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In Vivo Selection and Characterization of Metastatic Variants from Human Pancreatic Adenocarcinoma by Using Orthotopic Implantation in Nude Mice

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Abstract

We determined whether the implantation of human pancreatic cancer cells into the pancreas of nude mice can be used to select variants with increasing metastatic potential. COLO 357 line fast-growing cells were injected into the spleen or pancreas of nude mice. Hepatic metastases were harvested, and tumor cells were reinjected into the spleen or pancreas. This cycle was repeated several times to yield cell lines L3.6sl (spleen to liver) and L3.6pl (pancreas to liver). The variant cells produced significantly higher incidence and number of lymph node and liver metastases than the parental cells. Their increased metastatic potential was associated with increased expression (mRNA and protein) of the proangiogenic molecules basic fibroblast growth factor, vascular endothelial growth factor, and interleukin-8. The metastatic cells also exhibited increased motility and invasiveness, which were associated with increased expression of collagenase type IV (MMP-9) and decreased expression of E-cadherin. Collectively, the data show that the orthotopic implantation of human pancreatic cancer cells in nude mice is a relevant model with which to study the biology of pancreatic cancer metastasis and to select variant cell lines with enhanced metastatic potential.

Keywords: pancreatic cancer, orthotopic, metastasis, selection, IL-8.

Introduction

Cancer of the exocrine pancreas is a major unsolved health problem with approximately 27,000 deaths per year in the United States and 50,000 deaths per year in Western Europe [1,2]. It is characterized by extensive local invasion as well as early lymphatic and hematogenous metastasis. Due to the inability to detect pancreatic cancer at an early stage, its aggressiveness, and the lack of effective systemic therapies, only 1% to 4% of all patients with adenocarcinoma of the pancreas will be alive 5 years after diagnosis [3–6]. Because most deaths from pancreatic cancer are caused by metastasis, improvements in treatment require a better understanding of the biology of this process.

The process of metastasis consists of sequential and selective steps that include proliferation, induction of angiogenesis, detachment, motility, invasion into the circulation, aggregation and survival in the circulation, cell arrest in distant capillary beds and extravasation into the organ parenchyma, response to local growth factors, induction of angiogenesis, and proliferation [7]. The outcome of metastasis is determined by multiple interactions between metastatic tumor cells and host factors [7,8]. To produce clinically relevant metastases, tumor cells must complete all steps in the metastatic cascade [7]. Thus, the failure to produce a metastasis can be due to different single or multiple deficiencies [9].

The process of metastasis is highly selective and its outcome depends on the interaction of metastatic cells that preexist within a heterogeneous primary neoplasm and a variety of host factors [7,8]. Critical to our studies has been the development of relevant *in vivo* models for cancer metastasis [10,11], which have produced conclusive evidence that the outcome of metastasis is regulated by the interaction of unique tumor cells with homeostatic mechanisms [7,8]. Indeed, studies from our laboratory and others have shown that malignant human tumors implanted into orthotopic organs are highly vascularized, grow progressively, and produce distant metastasis, whereas the implantation of the same tumors at an ectopic organ does not lead to extensive angiogenesis or production of metastasis [12–20]. Similarly, the isolation of multiple variants (from biologically heterogeneous neoplasms) that differ in metastatic properties has greatly advanced our understanding of the genetic and epigenetic determinants of cancer metastasis [7–9].

Two general methods have been used to select metastatic variants *in vivo*. In the first, tumor cells are implanted into orthotopic organs, and *spontaneous metastases* from distant organs are isolated and reinjected into orthotopic or-

Abbreviations: matrix metalloproteinase-2 (MMP-2); bFGF, basic fibroblast growth factor; DAB, diaminobenzidine; FBS, fetal bovine serum; FG, fast growing line; HBSS, Hank's balanced salt solution; HUVEC, human umbilical vein endothelial cells; IHC, immunohistochemistry; ISH, in situ hybridization; MMP, matrix metalloproteinase; OD, optical density; PCNA, proliferating cell nuclear antigen; VEGF/VPF, vascular endothelial growth factor/vascular permeabilit factory

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gans. In the second method, tumor cells are introduced into the circulation to produce lesions in distant organs, or *experimental metastases*. Spontaneous metastases then occur subsequent to completion of all steps of the metastatic process, whereas experimental metastases reflect the ability of tumor cells to survive in the circulation, arrest in a distant capillary bed, and grow in a distant organ parenchyma [7–11].

Unfortunately, such models do not exist for pancreatic cancer. The purpose of the present study was, therefore, to develop an orthotopic model for metastasis of human pancreatic cancer, to select (*in vivo*) variant lines with increasing metastatic capacity, and to begin detailed characterization of their properties. To do so, we used the COLO 375 human pancreatic cancer cell line originally established by Morgan et al. [21] from a *celiac axis* lymph node that had been partially replaced by neoplastic *foci* of well-differentiated, mucin-containing pancreatic ducts. Vezeridis et al. subsequently injected the fast-growing (FG) variant line of the COLO 375 cells into the spleen of nude mice. The *in vivo* isolated cells designated as L3.3 produced liver lesions at a higher incidence than the original COLO 375 cells [22].

In the present study, we show that orthotopic implantation of heterogeneous human pancreatic adenocarcinoma into nude mice results in rapid growth and production of lymph node and liver metastases. Cells isolated from the metastases were more metastatic because they expressed several genes that correlate with production of metastasis.

Materials and Methods

Animals

Male athymic nude mice (BALB/c background) were purchased from the Animal Production Area of the National Cancer Institute-Frederick Cancer Research and Development Center (Frederick, MD). The mice were housed and maintained in laminar flow cabinets under specific pathogen-free conditions in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the US Department of Agriculture, US Department of Health and Human Services, and the National Institutes of Health. The mice were used in accordance with institutional guidelines when they were 8 to 12 weeks old.

