RESEARCH ARTICLE

Induction of MicroRNA-9 Mediates Cytotoxicity of Curcumin Against SKOV3 Ovarian Cancer Cells

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Abstract

Background: Curcumin, a phenolic compound extracted from the rhizomes of *Curcuma longa*, has shown cytotoxic effects against a variety of cancers. The aim of this study was to identify potential microRNA (miRNA) mediators of the anticancer effects of curcumin in ovarian cancer cells. Materials and Methods: SKOV3 ovarian cancer cells were treated with curcumin (10-60 μM) and miR-9 expression, cell proliferation, and apoptosis were assessed. The effects of miR-9 depletion on curcumin-mediated growth suppression were also examined. Phosphorylation of Akt and forkhead box protein O1 (FOXO1) was measured in cells with miR-9 overexpression or curcumin treatment. Results: Curcumin caused a significant and dose-dependent increase of miR-9 expression in SKOV3 cells, while significantly impeding cell proliferation and stimulating apoptosis. Depletion of miR-9 significantly (p<0.05) attenuated the growth-suppressive effects of curcumin on SKOV3 cells, coupled with reduced percentages of apoptotic cells. In contrast, overexpression of miR-9 significantly enhanced the cleavage of caspase-3 and poly(ADP-ribose) polymerase and promoted apoptotic death in SKOV3 cells. Western blot analysis showed that both miR-9 overexpression and curcumin similarly caused a significant (p<0.05) decline in the phosphorylation of Akt and FOXO1, compared to untreated cells. Conclusions: The present study provided evidence that curcumin exerts its cytotoxic effects against SKOV3 ovarian cancer cells largely through upregulation of miR-9 and subsequent modulation of Akt/FOXO1 axis. Further studies are needed to identify direct targets of miR-9 that mediate the anticancer effects of curcumin in ovarian cancer cells.

Keywords: Cancer therapy - microRNA - phytochemical - survival signaling - ovarian cancer cells

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Introduction

Bioactive phytochemicals from medicinal herbs and dietary plants are gaining increasing attention as anticancer agents (Shu et al., 2010). They are capable of regulating multiple signaling pathways associated with aggressive phenotypes. Because of their pharmacological safety, these phytochemicals can be applied in cancer chemoprevention or adjuvant chemotherapy. Curcumin (1, 7-bis (4-hydroxy-3-methoxyphenyl)-1, 6-heptadiene-3, 5-dione) (Figure 1A), is a dietary phytochemical derived from the rhizome of Curcuma longa. This natural compound exhibits growth-suppressive activity in a variety of cancer cells (Shehzad et al., 2013). Ovarian cancer is a common malignancy among women, with high mortality due to asymptomatic nature of the disease and advance stage at presentation (Itamochi and Kigawa, 2012). A preclinical study has indicated that curcumin inhibits ovarian cancer growth in athymic mice, which is associated with induction of apoptosis and suppression of angiogenesis (Lin et al., 2007). Mechanistic studies revealed that the apoptosis-inducing activity of curcumin against ovarian cancer cells is mediated through ablation of prosurvival Akt signaling and downregulation of Bcl-2 and survivin expression (Watson et al., 2010). Phosphorylation and inactivation of forkhead box protein O1 (FOXO1) is an important mechanism by which Akt activation promotes cell survival (Rena et al., 1999). FOXO1 acts as a tumor suppressor in a variety of cancers including ovarian cancer (Goto et al., 2008; Xie et al., 2012). Several studies have demonstrated that the anticancer effect of curcumin is associated with the modulation of FOXO1 expression (Li et al., 2014).

microRNAs (miRNAs) are a class of endogenous, small, non-coding RNAs, ~21-25 nucleotides in length (Ling et al., 2013). Mature miRNAs are produced by sequential processing of primary transcripts (pri-miRNAs) mediated by two RNase III enzymes, Drosha and Dicer (Siomi and Siomi, 2010). Typically, they negatively regulate mRNA stability and/or protein expression by imperfect base pairing to the 3'-untranslated regions of target mRNAs (Bartel, 2009). A single miRNA can

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modulate the expression of hundreds of different targets and is thus implicated in a broad range of physiological and pathological processes (Bartel, 2009; Guo et al., 2010). miRNAs are known to play complex roles in cancer development and progression, acting as oncogenes or tumor suppressor genes (Farazi et al., 2013).

