

All-*trans* retinoic acid upregulates the expression of p53 via Axin and inhibits the proliferation of glioma cells

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Abstract. All-*trans* retinoic acid (ATRA) is a potent chemopreventive and therapeutic agent and exerts its effects by inducing growth arrest. In the present study, we demonstrated that ATRA activated the expression of p53 via Axin and induced cell cycle arrest at the G1/S phase and apoptosis of glioma cells. Briefly, C6 cells were treated with ATRA, and the levels of p53 mRNA and protein were determined by RT-PCR, western blotting and immunohistochemistry. The results showed that ATRA activated the expression of p53. In addition, ectopic expression of Axin by transient transfection of C6 cells with rAxin revealed that overexpression of Axin induced cell cycle arrest and apoptosis with an upregulation of p53. Furthermore, loss-of-function of Axin in glioma cells by RNAi blocked ATRA-induced cell cycle phase arrest and apoptosis via downregulation of p53. The present study revealed a novel function of Axin and identified it as an important regulator of ATRA-activated p53 expression.

Introduction

All-*trans* retinoic acid (ATRA) is a member of the retinoid family which includes substances structurally or functionally related to retinol (vitamin A). ATRA exerts a profound effect on the survival, growth and differentiation of many types of cells during prenatal and adult life (1). Since 1989, when ATRA was introduced as a targeted therapy against acute promyelocytic leukemia (APL), it has been used extensively as an antitumor agent for many types of tumors (2). Several molecular mechanisms of ATRA have been identified in the induction of tumors undergoing cell cycle arrest and apoptosis by activating RAR and RXR (3-5). ATRA regulates the fate of cells also by other pathways, including p53 (6-10). These

studies revealed that ATRA induces cell cycle arrest and apoptosis by regulating the transcriptional activity of p53 and/or stability of its protein. However, it remains unclear whether p53 take part in ATRA-induced cell death in glioma cells.

Axin is a negative regulator of axis formation in the development of mouse embryos. Its deficiency leads to axis duplication (11). Overexpression of Axin blocked embryo axis formation in *Xenopus* and caused apoptosis in transgenic mice and certain cell lines (12,13). Accumulating data show that Axin emerges as a major scaffold for many pathways, including Wnt signaling, JNK mitogen-activated protein kinase signaling and transforming growth factor (TGF) β signaling (14-16). Notably, a previous study revealed that Axin participates in ATRA-induced differentiation of embryonic carcinoma cells (17), suggesting that Axin plays an important role in retinoid-mediated cell fate. Furthermore, we previously found that ATRA activates the transcription of Axin in glioma cell lines (18). Studies have demonstrated that Axin forms a complex with p53 and induces p53-mediated apoptosis by stimulating p53 transcriptional activity (19,20).

In the present study, the expression of p53 was found to be highly upregulated by ATRA treatment in the glioma C6 cell line. Ectopic expression of Axin induced cell cycle arrest and apoptosis and activated the expression of p53. Furthermore, the knockdown of Axin blocked ATRA-induced cell cycle arrest and apoptosis with downregulation of p53. These findings indicate that ATRA activated the intrinsic upregulation of p53 by a novel mechanism involved in the activation of Axin.

Materials and methods

Cell cultures and transfection. The rat C6 glioma cell line was cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum in a humidified atmosphere with 5% CO₂. The glioma C6 cells were transfected with pIRES2-EGFP-Axin and pIRES2-EGFP using Lipofectamine™ 2000 reagent (Invitrogen) according to the manufacturer's protocol. After an 8-h transfection, cells were cultured with DMEM for 24 h.

Growth curve assay. For the growth curve assay (MTT methods), C6 cells were plated in a 96-well plate at a density

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of 1×10^3 cells/well, and incubated in a humidified 5% CO₂ at 37°C and transfected with Axin siRNAs for 8 h. After 24, 48 and 72 h, methyl thiazolyl tetrazolium (MTT) (20 μ l/well) was added to the cells. After a 4-h incubation, the supernatants were removed, and 150 μ l dimethyl sulfoxide (DMSO) was added to each well and swirled for 10 min to solubilize the crystals. The absorbance was measured at 490 nm.

