Extracellular DNA in Pancreatic Cancer Promotes Cell Invasion and Metastasis

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Abstract

Aggressive metastasis is the chief cause of the high morbidity and mortality associated with pancreatic cancer, yet the basis for its aggressive behavior remains elusive. Extracellular DNA (exDNA) is a recently discovered component of inflammatory tissue states. Here, we report that exDNA is present on the surface of pancreatic cancer cells where it is critical for driving metastatic behavior. exDNA was abundant on the surface and vicinity of cultured pancreatic cancer cells but absent from normal pancreas cells. Strikingly, treatment of cancer cell cultures with DNase I to degrade DNA nonspecifically reduced metastatic characters associated with matrix attachment, migration, and invasion. We further assessed the role of exDNA in pancreatic cancer metastasis in vivo using an orthotopic xenograft model established by implantation of pancreatic cancer cells expressing firefly luciferase. Noninvasive bioluminescent imaging confirmed that DNase I treatment was sufficient to suppress tumor metastasis. Mechanistic investigations suggested the existence of a positive feedback loop in which exDNA promotes expression of the inflammatory chemokine CXCL8, which leads to higher production of exDNA by pancreatic cancer cells, with a significant reduction in CXCL8 levels achieved by DNase I treatment. Taken together, our results strongly suggest that exDNA contributes to the highly invasive and metastatic character of pancreatic cancer. Cancer Res; 73(14); 4256–66. ©2013 AACR.

Introduction

Pancreatic cancer is the fourth leading cause of cancer-related death in the United States and one of the most lethal malignancies, with a 5-year survival rate less than 5% and a death/incidence ratio of approximately 0.99 (1). Of note, 80% to 90% of patients with pancreatic cancer have already developed metastatic cancer at the time of diagnosis (2). The mechanisms underlying the development of metastases during pancreatic carcinogenesis are poorly understood (3). Understanding these mechanisms may provide novel approaches to pancreatic cancer treatment and/or prevention.

The development of metastasis is determined by both genetic alterations in tumor cells and by the surrounding microenvironment (4, 5). Inflammation has been shown to facilitate tumor metastasis, from dissemination of cancer cells at the primary tumor site to their implantation at secondary sites (6–8). The link between inflammation and pancreatic cancer development has been well recognized. Chronic pancreatitis/inflammation is among the few high risk factors of developing pancreatic cancer (1, 9–13).

Inflammation mediators including CXCL8 have been shown to induce the production of extracellular DNA (exDNA)—containing fibers by neutrophils (nicknamed neutrophil extracellular traps or NET; ref. 14). exDNA traps have been associated with several chronic inflammatory diseases, including cystic fibrosis, small-vessel vasculitis, and deep vein thrombosis (14–18). It has been recently reported that cytokine-induced production of NETs contributes to cancer-associated thrombosis (19). Production of exDNA has been reported in a number of inflammatory cell types in addition to neutrophils, including macrophages, mast cells, platelets, dendritic cells, B, and T lymphocytes. These inflammatory cells have all been found in the stroma of pancreatic cancer (20, 21) and have been implicated in angiogenesis and tumor metastasis by secreting cytokines and chemokines (22).

Others have shown significantly elevated production and secretion of the chemokine CXCL8 in the highly liver-metastatic pancreatic cancer cell line BxPC3 in comparison with the immortalized normal human pancreatic ductal epithelium human pancreatic ductal epithelial (HPDE) cell line (23). In this study, we asked whether normal or neoplastic pancreatic cells produced exDNA. We observed a large amount of exDNA on the surfaces and in the vicinity of the cultured BxPC3 and MiaPaCa-2 pancreatic cancer cell lines but not the normal pancreas HPDE cell line. We went on to assess the relationship between exDNA, CXCL8, and features of metastasis in both cell and tumor models of pancreatic cancer. This is the first report of the association of an abundant amount of surface exDNA...
with pancreatic cancer cells and its possible role in pancreatic cancer metastasis. This discovery may provide a new target and novel strategies for prevention, diagnosis, and treatment of this lethal cancer.

