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*Original Article*

# Involvement of hedgehog signaling in all-trans retinoic acid-mediated suppression of colon cancer

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**Abstract:** All-trans retinoic acid (ATRA) exerts tumor-inhibitory effects on acute leukemia and certain types of solid tumors. This study was designed to evaluate the mechanism on ATRA-mediated suppression of colon cancer based on the sonic hedgehog (Shh) signaling pathway. Methods: Normal intestinal epithelial cells and three colon cancer cell lines were studied to evaluate the inhibitory effect of ATRA on tumor cell activity. The inhibitory effect of ATRA on colon cancer was evaluated by cell invasion, migration, and apoptosis of HCT116 cells. Retinoic acid receptor (RAR)- and Shh-related protein expression was assessed. Results: ATRA administration inhibited the activity of three different colon cancer lines, but did not inhibit the activity of normal intestinal epithelial cells. Administration of ATRA induced apoptosis and restricted invasion and migration of HCT116 colon cancer cells. Administration of ATRA also increased expression of RAR and transmembrane receptor patched 1 (Ptch1), and decreased expression of the smoothened (Smo) and glioma-associated oncogene homolog1 (Gli-1). $RAR_{\alpha}$ and $RAR_{\beta}$ agonists inhibited Shh signaling, and the mediating effect of ATRA on Shh signaling was abolished by $RAR_{\alpha}$ or $RAR_{\beta}$ antagonists. The combination of purmorphamine (Smo agonist) and ATRA partially abolished the inhibitory effect of ATRA on the proliferation of colon cancer cells. In vivo studies showed that ATRA inhibited tumor growth, which was accompanied by downregulation of the Shh signaling pathway. Conclusions: ATRA inhibits the growth of colon cancer by downregulating the Shh pathway, which further verifies the anticancer activity of ATRA.

**Keywords:** Patched 1, colon cancer, all-trans retinoic acid, hedgehog

## Introduction

Colon cancer is a common malignant tumor with a high incidence worldwide. More than 1 million patients with colon cancer are diagnosed every year [1]. According to statistics, the global prevalence of colorectal cancer continues to grow, and the burden will increase by 60% by 2030 [2]. Although clinical treatment has made rapid progress in tumor surgery and the application of new radiotherapy and chemotherapy in recent decades, the mortality of colon tumor is still high due to drug resistance and metastasis. Therefore, it is of great significance to study the pathogenesis and therapeutic approach to colon cancer.

Retinoic acid (RA), the major biologically active form of vitamin A, plays an important role in regulating cell growth, differentiation, proliferation, and programmed apoptosis [3, 4]. It is involved in embryonic development, tissue differentiation, and tumor cell growth. This pleiotropy of RA is mainly mediated by two kinds of receptors belonging to the steroid/thyroxine superfamily, namely, retinoic acid receptors (RARs) and retinol-x receptors (RXRs), which are expressed to varying degrees at different stages of tissue development. There are three different subtypes of RAR and RXRs in humans. All-trans RA (ATRA) and 9-cis RA are common ligands involved in RA signaling. 9-cis RA can affect both RARs and RXRs, while ATRA is selective for RARs [5]. ATRA has been used as a drug to treat acute promyelocytic leukemia (APL) for more than 20 years [6, 7]. In addition, ATRA alone also has a certain inhibitory effect on other solid tumors, and ATRA combined with other drugs can increase their efficacy and reduce drug resistance [8-11]. For instance, treatment in combination with ATRA arrests

gefitinib-induced enrichment of aldehyde dehydrogenase 1A1 (ALDH1A1)/CD44 cancer stem cell-like cells and increases the antitumor effect of gefitinib on non-small cell lung adenocarcinoma (NSCLC/ADC) cells [12]. It is generally believed that ATRA plays an antitumor role by inducing apoptosis, preventing tumor cell proliferation and inducing abnormal cells to differentiate back into normal cells [13-15]. Some reports show that ATRA can arrest the progression and migration of colon cancer, and its mechanism may be related to reducing myosin light chain kinase (MLCK) expression by the extracellular signal-regulated kinase 1/Mitogen-activated protein kinase (ERK1/MAPK) pathway [16]. It is worth noting that ATRA antagonizes the hedgehog (Hh) pathway in embryonal carcinoma cells [17]. The Hh signaling pathway is a pivotal regulator of development and stem cell fate in animals. Abnormal up-regulation of the Hh pathway contributes to many kinds of cancer, such as gastric cancer, lung cancer, liver cancer, mammary cancer, prostate cancer, pancreatic cancer and colon cancer [18-20]. Vertebrates have three homologous Hh genes: sonic Hh (Shh), Indian Hh, and desert Hh. Classical Shh pathway includes transmembrane receptor patched 1 (Ptch1), smoothened (Smo), and glioma-associated oncogene homolog1 (Gli-1). At the molecular level, it has been shown that Shh signaling drives the progression of cancers by regulating cancer cell proliferation, malignancy, metastasis, and the expansion of cancer stem cells (CSCs) [21, 22]. Tazarotene, a retinoid with RAR β/γ specificity, inhibited a murine basal cell carcinoma (BCC) keratinocyte cell line, ASZ001, and the inhibitory effect of tazarotene on ASZ001 cells was accompanied by down-regulation of Gli-1 expression [23]. In addition, the mRNA and protein expression of Ptch1 was significantly upregulated by ATRA treatment in embryonal carcinoma NT2/D1 cells, which contributes to a decreased Shh pathway [17]. However, it is unclear whether Shh is involved in growth inhibition by ATRA on colon cancer.

In the current study, normal intestinal epithelial cells and three colon cancer cell lines were studied to evaluate the inhibitory effect of ATRA on tumor cell activity. We demonstrated that ATRA enhanced Ptch1 activity and downregulated Smo and Gli-1 protein expression, thereby inducing apoptosis and inhibiting colon cancer cell proliferation.

## Material and methods

### *Drugs and reagents*

ATRA was the from Sigma-Aldrich (St. Louis, MO, USA). Cyclopamine and purmorphamine were the products of Selleck Chemicals (Houston, TX, USA). AM580, AC-55649 and LE135 were obtained from MedChemExpress. Ro41-5253 was purchased from Enzo Life Sciences. Bovine serum (FBS), Dulbecco modified Eagle medium (DMEM) and Roswell Park Memorial Institute 1640 (RPMI 1640) medium were obtained from Gibco. The TUNEL Apoptosis Detection Kit (FITC) and Annexin V-FITC/PI apoptosis detection kits were obtained from Vazyme Biotech Co., Ltd. The EdU-488 Kit and JC-1 Kit were the products of Beyotime Biotechnology (Shanghai China). Ki67 antibody, Bax antibody, Bcl-2 antibody and Ptch1 antibody were purchased from ABclonal Biotechnology. β-actin antibody, Gli-1 antibody, and Smo antibody were purchased from Proteintech Biotechnology.

### *Cell viability assay*

In this study, NCM460, SW480, HCT116, and Caco2 cells were studied to evaluate the inhibition of ATRA on tumor cell viability. The culture conditions of the four kinds of cells were the same, except for the medium. SW480, HCT116 and Caco2 colon cancer cells were cultured with RPMI 1640, and the medium for NCM460 cells was DMEM. The viability of different cells was investigated by MTT assay. Different cells were cultured for 24 h before ATRA treatment (0, 0.01, 0.1, 0.5, 1, 5, 10 or 20 μM) at 37°C. The observation times were 24, 48 and 72 hours. Two hundred μl of MTT solution (5 g/L) was added to each well of 96-well plate, and the cells were incubated in the incubator for 4 h, followed by 150 μl of DMSO for 10 minutes. The OD value at 490 nm was observed to evaluate the cell viability.