Analysis of apoptosis. TUNEL method was used for analysis of apoptosis using an apoptosis detection kit (Maixin, China) according to the manufacturer's protocol. Briefly, cell slices were washed with PBS and digested with proteinase K for 3 min. Then, slices were labeled with TdT and DIG-d-UTP for 2 h at 37°C, and blocked with the blocking reagents at room temperature for 30 min. The anti-Dig-antibody was applied to the cells and incubated at 37°C for 30 min. After washing with PBS, slices were incubated with SABC at 37°C for 1 h, and visualized with DAB, and counterstained with hematoxylin. The apoptotic cells in five different fields were counted, and the apoptotic index was calculated as the ratio of apoptotic cells over total cells.

Flow cytometric analysis. Cells (1×10^6) were washed twice with PBS and pelleted at $1,000 \times g$ for 5 min. Cells were trypsinized and fixed with 70% ethanol and washed with PBS and resuspended in PBS containing RNAase A and propidium iodide (PI) in the dark for 10 min at 4°C. The DNA contents of the stained nuclei were analyzed on a Becton-Dickinson FACSscan flow cytometer. The distribution of DNA content categorized cells as being in the G1 and S phases. The cells with DNA content less than G1 were categorized in pre-G1 (hypodiploid cells) and determined as apoptotic phase cells.

Immunohistochemical staining. The cultured cells were incubated with 5% normal goat serum (Sigma) and incubated overnight at 4°C with a mouse monoclonal antibody for p53 (Sigma). Following washing with PBS, cells were incubated at room temperature with biotinylated goat anti-mouse/rabbit IgG followed by streptavidin enzyme conjugate (Zhongshan, China) at room temperature. The reaction product was visualized by diaminobenzidine tetrahydrochloride (DAB). All sections were counterstained with hematoxylin. Quantification of the percentage of immunoreactive cells was determined by capturing images from random fields.

RNA interference. RNA interference (RNAi) was employed in this study to inhibit Axin expression and analyze the effect of Axin on ATRA-inhibited cell growth and the expression of p53. Duplex RNAi oligos with a two-nucleotide overhang at the end of the sequence were designed and synthesized at GenePharm Co. The sequence of RNA interference was as follows (Axin1-2253): sense sequence, 5'-GUAUCGUUGUGGCCUACUATT-3' and antisense sequence, 5'-UAGUAGCCACAACGAUACTG-3'. The negative control siRNA has no known specific effects on gene expression: sense sequence, 5'-UUCUCCGAACGUGUCACGUTT-3' and antisense sequence, 5'-ACGUGACACGUUCGGAGAATT-3'. C6 cells were transfected with siRNAs at a final concentration of 100 nM using Lipofectamine 2000 reagent according to the manufacturer's instructions. At 8 h post-transfection, cells

were cultured by administration of 2.5 μ M of ATRA for 24 h. The interference efficiency exceeded 60% by comparison with the negative control.

RT-PCR. Total RNA was isolated using TRIzol (Invitrogen) according to the manufacturer's instructions. Single-stranded cDNA was synthesized from 1 μ g of total RNA using the PrimeScript™ RT reagent kit following the protocol recommended by the manufacturer (Takara). PCR primers were as follows: Axin forward, 5'-AGGGTCTGGAACAGGGAA-3' and reverse, 5'-GGATAGCGTGTGTCAGCATCA-3'; p53 forward, 5'-GCGTTGCTCTGATGGTGA-3' and reverse, 5'-CAGCGTGATGATGGTAAGGA-3'; β -actin forward, 5'-TCACCCACACTGTGCCCATCTA-3' and reverse, 5'-CATCGGAACCGCTCATTGCCGATAG-3'. A template-free negative control was included in each experiment. The PCR cycle was preceded by an initial denaturation of 5 min at 94°C followed by a final extension of 10 min at 72°C. Each PCR regime was followed by 30 cycles at 94°C for 30 sec, 58°C for 30 sec, and 72°C for 45 sec. Band intensity was quantified by Bandscan software. The gray values were expressed in relation to that of the control and presented as means \pm SD of three independent experiments.