Materials and Methods

Cell culture and DNase I treatment of cells

We obtained MiaPaCa-2, BxPC3, and Panc-1 human pancreatic cancer cell lines from American Type Culture Collection. The cells were cultured at 37°C with 5% CO2 in RPMI-1640 medium (Mediatech, Inc.), supplemented with 10% FBS (Omega Scientific, Inc.), 2.5 mg/mL glucose, 1% L-glutamine, and 1% penicillin/streptomycin (Invitrogen). Immortalized normal HPDE cells and HPDE-KRASG12D cells expressing mutated KRAS were kindly provided by Dr. Ming-Sound Tsao (University of Toronto, Toronto, Canada; ref. 24). HPDE cells were cultured in keratinocyte serum-free medium supplemented with EGF and bovine pituitary extract (Invitrogen). DNase I treatment of 10^5 cells was at the concentration of 3 U/100 µL. All the in vitro DNase I treatments lasted for 24 to 72 hours depending on different assays.

MTT cell viability and cell growth assay

Cell survival and growth was measured by MTT assay as previously described (25, 26). HPDE control cell line and pancreatic cancer cell lines BxPC3 and MiaPaCa-2 were examined with or without DNase I treatment (3 U/well/10,000 cells) 24 hours after cells were treated with DNase I.

Wound-healing assay

Cells were grown in 24-well plates in 500 µL medium per well until confluence was reached. A wound was made by scratching the cells with a 10-µl pipette tip in PBS, followed by replacement by culture media with and without DNase I (15 U/well for up to 3 days). The wounded monolayer was photographed over time and cell migration was assessed by measuring gap sizes at multiple fields using ImageJ (National Institute of Mental Health, Bethesda, MD).

Cell migration assay

Cell migration assays were conducted using a modified 24-well Boyden chamber. The top chamber (Transwell) with 8.0-µm pores on the filter membrane (BD Labware) was inserted into a 24-well plate (bottom chamber). Ten percent FBS-containing medium was placed in the bottom chambers to be used as a chemoattractant. Cells (3 × 10^5) in a 300 µL volume of serum-free medium with or without DNase I were placed in the top chambers and incubated at 37°C for 24 hours. Migrated cells on the bottom surface of the filter were fixed and stained with crystal violet.

Crystal violet staining

Twenty-four hours after culturing cells in the top chamber, medium in the Transwell was siphoned off and the chamber was moved to the bottom chamber containing 4% paraformaldehyde to fix cells for 10 minutes. Top chamber was rinsed in PBS and inverted for staining. Fifty microliter of 5% crystal violet (Sigma-Aldrich) in 25% methanol was applied onto the bottom of the filter of the top chamber and cells were stained for 10 minutes. Excess crystal violet was washed off by plunging the top chamber into distilled water in a beaker several times. Finish washing in a second beaker till water is clear. Cells on top side of filter (cell that did not migrate) were removed using a moist cotton swab. The filter was then air-dried. Cells in five to seven random fields were counted at ×40 objective lens under an inversion microscope.

Cell invasion assay

The system setup for invasion assay using Boyden chamber was exactly the same as for cell migration assay, except that the Transwell filter was coated with 40 µL Matrigel (BD Biosciences) and cells were stained 48 hours instead of 24 hours after culture.

Fluorescent dye staining

Cells (1 × 10^5/well) were seeded on sterile cover slips that were placed in 6-well plate. Two days later, culture medium was aspirated and the cover slips were rinsed with PBS. Cells that grew on the cover slips were then fixed in 4% paraformaldehyde for 10 minutes, followed by rinsing with water. Cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) or SYTOX Green by mounting the cover slip with the mounting medium containing DNA dye DAPI (Vector Laboratories), or by mounting the cover slip on a regular glass slide with KPL mounting medium containing another DNA fluorescent dye SYTOX Green (Molecular Probes), a nonliving-cell-permeant DNA-binding dye. For staining exDNA induced by CXCL8, SYTOX Green was added to cells cultured in a 24-well culture plate at 1 µmol/L, final concentration.

