression of Foxo3a and the phosphorylation status of Akt and GSK-3 β . As shown in the Fig. 5c,d, knockdown of SIRP α in HL-60 or NB4 cells treated with ATO significantly enhanced the phosphorylation of Akt and GSK-3 β , leading to increase of β -catenin level but decrease of Foxo3a expression.

Induction of SIRP α by ATO is through suppression of miR-17, miR-20a and miR-106a. As our previous study showed that SIRP α was post-transcriptionally regulated by a cluster of miRNAs, viz., miR-17, miR-20a, and miR-106a²⁵, we next determined whether these miRNAs were involved in modulating APL cell SIRP α protein levels in response to ATO. To address this, we assayed the level of these miRNAs in both HL-60 and NB4 cells treated with ATO. The expression of miR-17, miR-20a and miR-106a was decreased in a time-dependent manner (Fig. 6a), while mRNA level of SIRP α was largely not affected by ATO treatment (Fig. 6b). As a control, the level of miR-24, a randomly selected miRNA, was largely unchanged (Fig. 6a). To confirm the role of miR-17, miR-20a and miR-106a in regulating SIRP α protein expression in APL cells during ATO-induced apoptosis, we

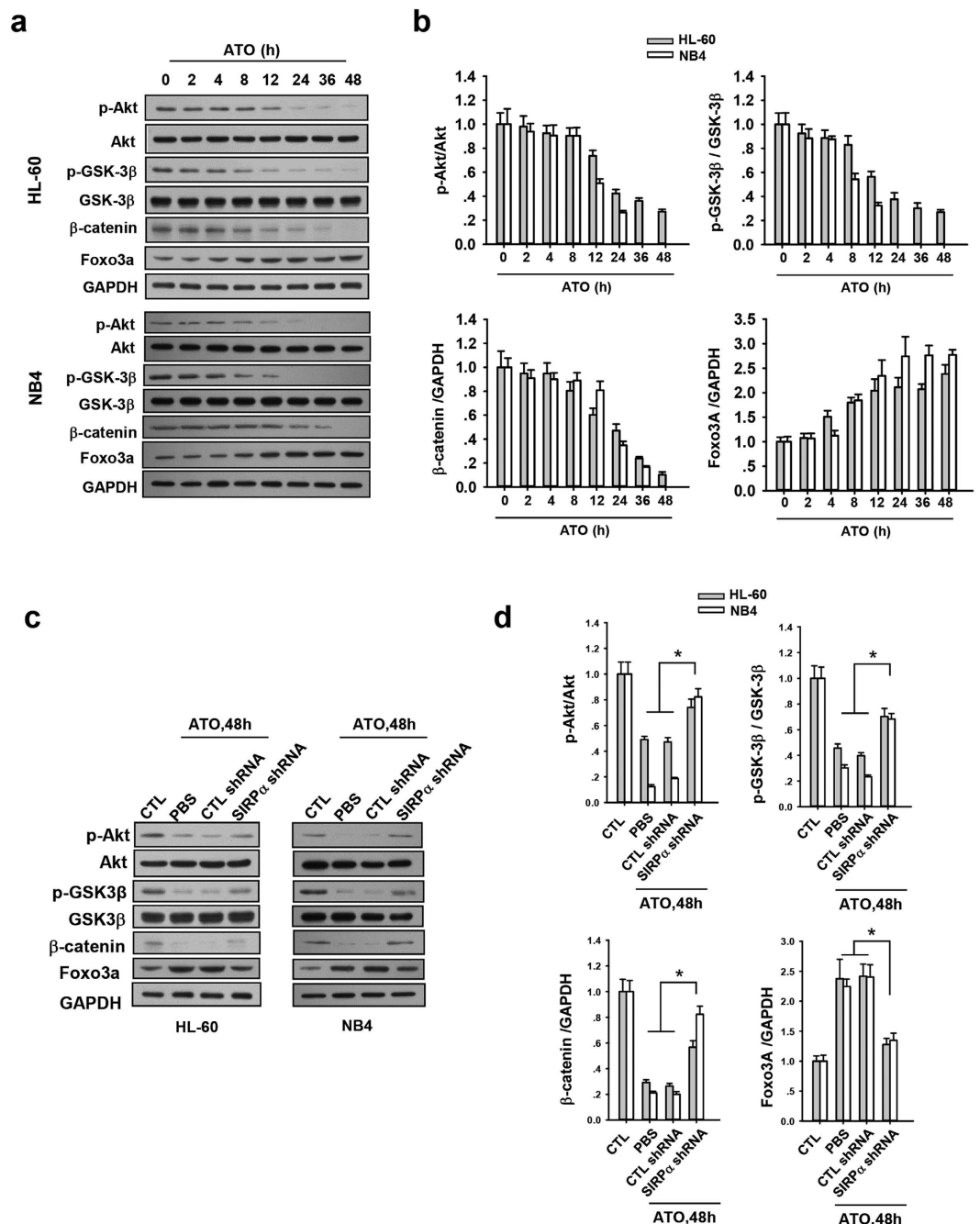


