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The effect of ALA-PDT on reversing the activation of cancer-associated fibroblasts in cutaneous squamous cell carcinoma

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ABSTRACT

Cancer-associated fibroblasts (CAFs) are important components of the tumor microenvironment, affecting the biological behavior of tumor cells and playing critical roles in tumor growth, invasion, and metastasis. Topical 5-aminolevulinic acid-mediated photodynamic therapy (ALA-PDT) is an established approach for the treatment of non-melanoma skin cancers. It is reported that ALA-PDT treating cutaneous squamous cell carcinoma(cSCC) also induce antitumor immune effect and has an impact on tumor microenvironment. However, the effect of ALA-PDT on CAFs is not well known. In this study, the data showed that the expression of α-smooth muscle actin (α-SMA) and fibroblast activation protein (FAP), as well as migratory ability, were elevated in 3T3 fibroblasts co-cultured with tumor cells. Western blot, qRT-PCR and transwell cell migration assay were performed to detect these findings. *In vivo*, the rate of tumor growth in mice injected with a mixture of tumor cells and 3T3 fibroblasts was higher than that in mice injected with tumor cells only. Furthermore, both in co-cultured 3T3 fibroblasts and CAFs, a reduction in the expression of a-SMA and FAP was observed after ALA-PDT. Same with migratory ability. The findings indicated an inhibitory effect of ALA-PDT on the activation of CAFs in cSCC.

1. Introduction

Cutaneous squamous cell carcinoma (cSCC) is one of the common skin cancers [1]. It predominantly occurs in the elderly and its incidence has been rising with the increasing elderly population [2]. More and more studies have demonstrated that the tumor microenvironment plays a vital role in the progression of cSCC. The tumor microenvironment is closely related to tumorigenesis, tumor progression and metastasis. Cancer-associated fibroblasts (CAFs) are the major components of the tumor microenvironment. Although the activation of cancer-associated fibroblasts is early event in the process of invasion of cSCC, it regulates growth factors and chemokines and provides a suitable environment that promotes the growth, invasion and metastasis of cSCC [3]. The interaction between CAFs and tumor cells has an important impact on tumor growth, invasion, and metastasis [4]. Compared to normal fibroblasts (NFs), CAFs are generally considered as activated fibroblasts which showing different molecular and functional phenotypes. Hallmarks of the CAFs phenotype include over-expressed α-smooth muscle actin (α-SMA) and fibroblast activation protein (FAP) [5,6]. In the past years, studies have focused exclusively on targeting tumor cells. Now, numerous studies have shown the importance of supporting cells (such as CAFs), which form tumor microenvironment and interact continuously with malignant cells to promote tumor growth [7].

Topical 5-aminolevulinic acid photodynamic therapy (ALA-PDT) is a non-invasive treatment showing promising effects on cSCC *in situ* [8]. Previously, the acute inflammatory reaction was observed after ALA-PDT treatment for cSCC. And detecting the tumor tissue after ALA-PDT, the expression of inflammatory cytokines such as IL-1βwas found up-regulated and the numbers of inflammatory cells such as neutrophils, DCs and T cells were also increased [9]. Moreover, IL-1β was found to be an important inflammatory factor for ALA-PDT treatment of cSCC and the main cells secreting IL1β were CAFs(paper in preparation). These findings suggested that the tumor microenvironment of cSCC changed after ALA-PDT, as well as the function of CAFs. However, it is not clear why the function of CAFs changes after ALA-PDT treatment of cSCC and whether ALA-PDT has an impact on CAFs, which need to be explored in future studies.

This study first investigated the effect of tumor cells on the activation of fibroblasts in cSCC and then assessed whether ALA-PDT could reverse the activation of CAFs, thereby slowing down tumor growth and metastasis.

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2. Methods

2.1. Cell culture

The murine SCC cell line XL50 was derived from cSCC induced by ultraviolet (UV) irradiation in SKH-1 hairless mice. This cell line was procured from the China Center for Type Culture Collection, Wuhan, China (CCTCC No: C201827). Primary cutaneous CAFs were collected from UV-induced SCC in SKH-1 mice by conventional tissue-adherent method [10], and the murine cell line NIH-3T3 was supplied by cell line service corporation (ZQ0096,Germany). All cells were cultured in DMEM medium (Gibco, Thermo Fisher Scientific, Germany) supplemented with fetal bovine serum (FBS; Biochrom AG, Germany) and 100 U/mL penicillin. The concentration of FBS used was 10% for XL50 cells, 20% for CAFs, and 15% for 3T3 fibroblasts. All cells were maintained under standard conditions (37 °C in a humidified atmosphere of 5% CO_2). The culture medium was changed every 2 days. After reaching confluence, cells were passaged with 1% trypsin/EDTA.