For staining cells in paraffin sections, tissue slides were deparaffinized with 100% xylene twice, 10 minutes each and hydrolyzed in 100% ethanol twice (5 minutes each), 95% ethanol twice (5 minutes each), 80% ethanol twice (5 minutes each), and water twice (5 minutes each). Then, DNA was stained by mounting the slides with the mounting medium containing DAPI (Vector Laboratories).

Immunofluorescence staining with DNA antibody

Cells (1 × 10^5/well) were seeded on sterile cover slips that were placed in 6-well plate. Two days later, culture medium was aspirated and the cover slips were rinsed with PBS. Cells that grew on the cover slips were then fixed in 4% paraformaldehyde for 10 minutes, followed by cold methanol and acetone treatment (5 minutes/each) for cell fixation and permeabilization, then by PBS rinsing. Some cells were not treated for permeabilization after the fixation in paraformaldehyde. Next, cells were blocked with 5% bovine serum albumin (BSA) in PBS for 1 hour at room temperature, followed by rinsing with PBS three times, 3 minutes/each. Then cells were probed with mouse DNA antibody (1:100 dilution in 5%BSA/PBS) either 1 hour at room temperature or at 4°C overnight. DNA antibody was purchased from Santa Cruz Biotechnology. After the primary antibody reaction, cells were rinsed with PBS three times, followed by secondary antibody (1:800 dilution in 5%BSA/PBS) reaction at room temperature for 40 minutes. The secondary antibody was Alexa Fluor 680–conjugated bovine
antimouse (Life Technologies). Following secondary antibody reaction, cover slips were rinsed in PBS and water separately, three time each solution. Finally, cover slips were mounted on regular glass slides either with KPL mounting medium or with the mounting medium containing DAPI.

**Immunofluorescence staining with integrin-β-1 antibody and DNA antibody**

Cells and cover slips were prepared the same way as above for staining with DNA antibody alone. Cells were not treated for permeabilization after the fixation. Here, 1:100 dilution of both mouse DNA and rat integrin-β-1 (a gift from Dr. Anne Cress, Arizona Cancer Center, University of Arizona, Tucson, AZ) primary antibodies were mixed to probe cells. Alexa Fluor 680/488-conjugated bovine anti-mouse/anti-rat secondary antibodies for DNA and for β-1 separately were mixed for secondary antibody reaction.

**Immunohistochemistry staining with DNA antibody**

Tissue slides were deparaffinized and hydrolyzed as described in the "Fluorescent dye staining" section. After the hydration, antigen retriever step was followed. Slides were emerged in the antigen retriever solution (0.01 mol/L sodium citrate, 0.05% Tween-20, pH 6.0) and heated in a microwave. The solution was brought to boil and the power was turned off. Then heat was turned on again to bring the solution to boil. The power "on and off" was repeated for 10 minutes to keep the solution to boil, but was kept from boiling out of the container. At the end of 10 minutes, the container was left on ice for 20 minutes. Then, slides were rinsed with PBS three times before they were subjected to immunoreaction. Procedures for blocking, primary antibody reaction, and secondary antibody reaction were the same as for the immunofluorescence stain as described earlier, except for using houseardish peroxidase–conjugated mouse secondary antibody (Santa Cruz Biotech). Alexa Fluor 680–conjugated secondary antibody. NovaRED substrate (Vector Laboratories) was used for detection. After secondary antibody reaction, slides were rinsed and mounted the same as for immunofluorescence stain.

