

# Secreted Interleukin-1 $\alpha$ Induces a Metastatic Phenotype in Pancreatic Cancer by Sustaining a Constitutive Activation of Nuclear Factor- $\kappa$ B

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## Abstract

Transcription factor nuclear factor- $\kappa$ B (NF- $\kappa$ B) is constitutively activated in most pancreatic cancer tissues and cell lines but not in normal pancreas nor in immortalized/nontumorigenic human pancreatic ductal epithelial cells. Inhibition of constitutive NF- $\kappa$ B activation in pancreatic cancer cell lines suppresses tumorigenesis and tumor metastasis. Recently, we identified autocrine secretion of proinflammatory cytokine interleukin (IL)-1 $\alpha$  as the mechanism of constitutive NF- $\kappa$ B activation in metastatic pancreatic cancer cell lines. However, the role of IL-1 $\alpha$  in determining the metastatic potential of pancreatic tumor remains to be further investigated. In the current study, we stably expressed IL-1 $\alpha$  in the nonmetastatic, IL-1 $\alpha$ -negative MiaPaCa-2 cell lines. Our results showed that the secretion of IL-1 $\alpha$  in MiaPaCa-2 cells constitutively activated NF- $\kappa$ B and increased the expression of NF- $\kappa$ B downstream genes involved in the different steps of the metastatic cascade, such as *urokinase-type plasminogen activator*, *vascular endothelial growth factor*, and *IL-8*. MiaPaCa-2/IL-1 $\alpha$  cells showed an enhanced cell invasion *in vitro* compared with parental MiaPaCa-2 cells and induced liver metastasis in an orthotopic mouse model. The metastatic phenotype induced by IL-1 $\alpha$  was inhibited by the expression of phosphorylation-defective I $\kappa$ B (I $\kappa$ B S32, 36A), which blocked NF- $\kappa$ B activation. Consistently, silencing the expression of IL-1 $\alpha$  by short hairpin RNA in the highly metastatic L3.6pl pancreatic cancer cells completely suppressed their metastatic spread. In summary, these findings showed that IL-1 $\alpha$  plays key roles in pancreatic cancer metastatic

behavior through the constitutive activation of NF- $\kappa$ B. Our findings further support the possible link between inflammation and cancer and suggest that IL-1 $\alpha$  may be a potential therapeutic target for treating pancreatic adenocarcinoma. (Mol Cancer Res 2009;7(5):624–33)

## Introduction

Pancreatic adenocarcinoma remains one of the most lethal and poorly understood human malignancies. It ranks fourth in the leading causes of cancer-related mortality among adults in the United States (1). At the time of diagnosis, two thirds of patients present with advanced disease characterized by early local regional spread and distant metastasis (2). Current chemotherapy, radiation therapy, and surgical procedures are largely ineffective in the treatment of this disease (3).

Nuclear factor- $\kappa$ B (NF- $\kappa$ B) is a family of pleiotropic transcription factors that control the expression of numerous genes involved in growth, tumorigenesis, tumor metastasis, apoptosis, and immune and inflammatory responses (4). Many tumors have acquired genetic alterations in the signaling pathways that regulate NF- $\kappa$ B activation. We previously reported that RelA, the p65 subunit of the NF- $\kappa$ B transcription factor, is constitutively activated in most pancreatic cancer tissues and human pancreatic cancer cell lines but not in normal pancreatic tissues or immortalized pancreatic ductal epithelial cells (5). In different models of pancreatic tumor, inhibiting constitutive NF- $\kappa$ B activation by expression of an I $\kappa$ B $\alpha$  phosphorylation mutant suppresses tumorigenicity by reducing expression of antiapoptotic proteins Bcl-x(L) and Bcl-2 and inhibits liver metastasis and angiogenic potential by reducing *in vivo* expression of vascular endothelial growth factor (VEGF; refs. 6–8).

Interleukin (IL)-1 $\alpha$  and IL-1 $\beta$  are between the most potent proinflammatory cytokines (9). They initiate and potentiate the inflammatory response working by themselves, but more importantly, they induce the expression of proinflammatory gene molecules mainly acting as potent activators of NF- $\kappa$ B.

Although only rarely secreted by normal cells other than macrophages, IL-1 $\alpha$  was shown to act as an autocrine growth stimulant for several malignant cell types (10, 11). We recently showed that an autocrine stimulation of IL-1 $\alpha$ , but not IL-1 $\beta$ , primarily induced by activator protein-1 activity, accounts for the constitutive activation of NF- $\kappa$ B in metastatic human pancreatic cancer cell lines but not in nonmetastatic ones. The constitutive NF- $\kappa$ B activity triggered by IL-1 $\alpha$  autocrine stimulation enhances the IL-1 $\alpha$  expression, initiating the formation of a

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**Note:** D. Melisi and J. Niu contributed equally to this work.

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positive feedback loop. Neutralization of IL-1 $\alpha$  activity suppressed the constitutive activation of NF- $\kappa$ B and the expression of its downstream target gene, *urokinase-type plasminogen activator (uPA)*, in metastatic pancreatic cancer cell lines (12). In an *in vitro* model of inflammatory breast cancer cells, Streicher et al. (13) more recently showed that amphiregulin, but not epidermal growth factor, induces an increased expression of IL-1, triggering a similar autocrine loop through a prompt activation of NF- $\kappa$ B.

In malignant cells, exogenous recombinant IL-1 induces secretion of growth, invasiveness promoting, and angiogenic factors (9). In an *in vitro* model of breast cancer cells, secreted IL-1 $\alpha$  of tumor origin was shown to induce expression of prometastatic genes in cancer as well as in stromal cells (14). In pancreatic cancer *in vitro* models, expression of IL-1 $\alpha$  mRNA and protein was observed only in the highly liver metastatic pancreatic cancer cell lines (15). Several studies showed *in vitro* that exogenous recombinant IL-1 $\alpha$  can favor the metastatic and invasive behavior of human pancreatic cancer cells (16-20). In a series of 30 pancreatic cancer patients, IL-1 $\alpha$  was found to be increased, whereas IL-1 $\beta$  decreased when liver metastases were present (21).

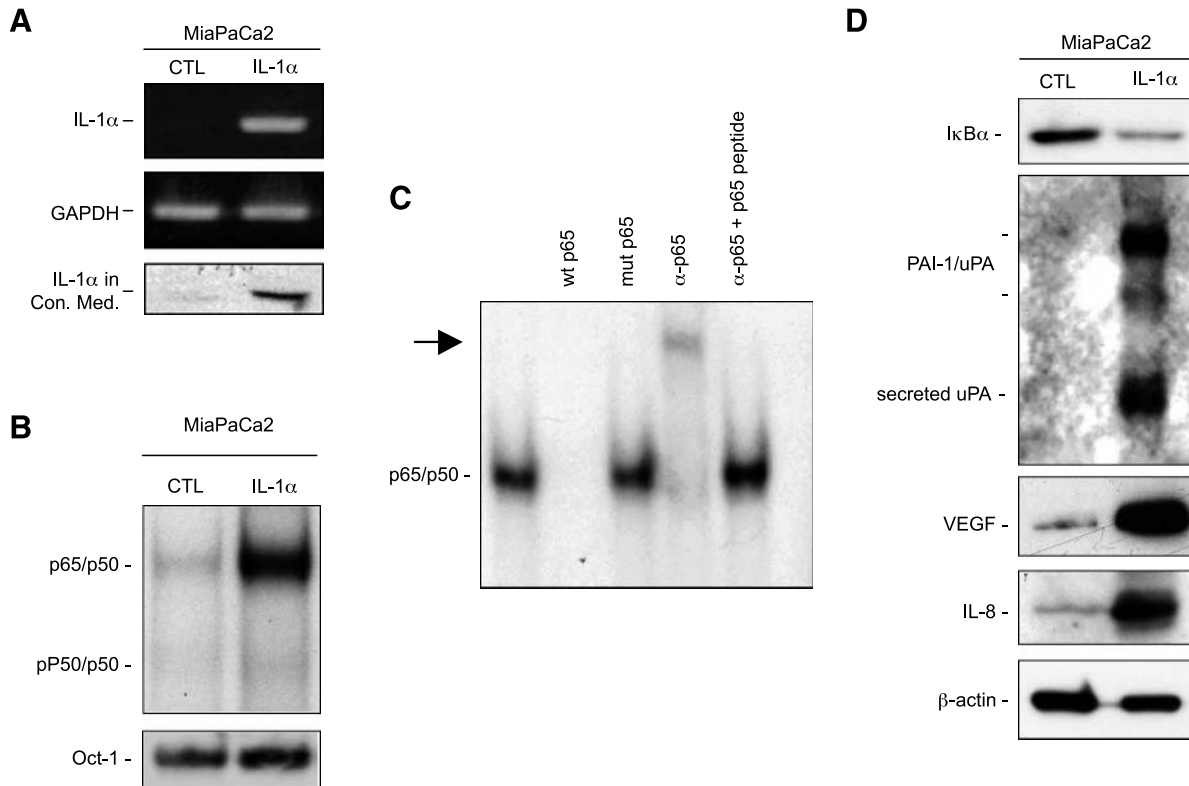
To show in an *in vivo* model that the IL-1 $\alpha$  autocrine loop is indeed responsible for the pronounced metastatic behavior of