2.2. SCC Mouse model and tumor volume regrowth

Female SKH-1 hairless mice (6–8 weeks of age) were provided by Shanghai Public Health Clinical [11] and housed at 24–26 °C with free access to a standard mouse chow and tap water. The mice were divided into two groups (n = 5/group) designated as: XL50group and XL50 + 3T3group. The XL50 cells were resuspended in 200 μl cell culture medium at 6×10^5 cells and injected subcutaneously into the backs of XL50 group mice. The XL50 cells (5×10^5) were resuspended in 100 μl cell culture medium and mixed with 3T3 fibroblasts (1×10^5,100 μl). This mixture was injected subcutaneously into the backs of XL50 + 3T3 group mice. After injection, mice were kept under observation and tumor volume was measured every alternate day to observe whether the size of tumor is increasing or decreasing.

2.3. Immunohistochemistry (IHC)

Mice were sacrificed at designated time points (12 h) after treatment with ALA-PDT and the tissue isolated was stored in formalin until immunohistochemical labeling for α-SMA (ab124964, Abcam).

2.4. Co-culture of normal 3T3 fibroblasts with XL50 cells

A protocol of transwell culture was used to induce CAFs [11]. XL50 cells were separated from 3T3 fibroblasts by using a six-well transwell plate (Corning, NY, USA) with a 0.4-μm pore polyester (PET) membrane insert. Although the cells could not pass through the membrane, soluble factors produced by XL50 cells could permeate this membrane to affect the fibroblasts [12,13]. 3T3 fibroblasts and XL50 cells were co-cultured for 7 days.

2.5. ALA-PDT treatment

CAFs and co-cultured 3T3 fibroblasts were incubated with 0.5 mM ALA(Shanghai Fudan-Zhangjiang Bio-Pharmaceutical Co, China) in serum-free medium for 5 h at 37 °C, then washed twice with phosphate buffered saline (PBS), and exposed to red light at the dose of 0.5 J/cm ^{2} (632.8 nm, 10 mW/cm^2) [14]. The whole process needed to avoid ambient light.

2.6. Cell viability assay

Changes in cell viability after ALA-PDT were investigated in all the groups using the CCK-8 assay according to the manufacturer's protocol. A spectrophotometer was used to measure the absorbance at 450 nm.

2.7. RNA isolation and PCR

Total RNA was extracted from CAFs and co-cultured 3T3 fibroblasts using TRIzol reagent (Invitrogen). Complementary DNA (cDNA) synthesis was synthesized from 1 μg of RNA using a cDNA reverse transcription kit (Thermo Fisher). Following reverse transcription, the samples were analyzed using a qPCR kit (Thermo Fisher) The following primers were used: α-SMA (forward primer: 5'-ACACGGCATCATCACC AACT-3' and reverse primer: 5'-GGTTCAGTGGTGCCTCTGTCA-3') and FAP (forward primer: 5'-ACCAGGAGATCCACCTTTTCA-3' and reverse primer: 5'-CTGGAGACCACCAAAGAGCAT-3').

2.8. Western blot analysis

The co-cultured 3T3 fibroblasts were lysed with RIPA buffer. These samples were adjusted to the same protein concentration by BCA protein assay kit. Equal amounts of protein were separated in SDS-PAGE gels and transferred to nitrocellulose membranes, which were subsequently incubated with the following primary antibodies: anti-α-SMA (ab124964, Abcam) and anti-FAP (ab28244, Abcam). Reactive bands were visualized using enhanced chemiluminescence.

2.9. Transwell cell migration assay

The migration assay was conducted using transwell inserts with 8-μm pores (Corning, NY, USA) according to the manufacturer's instructions. CAFs and co-cultured 3T3 fibroblasts were suspended in 100 μl DMEM, at a density of 5×10^4cells/ml, and were then seeded into the upper chamber. Cell culture medium (500 μl) was added into the lower chamber. The cells were allowed to migrate for 12 h at 37 °C. The chambers were then washed with PBS, and cells on the lower surface of the chamber were stained with 0.1% crystal violet. Images of migrating cells were acquired with a DMR inverted microscope.

2.10. Statistical analysis

Statistical analysis was conducted with GraphPad Prism 7.0. Quantitative data were expressed as mean ± standard deviation unless specified otherwise. Means were compared using paired and unpaired t-tests. P-values < 0.05 were considered statistically significant.