### *Colony formation and cell proliferation assays*

Colony formation was assessed to determine cell cloning ability. Two hundred HCT116 cells were inoculated into each well of the 6-well culture plate. These cells were treated with ATRA and cyclopamine for 24 h. Then, the cell supernatant was regularly replaced with fresh medium until day 10. The cells in the 6-well culture

plate were fixed and stained with crystal violet. The EdU incorporation method was performed to investigate HCT116 cell proliferation [24]. HCT116 cells were cultured in EdU working solution for 4 h after ATRA and cyclopamine treatment. The HCT116 cells were then washed with PBS and fixed with paraformaldehyde. After paraformaldehyde fixation for 25 min, the HCT116 cells were incubated with 0.1% Triton X-100, click reaction solution and Hoechst 33342 in turn, and photographed under fluorescence microscopy.

*Wound healing assay*

HCT116 cells were inoculated in a round-bottom cell culture dish and cultured for 24 h. The bottom of the culture dish was then scratched with a 200 μl pipette tip. After washing off the exfoliated cells, the cells were cultured for 48 hours. These scratches were observed with a microscope and the scratch distance was measured.

*Apoptosis detection*

After 48 hours of treatment with ATRA and cyclopamine, the HCT116 colon cancer cells were digested and centrifuged. 500 μl buffer reagent was added to the cells, and then 10 μl of Annexin V and 10 μl of Propidium Iodide were added to the HCT116 colon cancer cells respectively in the dark for 10 min.

*Mitochondrial membrane potential (MMP) measurement*

After drug incubation, the HCT116 cells were washed with JC-1 staining buffer, and then 2 μM of JC-1 staining working solution was added and they were incubated for 30 minutes. After washing again, the HCT116 cells were photographed and analyzed to evaluate cell MMP.

*Invasion and migration assays*

The migration and invasion of HCT116 cells were assayed by Transwell experiments.

The methods used for the cell migration and invasion experiments were the same, except that the upper chambers (24 well) in the invasion experiment were covered with Matrigel. The cells were cultured in the upper chamber of each Transwell with 150 μl serum-free medium. The culture system in the lower chamber is 600 μl complete medium containing 10% FBS (v/v). The cells were stained with 0.5% of crystal violet after fixing with paraformaldehyde. The cells that failed to pass through the membrane were gently removed with a cotton swab. The cells that passed through and remained at the bottom of the membrane were counted and photographed under a microscope. The number of cells passing through the membrane represents the ability of HCT116 cells to invade and migrate.

*In vivo tumor xenograft model and drug administration*

All animal experiments in this study were approved by the ethics committee of Jinzhou Medical University. The nude mice used in this experiment were 6-7 weeks old and weighed 18-20 g. After three days of preconditioning, HCT116 ($1\times10^7$) cells were inoculated on the backs of the nude mice at an inoculation volume of 0.1 ml. The size of the inoculated tumors in the nude mice was observed and measured regularly. When the diameter of most tumors reached 0.7 mm, the nude mice were gavaged with or without ATRA (1 mg/kg, 5 mg/kg). ATRA was dissolved in saline and gavaged for 14 days. The general state and food and water consumption by the nude mice were observed every day, and their weight was obtained every two days.

*TUNEL staining*

The tumor tissues fixed in formaldehyde were made into paraffin sections. The paraffin sections were routinely dewaxed and dehydrated and then incubated with trypsin at 37°C for 30 minutes. The TUNEL reaction solution was added to each slice and reacted for 1 hour. After washing with PBS, images were taken under a fluorescence microscope, and the number of green-positive nuclei was analyzed.

*Western blot analysis*

A portion of HCT116 cells and tumor tissues were collected and the protein content was measured by BCA method. The corresponding concentration of polyacrylamide gel was prepared, and 30 μg of protein extracts were collected on each pore between two layers of glass plates. The proteins separated by electric current were transferred to PVDF membranes

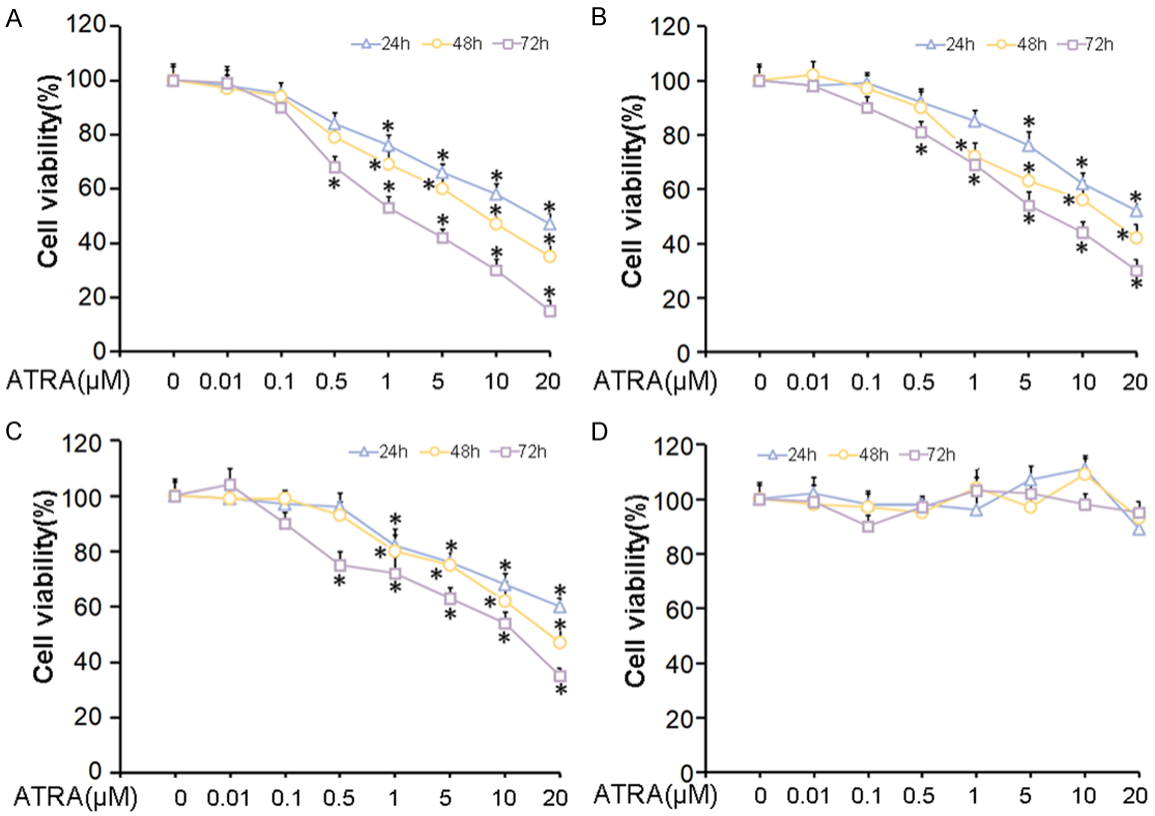


**Figure 1.** ATRA inhibits cell activity of colon cancer cells. Viability of the different cell lines was measured by MTT assay. HCT116 (A), SW480 (B), Caco2 (C), and NCM460 (D). Values are presented as mean ± SD, n = 3. (*P < 0.05 vs. 0 h group).

by semidry transfer initiation and blocked with 1% BSA for 1.5 hours. The primary antibody was diluted according to different manufacturer's instructions. The blocked PVDF membrane was incubated with different antibodies for 4°C overnight. The next day, the PVDF membrane was removed, washed with TBST and incubated with the secondary antibody for 1.5 hours. The blot was developed with ECL and a gel image was taken.

*Statistical analysis*

All data involved in the current study are presented as the mean ± SD and were analyzed and imaged with GraphPad Prism v9. The comparisons between different groups were analyzed by Student's t-test.

## Results

*ATRA inhibits the viability of colon cancer cells*

The viability of HCT116, SW480, Caco2 and NCM460 cells was determined by a colorimetric MTT assay. Cells were treated with or without ATRA (0.01, 0.1, 0.5, 1, 5, 10 and 20 μM) for 24, 48 or 72 h. ATRA inhibited the viability of all three colon cancer cell lines, but the toxic effect on HCT116 cells was more dramatic. Approximately 7.3 μM ATRA resulted in more than 50% death of HCT116 cells after 48 h of ARTA treatment. However, ATRA had no toxic effect on NCM460 cells at the same concentration. These results suggest that ATRA has an obvious toxic effect on colon cancer cells, especially HCT116 cells, but it has no toxic effect on normal colon epithelial cells (**Figure 1**). Therefore, HCT116 cells and 5 μM and 10 μM ATRA were used in the next experiments.

