Curcumin inhibits pancreatic cancer cell invasion and EMT by interfering with tumor-stromal crosstalk under hypoxic conditions via the IL-6/ERK/NF-κB axis

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Abstract. Hypoxic microenvironment and pancreatic stellate cells (PSCs) play important roles in pancreatic cancer progression. PSCs secrete a number of soluble factors, such as interleukin (IL)-6, to facilitate cancer metastasis. Our previous study revealed that curcumin inhibited the invasive ability of pancreatic cancer cells by modulating epithelial-to-mesenchymal transition (EMT)-related factors. However, whether curcumin could suppress tumor-stromal crosstalk in pancreatic cancer and the underlying mechanisms have yet to be fully elucidated. The aim of the present study was to evaluate whether curcumin could affect pancreatic cancer cell invasion and EMT by interfering with tumor-stromal interaction under hypoxic conditions. The PSCs were treated with curcumin under hypoxic conditions. The activation of PSCs was detected by testing the expression of α-smooth muscle actin by western blotting and immunofluorescence analysis. The wound healing assay was used to evaluate the migratory potential of PSCs. The secretion and expression of IL-6 by PSCs was detected by ELISA and reverse transcription-quantitative PCR (RT-qPCR) analysis. BxPC-3 and Panc-1 cells were treated with PSC-conditioned media (PSC-CM), IL-6, IL-6-neutralizing antibody or curcumin under conditions of normoxia or hypoxia. Transwell invasion assay was used to examine the invasive potential of pancreatic cancer cells. The activation of phosphorylated (p-) extracellular signal-regulated kinase (ERK) and p-nuclear factor (NF)-κB were measured by western blot analysis. The expression of EMT-related genes at the mRNA and protein levels was detected by RT-qPCR and western blot analysis, respectively. The results of the present study demonstrated that curcumin inhibited the activation and migration of PSCs under hypoxic conditions. Curcumin also suppressed the secretion and expression of IL-6 in PSCs. In addition, curcumin and IL-6-neutralizing antibody treatment suppressed PSC-CM-modulated pancreatic cancer invasion, EMT and the changes in the expression of E-cadherin, vimentin and matrix metallopeptidase-9. Furthermore, the increase in the levels of p-ERK and p-NF-κB induced by PSC-CM could be counterbalanced by both curcumin and IL-6-neutralizing antibody treatment under hypoxic conditions. Taken together, these data indicate that curcumin plays an important role in suppressing tumor-stromal crosstalk and pancreatic cancer metastasis by inhibiting the IL-6/ERK/NF-κB axis. Blocking the IL-6/ERK/NF-κB axis by curcumin may be a promising therapeutic strategy for the treatment of pancreatic cancer.

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Introduction

Pancreatic cancer is a one of the most highly malignant tumors of the digestive tract and has an extremely poor prognosis, with a 5-year relative survival rate of 9%. In 2019, an estimated 56,770 individuals were newly diagnosed with pancreatic cancer, which may result in ~45,750 cancer-related deaths in the United States (1). In China, the incidence rate of pancreatic cancer has also increased from 2000 to 2011. Pancreatic cancer accounts for the second leading upward trend of age-standardized mortality rates (2). Metastasis is the leading cause of pancreatic cancer-related mortality, as ~80% of the patients are not deemed suitable for surgical resection due to early relapse or advanced metastasis at diagnosis (3). Therefore, it is crucial to elucidate the molecular mechanisms underlying the invasion and metastasis of pancreatic cancer, and novel comprehensive and effective therapeutic interventions are urgently needed to improve the treatment outcome.

Pancreatic cancer is characterized by an excessive desmoplastic reaction, which favors hypoxia, further inducing epithelial-to-mesenchymal transition (EMT) and tumor metastasis (4). It has become obvious that the tumor microenvironment, which includes several types of stromal cells, such as immune, inflammatory and endothelial cells, as well

as pancreatic stellate cells (PSCs), plays a key role in tumor invasion and metastasis (5). PSCs generally exist in one of two statuses: Quiescent and activated. Under physiological conditions, non-activated PSCs contain abundant cytoplasmic vitamin A-containing lipid droplets, which produce very low levels of extracellular matrix (ECM). After activation, PSCs are characterized by a decrease in lipid droplets and increased expression of α-smooth muscle actin (α-SMA) and collagen-I, which are crucial for tumor development, evasion of immune surveillance, invasion and metastasis (6). In addition, activated PSCs can produce a number of soluble factors, such as interleukin (IL)-6, transforming growth factor (TGF)-β and stromal cell-derived factor (SDF)-1, which promote the malignant behavior of pancreatic cancer cells (7). IL-6 is a potent pro-inflammatory cytokine secreted by PSCs in the pancreatic cancer microenvironment. In addition to the inflammatory response, IL-6 is also associated with tumor progression, including proliferation, angiogenesis, chemoresistance, invasion, EMT and metastasis (7,8). Our previous study demonstrated that PSCs cultured under hypoxic conditions display higher levels of IL-6, vascular endothelial growth factor (VEGF)-A and SDF-1 transcription and secretion (9).

Low oxygen tension (hypoxia), which is correlated with poor survival of the patients, is most commonly present in the microenvironment of pancreatic cancer. The adaptation of pancreatic cancer cells to limited oxygen delivery promotes tumor invasion, angiogenesis and distant metastasis at an early stage of tumor development (10). Our previous studies have demonstrated that hypoxic conditions can promote the proliferation, migration, invasion and EMT of pancreatic cancer cells *in vitro* by activating the Hedgehog (Hh) signaling pathway (11). Using a three-dimensional (3D) matrices model, Sada *et al* demonstrated that the expression of hypoxia-induced 2-oxoglutarate 5-dioxygenase 2 in PSCs creates a permissive microenvironment for cancer cell migration through architectural regulation of stromal ECM in pancreatic cancer (12).

Curcumin, a natural polyphenol derived from turmeric, is well known for its potential applications in the treatment of multiple tumors, as well as for its anti-infectious, anti-inflammatory, antioxidant and chemopreventive properties (13). It has been confirmed that curcumin can exert its antitumor effects via targeting multiple signaling pathways (14). Our previous study demonstrated that curcumin inhibited high glucose-induced proliferative and invasive abilities of pancreatic cancer cells via inhibiting the epidermal growth factor (EGF)/extracellular signal-regulated kinase (ERK) and EGF/Akt signaling pathways (15). Curcumin has also been found to suppress hypoxia-induced proliferation, migration and EMT by inhibiting the Hh signaling pathway in pancreatic cancer cells (11). However, whether curcumin can suppress the tumor-stromal crosstalk in pancreatic cancer and the underlying mechanisms have yet to be fully elucidated.

The aim of the present study was to investigate the role of hypoxia in PSCs and pancreatic cancer cells, and examine the potential protective effect of curcumin against hypoxia-induced pancreatic cancer progression. It was also investigated whether curcumin acts by suppressing tumor-stromal crosstalk and pancreatic cancer metastasis through inhibiting the IL-6/ERK/nuclear factor (NF)-κB axis, and whether it may be considered as a novel option for the treatment of pancreatic cancer.

