

# Upregulation of autophagy by hypoxia-inducible factor-1 $\alpha$ promotes EMT and metastatic ability of CD133<sup>+</sup> pancreatic cancer stem-like cells during intermittent hypoxia

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**Abstract.** Epithelial-to-mesenchymal transition (EMT) facilitates the escape of pancreatic cancer cells from the primary tumor site, which is a key early event in metastasis. In the present study, we examined if intermittent hypoxia facilitates the invasiveness of human pancreatic cancer cell lines (Panc-1 and BxPC-3) by Transwell assay. We used western blotting and flow cytometry analysis to quantify stem-like cells in the migratory cells during intermittent hypoxia in the human pancreatic cancer cells. Under normoxia or intermittent hypoxia, the expression of autophagy-related proteins (LC3-II and Beclin), hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) and EMT-related markers (E-cadherin, Vimentin and N-cadherin) was examined by western blotting. siRNA and the autophagic inhibitor were used to access the role of HIF-1 $\alpha$  and autophagy in promoting metastasis and EMT. Under intermittent hypoxia, pancreatic cancer cells demonstrated enhanced invasive ability and enriched stem-like cells. The migratory cells displayed stem-like cell characteristics and elevated the expression of LC3-II and Beclin-1, HIF-1 $\alpha$ , E-cadherin, Vimentin and N-cadherin under intermittent hypoxia conditions. Moreover, enhanced autophagy was induced by the elevated level of HIF-1 $\alpha$ . The metastatic ability and EMT of pancreatic cancer

stem cells was associated with HIF-1 $\alpha$  and autophagy. This novel finding may indicate the specific role of HIF-1 $\alpha$  and autophagy in promoting the metastatic ability of pancreatic cancer stem cells. Additionally, it emphasizes the importance of developing therapeutic strategies targeting cancer stem cells and autophagy to reduce metastasis.

## Introduction

Pancreatic cancer is the fourth leading cause of cancer-related mortality in the US. Metastasis is the cause of pancreatic cancer fatality. Approximately 80% of patients are diagnosed with pancreatic cancer at a locally advanced or metastatic stage (1,2). An approach that inhibits the metastatic, invasive or cell migratory abilities of this cancer may facilitate the development of an effective strategy for changing the natural progress of this malignancy and producing marked improvements in patient survival rates.

A common feature of the environment is the presence of hypoxic areas within the tumor mass that develop when the high proliferation rate of tumor cells outstrips vasculature development. Hypoxia may be associated with the generation of a more invasive phenotype of tumor cells and tumor cell dissemination (3-5). Hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) is a central transcription factor that mediates hypoxia responsive genes and has been widely accepted to play a critical role in tumor invasion, metastasis, due to its increased cell survival, angiogenesis and cell migration and invasion. However, HIF-1 $\alpha$  has broad influence on tumor biology and its new roles in the malignant progression are still under investigation.

Hypoxia commonly results in autophagy which is involved in the process whereby cells deliver their own protein and organelle to lysosomes for degradation (6,7). Autophagy is an evolutionarily conserved catabolic pathway and facilitates the removal of ROS-altered mitochondria, and reduces oxidative stress (8). It is well known that upregulation of autophagy during hypoxia can favor tumor cell survival and growth.

Epithelial-to-mesenchymal transition (EMT) phenomena endow epithelial cells with enhanced migratory and invasive potential and, as such, have been implicated in many

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*Abbreviations:* CSCs, cancer stem cells; HIF-1 $\alpha$ , hypoxia-inducible factor-1 $\alpha$ ; PCR, polymerase chain reaction; CD133, cluster of differentiation 133; CD44, cluster of differentiation 44; 3-MA, 3-methyladenine; OCT-4, octamer-binding transcription factor-4

*Key words:* pancreatic cancer stem cell, HIF-1 $\alpha$ , autophagy, Epithelial-to-mesenchymal transition

physiological and pathological processes requiring cell migration (9,10). In cancer, EMT is a key process that contributes to cancer metastasis and is marked by a loss of epithelial features (E-cadherin) and an upregulation of mesenchymal properties (Vimentin and N-cadherin). Recent studies have implicated EMT as a process that is associated with cancer stem cells (CSCs) (11,12). It is now evident that sustained metastatic growth requires the dissemination of CSCs from the primary tumor followed by their re-establishment in a secondary site.

In the present study, we examined the effect of intermittent hypoxia on cell migration and expression of CSC-related markers in pancreatic carcinoma cells under intermittent hypoxic conditions including LC3-II and Beclin, HIF-1 $\alpha$ , E-cadherin, Vimentin and N-cadherin. We also investigated the role of HIF-1 $\alpha$  in the regulation of autophagy. Finally, we examined the effect of HIF-1 $\alpha$  induced autophagy on the expression of CSC-related markers including E-cadherin, Vimentin and N-cadherin.