Pancreatic Cancer Cell Lines and Culture Conditions

The FG and L3.3 [21,22] human pancreatic cancer cell lines were maintained as monolayer cultures in Dulbecco's minimal essential medium (DMEM), supplemented with 10% fetal bovine serum (FBS), sodium pyruvate, nonessential amino acids, L-glutamine, and a 2-fold vitamin solution (Life Technologies, Rockville, MD). The cultures were incubated at 37°C in a mixture of 5% carbon dioxide and 95% oxygen. The cultures were tested and found to be free of *Mycoplasma* and the following pathogenic murine viruses: reovirus type 3, pneumonia virus, K virus, Theiler's encephalitis virus, Sendai virus, minute virus, mouse adenovirus, mouse hepatitis virus, lymphocytic choriomeningitis virus, ectromelia virus, and lactate dehydrogenase virus (assayed by M. A. Bioproducts, Walkersville, MD). The cultures were maintained for no longer than 12 weeks after recovery from frozen stocks.

Tumor Cell Injection Techniques

For *in vivo* injection, cells were harvested from culture flasks by a 2 to 3 minutes treatment with trypsin and were transferred to serum-free Hanks' balanced salt solution (HBSS). Only single-cell suspensions of greater than 90% viability (trypan blue exclusion) were used for injection.

Orthotopic injection Male nude mice were anesthetized with methoxyflurane. A small left abdominal flank incision was made and the spleen exteriorized. Tumor cells (1×10^6/40 μL HBSS) were injected subcapsularly in a region of the pancreas just beneath the spleen. We used a 30-gauge needle, a 1-mL disposable syringe, and a calibrated, push-button-controlled dispensing device to inject the tumor cell suspension (Hamilton Syringe Co, Reno, NV). A successful subcapsular intrapancreatic injection of tumor cells was identified by the appearance of a fluid bleb without intraperitoneal leakage. To prevent such leakage, a cotton swab was held for 1 minute over the site of injection. One layer of the abdominal wound was closed with wound clips (Autoclip; Clay Adams, Parsippany, NJ). The animals tolerated the surgical procedure well, and no anesthesia-related deaths occurred.

Intrasplenic inoculation Tumor cells were injected into the spleen by methods previously described in detail [11,12]. Briefly, mice were anesthetized with methoxyflurane, a small left abdominal flank incision was created, and the spleen was exteriorized. Tumor cells (1×10^6/40 μL HBSS) were injected into the spleen with a 30-gauge needle. The spleen was returned to the abdomen, and the wound was closed in one layer with wound clips.

Intravenous injection Tumor cells (1×10^6 cells/200 μl HBSS) were injected into the lateral tail vein of unanesthetized nude mice with a 27-gauge needle.

Necropsy Procedure and Histopathological Studies [1]

Mice were euthanized by methoxyflurane. The presence of tumor lesions in the pancreas, spleen, lymph nodes, liver, lung, and other peritoneal organs was confirmed with a dissecting microscope. The number of visible liver and lung metastases, as well as enlarged regional lymph nodes, was determined with the aid of a dissecting microscope. Tumor lesions were harvested. Some were snap-frozen in liquid nitrogen for mRNA extraction; some were fixed in 10%

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buffered formalin and embedded in paraffin, and some were embedded in OCT compound (Miles, Inc, Elkhart, IN), snap-frozen in liquid nitrogen, and stored at −70°C.

In Vivo Selection of Metastatic Variants from the L3.3 Human Pancreatic Cancer Cell Line

L3.3 cells were injected into the pancreas or spleen of nude mice. Experimental (spleen injection) or spontaneous (pancreas injection) liver metastases were harvested and treated with DNase and collagenase [12,13]. Cells were established in culture. Primary cultures were passaged *in vitro* 2 or 3 times, and then cells were harvested by trypsinization and injected into the spleen or pancreas of another set of nude mice. The selection cycle was repeated 3 times to yield lines designated as L3.6sl (spleen to liver selection) or L3.6pl (pancreas to liver selection).

Immunohistochemistry

Paraffin-embedded tissues were sectioned at 4 to 6 μm thickness, mounted on positively charged Superfrost slides (Fisher Scientific Co, Houston, TX), and allowed to dry overnight at room temperature. Sections were deparaffinized in xylene followed by a graded series of ethanol (100%, 95%, 80%), and rehydrated in phosphate-buffered solution, pH 7.5). Paraffin-embedded tissues were used for localization of vascular endothelial growth factor/vascular permeability factor (VEGF/VPF), basic fibroblast growth factor (bFGF), matrix metalloproteinase-9 (MMP-9), and proliferating cell nuclear antigen (PCNA) proteins. Sections analyzed for PCNA and MMP-9 were microwaved 5 minutes for antigen retrieval [23]. All other paraffin-embedded tissues were treated for 5 minutes with pepsin (Biomeda, Foster City, CA) at 37°C and then washed 3 times with PBS. To identify CD-31/PECAM-1 [24], frozen tissues were sectioned (8–10 μm), mounted on positively charged Plus slides (Fisher Scientific), and air-dried 30 minutes. Frozen tissues were fixed in cold acetone (5 min), acetone/chloroform 1:1 (5 min), and acetone (5 min) and washed 3 times with PBS.