pancreatic cancer mediated by a constitutive activation of NF- $\kappa$ B, we stably expressed IL-1 $\alpha$  in the nonmetastatic pancreatic cancer cell line MiaPaCa-2 and used phosphorylation-defective mutant I $\kappa$ B $\alpha$ M (S32, 36A) to completely inhibit the activation of NF- $\kappa$ B. Our results showed that autocrine secretion of IL-1 $\alpha$  constitutively activated NF- $\kappa$ B in MiaPaCa-2 cells and endowed the cells with metastatic features. The inhibition of NF- $\kappa$ B by I $\kappa$ B $\alpha$ M (S32, 36A) suppressed the metastatic potential of the MiaPaCa-2/IL-1 $\alpha$  cells. Short hairpin RNA (shRNA) to knock down IL-1 $\alpha$  completely suppressed the spread of the highly metastatic L3.6pl pancreatic cancer cells.

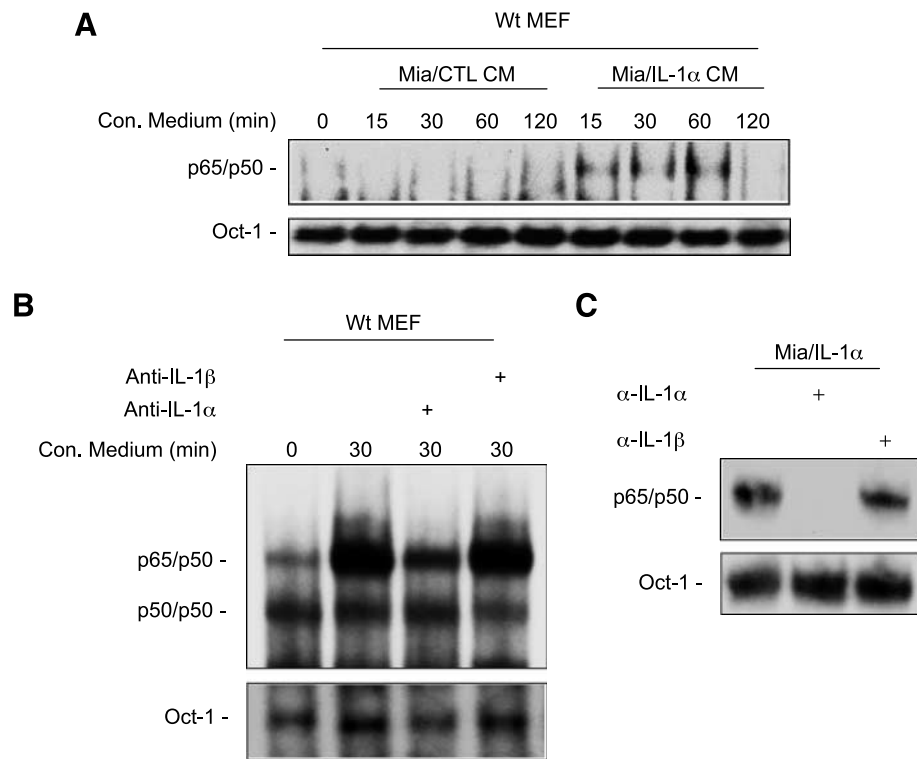
**Results**

*Expression of IL-1 $\alpha$  in Pancreatic Cancer Cell Line MiaPaCa-2 Activated NF- $\kappa$ B for the Transcription of Prometastatic Genes*

To show our hypothesis, we used the nonmetastatic MiaPaCa-2 pancreatic cancer cell line, which is characterized by a minimal basal NF- $\kappa$ B activation, and no IL-1 $\alpha$  expression. We used retrovirus infection to generate a variant of MiaPaCa-2 cell line that stably expressed IL-1 $\alpha$  (MiaPaCa-2/IL-1 $\alpha$ ). As shown in Fig. 1A, MiaPaCa-2/IL-1 $\alpha$  cells showed



**FIGURE 1.** Expression of IL-1 $\alpha$  in MiaPaCa-2 cells induces constitutive NF- $\kappa$ B activation. **A.** Reverse transcription-PCR and Western blot analysis were done to determine the expression and secretion of IL-1 $\alpha$  in conditioned medium collected in the same volume/cell number ratios and subjected to dialysis and concentration before the analysis. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. **B.** NF- $\kappa$ B activity was determined by EMSA using nuclear extracts (15  $\mu$ g) in this analysis with a HIV  $\kappa$ B probe. An Oct-1 probe was used as a loading control for quality and quantity of cell nuclear extracts. **C.** The nuclear extracts (15  $\mu$ g) from MiaPaCa-2/IL-1 $\alpha$  cells were used for the competition and supershift assays as indicated. Arrow, supershift band. **D.** For Western blot analysis, conditioned medium and cytoplasmic protein extracts were isolated as described and probed with anti-I $\kappa$ B $\alpha$ , anti-uPA, anti-IL-8, anti-VEGF, or anti- $\beta$ -actin antibodies.



**FIGURE 2.** Autocrine secretion of IL-1 $\alpha$  is responsible for constitutive NF- $\kappa$ B activation in MiaPaCa-2/IL-1 $\alpha$  cells. **A.** EMSA was done to determine the NF- $\kappa$ B activity in WT MEF cells stimulated by the conditioned medium isolated from MiaPaCa-2/CTL and MiaPaCa-2/IL-1 $\alpha$  cells at the time intervals indicated and an Oct-1 probe was used as a loading control for quality and quantity of cell nuclear extracts. **B.** Conditioned media from MiaPaCa-2/IL-1 $\alpha$  cells treated with anti-IL-1 $\alpha$  or anti-IL-1 $\beta$  antibodies (2  $\mu$ g/mL for 2 h) were used to stimulate WT MEF cells for 30 min. EMSA was done to determine the NF- $\kappa$ B activity with Oct-1 probe as a loading control. **C.** EMSA was done to determine the NF- $\kappa$ B activity in MiaPaCa-2/IL-1 $\alpha$  cells treated with anti-IL-1 $\alpha$  or anti-IL-1 $\beta$ . Indicated neutralizing antibodies (2  $\mu$ g/mL) were applied for 1 h and an Oct-1 probe was used as a loading control for cell nuclear extracts.

a constitutive expression and secretion of IL-1 $\alpha$  in their conditioned medium. IL-1 $\alpha$  expression constitutively activated NF- $\kappa$ B, but not Oct-1, as shown by electrophoretic mobility shift assay (EMSA; Fig. 1B). To confirm the specificity of NF- $\kappa$ B binding, we also did a supershift assay. As shown in Fig. 1C, unlabeled wild-type (WT) but not mutant NF- $\kappa$ B oligonucleotides completely blocked NF- $\kappa$ B activation by competition, and p65 antibody further retarded NF- $\kappa$ B shift in the gel, as antibody binding resulted in a larger complex. This supershift was inhibited when p65 peptide was added, which prevented the antibody from binding to the NF- $\kappa$ B complex. Consistent with the NF- $\kappa$ B activation, the I $\kappa$ B $\alpha$  level decreased in MiaPaCa-2/IL-1 $\alpha$  cells, indicating the degradation of I $\kappa$ B $\alpha$ , whereas the expression of some of the NF- $\kappa$ B downstream genes involved in the metastatic processes, *uPA*, *VEGF*, and *IL-8*, was elevated by the IL-1 $\alpha$  expression (Fig. 1D).