Materials and methods

Cell culture and reagents. Human PSCs were isolated from pancreatic tumor tissue surgically resected from patients at the Department of Hepatobiliary Surgery of the First Affiliated Hospital of Xi'an Jiaotong University. PSCs were obtained and cultured according to methods described in a previous study (16). The purity of the PSCs was evaluated by morphology, Oil red O staining of intracellular fat droplets and immunofluorescence of α-SMA, as described in our previous studies (9,17). The experimental protocol and patient consent forms were approved by the Ethics Committee of the First Affiliated Hospital of Xi'an Jiaotong University (Xi'an, China). The human pancreatic cancer cell lines, BxPC-3 and Panc-1, were obtained from the American Type Culture Collection. The culture medium was Gibco™ DMEM (Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 μg/ml streptomycin. When the experiment was performed, the cells were cultured under normoxic conditions (20% O_2) or controlled hypoxic conditions (1% O_2) at 37˚C. Exponentially growing cells in complete medium were pretreated for 1 h with 20 μM curcumin, followed by continual incubation with different O_2 concentrations according to the purpose of the experiment. Curcumin, MTT, IL-6 and PD 98059 were purchased from Sigma-Aldrich; Merck KGaA. Curcumin was dissolved in dimethylsulfoxide (DMSO) and then diluted to appropriate concentrations in culture medium. DMSO was used as the control group. Millicell culture plate inserts for the Transwell invasion assays were obtained from EMD Millipore. Matrigel was purchased from BD Biosciences. Primary antibodies [dilution 1:100 in phosphate-buffered saline (PBS)-Tween-20 (0.1%)] against E-cadherin (cat. no. sc-52328), vimentin (cat. no. sc-66002), IL-6 (cat. no. sc-130326) and matrix metallopeptidase (MMP)-9 (cat. no. sc-12759) were obtained from Santa Cruz Biotechnology, Inc. The anti-ERK (cat. no. 9102), anti-phosphorylated (p)-ERK (Thr202/Tyr204, cat. no. 9106), anti-NF-κB (cat. no. 6956), anti-p-NF-κB p65 (anti-p-NF-κB, Ser468, cat. no. 3039) and anti-α-SMA (cat. no. 48938) antibodies (dilution 1:200 in PBS-Tween-20) were obtained from Cell Signaling Technology, Inc. Other reagents were purchased from common commercial sources. All drug solutions were freshly prepared on the day of testing.

MTT assay. PSCs were seeded in 96-well plates at a density of $1x10^4$ cells/well. The cells were then treated with different concentrations (0-40 μM) of curcumin. After incubation for 24, 48 and 72 h at 37˚C, 15 μl of the MTT solution was added to each well, and the cells were incubated for 4 h at 37˚C. Subsequently, 100 μl of DMSO were added to each well. The optical density (OD) value at 490 nm was determined using a spectrophotometer (Bio-Rad Laboratories, Inc.). The proliferation inhibition rate was calculated as (1-ODsample/ODcontrol) x100%.

Wound healing assay. PSCs ($5x10^4$ cells/500 μl) were seeded in 24-well plates. A 1-μl sterile pipette tip was used to produce a linear wound in the cell monolayer after the cells had grown to ~90-100% confluence. After removing the cellular debris, PSCs were allowed to migrate for 24 h under normoxic or

hypoxic conditions, with or without curcumin. Images were captured at 0 and 24 h post-wounding using a Diaphot TMD inverted microscope (Nikon Corporation) at a magnification of x10. The relative distance migrated by the leading edge was assessed using Photoshop software (n=5).

Transwell Matrigel invasion assay. A chamber-based Transwell invasion assay was performed to evaluate the invasive ability of pancreatic cancer cells. Briefly, the 8.0-μm pore inserts were coated with 30 μl of Matrigel. After serum starvation for 24 h, cancer cells ($5x10^4$) were suspended in the upper chamber in DMEM containing 1% FBS and allowed to migrate toward a serum gradient (10%) in the lower chamber for 48 h. The non-invading cells were then removed from the upper surface with a cotton swab. After staining with 0.1% crystal violet solution for 10 min at room temperature, the stained cells on the bottom surface were counted on each membrane to test the invasion ability of cancer cells. Three random fields were captured at a magnification of x20 (n=3).

Reverse transcription-quantitative PCR (RT-qPCR) assay. Total RNA was extracted from PSCs and pancreatic cancer cells using the Fastgen200 RNA isolation system (Fastgen) and reverse transcription was performed using the PrimeScript RT Reagent Kit (Takara Biotechnology Co., Ltd.) according to the manufacturer's recommendations.

The primer sequences were as follows: E-cadherin, forward 5'-ATTCTGATTCTGCTGCTCTTG-3' and reverse 5'-AGTCCTGGTCCTCTTCTCC-3'; vimentin, forward 5'-AATGACCGCTTCGCCAAC-3' and reverse 5'-CCGCATCTCCTCCTCGTAG-3'; MMP-9, forward 5'-GCAATGCTGATGGGAAACCC-3' and reverse 5'-AGAAGCCGAAGAGCTTGTCC-3'; IL-6, forward 5'-AGTTCCTGCAGTCCAGCCTGAG-3' and reverse 5'-TCAAACTGCATAGCCACTTTCC-3'; and β-actin, forward 5'-GACTTAGTTGCGTTACACCCTTTCT-3' and reverse 5'-GAACGGTGAAGGTGACAGCAGT-3'.

The following PCR program was used: Denaturation at 95˚C for 30 sec, followed by 40 cycles of 95˚C for 5 sec, 60˚C for 30 sec and 72˚C for 30 sec. After each RT-qPCR experiment, a dissociation curve analysis was conducted. The relative gene expression was calculated using the previously described $2^{-\Delta\Delta Cq}$ method (18).

ELISA. The PSCs from the indicated groups were conditioned in serum-free medium for 72 h. The cellular culture media were then collected and centrifuged at 1,000 x g for 5 min at 4˚C to remove particles. The production of IL-6 in the supernatants of PSCs was detected by ELISA using the commercially available ELISA kit (cat. no. D6050; R&D Systems, Inc.) according to the manufacturer's instructions.

Immunofluorescence microscopy. After the designated treatment (hypoxia or normoxia), PSCs were fixed with 4% paraformaldehyde for 10 min at room temperature, permeabilized in 0.5% Triton X-100 for 10 min and blocked in 1% BSA (Beyotime Institute of Biotechnology) for 1 h. Fixed cells were incubated with mouse anti-human-α-SMA antibodies (1:200, cat. no. 48938, Cell Signaling Technology, Inc.) at 4˚C overnight. Cells were washed and incubated with goat anti-mouse DyLight 594 (red) IgG antibody (1:1,000, cat. no. ab96873, Abcam) for 1 h in a dark room. Nuclei were then stained with DAPI for 5 min at room temperature. The cells were visualized by a fluorescence microscope (Nikon Corporation) using appropriate excitation and emission spectra at a magnification, x400.

Western blotting. Proteins were electrophoretically resolved on a denaturing SDS-polyacrylamide gel (10-12%) and electrotransferred onto nitrocellulose membranes (EMD Millipore). The membranes were initially blocked with 5% non-fat dry milk in Tris-buffered saline for 2 h at room temperature and then probed with antibodies against α-SMA, E-cadherin, vimentin, MMP-9, ERK, p-ERK, NF-κB, p-NF-κB or β-actin (loading control). Following co-incubation with the primary antibodies at 4˚C overnight, the membranes were incubated with HRP-conjugated secondary antibodies for 2 h at 37˚C. The results were visualized using the ECL western blotting substrate (Thermo Fisher Scientific, Inc.) and photographed by GeneBox (CHEMI-XT16; SynGene).

