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Original Article

Radiation promotes epithelial-to-mesenchymal transition and invasion of pancreatic cancer cell by activating carcinoma-associated fibroblasts

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Abstract: The tumor microenvironment is of crucial importance affecting treatment and prognosis. High degree of carcinoma-associated fibroblast (CAF) infiltration occurs in pancreatic cancer, though its effect on radiotherapy remains unclear. In this study, we demonstrated that radiation enhanced the migration- and invasion-promoting capacity of CAFs both in vitro and in vivo in a lung metastasis model. Radiation exposure increased the expression of CXCL12 by CAFs. CAF-derived CXCL12 promoted tumor cell EMT and invasion directly, acting through CXCR4 on pancreatic cancer cells. In addition, we showed that CXCL12-CXCR4 signaling promoted pancreatic cancer cell EMT and invasion by activating the P38 pathway. Therefore, our study concluded that radiation promoted pancreatic cancer cell invasion and EMT by activating CAFs, while inhibiting the CXCL12/CXCR4 interaction between pancreatic cancer cells and CAFs could potentially attenuate tumor cell invasion induced by radiation, which provides an opportunity for the development of novel therapeutic targets to improve the prognosis for human pancreatic cancer treated with radiation therapy.

Keywords: Radiation, carcinoma-associated fibroblasts, pancreatic cancer, invasion, epithelial-mesenchymal transition

Introduction

Pancreatic carcinoma (PC) is nearly uniformly lethal and is the fourth leading cause of cancer-related deaths in industrialized countries. The 5-year survival rate of patients with pancreatic cancer is estimated at 3%-5% [1]. While surgical resection is the most effective treatment option, disease is locally advanced or has undergone distant metastasis at the time of diagnosis in the vast majority of cases. The prognosis for advanced or metastatic PC is extremely poor, with a median survival time of only 2-4 months [2]. Chemoradiation is a potential treatment option, though combined modality therapy has only a modest effect [3-5]. Therefore, a better understanding of the mechanisms underlying pancreatic cancer dissemination and metastasis may lead to the development of novel therapeutic strategies to improve patient outcomes.

Radiotherapy to induce DNA damage in tumor cells has been the mainstay of treatment for many primary neoplasms. Radiotherapy has been recommended as a standard treatment for pancreatic cancer, especially locally advanced pancreatic cancer. However, the anatomical location and fibrotic nature of pancreatic cancer has hindered progress in radiotherapy despite gradual dose adjustments and advances in therapeutic techniques. A better understanding of the mechanisms underlying pancreatic carcinogenesis may facilitate development of new targeted therapies to improve patient prognosis.

Recently, several studies have described how the tumor microenvironment can provide a niche that enables cancer cells to escape the cytotoxic effects of chemoradiotherapy [6-8]. Pancreatic cancer is typically associated with a high degree of stromal cell infiltration, with

fibroblasts constituting a major proportion of the stromal cell population [9, 10]. Carcinoma-associated fibroblasts (CAFs) are believed to promote tumor progression by sustaining proliferative signaling, enabling evasion of growth suppressors, promoting angiogenesis, and inducing epithelial-mesenchymal transition (EMT) [11-15]. Recent studies have suggested that CAFs act as a physical barrier to the delivery of cytotoxic chemotherapeutic agents to the peritumoral milieu. Another study has revealed that irradiation can convert fibroblasts into CAFs and stimulate pro-invasive activity [16-18]. However, the role of CAFs in radiotherapy remains unclear.

In this study, we investigated the effects of CAFs on pancreatic cancer cells following exposure to radiation. An *in vitro* model was generated by co-culturing ionizing radiation (IR)-exposed CAFs with PC cells and revealed that irradiation can significantly enhance the invasion-promoting capacity of CAFs in pancreatic cancer. We also identified that CAFs secret high concentrations of CXCL12 following radiotherapy. Our functional studies revealed that IR-exposed CAFs can promote pancreatic cancer cell migration, invasion, and ultimately EMT. Furthermore, CXCL12 plays an essential role in this process. Our results indicate that while radiotherapy can kill pancreatic cancer cells it may also induce CAFs to promote tumor cell invasion and EMT. Therefore, concurrent targeting of CAFs may be a promising new adjunct treatment to enhance radiotherapy for pancreatic cancer.

Materials and methods

Cell Culture

The human pancreatic cancer cell lines PANC-1 and MIA PaCa-2 were purchased from the American Type Culture Collection (Manassas, VA). Pancreatic normal fibroblasts (NF) and pancreatic CAFs were isolated from surgically-resected pancreatic cancer tissues in Fudan University Shanghai Cancer Center (Shanghai, China). Written informed consent was obtained before tissue collection. The establishment and confirmation of NF and CAF have been reported previously [19]. All cell lines were maintained in culture in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Scientific, Logan, UT) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA) and 100 μg/mL each of penicillin and streptomycin (Invitrogen, Carlsbad, CA). Cells were grown at 37°C with 5% CO_2 in a humidified atmosphere. All cell lines used in this study were tested for absence of mycoplasma contamination and authenticated by morphologic observation (MycoAlert, Lonza, Rockland, ME) 3 months ago.

RNA isolation and quantitative real-time PCR

Total RNA was isolated from pancreatic stromal fibroblasts and PC cells using Trizol reagent (Invitrogen) according to the manufacturer's protocol. Reverse transcription was performed using a PrimeScript RT reagent Kit (TaKaRa, Dalian, China). Semi-quantitative real-time PCR with SYBR green II was used to compare the relative expression of specific mRNAs. All primers were purchased from Sangon Biotech (Shanghai, China). Primer sequences are listed in Supplementary Table 1. All reactions were performed in triplicate. The $2^{-\Delta\Delta Ct}$ method was used to determine relative gene expression levels.

Co-culture system

An *in vitro* co-culture model was established using a 6-well transwell cell culture system (BD Biosciences, MA). CAFs were seeded at a density of 3×10^5 cells per well into the lower wells. After 12 h incubation, CAFs were irradiated with a 4-Gy dose at the Institute of Radiation Medicine, Fudan University (Shanghai, China). The dose of 4Gy used in this study was according to previous report [20]. Next, 2×10^5 PC cells were seeded into the upper chambers. Cells were cultured for the indicated times 24 h, and total RNA and protein was extracted from PC cells at each time point.

Immunofluorescence

PANC-1 and MIA PaCa-2 cells grown on cover slips were washed three times with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde for 30 min at room temperature. Cells were then washed three times with PBS. Specimens were permeabilized with 0.2% Triton X-100 for 15 min and blocked for 30 min with bovine serum albumin at 37°C. Samples were then incubated with primary antibody overnight at 4°C, followed by further washing

and incubation with fluorescein-conjugated secondary antibodies for 60 min. Finally, slides were incubated with propidium iodide for 10 min and embedded in 50% glycerol. Stained cells were mounted and visualized using an Olympus Instruments confocal microscope.

