# Original Research

# *Pseudomonas aeruginosa*-mannose-sensitive hemagglutinin inhibits chemical-induced skin cancer through suppressing hedgehog signaling

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**Impact statement**

*Pseudomonas aeruginosa*-mannose-sensitive hemagglutinin (PAM) restrained the chemical-induced skin cancer cells *in vitro* and *in vivo* partly through suppressing the Hh signaling pathway, indicating that PAM may be a promising anticancer agent for treating skin cancer.

## Abstract

*Pseudomonas aeruginosa*-mannose-sensitive hemagglutinin (PAM) is an inactivate *P. aeruginosa* with mannose-sensitive hemagglutinin. Recently, the anticancer properties of PAM against many cancers have been reported across a range of studies. However, the exact mechanism through which PAM prevents skin cancer remains unclear. The aim of this study is to show to what extent PAM could inhibit the dimethylbenzanthracene (DMBA)/12-O-tetradecanoylphorbol-13-acetate (TPA)-induced skin cancer. JB6 cells were treated by TPA so as to establish an *in vitro* model. The effects of PAM on proliferation of the cells were analyzed using cell counting kit-8 assays. Effects on epithelial–mesenchymal transition (EMT) were assayed by real-time PCR and Western blotting. A DMBA/TPA-induced skin cancer mouse model was also established. The results showed that TPA promoted EMT changes through the activation of the hedgehog (Hh) pathway, which was reversed by PAM. Moreover, PAM inhibited the cancer growth and Hh pathway *in vivo*. These data indicate that PAM may serve as a potential anticancer agent for the treatment of skin cancer.

**Keywords:** Skin cancer, epithelial–mesenchymal transition, *Pseudomonas aeruginosa*-mannose-sensitive hemagglutinin, hedgehog

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## Introduction

Skin cancer is a malignant and lethal tumor increasing every year, in spite of advanced technology and therapy.[1,2] According to statistics, more than 10,000 cases of skin cancer are diagnosed each year in China which amounts to nearly 40% of all new cancers. *Pseudomonas aeruginosa* is a Gram-negative bacterium that has been combined with mannose-sensitive hemagglutination to chemically inactivate *Pseudomonas aeruginosa*-mannose-sensitive hemagglutinin (PAM). It has been proven by related experiments that it has a broad spectrum immunomodulatory effect and has an obvious killing effect on tumor cells.[3,4] Li *et al.*[5] reported that PAM showed a significant inhibition to invasion and metastasis of hepatocellular carcinoma via EGFR/Akt/I$\kappa$B$\beta$/NF-$\kappa$B pathway. Cheng *et al.*[6] reported that PAM inhibits pancreatic cancer cell proliferation and induces apoptosis via the EGFR pathway and caspase signaling. Therefore, it has been used in the adjuvant treatment of various cancers such as breast cancer.[7]

Epithelial–mesenchymal transition (EMT) is an important process in which epithelial cells are changed into mesenchymal ones which is a key step in the development of cancerous cells. This process is defined by a loss of cell polarity and adhesion, enhanced invasive and migratory properties, and is an important mode for cancer cells to respond to the tumor microenvironment. A range of intracellular signaling pathways have been found to initiate EMT. The hedgehog (Hh) signaling pathway has been



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shown to regulate EMT, so it plays an important role in the creation of many kinds of tumors.[8,9] Sonic hedgehog (SHH), which combines with the smoothened (SMO) receptor on the membrane of the target cell and activates the transcription of the Gli protein family,[10] deregulates cell cycle control. GLI family zinc finger 1 (Gli-1) is an important transcription factor of the Hh signaling pathway reported to be overexpressed in skin cancers and is related with skin cancer invasiveness.[11] An overexpression of Gli-1 has been found to be connected with poor prognosis and is common in the advanced stages of cancer.[12]

Dimethylbenzanthracene (DMBA) and 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced mouse papillomas is suitable for the whole occurrence and development of skin cancer. DMBA can cause DNA damage in normal cells because of mutation. TPA is an accelerator in the development of cancer, which can induce inflammation, oxidative imbalance, and excessive cell proliferation.[13] In this study, the protective effects of PAM against skin cancer were detected *in vivo* and *in vitro*. Furthermore, the mechanisms of PAM against skin cancer were investigated by regulating the Hh signaling pathway. Our study suggests that PAM could be a novel option for treating skin cancer by suppressing the Hh signaling pathway.