3. Results

3.1. cSCC cells transformed 3T3 fibroblasts to CAFs in vitro and in vivo

3T3 fibroblasts and XL50 cells were co-cultured *in vitro* to induce activation of 3T3 fibroblasts into a carcinoma-associated phenotype.The control cells were grown under the same culture conditions, but were not co-cultured with the partners. Compared with the expression profile of control cells, the changes in expression of markers of 3T3 fibroblasts co-cultured with XL50 cells were then assessed. After 7 days of co-cultivation, the expression of α-SMA and FAP at the gene level was higher in 3T3 fibroblasts co-cultured with XL50 cells than that in control 3T3 fibroblasts and the same was true at the protein level (Fig. 1A–B). The migratory ability of 3T3 fibroblasts co-cultured with XL50 cells was visibly greater than that of the corresponding control groups (Fig. 1C). This indicated that 3T3 fibroblasts co-cultured with tumor cells were developing to a carcinoma-associated phenotype. To verify whether 3T3 fibroblasts transformed into CAFs after co-cultured with XL50 cells, tumor volume of the XL50 group and XL50 + 3T3 group were measured every alternate day for a month. Tumor volumes reached 5 mm in diameter 10 days after mice were injected. Though tumors of two groups grew continuously, the growth rate in the XL50 + 3T3group was higher than that in the XL50group. This resulted in a significantly different tumor size at day 25 after inoculation (Fig. 1D–E). Collectively, the results suggested that tumor cells could