Enzyme-linked immunosorbent assay (ELISA)

NF and CAF cells were seeded at a density of 1 × 10^6 cells/T25 flask in complete medium. Twenty-four h later, cells were treated with a 4-Gy dose of radiation. Culture medium was collected at 12, 24, 48 h time points following irradiation. Samples were centrifuged at 3000 rpm for 5 min to remove particles, and supernatants frozen at -80°C until use. CXCL12 was measured using an ELISA kit according to the manufacturer's instructions.

Transwell migration and invasion assays

For transwell migration assays, IR-exposed NF and CAF cells were suspended in DMEM containing 10% FBS. Cell suspensions (600 μL containing 6 × 10^5 cells) were placed in the lower chambers (24-well insert; pore size, 8 mm; Corning Costar). Next, PC cells were resuspended in DMEM containing 1% FBS and seeded at a density of 5 × 10^4 cells per well into the upper chambers. Plates were incubated for 24 h in a 5% CO_2 incubator. Invasion assays were performed using transwell cell migration plates (Corning, NY) and Matrigel invasion chambers (Matrigel-coated membrane, BD Biosciences). Treated PC cells were trypsinized and resuspended in serum-free medium and seeded at a density of 2 × 10^5 cells per well into the top chambers. DMEM supplemented with 10% FBS was used as a chemo-attractant in the bottom chambers. Following incubation at 37°C with 5% CO_2 for 24 h, migrated and invaded cells on the upper surface were gently removed with a cotton swab. Cells on the lower surface were fixed with methanol and then stained with crystal violet for 30 min. The number of cells that had migrated or invaded through the membrane was counted at a magnification of 100 × using a light microscope (Olympus). All assays were repeated at least three times.

Western blot analysis

Cultured cells were washed twice with PBS and scraped into NP-40 lysis buffer containing protease and phosphatase inhibitors. Supernatants were collected following centrifugation at 12 000 rpm for 30 min at 4°C. The bicinchoninic acid assay kit (Pierce, Rockford, IL) was used to determine protein concentrations. Equal amounts of protein from different cell samples were fractionated using sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane. Membranes were blocked in 5% skim milk in tris-buffered saline containing 0.1% Tween-20 (TBST), and then incubated with indicated primary antibodies for 2 h at room temperature. Following further washing in TBST, membranes were incubated with HRP-conjugated secondary antibodies for 1 h. Immunoreactive bands were visualized using an enhanced chemiluminescence kit (Millipore). The following primary anti-bodies were used: anti-Vimentin, anti-E-cadherin, anti-P38, anti-phosphorylated P38, and anti-β-actin (Protein-Tech Group, Chicago, IL).

In vivo tumorigenesis assays

Male BALB/c nude mice (4-6 weeks old) were obtained from obtained from the Shanghai Institute of Materia Medica, Chinese Academy of Sciences (Shanghai, China) and housed in laminar flow cabinets under specific pathogen-free conditions. All experiments involving animals were in accordance with the Guides for the Care and Use of Laboratory Animals and were performed according to the institutional ethical guidelines of the NIH. The study protocol was also approved by the Committee on the Use of Live Animals in Teaching and Research, Fudan University, Shanghai. PANC-1 cells (2 × 10^6 cells/mouse) were collected, enzymatically dissociated, and injected into the tail vein of each mouse. Mice were sacrificed 10 weeks after injection, and the lungs removed and weighed. Hematoxylin and eosin staining was performed on sections of metastatic tumors and normal lung tissues and the number of colonies and the ratio of the metastatic area were quantified. Each group contained a minimum of 6 mice.

Statistical analyses

Each experiment consisted of at least three replicates and the results are presented as

mean ± standard error. The differences among the groups were compared using one-way ANOVA and Student's t-tests. All statistical analyses were conducted with SPSS version 19.0 software. A $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Irradiation enhanced the invasion-promoting capacity of CAFs in pancreatic cancer

Recently, several studies have identified that the large-associated stromal components and its quantity in pancreatic cancer are supposed to be a dominant contributor of metastasis. To assess the effects of fibroblasts on the biological functions of PC cells after irradiation. CAFs were exposed to 4-Gy IR prior to co-culture with PC cells, mono-culture pancreatic cells had been regarded as blank control. IR-exposed fibroblasts significantly increased the migration capacity of PC cells compared with control, most notably at 12-h IR exposure (**Figure 1A**). Furthermore, we conducted an invasion assay to examine whether irradiation of fibroblasts could affect PC cell invasiveness. The number of invading PC cells was significantly increased in the IR group compared with the control group (**Figure 1B**).

We next co-cultured PC cells with IR-exposed fibroblasts to examine whether IR-exposed fibroblasts can affect EMT in PC cells. **Figure 1C** demonstrates the morphological changes, including the depletion of cell-cell junctions and presence of needle-like extensions, obvious in PC cells co-cultured with IR-exposed fibroblasts. Furthermore, immunofluorescence revealed that IR-exposed fibroblasts can promote expression of Vimentin and reduce E-cadherin levels in PC cells (**Figure 1D**). We also performed real-time PCR and western blotting to assess EMT marker expression at both the mRNA and protein levels. Increased expression of Vimentin and downregulation of E-cadherin was observed in PC cells cultured with irradiated CAFs (**Figure 1E**, **1F**). Collectively, these results indicate that irradiation can enhance the invasion-promoting capacity of CAFs in pancreatic cancer.

Irradiation induced CXCL12 secretion by fibroblasts.

After co-culture with IR-induced fibroblasts, the observed increase migration and invasion in PC cells led us to hypothesize that fibroblasts may secrete some kind of Chemokines to promote PC cell invasiveness and metastasis. To test this hypothesis, we next determined the expression levels of several chemokines previously reported to play roles in pancreatic cancer. We collected the culture supernatant from IR-exposed fibroblasts 12 h after irradiation and measured levels of chemokine mRNA. CXCL12 mRNA was the most significantly elevated species compared with control groups (**Figure 2A**). We next determined how CXCL12 secretion by CAFs was induced in the co-culture system. CAFs were first activated by co-culture with panc-1 cells before irradiation. These conditions supported cell interaction and mutual activation, and the continuous activation of stromal fibroblasts by pancreatic cancer cells prominently promoted CXCL12 secretion at both ELISA and mRNA levels (**Figure 2B**, **2C**). We next assessed levels of CXCL12 mRNA in supernatants at 12, 24, and 48 h following exposure of fibroblasts to IR. Irradiation significantly enhanced CXCL12 secretion by CAFs, especially at 12 h after IR (**Figure 2D**). These findings reveal that irradiation of fibroblasts induces higher concentrations of CXCL12. Therefore, CAF-derived CXCL12 may play an essential role during radiotherapy.