## Materials and methods

### Cell culture

The JB6 Cl 41-5a cell line (JB6, epidermis) was acquired from ATCC, USA. The cells were cultured in 75 $cm^2$ culture dishes using DMEM from Gibco (USA), which contained 10% heat-inactivated fetal bovine serum from Gibco (USA), ampicillin (100 units/mL), and streptomycin (100 μg/mL) at 37°C in a humidified atmosphere containing 5% $CO_2$.

### Cell counting kit-8 (CCK-8) assays of cell viability

The effects of TPA and/or PAM on proliferation of the cells were examined using CCK-8 assays (Dingguo, Beijing, China). Cells ($10^4$/well) were exposed to TPA at 0, 20, 60, 40, and 80 ng/mL for seven days to choose the suitable concentration to detect the protective effects of PAM. Cells ($10^4$/well) were exposed to PAM at $10^6$–$10^9$ cfu/mL for two days to detect the cell cytotoxicity. Then, PAM at 0, $10^6$, $10^7$, and $10^8$ cfu/mL were added to the cells 30 min prior to the treatment of TPA every day for seven days. After treatment, CCK-8 reagent was added and the absorbance (OD492) was measured in a plate reader.[14] PAM was supplied by Wanter Bio-Pharmaceutical Co., Ltd, Beijing, China ($2 \times 10^9$ cfu/mL).

### Real-time PCR

Total RNA was extracted from the cells using Trizol (Qiagen, China) and cDNAs were obtained by reverse transcription using SuperscriptII reverse transcriptase (Life Technologies, Grand Island, NY, USA). An Applied Biosystems 7500 Sequence Detection System (Thermo Fisher Scientific, Inc., USA) was used to perform qPCR (Thermo Fisher Scientific, Inc., USA), based on the guidelines indicated by the supplier. The thermo cycling reaction involved 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 60 s. Calculated ΔCt values normalized against a GAPDH control. Fold changes were calculated using ΔΔCt method.

### Western blot analysis

JB6 cells were obtained after different treatments and the proteins were extracted using a RIPA buffer on ice for 20 min (Dingguo, Beijing, China). The cell lysate was quantified using BCA method, and total protein (100 μg) was separated in 10% SDS-PAGE and transferred to nylon membranes (Roche, Germany). The membranes were then inhibited utilizing 5% non-fat dry milk in TBST. Subsequently, primary antibodies including rabbit-anti E-cadherin, N-cadherin, SHH, SMO, and Gli-1 were used to incubate the membranes overnight at 4°C. The membranes then combined with HRP-labeled goat anti-rabbit secondary antibodies after five times washes at room temperature for 2 h. A detailed chemiluminescence detection system was used to image the immunoreactive bands. The bands were analyzed with Image-Pro plus 5.0 software (Media Cybernetics, USA), with GAPDH utilized as an internal control.

### Immunofluorescence

After being cultured under various treatments, the JB6 cells were fixed with 4% paraformaldehyde, permeabilized in 0.5% Triton X-100, and inhibited in 1% BSA. The fixed cells were incubated overnight at 4°C using a primary antibody opposed to Gli-1 (1:100). The cells were then incubated in darkness at room temperature using a FITC-conjugated goat anti-rabbit IgG for 1 h. Nuclei were stained using 4′,6-diamidino-2-phenylindole for 5 min. A Zeiss instrument confocal microscope was used to image the cells at a magnification of 400×.

### Hh pathway inhibition and activation

Cyclopamine and purmorphamine were used to evaluate the inhibition and activation of Hh pathway. Cyclopamine was supplied by MedChemExpress USA, which is an Hh pathway antagonist with IC50 of 46 nM in the Hh cell assay. Purmorphamine was supplied by ApexBio USA, which directly activates smoothened and Hh signaling with IC50 of 1.5 μM.