Several lines of evidence indicate that miR-9 facilitates tumor cell motility and metastasis in some cancer types such as breast cancer (Ma et al., 2010) and hepatocellular carcinoma (Sun et al., 2013). However, in ovarian cancer (Corney and Nikitin, 2008; Guo et al., 2009), miR-9 functions as a tumor suppressor, causing tumor cell growth suppression. A recent study has shown that miR-9 has the potential of improving chemotherapeutic efficacy in ovarian cancer, as targeted inhibition of miR-9 sensitizes ovarian xenograft tumors to cisplatin and PARP inhibitor (Sun et al., 2013). Tang et al. (2013) reported that miR-9 inhibits ovarian cancer cell proliferation, migration, and invasion through suppression of talin 1/FAK/Akt pathway. These studies combined with the finding that curcumin can alter miRNA expression profiles in cancer cells (Sun et al., 2008) suggest the possibility that miR-9 may be involved in the anticancer effects of curcumin against ovarian cancer. To test this hypothesis, we examined the effects of curcumin treatment on miR-9 expression in ovarian cancer cells and explored the potential roles of miR-9 in curcumin-mediated cytotoxicity.

Materials and Methods

Cell culture and treatment

SKOV3 cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA), penicillin (100 U/mL) and streptomycin (100 μg/mL) in a humidified atmosphere of 5% CO_2 at 37°C. Cells were treated with curcumin (Sigma, St. Louis, MO, USA) at varying concentrations (10-60 μM) for 72h. If not stated otherwise, cells were exposed to 60 μM curcumin. After treatments, cells were tested for cell proliferation, apoptosis, and gene expression.

Oligonucleotides and cell transfection

For overexpression of miR-9, human pre-miR-9 mimic (Wang et al., 2010) and negative control oligonucleotides were purchased from Ambion (Austin, TX, USA). For knockdown of miR-9, locked nucleic acid (LNA)-based anti-miR-9 and universal LNA-based negative control were purchased from Exiqon (Vedbaek, Denmark). The final concentrations of the oligonucleotides used in this study were 30 nM.

Cells were plated overnight at 2×10^5 cells in 6-well plates. All transfections were performed using Lipofectamine 2000 (Invitrogen) according to the instructions of the manufacturer. Curcumin (60 μM) was added to the culture 24h after transfection. Following incubation for another 72h, cells were harvested for further analyses.

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis of miR-9

Total RNA was isolated from cells using the mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA). The level of mature miR-9 in cells after treatments was determined using Taqman miRNA Assays (Applied Biosystems, Foster City, CA, USA). After cDNA was synthesized with a miRNA-specific stem-loop primer, a quantitative PCR was performed using specific TaqMan MicroRNA Assay primers. The relative miR-9 amount normalized to the U6 snRNA level was calculated using the comparative cycle threshold ($^{\Delta\Delta Ct}$) method (Livak and Schmittgen, 2001). Each assay was carried out in triplicate.

MTT assay

After treatment with curcumin for 72 h, cell viability was assessed using the 3- (4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Cell samples were incubated with 5 mg/ml MTT (Sigma) for 4 h, and after removal of the MTT solution, formazan crystals were dissolved in dimethyl sulfoxide. The absorbance was measured at a wavelength of 570 nm.

Apoptosis analysis by annexin-V-staining

Apoptotic cell death was assessed using the annexin-V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) staining kit (BD Pharmingen, San Diego, CA), according to the manufacturer's recommendations. Briefly, cells were trypsinized, washed in phosphate buffered saline, and resuspended. Cell suspensions were stained with FITC-conjugated annexin V and PI, and analyzed by flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA). Apoptotic cells were defined as annexin V positive.