CXCL12/CXCR4 signaling promoted pancreatic cancer cell EMT and invasion

We next assessed the influence of CXCL12-CXCR4 signaling on cell migration and invasion using transwell chambers with or without Matrigel coating. Transwell assays revealed a significantly increased number of migrating cells in the CXCL12-treated group compared with the control group. Meanwhile, cellular migration is dramatically inhibited by addition of the CXCR4 antagonist AMD3100 (4 μg/ml). Additionally, CXCL12 significantly increased the invasive capacity of PC cells, while AMD3100 reversed this effect (**Figure 3A**, **3B**). We then explored the whether CXCL12-mediated stimulation of CXCR4 influenced EMT in PC cells. CXCL12 significantly upregulated expression of the mesenchymal marker Vimentin and abrogated expression of the epithelial marker E-cadherin at both mRNA and protein levels (**Figure 3C**, **3D**). These data suggest that CXCL12 may play a critical role in facilitating migration, invasion, and EMT in pancreatic cancer cells.

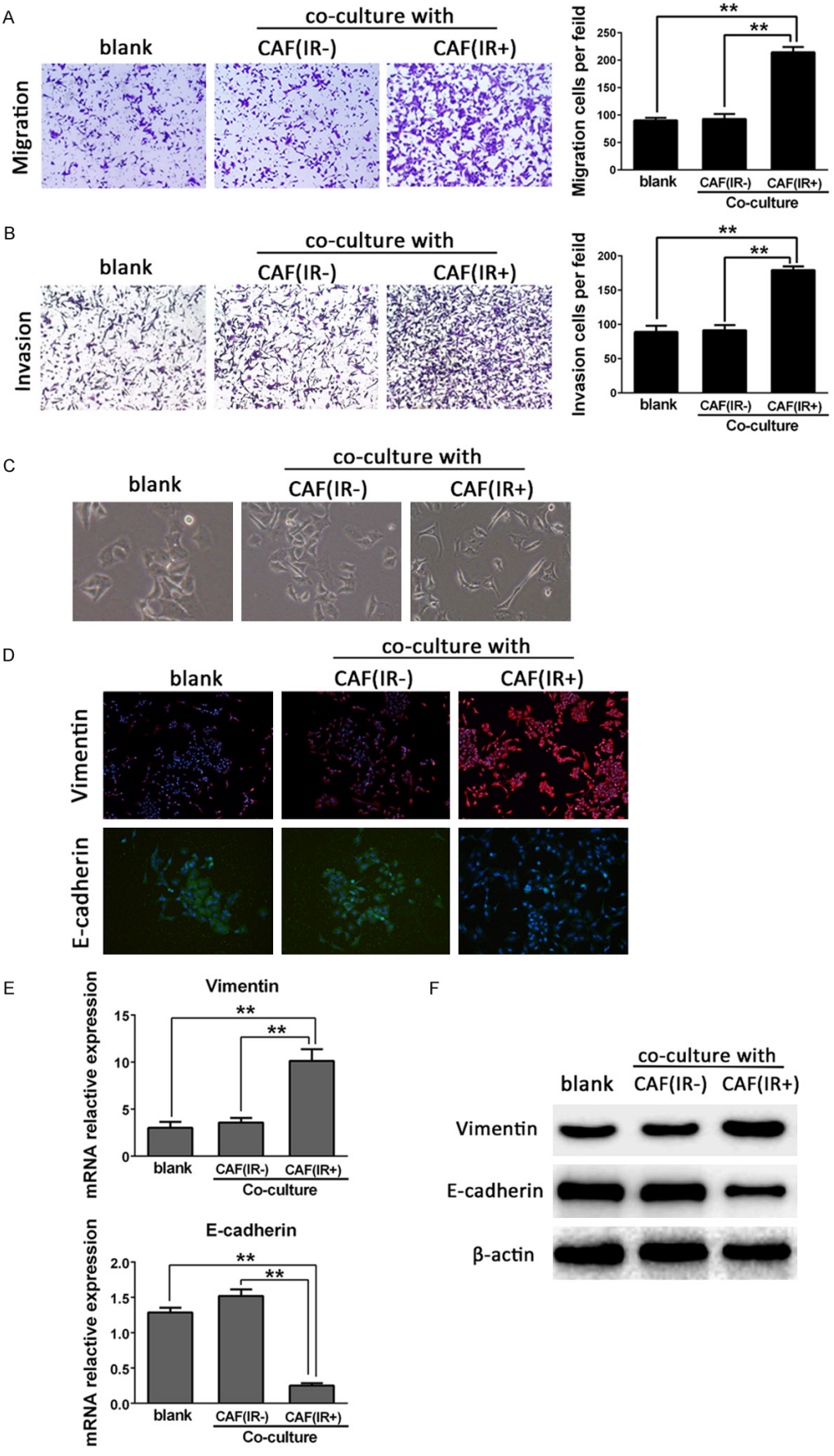
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Figure 1. Irradiation enhances the invasion-promoting capacity of CAFs which promote pancreatic cancer cell EMT. A. Migration of PANC-1 cells at 12 h after co-culture with pancreatic fibroblasts subjected to 4-Gy irradiation. B. Invasion of PANC-1 cells at 12 h after co-culture with pancreatic fibroblasts subjected to 4-Gy irradiation. C. Morphological changes in PANC-1 cells after co-culture with pancreatic fibroblasts subjected to 4-Gy irradiation. D. Immunofluorescence analysis of Vimentin and E-cadherin expression in PANC-1 cells treated as described in C. E. qRT-PCR analysis of Vimentin and E-cadherin mRNA levels in PANC-1 cells treated as described in C. F. Western blot analysis of Vimentin and E-cadherin protein levels in PANC-1 cells treated as described in C. An ANOVA test and a Student's t-test were used to determine the statistical significance of differences between the groups and two groups. Results are presented as the means ± SD of values obtained in three independent experiments. *$P < 0.05$, **$P < 0.01$.