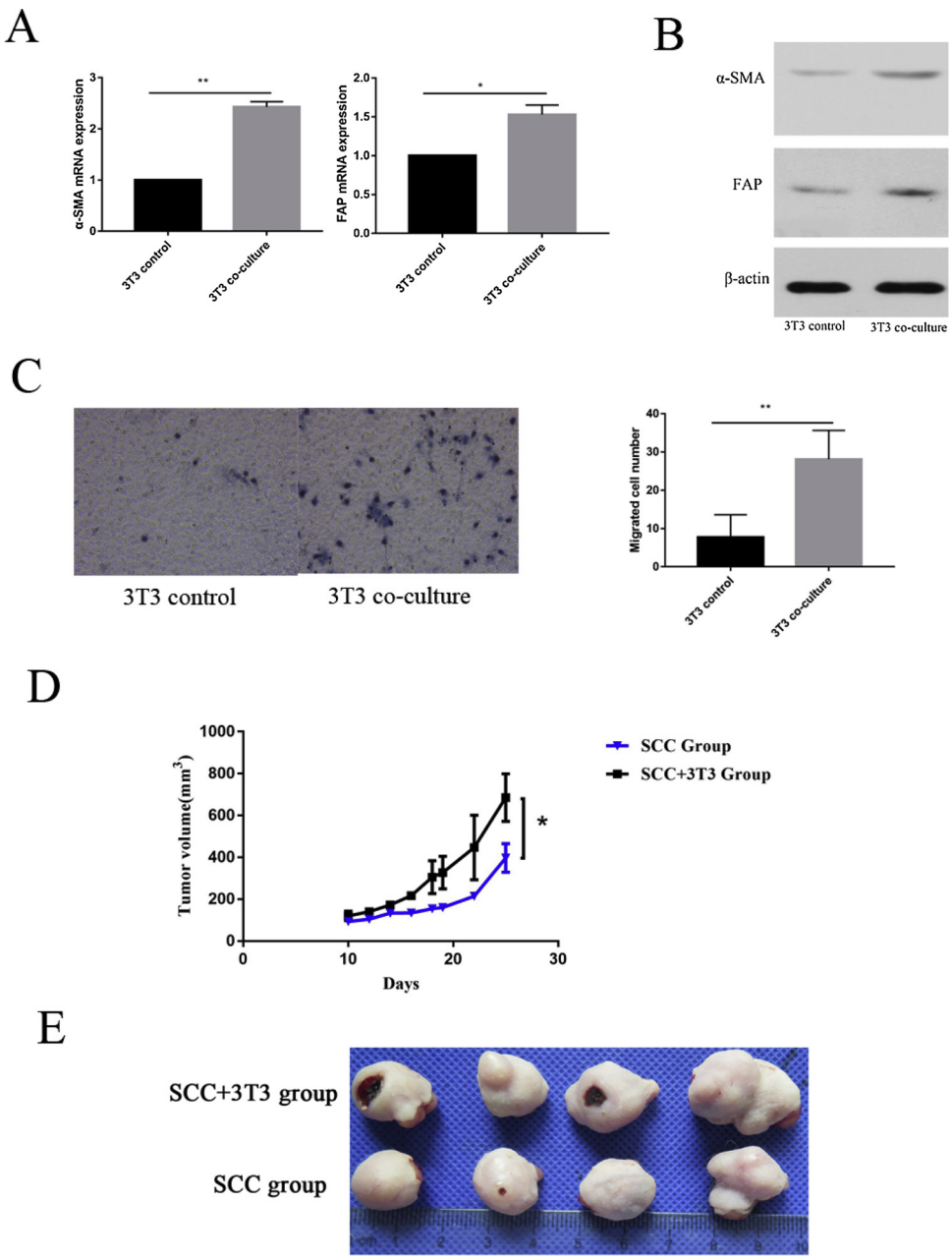

Fig. 1. Tumor cells activated 3T3 fibroblasts to CAFs *in vitro* and *in vivo*. (A) and (B) qRT-PCR and Western blotting showed higher expression of α-SMA and FAP in 3T3 fibroblasts after co-cultured with tumor cells. (C) Cell migration ability was measured using a transwell cell migration assay. The migration ability of co-cultured 3T3 fibroblasts was significantly greater than that of the corresponding control cells. (D) and (E) Tumor volume curve and tumor size showed the growth rate of tumors in XL50 group was slower than XL50 + 3T3 group.Note: *P < 0.05, ** P < 0.01.

transform 3T3 fibroblasts to CAFs, thereby promoting tumor growth.

3.2. ALA-PDT reversed the activation status of co-cultured 3T3 fibroblasts

To investigate the effects of ALA-PDT on 3T3 fibroblasts after co-cultured with XL50 cells, the dose dependent effect of ALA-PDT on co-cultured 3T3 fibroblasts was first examined. Co-cultured 3T3 fibroblasts were incubated with 0.5 mM ALA solution for 5 h, and then exposed to red light(10 mW/cm^2), using energy density in the range of 0.5-16 J/cm^2. The data showed that ALA-PDT exerted no significant inhibitory effects on the growth of co-cultured 3T3 fibroblasts at the dose of 0.5 J/cm^2 (Fig. 2A).

Next, ALA-PDT was employed to treat the co-cultured 3T3 fibroblasts at the dose of 0.5 J/cm^2. The data indicated that ALA-PDT decreased the expression of α-SMA and FAP in co-cultured 3T3 fibroblasts compared with that in non-treated controls (Fig. 2B). As shown in Fig. 2C, ALA-PDT decreased the migratory ability of co-cultured 3T3 fibroblasts compared with that of non-treated (control) fibroblasts. These results suggested that ALA-PDT could reverse the transformation of 3T3 fibroblasts into CAFs.

3.3. Phenotypic characterization of CAFs from cSCC

The primary CAFs were isolated from several mice with cSCC. IHC showed that the expression of α-SMA in the tumor tissue of cSCC was higher than that in normal tissue (Fig. 3A), indicating the existence of CAFs in the tumor tissue of cSCC. Then primary CAFs were isolated from the tumor tissue of cSCC (Fig. 3B) and qRT-PCR and Western blot