Figure 5. Induction of SIRP α contributed to the downregulation of β -catenin and upregulation of Foxo3a in APL cell lines treated with ATO. (a,b) Western blotting of p-Akt, Akt, p-GSK-3 β , GSK-3 β , β -catenin, Foxo3a and GAPDH in HL-60 or NB4 cells treated with ATO for indicated time: representative Western blotting (a) and quantitative analysis of protein level (b), the protein level of GAPDH was used as an internal control, the p-Akt (Ser473) and p-GSK-3 β (Ser9) were normalized to the total Akt and GSK-3 β , respectively. (c,d) Western blotting of p-Akt, Akt, p-GSK-3 β , GSK-3 β , β -catenin, Foxo3a and GAPDH in SIRP α shRNA lentivirus infected HL-60 or NB4 cells after the treatment of ATO for 48 h: representative Western blot (c) and quantitative analysis (d). Values were shown as the mean \pm SEM (n = 3). *P < 0.05. **P < 0.01.

transfected both HL-60 and NB4 cells with pre-miR-17 48 hours prior to the ATO treatment. As shown in Fig. 6c, HL-60 or NB4 cells transfected with pre-miR-17 expressed significantly less SIRP α compared to non-transfected cells or cells transfected with scramble oligonucleotide. Accordingly, pre-miR-17-transfected HL-60 or NB4 cells displayed a significantly delayed and attenuated apoptosis compared to the non-transfected cells or cells transfected with scramble oligonucleotide under the same treatment with ATO (Fig. 6d).

Our previous report also showed that c-Myc can promote the expression of the miR-17~92 cluster in promyelocytic cells²⁵. To test whether ATO suppresses the expression of miR-17, miR20a and miR-106a in promyelocytic