Western blot analysis. For western blot analysis, cells were lysed in 2X SDS loading buffer. Total extracts were obtained in the supernatant. Approximately 20 μ l of the samples was resolved on 10% SDS-PAGE, and transferred to PVDF membranes. For western blot analysis a mouse monoclonal antibody for p53 (Sigma), a mouse polyclonal antibody for Axin (Santa Cruz Biotechnology) and a rabbit polyclonal antibody for β -actin (Sigma) were used to detect the corresponding proteins. Peroxidase-conjugated goat anti-mouse/rabbit secondary antibody (Sigma) was used, and then the proteins were detected using an enhanced chemiluminescence reagent (Pierce). Band intensity was quantified by Bandscan software. Protein expression was normalized by the quantity of β -actin and presented as means \pm SD of three independent experiments.

Statistical evaluation. Data are reported as means \pm SD of three independent experiments. One-way ANOVA was used to assess statistical significance between the means. Significant differences were established at $P < 0.05$.

Results

ATRA activates the expression of p53. We previously cultured glioma C6 and U251 cell lines with or without ATRA for 24 h, and the levels of mRNA and protein of Axin were examined by RT-PCR and western blotting, respectively. A marked increase in Axin was observed after ATRA treatment in the cell lines accompanied with significant growth inhibition of the glioma cells (18). These results indicates that ATRA affects tumor cell survival via activation of Axin. To ascertain whether p53 is involved in ATRA-induced cell cycle arrest and apoptosis in C6 cells, immunohistochemistry was employed to investigate the expression of p53 in ATRA-treated C6 cells. ATRA treatment increased the expression of p53 protein from 9.8 ± 1.2 to $19.6 \pm 2.2\%$ (Fig. 1A). We then investigated the levels of p53