## Materials and methods

**Cell culture.** Panc-1 and BxPC3 were obtained from the Cell Bank of China Academy of Sciences (Shanghai, China). They were maintained in Dulbecco's modified Eagle's medium (DMEM)-F12 supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 100 U/ml penicillin and 100 U/ml streptomycin. To propagate the CSC-like fraction of the pancreatic cancer cells, culture conditions favoring proliferation of undifferentiated cells were used. We cultured the cells in serum-free DMEM-F12 medium containing insulin (Gibco), EGF and FGF (PeproTech, Rocky Hill, NJ, USA), B-27 (Gibco), in low-attachment dishes (Corning, Corning, NY, USA). Cells were passaged with 0.25% trypsin/EDTA every 3 days.

**Transwell assay.** Cell migration assay was performed in Boyden chamber using 8- $\mu$ m pore size filters. Briefly, 1x10<sup>4</sup> viable cells suspended in serum-free DMEM-F12 were allowed to migrate for 12 h toward DMEM-12 containing 10% FBS under the intermittent hypoxia and normoxia conditions, respectively. At the end of the assay, cells in the upper chamber and on the upper filter surface were removed, whereas cells on the lower filter surface were fixed with ethanol and stained with Giemsa. The number of migrating cells was determined by counting cells in 10 random fields at x200 magnification. All experiments were performed in triplicates.

**Intermittent hypoxia and normoxia environmental exposure.** The bulk and migrated pancreatic cancer cells were exposed to 5 cycles of hypoxia and normoxia. Each cycle consisted of a period of 12 h in hypoxia followed by 12 h recovery under normoxic conditions. The medium was changed during the re-oxygenation period. For hypoxia induction, cells were cultured in hypoxia chambers (Sanyo; containing 1% O<sub>2</sub>, 5% CO<sub>2</sub>, 94% N<sub>2</sub>). Nitrogen gas was supplied to the chambers to induce a controlled reduced percentage of oxygen. For normoxia, cells were cultured in incubators containing 5% CO<sub>2</sub> and ~20% O<sub>2</sub>.

**siRNA knockdown of HIF-1 $\alpha$  gene and chemical treatment.** Pancreatic cancer cells were seeded in 12-well plates. When

the cell density reached 50% confluence, two experiment conditions were established: i) the cells were treated with increasing concentrations of deferoxamine CoCl<sub>2</sub> (0, 200 and 400  $\mu$ M; Sigma, United Kingdom) for 48 h; ii) the cells were transfected with either 40 nmol/l control siRNA or HIF-1 $\alpha$ -specific siRNA (Suzhou Ribo Life Science Co., Ltd.). Transfections were carried out according to the manufacturer's instructions. Then, the cells were put in intermittent hypoxic and normoxic conditions, respectively. For the autophagy inhibition experiment, the cells were treated with 30  $\mu$ l of 3-methyladenine (3-MA) at the concentration of 10 mM and continually cultured for 24 h.

**Western blot analysis.** Protein concentrations were determined by the BCA method. Cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Merck Millipore, USA). Membranes were blocked with 5% (w/v) bovine serum albumin (BSA) in TBST for 1 h at room temperature and incubated overnight with primary antibodies at 4°C. They were subsequently incubated with horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature. Cell results were normalized to  $\beta$ -actin as appropriate. The immunoreactive bands were detected by chemiluminescence (ECL Plus; Merck Millipore) and relevant blots were quantified by densitometry using Lane 1D software. Immunoblotting with primary antibodies was as follows: rabbit anti-human-E-ca, rabbit anti-human-Vimentin, rabbit anti-human-N-ca, rabbit anti-human-Oct-4, mouse anti-human-Sox-2, rabbit anti-human-HIF-1 $\alpha$ , rabbit anti-human-LC3 and rabbit anti-human-MMP-9. All antibodies were obtained from Cell Signaling Technology, Inc. (Boston, MA, USA). Anti- $\beta$ -actin was obtained from Abcam (Cambridge, MA, USA). The secondary antibody preparations either anti-rabbit or anti-mouse were purchased from Boster Biotechnology Company (Wuhan, China).

**Real-time PCR.** Real-time quantitative PCR was carried out with SYBR-Green qPCR SuperMix using the CFX-96 system (both from Bio-Rad, Hercules, CA, USA). Total cellular RNA was isolated using TRIzol reagent, and cDNA was synthesized from 1  $\mu$ g of total RNA using oligo(dT) and murine Moloney leukemia virus reverse transcriptase (Toyobo, Japan). Relative expression levels of the genes were calculated using the 2<sup>- $\Delta\Delta$ CT</sup> method.

**Flow cytometry analysis.** To quantify the stem-like cells of the migrated pancreatic cancer cells under intermittent hypoxic and normoxic conditions, we measured the expression of the stem-related molecular marker CD133 using anti-CD133-PE (Miltenyi Biotec Ltd., Surrey, UK). Cells (5x10<sup>6</sup>) were harvested, disaggregated to a single cell suspension and stained with mouse anti-human CD133 PE. The antibody was incubated for 30 min at 4°C in the dark. Following incubation, the samples were washed with PBS and analyzed by FACSAria II (Becton-Dickinson, USA).