After the specific pretreatment procedures, all samples were incubated with a 3% H_2O_2 in methanol solution for 12 minutes at room temperature to block endogenous peroxidases. Sections were then washed 3 times with PBS and then incubated for 20 minutes at room temperature in a protein-blocking solution consisting of PBS supplemented with 1% normal goat serum and 5% normal horse serum. The primary antibodies were diluted in protein blocking solution to the desired concentration and applied to the sections overnight at 4°C. The sections were then rinsed in PBS and incubated for 10 minutes in protein-blocking solution before the addition of peroxidase-conjugated secondary antibody. After incubation in secondary antibody for 1 hour at room temperature, the samples were washed and incubated with stable diaminobenzidine (DAB, Research Genetics, Huntsville, AL). Staining was monitored under a bright-field microscope, and the reaction was stopped by washing with distilled water. Sections were counterstained with Gill's 3 hematoxylin (Sigma Chemical Co, St. Louis, MO) and mounted with Universal Mount (Research Genetics). The concentration of primary and secondary antibodies was determined by using separate specimens. Control specimens exposed to secondary antibody alone showed no specific staining.

Quantification of Microvessel Density and Immunohistochemistry

For the quantification of microvessel density (MVD), frozen sections of the different pancreatic tumors were fixed and stained with antibodies against CD31/PECAM-1 [24]. For each tumor, four fields of 1 mm^2 each were captured at 40 × magnification with highest intensity [25–27] by using a Sony 3-chip camera (Sony Corporation of America, Montvale, NJ) mounted on a Zeiss universal microscope (Carl Zeiss, Thornwood, NY) and Optimas Image Analysis software (Bioscan, Edmond, WA), installed in a Compaq computer with Pentium chip, a frame grabber, an optical disk storage system, and a Sony color printer.

For quantification of the staining intensity, the optical density (OD) at 200 × magnification of 100 VEGF/VPF, bFGF, and MMP-9 positive cells from each tumor tissue was measured by using the Optimas Image Analysis soft ware (Bioscan). The samples were not counterstained, so the OD was due solely to the product of the immunohistochemical (IHC) reaction [27]. To minimize changes in background and color intensities that may be encountered with fluctuation in illumination due to filament life or optical alignment, all images to be used for analysis were digitized and stored for further analysis. The intensity of staining with the different antibodies was standardized to that of the integrated OD and determined by comparison with the integrated OD of the FG tumor, which was set at 100 [27].

For quantification of PCNA expression, the number of PCNA + cells was counted in four fields of 0.161-mm^2 each at 100 × magnification at the areas of most intense PCNA expression.

Antibodies and Oligonucleotide Probes

All antibodies were purchased as listed: rabbit anti-bFGF (Sigma), rabbit anti-VEGF/VPF (Santa Cruz, Santa Cruz, CA), monoclonal mouse anti-MMP-9 AB3 (Oncogene Research, Uniondale, NY), rat anti-mouse CD31/PECAM-1 and peroxidase-conjugated rat anti-mouse IgG1 (Pharmingen, San Diego, CA), mouse anti-PCNA clone PC 10 (DAKO A/S, Copenhagen, Denmark), peroxidase-conjugated $F(ab')_2$ goat anti-rabbit IgG $F(ab')_2$ and peroxidase-conjugated goat anti-rat IgG [H + L] (Jackson Research Laboratories, West Grove, CA), peroxidase-conjugated rat anti-mouse IgG2a (Serotec, Harlan Bioproducts for Science, Inc., Indianapolis, IN), and peroxidase-conjugated $F(ab')_2$ anti-mouse IgG1 (Oncogene).

Specific antisense oligonucleotide DNA probes were designed complementary to the mRNA transcripts of metastasis and angiogenesis-related genes (MMP-2, MMP-9, E-cadherin, VEGF, bFGF, interleukin-8 [IL-8]) based on

published reports of the cDNA sequence: VEGF/VPF (TGG′TGA′TGT′TGG′-ACT′CCT′CAG′TGG′GCU), 57.7% guanosine-cytosine (CG) content [28]; bFGF (CGG′GAA′-GGC′GCC′GCT′GCC′GCC′), 85.7% GC content [27]; IL-8 (CTC′CAC′AAC′CCT′CTG′CAC′CC), 65% GC content [29]; MMP-2 (TGG′GCT′ACG′GCG′CGG′CGG′CGT′GGC′), 88.9% GC content [30]; MMP-9 (CCG′GTC′CAC′CTC′-GCT′GGC′GCT′CCG′GU), 80.0% GC content [31]; E-cadherin (mixture) (TGG′AGC′GGG′CTG′GAG′TCT′-GAA′CTG′), 62.5% CG content and (GAC′GCC′GGC′GGC′-CCC′TTC′ACA′GTC′), 75% CG content [32]. The specificity of the oligonucleotide sequence was initially determined by Gene Bank European Molecular Biology Library database search with the use of Genetics Computer Group sequence analysis program (GCG, Madison, WI) based on the FastA algorithm [33]. These sequences showed 100% homology with the target gene and minimal homology with nonspecific mammalian gene sequences.

In Situ Hybridization

In situ hybridization (ISH) was carried out according to the microprobe manual staining system (Fisher Scientific Co, Pittsburgh, PA) [34,35]. Tissue sections (5 μm) of formalin-fixed, paraffin-embedded specimens were mounted on Silane-coated ProbeOn slides (Fisher Scientific). The slides were placed in the microprobe slide holder, dewaxed, and dehydrated with Autodewaxer and Autoalcohol (Research Genetics), followed by an enzymatic digestion with pepsin [36]. Hybridization of the biotinylated probe was carried out sequentially for 3 minutes at 100°C, 4 minutes at room temperature, and 45 minutes at 45°C. The samples were then washed 3 times with 2× standard saline citrate (SSC) for 2 minutes at 45°C. The samples were incubated for 30 minutes in alkaline phosphatase–labeled avidin (DAKO, Carpineria, CA) at 45°C, briefly rinsed in Tris-buffered saline (50 mmol/L Tris–HCl [pH 7.6], 150 mmol/L NaCl), rinsed for 1 minute in alkaline phosphatase enhancer (Biomeda Corp, Foster City, CA), and finally incubated for 30 minutes with chromogen substrate FastRed (Research Genetics) at 45°C. Slides were washed several times with distilled water, counterstained with Gill's 3 hematoxylin (Sigma), and mounted with Universal Mount (Research Genetics). A positive reaction in this assay stained red. Control for endogenous alkaline phosphatase included treatment of the samples in the absence of the biotinylated probe and use of chromogen in the absence of any oligonucleotide probes. To check the specificity of the hybridization signal, the following controls were used: 1) RNase pretreatment of tissue sections; 2) a biotin-labeled sense probe; and 3) competition assay with unlabeled antisense probe [37]. A markedly decreased or absent signal was obtained after all of these treatments.