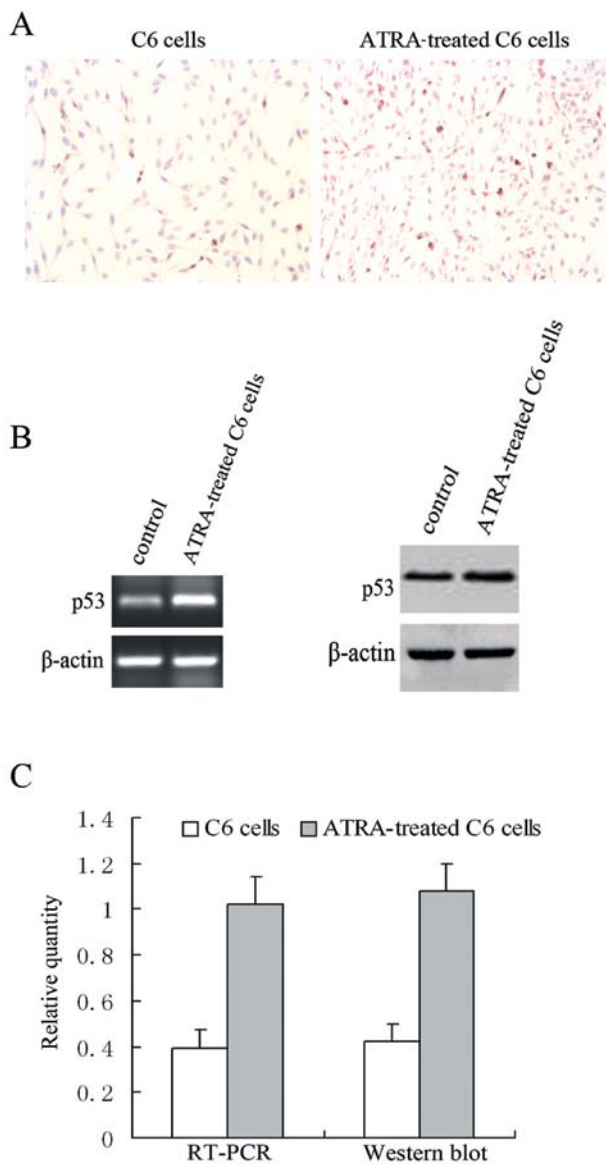


Figure 1. ATRA activates the expression of p53. C6 cells were cultured with or without ATRA for 24 h. (A) Immunohistochemistry revealed that ATRA treatment increased the expression of p53 protein in glioma C6 cells (n=3, $P<0.05$). (B) RT-PCR and western blotting indicated that levels of p53 mRNA and protein were increased in the C6 cells following ATRA treatment (n=3, $P<0.05$). (C) Expression of mRNA and protein was normalized, respectively, to the quantity of β -actin.

mRNA and protein by RT-PCR and western blotting, respectively (Fig. 1B). The level of total p53 protein was significantly elevated in correspondence with an increase in its mRNA after ATRA treatment (Fig. 1C). These data suggest that the activation of p53 participates in ATRA-induced cell cycle arrest and apoptosis in C6 cells.

Overexpression of Axin activates the expression of p53 and induces G1/S phase arrest and apoptosis. To identify whether overexpression of Axin induces the expression of p53, rat glioma C6 cells were transiently transfected with rat wild-type Axin. RT-PCR and western blotting were performed to detect the levels of p53 mRNA and protein (Fig. 2A and B). The levels of p53 mRNA and its protein in cells transfected with rAxin were increased compared to the levels in the cells