*ATRA inhibits HCT116 cell proliferation and colony formation*

The inhibition of ATRA on HCT116 cell proliferation was verified by EdU incorporation assays. ATRA and the Smo inhibitor cyclopamine significantly inhibited HCT116 cell proliferation, which was manifested by increased numbers

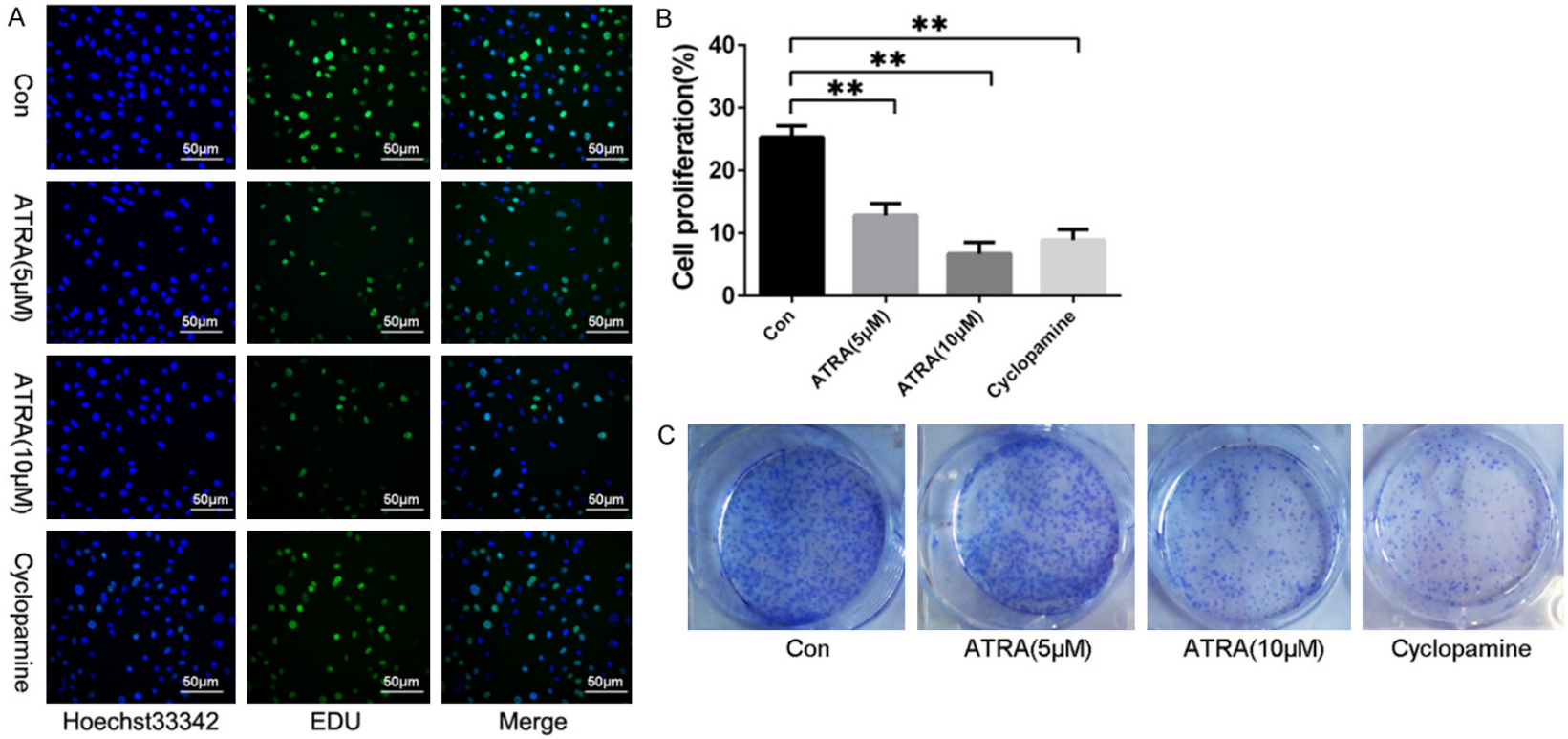


**Figure 2.** ATRA inhibits HCT116 cell proliferation. A, B: Proliferation was determined by EdU incorporation assay. C: Crystal violet staining of the colony formation of HCT116 cells. Data are presented as mean ± SD, Con, Control, n = 3. **P < 0.01 vs. the Con group.

of green nuclei in the EdU incorporation assay (**Figure 2A**, **2B**). Similarly, results from the colony formation assay showed that the clonogenic ability of HCT116 cells was significantly inhibited by ATRA and cyclopamine (**Figure 2C**).

*ATRA induces apoptosis of colon cancer HCT116 cells*

Results from flow cytometry and JC-1 staining showed that ATRA and cyclopamine caused significant apoptosis, as represented by increased apoptotic rate and MMP collapse (**Figure 3A-D**). Consistent with the above results, ATRA and cyclopamine increased pro-apoptotic protein Bax expression and decreased anti-apoptotic protein Bcl-2 expression (**Figure 3E-G**).

*ATRA inhibits the migration and invasion of HCT116 cells*

The effect of ATRA on the migration and invasion of HCT116 cells was assayed by Transwell assays. The results showed that ATRA and cyclopamine inhibited HCT116 cells from penetrating the membrane (**Figure 4A-C**). Similarly, the scratch wound healing assay confirmed the inhibition of ATRA and cyclopamine on HCT116 cells migration (**Figure 4D**, **4E**).

*ATRA inhibits Shh pathways dependent on RAR*

In the current study, we investigated whether the Shh pathway participated in ATRA-mediated suppression of colon cancer by WB. The results showed that the protein expression of Ptch1, a negative regulator of the Shh pathway, was significantly upregulated, while the protein expression of Smo and Gli-1 were downregulated after ATRA treatment. These effects of ATRA on Shh signaling were associated with increased protein expression of $RAR_{\alpha}$ and $RAR_{\beta}$, suggesting that ATRA treatment could regulate the Shh pathway in HCT116 cells (**Figure 5A-F**). To investigate the regulatory effect of ATRA on the Shh pathway in colon cancer cells rather than normal intestinal epithelial cells, the protein expression of Smo and Gli-1 were detected in HCT116 cells and NCM460 cells. The results showed that ATRA had no effect on the protein expression of Smo and Gli-1 in NCM460 cells (**Figure 5G-I**).

*The interaction of RAR and Shh*

In order to evaluate the mediating effect of ATRA on the Shh pathway, $RAR_{\alpha}$, $RAR_{\beta}$ and Smo agonists and antagonists were used in the current study. Activation of $RAR_{\alpha}$ or $RAR_{\beta}$ by AM580 ($RAR_{\alpha}$ agonist) or AC-55649 ($RAR_{\beta}$ agonist) showed similar inhibition of the Shh signaling pathway by ATRA (**Figure 6A-E**). Inhibition of RAR by Ro41-5253 ($RAR_{\alpha}$ antagonist) or LE135 ($RAR_{\beta}$ antagonist) abolished the mediating effect of ATRA on the Shh pathway (**Figure 6F-J**). In addition, the combination of ATRA with cyclopamine further downregulated the Shh pathway, except for the Ptch1 expression. These results demonstrated that the mediating effect of ATRA on the Shh pathway was dependent on RAR, while the combination of purmorphamine (Smo agonist) and ATRA partially abolished the suppressive inhibition of ATRA on cell proliferation of HCT116 colon cancer cells.

*ATRA showed antitumor effect in nude mice*

The suppressive effect of ATRA on colon tumor was further verified in nude mice in this study. Consistent with the in vitro results, ATRA significantly inhibited the growth of tumors, manifested as reduced tumor size and weight (**Figure 7A**, **7B**). The results showed that ATRA increased tumor tissue cell apoptosis, enhanced pro-apoptotic protein Bax expression, and decreased Bcl-2 and Ki-67 protein expression (**Figure 7D-J**). Compared to the control group, ATRA administration did not increase or reduce the experimental weight of nude mice (**Figure 7C**).