**Orthotopic xenograft mouse model**

All procedures involving animal were approved by the University of Arizona Institutional Animal Care and Use Committee (IACUC) protocol #07029. Pancreatic cancer cell line MIA PaCa-2/LucE expressing firefly luciferase was used. A total of 2 × 10^5 cells in 100 μl Matrigel (Becton Dickinson) were injected into severe combined immunodeficient (SCID) donor mice. After 4 days, Matrigel plugs were removed from the donor mice and implanted in the body-tail of the pancreases of receiving SCID mice at age of 6 to 8 weeks. Two groups of SCID mice were used. Group A: 10 SCID mice were injected with MIA PaCa-2/LucE cells and received an intraperitoneal injection of saline every other day as a control treatment. Group B: 14 SCID mice were injected with MIA PaCa-2/LucE cells together with DNase I at a concentration of 1 U/mouse in subgroup 1 (n = 7) and at a concentration of 50 U/mouse in subgroup 2 (n = 7). Group B mice received intraperitoneal injections of DNase I every other day at the same dosage as used in the first injection. Mice were purchased and maintained through the Experimental Mouse Shared Service in University of Arizona Cancer Center (EMSS/UACC). Mouse xenograft and DNase I application were carried out by specialists at the EMSS facility. In vivo tumor growth and spontaneous metastasis were monitored and recorded noninvasively every week by bioluminescence imaging (BLI) measuring the bioluminescence from tumor cells using an In Vivo Imaging System (AMI-1000; Spectral Instrument Imaging). At the end of the project, the mice were sacrificed and various organs were harvested for hematoxylin and eosin (H&E) staining and histology, and ex vivo bioluminescence analysis to confirm the tumor metastasis.

**Histology of pancreatic xenografts**

The pancreas, livers, spleens, diaphragms, and lungs were harvested, visually inspected, fixed in 10% formalin buffer, processed, embedded, sectioned, H&E, and immunohistochemistry (IHC) stained with DNA antibody, and analyzed by a pathologist for the presence of tumors.

**Cell adhesion assay**

The 96-well microtiter plate was coated with Matrigel to assess cell adhesion to extracellular matrix as described before (27). A 150-μL cell suspension of MiaPaCa-2, BxPc3, and HPDE cells were added to the coated wells (1 × 10^5/well) with or without the presence of 7 U DNase I/well and incubated at 37°C for 1 hour. After washing off unattached cells, attached cells were stained with crystal violet and counted under an inverting microscope.

**Hanging drop assay**

Cells were harvested and suspended in corresponding media as single cells. Drops (30-μL/drop) of media containing 20,000 cells per drop of HPDE or MiaPaCa-2 cells were pipetted onto the inner surface of a lid of a 24-well plate. The lid was placed upright on the plate so that the drops were hanging from the lid with cells suspended within them. The corresponding bottom wells contained media to maintain humidity. After culturing cells at 37°C overnight, hanging drops were transferred to microfuge tubes and were pipetted seven times up and down with a 200-μL standard tip, fixed with 2% paraformaldehyde, and aliquots were spread on regular microscope glass slides covered with cover slips. Images of five random fields were taken and clusters containing 10 or more cells were counted.

**Real-time PCR and CXCL8 ELISA**

Cells (3 × 10^5 in 500 μL/well) were seeded in 24-well plate. In 24 hours, media were changed and DNase I was added (15 U/wells) to treatment wells. Cells without DNase I addition were controls. Two days later, culture media were collected for CXCL8 ELISA, whereas cells were lysed for total RNA extraction and real-time PCR. Media were pulse-centrifuged at 1,000 rpm. Supernatant were transferred to new tube and stored at −80°C for later ELISA analysis. The secreted CXCL8 level was measured by using a CXCL8 ELISA assay kit (QuantiGlo Chemiluminescent Immunoassay; R&D Systems). Total RNA extraction and real-time PCR were conducted as described before (26). CXCL8 forward primer sequence...
is ATGACTTCCAAGCTGGCCGTGGCT; and CXCL8 reverse primer sequence is TCTCAGCCCTCTGAAAACTTCTC.