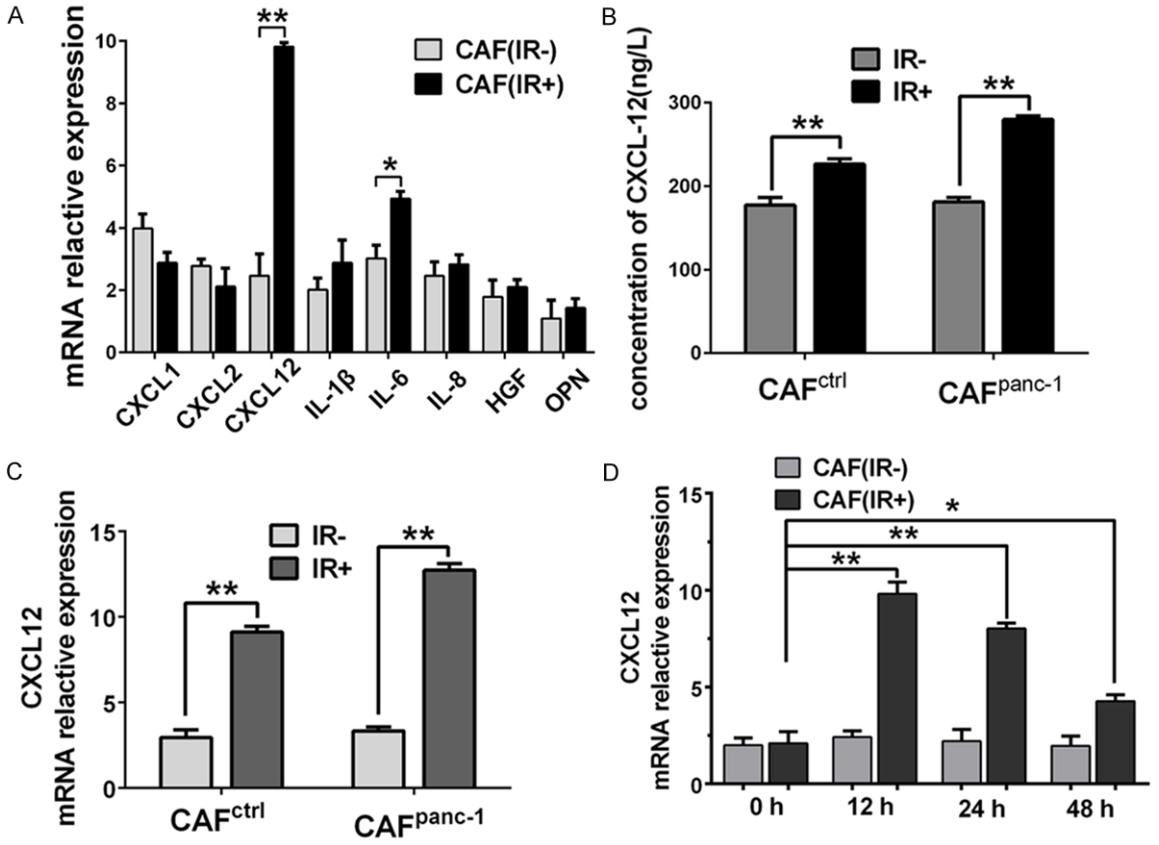

Figure 2. Irradiation induces high concentrations of fibroblast-derived CXCL12. A. qRT-PCR analysis of chemokine expression levels in pancreatic fibroblasts 12 hr after 0 or 4 Gy irradiation. B. Panc-1 cells cocultured with pancreatic fibroblasts. CAFs were first cocultured with Panc-1 cells growing in transwell inserts for 3 days (CAF^{Panc-1}) or cultured alone (CAF^{ctrl}) before being transferred to Transwell inserts. ELISA was performed on the supernatant from pancreatic fibroblasts at 24 h after 0- or 4-Gy irradiation. C. Relative expression of CXCL12 mRNA in pancreatic fibroblasts was determined by qRT-PCR. Pancreatic fibroblasts were treated as described in B. D. CXCL12 mRNA expression was measured by RT-PCR in pancreatic fibroblasts 12, 24, and 48 h after 0- or 4-Gy irradiation. An ANOVA test was used to determine the statistical significance of differences between the groups. Results are presented as the means ± SD of values obtained in three independent experiments. *$P < 0.05$, **$P < 0.01$.

Since P38 MAP Kinases pathway has been highlighted a pivotal role in tumorigenesis, proliferation and metastasis. We next investigated the effect of CXCL12 on the activation of P38 pathway. Examination of the immunoblotting profile of key P38 pathway proteins in PC cells following treatment with CXCL12. CXCL12 increased levels of phosphorylated P38, and this change was prevented by pre-treating cells with AMD3100 (**Figure 4A**). These findings suggest that CXCL12 induced activation of the P38-MAPK pathway. We next assessed whether CXCL12-mediated activation of the P38 pathway could promote EMT. Exposing PC cells