Western blot analysis

Primary antibodies used were as follows: rabbit anti-cleaved caspase-3 (#9664), anti-cleaved poly (ADP-ribose) polymerase (PARP; #5625), anti-Akt (#9272), anti-phospho-Akt (Ser473) (#4058), anti-FOXO1 (#2880), anti-phospho-FOXO1 (Ser256) (#9461), and anti-β-actin (#8457). These antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Cells were lysed in a 10 mM Tris buffer (pH 7.4) containing 1% sodium dodecyl sulfate (SDS) and complete protease inhibitors (Roche, Indianapolis, IN, USA). Samples of the protein lysates (50 μg) were loaded and fractionated by 10% SDS-polyacrylamide gel and transferred to polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA). The membrane was incubated overnight at 4°C with primary antibodies. Horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as the secondary antibody. The antibody complexes were visualized using the enhanced chemiluminescence system (Amersham Pharmacia Biotech, Arlington Heights, IL, USA). The intensity of each band was measured by densitometric analysis using the QuantityOne software (Bio-Rad, Hercules, CA, USA).

Statistical analysis

Statistical analysis was performed by SPSS software version 12.0 (SPSS Inc., Chicago, IL, USA). Data are presented as mean±standard deviation (SD). Differences among multiple groups were performed with one-way analysis of variance followed by Tukey's multiple comparison test. $p<0.05$ was considered to indicate statistically significant differences.

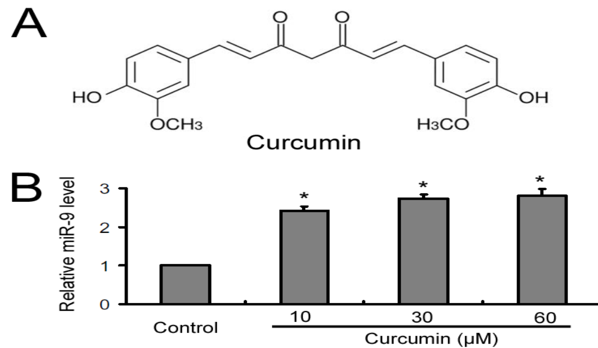

Figure 1. Curcumin Upregulates the Expression of miR-9 in Ovarian Cancer Cells. A) Chemical structure of curcumin. **B)** SKOV3 cells were treated with different concentrations of curcumin for 72 h, and the expression of miR-9 was measured using qRT-PCR analysis. Results are expressed as fold change relative to untreated control cells (set to 1). Error bars represent SD from three independent experiments. *$p<0.05$ *vs* the control

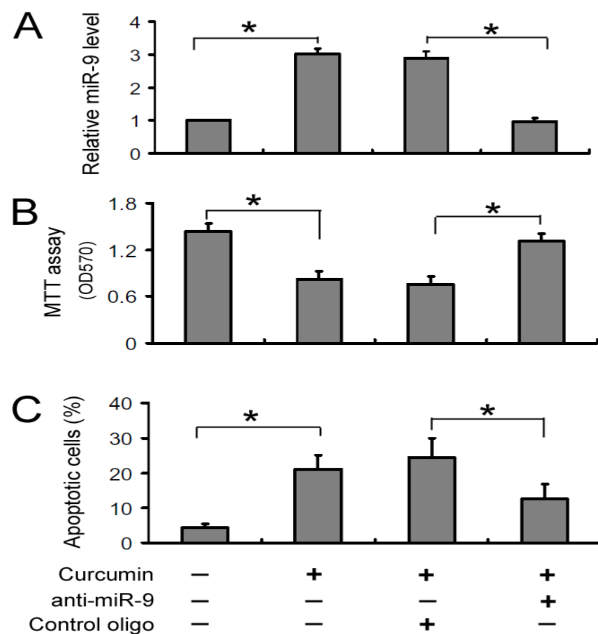

Figure 2. Effects of Depletion of miR-9 on the Cytotoxicity of Curcumin in SKOV3 Cells. Cells were transfected with anti-miR-9 or control oligonucleotides (oligo) 24h before the treatment with 40 μM curcumin. **A)** qRT-PCR analysis of miR-9 levels in each condition. Results are expressed as fold change relative to untreated control cells (set to 1). **B)** Cell proliferation was assessed using the MTT assay 72h after treatment. Data represent the mean±SD of three independent experiments. **C)** Apoptosis detection by annexin-V/PI staining. After treatment, cells were stained with annexin-V and PI and subjected to flow cytometric analysis. The percentage of annexin-V-positive apoptotic cells in each condition was determined. Graphs represent average values from three independent experiments, and error bars indicate SD. *$p<0.05$