### Animal models

The study was approved by the Institutional Ethic Committee of Jilin University (JLU-2016-190). The clean grade Institute of Cancer Research mice (female, 18–22 g, 6–8 weeks old) were purchased from the Experimental Animals Center of Jilin University. The mice were divided into three groups and were reared in a plastic cage at a temperature of 20 ± 2°C with 12 h of day/night cycle and free access to water and food. The experiment complied with animal welfare and ethics, and was strictly designed to reduce the pain of animals and the number of animals used. All the animals were adapted for seven

days, and then the later experiment was carried out. DMBA and TPA were prepared in the biosafety cabinet-II and the operators wore double gloves, caps, masks, and protective clothing. Shaving the hair of mice for about 2 cm × 3 cm size on the back, and then applying shaving cream evenly to the shaved back of the mice with hair removal once a week. The model group smeared DMBA (60 μg in 200 μL acetone/each) on the hairless back of mice in the first week and TPA (4 μg in 20 μL acetone/each) two times a week from the 1st to 20th week. PAM group was injected weekly ($10^6$ cfu/each). The control group received normal feeding without any modeling and drug administration. The number and size of papilloma like tumor on the back skin of mice were recorded every week.

### Immunohistochemistry

Mice skin tumors were fixed with 10% paraformaldehyde for 24 h. Tissues were embedded in paraffin, sectioned (5 μm), and stained with immunohistochemistry with the antibody proliferating cell nuclear antigen (PCNA) and Gli-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The images were photographed with different magnification for IHC staining (400×). The integral optical density values of PCNA and Gli-1 were measured by CMIAS-8 color pathological image analysis system.

### Statistics

The data generated in the present study were shown as mean ± standard deviation. A Student's *t*-test, one-way analysis of variance, and post hoc least significant difference test or Mann–Whitney U test (when applicable) were performed to analyze differences using SPSS 13.0 software (Chicago, IL, USA). A p-value of ($p < 0.05$) was considered statistically significant.