Statistical analysis. Statistical analysis was performed using SPSS software (version 17.0, SPSS Inc.). Data are presented as the means ± standard error of the mean from at least three independent experiments. Comparisons between two groups were performed using Student's t-test. Differences among three or more groups were evaluated by analysis of variance followed by the least significant difference test. $P<0.05$ was considered to indicate statistically significant differences. All experiments were repeated independently at least three times.

Results

Curcumin inhibits hypoxia-induced α-SMA expression in PSCs. A typical characteristic of activated PCSs is to obtain a myofibroblast-like phenotype with increasing expression of α-SMA (19). Our previous study demonstrated that hypoxia is able to increase the activation of PSCs (9). In the present study, the cytotoxic effect of curcumin on PSCs was first investigated. As shown in Fig. 1A, PSCs were treated with curcumin at various concentrations (0-40 μM) for 24, 48 and 72 h (DMSO was used as the control group). The proliferative abilities of PSCs decreased in response to curcumin treatment in both a time- and dose-dependent manner. Curcumin had a 50% inhibitory concentration (IC_{50}) of ~20 μM, and this concentration was used for the subsequent experiments. This result also proved that hypoxia could enhance the expression of α-SMA in PSCs. Treatment with 20 μM curcumin for 48 h reduced PSC activation, as revealed by α-SMA expression (Fig. 1B and C).

Curcumin abrogates hypoxia-activated migratory ability and IL-6 secretion in PSCs. A hypoxic microenvironment plays a key role in pancreatic cancer progression. It has been suggested that a hypoxic microenvironment could affect cancer cells as well as the surrounding PSCs (20). The present study demonstrated that hypoxia induced migration of PSCs, as assessed by the wound healing assay. Delayed wound closure was observed following treatment of PSCs with curcumin (Fig. 2A).

IL-6 is a potent pro-inflammatory cytokine secreted by PSCs in the pancreatic cancer microenvironment. Previous

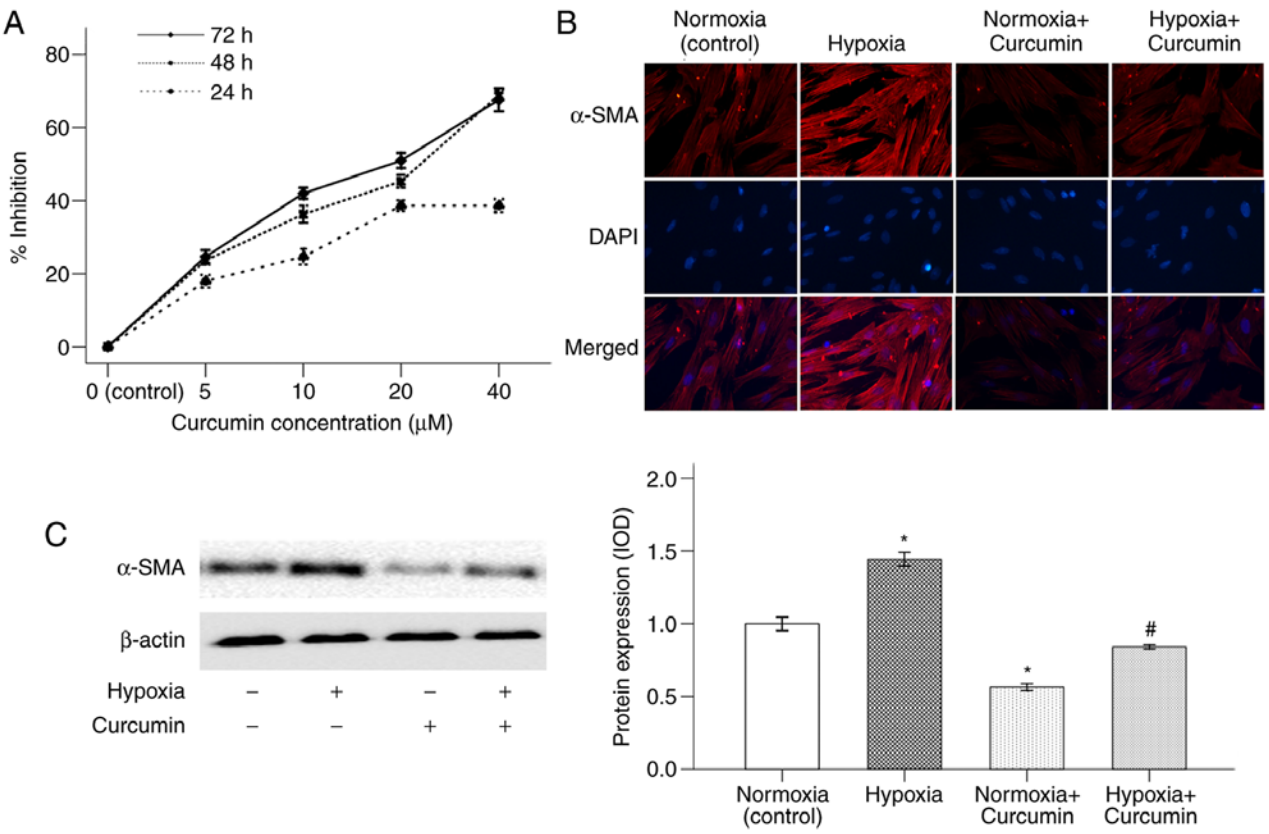

Figure 1. Curcumin inhibits hypoxia-induced PSC activation. (A) PSCs were treated with increasing concentrations of curcumin (5, 10, 20 and 40 μM) for 24, 48 and 72 h. The proliferative abilities of PSCs decreased in response to the curcumin treatment in both a time- and dose-dependent manner. (B) The activation of PSCs were tested by immunofluorescence microscopy analysis. (C) Curcumin suppressed hypoxia-induced PSC activation, as revealed by α-SMA expression using western blotting. DMSO was used as the control group. *P<0.05 compared with the untreated group under normoxia; #P<0.05 compared with the untreated group under hypoxia. The data are representative of 3 independent experiments. PSC, pancreatic stellate cell; SMA, smooth muscle actin; DMSO, dimethyl sulfoxide.

results have indicated that the activated stroma is able to secrete large amounts of IL-6, which could further promote the invasive capacity of the surrounding tumor cells (21). To verify whether hypoxia-activated PSCs have higher expression of IL-6, RT-qPCR and ELISA were performed. As shown in Fig. 2B and C, PSCs cultured under hypoxic conditions displayed higher levels of IL-6. However, treatment with 20 μM curcumin for 72 h counterbalanced the effect of hypoxia on the expression of IL-6 in PSCs.

Curcumin suppresses PSC-CM-induced invasive ability of pancreatic cancer cells. PSCs do not only form a dense fibrotic stroma and interact with cancer cells, but may also be able to induce distant metastasis (19). The present study focused on whether media from the PSCs cultured under hypoxic conditions could promote the metastatic potential of BxPC-3 cells. The results demonstrated that both hypoxia and IL-6 increased the invasion rates of cancer cells. PSC-CM also promoted the invasive ability of pancreatic cancer cells. To elucidate the role of IL-6 in the PSC-CM-induced alterations in the function of pancreatic cancer cells, a neutralizing antibody against IL-6 was employed. It was demonstrated that treatment with anti-IL-6 antibody was able to suppress the PSC-CM-induced invasion of BxPC-3 cells. Therefore, PSC-CM-induced invasion may be attributed to the production of IL-6. Our previous studies indicated that curcumin has an IC_{50} of 20 μM in BxPC-3 cells (11). Thus, 20 μM of curcumin was used in the present experiments. The mean number of cells invading into the lower chamber, which was induced by PSC-CM, was also lower following 48 h of co-treatment with curcumin under both hypoxic and normoxic conditions (Fig. 3). Therefore, curcumin may be considered as potential therapy against IL-6-induced pathological conditions (22).