**Statistical analysis.** The significance of differences between groups was analyzed using the Student's t-test, one-way or two-way ANOVA. Values of P<0.05 were considered to indi-

cate a statistically significant difference. All experiments were performed at least in triplicate.

## Results

*Intermittent hypoxia increases the in vitro migration potential and promotes EMT in pancreatic cancer cells.* It was reported that hypoxia is strongly associated with tumor metastasis. Firstly, we evaluated the migration ability of pancreatic cancer cells under intermittent hypoxic conditions. Transwell assay results revealed that the number of Panc-1 and BxPC-3 cells under intermittent hypoxic conditions that passed through the membrane was 4-fold higher than the number of cells under normoxic conditions (Fig. 1A), suggesting that intermittent hypoxia promotes the invasive activity of pancreatic carcinoma cells. Secondly, to illustrate whether initiation of EMT was induced by intermittent hypoxia, we evaluated the expression level of EMT-related markers altered in the cells. The results demonstrated that expression of E-cadherin was significantly reduced in Panc-1 and PC-3 cells. In contrast, expression of Vimentin and N-cadherin were significantly increased in these cells (Fig. 1B). Furthermore, the percentage of fibroblast-like cells of Panc-1 and BxPC-3 cells under hypoxic conditions was higher than the cells cultured under normoxic conditions which often show an epithelial-like appearance (Fig. 1C).

*Intermittent hypoxia maintains stem cell properties and the expression of CSC markers in pancreatic carcinoma cells.* It is widely accepted that CSCs are highly associated with tumor growth, invasion and metastasis, and are commonly considered one of the major causes of tumor recurrence and relapse. The surface markers CD133 have been well defined for isolating and identifying CSCs from pancreatic cancer cells (13). The migrated Panc-1 cells were digested and re-cultured in the serum-free medium (SFM) under the intermittent hypoxic and normoxic conditions, respectively. The total Panc-1 cells cultured in normal medium (DMEM-F12 plus 10% FBS) under the normoxic conditions were used as the control group. Flow cytometry analysis assay was used to quantify the CD133<sup>+</sup> subpopulation. Cell quantification indicated that 65±5% of the migrated cells cultured in the serum-free medium under the intermittent hypoxic conditions were positive for CD133, whereas 30±3% of the migrated cells cultured in the serum-free medium under normoxic conditions were positive for CD133 and 10±3% for the control group (Fig. 2A). We next evaluated the role of intermittent hypoxia in the self-renewal capacity of the migrated cells using the sphere-formation assay. It successfully obtained the percentage of the spheres in the intermittent hypoxic conditions was much higher than the cells under the normoxic conditions. The migrated cells cultured in the serum-free medium under normoxic conditions formed only small irregular aggregates. Also, the total cells cultured in the normal medium under normoxic conditions were grown in the adherent way and no sphere was found (Fig. 2B). The western bolt assay was conducted to examine the effect of intermittent hypoxia on CSC signature proteins in the migrated cells. It was clearly found that expression of Oct-4 and Sox-2 was upregulated in intermittent hypoxia conditioned tumor cells (Fig. 2C). Similar results were acquired in the BxPC-3 cell line (data not shown). These data indicate that the migrated

