

Original Article

Alpinumisoflavone induces apoptosis in esophageal squamous cell carcinoma by modulating miR-370/PIM1 signaling

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Abstract: Esophageal squamous cell carcinoma (ESCC) is the most prevalent type of esophageal cancer and accumulating evidence has confirmed the role of miRNAs in ESCC. One such miRNA, miR-370, was found to be aberrantly downregulated in various human malignancies. This study showed that the expression of miR-370 was significantly lower in ESCC tissues and cell lines, and miR-370 functioned as a tumor suppressor in ESCC. Moreover, this is the first report that showed miR-370 suppresses cell proliferation and tumor growth by directly targeting Pim family kinases 1 (PIM1). Furthermore, alpinumisoflavone, a naturally occurring flavonoid, could inhibit tumor growth of ESCC by targeting miR-370/PIM1 signaling.

Keywords: ESCC, miR-370, PIM1, alpinumisoflavone

Introduction

Esophageal cancer is the sixth leading cause of cancer-related deaths worldwide [1]. Adenocarcinoma is the most prevalent type of esophageal cancer in the western countries, while 90% of the esophageal cancers in the region stretching from northern Iran to North-Central China are known to arise from the squamous cells [2]. Despite the use of multiple therapeutic strategies, esophageal squamous cell carcinoma (ESCC) has been associated with a low 5-year survival rate of 15-20% [3]. Therefore, novel therapeutic targets and agents are urgently needed.

MicroRNAs (miRNAs), a family of small non-coding RNAs, function as oncogenes or tumor suppressors and regulate various cellular processes, such as differentiation, proliferation, apoptosis, migration and invasion by repressing the expression of target genes [4]. Accumulating evidence has shown that miR-370 is commonly downregulated in various human malignancies, including ovarian cancer [5], gastric cancer [6], liver cancer [7] and non-small

cell lung cancer [8], and plays a regulatory role in the proliferation of various cancerous cells, cell differentiation, apoptosis, and chemosensitivity. However, its role in the development of ESCC has not yet been explored.

Pim family kinases, a family of small, constitutively active, and highly evolutionarily conserved serine/threonine-specific kinases, includes three members, PIM1, PIM2, and PIM3. As oncogenes, PIMs are found to be aberrantly highly expressed in both solid tumors and hematological malignancies [9]. Particularly, PIM1 has been reported to play regulatory roles in cellular proliferation, cell cycle, apoptosis, and metabolism in human malignancies [9]. Overexpression of PIM1 protein has been implicated in the tumorigenesis of ESCC [10]. Moreover, PIM1 expression was associated with lymphatic metastasis of ESCC, highlighting its potential as a therapeutic target for ESCC.

Alpinumisoflavone (AIF) is one of the major active ingredients of a traditional Chinese medicine *Derris eriocarpa*, which is widely distributed mainly over Yunnan, Guangxi and Guizhou in

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China. Previous studies have found that AIF has numerous pharmacological activities, including atheroprotective [11], estrogenic [12], and anti-bacterial [13]. Recent studies have identified AIF as a potential effective antineoplastic drug [13, 14]. However, the mechanisms by which AIF exerts anti-tumor effects remain unknown. The present study showed that miR-370 was downregulated in ESCC tissue and correlated with TNM stage. Ectopic overexpression of miR-370 in ESCC cell lines led to the suppression of proliferation and apoptosis induction by directly targeting PIM1. Furthermore, AIF could inhibit proliferation and induce apoptosis *in vitro* and *in vivo* through regulation of miR-370/PIM1 signaling.

Materials and methods

Patient information and specimen collection

Sixty-three ESCC patients from the General Surgery Department and Digestive Disease Department, Qingdao University Affiliated Hospital (Qingdao, China) and the First Affiliated Hospital of Chongqing Medical University (Chongqing, China), between January 2013 and December 2013, were included in this study. All patients who had received radiotherapy, chemotherapy, or other esophageal surgery before esophagectomy were excluded. ESCC and matched normal esophageal tissues (5 cm away from the tumor lesion according to the NCCN guideline of esophageal cancer) were collected immediately after resection, treated with RNAlater reagent (TaKaRa, Dalian, China), and stored at -80°C until use. This study was approved by the Ethics Committee of Qingdao University Affiliated Hospital and the First Affiliated Hospital of Chongqing Medical University, and a written consent form was signed by each patient.

Cell lines and cultures

Human esophageal immortalized cell line, Het-1A, was purchased from the American Type Culture Collection (Manassas, VA) and cultured in bronchial epithelial basal medium with growth supplements (Clonetics, Allendale, NJ). Human ESCC cell lines EC9706 and KYSE30 were purchased from the Chinese Academy of Medical Science (Beijing, China) and maintained in RPMI-1640 medium (Invitrogen, Carlsbad, CA) containing 10 units/mL penicillin and

streptomycin (Hyclone, USA) and 10% fetal bovine serum (FBS; Hyclone, Logan, UT). Cells were maintained in a 5% CO_2 humidified incubator at 37°C .

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using Trizol reagent (Tiangen Biotech, Beijing, China), and miRNAs were obtained using the miRcute miRNA isolation kit (DP501) (Tiangen Biotech, Beijing, China) from cultured cells or human tissues. MiR-370 expression was quantified by real-time PCR with a TaqMan probe (Applied Biosystems, Carlsbad, CA) according to the manufacturer's instructions. Briefly, cDNA was obtained by High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA), and qRT-PCR was performed using a TaqMan PCR kit and the ABI 7500 system (Thermo Fisher Scientific, Waltham, MA). The relative expression of miR-370 in cells and tissues was normalized to that of U6. For PIM1 mRNA detection, the primer was synthesized based on published sequence [15]. First-strand cDNA was reverse transcribed from 1 μg total RNA using the Super M-MLV Reverse transcriptase (BioTeke Co., Beijing, China). PCR reaction solution included SYBR GREEN mastermix (Solarbio Co., Beijing, China), forward primer, reverse primer, and 10 ng template cDNA. GAPDH was used as an internal control for normalization. PCR results were analyzed using the comparative ΔCt method (ABPrism software, Applied Biosystems, Foster City, CA).

Western blot

Proteins were isolated by lysing frozen tissues in RIPA buffer (Sigma, St. Louis, MO) and cells in lysis buffer (Beyotime, Shanghai, China) containing protease inhibitors (Sigma, St. Louis, MO). The protein concentration was measured by the BCA protein assay kit (Beyotime, Shanghai, China). Extracted proteins were separated on SDS-PAGE and electrophoretically transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA). Proteins were probed with specific antibodies using a standard protocol. Specific primary antibodies against PIM1 and β -actin were purchased from Abcam (Shanghai, China). The secondary antibodies used in this study were goat anti-rabbit IgG-HRP, goat anti-mouse IgG-HRP and donkey anti-goat IgG-HRP (Beyotime Institute

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of Biotechnology, Shanghai, China). Signals were detected using chemiluminescent substrate (KPL, Guildford, UK) and the blot intensities were quantified using BandScan software (Glyko, Novato, CA).

Construction of reporter plasmids and luciferase assay

The reporter plasmid was constructed as previously described [16]. Briefly, a fragment containing PIM1 3'UTR was amplified by PCR from human genomic DNA utilizing specific primers and then inserted into a pGL3 vector (Promega, Madison, WI) downstream the stop codon of firefly-luciferase reporter gene, thus resulting in the pGL3-3'UTR/PIM1 construct. For the luciferase assay, 293 T recipient cells were transiently co-transfected with 0.2 μg of pGL3-3'UTR/PIM1 constructs, 0.02 μg of pRL-TK-Renilla luciferase reporter plasmids (Promega, Madison, WI) containing the Renilla-luciferase for normalization, and 5 pmol of miR-370 overexpression construct or control. At 24 hours after transfection, the cells were lysed and the luciferase activity was measured with a luminometer using the dual-luciferase reporter assay system, according to the manufacturer's instructions.

MiR-370 knockdown or overexpression

The lentiviral constructs miR-370 mimic and miR-con, and miR-370 inhibitor were obtained from Qiagen (Gene Copoeia, Rockville, MD). Cells were seeded into a 96-well plate, incubated overnight, and then transfected with miR-370 mimic and miR-con, or miR-370 inhibitor according to the manufacturer's instructions. The transfection efficiency was confirmed by qPCR analysis.