Curcumin restrains PSC-CM-induced IL-6/ERK/NF-κB axis activation in pancreatic cancer cells under hypoxic conditions. Previous studies have demonstrated that hypoxic conditions (10) and PSC-CM (23) activate multiple signaling pathways in pancreatic cancer cells. The ERK/NF-κB pathway plays an important role in numerous cellular processes, including tumor cell survival, migration and invasion (24). Our previous study demonstrated that curcumin plays an important role in inhibiting the proliferation, migration and invasion of pancreatic cancer cells via the reactive oxygen species/ERK/NF-κB signaling pathway (25). In the present study, BxPC-3 cells were treated with PSC-CM activated by hypoxia in the presence of curcumin or anti-IL-6 antibody, and the activation of the ERK/NF-κB signaling pathway was tested via western blotting. As shown in Fig. 4A, hypoxia significantly increased the expression of p-ERK and p-NF-κB in BxPC-3 cells. Treatment with PSC-CM greatly

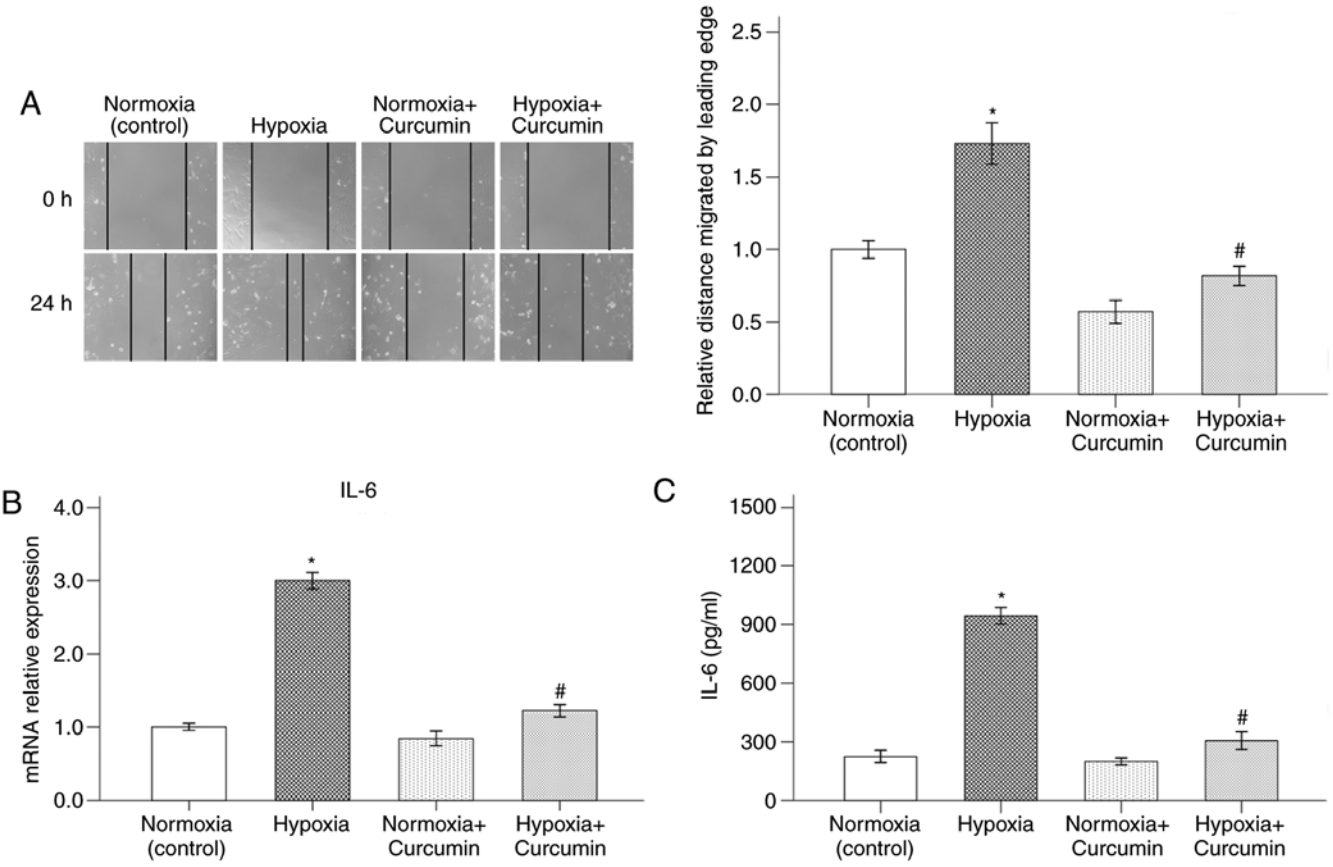

Figure 2. Curcumin abolishes hypoxia-induced migration and IL-6 secretion in activated PSCs. (A) PSCs were allowed to migrate for 24 h under normoxic or hypoxic conditions, with or without curcumin. The migratory ability of PSCs increased under hypoxic conditions, and this effect was suppressed by curcumin. Hypoxia-induced (B) mRNA expression and (C) secretion of IL-6 were also counterbalanced by curcumin. DMSO was used as the control group. *P<0.05 compared with the untreated group under normoxia; $^{\#}$P<0.05 compared with the untreated group under hypoxia. The data are representative of 3 independent experiments. PSC, pancreatic stellate cell; IL, interleukin; DMSO, dimethyl sulfoxide.

enhanced this effect. PSC-CM was also able to activate the ERK/NF-κB signaling pathway in BxPC-3 cells, even under normoxic conditions. The ability of the anti-IL-6 antibody to abolish the effects on hypoxia and PSC-CM-induced p-ERK and p-NF-κB expression demonstrated that IL-6 played an important role in ERK/NF-κB pathway activation. In addition, the PSC-CM-induced phosphorylation of ERK and NF-κB also strongly decreased following the addition of curcumin. Moreover, the ERK inhibitor PD 98059 (50 μM) inhibited hypoxia-induced activation of p-ERK and p-NF-κB, indicating that the NF-κB transcription factor is modulated by the ERK pathway (Fig. 4B). PD 98059 also decreased pancreatic cancer cell invasion under hypoxic conditions, indicating that the activation of the ERK signaling pathway is involved in hypoxia-induced pancreatic cancer cell invasion (Fig. 4C).