**Measurement of exDNA induced by CXCL8**

Of note, 100 μL of 10,000 cells per well were seeded in a 96-well plate together with 80 ng/mL of CXCL8 protein (BD Biosciences). Cells without CXCL8 treatment are control cells. After overnight incubation, 1 μmol/L of SYTOX Green was added to the cells to detect exDNA. The plates were read within 15 minutes in a Spectra MAX Gemini fluorescence microplate reader with a filter setting of 485 nm (excitation)/538 nm (emission). For nonquantitative observation of exDNA after CXCL8 treatment, cells were seeded in a 24-well plate with or without 80 ng/mL CXCL8. After overnight incubation, 1 μmol/L SYTOX Green was added to the cells. Cells were observed under a Carl Zeiss fluorescent microscope. Images were captured using the AxioVisionE software.

**Statistical analyses**

All data are reported as mean ± SD. Statistical comparisons between treatment groups were done by the Student t test and by the single factor ANOVA analysis. For all analyses, differences were considered significant at P < 0.05.

**Results**

**exDNA on the surface of pancreatic cancer cells in vitro**

We detected exDNA on the surface and in the vicinity of cultured pancreatic cancer cell lines. We used fluorescent DNA dyes DAPI and SYTOX Green to stain cells cultured on cover slips. DAPI is a fluorescent stain that binds strongly to A–T-rich regions in DNA. To validate the DAPI stain, we used another DNA fluorescent dye: SYTOX Green. Unlike DAPI, this dye shows little base selectivity. exDNA associated with cancer cell lines BxPc3 and MiaPaCa-2 was observed (Fig. 1B–D), but no exDNA was visible associated with the immortalized normal HPDE cell line (Fig. 1A). All cells were tested Mycoplasma-free.

To confirm that the fluorescent dyes were detecting DNA molecules, we used a DNA antibody (Santa Cruz Biotechnology) to carry out the immunofluorescence assay (IFA) with a fluorochrome Alexa Fluor 594–conjugated secondary antibody. In agreement with the fluorescent dye staining, abundant exDNA associated with pancreatic cancer cell lines was observed (Fig. 1F) by IFA, whereas no exDNA but only nuclei DNA positively reacted to the DNA antibody in the control cell line HPDE (Fig. 1E). The IFA results were examined under both regular fluorescence microscope (Fig. 1E–G) and the confocal microscope (Fig. 1H–J). When MiaPaCa-2 cells were treated with DNase I before IFA, only trace amount of extracellular fluorescent materials were seen (Fig. 1G, arrows). The exDNA associated with cancer cells does not seem to be produced by dying cells, because HPDE has a higher apoptosis level than BxPc3 cells but no associated exDNA (Supplementary Fig. S1). We also used fluorescein diacetate to conduct a viability assay and showed that DNase I treatment did not affect cell viability.
Inactivated DNase I had no effect on exDNA association with metastasized pancreatic cancer cells in situ. To examine the presence of exDNA in pancreatic cancer tissue in vivo, we conducted IFA using the DNA antibody on mouse tissue sections. Figure 2A shows IFA of exDNA in metastatic pancreatic cancer in diaphragm of an orthotopic xenograft mouse model. Figure 2B shows exDNA detected by IHC in metastatic pancreatic cancer in liver. These results suggest exDNA may be related to pancreatic cancer metastasis.

exDNA does not affect cell growth in vitro

MTT colorimetric assay is an established method of determining cell viability and cell growth (25). To assess if exDNA affect cell proliferation, we used MTT assay to measure the cell growth of different cell lines with and without DNase I treatment. Our results show that cell growth and cell viability were not affected by DNase I treatment (Fig. 3A). The degradation of cell surface exDNA by DNase I is shown in Fig. 1G by arrows. These data indicate that the presence or absence of cell surface exDNA does not affect cell viability and growth. Also, these results show that at the concentration we used in our experiments, DNase I can digest exDNA but does not affect cell viability and cell growth, which is important for using DNase I to study cancer cell metastasis in vitro.