PIM1 knockdown

The shRNA oligos targeting PIM1 gene were obtained from GenePharma (Shanghai, China). PIM1 targeting shRNA sequence and one scramble sequence as the control were inserted into the plasmid vector pGCsi-H1. The resulting plasmids were named pGCsi-H1-PIM1 and -control, respectively. For transfection, cells in logarithmic growth phase were seeded in a 6-well plate and transfected with the constructed plasmids as per the manufacturer's protocol (Invitrogen, Carlsbad, CA). Transfected cells were incubated for another 48 hours and the knockdown was verified by western blot.

PIM1 overexpression

PIM1 was overexpressed by transfection with an expression construct using Lipofectamine 3000 reagent (Invitrogen, Grand Island, NY), according to the manufacturer's instructions. PIM1 overexpressing vector was constructed as previously described using a plasmid vector pGCsi-H1 [17]. Cells transfected with an empty vector were used as controls. At 48 hours after transfection, the cells were rinsed, resuspended in fresh culture media and the overexpression was verified by western blot.

Cell proliferation assay

Cell proliferation was determined by MTT assay (Sigma, MO, USA). Briefly, 5×10^3 cells/well were plated in 96-well plates. After treatment, 20 μl of MTT solution (5 mg/ml in PBS) was added to each well and incubated for 2 hours. MTT formazan was dissolved in 150 μl of isopropanol and the absorbance was measured at 595 nm with an ELISA reader (Tecan Group Ltd, Männedorf, Switzerland).

Colony formation

Cells suspended in RPMI-1640 agarose medium were seeded in each well of a 6-well plate over a layer of solidified RPMI-1640 agarose medium. Cultures were maintained for 14 days without fresh medium feeding at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Then, colonies with over 50 cells were enumerated, stained with crystal violet and photographed using a digital camera (Nikon DXM-1200, Tokyo, Japan).

Apoptosis analysis

Apoptosis was evaluated by flow cytometry using a FITC Annexin V apoptosis kit (BD Pharmingen, Franklin Lakes, NJ) according to the manufacturer's instructions. Briefly, 1×10^6 cells/ml were stained with annexin V-FITC and propidium (PI) for 15 minutes in the dark before analysis with a flow cytometer (Beckman Coulter Inc., Miami, FL).

Xenograft models and immunohistochemistry analysis

The animal study was performed according to the regulations of the State Food and Drug Administration (SFDA) of China on Animal Care, and the study protocol was approved by the Medical Ethics Committee of our hospital. Female 5-week-old BALB/c nude mice were

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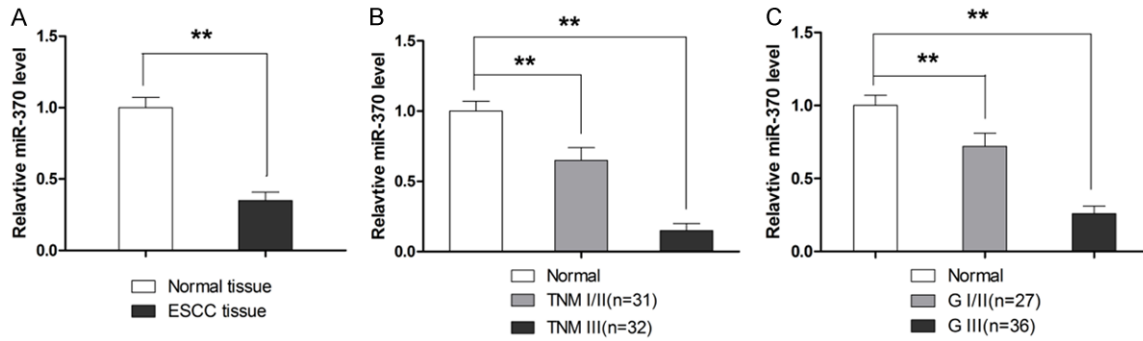


Figure 1. MiR-370 level in ESCC tissues. A. MiR-370 expression is lower in ESCC tissue as compared to adjacent normal tissue. B. MiR-370 expression is associated with TNM stage. C. MiR-370 expression is associated with pathological stage. ** $P < 0.01$.

Table 1. Correlation between miR-370 expression and clinicopathological features of ESCC patients

Characteristics	MiR-370 expression		P value
	High	Low	
Age (years)			
Over 60	21	17	0.5723
Less than 60	12	13	
Gender			
Male	32	20	0.3249
Female	5	6	
Tumor localization			
Upper third	3	5	0.7351
Middle third	13	16	
Lower third	9	17	
Tumor length (cm)			
<3	11	12	0.1008
3-5	12	10	
>5	4	14	
Pathological			
GI	3	9	0.0017
GII	12	10	
GIII	24	5	
TNM			
I	6	12	0.0213
II	10	11	
III	18	6	

bought from the Experimental Animal Center of Shanghai Laboratory Animal Center, Chinese Academy of Sciences (Shanghai, China). Approximately 1×10^6 KYSE30 cells were suspended in 100 μ l PBS and subcutaneously inoculated into the right side of the posterior flank of mice. Tumor diameters were measured every other day. The mice were sacrificed at 30 days after inoculation, and solid tumors were re-

moved and measured. Tumor volume was calculated using the equation: $V = A \times B^2 / 2$ (mm^3), where A is the largest diameter and B is the perpendicular diameter. The primary tumors were excised and analyzed by immunohistochemistry and TUNEL staining. Briefly, apoptotic cells in tumor tissue were measured by the TUNEL apoptosis detection kit (Beyotime, Shanghai, China) as per the manufacturer's protocol. Three equal-sized fields were randomly chosen and the mean number of green fluorescence-positive cells was counted. Immunohistochemistry was performed using mouse anti-human PIM1 and cleaved caspase-3 for detecting tumor cell proliferation and apoptosis in the paraffin sections, respectively.

Statistical analysis

The data are presented as mean \pm SD (Standard Deviation) and represent the results of three separate experiments, each conducted in quadruplicate unless otherwise stated. All statistical analysis was performed using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA). Values are presented as the mean \pm SD. Comparisons were performed by one-way ANOVA followed by Dunnett's t-test. Clinical characteristics were compared with the Chi-square test. Any difference with a P value < 0.05 was defined as statistically significant.

Results

MiR-370 is downregulated in ESCC tissue and correlates with clinicopathological stage of ESCC

To examine the role of miR-370 in the development of ESCC, qRT-PCR analysis was used to determine the expression of miR-370 in ESCC

tissues. As shown in **Figure 1A**, miR-370 was significantly downregulated in ESCC tissue samples as compared to the matched distant normal tissues. To further investigate the clinicopathological significance of miR-370 expression in ESCC patients, the mean fold decrease of miR-370 expression in ESCC samples was taken as the cut-off point of low and high expression of miR-370. MiR-370 level was significantly negatively associated with ESCC TNM stage and pathologic grade (**Figure 1B** and **1C**), but not with other clinicopathological data, such as age, gender, tumor location, and tumor length (**Table 1**).

MiR-370 functions as a tumor suppressor in ESCC cells

The role of miR-370 in ESCC was studied in two ESCC cell lines EC9706 and KYSE30. As shown in **Figure 2A**, the expression of miR-370 in both cell lines was significantly lower as compared to human esophageal immortalized Het-1A cells. Then miR-370 mimic and inhibitor as well as negative control (NC) were transfected into ESCC cells to investigate the effect of miR-370 on cell viability. As shown in **Figure 2B**, miR-370 mimic significantly increased the expression of miR-370, while miR-370 inhibitor markedly decreased the level of miR-370. MTT assay showed that overexpression of miR-370 significantly inhibited the proliferation of ESCC cells as compared to the NC cells, while miR-370 inhibitor enhanced the proliferation of both tested cell lines (**Figure 2C**). The anti-proliferative effect of miR-370 was also confirmed by colony formation assay, as shown in **Figure 2D**. Next, we examined whether miR-370 induces apoptosis in ESCC cells. As shown in **Figure 2E**, miR-370 overexpression significantly increased the number of apoptotic cells, while miR-370 inhibitor resulted in decreased apoptotic cell population. The apoptosis-inducing role of miR-370 was also confirmed since its overexpression significantly elevated the levels of activated caspase-3, the key marker for apoptosis (**Figure 2F**).