Curcumin inhibits PSC-CM-promoted EMT in pancreatic cancer cells. EMT, a critical event during tumor invasion and metastasis, includes four important steps: Loss of cellular polarized epithelial traits, expression of mesenchymal proteins (such as vimentin and N-cadherin), degradation of the basement membrane, and enhancement of the cell invasive ability and entry into the circulation (26). Our previous study demonstrated that hypoxia is able to induce invasion and EMT of pancreatic cancer cells (11). The focus of the present study was to determine whether media from PSCs cultured under hypoxic conditions could promote the EMT of pancreatic cancer cells. The results demonstrated that cancer cell morphology changed from a typical epithelial phenotype to a mesenchymal phenotype following PSC-CM treatment for 48 h, which was counterbalanced by curcumin (Fig. 5A). In addition, PSC-CM significantly decreased the E-cadherin level and increased the expression of vimentin and MMP-9 in pancreatic cancer cells at both the mRNA and protein levels, under either normoxic or hypoxic conditions, which indicates that PSC-CM can promote EMT in pancreatic cancer cells. These effects of hypoxia and PSC-CM were counterbalanced by either 20 μM curcumin or anti-IL-6 antibody (5 μg/ml) (Fig. 5B and C).

Moreover, the shape of Panc-1 cells changed into myofibroblast-like phenotype following PSC-CM treatment for 48 h (Fig. 6A). Hypoxic conditions and hypoxia-induced PSC-CM promoted the invasive ability of Panc-1 cells (Fig. 6B). The expressions of E-cadherin, vimentin, MMP-9 were also modulated by PSC-CM (Fig. 6C). As shown in Fig. 4D, hypoxia significantly increased the expression of p-ERK and p-NF-κB in Panc-1 cells. Treatment with PSC-CM was also able to activate the ERK/NF-κB signaling pathway under both hypoxic and normoxic conditions, whereas these effects of PSC-CM were suppressed by curcumin.

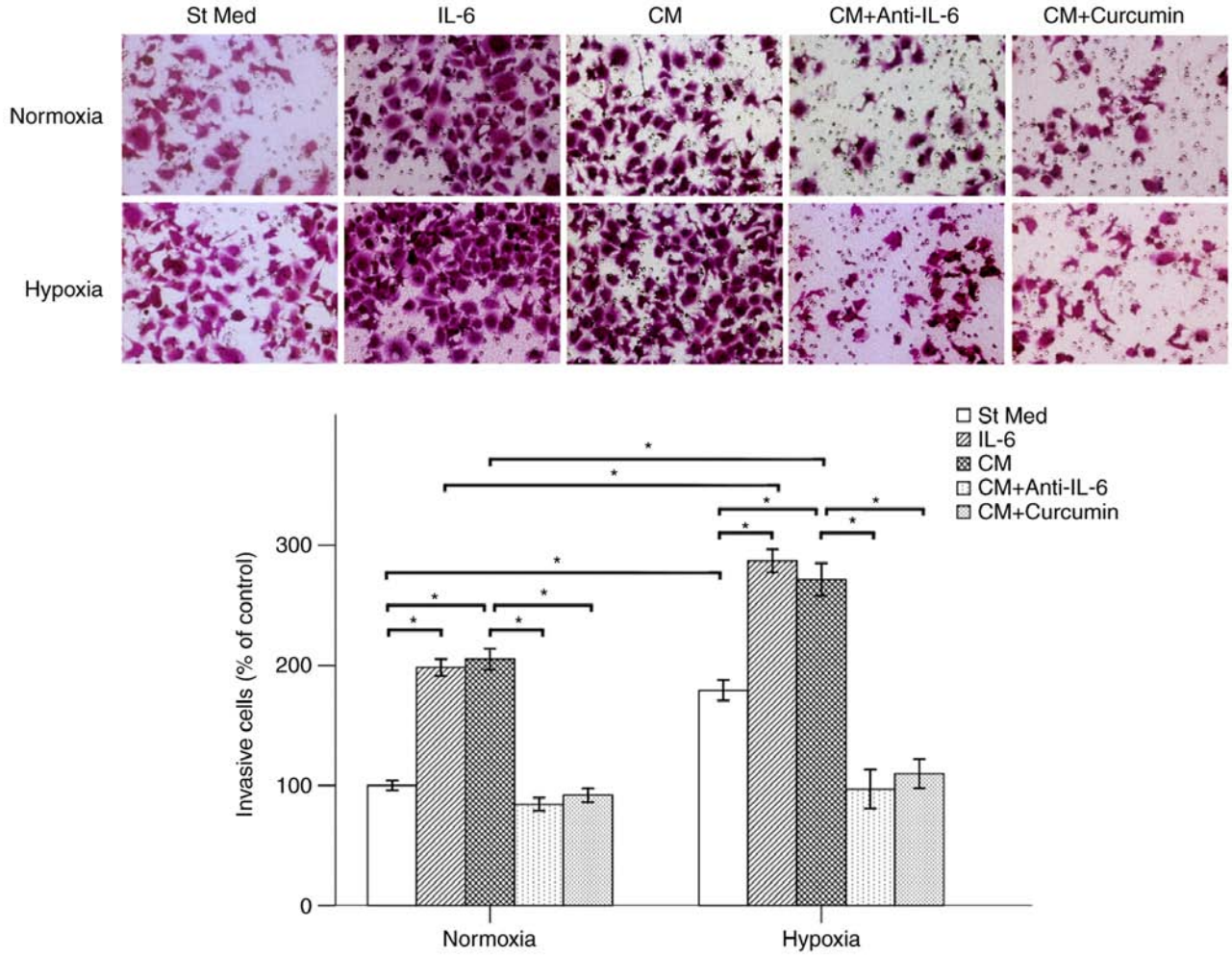

Figure 3. Effect of curcumin on cancer cell invasive ability under hypoxic conditions. BxPC-3 cells were allowed to migrate for 48 h under normoxic or hypoxic conditions. Hypoxia, IL-6 as well as PSC-CM promoted the invasion of pancreatic cancer cells. Furthermore, hypoxia-enhanced cancer cell invasion was counterbalanced by neutralizing antibody against IL-6 and curcumin. The number of migrated cells was quantified by counting the cells from 3 random felds at a magnification, x200. *P<0.05. The data are representative of 3 independent experiments. IL, interleukin; PSC, pancreatic stellate cell; CM, conditioned medium.

Taken together, these results demonstrated that curcumin plays an important role in suppressing tumor-stromal crosstalk and pancreatic cancer invasion and EMT by inhibiting the IL-6/ERK/NF-κB axis.

Discussion

Pancreatic cancer is one of the most lethal types of cancer, characterized by the lack of an effective therapeutic strategy and poor life expectancy, mainly due to its early metastasis and rapid progression. As pancreatic cancer is clinically known as a hypovascular tumor, the pancreatic tumor microenvironment is characterized by lack of oxygen and cells are continuously exposed to hypoxia (27). A number of studies have indicated that a hypoxic microenvironment is highly associated with poor clinical outcome, which is attributed to the enhanced cancer cell invasion and metastasis (28). Our previous study also demonstrated that hypoxic conditions promote the proliferation, migration and EMT in pancreatic cancer cells (11). The pancreatic cancer microenvironment contains several types of stromal cells, including PSCs. Hypoxia activates PSCs, which further secrete a variety of cytokines and growth factors, which in turn promote the progression of pancreatic cancer, particularly cell migration and invasion (9). The focus of the present study was the underlying mechanisms through which curcumin inhibits hypoxia-induced tumor-stromal crosstalk, as well as the invasion and EMT of pancreatic cancer cells.