exDNA affects pancreatic cancer cells’ metastatic potential in vitro

We used wound-healing and Transwell cell migration assays to assess the effect of exDNA on cell migration. We used Transwell with Matrigel-coated membrane to examine cell invasion. At the concentration of 10 U/5 x 10^5 cells, DNase I treatment did not affect cell viability, but significantly reduced the rate of pancreatic cancer cell migration (Fig. 3B–E) and invasion (Fig. 3F and G). The same DNase I treatment did not affect cell migration and invasion of the normal pancreas cells (HPDE). In addition to cell migration and invasion, cancer cell adhesion to each other and to cell matrix is another important characteristic correlated with their metastatic potential (28). We used hanging drop assay to examine the cell–cell adhesion ability. Figure 4A and B shows that exDNA increased MiaPaCa-2 cancer cell aggregation ability, which may help them to...
survive circulating system during the metastasis and promote metastasis by increase cancer cell arrest in microvasculature (29). To assess the effect of exDNA on cell’s attachment ability to extracellular matrix, we conducted the cell attachment assay on Matrigel that consists of assortment of extracellular matrix proteins and is used in vitro as basement membrane (30). Figure 4C and D showed that exDNA aids pancreatic cancer cell attachment to Matrigel, which may facilitate the invasion of metastatic cancer cells through the basement membrane in vivo. Furthermore, we examined the effect of DNase I on integrin-β-1, an integrin protein important for cell adhesion and metastasis, to see if DNase I affect other surface proteins. Supplementary Fig. S3 showed DNase I did not abolish β-1 on cell surface.

**DNase I inhibits pancreatic tumor cells metastasis in orthotopic xenograft mouse model**

We established an orthotopic pancreatic cancer mouse model, using cancer cells genetically modified to express luciferase. This cell line was established by the EMSS/UACC and has the unique feature that it spontaneously metastasizes. Using an In Vivo Imaging System, a bioluminescence
technology, we were able to noninvasively monitor the orthotopic tumor growth/metastasis in the whole mouse and to confirm the distant metastasis \textit{ex vivo} after organs were harvested at the end of the experiment. BLI allowed us to quantitatively measure the tumor development, as bioluminescence intensity measured in photons/second/region of interest (ph/s/ROI) is proportional to tumor burden. Figure 5A and B shows that tumor burden was significantly inhibited by DNase I treatment at weeks 4 and 5 after tumor implantation.

At the end of the mouse experiment, the \textit{In Vivo} Imaging System allowed us to detect luciferase-expressing tumor cells in each harvested organs quickly and quantitatively. Figure 5C and D shows that DNase I treatment significantly inhibited tumor metastasis from pancreas to liver and to the diaphragm. Although all pancreases developed high level of pancreatic cancer reflected by the highest level of the bioluminescence in BLI, there was no difference in the primary pancreatic tumor growth between control mouse group and DNase I–treated mouse group.

We recorded tumor growth and metastasis in different organs by gross examination. Table 1 shows visible tumor in mice organs from control group and from DNase I–treated group. Positive (+) indicates that tumor(s) was/were visible, whereas negative (−) indicates that no visible tumor on the organ examined. Although not as sensitive as bioluminescence imaging, data from gross examination in Table 1 also showed DNase I–inhibited orthotopic pancreatic tumor metastasizing to different organs.