Results

Curcumin elevates the expression of miR-9 in SKOV3 cells

qRT-PCR analysis revealed that curcumin treatment caused a significant increase in the expression of miR-9 in SKOV3 cells, as compared to untreated control cells ($p<0.05$; Figure 1B). Moreover, this induction was in a dose-dependent manner.

miR-9 mediates the cytotoxicity of curcumin in SKOV3 cells

Having identified the upregulation of miR-9 by curcumin, we next checked the role of miR-9 in curcumin-mediated cytotoxicity in SKOV3 cells. As shown in Figure 2A, the delivery of specific anti-miR-9 but not control oligonucleotides significantly ($p<0.05$) blocked the upregulation of miR-9 by curcumin. MTT assay revealed that treatment with 60 μM curcumin for 72h significantly impeded cell proliferation of SKOV3 cells by about 50% ($p<0.05$ relative to untreated control; Figure 2B). Notably, downregulation of miR-9 significantly attenuated the growth-suppressive effects of curcumin on SKOV3 cells (Figure 2B). Flow cytometric analysis demonstrated that exposure to 60 μM curcumin for 72 h resulted in

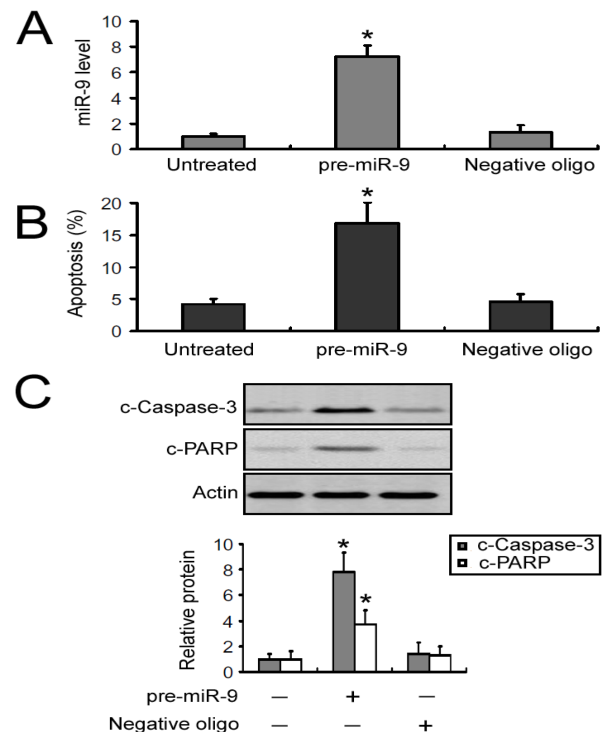

Figure 3. Enforced Expression of miR-9 Induces Apoptosis in SKOV3 Cells. Cells were transfected with pre-miR-9 mimic or negative control oligonucleotides (oligo). After incubation for 48 h, the cells were harvested for analysis of gene expression and apoptosis. **A)** The expression of miR-9 was measured using qRT-PCR analysis. Results are expressed as fold change relative to untreated cells (set to 1). **B)** Flow cytometric analysis of apoptotic cells stained with annexin-V and PI. The percentage of annexin-V-positive apoptotic cells in each condition was determined. **C)** Total cell lysates were subjected to Western blot analysis using antibodies against cleaved caspase-3 (c-Caspase-3) and PARP (c-PARP). Representative blots are shown in top panels. Bar graphs (bottom panels) represent densitometric analysis of three independent experiments. *$p<0.05$ *vs* untreated cells