The data of the present study demonstrated that curcumin inhibited the activation of PSCs under hypoxic conditions. Hypoxia-induced migration and IL-6 secretion of PSCs were also suppressed by curcumin. IL-6 and PSC-CM stimulated the invasive ability of pancreatic cancer cells under hypoxic conditions, while IL-6-neutralizing antibody treatment suppressed the PSC-CM-modulated pancreatic cancer cell invasion, indicating that hypoxia-induced IL-6 secretion of PSCs may promote the progression of pancreatic cancer. Curcumin counterbalanced these effects of hypoxia and PSC-CM treatment. PSC-CM-modulated cancer cell morphology, and the changes in the expression of E-cadherin, vimentin and MMP-9, were inhibited by curcumin and IL-6-neutralizing antibody. In addition, hypoxia and PSC-CM-induced activation of the ERK pathway and the transcription factor NF-κB were also suppressed by IL-6-neutralizing antibody and curcumin. These results indicated that curcumin inhibits hypoxia-induced

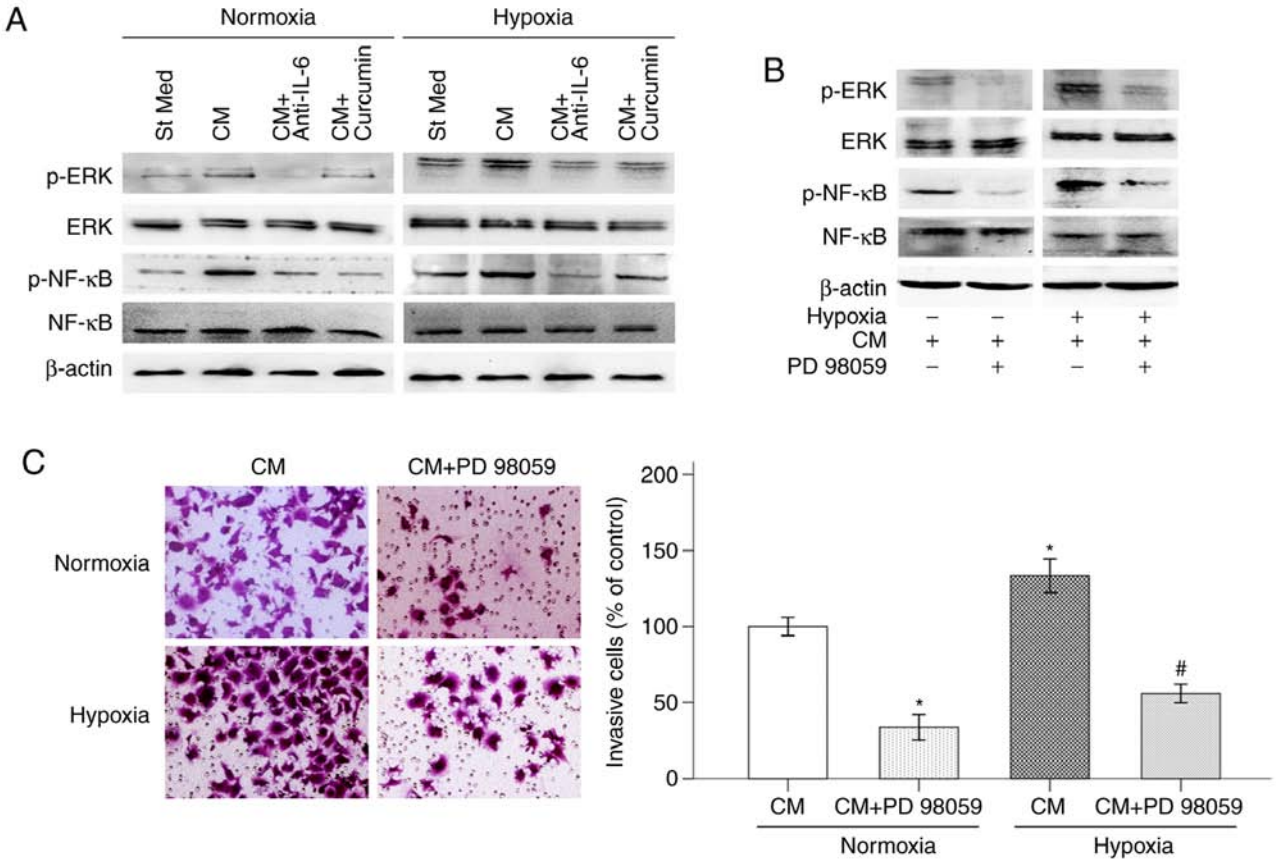

Figure 4. Curcumin attenuates hypoxia- and hypoxia-induced PSC-CM-mediated activation of the ERK/NF-κB pathway in BxPC-3 cells. (A) Hypoxia and hypoxia-induced PSC-CM increased the expression of p-ERK and p-NF-κB in BxPC-3 cells. These effects were counterbalanced by neutralizing antibody against IL-6 and curcumin. (B) PD 98059 (50 μM), an ERK inhibitor, inhibited hypoxia-induced expression of p-ERK and p-NF-κB. (C) PD 98059 also decreased hypoxia-induced invasive ability of BxPC-3 cells. The number of migrated cells was quantified by counting the number of cells from 3 random fields at a magnification, x200. *P<0.05 compared with the normoxic group; #P<0.05 compared with the hypoxic group. The data are representative of 3 independent experiments. PSC, pancreatic stellate cell; CM, conditioned medium; ERK, extracelular signal-regulated kinase; NF, nuclear factor.

tumor-stromal crosstalk and pancreatic cancer EMT and metastasis via the IL-6/ERK/NF-κB axis.

The pancreatic cancer microenvironment includes not only tumor cells, but also other cell types, including PSCs, immune cells and inflammatory cells, ECM, blood vessels, and other signaling molecules (5). Our previous studies have focused on the effect of hypoxia on the tumor cells (11). The present study, however, investigated the effect of curcumin on hypoxia-induced tumor-stromal interaction. The normal function of PSCs includes immune responses, phagocytosis, and stimulating the secretion of amylase in the healthy pancreas (29). However, PSCs also act as the sprouted seed for tumor progression, due to their important role in inducing a fibrotic environment that facilitates tumor metastasis (30). Several cytokines and growth factors derived from PSCs are also involved in malignant tumor progression. It has been proven that PSCs promote pancreatic cancer cell invasion and migration through the hepatocyte growth factor (HGF)/c-Met/survivin pathway, which is negatively regulated by P53/P21 (31). PSCs may also enhance pancreatic cancer cell invasion of the dorsal root ganglia (DRG), promote the outgrowth of DRG and further facilitate tumor cell perineural invasion via the HGF/c-Met pathway (32). In addition, paracrine IL-6 from PSCs promotes the EMT of pancreatic cancer cells via the signal transducer and activator of transcription 3 (STAT3)/nuclear factor erythroid 2-related factor 2 (Nrf2) pathway (33). In the present study, it was observed that hypoxia could activate PSCs and promote the paracrine secretion of IL-6, thereby further promoting the invasion and EMT of pancreatic cancer cells via the ERK/NF-κB signaling pathway.