#### Autocrine Secretion of IL-1 $\alpha$ , but not IL-1 $\beta$ , Accounted for the NF- $\kappa$ B Activation

To determine if the constitutive NF- $\kappa$ B activation in MiaPaCa-2/IL-1 $\alpha$  cells is the result of expression of IL-1 $\alpha$ , we collected the conditioned medium from MiaPaCa-2/CTL cells and MiaPaCa-2/IL-1 $\alpha$  cells and used them to treat WT mouse embryonic fibroblast (MEF) cells at the time intervals indicated (Fig. 2A). These results showed that the conditioned medium from MiaPaCa-2/IL-1 $\alpha$  cells but not MiaPaCa-2/CTL cells

was able to activate NF- $\kappa$ B (Fig. 2A). When conditioned medium from MiaPaCa-2/IL-1 $\alpha$  cells was pretreated by IL-1 $\alpha$  neutralizing antibody, it failed to activate NF- $\kappa$ B in MEF cells, whereas IL-1 $\beta$  neutralizing antibody did not block its NF- $\kappa$ B-inducing activity (Fig. 2B), indicating that IL-1 $\alpha$  is the cytokine secreted by MiaPaCa-2/IL-1 $\alpha$  cells responsible for the NF- $\kappa$ B activation. We also treated MiaPaCa-2/IL-1 $\alpha$  cells directly with neutralizing antibodies against IL-1 $\alpha$  and IL-1 $\beta$  for 30 minutes and isolated the nuclear extracts for EMSA. Anti-IL-1 $\alpha$  neutralizing antibody completely inhibited constitutive NF- $\kappa$ B activity in these cells, whereas anti-IL-1 $\beta$  neutralizing antibody had no effects on this activity (Fig. 2C). Together, these results suggest that autocrine secretion of IL-1 $\alpha$  conferred the induction of constitutive NF- $\kappa$ B activation in MiaPaCa-2/IL-1 $\alpha$  cells.

#### IL-1 $\alpha$ Autocrine Activity Enhanced the Low-Anchorage Colony Formation and Invasion of Pancreatic Cancer Cells

Because low-anchorage growth is one of the most common characteristics of metastatic cancer cells, we evaluated the ability of MiaPaCa-2/CTL and MiaPaCa-2/IL-1 $\alpha$  cells to form colonies in soft agar. Although MiaPaCa-2/IL-1 $\alpha$  cells did not show a significant growth advantage over the control cell line in normal culture conditions (Fig. 3A), IL-1 $\alpha$  expression significantly increased the low-anchorage growth for MiaPaCa-2

cells, especially in the less permissive soft agar higher concentration (Fig. 3B).

Cell invasion is an initial key step in the development of metastasis. Using a Matrigel-coated Boyden chamber method, MiaPaCa-2/IL-1 $\alpha$  cells showed a significantly higher invasive activity than that of control cells (Fig. 3B and C). Thus, their invasive phenotype also seems to be dependent on autocrine IL-1 $\alpha$  signaling.

#### IL-1 $\alpha$ Secretion Induced a Metastatic Phenotype in a Pancreatic Cancer Orthotopic *In vivo* Model

To determine the metastatic potential induced by the secretion of IL-1 $\alpha$  in a preclinical *in vivo* model that could be predictive of similar activity in humans, allowing also to monitor tumor growth and dissemination over time, MiaPaCa-2/CTL cells and MiaPaCa-2/IL-1 $\alpha$  cells infected by lentivirus to stably express high levels of luciferase were orthotopically injected into the pancreas of severe combined immunodeficient (SCID) mice. Although the mice in both groups showed a similar primary tumor volume (Fig. 4A), only mice bearing the MiaPaCa-2/IL-1 $\alpha$  tumors (mice no. 6, 8, and 10) had a diffuse pattern of abdominal metastases and developed ascites (Fig. 4B-D). At necropsy, liver metastases of pancreatic adenocarcinoma were observed only in the livers from the mice injected with MiaPaCa-2/IL-1 $\alpha$  cells (Fig. 4D and E). Thus, our results indicate that independently of any activity on the orthotopic primary,

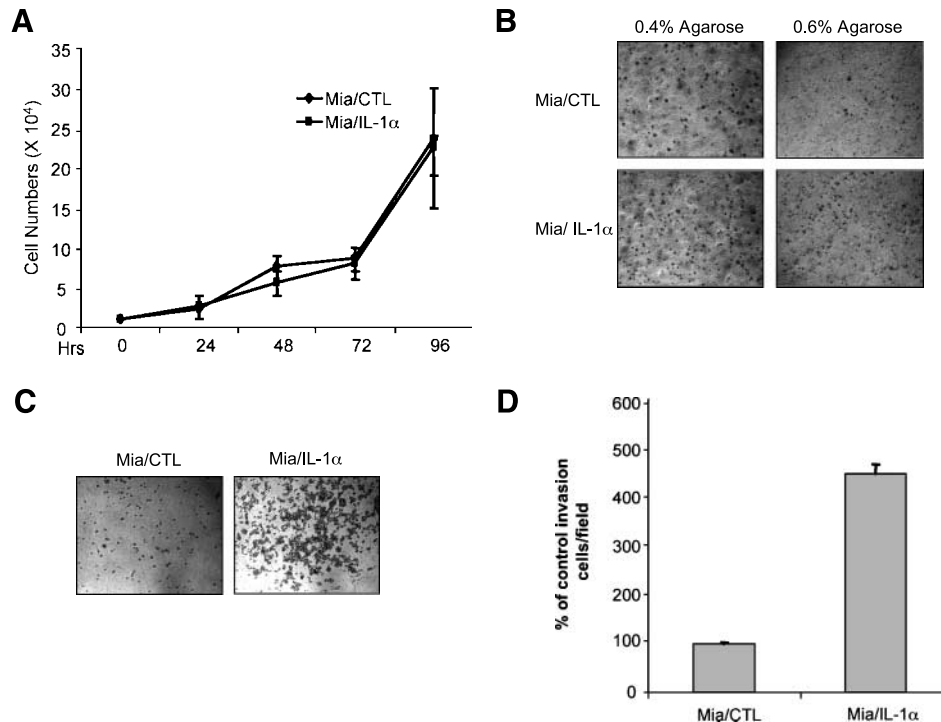
the secretion of IL-1 $\alpha$  induces the development of abdominal metastases from pancreatic cancer cells.