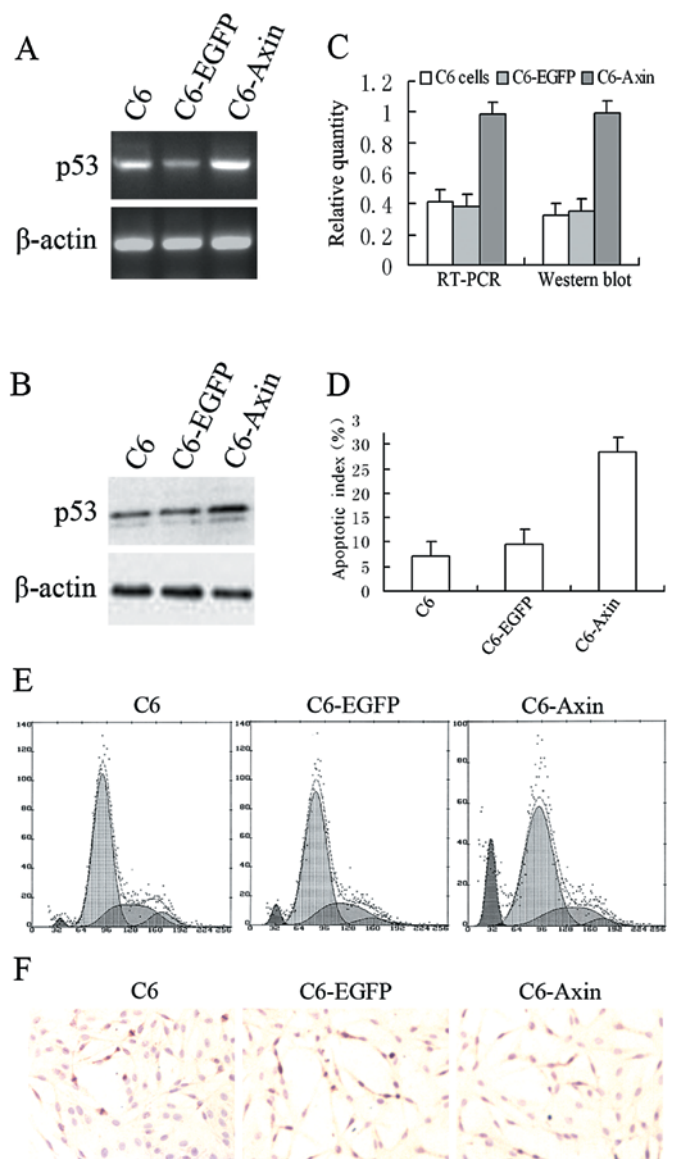


Figure 2. Overexpression of Axin activates the expression of p53 and induces G1/S phase arrest and apoptosis. C6 cells were transiently transfected with rat wild-type Axin (C6-Axin) or empty vector (C6-EGFP). Accompanied by the overexpression of Axin, the levels of p53 (A) mRNA and (B) protein in cells transfected with rAxin were increased compared to the levels in the cells transfected with the empty vector (n=3, $P<0.05$). (C) Expression of mRNA and protein was normalized, respectively, to the quantity of β -actin. (E) Concerning C6 cells transfected with rAxin, flow cytometric analysis showed that the percentages of cells in pre-G1 and G1 phases were increased and the percentage of cells in the S phase was decreased compared to cells transiently transfected with the empty vector (n=3, $P<0.05$). (D and F) The apoptotic index of C6 transfected with Axin was significantly increased compared to that of C6 cells transfected with the empty vector and the C6 cells (n=5, $P<0.05$).

transfected with the empty vector (Fig. 2C). These results confirmed that the overexpression of Axin was required for the activation of p53. To identify whether overexpression of Axin induces cell cycle arrest and apoptosis, flow cytometry (Fig. 2E) and TUNEL analysis (Fig. 2D and F) were used to determine the DNA content and apoptotic index, respectively. Flow cytometric analysis showed that the percentages of C6 cells transiently transfected with the empty vector in pre-G1, G1 and S phases were 4.8 ± 1.4 , 63.6 ± 2.8 and $25.8\pm2.4\%$,