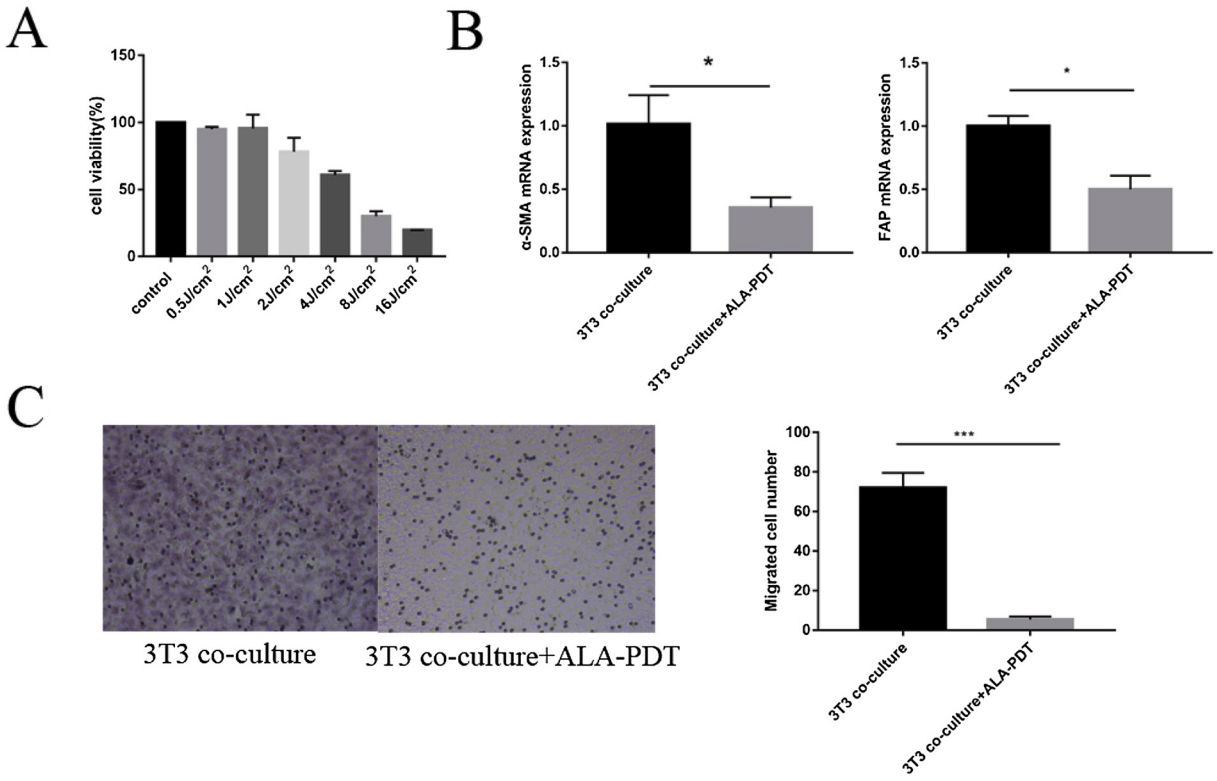

Fig. 2. Effects of ALA-PDT on 3T3 fibroblasts after co-cultured with tumor cells. (A)The co-cultured 3T3 fibroblasts were pretreated with different doses of ALA-PDT (0.5,1,2,4,8 and 16 J/cm^2).The viability of **co-**cultured 3T3 fibroblasts were detected by CCK-8 assay. ALA-PDT had no significant growth inhibitory effect on **co-**cultured 3T3 fibroblasts at the light dose of 0.5 J/cm^2.(B) qRT-PCR showed lower expression of α-SMA and FAP in co-cultured 3T3 fibroblasts after ALA-PDT. (C) The migration ability of co-cultured 3T3 fibroblasts after ALA-PDT was significantly lower than that of the corresponding control cells. Note: *P < 0.05, ** P < 0.01.

were used to analyze and identify their phenotype. Fig. 3C–D showed that the expression of α-SMA and FAP in CAFs is significantly higher than that in 3T3 fibroblasts, suggesting that CAFs were activated fibroblasts.

3.4. ALA-PDT reversed the activation of CAFs in cSCC

To assessed the effect of ALA-PDT on the activation of CAFs in cSCC, qRT-PCR was used to analyze the expression of α-SMA and FAP in CAFs treated with ALA-PDT *in vitro* and the expression of α-SMA and FAP was found decreased in CAFs after ALA-PDT compared with that in non-treated control fibroblasts (Fig. 4A). As shown in Fig. 4B, ALA-PDT decreased the migratory ability of CAFs compared with that of non-treated control fibroblasts *in vitro*. IHC and qRT-PCR showed that the expression of α-SMA in tumor tissue of cSCC decreased after ALA-PDT (Fig. 4C–D).Taken together, the data indicated that ALA-PDT could reverse the activation of CAFs in cSCC, rendering them into NFs.

4. Discussion