PIM1 is directly targeted by miR-370

MiRNAs are known to influence gene expression through post-transcriptional mechanisms. Therefore, two computational algorithms, TargetScan [18] and microRNA.org [19], were used in combination to search for potential tar-

gets that mediate the anti-tumor effect of miR-370 in ESCC. Both algorithms predicted an interaction between miR-370 and the target sites in PIM1 3'-UTR, as shown in **Figure 3A**. The association between miR-370 expression and mRNA and protein levels of PIM1 was further examined in the tissues of 20 randomly chosen patients from our hospital. According to Pearson's correlation scatter plots analysis, an inverse correlation between the protein or mRNA levels of PIM1 and miR-370 was observed in ESCC tissues, which indicated that miR-370 might affect the stability of PIM1 mRNA in addition to regulating its expression post-transcription (**Figure 3B** and **3C**). To further examine the regulating mechanism of miR-370 on PIM1, the mRNA and protein expressions of PIM1 were measured in cells transfected with miR-370 mimic and miR-370 inhibitor. As shown **Figure 3D** and **3E**, overexpression in ESCC cells resulted in significant decrease of both mRNA and protein expression of PIM1. In contrast, knockdown of miR-370 was associated with significantly higher expression of PIM1 mRNA and protein, suggesting that miR-370 might degrade PIM1 mRNA as well as suppress protein translation. Next, luciferase assay was used to determine whether miR-370 regulates the expression of PIM1 by directly targeting the 3'-UTR of PIM1. As shown in **Figure 3F**, miR-370 mimic significantly decreased the luciferase reporter activity, while miR-370 inhibitor significantly increased the reporter activity as compared to controls, indicating that PIM1 was directly targeted by miR-370. Taken together, our findings showed that miR-370 regulated the expression of PIM1 at both the transcript and protein levels.

MiR-370 functions as a tumor suppressor by targeting PIM1 in ESCC cells

To verify the role of PIM1 in the anti-tumor effect of miR-370, its expression was manipulated in EC9706 and KYSE30 cells using shRNA or overexpression plasmid. As shown in **Figures 4A** and **5A**, the expression of PIM1 was efficiently suppressed by shRNA, while the level of PIM1 protein was markedly increased in cells transfected with the overexpression plasmid. Next, the effect of PIM1 knockdown on cell proliferation and apoptosis was examined in cells transfected with miR-370 inhibitor. As shown in **Figure 4B-E**, the promoting effect of miR-370 inhibitor on cell proliferation as well as the sup-

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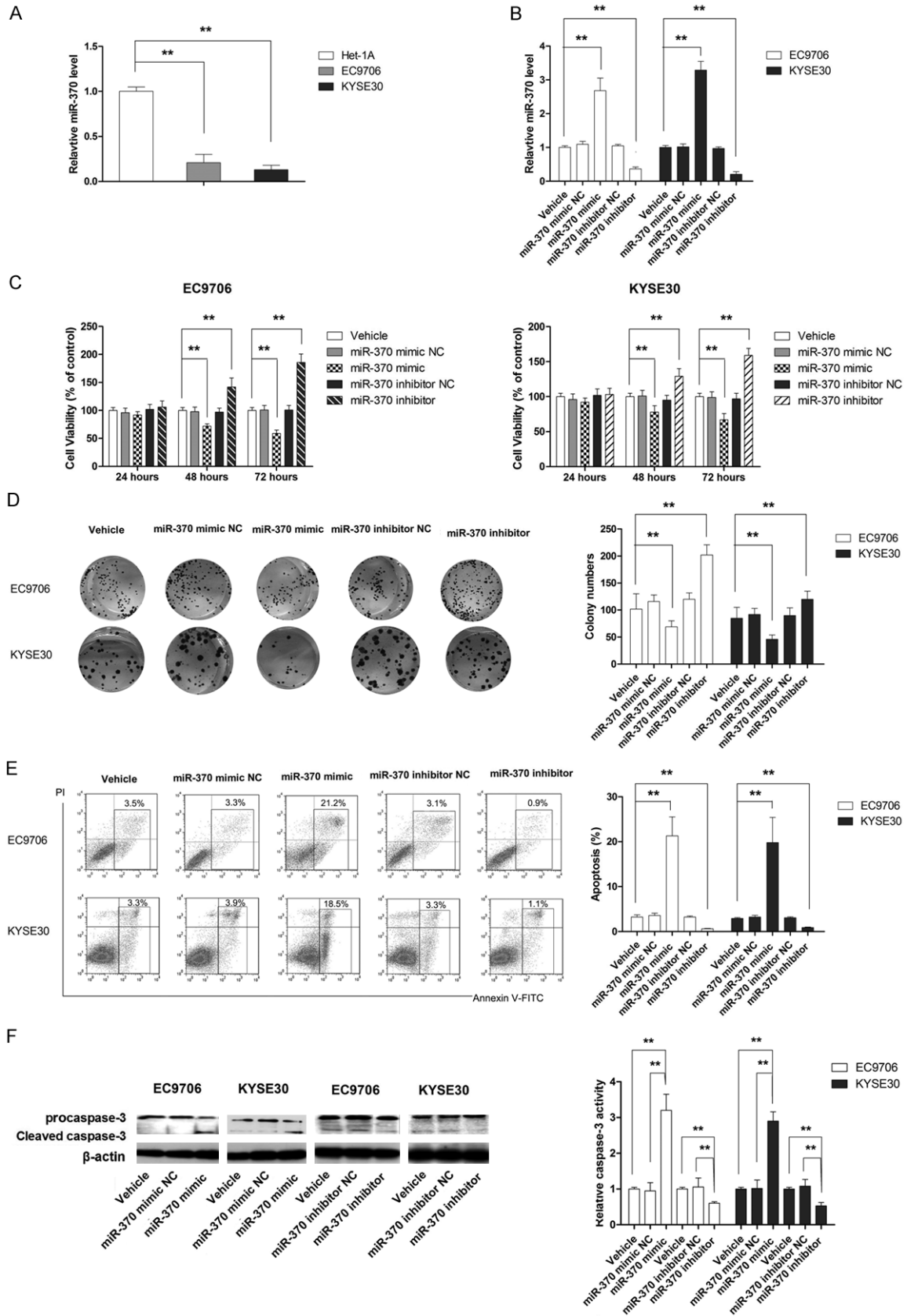


Figure 2. MiR-370 functions as a tumor suppressor in ESCC cells. A. MiR-370 is significantly downregulated in ESCC cells. B. The level of miR-370 is manipulated using miR-370 mimic and miR-370 inhibitor. C. MiR-370 mimic and

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inhibitor affected the viability of ESCC cells. D. Mi-370 mimic suppresses cell proliferation, while miR-370 inhibitor promotes cell growth. E. MiR-370 mimic induces apoptosis, while miR-370 inhibitor suppresses apoptosis in ESCC cells. F. MiR-370 mimic activates caspase-3, while miR-370 inhibitor suppresses caspase-3 activation in ESCC cells. **P<0.01.