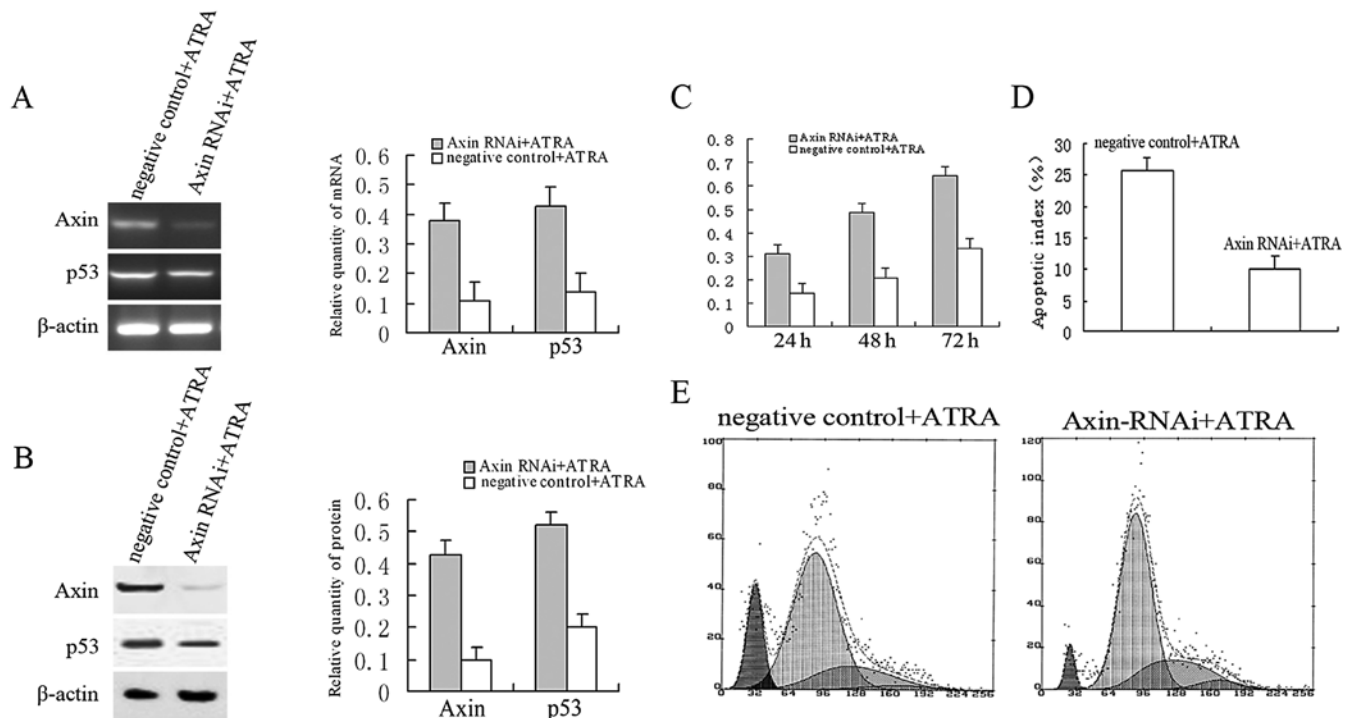


Figure 3. Axin RNAi decreases the expression of p53 and attenuates ATRA-induced G1/S phase arrest and apoptosis. C6 cells were transfected with siRNAs at a final concentration of 100 nM using Lipofectamine 2000 according to the manufacturer's instructions. At 8 h post-transfection, cells were cultured with 2.5 μ M of ATRA for 24 h ($n=3$, $P<0.05$). (A) RT-PCR and (B) western blotting indicated that Axin mRNA was barely detected and protein expression was decreased significantly in cells transfected with the RNAi sequence against Axin following ATRA treatment for 24 h. As compared with the control, Axin-RNAi caused a decrease in p53 mRNA and its protein following ATRA treatment for 24 h ($n=3$, $P<0.05$). The expression of mRNA and protein was normalized, respectively, to the quantity of β -actin. (C) MTT assay indicated that the growth rate of the Axin RNAi group was significantly decreased compared to the negative control group after transfection ($n=3$, $P<0.05$). (D) TUNEL analysis revealed that the apoptotic index of cells with knockdown of Axin was significantly decreased compared to the negative control ($n=3$, $P<0.05$). (E) Flow cytometric analysis revealed that Axin RNAi attenuated ATRA-induced G1/S phase arrest and apoptosis ($n=3$, $P<0.05$).

respectively, while the percentages of C6 cells transfected with rAxin in pre-G1, G1 and S phases were 16.0 ± 2.3 , 74.8 ± 3.2 and $11.6\pm1.7\%$, respectively. The apoptotic index of C6 cells transfected with Axin ($28.4\pm3.3\%$) was significantly increased compared to the C6 cells transfected with the empty vector ($9.6\pm2.4\%$) and the C6 cells ($6.8\pm1.8\%$). These results revealed that overexpression of Axin inhibits cell proliferation and induces apoptotic cell death.

Axin RNAi attenuates ATRA-induced G1/S arrest and apoptosis accompanied by inhibition of the expression of p53. We performed RNA interference to further investigate the role of Axin in ATRA-induced cell cycle arrest and apoptotic cell death of C6 cells. Axin mRNA (Fig. 3A) was barely detected, and its protein expression (Fig. 3B) was downregulated significantly in cells transfected with the RNAi sequence against Axin following ATRA treatment. These data demonstrated that Axin-RNAi effectively silenced the ATRA-induced increase in Axin. Interestingly, Axin-RNAi caused only a moderate decrease in p53 mRNA and its protein following ATRA treatment (Fig. 3A and B). These data revealed that the inactivation of p53 is associated with the silencing of Axin and implies that there are other mechanisms participating in ATRA-induced activation of p53. We next examined the effect of silencing Axin expression on ATRA-mediated cell growth. As compared with the control, the percentages of cells in the pre-G1 and G1 phases of the cell cycle were decreased