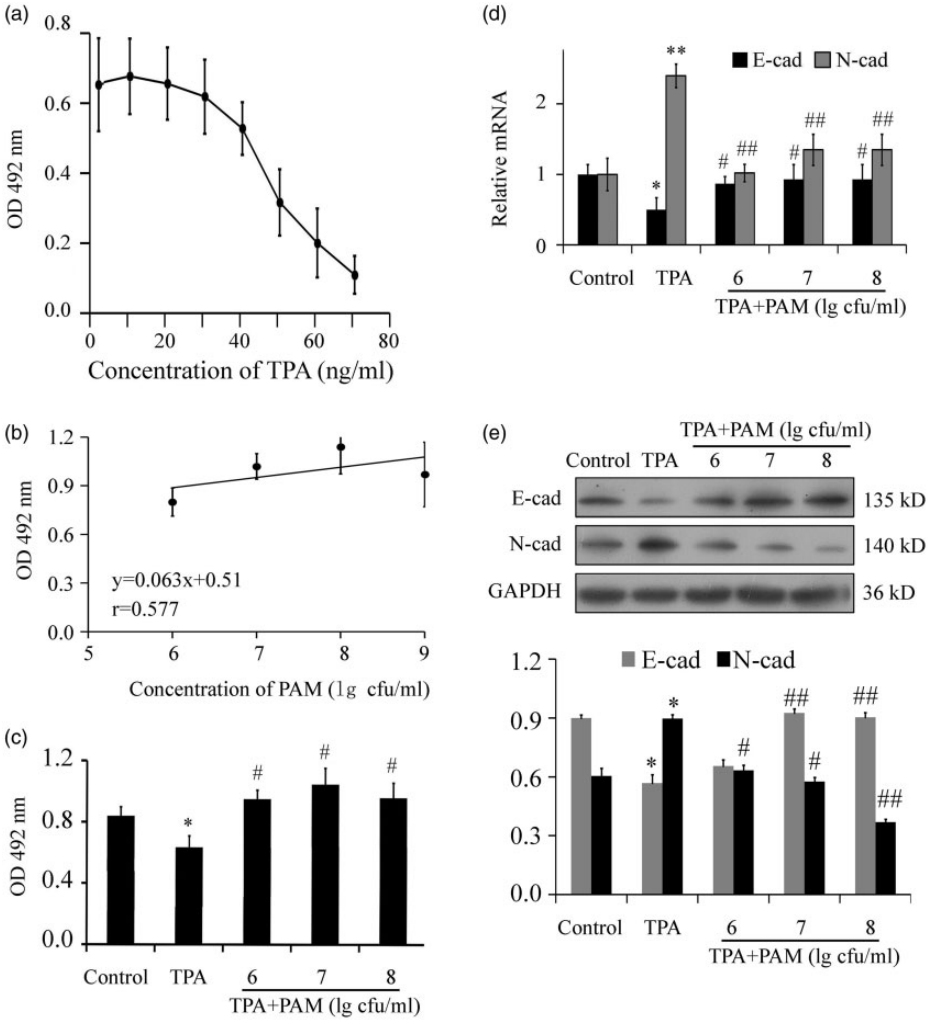


**Figure 1.** PAM promotes proliferation and inhibits EMT of JB6 cells induced by TPA (n = 5). (a) Proliferation of JB6 cells was determined by CCK-8 assay under different concentration of TPA for seven days. (b) Proliferation of JB6 cells was determined by CCK-8 assay under different concentration of PAM for two days. (c) Proliferation of JB6 cells was determined by CCK-8 assay under TPA (20 ng/mL) and different concentration of PAM for seven days. TPA inhibited the proliferation which was reversed by PAM. (d) EMT was assayed by real-time PCR under TPA (20 ng/mL) and different concentration of PAM for seven days. (e) EMT was assayed by Western blot under TPA (20 ng/mL) and different concentration of PAM for seven days. TPA promoted the EMT which was reversed by PAM. $^{*}p < 0.05$ and $^{**}p < 0.01$ versus Control and $^{\#}p < 0.05$ versus $^{\#\#}p < 0.01$ versus TPA.

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; PAM: *Pseudomonas aeruginosa*-mannose-sensitive hemagglutinin; TPA: 2-O-tetradecanoylphorbol-13-acetate.

## Results

### PAM promotes proliferation and inhibits EMT induced by TPA

We treated JB6 cells with different dosage of TPA for seven days and found that TPA inhibited the proliferation of cells in a concentration-dependent manner, indicating that the *in vitro* model was successfully established (Figure 1(a)). The concentration of TPA (20 ng/mL) was chosen because it did not affect the normal growth of JB6 cells. Then, the protective effects of PAM on proliferation and EMT were examined. The results showed that PAM promoted the proliferation of JB6 cells in a concentration-dependent manner (Figure 1(b)). TPA (20 ng/mL) inhibited the cell proliferation, which was abrogated by different concentration of PAM ($p < 0.01$, Figure 1(c)). The results of qRT-PCR and Western blot to detect E-cadherin and N-cadherin also showed that TPA (20 ng/mL) inhibited E-cadherin and