*ATRA inhibited the Shh pathway in vivo*

The mediating effect of ATRA on the Shh pathway was measured by an in vivo study. Not surprisingly, the protein expressions of Ptch 1, $RAR_{\alpha}$ and $RAR_{\beta}$ were upregulated, and the expression of Smo and Gli-1 was downregulated in the tumor tissue after ATRA treatment (**Figure 8**), which further verified the experimental results of the in vitro study.

## Discussion

The Shh and RAR signaling pathways both play a pivotal role in regulating the differentiation

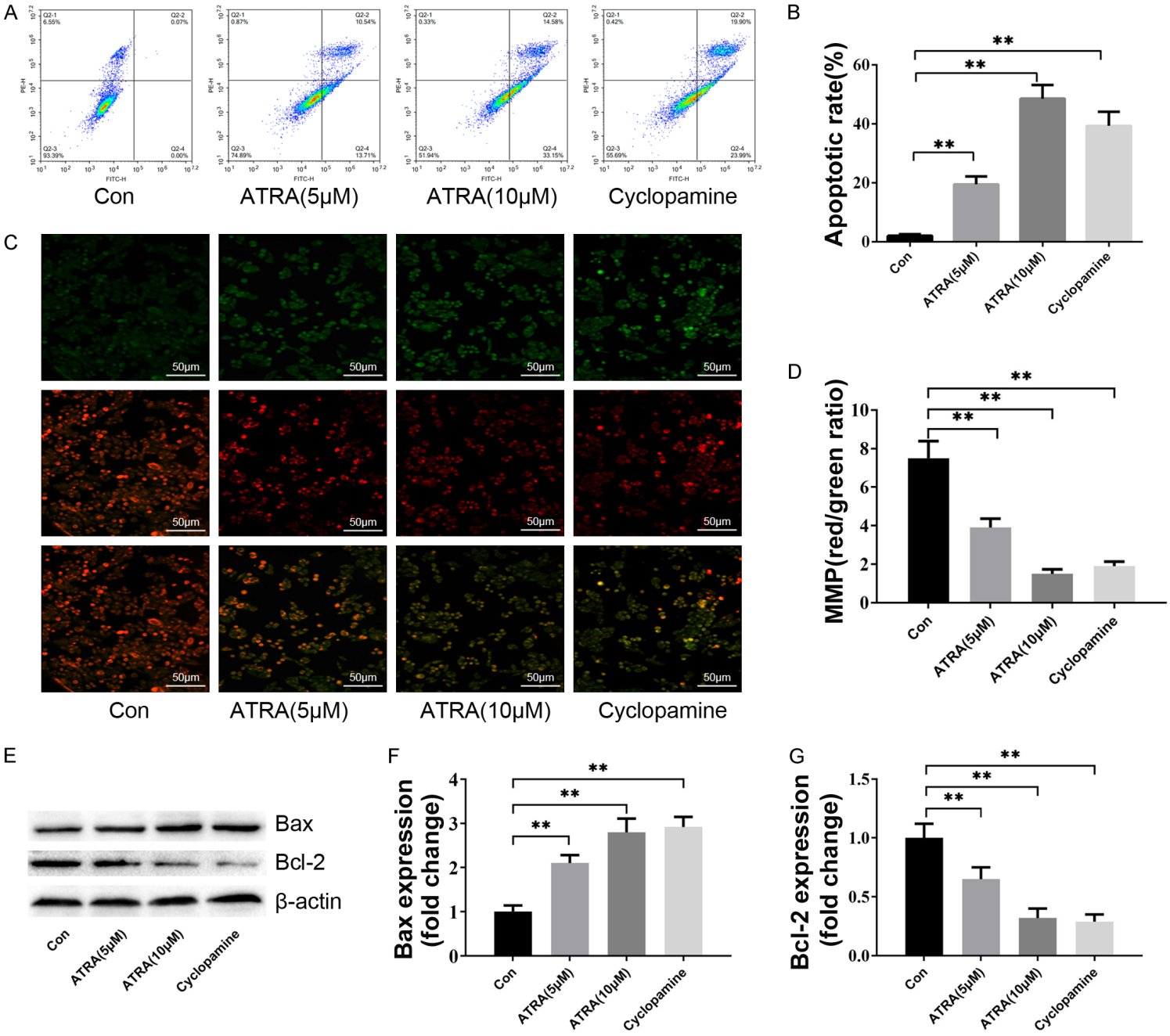
A
Con
ATRA(5μM)
ATRA(10μM)
Cyclopamine
B
Apoptotic rate(%)
C
50μm
Con
ATRA(5μM)
ATRA(10μM)
Cyclopamine
D
MMP(red/green ratio)
E
Bax
Bcl-2
β-actin
F
Bax expression (fold change)
G
Bcl-2 expression (fold change)

**Figure 3.** ATRA induces apoptosis of HCT116 cells. A, B: The apoptotic rate of HCT116 cells was determined by flow cytometry. C, D: MMP was measured by JC-1 staining. E-G: Apoptosis-related protein Bcl-2 and Bax expression. Data are presented as mean ± SD, n = 3. $^{**}P < 0.01$ vs. the Con group.