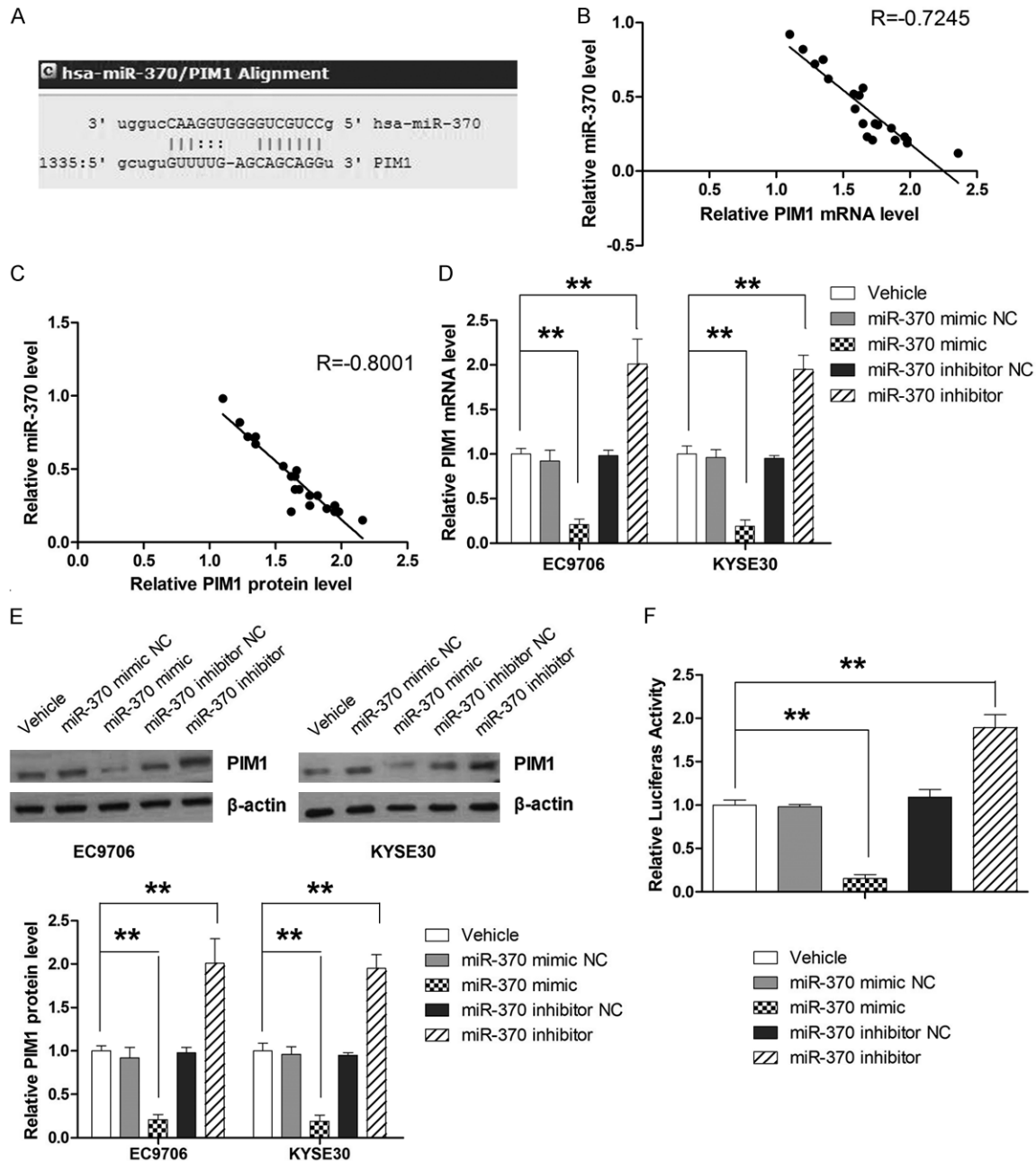


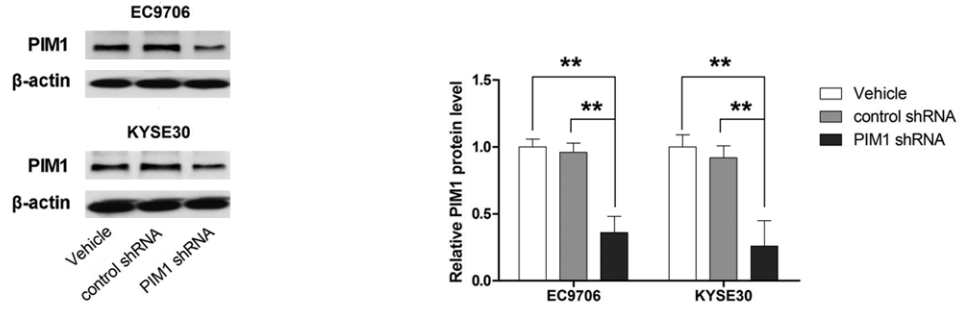
Figure 3. MiR-370 directly targets the PIM1 gene. A. Schematic illustration of the hypothesized duplexes formed from interactions between the PIM1 3'-UTR binding sites and miR-370. B. Inverse correlation between miR-370 and PIM1 mRNA level in ESCC tissues. C. Inverse correlation between miR-370 and PIM1 protein level in ESCC tissues. D. Quantitative analysis of PIM1 mRNA levels after transfection with miR-370 mimic and miR-370 inhibitor. E. Quantitative analysis of PIM1 protein levels after transfection with miR-370 mimic and miR-370 inhibitor. F. Luciferase activity is increased by miR-370 inhibitor and suppressed by miR-370 mimic. **P<0.01.

pressing effect of miR-370 inhibitor on apoptosis was almost completely abrogated by PIM1

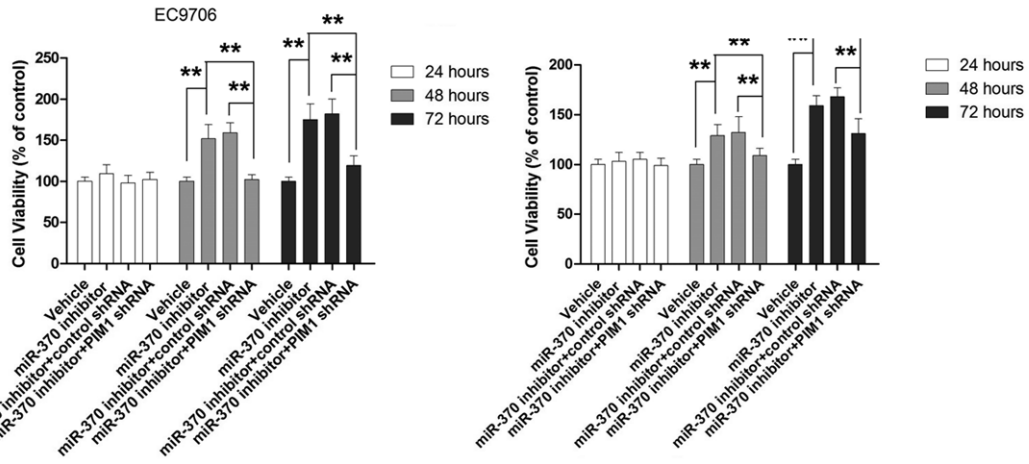
shRNA. Moreover, ectopic overexpression of PIM1 significantly attenuated the suppression

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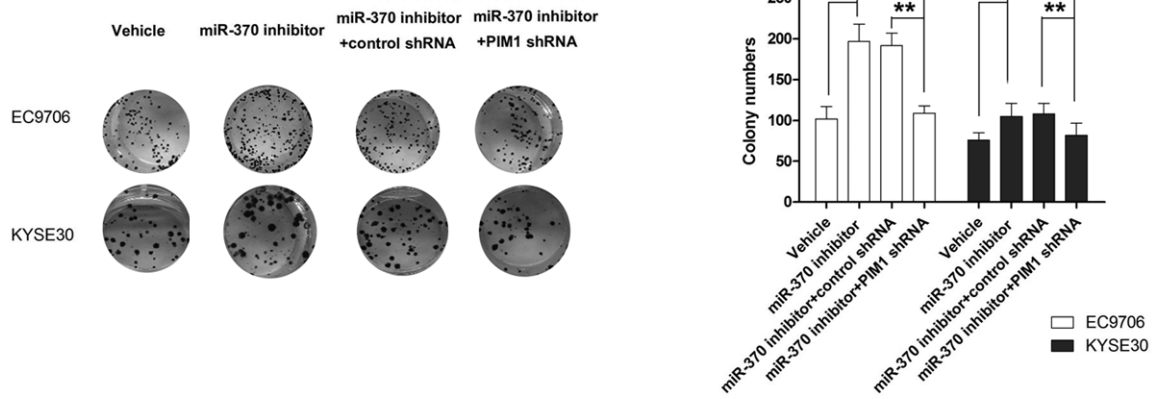
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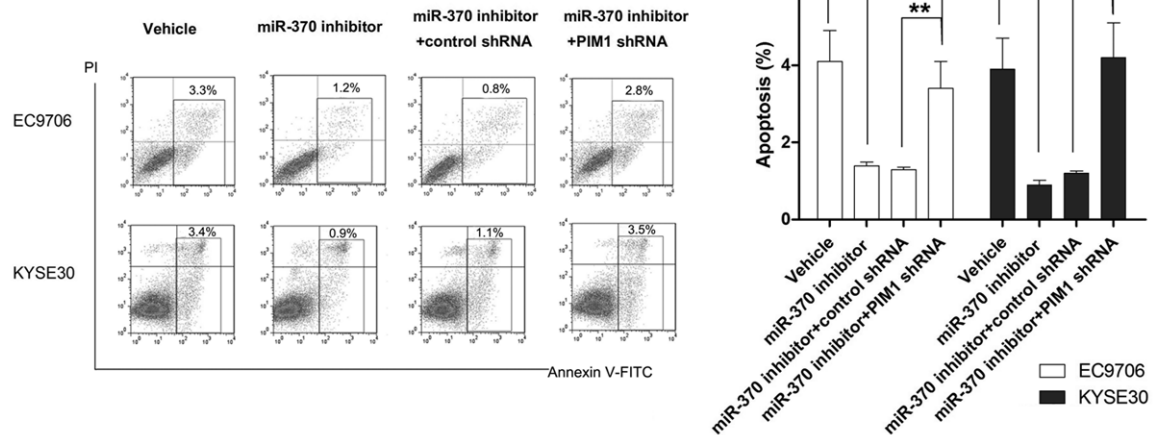
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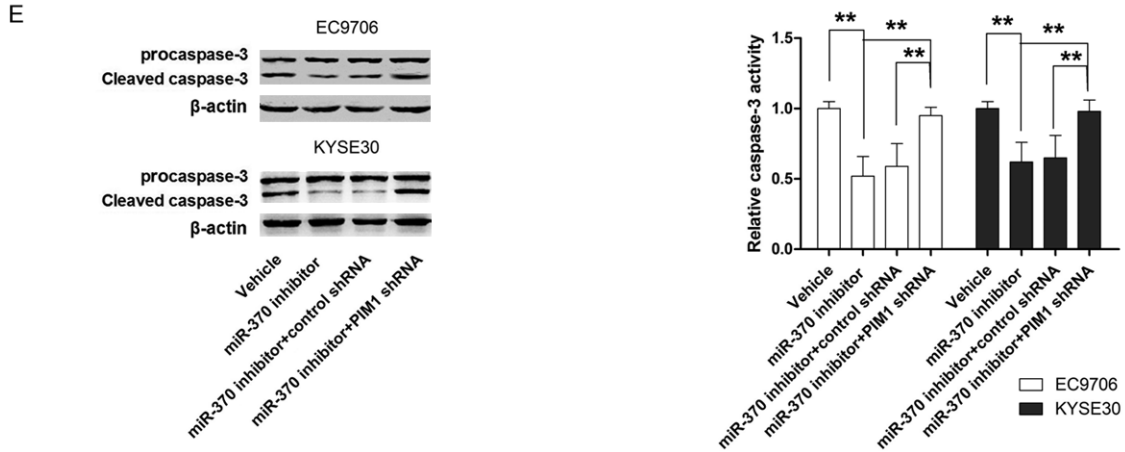