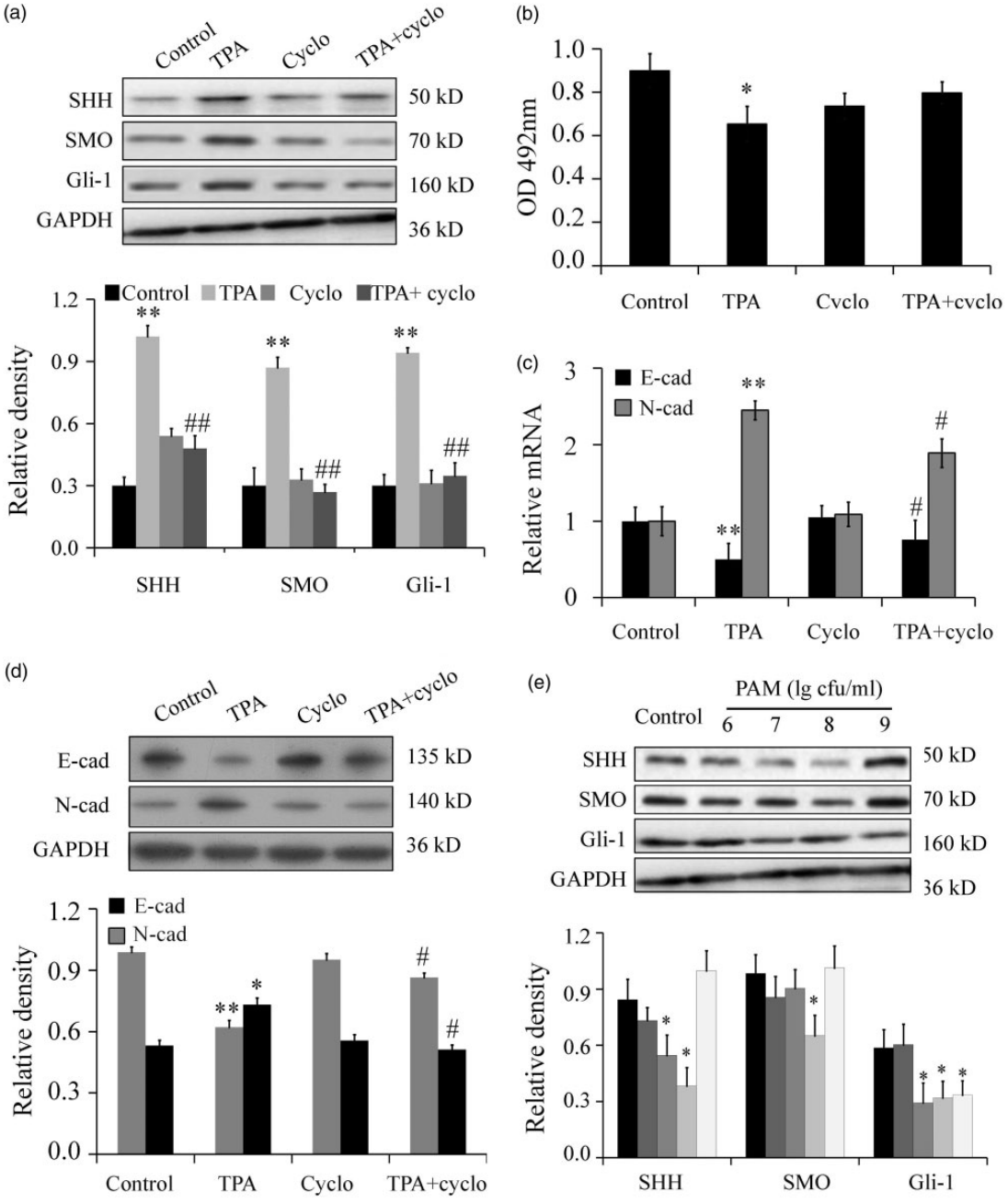


**Figure 2.** Inhibiting Hh signaling abolishes TPA-induced EMT. (a) The expression levels of SHH, SMO, and Gli-1 were increased under TPA (20 ng/mL) using Western blot analysis, which were abrogated by Hh pathway antagonist cyclo (n = 4). (b) Proliferation of JB6 cells was inhibited under TPA (20 ng/mL) using CCK-8 assay, which were abrogated by cyclo (n = 5); (c) and (d) EMT was induced under TPA (20 ng/mL) using real-time PCR assay and Western blot, which were abrogated by cyclo (n = 5). (e) The expression levels of SHH, SMO, and Gli-1 under PAM were assayed by Western blot (n = 4). $^{*}p < 0.05$ and $^{**}p < 0.01$ versus Control; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ versus TPA.

Cyclo: cyclopamine; Gli-1: GLI family zinc finger 1; PAM: *Pseudomonas aeruginosa*-mannose-sensitive hemagglutinin; SHH: sonic hedgehog; SMO: smoothened; TPA: 2-O-tetradecanoylphorbol-13-acetate.

promoted N-cadherin mRNA and protein expression, which were abrogated by different concentration of PAM ($p < 0.01$, Figure 1(d) and (e)). Hence, the results suggest that PAM promotes proliferation and inhibits EMT induced by TPA. However, there were no significant differences among different concentrations.

### Inhibiting Hh signaling abolishes TPA-induced EMT

To determine whether the effects of TPA were mediated by Hh signaling, we detected the expression levels of SHH, SMO, and Gli-1 using Western blot. The results indicated that SHH, SMO, or Gli-1 expression in the cells