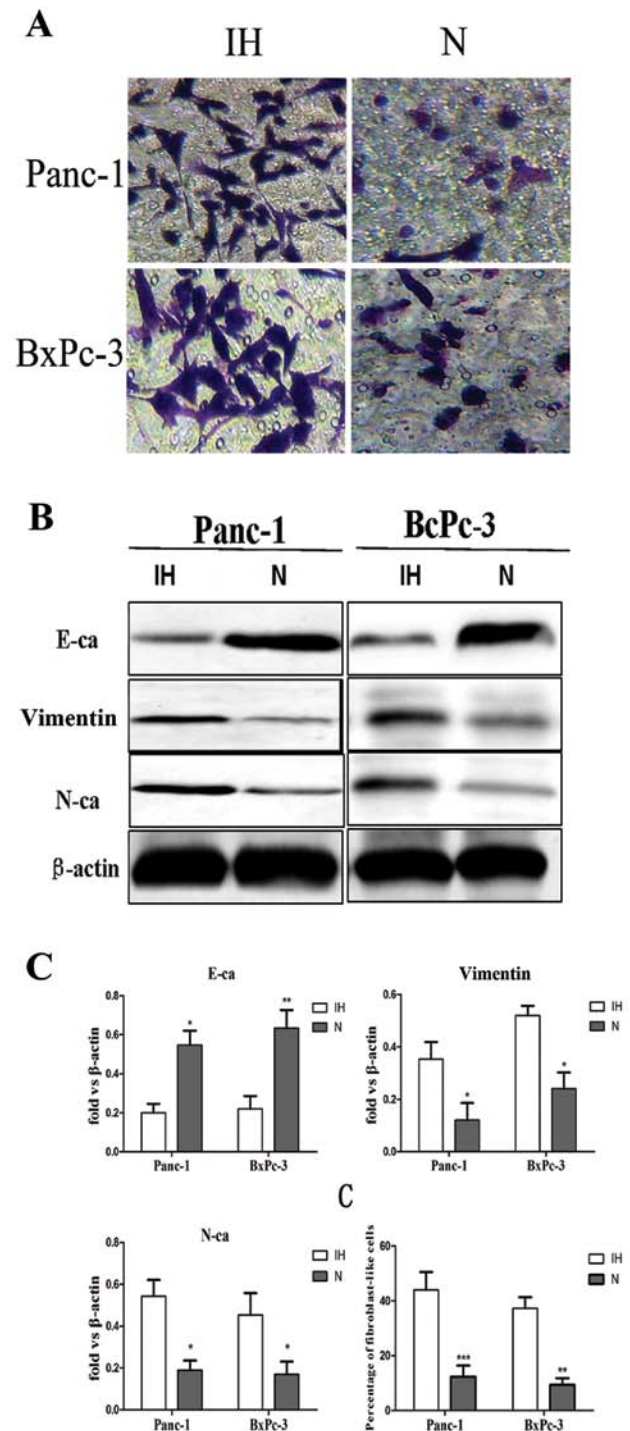


Figure 1. Migration potential and EMT of pancreatic cancer cells evaluated under intermittent hypoxia. (A) Phase-contrast images of the number of Panc-1 and BxPC-3 cells (x200) under intermittent hypoxic conditions passed through the membrane by the Trypan Blue staining. The number of cells under intermittent hypoxia was 4-fold higher than that under normoxia. (B) Expression of E-ca, Vimentin and N-ca proteins in the cells cultured under the intermittent hypoxic and normoxic conditions were detected by western blotting. Data were normalized to  $\beta$ -actin levels. Experiments were repeated three times with similar data. (C) The percentage of fibroblastic-like cells was assessed. Data are presented as the means  $\pm$  SD for three independent experiments. IH, the intermittent hypoxic conditions group; N, the normoxic conditions group. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

pancreatic cancer cell lines with stem cell-like properties were enriched and maintained under intermittent hypoxic conditions.