Tumor development is a complex process, which involves dynamic interactions between tumor cells and their surrounding stroma. In this process, tumor cells first recruit NFs to the tumor mass and transform them into activated fibroblasts called CAFs, which in turn, support the progression and metastasis of tumor cells [15]. CAFs are found in high quantity in tumor stroma and are characterized by increased expression of α-SMA, FAP, PDGFRs and FSP1 [16,17]. Molecular and functional phenotype between CAFs and NFs differs and previous studies have shown that CAFs highly express α-SMA and FAP, which are weakly expressed in NFs [18]. In the present study, we simulated the tumor microenvironment *in vitro* and co-cultured cSCC cells and normal fibroblasts through a transwell chamber to induce the formation of CAFs. The differences in the expression of α-SMA and FAP between 3T3 fibroblasts and co-cultured 3T3 fibroblasts were assessed to determine the formation of CAFs. The studies *in vitro* showed that the expression of α-SMA and FAP was higher in co-cultured 3T3 fibroblasts than that in 3T3 fibroblasts. The results *in vivo* showed that the growth rate of cSCC in mice injected with a mixture of cSCC cells and 3T3 fibroblasts was higher than that in mice injected with cSCC cells alone. These findings indicated that tumor cells could activate NFs to CAF-like phenotypes and CAFs could promote tumor growth and progression. Although the activation of cancer-associated fibroblasts is early event in the process of invasion of cSCC, CAFs, as dominant components of the tumor microenvironment, have been shown to facilitate tumor growth and invasiveness [6,19,20]. Meng Sha et al previously reported that CAFs promoted the proliferation and migration of cholangiocarcinoma cells *in vitro* and the growth of tumors *in vivo* [21]. Hajime Kashima also found that CAFs enhanced invasion and the lymph node metastasis of esophageal cancer [22]. Yongchen Ma et al also reported that CAFs could promote migration and invasion of gastric cancer [23]. The above results showed that CAFs played important roles in the progression of tumor, which were consistent with our findings.