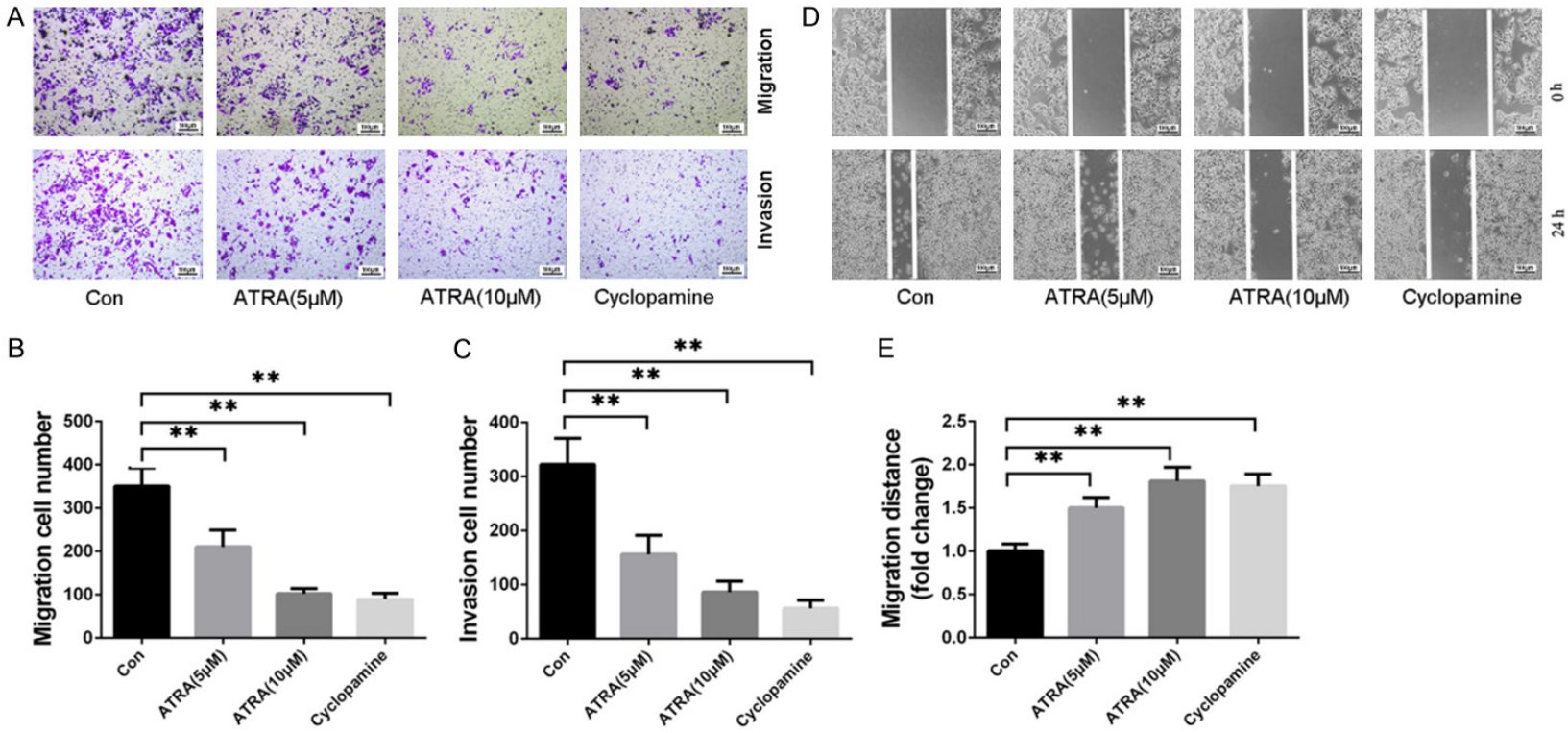


**Figure 4.** ATRA inhibits the migration and invasion of HCT116 cells. A-C: Migration and invasion of HCT116 cells were detected by Transwell experiments. D, E: The migration distance of HCT116 cells was reflected by a wound healing assay. Data are presented as mean ± SD, n = 4. $^{**}P < 0.01$ vs. the Con group.

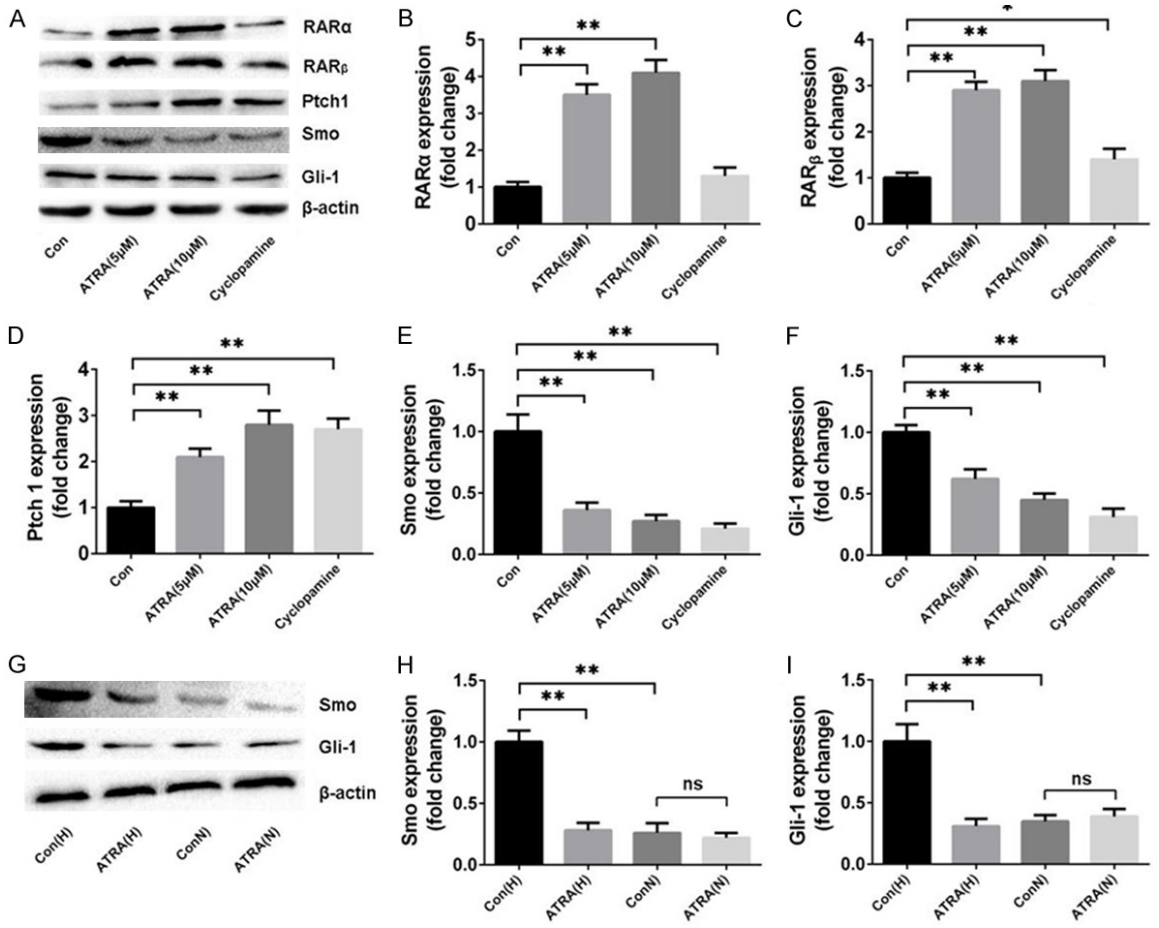


**Figure 5.** ATRA inhibits Shh pathways dependent on RAR. A: The protein expression of Ptch1, $RAR_{\alpha}$, $RAR_{\beta}$, Gli-1, and Smo was measured by western blot in HCT116 cells. B-F: Statistical results of protein expression by Image J software. G: The protein expression of Gli-1 and Smo was measured by western blot in HCT116 and NCM460 cells. H and I: Statistical results of protein expression by Image J software. H: HCT116 cells, N: NCM460 cells. Data are presented as mean ± SD, n = 3. $^{**}P < 0.01$ vs. the Con or Con (H) group.

and proliferation of many types of cells. Interaction between RA signaling and Shh signaling is complicated in embryonal carcinomas and other cell types, but the interaction between the two signaling pathways in regulating colon cancer cell fates was unclear. This study found that ATRA induced cell apoptosis and restrained cell proliferation and migration of HCT116 colon cancer cells. The nude mice study showed that ATRA caused tumor shrinkage and induced significant cell apoptosis, which was related to downregulation of the Shh pathway. Moreover, $RAR_{\alpha}$ or $RAR_{\beta}$ agonists downregulated the Shh pathway, while $RAR_{\alpha}$ or $RAR_{\beta}$ antagonists abrogated the mediating effect of ATRA on the Shh pathway. The combination of Shh inhibitor with ATRA further enhanced the inhibition of ATRA on HCT116 colon cancer cell proliferation, while the combination of Shh agonist with ATRA partially abolished the inhibition of ATRA on HCT116 colon cancer cell proliferation.

The typical pathway of Shh is initiated by the binding of the Hh ligand to Ptch1. The activation of Shh signaling can either occur through the stimulation of Shh protein or the loss of Ptch1 activity. Abnormal activation of the Shh signaling pathway or interaction with other signaling pathways plays a pivotal role in tumorigenesis, growth, and metastasis of many human solid tumors, such as gastric, breast, colorectal, and liver. Therefore, Shh signaling has also become a target of a variety of antitumor drugs. Crocetin suppresses angiogenesis and metastasis of gastric cancer by restraining the Shh signaling pathway [25]. Berberine sup-