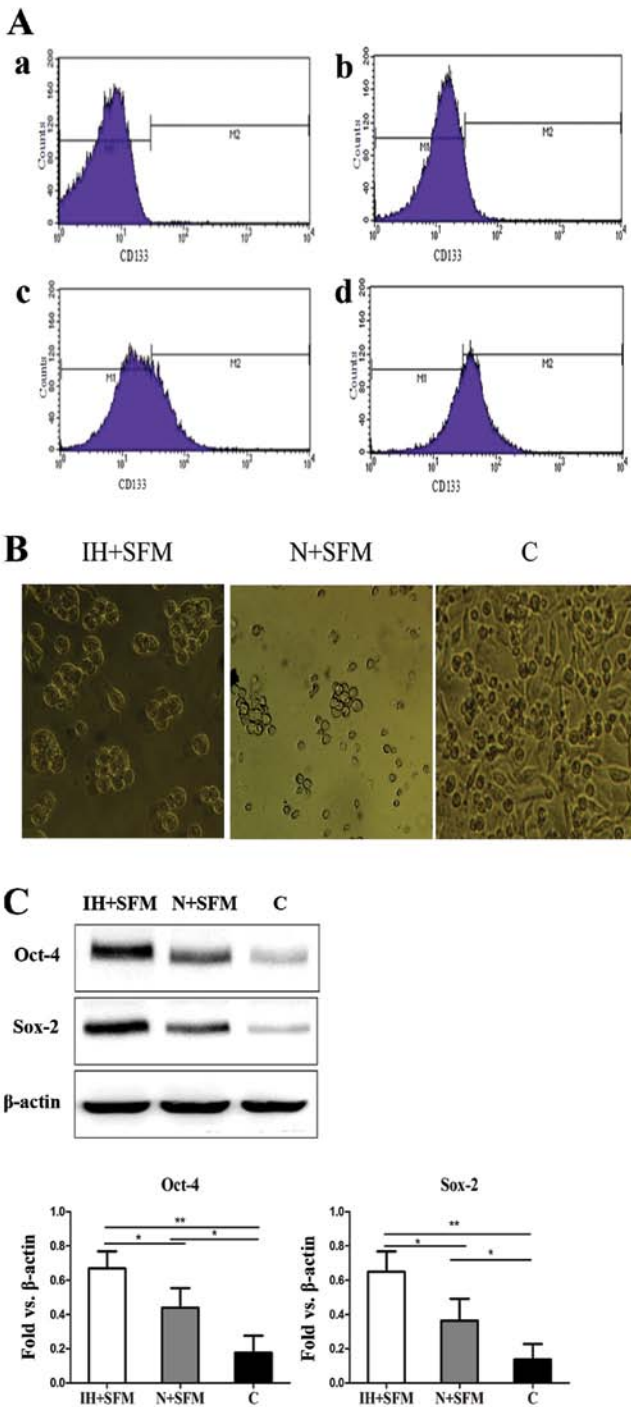


Figure 2. The CD133<sup>+</sup> stem-like cells enriched and maintained under intermittent hypoxic conditions. (A) The percentage of CD133<sup>+</sup> subpopulation was analyzed by flow cytometry. M2 represents the percentage of the cells that were positive for CD133. (A-a) Isotype control. (A-b) total pancreatic cancer cells cultured under the normoxic conditions group, (A-c) migrated cells cultured in the SFM under normoxic conditions group, (A-d) migrated cells cultured in the SFM under intermittent hypoxic conditions group. (B) The cells' self-renewal ability in the SFM was assessed. More regular spheres were detected when the migrated cells were cultured in SFM under intermittent hypoxic conditions. (C) Expression of Oct-4 and Sox-2 proteins in the cells cultured in the SFM under the intermittent hypoxic and normoxic conditions were detected by western blotting. Data were normalized to  $\beta$ -actin levels. Experiments were repeated three times with similar data. IH+SFM, the migrated cells cultured in the SFM under intermittent hypoxic conditions group; N+SFM, the migrated cells cultured in the SFM under normoxic conditions group; C, total pancreatic cancer cells cultured under the normoxic conditions group. \* $P < 0.05$ , \*\* $P < 0.01$ . SFM, serum-free medium; IH, intermittent hypoxia.

*Intermittent hypoxia upregulates the expression level of HIF-1 $\alpha$  and induces autophagy in pancreatic cancer cells.* The migrated Panc-1 cells were cultured under intermittent hypoxic conditions for various time periods (0, 12, 24, 48 and 72 h). The results confirmed that HIF-1 $\alpha$  was rapidly induced in migrated cells under intermittent hypoxic conditions by western blotting. Also, HIF-1 $\alpha$  protein level increased in a time-dependent manner (Fig. 3A). To assess whether intermittent hypoxia affects the level of autophagy, we evaluated mRNA expression of Beclin-1, ATG5 in pancreatic cancer cells under intermittent hypoxic conditions by RT-PCR. The pancreatic cancer cells cultured under normoxic conditions were used as control. Beclin-1 and ATG5 are all involved in autophagosome formation. Higher expression of Beclin-1 and ATG5 was detected by RT-PCR in the intermittent hypoxia group (Fig. 3B). LC3 was used as a measure of downstream autophagy activation. Both cell lines under intermittent hypoxic conditions showed enhanced expression of LC3-II protein and expressed a higher LC3-II/LC3-I ratio compared to the cells under the normoxic conditions (Fig. 3A).

*Higher level autophagy is associated with upregulated HIF-1 $\alpha$ .* It was confirmed that HIF-1 plays a critical role in the cellular transcriptional response to hypoxia. Having demonstrated that the level of autophagy and HIF-1 $\alpha$  was elevated together under the intermittent hypoxic conditions, we further investigated the association between HIF-1 $\alpha$  induction and autophagy upregulation. To examine whether HIF-1 $\alpha$  mediates hypoxia-induced autophagy enhancement, pancreatic cancer cells were treated with increasing concentrations of Cobalt chloride (CoCl<sub>2</sub>, hypoxia surrogates) for 48 h. Western blot assay was employed to assess the protein level changes. Results confirmed that CoCl<sub>2</sub> treatment induced HIF-1 $\alpha$  and autophagy-related protein expression level upregulation. The change occurred in a dose-dependent manner (Fig. 4A). Furthermore, suppression of HIF-1 $\alpha$  expression by siRNA in pancreatic cancer cells abolished hypoxia-induced autophagy upregulation (Fig. 4B).

*Hypoxia-induced EMT phenotype is mediated by HIF-1 $\alpha$  and autophagy.* Given that intermittent hypoxia can promote the migration ability of pancreatic cancer cells and induce EMT, we next investigated whether HIF-1 $\alpha$ -induced autophagy has an effect on invasion and EMT in pancreatic cancer cells. Under intermittent hypoxic conditions, we carried out the knockdown of HIF-1 $\alpha$  expression in pancreatic cancer cells by siRNA and inhibition of autophagy with 3-MA (the inhibitor of autophagy). As shown in Fig. 5A, HIF-1 $\alpha$  siRNA with or without 3-MA treatment inhibited the capacity of intermittent hypoxia-induced invasion of both Panc-1 and BxPC-3 cells by *in vitro* invasion assay. Results demonstrated that the percentage of the cells whose morphology appeared to change from a fibroblast-like to an epithelial-like appearance was decreased after the inhibition. Also, the expression level of E-cadherin was upregulated, and Vimentin and MMP-9 protein levels were downregulated (Fig. 5B). These data suggest that HIF-1 $\alpha$ -induced autophagy promotes hypoxia-induced cell migration and invasion of human pancreatic cancer cells.