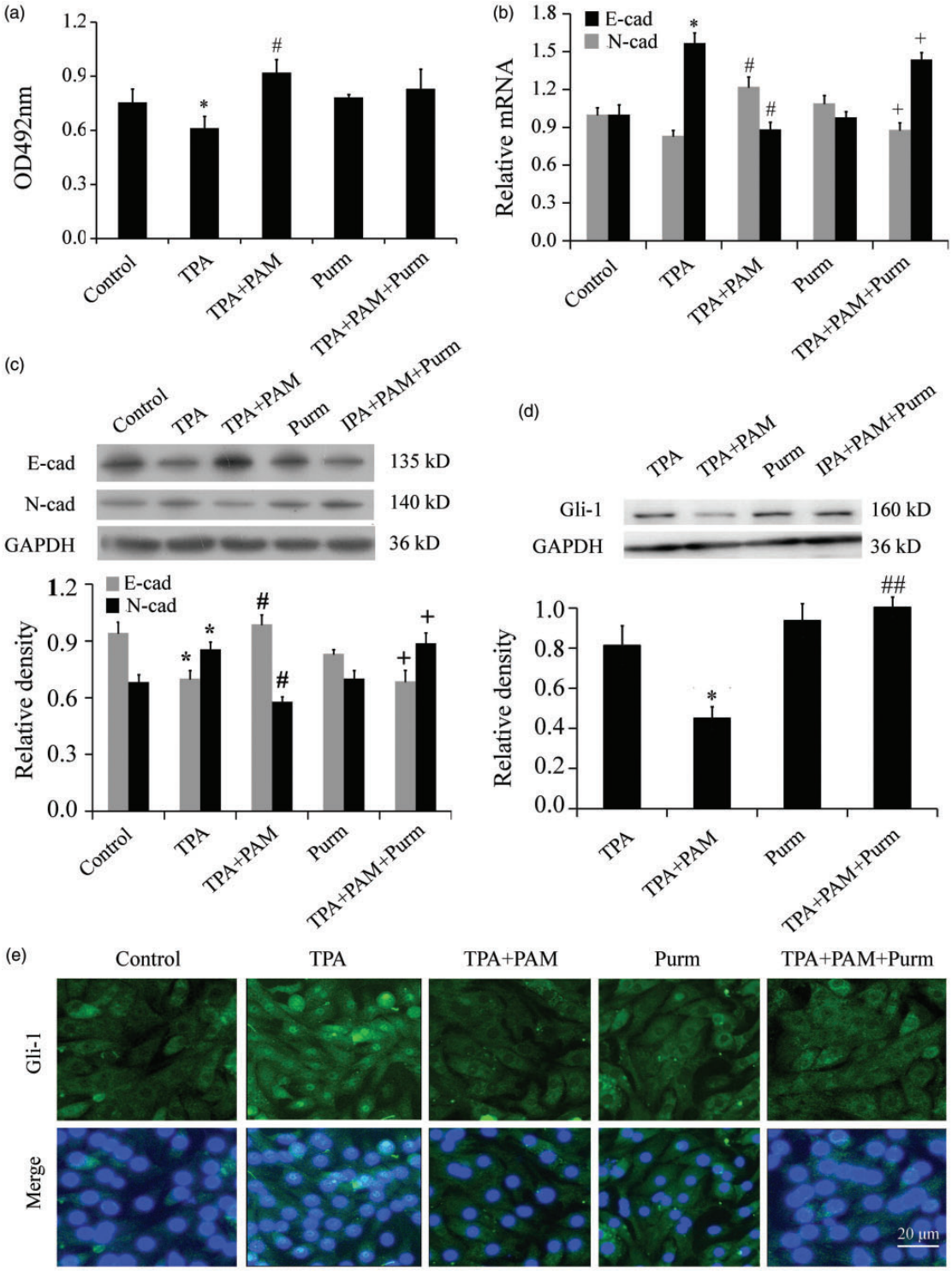


**Figure 3.** PAM suppresses TPA-induced EMT through Hh signaling. (a) Proliferation of JB6 cells was determined by CCK-8 assay (n = 5); (b) and (c) EMT was assayed by real-time PCR and Western blot (n = 5). (d) The expression levels of Gli-1 were assayed by Western blot (n = 4). (e) Translocation of Gli-1 was assayed by immunofluorescence. $^{*}p < 0.05$ versus Control; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ versus TPA. (A color version of this figure is available in the online journal.)
GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; PAM: *Pseudomonas aeruginosa*-mannose-sensitive hemagglutinin; Purm: purmorphamine; TPA: 2-O-tetradecanoylphorbol-13-acetate.

experiencing TPA (20 ng/mL) treatment was significantly greater than that in normal cells ($p < 0.01$, Figure 2(a)), which was reversed by cyclopamine (Hh pathway antagonist). However, cyclopamine had no effect on normal cells. Furthermore, in comparison to the TPA-treated cells, cyclopamine significantly inhibited the EMT induced by TPA (20 ng/mL), but had no influence on proliferation (Figure 2(b) to (d)). Therefore, inhibiting Hh signaling was found to abolish TPA-induced EMT of JB6 cells. Moreover, Western blot analysis indicated that SHH with PAM ($10^7$ and $10^8$ cfu/mL), SMO with PAM ($10^8$ cfu/mL), or Gli-1 with PAM ($10^7$–$10^9$ cfu/mL) in the cells was significantly decreased than those in the normal cells ($p < 0.05$, Figure 2(e)). However, PAM ($10^9$ cfu/mL) seemed to have no inhibitory effects on the expression of SHH and SMO.