(12.6 ± 2.3 and $14.9\pm2.8\%$, respectively), while the percentage of cells in the S phase was increased ($15.6\pm2.1\%$) (Fig. 3E). Forty-eight hours after transfection, the viability of the Axin RNAi-transfected C6 cells decreased by ~60% compared with the control according to the MTT assay (Fig. 3C). TUNEL analysis revealed that the apoptotic index of cells with knockdown of Axin significantly decreased compared to the negative control (from 25.8 ± 2.5 to $10.1\pm2.3\%$) (Fig. 3D). These data reinforced the finding that Axin is an efficient regulator of ATRA-induced G1/S phase arrest and apoptosis, and silencing of its expression compromised the susceptibility to the effects of ATRA.

Discussion

The aggressiveness of cancer cells results from their proliferative advantage over their normal counterparts and from their inability to undergo apoptosis. Consistent with previous research (21-24), our results demonstrated that ATRA induced cell cycle arrest as well as apoptosis in glioma cells. The present study for the first time demonstrated that ATRA-mediated cell cycle arrest and apoptosis were associated with the activation of p53 resulting from the activation of Axin and this activation may be a general mechanism contributing to ATRA-inhibited tumor cell growth.

The tumor suppressor protein p53 is a nuclear phospho-protein that can potentially regulate the growth of mammalian

cells (25). Activation of p53 results in altered transcription of a wide variety of genes that are involved in many aspects of cell metabolism, cell cycle regulation and apoptosis. Moreover, the introduction of a wild-type p53 expression vector into tumor cells including glioma cells suggests its ability to halt the cell cycle, trigger apoptosis, and prevent undue cell proliferation (26-29). ATRA regulation of the fate of cells by p53 has been reported (30). These studies revealed that ATRA induces cell cycle arrest and apoptosis by regulating the transcriptional activity of p53 and/or stability of its protein. However, whether p53 participates in ATRA-mediated growth control in glioma cells remains unclear. Current evidence showed that G1/S arrest and apoptosis induced by ATRA were associated with the activity of p53 in the C6 glioma cell line.

Previous research revealed that Axin participates in ATRA-mediated differentiation of embryonic carcinoma cells (17) and overexpression of Axin causes apoptotic cell death in transgenic mice and certain cell lines (12,13). Recently, our group found that the activation of Axin was required for ATRA-inhibited cellular proliferation in glioma cell lines (18). Here, we found that overexpression of Axin by transiently transfecting C6 cells with rAxin inhibited the G1/S phase progression in addition to inducing apoptotic cell death. These results were further confirmed by Axin gene silencing. Herein, we propose that the activation of Axin, as well as the activation of p53, inhibited cell growth by cell cycle arrest and apoptotic cell death in the C6 glioma cell line following ATRA treatment.

Axin has been reported to stimulate p53 and p53-dependent transcriptional activity by the formation of a complex with p53 (19). This raised an intriguing possibility that ATRA-activated expression of Axin may inhibit tumor cell growth and activate the expression of p53. In the present study, we provide evidence that ATRA increases the expression of p53 through the activation of Axin. As mentioned above, p53, widely expressed in normal adult tissues, tumors, and embryos, is ubiquitously involved in growth arrest and/or apoptosis via a multitude of molecular pathways involving transactivation of target genes and direct signaling events (31-33). Based on these results, it is legitimate to suggest that Axin is an important regulator of ATRA-induced growth arrest and apoptosis of C6 glioma cells by mediating the p53-dependent cell death pathway, and this may be a general mechanism contributing to ATRA-mediated cell cycle arrest and apoptotic cell death.

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