Figure 4. PIM1 shRNA impairs the pro-growth effect of miR-370 inhibitor in ESCC cells. A. PIM1 expression is suppressed by targeted shRNA. B. Knockdown of PIM1 in cells transfected with miR-370 inhibitor results in loss of cell viability. C. Knockdown of PIM1 in cells transfected with miR-370 inhibitor leads to growth inhibition. D. Knockdown of PIM1 in cells transfected with miR-370 inhibitor results in increased apoptosis. E. Knockdown of PIM1 in cells transfected with miR-370 inhibitor results in caspase-3 activation. ** $P < 0.01$.

of proliferation and apoptosis induced by miR-370 mimic (**Figure 5B-E**). Our results provided strong evidence that miR-370 functioned as a tumor suppressor by targeting PIM1 in ESCC.

AIF induces apoptosis in EC9706 and KYSE30 cells

As shown in **Figure 6A** and **6B**, AIF treatment led to a dose-dependent loss of viability in EC9706 and KYSE30 cells, although the two cell lines displayed different sensitivities to AIF. Additionally, AIF at 10 and 20 μM significantly induced apoptosis in both cell lines (**Figure 6C** and **6D**).

AIF induces apoptosis in ESCC cells by modulating miR-370/PIM1 signaling

To further investigate the mechanism by which AIF exerted its anti-tumor effect, we examined its effect on the expression of miR-370 and PIM1. As shown in **Figure 7A-C**, AIF dose-dependently increased the expression of miR-370 and repressed the mRNA and protein expression of PIM1. Next, we examined whether AIF exerted its apoptosis-inducing effect by targeting miR-370/PIM1 signaling. Our results showed that miR-370 inhibitor or PIM1 overexpression reversed the loss of cell viability caused by AIF (**Figure 7D** and **7E**). Correspondingly, miR-370 inhibitor or PIM1 overexpression significantly rescued apoptosis and caspase-3 activation induced by AIF in both

EC9706 and KYSE30 cells (**Figure 7F** and **7G**). Taken together, our findings suggested that AIF exerted apoptosis-inducing effect by targeting miR-370/PIM1 signaling.

AIF exerts anti-tumor effect in vivo

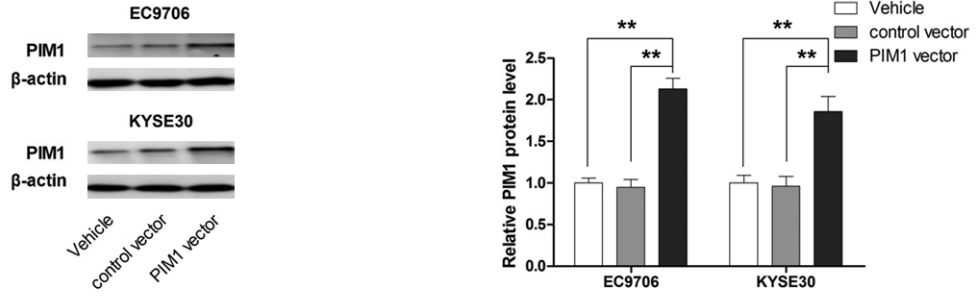
The anti-tumor effect of AIF was evaluated in the xenograft model of ESCC. As shown **Figure 8A** and **8B**, AIF significantly suppressed tumor growth in a dose-dependent manner without affecting the body weight of the animal model. Similar to the observation in tumor growth, TUNEL assay and immunohistochemistry assay of cleaved caspase-3 showed that AIF treatment resulted in apoptosis *in vivo* (**Figure 8C** and **8D**). Moreover, tumor growth inhibition by AIF correlated with decreased expression of PIM1 and upregulation of miR-370 in tissues (**Figure 8E** and **8F**), suggesting that AIF inhibited tumor growth by inducing apoptosis through modulation of miR-370/PIM1 signaling.

Discussion

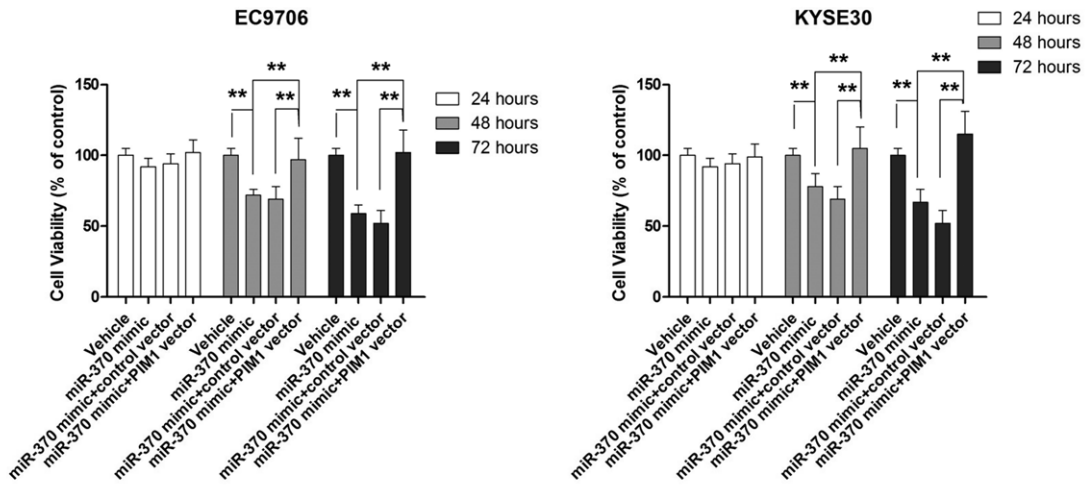
Although accumulating evidence has demonstrated that miR-370 dysregulation occurs in various types of cancer, its functional role in cancer remains controversial. MiR-370 may play a key role in tumorigenesis and progression by acting as an oncogene or a tumor suppressor gene. For example, miR-370 was down-regulated and functioned as a tumor suppressor gene in laryngeal squamous cell carcinoma