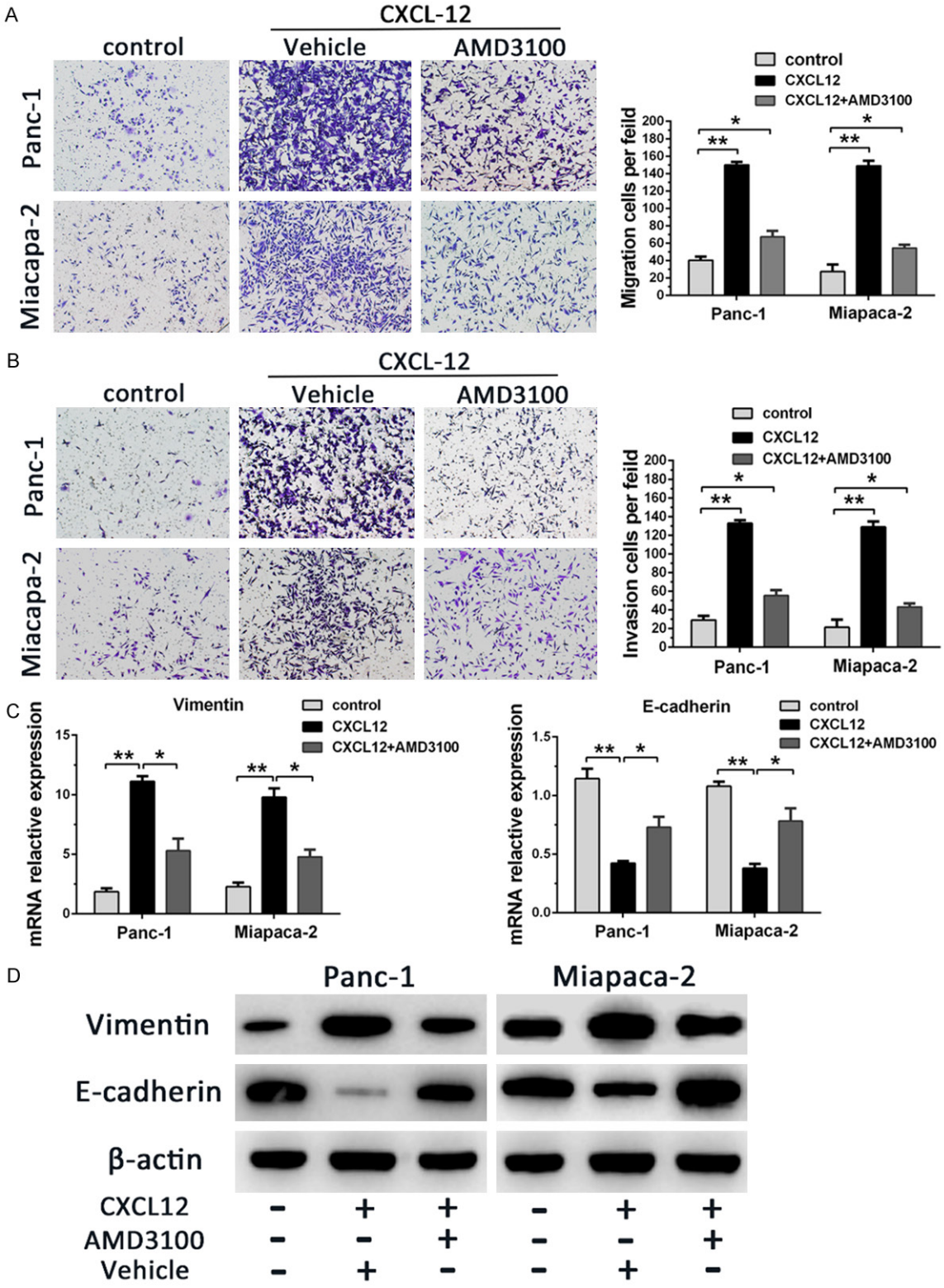

Figure 3. CXCL12-CXCR4 signaling promotes pancreatic cancer cell EMT and invasion. A. Effects of the CXCL12 on pancreatic cancer cell migration capacity. The number of migrated cells was quantified by counting the number of cells from ten random fields at 200× magnification. B. Effects of the CXCL12 on pancreatic cancer cell invasion capacity. The number of invaded cells was quantified by counting the number of cells from ten random fields at 200× magnification. C. qRT-PCR analysis of Vimentin and E-cadherin mRNA levels following treatment with CXCL12. D. Western blot analysis of Vimentin and E-cadherin protein levels following treatment with CXCL12. Results are presented as the means ± SD of values obtained in three independent experiments. *$P < 0.05$, **$P < 0.01$.

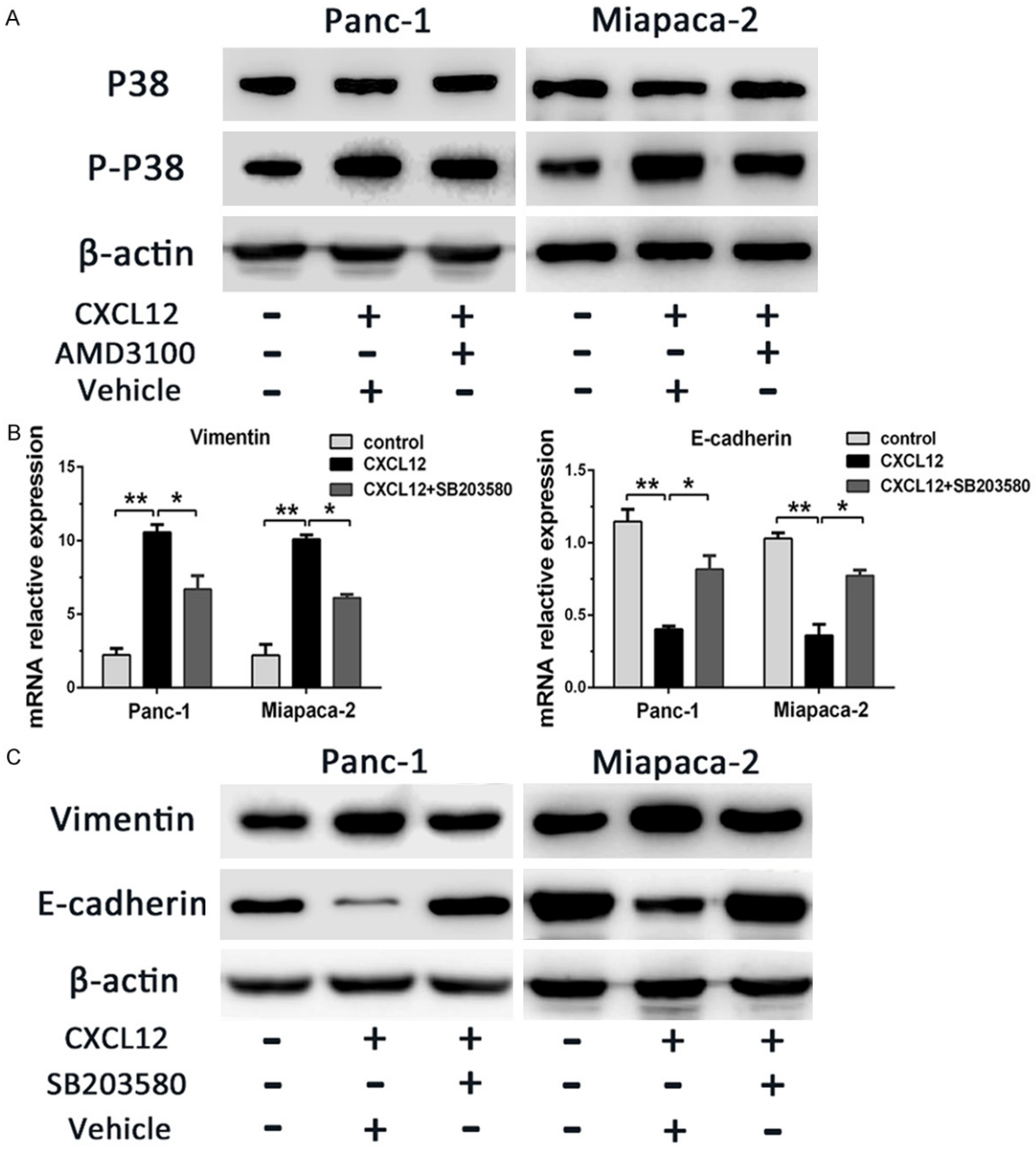

Figure 4. CXCL12 induces pancreatic cancer cell EMT by activating the P38 pathway. A. Western blot analysis of P38 and P-P38 in PANC-1 and MIA PaCa-2 cells following treatment with CXCL12 for 48 h with or without AMD3100 pretreatment. B. qRT-PCR analysis of Vimentin and E-cadherin mRNA levels in PC cells treated with CXCL12 for 48 h with or without SB203580 pretreatment. C. Western blot analysis of Vimentin and E-cadherin protein levels in PC cells treated with CXCL12 for 48 h with or without SB203580 pretreatment. Results are presented as the means ± SD of values obtained in three independent experiments. $*P < 0.05$, $**P < 0.01$.

to the P38 inhibitor SB203580 (5 μg/ml) impaired CXCL12-mediated down-regulation of E-cadherin and up-regulation of Vimentin (**Figure 4B**, **4C**). Therefore, CXCL12/CXCR4 signaling promoted pancreatic cancer cell EMT and invasion by activating the P38 pathway.