Stained sections were examined under a Zeiss photomicroscope (Zeiss) equipped with a 3-chip-charged coupled device color camera (model DXC-960 MD, Sony). The images were analyzed by using the Optimas Image Analysis software (version 5.2, Bothell, WA). The samples were not counterstained, so the OD was due solely to the product of the ISH reaction. For each section, we determined the OD in several 2 × 2 mm zones located at the center and periphery of the tumor. Three to five different fields in each 2 × 2 mm zone were quantified to derive an average value. The intensity of staining was standardized to that of the integrated OD of poly $d(T0)_{20}$ and determined by comparison with the integrated OD of nonpathological pancreatic epithelial cells, which was set at 100.

In Vitro Growth Kinetics

Pancreatic cancer cells were harvested from 75% confluent monolayer cultures by a brief trypsinization with 0.1% trypsin and 0.1% EDTA. The cells were washed in cold HBSS, plated at a density of 1500 cells per 38-mm^2 well, and then given supplemented DMEM containing 10% FBS. Cell growth was monitored after 8 hours and 1, 2, 3, and 4 days by using the MTT assay as described previously [38]. *In vitro* doubling times were calculated from the log phase growth curve [39].

In Vitro Migration Assay

A modified Boyden chamber assay was used to examine the *in vitro* motility of the different pancreatic cancer cells. Polycarbonate filters (pore size, 8 μm; diameter, 13 mm) were glued to inserts, which were placed in the wells of 24-well tissue culture plates. The lower part of the chamber was filled with fibronectin (100 ng/mL) as a chemoattractant. Cells (2×10^4) were suspended in 100-μl HBSS and placed in the upper part of the chamber. After 6 hours incubation at 37°C, the filters were carefully removed from the inserts, stained with hematoxylin = losin for 10 minutes, and mounted on microscopic slides. The number of stained cells was counted in 10 microscopic fields (each 0.161 mm^2) at 100× magnification. Migratory activity was quantified by the average number of cells per microscopic field.

Endothelial Cell Proliferation Assay

Primary human umbilical vein endothelial cells (HUVEC) were cultured in complete DMEM containing 15% FBS and 10 ng/mL bFGF. Cell culture flasks were coated with 4 mg/mL gelatin. Cells were plated in 96-well plates at a density of 3×10^3 cells per well. After 24 hours, the supernatant in each well was removed, and 200 μl of conditioned medium of the different pancreatic cancer cells was added in various concentrations (10% and 50% conditioned medium). Conditioned medium was prepared by culturing 5×10^5 pancreatic cancer cells in a 6 well plate in supplemented DMEM containing 10% FBS for 5 days. After 4 days of incubation, the growth of HUVECs in various concentrated conditioned media of the different pancreatic cancer cells was monitored by MTT assay and compared to the growth of HUVECs treated with supplemented DMEM containing 15% FBS and 10 ng/mL bFGF only.

Statistical Analysis

The unpaired Student *t* test was used to compare the *in vitro* and *in vivo* results. Survival analysis was computed by the Kaplan–Meier method and compared by the log-rank

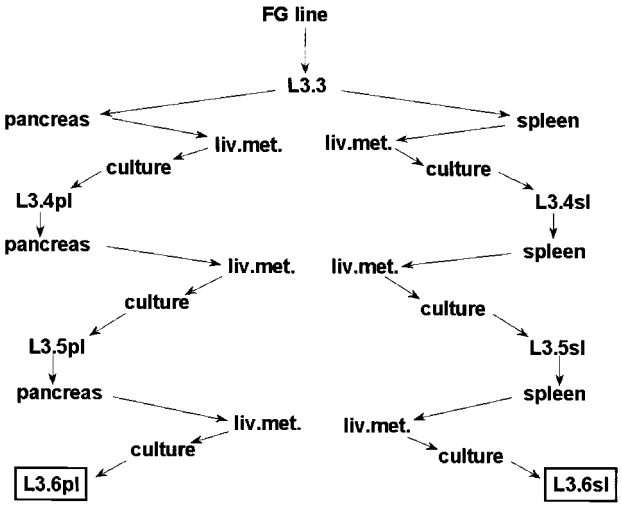

Figure 1. *In vivo selection of metastatic human pancreatic cancer cells. L3.3 cells (derived from the FG line) were injected into the spleen or the pancreas of nude mice. Experimental liver metastases (spleen injection) or spontaneous liver metastases (pancreas injection) were harvested, established in culture, and designated L3.4sl (spleen–liver) and L3.4pl (pancreas–liver). The cultures were harvested and injected into the spleen or pancreas of another set of nude mice. The cycles were repeated twice more to yield lines L3.6sl and L3.6pl.*