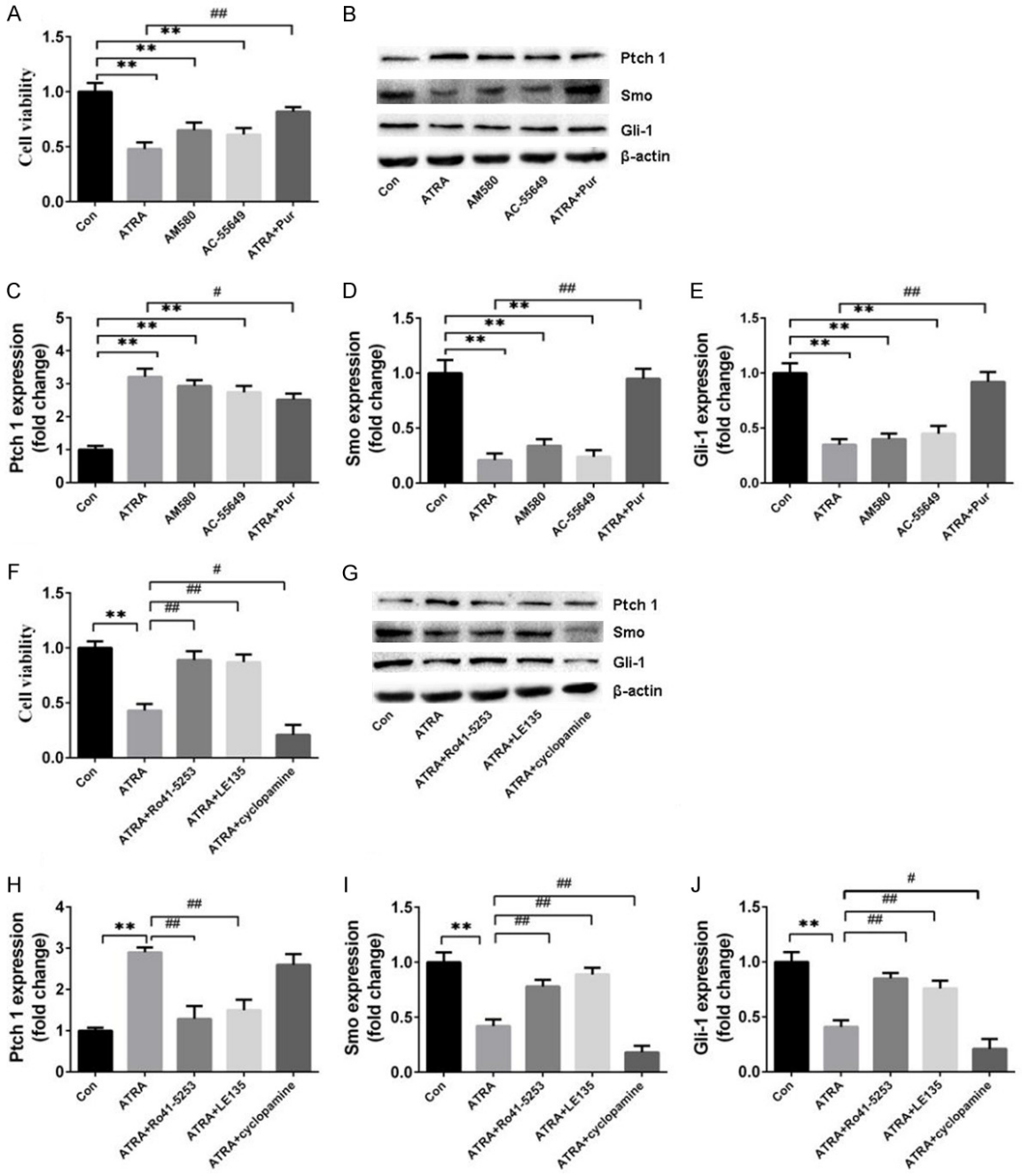


**Figure 6.** Interaction of RAR and Shh. A and F: HCT116 cell viability assay. B and G: Images of different protein expressions by western blot in HCT116 cells. C-E and H-J: Statistical results of protein expression by Image J software. Pur, Purmorphamine. N = 3. $^{**}P < 0.01$ vs. the Con group, $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ vs. the ATRA group.

presses colorectal cancer growth by interrupting activation of the paracrine hedgehog pathway [26]. Recently, the US Food and Drug Administration approved GDC-0449, an antagonist of Smo for the treatment of skin basal cell carcinoma, and GDC-0449 showed an inhibitory effect on colon cancer by upregulation of p21 and downregulation of CycD1 and Bcl-2, preventing the replication of colon cancer cells and triggering apoptosis [27, 28]. Consistent with previous results, the current study showed that cyclopamine, an inhibitor of Smo, exerted an inhibitory effect on colon cancer cells, including decreased cell activity, MMP, migration, and invasion, and an increased apoptotic rate; and the effect was similar to that of ATRA.

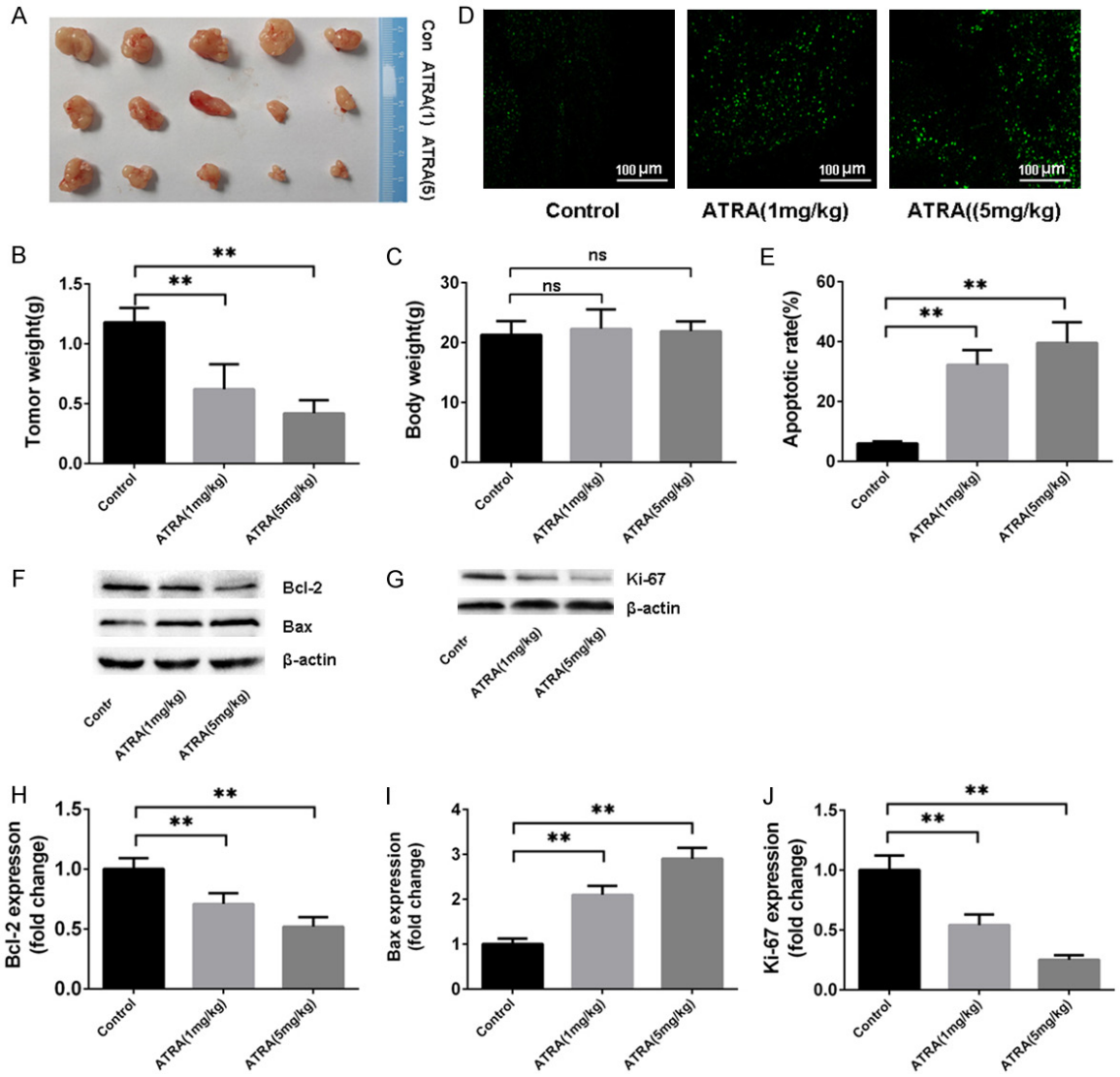


**Figure 7.** Suppressive effect of ATRA on colon tumor in mice. (A and B) Tumor tissue and tumor weight statistics of the different groups. (C) Body weight statistics of the mice in different groups. (D and E) TUNEL staining was used to observe the apoptosis rate of the tumor tissue. (F-J) Expression of different proteins assayed by western blot. n = 5 for (A-D); n = 3 for (F-J). $^{**}P < 0.01$ vs. the Control group.