Irradiation promotes EMT and invasion of pancreatic cancer cells by activating CAFs

The elevated levels of CXCL12 secreted by IR-exposed CAFs suggested that irradiation may induce pancreatic cancer cell migration,

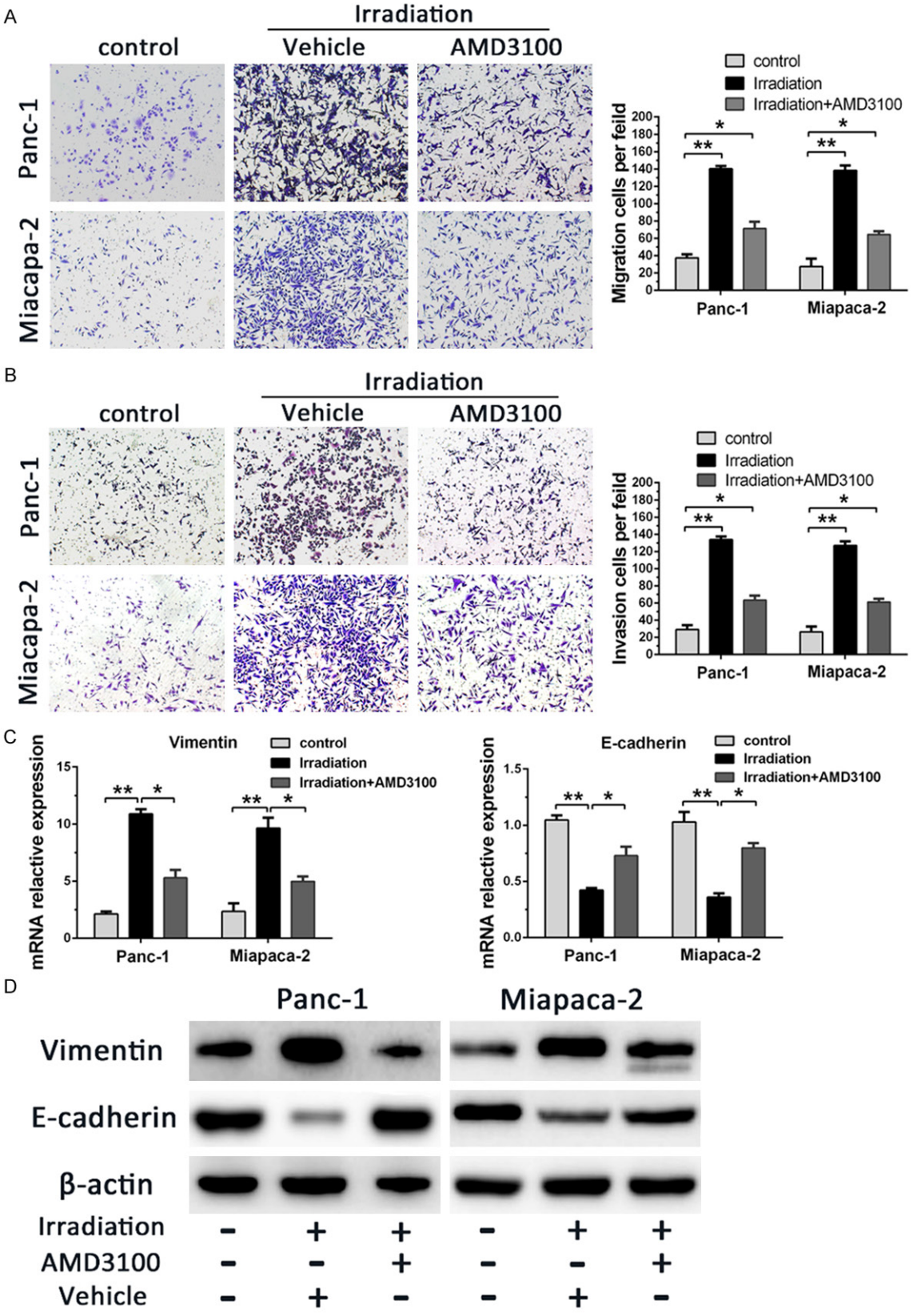
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Figure 5. Radiation promotes EMT and invasion of pancreatic cancer cells by activating CAFs. A. Migration in PANC-1 and MIA PaCa-2 cells co-cultured with CAFs subjected to 0- or 4-Gy irradiation, and in the presence or absence of AMD3100. The number of cells from ten random fields at 200× magnification was counted. B. PANC-1 and MIA PaCa-2 cells were treated as described in 5A and subjected to an invasion assay. The number of invaded cells was quantified by counting the number of cells from ten random fields at 200× magnification. C. PANC-1 and MIA PaCa-2 were treated as described in 5A and qRT-PCR conducted to evaluate Vimentin and E-cadherin mRNA levels. D. PANC-1 and MIA PaCa-2 cells were treated as described in 5A and western blotting was performed to evaluate Vimentin and E-cadherin protein levels. Results are presented as the means ± SD of values obtained in three independent experiments. $*P < 0.05$, $**P < 0.01$.

invasion, and EMT via CXCL12. Therefore, we co-cultured PC cells with CAFs that had been exposed to 4-Gy IR and performed transwell assays to assess the migration and invasive capacity of PC cells. IR-exposed CAFs promoted both the migration and invasion of PANC-1 and MIA PaCa-2 cells, and both effects were prevented by blockade of CXCL12 with AMD3100 (**Figure 5A**, **5B**).

We further investigated the influence of irradiation on CXCL12-induced EMT in PC cells. RT-PCR findings revealed significantly increased levels of vimentin and decreased E-cadherin 48 h following irradiation (**Figure 5C**). Similar results were obtained from western blotting experiments. Moreover, suppression of CXCL-12-CXCR4 signaling with AMD3100 reduced the expression of vimentin and rescued E-cadherin expression (**Figure 5D**). Taken together, these findings indicate that irradiation induced pancreatic cancer cell invasion and EMT by activating CAFs.