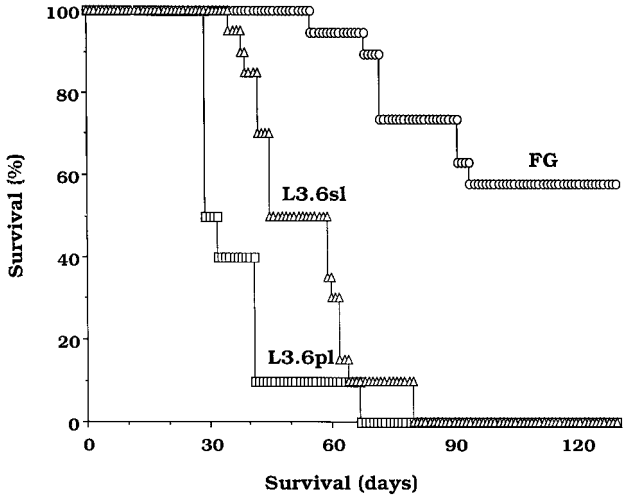

Figure 2. *Percent cumulative survival. The pancreases of nude mice were injected with 1×10^6 viable FG, L3.6sl, or L3.6pl cells. Moribund mice were killed and necropsied. Survival analysis was computed by the Kaplan–Meier method, and the unpaired Student t test was used to compare the results among the three different cell lines. FG versus L3.6sl, $P < .002$; FG versus L3.6pl, $P < .0001$; L3.6sl versus L3.6pl, $P < 0.02$.*

test [40]. The Mann–Whitney test was used to compare the level of gene expression among the different cell lines (univariate analyses). Significance was defined as a two-sided $P < .05$ [40].

Results

Selection of Highly Metastatic Variant Lines

In the first set of experiments, we injected cells from the FG line derived from COLO357 human pancreatic adenocarcinoma cell line [21,22] into the spleen or pancreas of nude mice. Intrasplenic injection allows cells to reach the liver parenchyma within minutes after injection [13,14]. Liver colonization can occur rapidly and without necessity to overcome the barrier of the primary site microenvironment. The liver lesions thus formed are termed *experimental metastases*. In contrast, to produce liver lesions, human pancreatic cancer cells implanted into the pancreas must complete all the steps of the process. The lesions then are designated as *spontaneous metastases*. Experimental or spontaneous liver metastases were harvested, established in tissue culture, and designated as L3.4sl and L3.4pl, respectively. Cells harvested from these cultures were injected into the spleen or pancreas of another set of nude mice. Liver lesions were again isolated, and cells were established in culture. After three such selection cycles, lines L3.6sl and L3.6pl were established in culture (Figure 1). Cytogenetic analysis confirmed the human origin of the cells.

To determine whether the isolated variants had increased metastatic potential, cells from the original FG line and from the L3.6sl, and L3.6pl lines were injected into the spleen and/or the pancreas of nude mice ($n = 20$). The mice were killed when moribund or by day 105 after injection. Tumorigenicity, production of metastases, and survival were determined (Table 1). In the first assay for experimental metastasis, we injected the cells into the spleen. In-

Table 1. Tumorigenicity and Production of Metastasis by Human Pancreatic Cancer Cells Subsequent to Implantation in Athymic Nude Mice.

Site of Implantation*	Cell Line	Median Survival (days)	Tumorigenicity	Liver Metastasis		Lymph Node Metastasis‡
				Incidence†	Median (range)	
Spleen	FG	92	5/20	4/20	0 (0–2)	2/10
	L3.6sl	71	19/20‖	10/19	3 (0–10)	7/19§
	L3.6pl	34	20/20‖	13/20	10 (0–75)	3/20
Pancreas	FG	78	20/20	1/20	0 (0–1)	3/20
	L3.6sl	56	20/20	5/20	0 (0–3)	12/20‖
	L3.6pl	36	20/20	10/20	1 (0–15)	9/20§

*Nude mice were injected into the spleen, pancreas or vein with 1×10^6 viable cells. The mice were killed when moribund and necropsied. The presence of tumor lesions in the spleen, pancreas, liver, lymph nodes, or lungs was determined with the aid of a dissecting microscope.
†Number of tumor-positive mice per number of injected mice.
‡Number of mice with macroscopically enlarged regional lymph node per number of injected mice.
§$P < .01$ versus FG.
‖$P < .001$ versus FG.

trasplenic injection of FG cells produced spleen tumors in 5 of 20 animals, experimental liver metastases in 4 of 20 mice, and a median survival time of 92 days. Injection of L3.6sl cells produced spleen tumors in 19 of 20 mice,