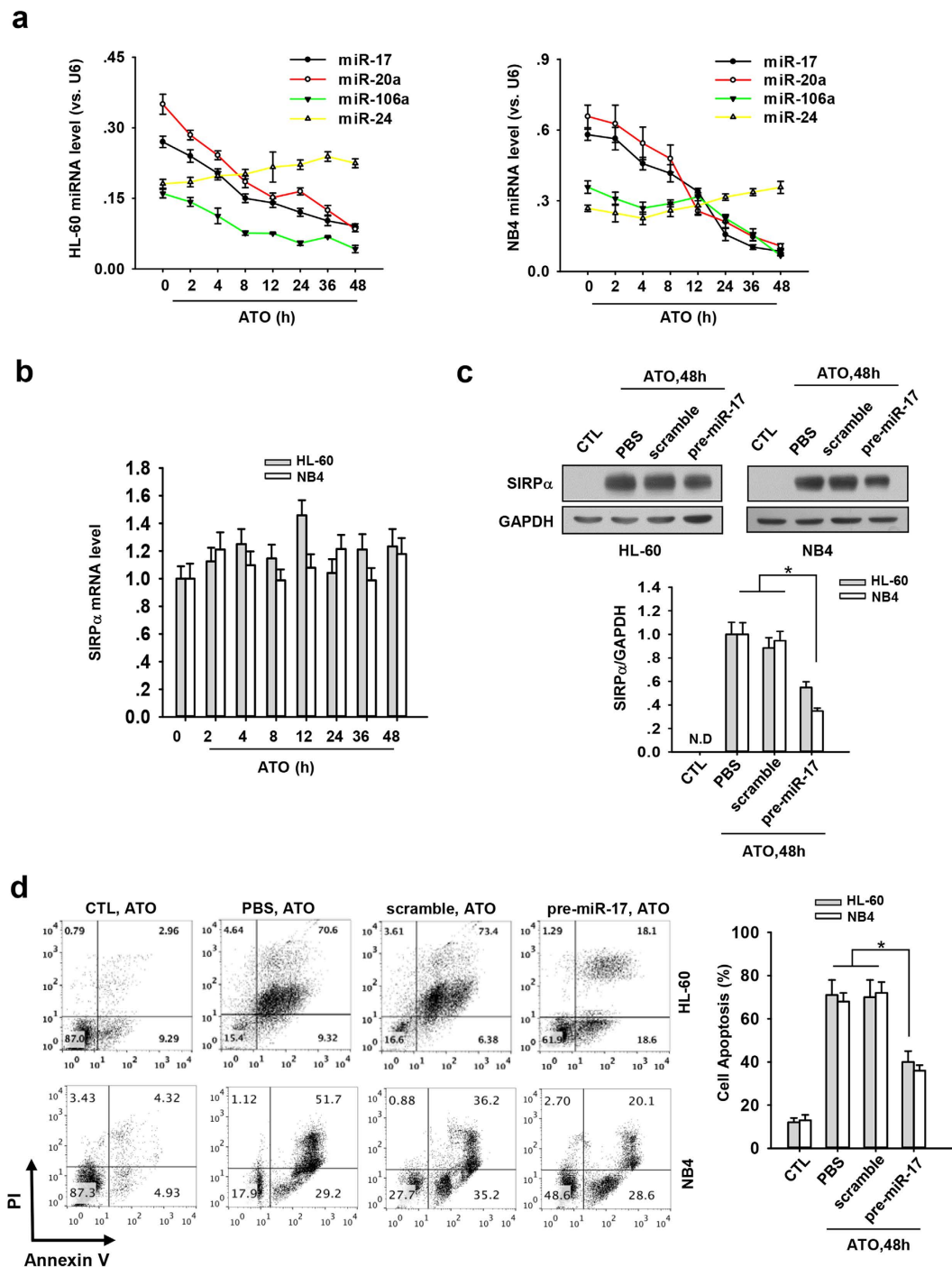


Figure 6. ATO induced expression of SIRP α through the suppression of miR-17, miR-20a, and miR-106a.

(a) The level of SIRP α -regulating miRNAs, miR-17, miR-20a, miR-106a in HL-60 or NB4 cells treated with ATO at indicated time. The level of all miRNAs was normalized to that of U6. (b) The relative mRNA level of SIRP α in HL-60 or NB4 cells treated with ATO for indicated time. Total RNA was extracted from the cells and analyzed with RT-qPCR. The mRNA level of GAPDH was used as an internal control. (c) Western blotting of SIRP α protein level in the HL-60 or NB4 cells treated with ATO for indicated time. Before ATO treatment, the cells were transfected with pre-miR-17. The mock-transfected cells (PBS) or cells transfected with scrambles oligonucleotide were used as a control: representative Western blot (upper panel) and quantitative analysis (lower panel). (d) Flow cytometry analysis of apoptosis of pre-miR-17-transfected HL-60 or NB4 cells after the treatment of ATO for 48 h: representative flow cytometer data (left panel) and quantitative analysis of apoptosis (right panel). The percentage of annexin V-positive cells was calculated. Values were shown as the mean \pm SEM (n = 3). *P < 0.05. **P < 0.01.