\textbf{exDNA affects CXCL8 production in pancreatic cancer cell lines}

To elucidate the mechanism of exDNA in cancer metastasis, we examined the effect of DNase I treatment on cell production of cytokines with the Human Cytokine ELISA Plate Array I (Signosis) by following the manufacturer’s instructions. The ELISA array test showed that only CXCL8, one of the major inflammatory mediator, was affected by the DNase I treatment (data not shown). We then focused on CXCL8 for further examination. After treating cells with DNase I for 48 hours, we measured secreted CXCL8 protein level in the culture media and CXCL8 mRNA level in cells. Figure 6 shows both the transcription level of CXCL8 mRNA (Fig. 6A) and secreted CXCL8 protein levels (Fig. 6B) in two cancer cell lines, and the DNase I treatment significantly reduced CXCL8 mRNA (Fig. 6A) and secreted CXCL8 protein levels (Fig. 6B) in two cancer cell lines, but not in the normal HPDE cell line. The high CXCL8 level correlates with the high amount of cell surface exDNA in these cancer cells.

\textbf{CXCL8 elevates exDNA production in cancer cell lines}

To further evaluate whether there is a feedback loop of CXCL8 on exDNA production, we then examined if CXCL8 can increase exDNA production in pancreatic cancer cells as well
as HPDE cells with mutated *KRAS* gene. We added CXCL8 to MiaPaCa-2 and HPDE-KRAS<sup>G12D</sup> cells cultured in 96- and 24-well plates. Figure 6C showed CXCL8 treatment significantly elevated the exDNA production quantitatively (Fig. 6C, a) and microscopically in both cell lines (Fig. 6C, b–e). DNase I treatment also reduced exDNA associated with HPDE-KRAS<sup>G12D</sup> cells the same as in pancreatic cancer cells (Supplementary Fig. S4).

### Discussion

Our study provides the first evidence that exDNA is associated with pancreatic cancer cells in culture and in tissue sections, but not with normal pancreas cells. Our data indicate that cancer cell–related exDNA is involved in cell metastatic potential *in vitro* and that DNase I treatment significantly decreased cancer metastasis in the orthotopic xenograft pancreatic cancer mouse model. Our data are consistent with

#### Table 1. Harvested organs with visible tumor (tm+) or without visible tumor (tm−) in each mouse (ms#)

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studies dating to the 1960s, when reports appeared implicating exDNA in cancer development (29, 31, 32). This area of research fell dormant until recent reports of the suppressive effect of DNase I on the metastasis of pulmonary and liver cancers in murine tumor models (33). Studies identifying NETs and other exDNA traps have provided a mechanistic rationale for exDNA in carcinogenesis (14–16, 18, 19, 34).

The exDNA in our cell culture experiments is found in pancreatic cancer cell cultures and is not dependent on microenvironmental factors. Thus, the results of our cell culture experiments are likely affected by extracellular traps made up of pancreatic cellular exDNA. The results from mouse models could involve traps containing tumor and/or stromal exDNA. Our current findings do not address the pathway(s) for producing exDNA in either tumor or stromal cells. Apoptosis, necrosis, inflammatory cell lysis, and active cell secretion all can release exDNA (35). Our apoptosis assessment, however, does not suggest that exDNA is associated with cell apoptosis. Supplementary Fig. S5 shows significant higher level of caspase activity in HPDE cells, but we did not observe cell surface exDNA in this cell line. Micronuclei or microparticles are another possible source of exDNA. These are small, extranuclear bodies that are formed during mitosis from lagging chromosomes, often related to DNA damages. DNA in micronuclei/microparticles is ultimately eliminated from cells (36). Levels of micronuclei in the blood are elevated in a wide variety of diseases (37). It has recently been shown that micronuclei in peripheral lymphocytes were associated with pancreatic cancer (38). We have frequently observed micronuclei in pancreatic cancer cells; micronuclei were often found connected with exDNA (Supplementary Fig. S5). Further examination of micronuclei would be important for tracing the origin and the fate of exDNA, further for understanding its function.