### PAM suppresses TPA-induced EMT through Hh signaling

Next, we aimed to investigate whether PAM suppresses TPA-induced cell proliferation and EMT through Hh signaling. We found PAM ($10^8$ cfu/mL) promoted proliferation and inhibited EMT induced by TPA (20 ng/mL). The inhibitory effects on EMT were reversed by administration of 1 μM of purmorphamine (Figure 3(a) to (c)). PAM ($10^8$ cfu/mL) also inhibited the protein expression of Gli-1 and its translocation, which could be abrogated by administration of 1 μM of purmorphamine (Figure 3(d) and (e)). These data thus imply that PAM blocks the Hh pathway induced by TPA.

### PAM suppresses skin cancer partly through Hh signaling in vivo

In order to evaluate the protective effect of PAM, we selected the classic DMBA/TPA mouse skin cancer model. According to the results of pre-test, DMBA/TPA can cause papilloma in the back skin of mice. Pretreatment with PAM ($10^8$ cfu/mL) can reduce the number of tumor induced by DMBA/TPA and the epidermal thickness (Figure 4(a) and (b)). PCNA was closely related to proliferative and invasive ability of skin cancer. Its staining was mainly located in the cell nuclei. Dramatic differences were found between the model group and PAM group, indicating PAM can effectively inhibit the proliferation of skin cancer (Figure 4(c)). PAM ($10^8$ cfu/mL) also inhibited the protein expression of Gli-1 (Figure 4(c)).

## Discussion

Studies have confirmed that aberrant Hh signaling activation is found to be involved in the initiation and/or maintenance of different cancer types such as breast and lung cancer.[15,16] Additionally, Hh pathway-related molecules are found to be essential in determining the prognosis of skin cancer.[17] Consistent evidence of the poor prognostic indicator of Gli overexpression in skin cancer has been