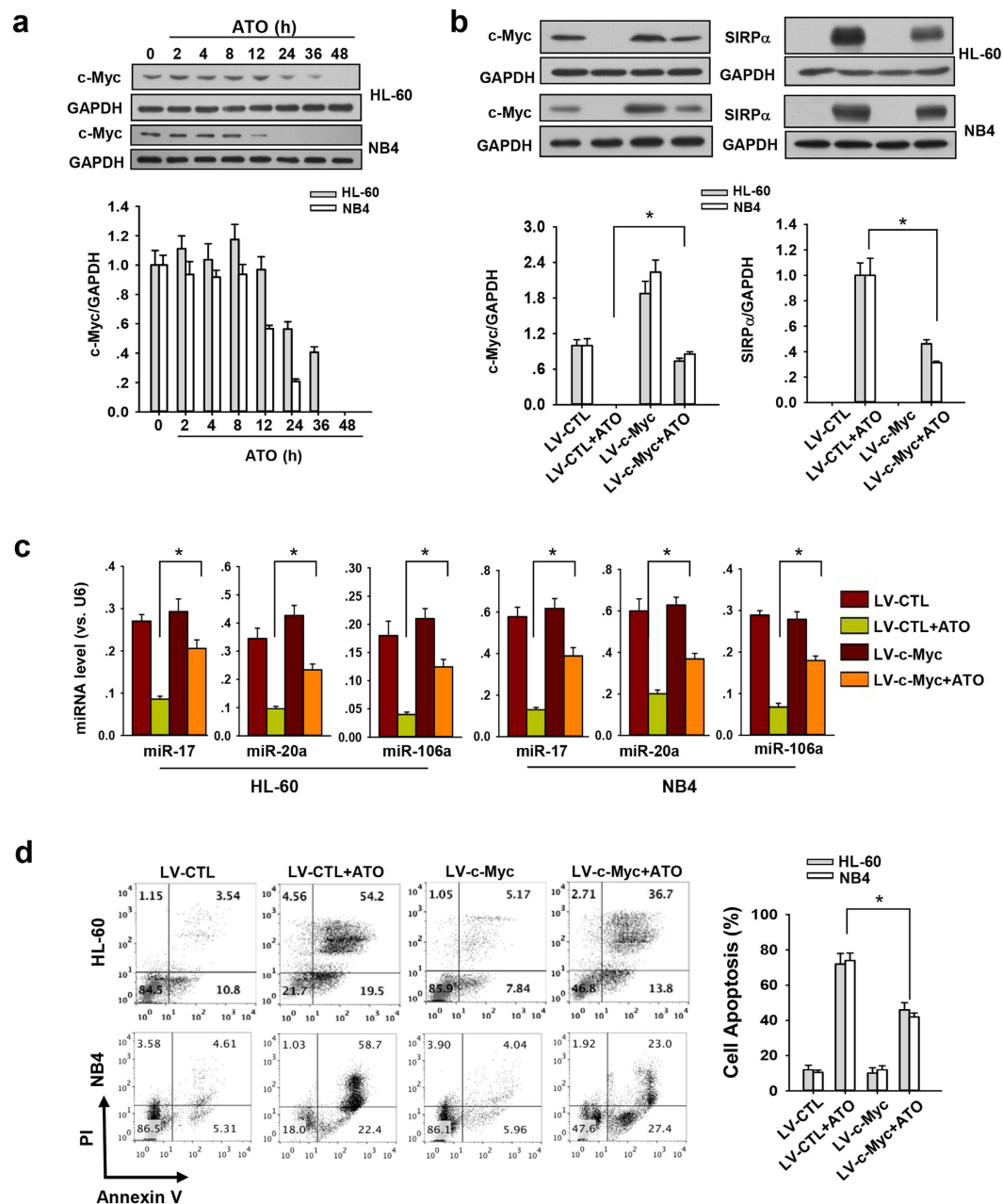


Figure 7. ATO suppressed miR-17, miR20a, and miR-106a through the inhibition of c-Myc expression.

(a) Western blotting analysis of c-Myc level in HL-60 or NB4 cells treated with ATO for indicated time: representative Western blot (upper panel) and quantitative analysis (lower panel). (b) Western blotting of c-Myc and SIRP α in c-Myc overexpression lentivirus (LV-c-Myc) infected HL-60 or NB4 cells after treated with ATO for 48 hours. The lentivirus (LV-CTL) was used as a control: representative Western blotting (upper panel) and quantitative analysis (lower panel). (c) The level of SIRP α -regulating miRNAs, miR-17, miR-20a, miR-106a in LV-c-Myc infected HL-60 or NB4 cells after treated with ATO for 48 h, LV-CTL was used as a control. (d) Flow cytometry analysis of apoptosis of LV-c-Myc infected HL-60 or NB4 cells after the treatment of ATO for 48 h: representative flow cytometer results (left panel) and quantitative analysis of apoptosis (right panel). The percentage of annexin V-positive cells was calculated. Values were shown as the mean \pm SEM (n = 3). *P < 0.05. **P < 0.01.