#### Expression of I $\kappa$ B $\alpha$ (S32, 36A) Phosphorylation Mutant (I $\kappa$ B $\alpha$ M) Inhibited the IL-1 $\alpha$ -Induced NF- $\kappa$ B Activity and the Expression of NF- $\kappa$ B-Regulated Genes

To determine the roles of the NF- $\kappa$ B activation in IL-1 $\alpha$ -induced pancreatic tumor metastatic processes, we used retroviral infection to generate stable clones (MiaPaCa-2/IL-1 $\alpha$ /I $\kappa$ B $\alpha$ M) that expressed I $\kappa$ B $\alpha$ M (I $\kappa$ B $\alpha$  S32, 36A) with mutated PEST domain to increase its stability (22) and these clones were pooled. As shown in Fig. 5A, the expression of I $\kappa$ B $\alpha$ M seemed as a slower migrating band, compared with WT I $\kappa$ B $\alpha$ , as I $\kappa$ B $\alpha$ M is Flag tagged. In the presence of I $\kappa$ B $\alpha$ M in MiaPaCa-2/IL-1 $\alpha$  cells, the endogenous I $\kappa$ B $\alpha$  protein was reduced to a minimum level due to the negative feedback regulation of I $\kappa$ B $\alpha$  by NF- $\kappa$ B (Fig. 5A; ref. 23). Constitutive NF- $\kappa$ B activation and tumor necrosis factor- $\alpha$ -mediated NF- $\kappa$ B activation were inhibited completely by I $\kappa$ B $\alpha$ M as determined by EMSA (Fig. 5B and C). The expression of the NF- $\kappa$ B downstream target genes *uPA*, *VEGF*, and *IL-8* was inhibited by I $\kappa$ B $\alpha$ M (Fig. 5D).

#### I $\kappa$ B $\alpha$ M Inhibited Tumor Aggressiveness of MiaPaCa-2/IL-1 $\alpha$ Cells

To show that the metastatic phenotype induced by IL-1 $\alpha$  is indeed mediated through the activation of NF- $\kappa$ B, we evaluated



**FIGURE 3.** IL-1 $\alpha$  expression in MiaPaCa-2 cells enhances anchorage-independent cell growth and cell invasion. **A.** Growth curve. MiaPaCa-2/CTL and MiaPaCa-2/IL-1 $\alpha$  cells ( $2 \times 10^4$ ) were cultured in regular DMEM. Cells were counted in triplicate at the time intervals indicated. Points, mean of three independent experiments; bars, SE. **B.** Soft agar assay. MiaPaCa-2/CTL and MiaPaCa-2/IL-1 $\alpha$  cells ( $5 \times 10^4$ ) were dispensed in either 0.4% or 0.6% top agarose on top of the 0.8% agarose-containing bottom agar. After 2 wk, the colonies were photographed under light microscopy. Magnification,  $\times 10$ . **C** and **D.** Cell invasion assay. MiaPaCa-2/CTL and MiaPaCa-2/IL-1 $\alpha$  cells were added to the top compartment of a Boyden chamber coated with Biocoat growth factor-reduced Matrigel basement membrane. After incubation for 48 h, cells that traversed the Matrigel-coated filters were stained, counted, and photographed. The representative fields are shown in **C**. Magnification,  $\times 10$ . The number of cells that traversed Matrigel-covered filters was determined by counting at least three randomized fields per insert. Columns, mean of three independent experiments; bars, SE.

the behavior of MiaPaCa-2/IL-1 $\alpha$  and MiaPaCa-2/IL-1 $\alpha$ /I $\kappa$ B $\alpha$ M cell lines *in vitro* and *in vivo*.

Whereas the inhibition of NF- $\kappa$ B by I $\kappa$ B $\alpha$ M had no significant antiproliferative effects on cells grown as a monolayer in cell culture dishes (Fig. 6A), the low-anchorage growth of MiaPaCa-2/IL-1 $\alpha$  cells was significantly inhibited by I $\kappa$ B $\alpha$ M expression, especially at the higher concentration of top layer agarose (Fig. 6B). Likewise, the *in vitro* cell invasion was also inhibited by the expression of I $\kappa$ B $\alpha$ M, as shown in Fig. 6C.

To test our *in vitro* findings in an *in vivo* setting, 20 mice were injected with MiaPaCa-2/IL-1 $\alpha$  or MiaPaCa-2/IL-1 $\alpha$ /I $\kappa$ B $\alpha$ M cells. Ten weeks after the injection, the mice injected with MiaPaCa-2/IL-1 $\alpha$  cells developed a diffuse pattern of abdominal metastases. Although 9 of the 10 mice injected with MiaPaCa-2/IL-1 $\alpha$ /I $\kappa$ B $\alpha$ M cells developed a primary pancreatic tumor, only 2 of them (no. 6 and 8) developed liver metastasis (Fig. 6D). Although the mice in both groups showed a similar primary tumor volume (Fig. 6E), at necropsy significantly fewer mice injected with MiaPaCa-2/IL-1 $\alpha$ /I $\kappa$ B $\alpha$ M cells developed liver nodules and ascites (Fig. 6F). Taken together, these results showed that the establishment of an IL-1 $\alpha$  autocrine loop in pancreatic cancer is sufficient to elicit an *in vivo* metastatic phenotype through a constitutive activation of NF- $\kappa$ B.

#### Silencing of IL-1 $\alpha$ Suppressed the Dissemination of the Highly Metastatic L3.6pl Pancreatic Cancer Orthotopic *In vivo* Model

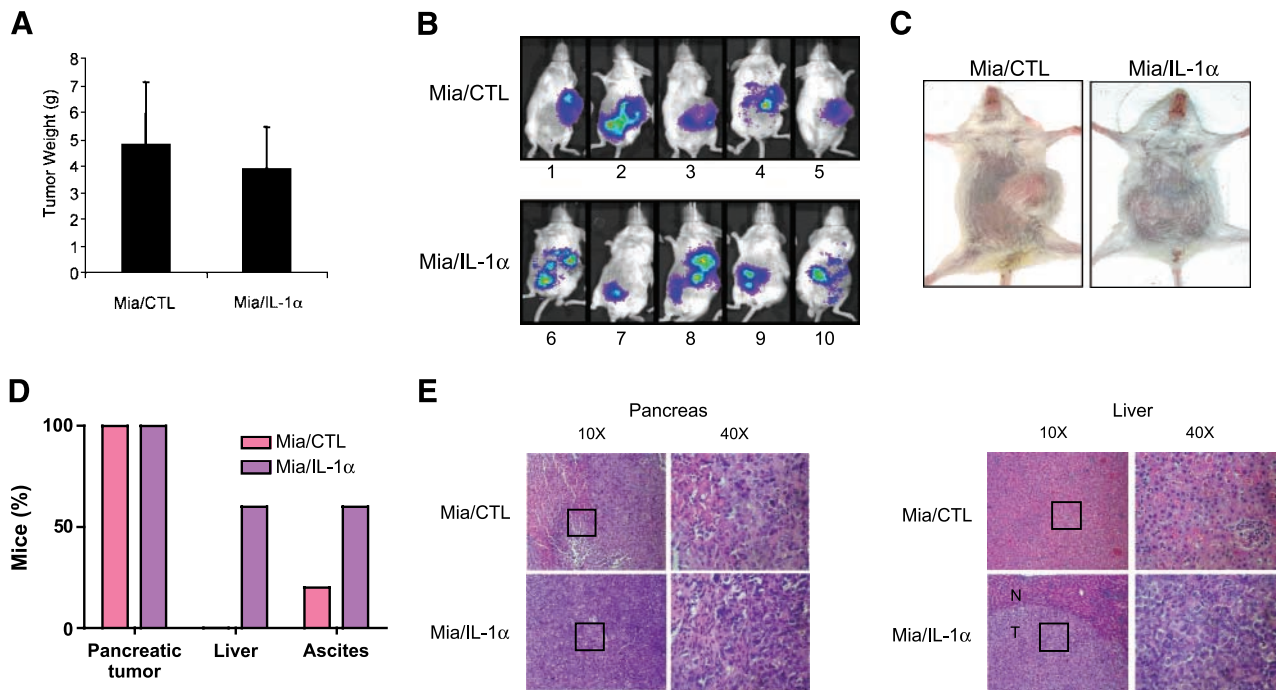
To further prove our hypothesis, we knocked down the expression of IL-1 $\alpha$  in the highly metastatic L3.6pl pancreatic

cancer cell line by using a lentivirus expressing green fluorescent protein and an IL-1 $\alpha$  shRNA (L3.6pl<sup>IL-1 $\alpha$ shRNA</sup>) or a scramble sequence (L3.6pl) as control (Fig. 7A). To determine the *in vivo* antimetastatic effect of silencing IL-1 $\alpha$ , a group of 20 mice was orthotopically inoculated with L3.6pl or L3.6pl<sup>IL-1 $\alpha$ shRNA</sup> cells. Although the mice in both groups showed a similar primary tumor volume (Fig. 7B), the mice bearing orthotopic L3.6pl pancreatic tumors had a diffuse pattern of abdominal metastasis at necropsy, and the mice bearing orthotopic L3.6pl<sup>IL-1 $\alpha$ shRNA</sup> pancreatic tumors developed significantly fewer metastatic lesions, and in some of them, no metastatic lesion as indicated by the green fluorescent protein signal could be identified in the abdomen (Fig. 6C-F). Thus, our results indicate that independently of any activity on the orthotopic primary, silencing of IL-1 $\alpha$  significantly reduces metastasis from pancreatic cancer cells.