Irradiation promotes PC cell metastasis in vivo

To further explore the effects of irradiation to CAF on metastasis in vivo, we next examined the metastatic nodules formed in the lungs of NOD/SCID mice injected in the tail vein with PC cells co-cultured with IR-exposed CAFs. CAFs were subjected to 4-Gy IR followed by collection of supernatant. This supernatant was added to PC cell medium every second day for 2 weeks. Mice were sacrificed 10 weeks after injection and the lungs harvested. Both the control and CAF supernatant-treated cells formed tumors in the lungs following tail vein injection. However, PC cells exposed to IR-exposed CAF supernatant significantly accelerated the growth of lung tumors in metastatic colonies and possessed a decreased ratio of metastatic area to total area. Moreover, addition of AMD-3100 to the IR-treated CAF supernatant prior to co-culture with PC cells reduced formation of lung metastases (**Figure 6A**, **6B**). Taken together, these results indicate that irradiation may promote PC cell invasiveness through CAF secretion of CXCL12. Therefore, CAF-mediated CXCL12 production may promote invasiveness in pancreatic cancer cells following radiotherapy.

Discussion

In this study, we have identified that irradiation can enhance the invasion-facilitating capacity of fibroblasts, thereby promoting pancreatic cancer cell migration, invasion, and EMT. We also found that irradiation can stimulate pancreatic fibroblasts to secrete high concentrations of CXCL12. Additionally, CAF-mediated promotion of PC cell migration, invasion, and EMT depends on CXCL12 secretion and activation of the P38 pathway. Most importantly, we demonstrated that irradiation promotes PC cell invasiveness by stimulating secretion of CXCL12 by CAFs both *in vitro* and *in vivo*. Collectively, our findings reveal that CAFs are involved in radiation-induced EMT and invasion in pancreatic cancer. This represents an attractive means by which to enhance the effectiveness of radiotherapy through targeting CAF activity to disrupt the CXCL12-CXCR4 axis.

Radiotherapy has made significant contributions to cancer treatment. For pancreatic cancer, radiotherapy has been recommended as a postoperative adjuvant and neoadjuvant treatment. However, despite ongoing improvements in radiotherapy technology, its benefits for pancreatic cancer remain limited. Recent studies have highlighted the importance of interactions between tumor cells and their microenvironment during tumor treatment [21]. Therefore, elucidating the precise mechanisms by which the tumor microenvironment influences radiotherapy may lead to the development of novel therapeutic strategies in pancreatic cancer. In our study, we have first described how irradia-

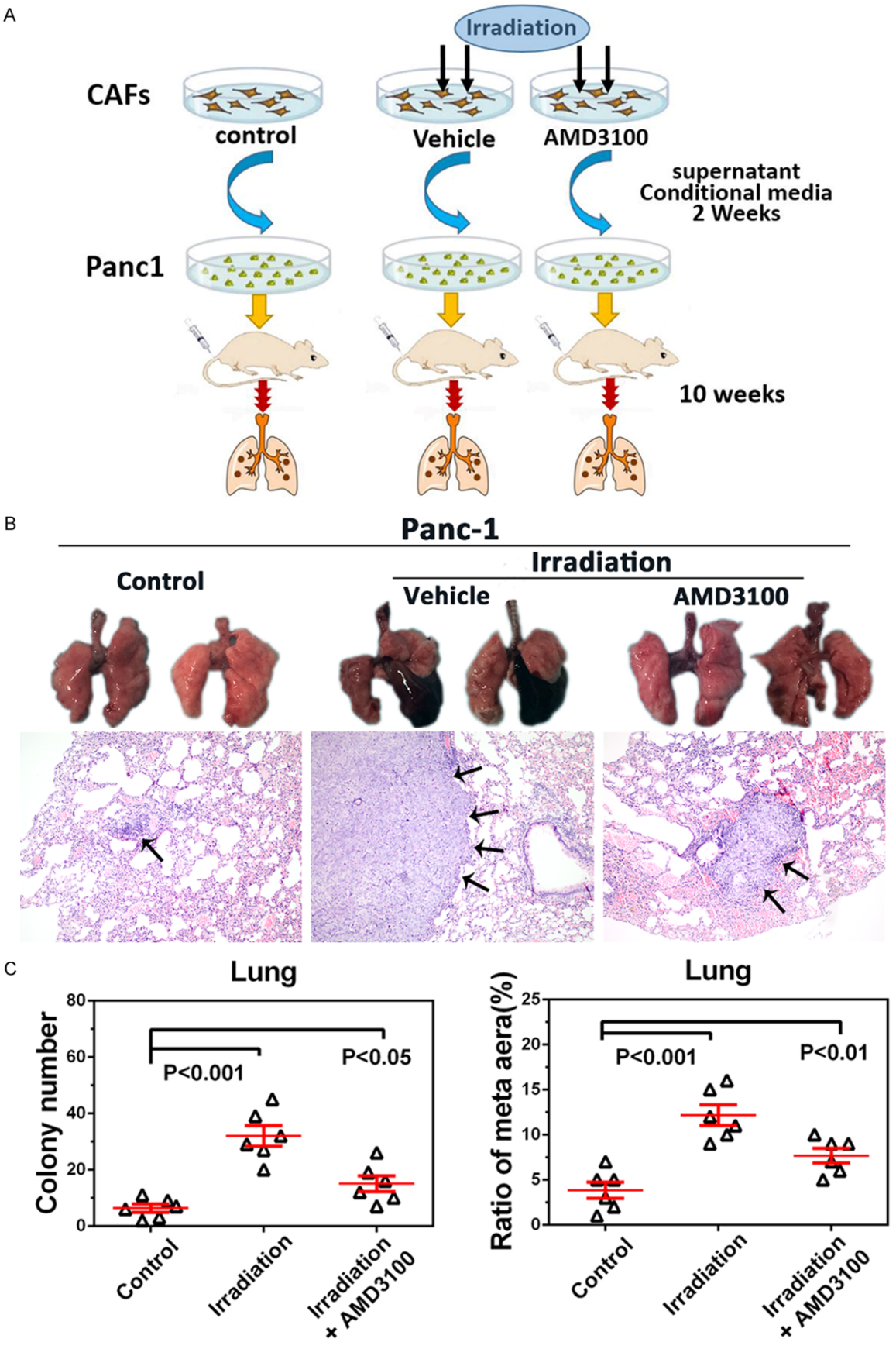
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Figure 6. Irradiation promotes PC cells metastasis *in vivo*. A. Flow chart of in vivo exploration of irradiation facilitates PC cells metastasis. B. CXCL12 secreted by IR-exposed CAFs influences tumor metastasis *in vivo*. Lungs were harvested from mice 10 weeks after tail vein injection of control, co-culture, or AMD3100-treated PANC-1 cells. Representative lungs from NOD/SCID mice are shown. Hematoxylin and eosin stain staining was performed on sections of metastatic tumor and normal lung tissues. Metastatic nodules are indicated by arrows at 200× magnification. C. Tumor metastases were quantified by counting the number of metastatic colonies in one histological section of the midportion of each sample of the lung from each mouse, and by determining the ratio of the metastatic area to the total area in histological sections from the midportion of each lung (n=6 per group). A Student's t-test was used to determine the statistical significance of the differences between two groups.

tion of pancreatic fibroblasts can significantly enhance their ability to promote cancer invasiveness.