ATRA is derived from vitamin A and has been approved for the treatment of acute leukemia. In addition, ATRA also regulates multiple cancer-associated processes in solid cancer cells in a RAR-dependent or RAR-independent manner [29, 30]. When colon cancer occurs, every step of ATRA biosynthesis is affected [31]. ATRA functions by binding to RAR/RXR heterodimers, which subsequently bind to retinoic acid response element (RARE) regions in the enhancer of targeted genes. Some studies reported that activation of RAR by ATRA could induce tumor stem cells to differentiate into normal cells, limit tumor cell overgrowth, and induce cancer cell apoptosis [32].

Our current study shows that oral ATRA can limit the tumor growth of xenografts in nude mice and induce tumor cell apoptosis. In vitro experiments further verified the above conclusions and proved that the effect of ATRA was receptor-dependent. Since the observed concentration of ATRA had no effect on the proliferation of NCM460 cells, we compared the protein expression of Smo and Gli-1 in HCT116 and NCM460 cells. The protein expression of Smo and Gli-1 in NCM460 cells was lower than that in HCT116 cells, and ATRA had no effect on their expression in NCM460 cells. These results suggest that Shh signal is highly expressed in tumor cells and may be a target of ATRA.

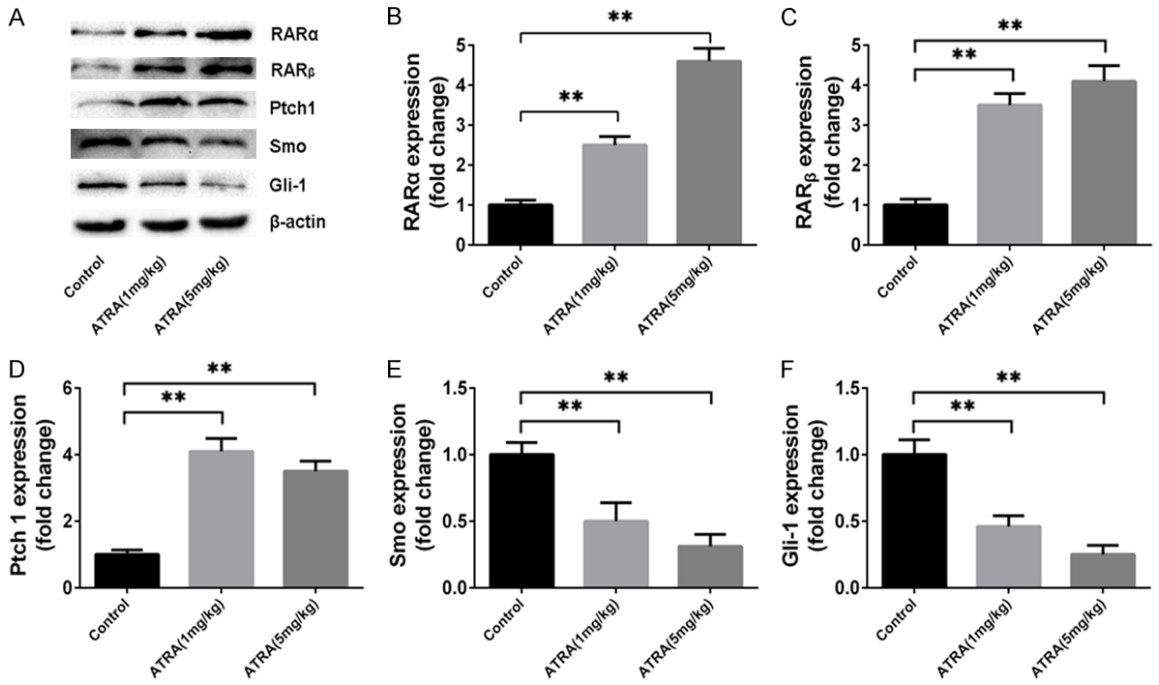


**Figure 8.** ATRA inhibited the Shh pathway in tumor tissue. A-F: The expression of different proteins assayed by western blot (Smo, Gli-1, Ptch1, $RAR_{\alpha}$, and $RAR_{\beta}$) of tumor tissue. n = 3. $^{**}P < 0.01$ vs. the control group.

Recently, several studies have addressed the crosstalk between the RA and Shh pathways [33-35]. Forskolin (FSK), an antagonist of of the hedgehog signaling pathway, showed a synergistic effect with ATRA against bleomycin-induced pulmonary fibrosis. Although FSK alone could not significantly increase in RARα and RXRα expression, combined with ATRA it could significantly upregulate RARα and RXRα expression. It is worth noting that ATRA treatment was more effective in upregulating Ptch1 expression than FSK treatment in a bleomycin-induced pulmonary fibrosis model [33]. Additionally, according to Alexander's report, the mRNA and protein expression of Ptch1 was significantly upregulated by ATRA treatment in embryonal carcinoma NT2/D1 cells, which contributes to decreased Gli-1 expression, and the mediating effect of ATRA on the Shh pathway was confirmed in the murine ES cell lines AB2.2, NB4, Tera-1, and P19 [17]. Treatment with inhibitors of Smo (GDC-0449 and cyclopamine) had no effect on Ptch1 induction despite RA treatment, suggesting that the observed effect of ATRA on Ptch1 did not depend on Smo activity [17]. In contrast to the above results, ATRA not only affected the expression of Ptch1 and Gli-1 but also decreased the expression of Smo in HCT116 cells in our current study. In addition, inhibition of RAR by an antagonist abolished the mediating effect of ATRA on the Shh pathway, and the combination of ATRA with cyclopamine further decreased Smo and Gli-1 expression but not Ptch1 expression, indicating that the mediating effect of ATRA on the Shh pathway in colon HCT116 cells occurred in a RAR-dependent manner. The reason for the discrepancy among the different studies may be attributed to different cell types. However, additional mechanistic studies are still required to fully explain the current research results.

In summary, downregulation of the Shh pathway mediated by ATRA was involved in inhibiting the cell proliferation, migration and invasion of colon cancer cells and inducing apoptosis. These results help us to understand the application of ATRA for the treatment of solid tumors, and its molecular mechanism.

## Acknowledgements

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## Disclosure of conflict of interest

None.

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## References

[1] Cerezo-Guisado MI, Zur R, Lorenzo MJ, Risco A, Martín-Serrano MA, Alvarez-Barrientos A, Cuenda A and Centeno F. Implication of Akt, ERK1/2 and alternative p38MAPK signalling pathways in human colon cancer cell apoptosis induced by green tea EGCG. Food Chem Toxicol 2015; 84: 125-132.

[2] Arnold M, Sierra MS, Laversanne M, Soerjomataram I, Jemal A and Bray F. Global patterns and trends in colorectal cancer incidence and mortality. Gut 2017; 66: 683-691.

[3] Forouzanfar F, Soleimannejad M, Soltani A, Sadat Mirsafaee P and Asgharzade S. Retinoic acid and taurine enhance differentiation of the human bone marrow stem cells into cone photoreceptor cells and retinal ganglion cells. J Cell Biochem 2021; 122: 1915-1924.

[4] Yoshioka H, Mikami Y, Ramakrishnan SS, Suzuki A and Iwata J. MicroRNA-124-3p plays a crucial role in cleft palate induced by retinoic acid. Front Cell Dev Biol 2021; 9: 621045.

[5] di Masi A, Leboffe L, De Marinis E, Pagano F, Cicconi L, Rochette-Egly C, Lo-Coco F, Ascenzi P and Nervi C. Retinoic acid receptors: from molecular mechanisms to cancer therapy. Mol Aspects Med 2015; 41: 1-115.

[6] Vu HT, Hoang TX and Kim JY. All-trans retinoic acid enhances matrix metalloproteinase 2 expression and secretion in human myeloid leukemia THP-1 cells. Biomed Res Int 2018; 2018: 5971080.

[7] Costantini L, Molinari R, Farinon B and Merendino N. Retinoic acids in the treatment of most lethal solid cancers. J Clin Med 2020; 9: 360.