cells by reducing c-Myc level, we determined the effect of ATO treatment on c-Myc level, as well as miR-17, miR-20a, and miR-106a expression, in both HL-60 and NB4 cells. As shown in Fig. 7a, c-Myc levels were strongly decreased in HL-60 cells treated with ATO in a time-dependent manner, suggesting a negative regulatory role of c-Myc in SIRP α protein expression in response to ATO treatment. Furthermore, when ATO-induced reduction of c-Myc in HL-60 or NB4 cells was reversed by transfection with lentivirus-mediated c-Myc expression vector (LV-c-Myc), the induction of SIRP α protein by ATO treatment was largely abolished (Fig. 7b). The downregulation of miR-17, miR-20a and miR-106a in ATO-treated HL-60 or NB4 cells was also reversed by overexpression

of c-Myc (Fig. 7c). As shown in Fig. 7d, restoration of c-Myc level in ATO-treated HL-60 or NB4 cells significantly reduced cell apoptosis. These results suggest that ATO downregulates miR-17, miR-20a, and miR-106a possibly by suppressing c-Myc subsequent to which SIRP α expression is induced in APL cells.

Discussion

As a critical signal transduction protein, SIRP α has been shown to be involved in regulating many aspects of cellular responses, particularly the inflammatory responses of leukocytes, including activation, chemotaxis and phagocytosis^{27–32}. A correlation between SIRP α and cell growth and survival was reported recently by Yan *et al.*³³ who showed that ectopic expression of SIRP α in hepatocellular carcinoma suppressed cell growth. In the present study, we demonstrated SIRP α 's role as a pro-apoptotic molecule that mediates ATO-induced apoptosis of APL cells.

Several pieces of evidence support this previously unrecognized role of SIRP α in ATO-induced apoptosis. First, SIRP α expression was induced by ATO treatment in APL cells in a time-dependent manner. Immunofluorescence staining showed that most of the SIRP α protein was on the cell surface, where SIRP α could bind to its ligand and initiate downstream signaling. More importantly, specific knockdown of ATO-induced SIRP α expression via lentivirus-mediated SIRP α shRNA largely blocked ATO-induced APL cell apoptosis. Second, although ATO treatment can effectively induce apoptosis of APL cells, it shows limited effect on other malignancies particularly solid tumors¹⁶. Here we also found that hepatocellular carcinoma Huh7 cells, unlike APL cells, were insensitive to ATO treatment. Serving a negative control, Huh7 cells displayed no induction of SIRP α by ATO.

Overexpression of SIRP α alone in APL cells (HL-60 and NB4) or hepatocellular carcinoma Huh7 cells significantly increased cell apoptosis, strongly arguing that SIRP α is a general pro-apoptotic molecule functioning in various tumor cells. As SIRP α can induce cell apoptosis, we speculate that induction of SIRP α in APL cells is not just sensitizing the cells to ATO treatment but is a novel mechanism underneath the ATO-mediated APL cell death. Failure to induce SIRP α in cancer cells such as Huh7 cells yielded the poor effect of ATO treatment on their apoptosis. In an effort to probe into the role of SIRP α in ATO-induced apoptosis in APL cells, we employed a lentivirus system to increase or knock down cellular SIRP α protein level by transfecting cells with lentivirus-mediated SIRP α mRNA or SIRP α shRNA. As shown by our results (Figs 4 and 5), the contribution of SIRP α to the ATO-induced APL cell apoptosis is produced possibly through its role in inhibiting β -catenin signal pathway. Treatment with ATO or overexpression of SIRP α alone decreased the levels of phosphorylated Akt and GSK-3 β , leading to suppression of β -catenin level but enhancement of Foxo3a expression. In contrast, knockdown of SIRP α in ATO-treated APL cells significantly rescued the expression of β -catenin, inhibited the increase of Foxo3a and alleviated the cell apoptosis induced by ATO.