As a hypovascular tumor, pancreatic cancer thrives under hypoxic conditions. Hypoxia-inducible factor (HIF)-1, a key mediator of the cellular response to hypoxia, is overexpressed in a variety of solid tumors, including pancreatic cancer (34). It has been proven that HIF-1α can recruit macrophages by promoting secretion of C-C motif chemokine ligand 2, which further accelerates the activation of PSCs (35). Hirakawa *et al* (27) identified a motility-stimulating effect of pancreatic cancer-associated fibroblasts on pancreatic cancer cells through the paracrine insulin-like growth factor-1 (IGF1)/IGF1R signaling axis, particularly under hypoxic conditions. Hypoxia is also able to induce PSCs to secrete connective tissue growth factor (CTGF), which in turn promotes the invasion of pancreatic cancer cells (36). Masamune *et al* (20) demonstrated that PSCs expressed a variety of angiogenesis-regulating molecules, including VEGF receptors, angiopoietin-1, and the angiopoietin receptor Tie-2. Hypoxia stimulates PSCs to induce fibrosis and angiogenesis in pancreatic cancer. PD 98059, a MEK inhibitor, has been shown to suppress the ERK pathway and invasion of pancreatic cancer

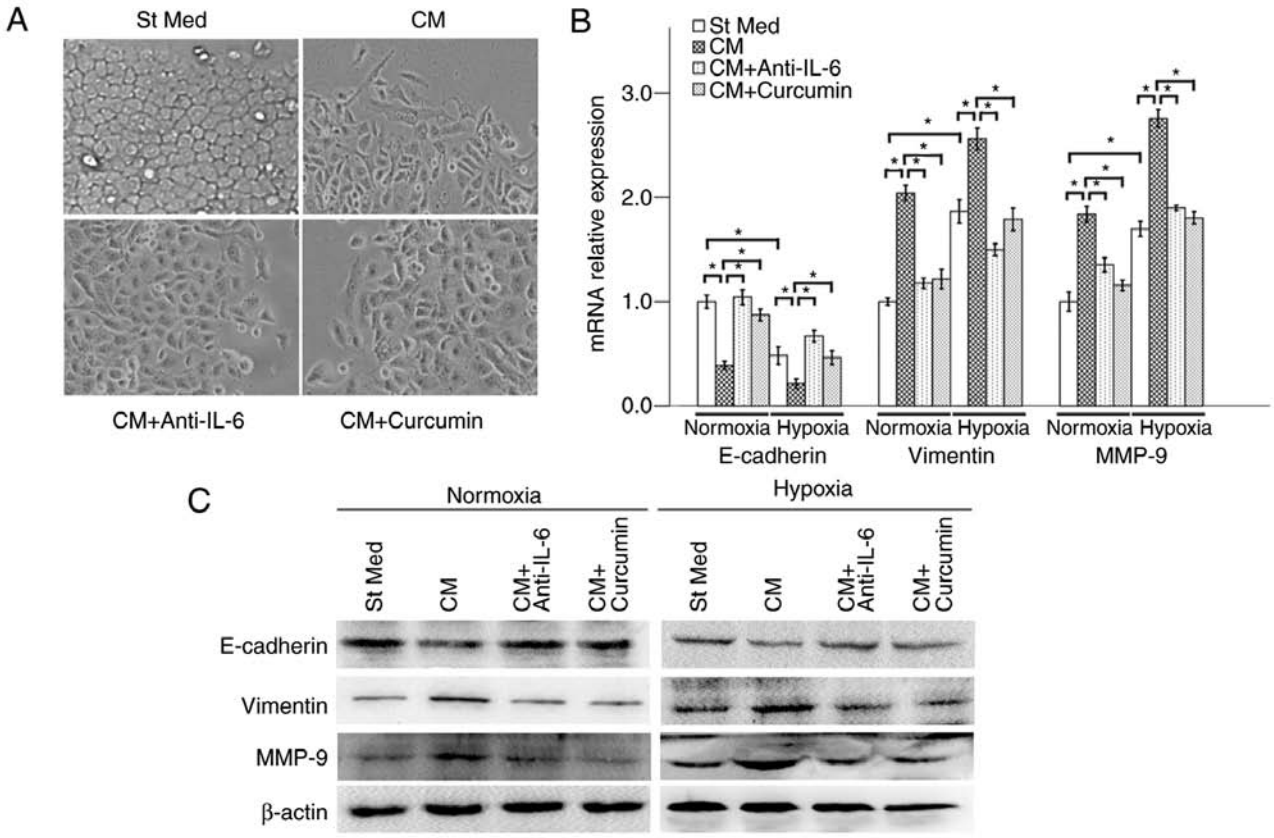

Figure 5. Curcumin inhibits hypoxia-induced PSC-CM-mediated EMT of pancreatic cancer cells. (A) BxPC-3 cells were treated with PSC-CM, IL-6 neutralizing antibody (5 μg/ml) or 20 μM curcumin, and the occurrence of EMT was evaluated based on the morphological changes displayed by cancer cells. Hypoxia and PSC-CM decreased the expression of E-cadherin and increased the expression of vimentin and MMP-9 at the (B) mRNA and (C) protein levels. These effects were counterbalanced by neutralizing antibody against IL-6 and curcumin. *P<0.05. The data are representative of 3 independent experiments. PSC, pancreatic stellate cell; CM, conditioned medium; EMT, epthelial-to-mesenchymal transition; MMP, matrix metallopeptidase.

cells in our previous study (37). PD 98059 was used to inhibit the ERK phosphorylation, in order to verify that this process occurs upstream of ERK signaling. The result demonstrated that PD 98059 not only inhibited hypoxia-induced expression of p-ERK and p-NF-κB, but also decreased pancreatic cancer cell invasion under hypoxic conditions. These results indicated that hypoxia can promote the invasion and EMT of pancreatic cancer cells by interfering with tumor-stromal crosstalk via the IL-6/ERK/NF-κB axis.

As a pro-inflammatory cytokine, IL-6 is involved in multiple biological processes, including tumor progression. In the cancer microenvironment, IL-6 is produced by a number of cell types, including fibroblast stromal cells, immune cells, and even tumor cells (38). It has been reported that IL-6 is closely associated with pancreatic cancer development, progression, invasion, EMT and metastasis by activating Janus kinase 2 (JAK2)/STAT3, mitogen-activated protein kinase (MAPK) and other signaling pathways that contribute to oncogenesis (39). High circulating levels of IL-6 have been associated with short overall survival in gastrointestinal cancer patients (38). In a study using an orthotopic xenograft model with pancreatic cancer cells in SCID/bg mice, Goumas *et al* (40) demonstrated that treatment with the anti-IL-6-receptor antibody tocilizumab resulted in a marked decrease in tumor weight and new distant metastases compared with the control group. The results of the present study also indicated that hypoxia can increase the production of IL-6 by PSCs. IL-6 treatment or hypoxia-induced PSC-CM promoted the invasion of pancreatic cancer cells. PSC-CM also promoted the EMT of BxPC-3 and Panc-1 cells, which was counterbalanced by the anti-IL-6 antibody or curcumin.