[8] Wang C, Zhao D, Wang K, Gao L, He Y, Wu H, Ruan L, Chen W, Zhang D, Xia T, Qian S, Liu Z, Yang Y, Yang W, Hu A and Zhao Q. All-trans retinoic acid rescues the tumor suppressive role of RAR-beta by inhibiting LncHOXA10 expression in gastric tumorigenesis. Nutr Cancer 2021; 73: 2065-2077.

[9] Wang S, Feng R, Shi Y, Chen D, Weng H, Ding H and Zhang C. Intracellular alpha-fetoprotein interferes with all-trans retinoic acid induced ATG7 expression and autophagy in hepatocellular carcinoma cells. Sci Rep 2021; 11: 2146.

[10] Bessone MID, Berardi DE, Cirigliano SM, Delbart DI, Peters MG, Todaro LB and Urtreger AJ. Protein kinase C alpha (PKCalpha) overexpression leads to a better response to retinoid acid therapy through retinoic acid receptor beta (RARbeta) activation in mammary cancer cells. J Cancer Res Clin Oncol 2020; 146: 3241-3253.

[11] Bolis M, Paroni G, Fratelli M, Vallerga A, Guarrera L, Zanetti A, Kurosaki M, Garattini SK, Gianni' M, Lupi M, Pattini L, Barzago MM, Terao M and Garattini E. All-trans retinoic acid stimulates viral mimicry, interferon responses and antigen presentation in breast-cancer cells. Cancers (Basel) 2020; 12: 1169.

[12] Yao W, Wang L, Huang H, Li X, Wang P, Mi K, Cheng J, Liu H, Gu C, Huang L and Huang J. All-trans retinoic acid reduces cancer stem cell-like cell-mediated resistance to gefitinib in NSCLC adenocarcinoma cells. BMC Cancer 2020; 20: 315.

[13] Fu F, Li LS, Li R, Deng Q, Yu QX, Yang X, Pan M, Han J, Zhen L, Zhang LN, Lei TY, Li DZ and Liao C. All-trans-retinoid acid induces the differentiation of P19 cells into neurons involved in the PI3K/Akt/GSK3beta signaling pathway. J Cell Biochem 2020; 121: 4386-4396.

[14] Chen XJ, He MJ and Zhou G. All-trans retinoic acid induces anti-tumor effects via STAT3 signaling inhibition in oral squamous cell carcinoma and oral dysplasia. J Oral Pathol Med 2019; 48: 832-839.

[15] Lokman NA, Ho R, Gunasegaran K, Bonner WM, Oehler MK and Ricciardelli C. Anti-tumour effects of all-trans retinoid acid on serous ovarian cancer. J Exp Clin Cancer Res 2019; 38: 10.

[16] Zuo L, Yang X, Lu M, Hu R, Zhu H, Zhang S, Zhou Q, Chen F, Gui S and Wang Y. All-trans retinoic acid inhibits human colorectal cancer cells rko migration via downregulating myosin light chain kinase expression through MAPK signaling pathway. Nutr Cancer 2016; 68: 1225-1233.

[17] Busch AM, Galimberti F, Nehls KE, Roengvoraphoj M, Sekula D, Li B, Guo Y, Direnzo J, Fiering SN, Spinella MJ, Robbins DJ, Memoli VA, Freemantle SJ and Dmitrovsky E. All-trans-retinoic acid antagonizes the hedgehog pathway by inducing patched. Cancer Biol Ther 2014; 15: 463-472.

[18] Hinshaw DC, Hanna A, Lama-Sherpa T, Metge B, Kammerud SC, Benavides GA, Kumar A, Alsheikh HA, Mota M, Chen D, Ballinger SW, Rathmell JC, Ponnazhagan S, Darley-Usmar V, Samant RS and Shevde LA. Hedgehog signaling regulates metabolism and polarization of mammary tumor-associated macrophages. Cancer Res 2021; 81: 5425-5437.

[19] Xu Z, Li M, Sun R, Chu B, Song B, Wang H, Su Y and He Y. Nanoparticles as a hedgehog signaling inhibitor for the suppression of cancer growth and metastasis. Nanoscale 2021; 13: 11077-11085.

[20] Liang Y, Yang L and Xie J. The role of the hedgehog pathway in chemoresistance of gastrointestinal cancers. Cells 2021; 10: 2030.
[21] Fernandez-Zapico ME. GLI1 finds a new role in cancer stem cell biology. EMBO Mol Med 2013; 5: 483-485.
[22] Cochrane CR, Szczepny A, Watkins DN and Cain JE. Hedgehog signaling in the maintenance of cancer stem cells. Cancers (Basel) 2015; 7: 1554-1585.
[23] So PL, Fujimoto MA and Epstein EH Jr. Pharmacologic retinoid signaling and physiologic retinoic acid receptor signaling inhibit basal cell carcinoma tumorigenesis. Mol Cancer Ther 2008; 7: 1275-1284.
[24] Wu H, Chen Y, Li B, Li C, Guo J, You J, Hu X, Kuang D, Qi S, Liu P, Li L and Xu C. Targeting ROCK1/2 blocks cell division and induces mitotic catastrophe in hepatocellular carcinoma. Biochem Pharmacol 2021; 184: 114353.
[25] Zang M, Hou J, Huang Y, Wang J, Ding X, Zhang B, Wang Y, Xuan Y and Zhou Y. Crocetin suppresses angiogenesis and metastasis through inhibiting sonic hedgehog signaling pathway in gastric cancer. Biochem Biophys Res Commun 2021; 576: 86-92.
[26] Shen ZQ, Wang J, Tan WF and Huang TM. Berberine inhibits colorectal tumor growth by suppressing SHH secretion. Acta Pharmacol Sin 2021; 42: 1190-1194.
[27] Wu C, Hu S, Cheng J, Wang G and Tao K. Smoothened antagonist GDC-0449 (vismodegib) inhibits proliferation and triggers apoptosis in colon cancer cell lines. Exp Ther Med 2017; 13: 2529-2536.
[28] Magistri P, Battistelli C, Strippoli R, Petrucciani N, Pellinen T, Rossi L, Mangogna L, Aurello P, D'Angelo F, Tripodi M, Ramacciato G and Nigri G. SMO inhibition modulates cellular plasticity and invasiveness in colorectal cancer. Front Pharmacol 2017; 8: 956.
[29] Dhandapani L, Yue P, Ramalingam SS, Khuri FR and Sun SY. Retinoic acid enhances TRAIL-induced apoptosis in cancer cells by upregulating TRAIL receptor 1 expression. Cancer Res 2011; 71: 5245-5254.
[30] Tsuchiya H and Oura S. Involvement of MAFB and MAFF in retinoid-mediated suppression of hepatocellular carcinoma invasion. Int J Mol Sci 2018; 19: 1450.
[31] Kropotova ES, Zinovieva OL, Zyryanova AF, Dybovaya VI, Prasolov VS, Beresten SF, Oparina NY and Mashkova TD. Altered expression of multiple genes involved in retinoic acid biosynthesis in human colorectal cancer. Pathol Oncol Res 2014; 20: 707-717.
[32] Modarai SR, Gupta A, Opdenaker LM, Kowash R, Masters G, Viswanathan V, Zhang T, Fields JZ and Boman BM. The anti-cancer effect of retinoic acid signaling in CRC occurs via decreased growth of ALDH+ colon cancer stem cells and increased differentiation of stem cells. Oncotarget 2018; 9: 34658-34669.
[33] Eleraky AF, Helal GK, Elshafie MF and Ismail RS. Concomitant inhibition of hedgehog signalling and activation of retinoid receptors abolishes bleomycin-induced lung fibrosis. Clin Exp Pharmacol Physiol 2021; 48: 1024-1040.
[34] Mich JK and Chen JK. Hedgehog and retinoic acid signaling cooperate to promote motoneurogenesis in zebrafish. Development 2011; 138: 5113-5119.
[35] England S, Batista MF, Mich JK, Chen JK and Lewis KE. Roles of hedgehog pathway components and retinoic acid signalling in specifying zebrafish ventral spinal cord neurons. Development 2011; 138: 5121-5134.