Previous studies have reported that a high level of stromal infiltration occurs in various tumors and CAFs constitute a major component of these infiltrating cells [22, 23]. CAFs play multiple roles in tumor behavior and influence patient outcomes through their effects on direct cell-cell adhesion and extracellular matrix remodeling [24, 25]. Furthermore, an increasing number of studies are providing evidence of the oncogenic properties of activated CAFs [26-28]. Growth factors and chemokines produced by CAFs facilitate angiogenic recruitment of endothelial cells and pericytes, which contribute to all stages of cancer growth and progression [29-32]. Although several studies have reported that CAFs enhance the invasive and migratory capacities of pancreatic tumor cells by secreting cytokines, the precise link between CAFs and irradiation have remained largely elusive until now [33, 34]. Here, we have described a role for CXCL12 as this link, and report that CXCL12 is obviously upregulated in the supernatant of IR-exposed CAFs.

CXCL12 is a ubiquitous chemokine that plays a critical role in hematopoietic stem cell maintenance and the migration of various cell types [35, 36]. CXCL12 binds to its G protein-coupled receptor CXCR4 and appears broadly effective in facilitating cancer initiation and progression [37-39]. CXCR4 overexpression is commonly identified in multiple primary neoplasms [40, 41]. Furthermore, CXCR4 is aberrantly overexpressed in pancreatic cancer tissues compared with paracarcinomatous tissues. Additionally, activation of the CXCL12-CXCR4 axis increased gene expression of invasion-related genes, enhancing PC cell migration and invasion [42, 43]. In this study, we showed that the CXCL12-CXCR4 axis is required for CAF-induced PC cell invasion. More importantly, we revealed that IR-exposed CAF-mediated PC cell migration and invasion was primarily dependent on CXCL12.

Multiple intracellular signaling pathways influence tumor cell proliferation and differentiation, including Wnt, Notch, NF-κB, JAK/STAT, and PI3K-AKT [44]. However, the precise mechanisms by which CXCL12 promotes pancreatic cancer cell proliferation remain unclear. In our study, we have explored the potential Signaling pathways which may influence the biological function of pancreatic cancer and identified an essential role for the P38 pathway in mediating CXCL12-CXCR4-driven invasiveness. Moreover, blocking the P38 pathway using an inhibitor prominently attenuated the invasive ability of pancreatic cancer.

EMT is characterized by the loss of cell-to-cell adhesion, increased cellular plasticity, and development of a mesenchymal phenotype [45]. EMT is recognized as an important determinant of tumor progression in solid tumors including pancreatic cancer [22]. Abnormal induction of EMT in cancer cells is closely related to the acquisition of stem cell-like properties, an increased migration and invasion capacity, and resistance to therapy [46, 47]. In our study, we first certified IR-exposed pancreatic cancer fibroblasts can promote EMT. We also addressed the cross-talk between the CXCL12/CXCR4 axis and the EMT, revealed that irradiation facilitated EMT and invasion of pancreatic cancer cells by activating CAFs to secrete CXCL12.

In conclusion, our study have elucidated that radiation exposure can promote EMT and invasion of pancreatic cancer cells by activating CAFs. Furthermore, CXCL12 plays a crucial role in this process. Inhibitors of the CXCL12-CXCR4 interaction could interfere with tumor cell growth, migration, and invasion. Further knowledge of how to best target CAFs in combination

with radiotherapy may facilitate development of innovative therapeutic regimes to improve patient survival and outcomes.

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Disclosure of conflict of interest

None.

Authors' contribution

D. Li performed the majority of experiments, acquisition of data, analysis and interpretation of data, statistical analysis, and drafting of the manuscript; C. Qu and Z. Ning conducted part of the experiment; H. Wang, K. Zang, and L. Zhuang for acquisition of data, analysis and interpretation of data, and statistical analysis; L. Chen for technical and material support; P. Wang for study concept and design; Z. Meng for critical revision of the manuscript for important intellectual content and study supervision.

Abbreviations

CAF, Carcinoma associated fibroblast; NF, Normal fibroblast; EMT, Epithelial to mesenchymal transition; PC, Pancreatic carcinoma; IR, ionizing radiation; PI, prodium iodide; Elisa, Enzyme-linked immunosorbent assay; DMEM, Dulbecco's Modified Eagle's Medium; FBS, Fetal bovine serum; PBS, phosphate-buffer saline; RT-PCR, reverse transcriptase-polymerase chain reaction.

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Supplementary Table 1. Primers used for qRT-PCR

Generic Primers	Forward	Reverse
CXCL1	5'-GACCAGAAGGGAGGAGGAAG-3'	5'-TGCTCAAACACATTAGGCACA-3'
CXCL2	5'-CTCAAGAATGGGCAGAAAGC-3'	5'-AGGAACAGCCACCAATAAGC-3'
CXCL12	5'-GGCTCCCTGTAACCTCTTCA-3'	5'-CAGACTCAATCCCAACACACA-3'
IL-1β	5'-CATCAGCACCTCTCAAGCAG-3'	5'-AGTCCACATTCAGCACAGGA-3'
IL-6	5'-TACATCCTCGACGGCATCTC-3'	5'-GCCATCTTTGGAAGGTTCAG-3'
IL-8	5'-GTGCAGTTTTGCCAAGGAGT-3'	5'-CTCTGCACCCAGTTTTCCTT-3'
HGF	5'-TGTCATTGTTCCTGGTCGTG-3'	5'-TTGTATTGGTGGGTGCTTCA-3'
OPN	5'-GCCGTGGGAAGGACAGTTAT-3'	5'-GCTCATTGCTCTCATCATTGG-3'
GAPDH	5'-GAGCTGAACGGGAAGCTCACTG-3'	5'-TGGTGCTCAGTGTAGCCCAGGA-3'