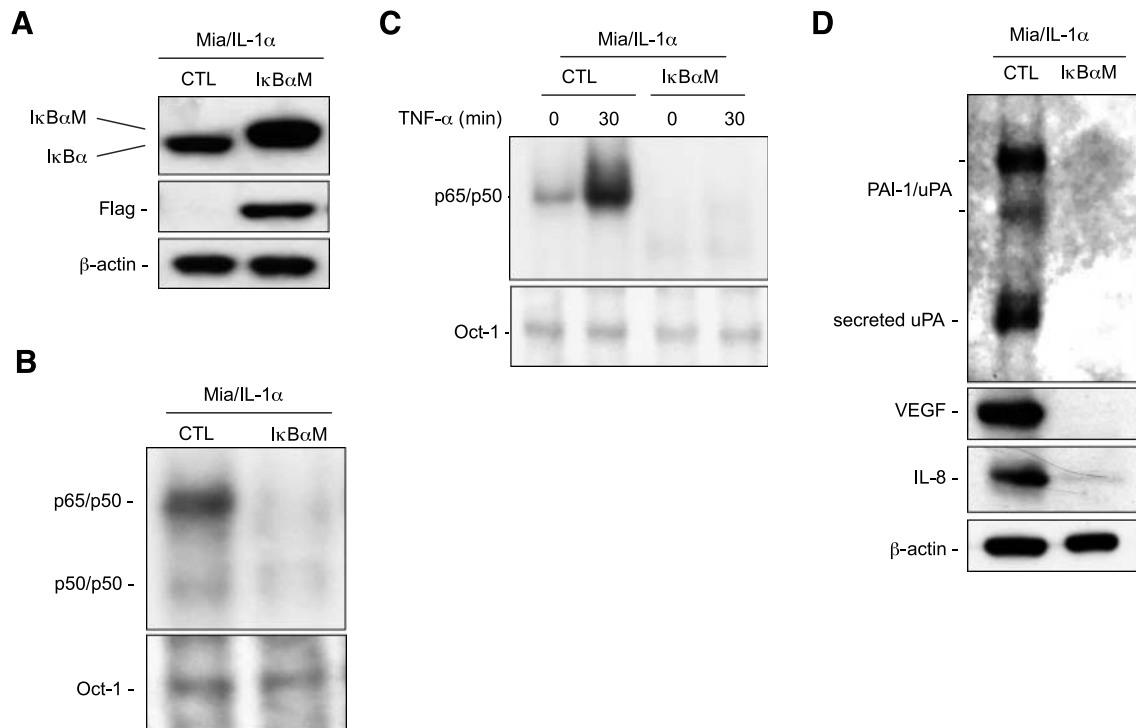
#### Discussion

The functional relationship between chronic inflammation and cancer is a widely accepted concept (24). However, many of the molecular mechanisms mediating this relationship remain unresolved. Of special relevance are different proinflammatory cytokine signaling mechanisms that are important mediators of chronic inflammatory responses and can be redirected to promote cancer cells invasion, migration, and metastasis (25).

IL-1 $\alpha$  and IL-1 $\beta$  are between the most potent cytokines that primarily affect inflammation, immunity, and hematopoiesis (26). Abundant at tumor site, microenvironment and tumor



**FIGURE 4.** IL-1 $\alpha$  secretion induces a metastatic phenotype in a pancreatic cancer orthotopic *in vivo* model. **A.** Tumors from mice injected with MiaPaCa-2/CTL or MiaPaCa-2/IL-1 $\alpha$  cells were weighed. Columns, mean of all individual tumors in the group; bars, SE. **B** and **C.** Tumor development, as indicated by the Lumi-light, was monitored and photographed in real time as shown by the color. **D.** Percentage of mice that developed pancreatic tumors, liver metastasis, and ascites. **E.** H&E staining of the pancreas and liver tissues from SCID/NCr mice injected with MiaPaCa-2/CTL and MiaPaCa-2/IL-1 $\alpha$  cells, either with  $\times 10$  or  $\times 40$  magnification. The square in  $\times 10$  magnification represents the field in the  $\times 40$  magnification. N, normal; T, tumor.



**FIGURE 5.** Phosphorylation-defective I $\kappa$ B $\alpha$  mutant (I $\kappa$ B $\alpha$ M) inhibited constitutive NF- $\kappa$ B activation and expression of NF- $\kappa$ B downstream genes in MiaPaCa-2/IL-1 $\alpha$  cells. **A.** Western blot analysis for expression of Flag-tagged phosphorylation-defective I $\kappa$ B $\alpha$  mutant (I $\kappa$ B $\alpha$ M) in MiaPaCa-2/IL-1 $\alpha$  cells was done using cytoplasmic extracts (20  $\mu$ g) with anti-Flag and anti-I $\kappa$ B $\alpha$  antibodies. Anti- $\beta$ -actin antibody was used as a loading control. **B.** Nuclear extracts (15  $\mu$ g) isolated from MiaPaCa-2/IL-1 $\alpha$ /CTL and MiaPaCa-2/IL-1 $\alpha$ /I $\kappa$ B $\alpha$ M cells were used in EMSA analysis with a HIV  $\kappa$ B probe to determine NF- $\kappa$ B activity and an Oct-1 probe was used as a loading control for quality and quantity of cell nuclear extracts. **C.** The I $\kappa$ B $\alpha$ M-mediated inhibition of cytokine-induced NF- $\kappa$ B activation was confirmed by NF- $\kappa$ B EMSA using MiaPaCa-2/IL-1 $\alpha$  and MiaPaCa-2/IL-1 $\alpha$ /I $\kappa$ B $\alpha$ M cells with and without tumor necrosis factor- $\alpha$  (*TNF- $\alpha$* ) stimulation as indicated and an Oct-1 probe was used as a loading control. **D.** The expression levels of NF- $\kappa$ B downstream genes *uPA*, *IL-8*, and *VEGF* were determined by Western blot analysis with the antibodies as indicated.  $\beta$ -Actin protein level was used as a loading control.

cell-derived IL-1 affect the process of carcinogenesis, tumor growth, and invasiveness and also the patterns of tumor-host interactions. In particular, IL-1 $\alpha$  has been suggested to regulate cancer cell invasion and metastasis, inducing invasiveness-promoting factors (i.e., matrix metalloproteinases) and adhesion molecules (27, 28). In an *in vitro* model of pancreatic cancer, recombinant IL-1 $\alpha$  was shown to enhance the adhesion of metastatic cells to extracellular matrix proteins by inducing the  $\alpha_5$ -integrin subunit (20).