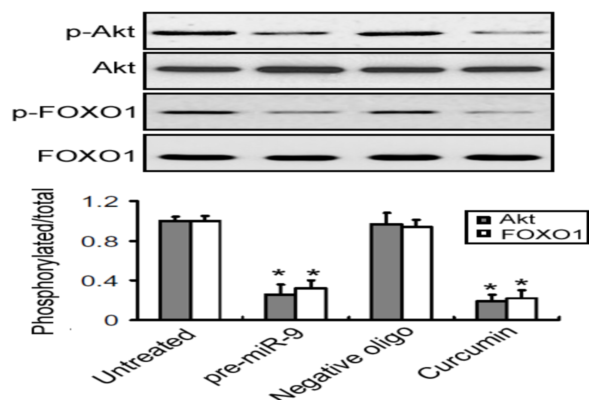

Figure 4. miR-9 Overexpression Inhibits Akt/FOXO1 axis. SKOV3 cells were transfected with pre-miR-9 mimic or negative control oligonucleotides (oligo) or treated with 60 μM curcumin for 48-72h. After the treatment, cells were subjected to Western blot analysis of indicated proteins. Representative blots are shown in top panels. Bar graphs (bottom panels) represent densitometric analysis of three independent experiments. *$p<0.05$ *vs* untreated cells

a significant increase in the percentage of apoptosis compared to untreated control (21.2±3.9% vs. 4.3±1.2%; $p<0.05$; Figure 2C). Such induction of apoptosis by curcumin was significantly ($p<0.05$) antagonized by the delivery of anti-miR-9.

Enforced expression of miR-9 induces apoptosis in SKOV3 cells

We next determined the effect of miR-9 overexpression on the survival of ovarian cancer cells. As shown in Figure 3A, transfection with pre-miR-9 mimic oligonucleotides led to a marked increase in the abundance of miR-9 in SKOV3 cells, compared to transfection with control oligonucleotides. Of note, enforced expression of miR-9 significantly ($p<0.05$) stimulated apoptotic death in SKOV3 cells, which resembled the effects of curcumin (Figure 3B). Western blot analysis demonstrated that miR-9 overexpression significantly ($p<0.05$) enhanced the cleavage of caspase-3 and PARP (Figure 3C).

miR-9 overexpression inhibits Akt activation and FOXO1 phosphorylation

Given the critical role for the Akt/FOXO1 axis in the regulation of cell survival (Rena et al., 1999; Xie et al., 2012), we examined the effect of miR-9 overexpression on Akt and FOXO1 phosphorylation in ovarian cancer cells. Western blot analysis showed that the phosphorylation levels of Akt and FOXO1 were significantly ($p<0.05$) lower in miR-9-overexpressing SKOV3 cells than in those transfected with control oligonucleotides (Figure 4). Similarly, curcumin treatment resulted in a significant ($p<0.05$) decline in the phosphorylation of Akt and FOXO1 in SKOV3 cells, compared to untreated cells (Figure 4). However, the total protein levels of Akt and FOXO1 remained unchanged by either curcumin treatment or miR-9 transfection.

Discussion

Curcumin exerts anticancer effects in many types of cancer cells such as non-small cell lung cancer cells (Li et al., 2013) and breast cancer cells (Nasiri et al., 2013). It has been shown to modulate the expression of large numbers of miRNAs in cancer cells that lead to either reduction of tumor growth or sensitization of cancer cells to chemotherapeutic agents (Sun et al., 2008; Sethi et al., 2013). miRNAs play an important role in the modulation of chemosensitivity of tumor cells (Song et al., 2013; Zhu et al., 2014). It has been reported that miR-186* is involved in curcumin-induced apoptosis in A549/DDP multidrug-resistant human lung adenocarcinoma cells (Zhang et al., 2010). Downregulation of miR-21 transcription via activator protein 1 mediates the suppressive effects of curcumin on tumor growth, invasion and in vivo metastasis in colorectal cancer (Mudduluru et al., 2011). Induction of tumor suppressive miRNAs is another mechanism linked to the cytotoxicity of curcumin against cancer cells. Curcumin was found to induce miR-203 transcription in bladder cancer cells that leads to downregulation of miR-203 target genes Akt2 and Src, culminating in decreased cell proliferation and increased apoptosis (Saini et al., 2011). Upregulation of the tumor suppressor let-7a miRNA is associated with curcumin-induced apoptosis in esophageal cancer cells (Subramaniam et al., 2012). Our present data revealed that curcumin treatment resulted in a dose-dependent and significant elevation of miR-9 expression in SKOV3 ovarian cancer cells, compared to untreated control cells. In contrast, curcumin treatment caused a marked decline in the expression of miR-9 in human-derived retinal pigment epithelial ARPE-19 cells (Howell et al., 2013). These findings suggest that the regulatory effects of curcumin on miR-9 expression may be cellular context dependent.