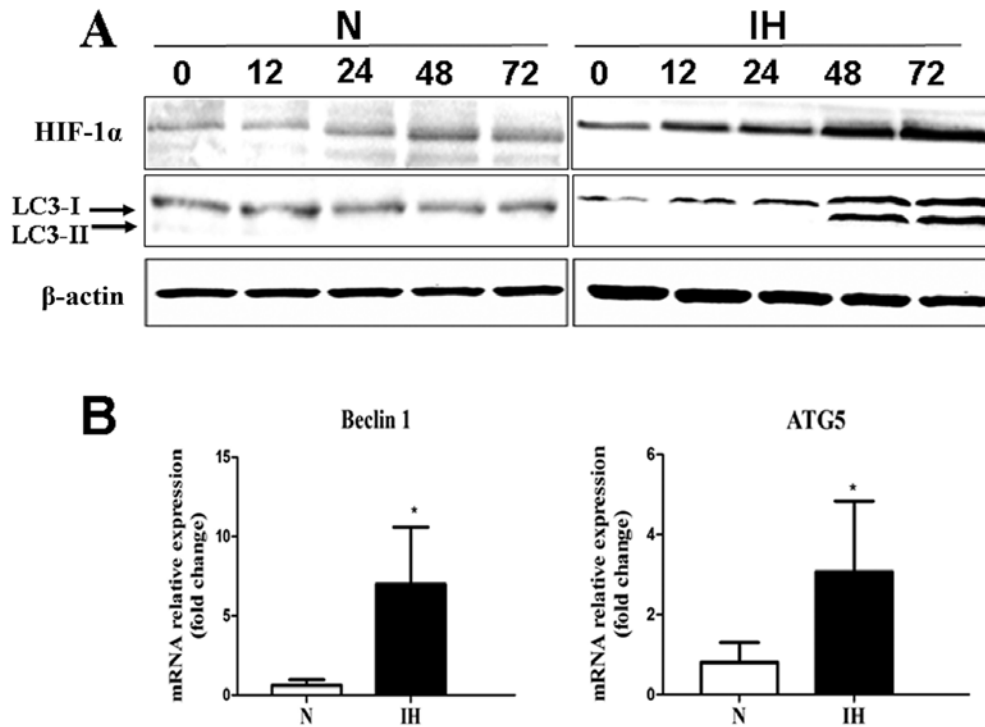


Figure 3. The expression level of HIF-1 $\alpha$  and autophagy-related genes and proteins is enhanced in pancreatic cancer cells under intermittent hypoxia conditions. (A) The migrated pancreatic cancer cells were treated with intermittent hypoxia (IH) and normoxia (N) for the indicated times (0, 12, 24, 48 and 72 h). Expression of HIF-1 $\alpha$ , LC3-II proteins in the migrated pancreatic cancer cells under the IH and N conditions were detected by western blotting. Data were normalized to  $\beta$ -actin levels. (B) Beclin-1, Atg-5 gene expression of the migrated pancreatic cancer cells under the IH and N conditions were detected by RT-PCR. Data were normalized to  $\beta$ -actin levels. Experiments were repeated three times with similar data. IH represents the intermittent hypoxic conditions group; N, represents the normoxic conditions group. \* $P < 0.05$ . HIF-1 $\alpha$ , hypoxia-inducible factor-1 $\alpha$ .

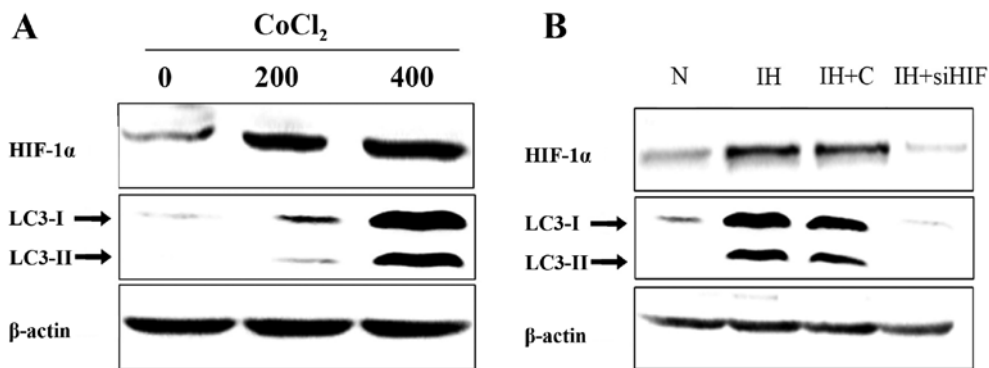


Figure 4. Elevated autophagy is associated with upregulated HIF-1 $\alpha$ . (A) Expression of HIF-1 $\alpha$  and LC3-II in the migrated pancreatic cancer cells treated with increasing concentrations of CoCl<sub>2</sub> was analyzed by western blotting. (B) The migrated pancreatic cancer cells were transfected with non-targeted control siRNA and siRNA directed against HIF-1 $\alpha$ . The levels of HIF-1 $\alpha$  and LC3-II were analyzed after 72 h of intermittent hypoxic conditions by western blotting. Data were normalized to  $\beta$ -actin levels. Experiments were repeated three times with similar data. N, the normoxic conditions group; IH, the intermittent hypoxic conditions group; IH+C, cells treated with non-targeted control siRNA under intermittent hypoxic conditions group; IH+siHIF, cells treated with HIF-1 $\alpha$  special targeted siRNA under intermittent hypoxic conditions group. HIF-1 $\alpha$ , hypoxia-inducible factor-1 $\alpha$ .