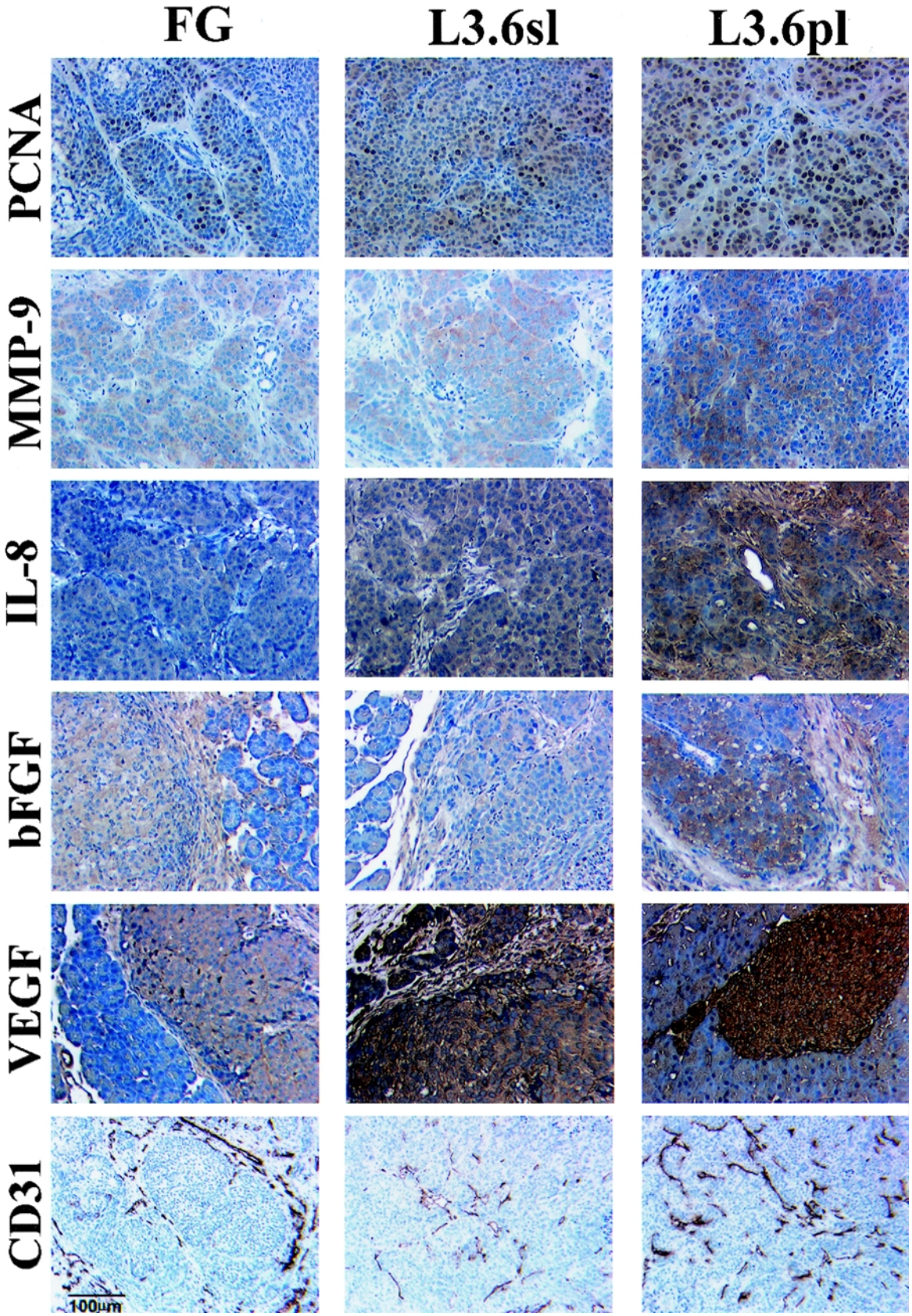

Figure 3. *Representative IHC staining of FG, L3.6sl, and L3.6pl human pancreatic cells growing in the pancreas of nude mice. Tissue sections were stained for expression of PCNA (to show cell division), MMP-9, IL-8, bFGF, VEGF/VPF, and CD31 (to show endothelial cells).*

experimental liver metastases in 10 of 19 mice ($P < .01$), and a median survival time of 71 days. For L3.6pl cells, 20 of 20 mice had spleen tumors and 13 of 20 had liver metastases ($P < .01$); the median survival time was 34 days ($P < .01$). The highest median number of liver metastases was 10 (3–75), found after intrasplenic injection of L3.6pl cells, suggesting that these cells have a higher capacity to colonize the liver.

In the second assay for spontaneous metastasis, we injected the cells into the pancreas. All three lines were 100% tumorigenic after intrapancreatic injection (Table 1). Liver metastases were produced in 1 of 20, 5 of 20, and 10 of 20 mice after injection of FG, L3.6sl, and L3.6pl cells, respectively. The median survival time was 84, 56, or 36 days after the intrapancreatic injection of FG, L3.6sl, and L3.6pl cells, respectively (Figure 2). These data show that selection of cells for experimental metastasis also increased their ability to form spontaneous metastasis. The selection of cells for spontaneous metastasis, however, yielded cells with the highest metastatic potential.

The intravenous (IV) injection of 1×10^6 FG cells produced a median of 75 experimental lung metastasis per mouse (range, 18–142), whereas the intravenous injection of L3.6sl cells produced a median number of 3 lung lesions. The IV injection did not yield a significant number of liver metastases.

Cell Growth in Vitro and in Vivo

The *in vitro* doubling time of FG, L3.6sl and L3.6pl was 25, 20, and 16 hours, respectively. The average number of PCNA + cells (per microscopic field) in the pancreatic tumors agreed with the *in vitro* results. The average number of PCNA + cells per microscopic field was 178 ± 42, 289 ± 48, and 399 ± 33 in 72- to 84-day-old pancreatic tumors of FG, 53- to 59-day-old pancreatic tumors of L3.6sl, and 35- to 37-day-old pancreatic tumors produced by L3.6pl cells (Figure 3; Table 2).

Cell Motility

To determine whether the increased metastatic potential was associated with increased motility, we used a modified Boyden chamber assay with fibrinogen (100 ng/mL) as a chemoattractant. The motility of L3.6sl and L3.6pl cells differed significantly from that of the FG cells. Specifically, the number of FG, L36sl, and L3.6pl cells per 10 microscopic fields was 3 ± 1, 38 ± 5, and 44 ± 7 cells, respectively ($P < .001$).

Relative Expression of MMP-2/MMP-9 and E-cadherin

Detachment of malignant cells from the primary tumor and penetration of host stroma including basement membranes and extracellular matrix (ECM) is an essential part of the metastatic process [7]. The degradation of the ECM components surrounding malignant tumors is primarily mediated by MMPs or type IV collagenases [41–46]. To determine whether the metastatic potential of the variant cells correlated with their production of MMPs, we examined the expression of MMPs by ISH.