Our previous study showed that SIRP α expression was modulated at posttranscriptional level by miR-17, miR-20a, and miR-106a²⁵. In the present study, we also observed that the induction of SIRP α was dependent on the suppression of these three miRNAs by ATO treatment. Overexpression of one such miRNA in ATO-treated APL cells significantly abolished the induction of SIRP α by ATO (Fig. 6). The involvement of miR-17, miR-20a and miR-106a in posttranscriptional regulation of SIRP α was also supported by the facts that ATO treatment did not affect SIRP α mRNA level but increased SIRP α protein and reduced c-Myc expression in APL cells (Fig. 7). Given that c-Myc strongly promotes the expression of miR-17, miR-20a, and miR-106a, as indicated by Fig. 6 as well as our previous report²⁵, suppression of c-Myc level in APL cells by ATO treatment may play a key role in downregulation of miR-17, miR-20a and miR-106a, and thus upregulation of SIRP α protein translation. Interestingly, since SIRP α protein can inhibit β -catenin signal pathway, which in turn, positively regulates c-Myc transcription^{34,35}, induction of SIRP α may downregulate c-Myc level, leading to downregulation of miR-17, miR-20a and miR-106a expression but even higher level of SIRP α protein. Therefore, through β -catenin and c-Myc signal pathways, a miRNA-based positive-feedback regulatory loop may be involved in ATO-induced SIRP α induction and cell apoptosis in APL cells.

Given that SIRP α can suppress tumor cell survival and more important, SIRP α is induced by ATO treatment in APL cells, the rapid induction of SIRP α in APL cells may serve as a novel prognostic marker for ATO treatment. In addition, as tumor cells express SIRP α mRNA but no SIRP α protein, induction of SIRP α protein expression in these tumor cells by suppressing the expression of SIRP α -targeting miRNAs also provide a potential anti-tumor strategy.

In conclusion, our results identify SIRP α as an important pro-apoptotic regulator and the induction of SIRP α may play a critical part in mediating ATO-induced apoptosis of APL cells.

Materials and Methods

Cell and Reagent. HL-60 and NB4 cells were maintained in RPMI1640 (GIBCO, Carlsbad, CA) supplemented with 10% (v/v) fetal bovine serum (FBS) (GIBCO), 1% penicillin and streptomycin (GIBCO). Huh7 cells were cultured in DMEM (GIBCO) supplemented with 10% (v/v) fetal bovine serum (FBS), 1% penicillin and streptomycin. Antibodies against cleaved caspase-3, Akt, p-Akt (Ser473), GSK-3 β , p-GSK-3 (Ser9) and β -catenin were purchased from Cell Signaling Technology (Danvers, MA). Antibodies against GAPDH, SIRP α , and secondary antibodies against mouse or rabbit IgGs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa Flour 488 (goat anti-rabbit IgG, green) and annexin V-PI apoptosis kit were purchased from Invitrogen (Carlsbad, CA). Arsenic trioxide (As₂O₃; ATO) was purchased from Sigma Aldrich (St Louis, MO). Cells in our experiment were treated with ATO at 5 μ M concentration where indicated. Lithium chloride (LiCl) and SB-216763 were purchased from Sigma Aldrich. 48 hours post-infection of lentivirus, cells were treated with 5 μ M or 10 μ M LiCl, 5 mM or 10 mM SB-216763 for 4 h and then lysed for westert blotting analysis..

MTT proliferation assay. Cells in each experiment were seeded in 96-well plates at a density of 1.0×10^4 cells/well and infected with SIRP α overexpressing lentivirus or control lentivirus. 48 hours post infection, cell proliferation was quantified in 3 days by MTT assay. In brief, 20 μ l of MTT (5 mg/ml; Sigma) was added to each well followed by incubation for 4 h at 37°C. The medium was then replaced with 150 μ l of dimethylsulphoxide (DMSO) (Sigma). The viability of the cells was assessed by the detection of absorbance at 492 nm using a spectrophotometer. The growth curves were plotted.