## Discussion

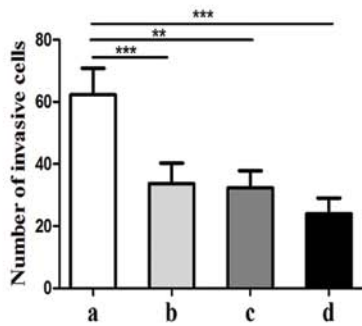
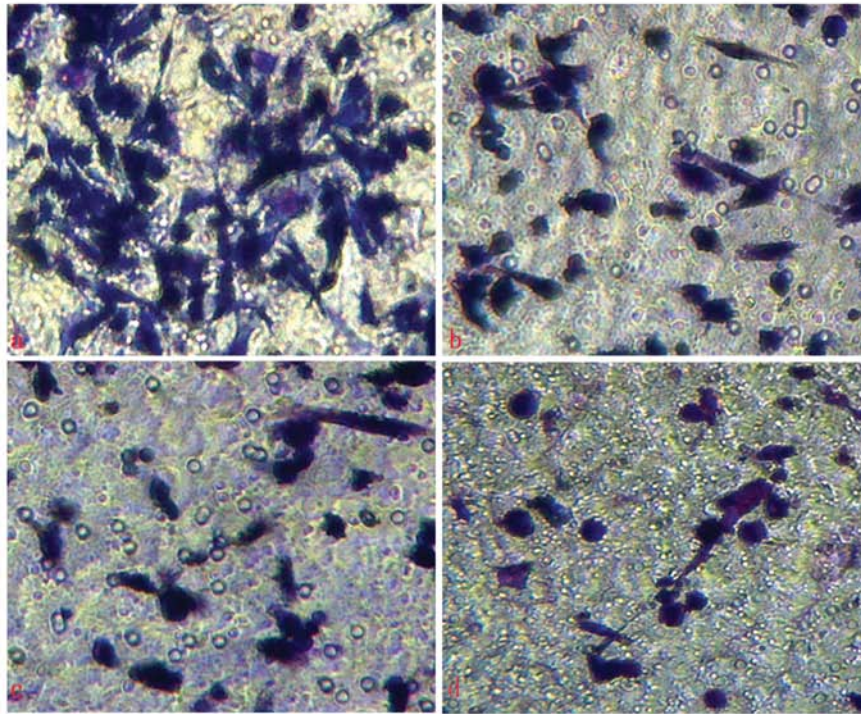
Pancreatic carcinoma remains one of the most aggressive diseases with only a modest improvement in 5-year survival rates with new and improved cancer therapies. Most deaths due to pancreatic cancer are correlated with metastasis of the primary tumor rather than development of the primary tumor, yet our understanding of this complex problem remains limited. As such, gaining an understanding of pancreatic

cancer metastasis could considerably assist in selecting the appropriate therapy strategy and may increase patient survival rates.

Metastasis mainly consists of four steps; namely, primary tumor cells enter the circulatory system, survival of circulating tumor cells (CTCs), movement from the circulation into a secondary tissue, and tumor growth at a secondary site (14). An emerging concept for metastasis is that cellular plasticity is associated with EMT. EMT is defined as a