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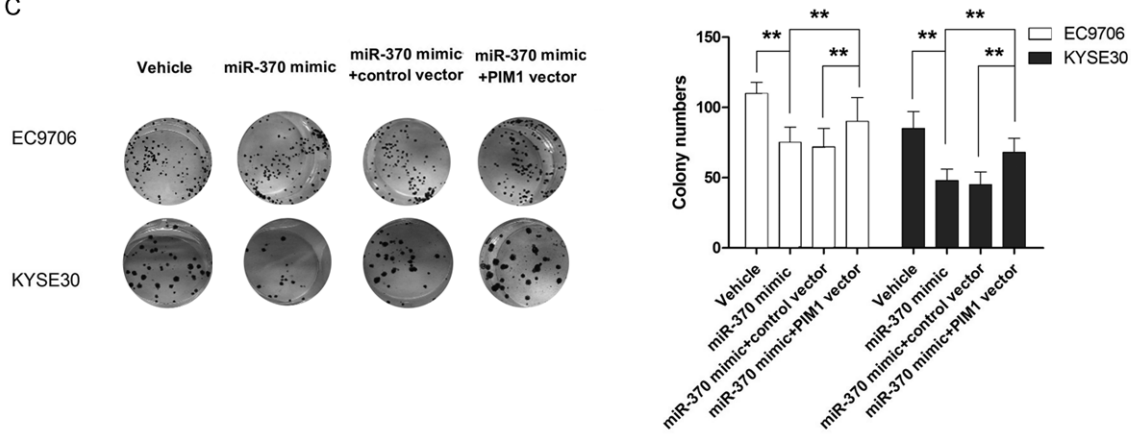
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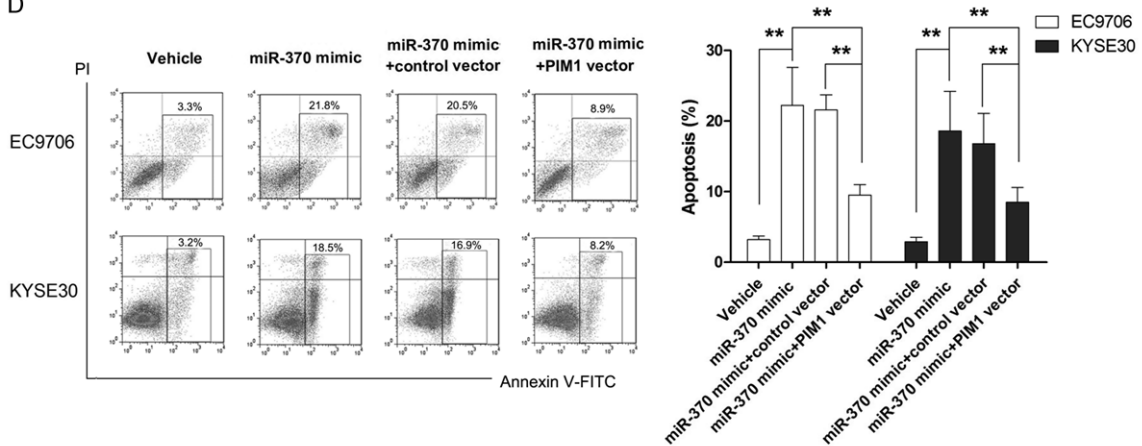
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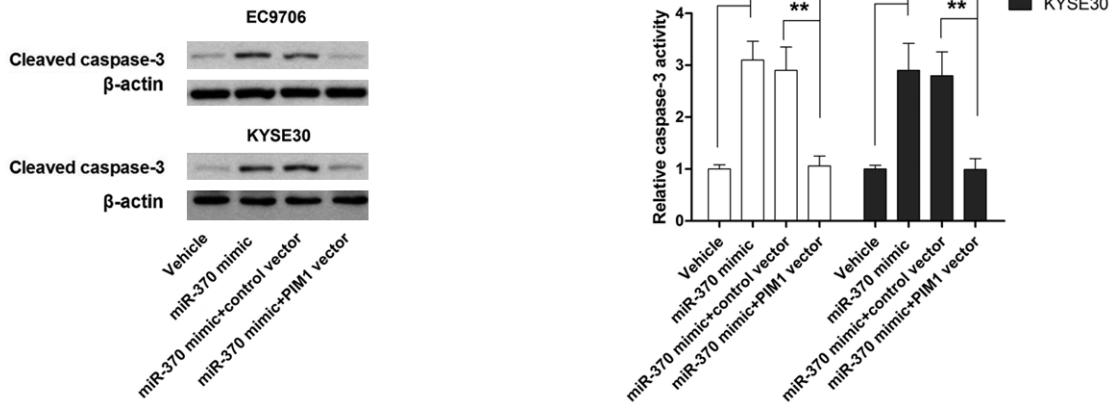
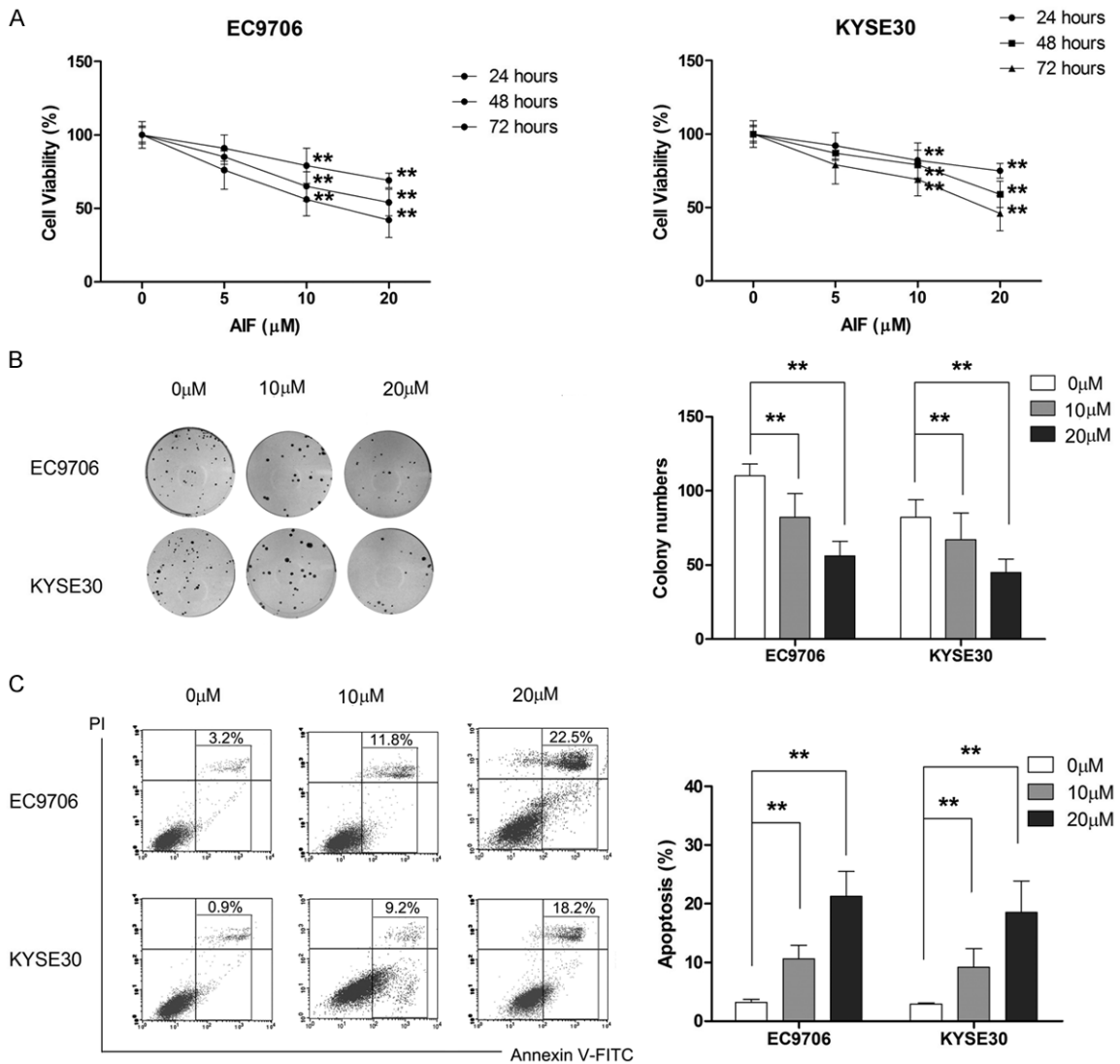


Figure 5. PIM1 overexpression compromises the anti-tumor effect of miR-370 mimic in ESCC cells. A. PIM1 expression is increased by PIM1 vector. B. Overexpression of PIM1 in cells transfected with miR-370 mimic leads to impaired anti-proliferative effect. C. Overexpression of PIM1 in cells transfected with miR-370 mimic leads to increased cell growth. D. Overexpression of PIM1 in cells transfected with miR-370 mimic leads to apoptosis inhibition. E. Overexpression of PIM1 in cells transfected with miR-370 mimic correlates with decreased caspase-3 activity. ** $P < 0.01$.