Cell lysis and Immunoblotting. Cells were treated with or without ATO for the indicated time and subsequently lysed in the RIPA lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitor cocktail (Amresco, Cleveland, Ohio) and/or phosphatase inhibitor cocktail (Cell Signaling Technology). The whole cell lysis was subjected to SDS-PAGE, transferred onto a PVDF membrane, and then immunoblotted with respective primary antibodies. The bound primary antibody were visualized with specific horseradish peroxidase-conjugated secondary antibodies (Santa Cruz) using an enhanced chemiluminescence (ECL) reagents (Thermo Scientific, Hudson, NH). The bar graphs corresponding to the Western blots were generated through densitometric analysis with Image J software.

Immunofluorescence Analyses. Cells were harvested after treatment in the presence or absence of ATO for indicated time by centrifugation at $300 \times g$ for 5 minutes at 4°C and subjected to Immunofluorescence. Briefly, the harvested cells were washed using cold phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 10 minutes at room temperature. Then the cells were washed using PBS and permeabilized with PBS containing 0.2% (v/v) Triton X-100 for 5 minutes at room temperature. After blocking permeabilized cells with bovine serum albumin (BSA) for 1 hour at room temperature, cells were washed using PBS and incubated with SIRP α antibody (1:100) in PBS containing 10 mg/ml BSA overnight at 4°C. Cells were washed using PBS and then incubated with Alexa Flour 488 (goat anti-rabbit IgG, 1:2000) for 1 h at room temperature. The cells were washed using PBS and then mounted and analyzed with an inverted confocal laser microscope (Nikon).

RNA isolation and RT-qPCR. Total RNA from cells was extracted using the TRIzol Reagent according to the manufacturer's protocol (Invitrogen). The relative expression of mRNAs was determined by RT-qPCR using LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's protocol. The mRNA of GAPDH was used as internal controls. The expression of miRNAs was determined by qRT-PCR using TaqMan miRNA probes (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. The levels of miRNAs in cells were normalized to U6 snRNA. All of the reactions were run in triplicate. A comparative threshold cycle (Δ CT) method was used and values were expressed as $2^{-\Delta\Delta CT}$.

Flow Cytometric Assays. Cells were treated for the respective time period and harvested by centrifugation at $300 \times g$ for 5 minutes at 4°C. The annexinV-propidium iodide kit (Invitrogen) was used to stain the cells for the evaluation of apoptosis according to the manufacturer's protocol.

Infection with shRNA SIRP α lentivirus, SIRP α -expressing lentivirus and c-Myc-expressing lentivirus. Lentivirus encoding SIRP α , shRNA SIRP α , and c-Myc were generated and confirmed by the GenePharma Company (Shanghai, China). An empty backbone lentivirus was used as a control. Cells were incubated with respective virus at a multiplicity of infection (MOI) of 5 along with 8 μ g/ml Polybrene for 48 h before the treatment with indicated dose of ATO. The selection marker was GFP. The infected cells were gated by GFP expression via flow cytometry analysis.

Statistical analyses. Data derived from at least three independent experiments are expressed as the mean \pm SEM. Normal distributed variables were compared using Student's *t*-test. The reported P value was 2-sided. $P < 0.05$ was considered to be statistically significant.

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Acknowledgements

The authors thank Dr. Jill Leslie Littrell (Georgia State University, Atlanta, GA) for critical reading and constructive discussion of the manuscript. This work was supported by grants from the National Basic Research Program of China (973 Program) (No. 2014CB542300, 2012CB517603 and 2011CB504803), the National Natural Science Foundation of China (No. 81250044 and 81401895), National Natural Science Foundation of China Grants (No. 31301061).

Author Contributions

K.Z., Y.L. and C.Y.Z. (study concept and design, analysis and interpretation of data); K.Z. (drafting of the manuscript); C.P., D.Z., J.Z., L.L. and D.W. (acquisition of data; analysis and interpretation of data; statistical analysis).

Additional Information

Supplementary information accompanies this paper at <http://www.nature.com/srep>

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Pan, C. *et al.* Role of Signal Regulatory Protein α in Arsenic Trioxide-induced Promyelocytic Leukemia Cell Apoptosis. *Sci. Rep.* **6**, 23710; doi: 10.1038/srep23710 (2016).



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