Whereas IL-1 $\alpha$  and IL-1 $\beta$  in their recombinant forms bind to the same receptors and induce the same biological functions in primary cells, the most recent models about the IL-1 system propose a different role for IL-1 $\alpha$  and IL-1 $\beta$  based on their compartmentalization within the producing cell or the microenvironment. Whereas IL-1 $\beta$  is solely active as a secreted product, IL-1 $\alpha$  is normally secreted only in a limited manner and therefore it is mainly active as an intracellular precursor or in its membrane-associated form (9). On the other hand, tumor cells were largely shown to generate and secrete IL-1 either constitutively or in response to cytokines. In particular, the overexpression of IL-1 $\alpha$  by different malignant cells can induce diametrically opposite effects in accordance with its compartmentalization. In a model of fibrosarcoma cells, the transfection of the active form of IL-1 $\alpha$  resulted in its cytosolic and membrane-associated expression and increased the immunogenicity of

malignant cells *in vivo*. Regression of IL-1 $\alpha$ -positive fibrosarcomas involved their early infiltration by mononuclear cells and replacement by a fibrotic scar tissue (29). However, the exertion of this antitumor immune responses by tumor cell-associated IL-1 $\alpha$  is not observed when the overexpression in malignant cells leads to the secretion of the cytokine. Thus, IL-1 $\alpha$ -transfected A375 melanoma cells were shown to actively secrete the cytokine and to induce *in vivo* the expression of vascular cell adhesion molecule-1 on lung microvascular endothelial cells, enhancing their adhesiveness for tumor cells and increasing experimental lung metastases in nude mice (30).

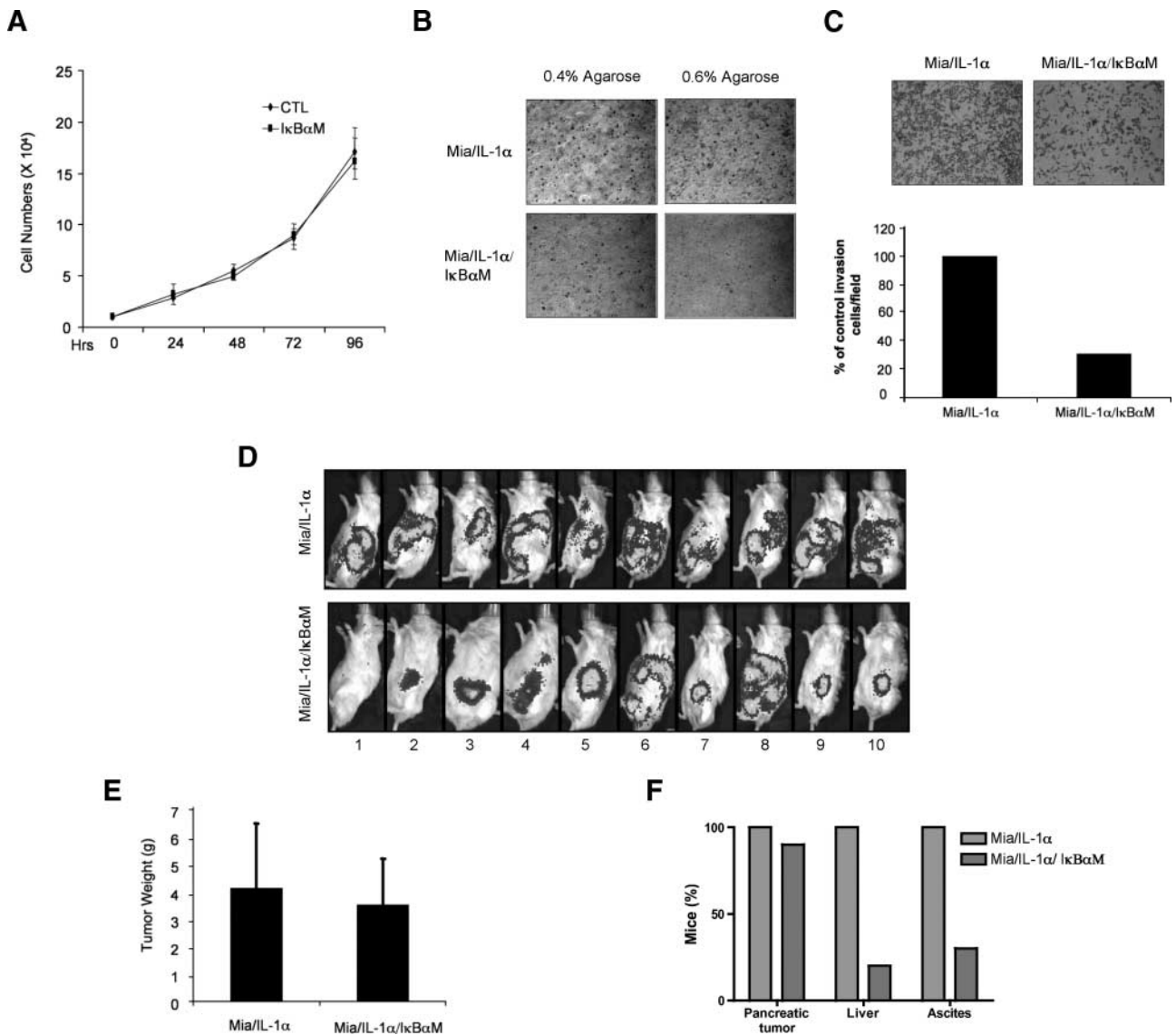
NF- $\kappa$ B activation was recently identified as a key modulator in driving inflammation to cancer (31). NF- $\kappa$ B is activated by inflammatory stimuli and its constitutive activation is found in cancer. Our laboratory previously reported that RelA, the p65 subunit of the NF- $\kappa$ B transcription factor, was constitutively activated in most pancreatic cancer tissues and cell lines but not in normal pancreatic tissues and immortalized pancreatic ductal epithelial cells (5, 32). Further studies showed that inhibition of constitutive NF- $\kappa$ B activity by a phosphorylation-defective mutant I $\kappa$ B $\alpha$  (S32, 36A) completely suppressed the liver metastasis and the tumorigenic phenotype of pancreatic cancer cell lines, suggesting that constitutive NF- $\kappa$ B activity plays a key role in pancreatic cancer metastasis and tumor progression (7, 33).

Noticeably, it has been shown that feedback loops exist between some proinflammatory cytokines and NF- $\kappa$ B activation (34). In particular, IL-1 $\alpha$  is not only a NF- $\kappa$ B inducer but also a downstream target gene of NF- $\kappa$ B (35, 36), whereas IL-1 $\beta$  production is negatively controlled by I $\kappa$ B kinase  $\beta$ -dependent NF- $\kappa$ B signaling (37).