A



B

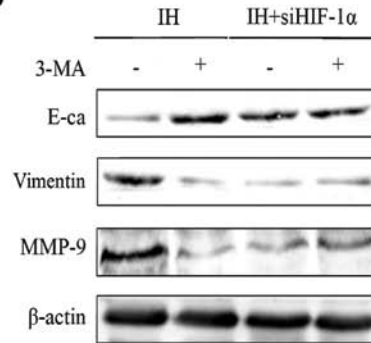


Figure 5. Blockade of HIF-1 $\alpha$  and autophagy reduce EMT and metastatic ability of pancreatic cancer cells. (A) Phase-contrast images of the number of Panc-1 and BxPC-3 cells (x200) that passed through the membrane under different conditions by the trypan blue staining. The number of cells under intermittent hypoxia was markedly reduced after the inhibition of HIF-1 $\alpha$  and autophagy. (A-a) The total pancreatic cancer cells cultured under intermittent hypoxia without HIF-1 $\alpha$  siRNA and 3-MA which was used as control; (A-b) the total pancreatic cancer cells treated with 3-MA cultured under intermittent hypoxia; (A-c) the total pancreatic cancer cells treated with HIF-1 $\alpha$  siRNA cultured under intermittent hypoxia; (A-d) the total pancreatic cancer cells cultured under intermittent hypoxia with HIF-1 $\alpha$  siRNA and 3-MA. (B) Western blot analyses for EMT-related protein (E-ca, Vimentin and MMP-9) level changes during intermittent hypoxia with or without HIF-1 $\alpha$  siRNA and 3-MA. Data were normalized to  $\beta$ -actin levels. The experiment was repeated three times and the data are expressed as means  $\pm$  standard deviation. The difference between these three groups was significant. \*\*P<0.01, \*\*\*P<0.001. HIF-1 $\alpha$ , hypoxia-inducible factor-1 $\alpha$ ; 3-MA, 3-methyladenine.

biologic process that allows a polarized epithelial cell, that normally interacts with the basement membrane via its basal surface, to undergo multiple biochemical changes that enable it to assume a mesenchymal cell phenotype, which includes enhanced migratory capacity, invasiveness, elevated resistance to apoptosis, and greatly increased production of extracellular matrix components (15-17). In addition to weakening cell-cell adhesion, EMT provides tumor cells with an enhanced ability to degrade the extracellular matrix, a property which favors cell invasion and intravasation. Indeed, EMT can induce the expression of proteases, such as different MMPs, that can

degrade the basement membrane (18). Although the involvement of EMT processes in the metastatic cascade remains a subject of debate, an increasing number of studies demonstrate their involvement in increased cell migration and invasion. Our results confirmed that intermittent hypoxia-induced EMT enhanced the pancreatic cancer cell migratory capacity, illustrating the importance of epithelial-mesenchymal plasticity as a metastasis-promoting property. CSCs, which comprise a small fraction of cancer cells, are believed to constitute the origin of most human tumors (19). Several studies also suggest that CSCs serve as the basis of metastases. Our results demon-

strated that CSCs were enriched in the migrated pancreatic cancer cells which were EMT-induced. EMT programs may provide a ready source of CSCs by enabling the dedifferentiation of more epithelial cells within carcinomas.

Tumor metastasis is driven not only by the accumulation of intrinsic alterations in malignant cells, but also by the interactions of cancer cells with the tumor microenvironment in which these cells are located, the niches (vascular proliferations, hypoxia/necrosis) (20-22). Hypoxia is a common phenomenon in malignant tumors including pancreatic carcinoma. Hypoxia is created in a tumor when the O<sub>2</sub> consumption outweighs the O<sub>2</sub> supply. Many studies have demonstrated that hypoxia generally correlates with tumor progression and metastasis (5,23,24). However, the molecular mechanism of hypoxia-mediated invasion and metastasis remains poorly understood. Several previous studies carried out experiments by acute or chronic hypoxia, but in the present study we used intermittent hypoxia which is characterized by cyclic periods of hypoxia and re-oxygenation. Intermittent hypoxia is described as more representative of the oxygen tension of the environment in tumors than a permanent exposure to low oxygen levels.

Tumor hypoxia has been reported to influence the tumor progression through the EMT process by regulation of a number of key factors. It has been observed that hypoxia-induced factor (HIF-1 $\alpha$ ), a key effector of hypoxia, activates Twist, Snail and SIP1 expression, thereby leading to E-cadherin repression (25-27). In the present study, we found that the upregulated HIF-1 $\alpha$  induced the EMT process in pancreatic cancer cells which was consistent with the previous study. However, the complex relationship between HIF-1 $\alpha$ , EMT and metastasis remains to be delineated. There are findings that implicate the importance of hypoxia and HIF-1 $\alpha$  in the induction of cancer metastasis beyond angiogenesis.

Cancer cells face diverse stresses, environmental and cellular, during every step of metastatic progression. To cope with this, tumor cells induce adaptive pathways, such as autophagy. Autophagy is an evolutionarily conserved catabolic pathway that degrades long-lived cellular macromolecules and can protect cells during various types of stress. It is involved in the process that cells deliver their own protein and organelle to lysosomes for degradation (28-30). A series of autophagy-related genes (Beclin-1, ATGs) regulate the process of autophagy (31). Signaling involves the conversion of LC3-I to LC3-II by binding to the membrane of autophagosomes after these vacuoles are formed (32). Indeed, autophagy has been found to be upregulated in cancer cells during many of the principal events directing metastasis, by which cells adapt their metabolism to the stresses induced by starvation, hypoxia, radiation or chemotherapeutic agents, thus allowing cells to evade apoptosis (32,33). In the present study, we found that autophagy can be induced by HIF-1 $\alpha$  under hypoxic stress. Upregulated autophagy further enhanced the EMT process and the migration ability in pancreatic cell lines. However, the function of HIF-1 $\alpha$  and their potential downstream targets in tumor autophagy require further clarification.

In the present study, we reported a model of EMT and metastasis that is generated by cooperation of tumor microenvironment with intrinsic genetic changes within a developing cancer cell.

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