Topical ALA-PDT is an effective and non-invasive therapy for skin cancer in situ. At present, it is mainly believed that ALA-PDT kills tumor cells by generating intracellular reactive oxygen species (ROS) [24]. Most studies of ALA-PDT, with respect to squamous cell carcinoma, focus on how ALA-PDT affects the behavior and characteristics of tumor cells rather than that of the tumor microenvironment. However, The previous studies have shown that ALA-PDT can affect the tumor microenvironment by promoting the secretion of inflammatory factors and the infiltration of immune cells [24]. Furthermore, the secretory function of CAFs was observed to be changed after ALA-PDT, which enhanced the effect of ALA-PDT by promoting the secretion of IL1β (The article has not been published yet.). Therefore, this study

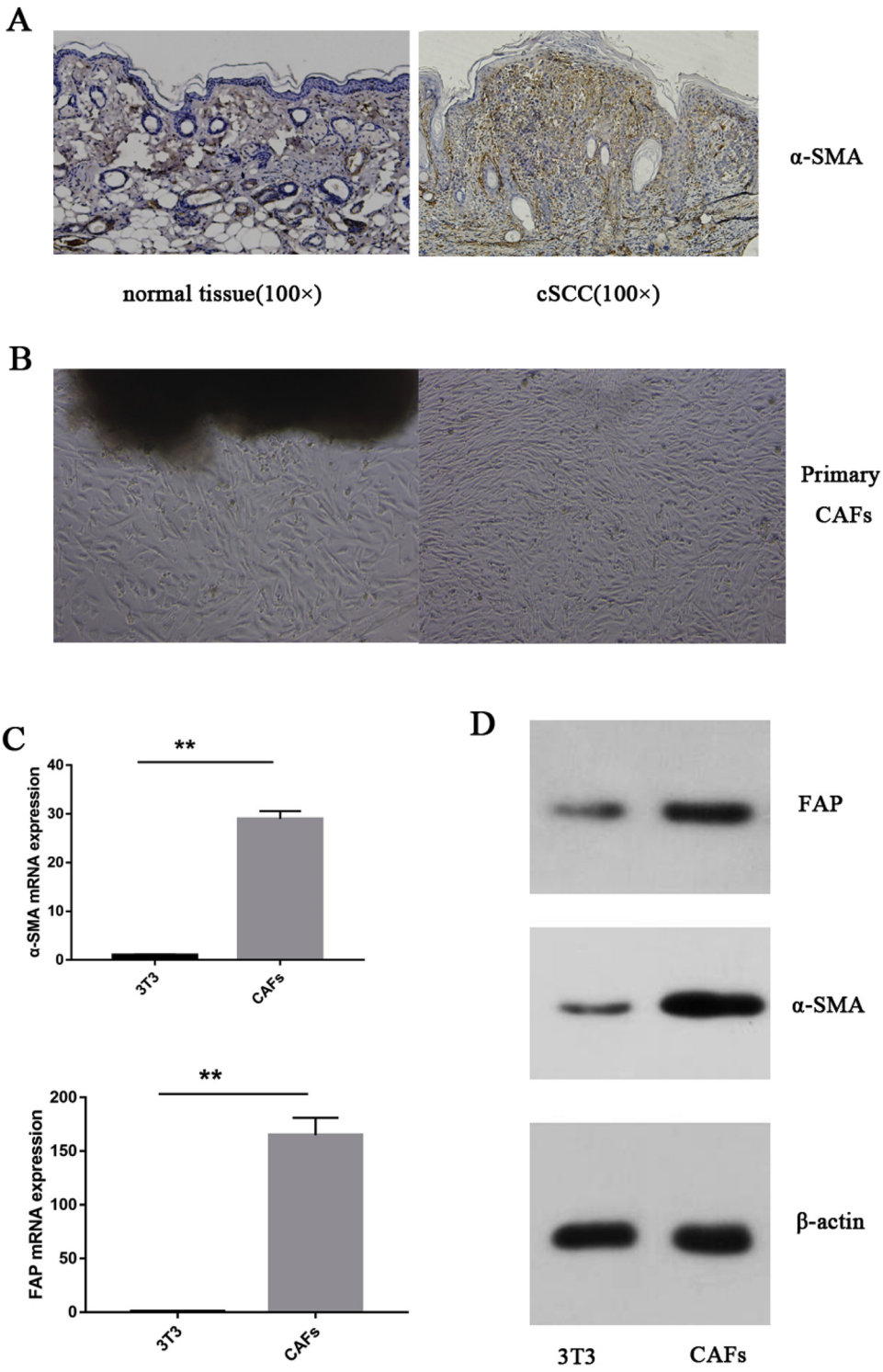

Fig. 3. Primary isolation and identification of CAFs.(A)IHC showed the expression of α-SMA in cSCC was higher than that in normal tissues. (B) It showed the cell morphology of primary CAFs isolated from cSCC. (C) and (D) The expression of α-SMA in CAFs was significantly higher than that of 3T3 fibroblasts. Note: *P < 0.05, ** P < 0.01.

examined the effects of ALA-PDT on characteristics and behavior of CAFs and investigated whether ALA-PDT could induce changes in the activation of CAFs. In our previous study, ALA-PDT at 0.5 J/cm^2 mainly induced apoptosis of cSCC cells and stimulated anti-tumor immunity, so the appropriate dose of ALA-PDT for cSCC cells *in vitro* was determined to be 0.5 J/cm^2. Since both tumor cells and fibroblasts are components of tumor tissue, they are considered as a whole in clinical treatment, so the therapeutic dose should be consistent. In this study, the dose to treat fibroblasts was also selected to be 0.5 J/cm^2. Moreover, the results of CCK-8 showed that this dose had no significant inhibitory effect on CAFs, which was suitable for our study. And the findings showed that ALA-PDT at 0.5 J/cm^2 reduced the expression of α-SMA and FAP, as well as migratory ability in co-cultured 3T3 fibroblasts and CAFs.These changes were not due to the inhibitory effect of ALA-PDT on the growth of fibroblasts and implied that CAFs were reversed to a phenotype comparable to that of NFs, which may result in a change of their