Cancer cell surface–associated DNA has been reported in other types of cancer cells (39–41). We found exDNA on the surfaces and in the vicinity of cultured pancreatic cancer cell lines, also observed abundant exDNA associated with metastasized pancreatic cancer cells in the tissue section from the xenograft mouse model (Fig. 2). We cross-examined the presence of pancreatic cancer cell–associated exDNA using DNA fluorescent dyes, immunofluorescent, and immunohistochemical stainings using DNA antibody both in cultured cells and mouse tissue sections. The extracellular nature of exDNA was clearly shown by immunofluorescent assays using DNA
antibody on permeabilized (Fig. 1F) or nonpermeabilized cells (Fig. II). DNA antibody reacted with both nuclei DNA and exDNA (Fig. 1F) in the permeabilized cells, whereas it reacted only with exDNA in the nonpermeabilized cells (Fig. II). The fact that we observed abundant cell surface–bound exDNA in pancreatic cancer cell lines, but not in normal pancreas cell line and the early studies of using DNase to control cancer metastasis prompted us to test the hypothesis that exDNA associated with pancreatic cancer cells plays a role in pancreatic cancer metastasis.

A significant higher level of CXCL8 mRNA and secreted CXCL8 protein were detected in pancreatic cancer cell lines compared with that in normal HPDE cells (Fig. 6), which is in agreement with earlier studies (23, 42). Our study showed that the levels of CXCL8 mRNA and secreted CXCL8 protein correspond to the level of exDNA in each cell lines, and DNase I treatment can reduce CXCL8 mRNA and secreted CXCL8 protein levels while degrading exDNA. The correlation between CXCL8 and exDNA has never been shown in cancer cells, although this relationship has been reported in inflammatory cells (14, 18). Our current study suggests that exDNA could very well be another mediator molecule common to cancer and inflammation. It has been shown that the production and secretion of cytokines in the inflammatory cells can be induced by bacterial DNA and by DNA-containing immune complex (43). In inflammatory neutrophils, production of exDNA in the form of NET has been reported to be induced by CXCL8 (14, 18). A possible exDNA and CXCL8 feedback loop may exist in inflammatory cells. Our results indicated for the first time an exDNA and CXCL8 feedback loop in pancreatic cancer cells.

The elevated production of CXCL8 has been recently shown to play a novel role in cancer cell metastasis potential, including cell migration and invasion, and to extracellular matrix. These are all vital steps in cancer metastasis (27, 48, 49). These findings provide a mechanistic explanation and insight into previous reports on the correlation between cell surface exDNA and circulating exDNA and cancer development (35, 39, 40, 50). The role of exDNA in pancreatic cancer metastasis was further delimited by the MTT assay, which showed that DNase I treatment did not affect the viability and proliferation of cancer cells. The study by Sugihara and colleagues (29) also showed that exDNA had no effect on primary tumor growth when tumor was treated with DNase I.

Here, we showed for the first time that DNase I inhibits pancreatic cancer metastasis. Our in vivo mouse model validated the role of exDNA in pancreatic cancer metastasis. Tumor cell metastasis is confirmed by ex vivo BLI measurement in different organs harvested at the end of the experiment. Higher concentration of DNase I treatment gave rise to similar result as that from the lower concentration DNase I treatment (data not shown). Our mouse data are in agreement with other mouse model studies showing that DNase I inhibits metastasis of other types of cancer (36, 39, 40). These findings unveiled that exDNA could be a potential novel hallmark in pancreatic cancer metastasis, which may lead to new drug discoveries and cancer detection methods.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: F. Wen, J. Shi
Development of methodology: F. Wen, J. Shi
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): F. Wen, A. Shen, A. Choi, J. Shi
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): F. Wen, E.W. Gerner
Writing, review, and/or revision of the manuscript: F. Wen, E.W. Gerner, J. Shi
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): F. Wen, A. Shen, A. Choi, J. Shi
Study supervision: F. Wen, J. Shi

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