Curcumin (diferuloylmethane) has been proven to suppress the proliferation, invasion, EMT and metastasis of a variety of tumors, including pancreatic cancer (11). Curcumin exerts its anticancer effects via targeting multiple signaling pathways, such as the MAPK and NF-κB signaling pathways. Our recent study demonstrated that curcumin attenuated hyperglycemia-driven epidermal growth factor (EGF)-induced invasive and migratory abilities of pancreatic cancer cells by inhibiting the EGF/EGFR signaling pathway and its downstream signaling molecules, including ERK and Akt (15). Curcumin may also play an important role in suppressing superoxide dismutase-driven hydrogen peroxide -induced pancreatic cancer EMT and metastasis by inhibiting the phosphoinositide 3 kinase/Akt/NF-κB pathway (41). In recent years, an increasing number of studies have been focusing on the role of curcumin in the tumor microenvironment. Shao *et al* (42) recently revealed that curcumin exerts a promising therapeutic effect on hepatic stellate cell-induced hepatocellular carcinoma invasion and angiogenesis via downregulating CTGF. Wang *et al* (43) also provided evidence that curcumin was able to suppress the migration, EMT and metastasis of pancreatic

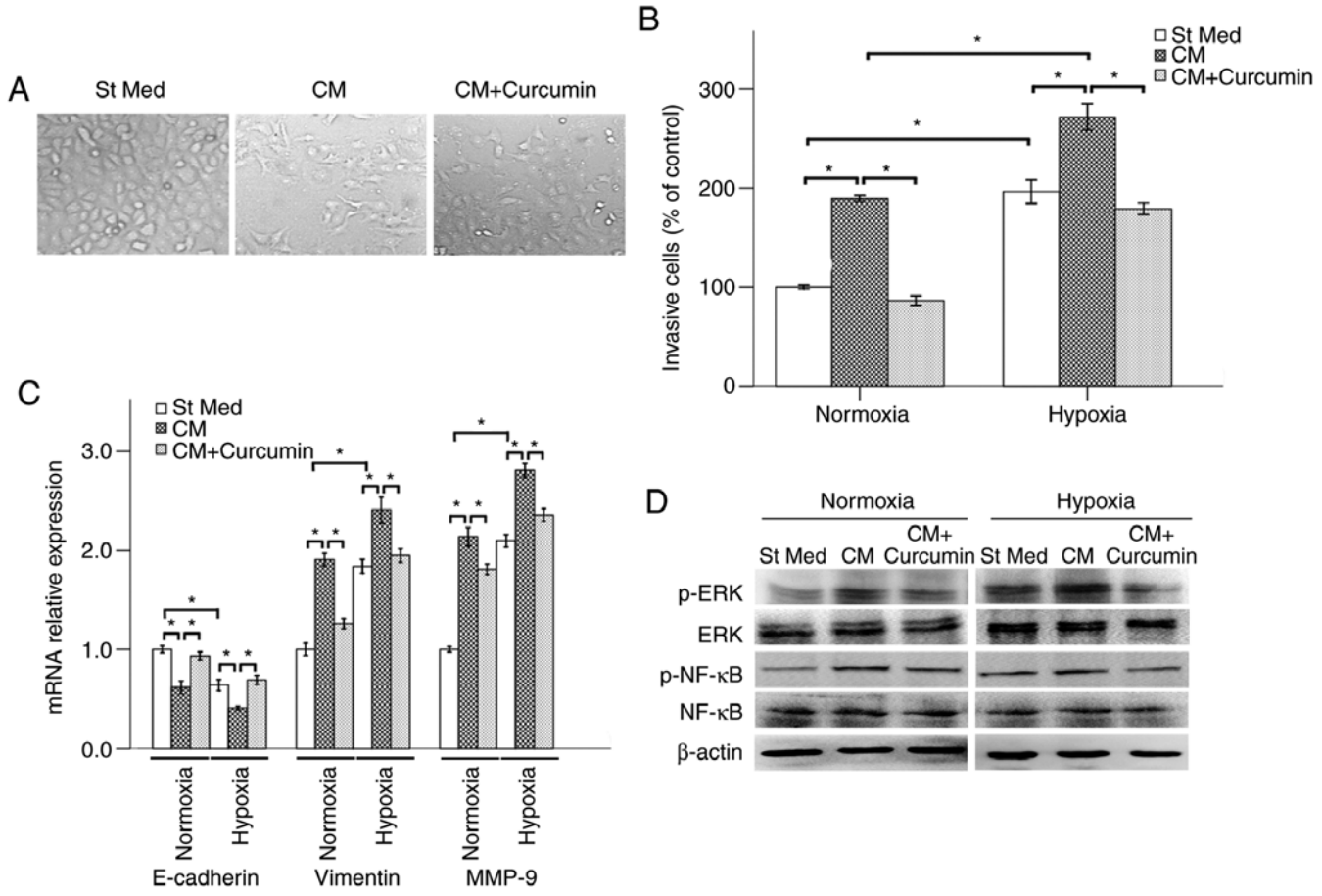

Figure 6. Effect of curcumin on hypoxia and PSC-CM induced EMT of Panc-1 cells. (A) Panc-1 cells were treated with PSC-CM or 20 μM curcumin, and the occurrence of EMT was evaluated based on morphological changes displayed by cancer cells. (B) Panc-1 cells treated with PSC-CM or 20 μM curcumin under both hypoxic and normoxic conditions were allowed to migrate for 48 h. Curcumin inhibited hypoxia- or PSC-CM-induced invasion of Panc-1 cells. The number of migrated cells was quantified by counting the cells from 3 random fields at a magnification of x200. (C) Hypoxia and PSC-CM modulated the expression of E-cadherin, vimentin and MMP-9 at the mRNA level, which was reversed by curcumin. (D) Hypoxia and hypoxia-induced PSC-CM increased the expression of p-ERK and p-NF-κB in Panc-1 cells, and these effects were counterbalanced by curcumin. *P<0.05. The data are representative of 3 independent experiments. PSC, pancreatic stellate cell; CM, conditioned medium; EMT, epthelial-to-mesenchymal transition; MMP, matrix metallopeptidase; ERK, extracelular signal-regulated kinase.

cancer cells by reducing the mesenchymal characteristics of cancer-associated fibroblasts. The data presented herein indicate that curcumin inhibited hypoxia-activated migratory ability and IL-6 secretion in PSCs. Curcumin may also abrogate hypoxia-induced PSC-CM-mediated invasive ability and EMT of pancreatic cancer cells. The focus of our future study will be to investigate the components of the pathway directly activated by IL-6, such as JAK2, STAT3 or Nrf2, which was a limitation of the present study. Interestingly, in the present study it was also observed that curcumin was able to inhibit α-SMA expression and cell migration under normoxic conditions. The effect of curcumin on these processes appeared to be independent of IL-6, since this molecule was not induced under normoxia in pancreatic cancer cells. As curcumin can inhibit several important factors secreted by stromal cells, including SDF-1 and CTGF (42), future work will also focus on these factors. Curcumin has also been applied in the clinical setting, either alone or in combination with other drugs. In a phase I/II study, gemcitabine-resistant patients with pancreatic cancer received oral curcumin daily (8 g) in combination with gemcitabine-based chemotherapy. The result demonstrated that curcumin was safe and able to increase the median survival time (161 days) when compared with the gemcitabine alone group, the mean survival rate of which was ~10 weeks (44). Our future research may also include more *in vivo* experiments as well as preclinical trials to further validate the present findings.

In conclusion, the present study demonstrated that curcumin plays an important role in suppressing tumor-stromal crosstalk and pancreatic cancer cell invasion and EMT by inhibiting the IL-6/ERK/NF-κB axis under hypoxic conditions. Therefore, curcumin may hold promise as a potential anticancer agent for the treatment of patients with pancreatic cancer.

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Availability of data and materials

The datasets generated and/or analyzed during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

WL, QM and ZWa conceived and designed the study; WL, LS and JL conducted the experiments; ZWu and JL performed the data analysis; WL wrote the paper; ZWa and QM reviewed and edited the manuscript. All the authors have read and approved the final version of the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The experimental protocol and patient consent forms were approved by the Ethics Committee of the First Affiliated Hospital of Xi'an Jiaotong University (Xi'an, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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