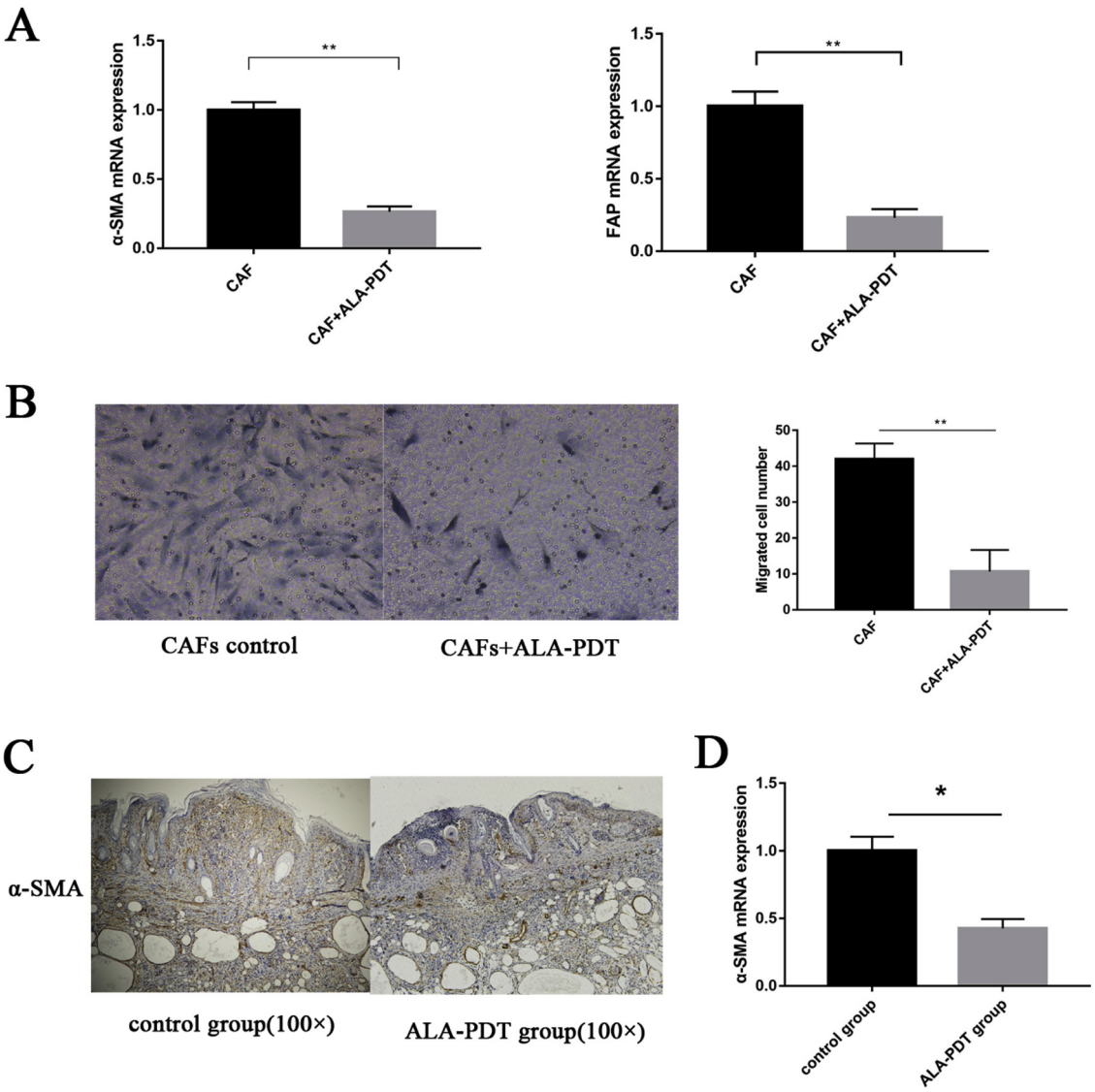

Fig. 4. Effect of ALA-PDT on CAFs *in vitro* and *in vivo*. (A) qRT-PCR showed the expression of α-SMA and FAP decreased in CAFs after ALA-PDT compared with that in non-treated control fibroblasts. (B) Cell migration ability was measured using a Transwell cell migration assay.The migration ability of CAFs after ALA-PDT was significantly lower than that of the corresponding control cells.(C) and (D) IHC and qRT-PCR showed that the expression of α-SMA in tumor tissue of cSCC decreased after ALA-PDT. *P $<$ 0.05, ** P $<$ 0.01.

secretion. Interestingly, our findings validated our previous results that the secretory function of CAFs changed after ALA-PDT and enhanced the effect of ALA-PDT by promoting the secretion of IL1β.

Both CAFs and NFs are components of the tumor microenvironment. However, CAFs are phenotypically and functionally different from NFs, which display a quiescent phenotype under physiological conditions. Several reports have indicated that NFs exert a suppressive effect against tumor growth via various mechanisms [25,26]. NFs can inhibit the growth and development of cancer cells through direct cell-cell interaction and by secreting paracrine factors [27]. The anti-tumorigenic functions of NFs have lately garnered more attention. This study provided the hypothesis that ALA-PDT could reverse the activation of CAFs in cSCC and induced a NF-like phenotype to inhibit the growth of tumor. In fact, this study is not the first to focus on reversing the activation of CAFs in tumors. Curcumin has been reported to reverse the activation of CAFs and normalized them in oral squamous cell carcinoma and pancreatic cancer [28,29]. Ligustilide was found to be able to reverse the immunosuppressive function of CAFs, from promoting tumors to suppressing tumors [30]. These results, combined with our results, proved that active fibroblasts could be inactivated and transformed into normal state, providing a new therapeutic direction for immunotherapy of tumors.

In conclusion, the importance of CAFs in the tumor microenvironment is becoming apparent, indicating that CAFs can be targeted cells in therapies against cancer. The results showed that ALA-PDT could reverse the activation of CAFs and induced a NF-like phenotype in cSCC. In the clinic, ALA-PDT can not only treat superficial skin cancers, but also has an effect on invasive squamous cell carcinoma, which may be related to its ability of reversing the activation status of CAFs. To our knowledge, this research provides preliminary evidence that ALA-PDT can affect CAFs in the tumor microenvironment. However, the effects of ALA-PDT on the secretory and immune functions of CAFs are not yet clear and need to be explored in future studies.

Conflict of interest

The authors have no conflict of interest to declare.

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