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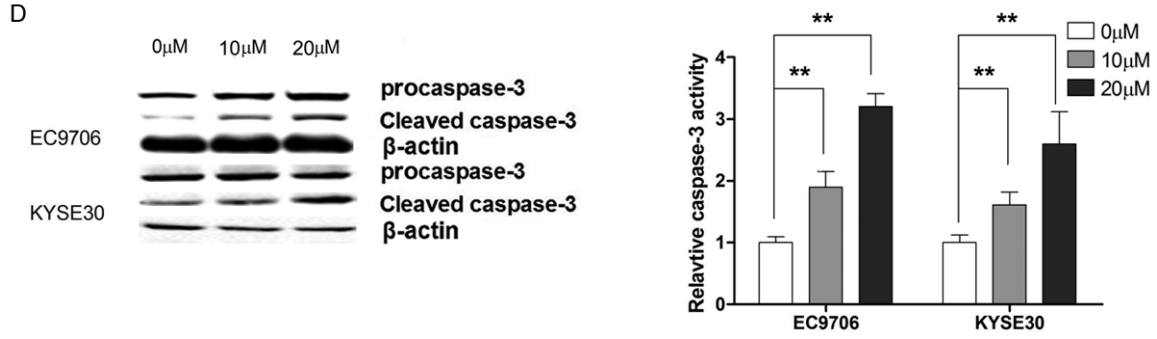
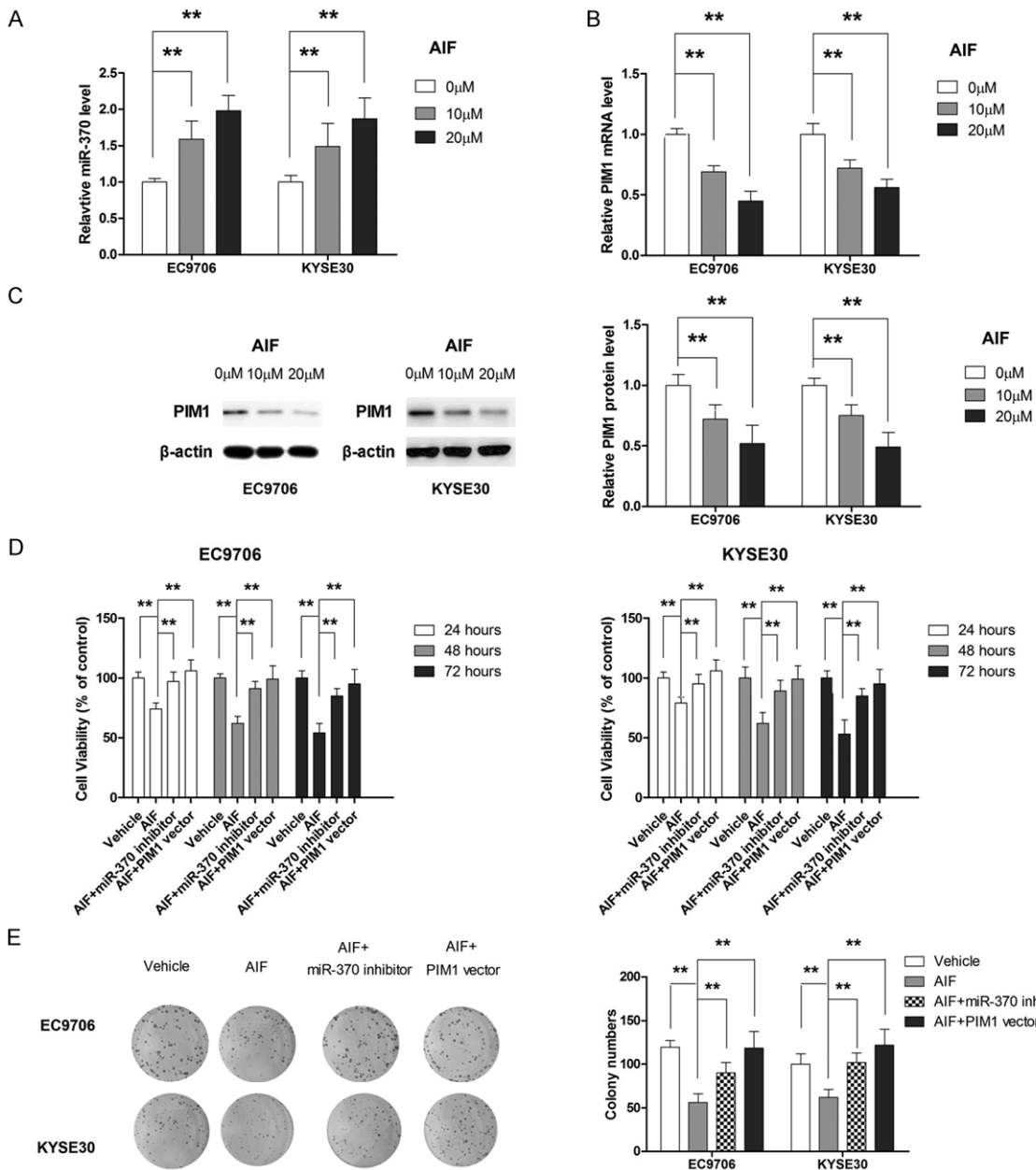


Figure 6. AIF exerts anti-tumor effect in ESCC cells. A. AIF dose-dependently inhibits the proliferation of ESCC cells. B. AIF suppresses cell growth in a dose-dependent manner. C. AIF induces apoptosis in ESCC cells. D. AIF activates caspase-3 in a dose-dependent manner. **P<0.01.



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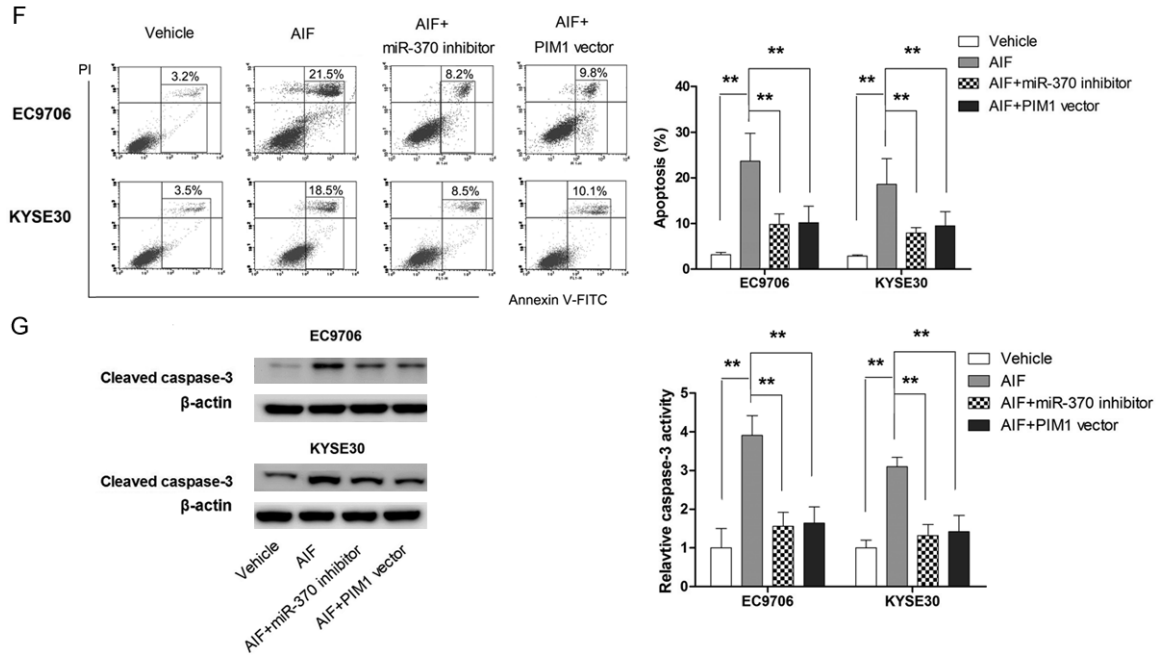


Figure 7. AIF upregulates miR-370 and represses the expression of PIM1. A. AIF dose-dependently increases the expression level of miR-370. B. AIF dose-dependently suppresses the mRNA level of PIM1. C. AIF dose-dependently represses the protein level of PIM1. D. MiR-370 inhibitor or PIM1 overexpression attenuates the anti-proliferative effect of AIF. E. MiR-370 inhibitor or PIM1 overexpression significantly abrogates the anti-growth effect of AIF. F. MiR-370 inhibitor or PIM1 overexpression compromises the apoptosis-inducing effect of AIF. G. MiR-370 inhibitor or PIM1 overexpression impairs the suppressing effect of AIF on invasion. ** $P < 0.01$.