It has been documented that miR-9 expression is decreased in ovarian cancer tissues relative to normal tissues (Guo et al., 2009). When compared to primary ovarian cancer, this miRNA is downregulated in recurrent ovarian cancer (Laios et al., 2008). Gain-of-function studies have demonstrated the growth-suppressive effects of miR-9 on ovarian cancer cells (Guo et al., 2009; Sun et al., 2013). miR-9 may thus represent a key target for the treatment of ovarian cancer. In this regard, we examined whether upregulation of miR-9 was involved in the anticancer effects of curcumin in ovarian cancer cells. We found that the delivery of anti-miR-9 significantly abolished curcumin-induced miR-9 elevation. Of note, the blockade of miR-9 upregulation significantly reversed the tumor-suppressive effects of curcumin on ovarian cancer cells, leading to restoration of cell proliferation and reduced cell death. In contrast, enforced expression of miR-9 caused a significant apoptosis in SKOV3 cells, as determined by annexin-v/PI staining. Activation of caspase-3 followed by PARP cleavage is critical for triggering apoptosis in many cells (Elmore, 2007). It has been reported that curcumin induces apoptosis in cisplatin-resistant human ovarian cancer cells via activation of caspase-3 and degradation of PARP (Weir et al., 2007). Similarly, our present data revealed that miR-9 overexpression resulted in activation of caspase-3 and cleavage of PARP in SKOV3 cells, which confirmed the pro-apoptotic activity of miR-9 at the molecular level.

Taken together, these data indicate that the cytotoxic activity of curcumin against SKOV3 ovarian cancer cells is largely/partially dependent on the induction of miR-9, which in turn stimulates caspase-3-mediated apoptosis.

Akt is constitutively activated and contributes to cell survival in ovarian cancer (Tang et al., 2006). Development of effective agents to limit Akt signaling is thus of clinical significance in treatment of this disease. Phenethyl isothiocyanate, acting as an inhibitor of EGFR-Akt axis, has been shown to suppress ovarian tumor growth in a preclinical mouse model (Loganathan et al., 2012). Curcumin has been shown to inhibit Akt phosphorylation in ovarian cancer cells (Weir et al., 2007). Consistently, we also observed the inhibitory effects of curcumin on Akt activation in SKOV3 cells. Moreover, the Akt downstream effector FOXO1 phosphorylation was significantly repressed by curcumin treatment. Most interestingly, miR-9 overexpression phenocopied the effect of curcumin treatment, impairing Akt activation and FOXO1 phosphorylation. A recent report shows that FOXO1 is a direct target of miR-9 in hematopoietic cells (Senyuk et al., 2013). Taken together, these data suggest that miR-9 mediates the suppression of Akt/FOXO1 axis by curcumin in ovarian cancer cells. Indeed, the inactivation of Akt signaling by miR-9 is also described in a previous study (Tang et al., 2013).

It is well accepted that each miRNA can regulate the expression of many target mRNAs (Jiang et al., 2013; Ling et al., 2013). Several direct targets of miR-9 such as cyclin D1, Ets1 (Zheng et al., 2013), and CXCR4 (Lu et al., 2013) have currently been identified. Repression of cyclin D1 and Ets1 is involved in miR-9-mediated suppression of the proliferation, invasion and metastasis of gastric cancer cells (Zheng et al., 2013). Targeting CXCR4 was found to mediate the suppressive effects of miR-9 in nasopharyngeal carcinoma (Lu et al., 2013). Identification of specific target genes is of great importance in clarifying the functions of miR-9 in curcumin-mediated cytotoxicity. Therefore, additional studies are needed to address this issue.

In conclusion, we demonstrate that curcumin is capable of inducing miR-9 expression in SKOV3 ovarian cancer cells, which in turn suppresses the Akt/FOXO1 axis and promotes caspase-3-associated apoptosis. These findings establish a mechanistic rationale for the use of curcumin in the treatment of ovarian cancer. However, miR-9 direct targets mediating the anticancer effects of curcumin in ovarian cancer need to be further identified.

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