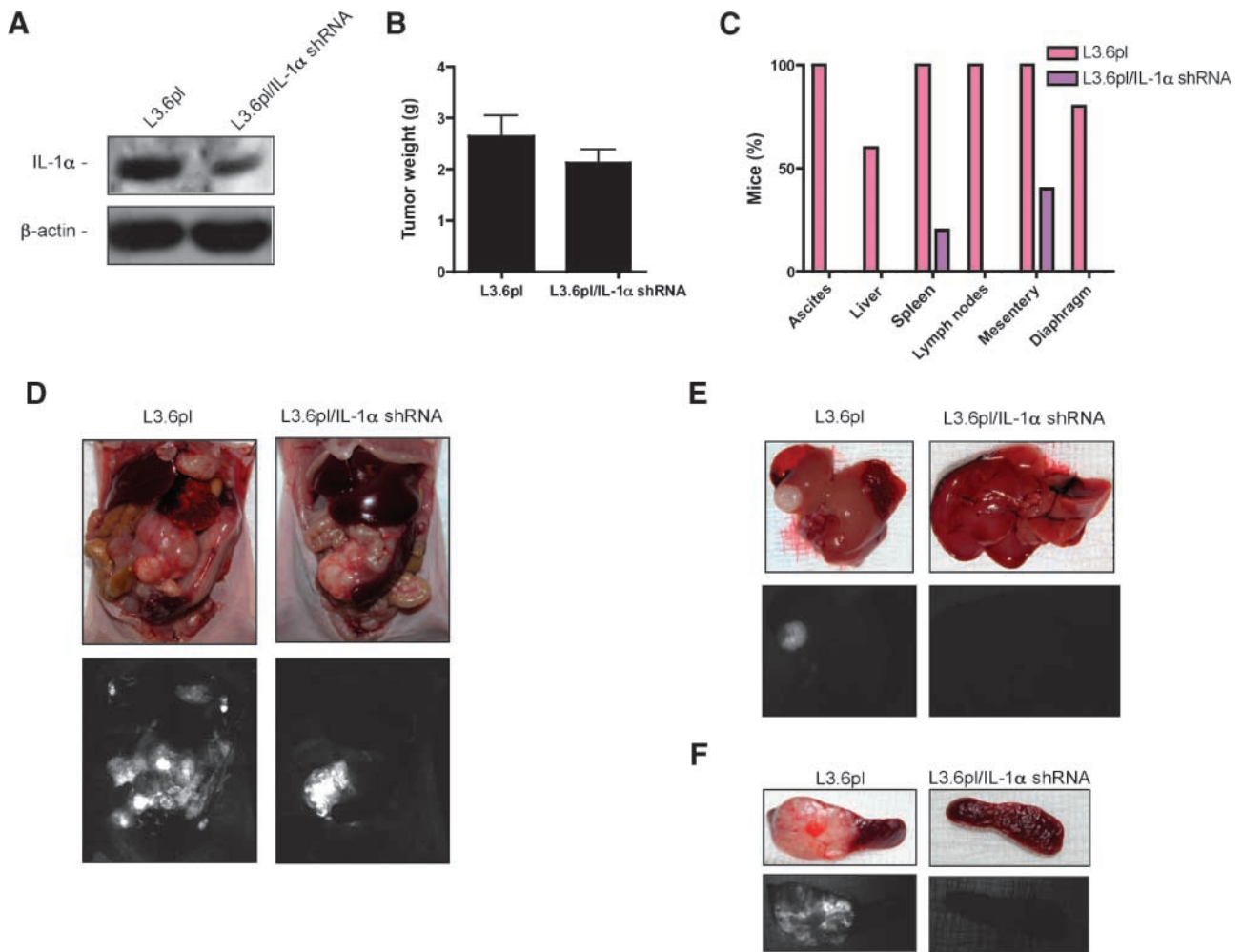
More recently, we showed that autocrine secretion of IL-1 $\alpha$ , but not IL-1 $\beta$ , leads to the activation of NF- $\kappa$ B in metastatic pancreatic cancer cell lines (12). In turn, NF- $\kappa$ B activation induces expression of IL-1 $\alpha$ , initiating the formation of a

positive feedback loop and establishing a mechanism for the constitutive NF- $\kappa$ B activation in this disease.

However, whether this IL-1 $\alpha$  autocrine loop plays a key role in pancreatic cancer metastasis was unclear. In the current study, we closed the circle showing that the autocrine secretion of IL-1 $\alpha$  in MiaPaCa-2 pancreatic cancer cell line constitutively activated NF- $\kappa$ B and in turn induced a metastatic behavior *in vivo* as shown by the higher incidence of liver metastases and ascites in an orthotopic mouse model. In particular, we found that the IL-1 $\alpha$  secreted by pancreatic cancer cells enhanced the



**FIGURE 6.** Inhibition of NF- $\kappa$ B in MiaPaCa-2/IL-1 $\alpha$  cells inhibited pancreatic tumor metastatic potential. **A.** Growth curve. MiaPaCa-2/IL-1 $\alpha$ /CTL and MiaPaCa-2/IL-1 $\alpha$ /I $\kappa$ B $\alpha$ M cells ( $2 \times 10^4$ ) were cultured in regular DMEM. Cells were counted in triplicate at indicated time intervals. Points, mean of three independent experiments; bars, SE. **B.** Soft agar assay. MiaPaCa-2/IL-1 $\alpha$ /CTL and MiaPaCa-2/IL-1 $\alpha$ /I $\kappa$ B $\alpha$ M cells ( $5 \times 10^4$ ) were dispensed in either 0.4% or 0.6% top agarose on top of the 0.8% agarose-containing bottom agar. After 2 wk, the colonies were photographed under light microscopy. Magnification,  $\times 10$ . **C.** Invasion assay. MiaPaCa-2/IL-1 $\alpha$ /CTL and MiaPaCa-2/IL-1 $\alpha$ /I $\kappa$ B $\alpha$ M cells were added to the top compartment of a Boyden chamber coated with Biocoat growth factor–reduced Matrigel basement. After incubation for 48 h, cells that traversed the Matrigel-coated filters were stained, counted, and photographed. The representative fields are shown in **C.** Magnification,  $\times 10$ . The number of cells that traversed Matrigel-covered filters was determined by counting at least three randomized fields per insert. Columns, mean of three independent experiments; bars, SE. **D.** SCID/NCr mice injected with MiaPaCa-2/IL-1 $\alpha$ /CTL or MiaPaCa-2/IL-1 $\alpha$ /I $\kappa$ B $\alpha$ M cells. The tumor development was monitored by Lumi-light display and photographed in real time. **E** and **F.** Tumor profiles for SCID/NCr mice injected with MiaPaCa-2/IL-1 $\alpha$ /CTL and MiaPaCa-2/IL-1 $\alpha$ /I $\kappa$ B $\alpha$ M cells. Tumors were weighed. Columns, mean of all individual tumors in the group; bars, SE. Percentage of mice that developed pancreatic tumors, liver metastases, and ascites.



**FIGURE 7.** Silencing IL-1 $\alpha$  suppressed the formation of secondary lesions from L3.6pl highly metastatic cells. **A.** Western blot analysis for the expression of IL-1 $\alpha$  was done using cytoplasmic extracts (20  $\mu$ g) from L3.6pl or L3.6pl<sup>IL-1 $\alpha$ shRNA</sup> cells. **B.** Athymic nude mice (NCI-nu) inoculated with L3.6pl or L3.6pl<sup>IL-1 $\alpha$ shRNA</sup> cells. Tumors were weighed. Columns, mean of all individual tumors in the group; bars, SE. **C** and **D.** At necropsy, the presence of ascites and fluorescent tumor lesions in the liver (**E**), spleen (**F**), intestinal mesentery, diaphragm, lymph nodes (celiac and para-aortic), and other peritoneal organs was confirmed with a stereoscopic dissecting fluorescence microscope.

expression of several NF- $\kappa$ B-regulated genes involved in the metastatic processes and angiogenesis, including *uPA*, *IL-8*, and *VEGF*. The inhibition of NF- $\kappa$ B by phosphorylation-defective I $\kappa$ B $\alpha$ M (I $\kappa$ B $\alpha$  S32A, S36A) inhibited these responses, suggesting that the effects of IL-1 $\alpha$  on pancreatic tumor progression are indeed mediated by the activation of NF- $\kappa$ B.

These findings provide us with a better understanding of the role for the secreted IL-1 $\alpha$ -NF- $\kappa$ B positive feedback loop as a fundamental molecular mechanism to support the malignant phenotype in pancreatic cancer. In our opinion, IL-1 $\alpha$  autocrine secretion remains a main therapeutic target for pancreatic cancer patient treatment.

## Materials and Methods

### Cell Lines and Reagents

The human pancreatic cancer cell line MiaPaCa-2 was purchased from the American Type Culture Collection. Human pancreatic cancer cell line L3.6pl was previously described (38). The

human IL-1 $\alpha$  cDNA fragment was cloned from the MDAPanc28 pancreatic cancer cell line with reverse transcription-PCR and cloned into the *Hpa*I site of retrovirus vector pLHCX. The stable MiaPaCa-2/IL-1 $\alpha$  and MiaPaCa-2/IL-1 $\alpha$ /I $\kappa$ B $\alpha$ M cell lines were established with retrovirus infection as previously described by Dong et al. (32). All MiaPaCa-2 cells were infected with lentivirus containing luciferase, making them suitable for *in vivo* imaging. WT MEF cells were established in our laboratory according to the report by Friess et al. (39). To silence the expression of IL-1 $\alpha$ , two human IL-1 $\alpha$  shRNA target sequences were chosen: 5'-gatccccAAGTATAATTCGAGCCAAT-tcaagagaATTGGCTC-GAATTATACTT3tttttc-3', 5'-tcgagaaaaAAGTATAATTC-GAGCCAATtctctttaaATTGGCTCGAATTATACTT3ggg-3', 5'-gatccccGGCCAAAGTTCCAGACATGtcaagagaCATGTCTGGAACCTTTGGCCtttttc-3', and 5'-tcgagaaaaGGCCAAAGTTCCAGACATGtctctttaaCATGTCTGGAA-CTTTGGCCggg-3'.