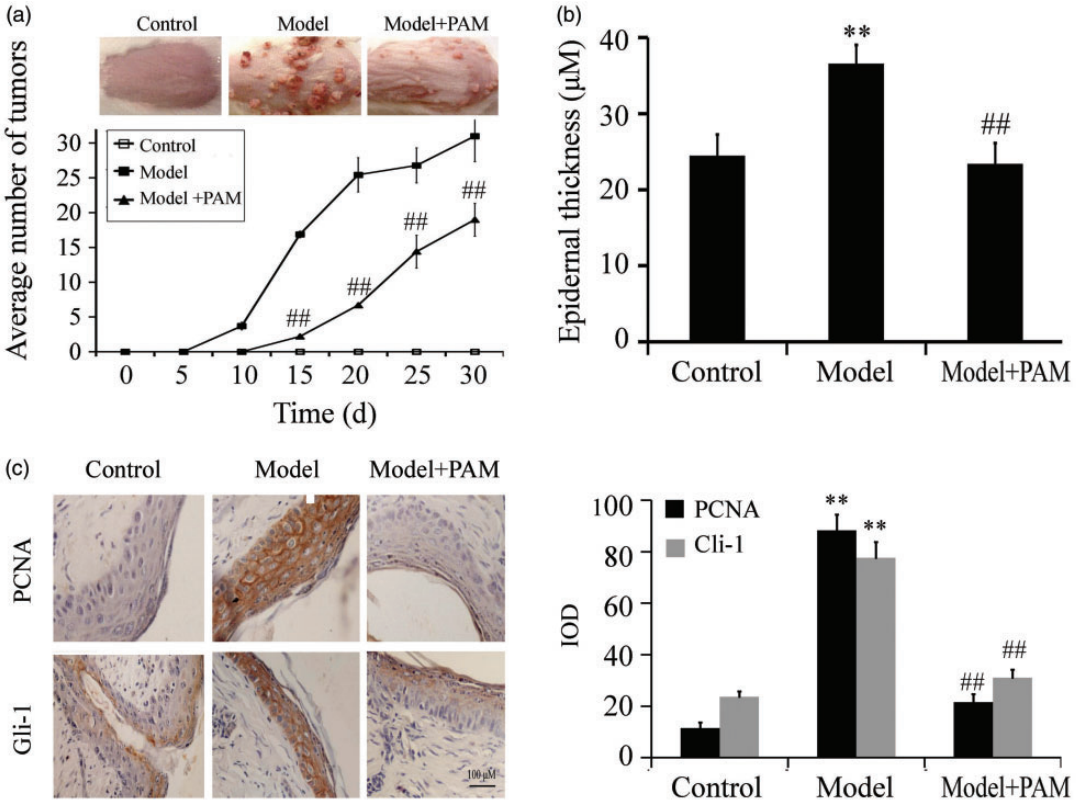


**Figure 4.** PAM suppresses skin cancer partly through Hh signaling *in vivo* (n = 6). (a) The number of tumor, (b) epidermal thickness, and (c) the expression levels of PCNA and Gli-1 were assayed by immunohistochemistry. $^{**}p < 0.01$ versus Control; and $^{\#\#}p < 0.01$ versus model. (A color version of this figure is available in the online journal.)

Gli-1: GLI family zinc finger 1; PAM: *Pseudomonas aeruginosa*-mannose-sensitive hemagglutinin; PCNA: proliferating cell nuclear antigen.

found in a wide range of research. Inhibiting the Gli with posaconazole, basal cell carcinoma proliferation and migration may be diminished significantly.[18] Gli synergizes with EGFR signaling which leads to increased transformation and tumorigenicity. We treated JB6 cells with different doses of TPA and found that TPA inhibited cell proliferation and promoted EMT. Moreover, the SHH, SMO, and Gli-1 expression in the TPA group was significantly higher than those in the normal group, which promoted the EMT of JB6 cells.

PA is a common normal flora of the human body, which is believed to recognize TLRs 2, 4, and 5 on epithelial cells through its LPS and flagellin and promote cell proliferation and inhibit EMT.[19,20] Recently PAM is known to inhibit invasion and metastasis of HCC through suppressing EMT via inhibiting EGFR/Akt/IκBβ/NF-κB pathway.[5] Meanwhile, PAM could efficiently inhibit proliferation and induce apoptosis in human bladder cancer cells *in vitro* and *in vivo*, which is associated with the inactivation of EGFR signaling pathway.[21] In the present study, we found PAM promoted the proliferation of JB6 cells in a concentration-dependent manner and the cell proliferation inhibited by TPA, indicating PAM has promoting proliferation effects, which is not associated with Hh signaling. Some reports show that PAM exhibits inhibitory effects on proliferation in some tumor cell lines.[6,21,22] On the other hand, PAM inhibits EMT induced by TPA through inhibition of Hh signaling. *In vivo*, pretreatment with PAM ($10^8$ cfu/mL) can reduce the number of tumor induced by DMBA/TPA and the epidermal thickness and inhibit the expression of Gli-1, indicating PAM inhibited EMT *in vitro* and *in vivo*, which is consistent with previous report.[5]

However, there are several limitations in the present study. First, the promoting effects on proliferation and inhibitory effects on EMT of skin cancer cell line JB6 cells are obvious, but the inhibitory effects still need to be validated in a variety of different tumor cell lines and normal cell lines. This study also found that PAM has no obvious protective effects at low concentrations and high concentrations, and the effective use concentration has yet to be further determined. But according to the effect of *in vivo* response, PAM will have a good application prospect in the future clinical treatment of skin cancer.

## Conclusion

Our study demonstrates that PAM is important in restraining the chemical-induced skin cancer cells *in vitro* and *in vivo* partly through suppressing the Hh signaling pathway. These results suggest that PAM may be a promising anticancer agent for treating skin cancer.

**Authors' contributions:** DX and MC contributed equally to this paper. All authors participated in the design, interpretation of the studies and analysis of the data, and review of the manuscript; DX, MC, WZ, and XM conducted the experiment. LL wrote the manuscript.

### DECLARATION OF CONFLICTING INTERESTS

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