Pancreatic tumors derived from L3.6pl and L3.6sl cells had significantly higher MMP-9 and MMP-2 mRNA levels than tumors derived from FG (Figure 4, Table 3). The MMP-9 mRNA expression level was significantly higher in pancreatic tumors derived from L3.6pl cells (spontaneous metastasis) than in L3.6sl cells (experimental metastasis). The expression of E-cadherin, a transmembrane glycoprotein that is directly related to cell-to-cell cohesion, was high in tumors derived from FG and low in tumors derived from L3.6sl and L3.6pl cells. IHC staining of pancreatic tumors with anti-MMP-9 antibody revealed a significant increase in the expression of MMP-9 protein in tumors derived from metastatic L3.6sl and L3.6pl cells (Figure 3, Table 2).

Gelatin zymography of tumor lysates revealed 5-fold and 3.5-fold increases in activity of the 92-kDa MMP-9 in pancreatic tumors (or culture supernatants) derived from L3.6sl and L3.6pl cells, respectively, as compared with FG cells. MMP-2 activity was indistinguishable among the 3 different tumors. Additionally, a 3-fold increase of MMP-9 activity was determined in lysates of pancreatic tumors from L3.6pl versus L3.6sl cells (Figure 5).

Expression of VEGF, bFGF, IL-8, and Quantification of Microvessel Density

In situ hybridization of tissue sections demonstrated a significant difference in VEGF/VPF, bFGF, and IL-8 mRNA

Table 2. Immunohistochemical Analysis of Pancreatic Cancers Produced by Orthotopic Implantation in Athymic Nude Mice.

Cell Line *	PCNA†	CD-31‡	VEGF/VPF§	bFGF§	IL-8§	MMP-9§
FG	180 (152–228)	7 (6–13)	100 (97–108)	100 (92–103)	100 (97–105)	100 (98–107)
L3.6sl	262 (244–350)	35 (33–57)	133 (130–139)	136 (131–147)	141 (137–145)	123 (118–124)
L3.6pl	374 (366–391)	73 (59–112)	178 (168–185)	176 (169–182)	157 (155–165)	145 (133–150)

* Tumor cells were injected into the pancreas of athymic nude mice. Tumors were harvested and processed for IHC as described in Materials and Methods.

† Median (range) number of PCNA + cells was determined by counting positive cells in four fields of 0.161 mm^2 each at 100 × magnification. FG *v* L3.6sl, $P < .004$; FG *v* L3.6pl, $P < .002$; L3.6sl *v* L3.6pl, $P < 0.002$.

‡ Median (range) number of CD31-positive cells was determined by counting positive cells in four fields of 1.0 mm^2 each at 40 × magnification at the area of most intense CD31 expression. FG *v* L3.6sl, $P < .01$; FG *v* L3.6pl, $P < .006$; L3.6sl *v* L3.6pl, $P < .01$.

§ Median (range) OD of VEGF/VPF, bFGF, and MMP-9 was determined by measurement of 100 cells per tumor at 200 × magnification with the Optimas Image Analysis software. The integrated OD was compared with the integrated OD in the FG tumors, which was set at 100. VEGF/VPF: FG *v* L3.6sl, $P < .05$; FG *v* L3.6pl, $P < .01$; L3.6sl *v* L3.6pl, $P < 0.05$. bFGF: FG *v* L3.6sl, $P < .05$; FG *v* L3.6pl, $P < .01$; L3.6sl *v* L3.6pl, $P < .05$. MMP-9: FG *v* L3.6al, $P < .05$; FG *v* L3.6pl, $P < .02$; L3.6sl *v* L3.6pl, $P < .04$.