These sequences were cloned into FG12 lentivirus vector (gift from Dr. Qin Xiaofeng, The University of Texas M. D.



Anderson Cancer Center, Houston, TX) with *Bam*HI and *Hind*III. The shRNA vector and packaging vectors were co-transfected into 293T cells. The virus-containing supernatants were collected after 72 h of transfection and filtered through a minipore 0.45- $\mu$ m filter. The L3.6pl cells were transduced by the lentivirus in the presence of the polycation Polybrene. The stably transduced cells were sorted by green fluorescent protein.

All cell lines were grown in the original cell culture medium specified by the American Type Culture Collection and DMEM containing 10% fetal bovine serum. IL-1 $\alpha$  and IL-1 $\beta$  neutralizing antibodies were obtained from R&D Systems.

#### *Electrophoretic Mobility Shift Assay*

Conditioned media were prepared as previously described (12). The harvested conditioned media were used to treat WT MEFs at different intervals. Neutralizing antibodies (2 mg/mL) were used to block the corresponding cytokines. The nuclear extracts were prepared according to the method of Andrews and Faller (40). DNA binding assays for NF- $\kappa$ B proteins were done with 10  $\mu$ g of nuclear extracts as described by Sclabas et al. (6). The WT double-stranded oligonucleotides containing the  $\kappa$ B site were obtained from Santa Cruz Biotechnology, Inc. and labeled with  $^{32}$ P to be used as probes. The mutant  $\kappa$ B site for HIV long terminal repeat (5'-CTCAACAGAGTTGACTTTTCGAGAGGCCAT-3') was used for competition studies. The competition was done with a 50-fold excess of unlabeled WT or mutant  $\kappa$ B oligonucleotides. Supershift experiments were done with anti-p65 antibody (Santa Cruz Biotechnology). The reactions were analyzed on 4% polyacrylamide gels containing 0.25 $\times$  Tris/borate/EDTA buffer.

#### *Western Blot Analysis*

MiaPaCa-2 cells were washed twice with cold PBS and lysed at 4°C into radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 8), 150 mmol/L NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS]. The lysates were cleared by centrifugation. To detect secreted IL-1 $\alpha$ , uPA, VEGF, and IL-8, conditioned media were collected, centrifuged at 15,000  $\times$  g for 5 min to remove cell debris, and then subjected to immunoblotting under nonreducing conditions. Twenty-four-hour conditioned media were dialyzed for 24 h and concentrated using Microcon YM-10 centrifugal filter devices from Millipore. The volumes of conditioned medium loaded on gels were normalized to the protein concentrations of cell lysates. The concentrated media or 50  $\mu$ g of protein extracts were resolved by SDS-PAGE, transferred to nylon membranes (Immobilon-P, Millipore), and detected with I $\kappa$ B $\alpha$ , IL-1 $\alpha$  (Santa Cruz Biotechnology), uPA (American Diagnostica), IL-8, VEGF (BioSource International), or  $\beta$ -actin antibody (Sigma Chemical). Subsequent Western blot analyses were carried out with Lumi-light Western blot substrate (Roche Diagnostics).

#### *Reverse Transcription-PCR*

IL-1 $\alpha$  mRNA level was detected with reverse transcription-PCR. In brief, total RNA from MiaPaCa-2 cells was extracted using Trizol reagent according to the manufacturer's protocol (Invitrogen Life Technologies). The RNA was then reverse transcribed into cDNA and amplified at 94°C for 5 min, then

30 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, and finally 72°C for 7 min. The primers used for IL-1 $\alpha$  were 5'-GGTAGTAGCAACCAACGGGA-3' and 5'-TGGGTATCTCAGGCATCTCC-3'. The PCR products were 419 bp long.

#### *Growth Curve*

MiaPaCa-2 cells ( $1 \times 10^4$ ) were seeded in six-well plates and kept in normal DMEM (contains 10% fetal bovine serum). Cells were counted every 24 h in triplicate for 4 d. Each experiment was done independently at least thrice with similar results each time. Results were expressed as the mean  $\pm$  SE of three independent experiments. All of the statistical analyses were done using StatView 5.0 (Abacus Concepts, Inc.).

#### *Cell Invasion Assay*

Invasion assay was done in 24-well plates by using a BD Biocoat growth factor-reduced Matrigel invasion chamber (BD Biosciences) with an 8.0- $\mu$ m pore size positron emission tomography membrane as previously described (41). In brief,  $5 \times 10^4$  MiaPaCa-2 cells were seeded in the invasion chambers and incubated at 37°C for 48 h. Noninvading cells from the interior of the inserts were removed by using cotton-tipped swabs. Invading cells on the under side of the membrane were fixed by 10% formaldehyde for 10 min and then stained with 1% crystal violet, and at least three random fields per insert were counted under an Olympus microscope. A field representative of each experiment was photographed on an Olympus DP 10 digital camera under Olympus IMT-2 light microscope with  $\times 4$  lens and  $\times 2.5$  magnification. Results are expressed as the mean  $\pm$  SE of three independent experiments.

#### *Soft Agar Assay*

A 24-well plate was coated with 0.8% agarose-containing DMEM as bottom agar;  $5 \times 10^4$  MiaPaCa-2 cells were dispensed in either 0.4% or 0.6% agarose-containing medium and placed on the top of the bottom agar. After 2 wk, the colonies were photographed under an Olympus IMT-2 light microscopy.

#### *Orthotopic Mouse Model*

Female SCID/NCI (BALB/c background) or female athymic nude mice (NCI-nu) were purchased from National Cancer Institute (Frederick, MD) at 4 to 6 wk old. Mice were housed in cabinets with laminar flow under specific pathogen-free conditions. Animals were maintained according to institutional regulations and the animal protocol was approved by the Institutional Animal Care and Use Committee at The University of Texas M. D. Anderson Cancer Center. The orthotopic injection of pancreatic cancer cells was done as previously described (42). Briefly, mice are anesthetized with methoxyflurane; a small left abdominal flank incision is made, and the spleen is exposed. One million of cells with  $>90\%$  viability in trypan blue exclusion assay, suspended in 50  $\mu$ L PBS, were injected subcapsularly in a region of the pancreas just beneath the spleen. A 30-gauge needle, a 1-mL disposable syringe, and a calibrated, push button-controlled dispensing device are used to inject the cell suspension. A successful subcapsular intrapancreatic injection of tumor cells is confirmed by the appearance of a fluid bleb without i.p. leakage. A cotton swab is held for

1 min over the site of injection to prevent such leakage. One layer of the abdominal wound is closed with wound clips. The animals usually tolerate the surgical procedure well, and no anesthesia-related deaths have occurred. Tumor development was monitored in real time with the IVIS imaging system, equipped with Living Imaging software (Xenogen).

At necropsy, the presence of ascites and fluorescent tumor lesions in the pancreas, spleen, lymph nodes (celiac and para-aortic), liver, diaphragm, and other peritoneal organs was confirmed with a Leica MZ16 stereoscopic dissecting fluorescence microscope equipped with a Hamamatsu Orca ER cooled charge-coupled device digital camera coupled to a data acquisition computer running the image acquisition software Image-Pro version 6.0. The mice were sacrificed when required by our institutional guidelines. Formalin-fixed, paraffin-embedded pancreas or liver tissues were dissected from the mice immediately after sacrifice and processed for H&E staining.

### Disclosure of Potential Conflicts of Interest

There are no potential conflicts of interest.

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