ma, *Helicobacter pylori*-induced gastric carcinoma, and acute myeloid leukemia [6, 20, 21]. In cholangiocarcinoma, ectopic overexpression of miR-370 was reported to exert an anti-proliferative effect [22]. In hepatocellular carcinoma cells, miR-370 suppressed metastasis by inhibiting migration and invasion [23]. Moreover, restoration of miR-370 was found to resensitize endometrioid ovarian cancer cells to cisplatin [5]. In contrast, numerous studies have demonstrated that miR-370 acts as an oncogenic miRNA. Cao et al. have found that miR-370 overexpression led to enhanced proliferation rate, upregulation of cyclin-dependent kinase inhibitors, and downregulation of cyclin D1 in the Wilms' tumor G401 cell line, suggesting a pro-proliferative role of miR-370 [24]. In human prostate and gastric cancers, miR-370 was upregulated and functioned as an oncogene by directly regulating FOXO1 [25, 26]. A recent study by Sim et al. also identified miR-370 as an oncogenic miRNA in breast cancer [27]. These studies suggested that miR-370 can function as an oncogene or tumor suppressor depending on its key target genes, which may be cell specific. To our knowledge, the clinical

significance of miR-370 and its functional role in ESCC have not been investigated. In the present study, the expression of miR-370 was first examined in ESCC tissues and compared with matched distant normal tissues. Our findings revealed that miR-370 was significantly downregulated in ESCC tissues. Next, we established the correlation between the expression pattern of miR-370 and clinicopathological features in ESCC patients, and found that miR-370 was negatively associated with aggressive ESCC. Moreover, ectopic overexpression of miR-370 inhibited proliferation and induced apoptosis, while miR-370 inhibitor promoted cell proliferation and suppressed apoptosis in ESCC cells *in vitro*. Collectively, our results suggested that miR-370 functioned as a tumor suppressor in ESCC.

Given its regulatory role in various biological activities of cancer cells, PIM1 has been considered a promising target for anticancer drug discovery [9]. Overexpression of PIM1 protein has been implicated in the tumorigenesis of ESCC [10]. Moreover, PIM1 expression was associated with lymphatic metastasis of ESCC,

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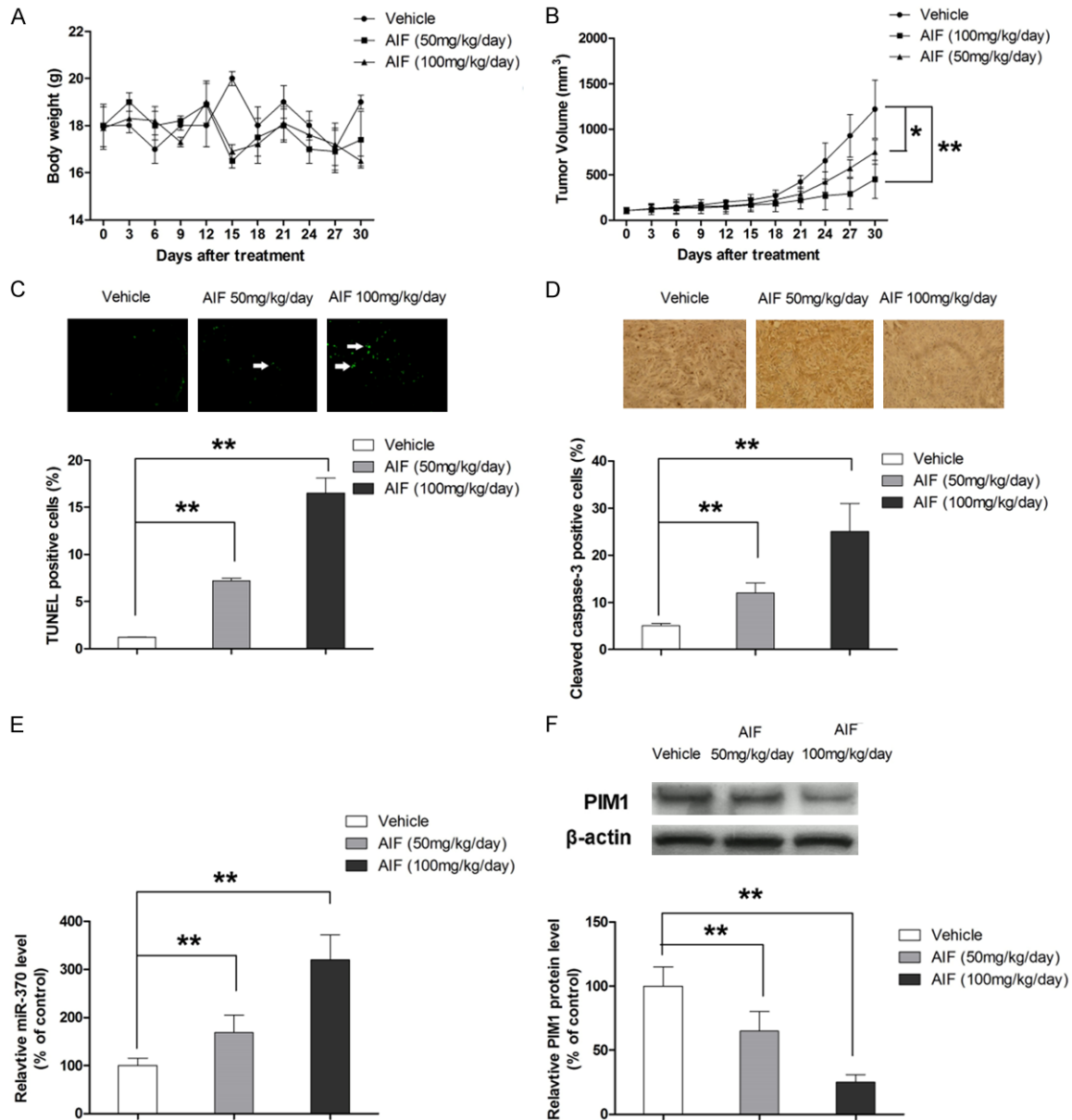


Figure 8. AIF exerts anti-tumor effect in xenograft model of ESCC. A. AIF treatment does not change the body weight of the animal model. B. AIF suppresses tumor growth in a dose-dependent manner. C. AIF induces apoptosis *in vivo*. D. AIF activates caspase-3 *in vivo*. E. AIF elevates the expression of miR-370 in tumor tissue. F. AIF suppresses the expression of PIM1 in tumor tissue. * $P < 0.05$, ** $P < 0.01$.

highlighting its potential as a therapeutic target for ESCC. The present study showed that silencing PIM1 expression using shRNA inhibited cell proliferation and induced apoptosis in ESCC cells, whereas overexpressing PIM1 had opposite effects, thus validating the role of PIM1 as an oncogene in ESCC. In addition to proliferation and apoptosis, PIM1 is also involved in other physiological behaviors of cancer cells. In hepatocellular carcinoma, PIM1 was involved in cell metabolism and promoted tumor progression

by enhancing glycolysis [17]. In nasopharyngeal carcinoma, breast cancer and melanoma, PIM1 has been found to promote cell migration and invasion, highlighting its role in metastasis [15, 28, 29]. Therefore, by targeting PIM1, miR-370 might also play a key role in cell metabolism and metastasis, although additional data is needed.

Flavonoids, a group of important naturally occurring compounds found in several edible fruits,

vegetables and medicinal plants, have been reported to protect against cardiovascular diseases, suppress cancer progression, lower the risk of diabetes, improve cognitive functions following stroke and help in neurodegenerative disorders [30, 31]. Interestingly, numerous flavonoids exert anti-cancer effects through modulating miRNAs. For example, quercetin, a flavonoid enriched in onions, apples, tea, and red wine, has been reported to suppress proliferation and invasive behavior by upregulating miR-146a in human breast cancer cells [32]. A later study in pancreatic ductal adenocarcinoma cells (MIA PaCa-2, Capan-1, and S2-013) has also shown that quercetin upregulated miR-142-3p and thus inhibited cell proliferation by reducing the expression of heat-shock protein 70 (HSP70), which is a direct target of miR-142-3p [33]. Another important flavonoid compound, genistein, modulated the expression of miR-23b, miR-27a and miR-223 in various cancerous cells [34-36]. This study showed that AIF inhibited proliferation and induced apoptosis in ESCC through upregulating tumor suppressor miR-370 and subsequently repressing the expression of PIM1. To our knowledge, this is the first experimental evidence showing that AIF could exert anti-tumor effect through modulating miRNA. Moreover, our findings highlight that miRNA signaling pathway might play a key role in regulating response of cancer cells to flavonoids.

In summary, the current study provides novel evidence that miR-370 functions as a tumor suppressor in ESCC and correlates with cancer development of ESCC. This is the first report that miR-370 regulates cell proliferation and apoptosis through directly targeting PIM1. Moreover, our results showed that AIF exerts anti-tumor effect on ESCC by inhibiting cell proliferation and inducing apoptosis via up-regulation of miR-370 and suppression of PIM1 signaling.

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Disclosure of conflict of interest

None.

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