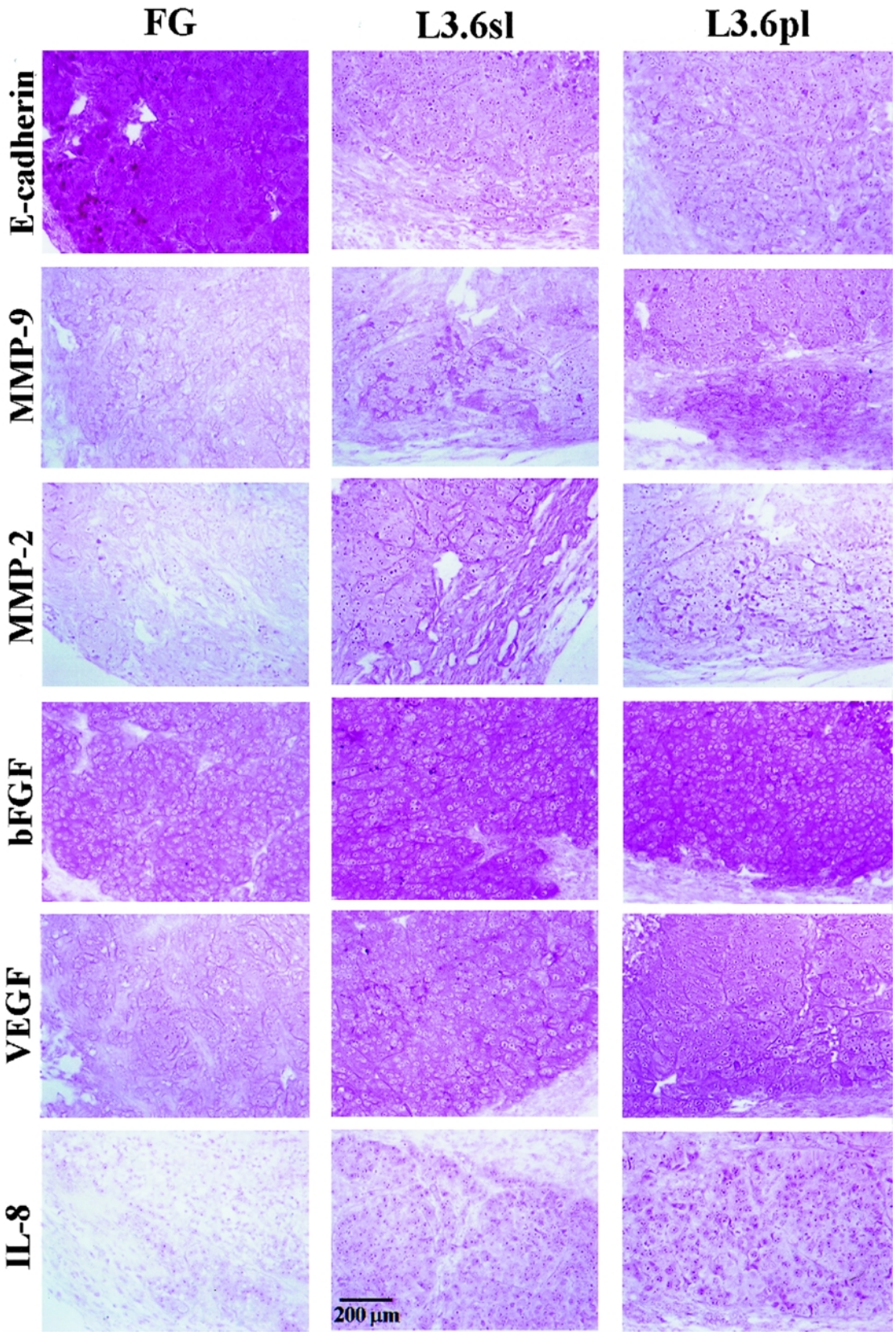

Figure 4. *Representative ISH of FG, L3.6sl, and L3.6pl human pancreatic cells growing in the pancreas of nude mice. Metastatic cells (L3.6sl, L3.6pl) concurrently expressed high levels of MMP-2, MMP-9, bFGF, VEGF/VPF, IL-8, and low levels of E-cadherin. The low metastatic cells expressed high levels of E-cadherin.*

expression level among pancreatic tumors derived from FG, L3.6sl, and L3.6pl cells (see Figure 4). IL-8 mRNA expression level was significantly higher in pancreatic tumors derived from L3.6pl than in L3.6sl or FG cells (see Figure 4).

Table 3. *In Situ* Hybridization Analysis for Relative Expression of Metastasis-Regulating Genes by Human Prostate Cancer Cell Lines Growing in the Pancreas of Nude Mice.

Cell Line	Median Expression Index (Range)						MMP/E-Cadherin Ratio
	VEGF/VPF	bFGF	IL-8	MMP-2	MMP-9	E-cadherin	
FG	146 (127–147)	166 (155–172)	84 (75–940)	119 (96–142)	92 (83–100)	73 (65–80)	1.5 (1.4–1.5)
L3.6sl	295 (163–353)	263 (223–285)	208 (210–215)	201 (180–225)	186 (167–195)	55 (46–56)	3.7 (3.3–4.0)
L3.6pl	280 (233–315)	240 (224–252)	198 (149–210)	200 (171–248)	146 (152–150)	48 (41–55)	3.8 (3.3–3.9)
P value							
FG *v* L3.6sl	.001	.0006	.001	.0006	.002	.006	.0006
FG *v* L3.6pl	.001	.0006	.001	.0006	.001	.006	.0006
L3.6sl *v* L3.6pl	NS	NS	.02	NS	.001	NS	NS

Note: Tumor cells were injected into the pancreas of athymic nude mice. Tumors were harvested and processed for *in situ* hybridization analysis as described in Materials and Methods. Relative expression of specific mRNA was determined by examining at least 7 different fields/specimen. The expression of each gene in normal epithelium of mouse pancreas was set at the value 100.

Similar results were obtained *in vitro* by Northern blot analysis of cultured cells (data not shown).

IHC staining with specific antibodies demonstrated increased protein expression of VEGF/VPF, bFGF, or IL-8 in L3.6sl and L3.6pl tumors (see Figure 3, Table 2) (FG *v* L3.6sl/L3.6pl, $P < .01$, L3.6sl *v* L3.6pl, $P < .03$). These *in vivo* results are supported by Western blot analysis of tissue culture lysates for bFGF as well as ELISA assay of culture supernatants for IL-8 and VEGF/VPF (data not shown).

To determine whether the proangiogenic molecules produced by the pancreatic tumor cells were biologically active, we incubated HUVEC with culture supernatants of the three cell lines and determined HUVEC proliferation by the MTT assay [38,39]. Conditioned media of FG, L3.6sl, and L3.6pl cells stimulated proliferation of HUVECs ($P < .02$). To further investigate angiogenesis in the orthotopic tumors, we quantified MVD after staining frozen sections of tumors with CD31/PECAM-1. In pancreatic tumors derived from FG, L3.6sl, and L3.6pl, the average number of CD31/PECAM-1 positive vessels (per 1-mm^2 field at 40$\times$ magnification) was 3 ± 1, 44 ± 11, and 89 ± 25, respectively ($P < .001$).

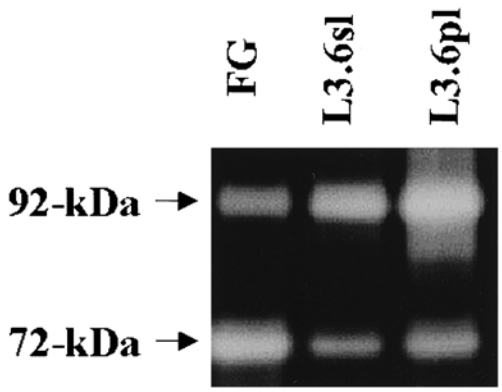

Figure 5. *Gelatin zymography. Lysates from FG L3.6sl and L3.6pl pancreatic tumors were prepared. Protein (1 μg) was loaded on a 7.5% polyacrylamide slab gel-impregnated with 1 mg/mL gelatin under nonreducing conditions.*

Discussion

Metastasis is a highly selective process that favors the survival and growth of unique subpopulations of metastatic cells that preexist within heterogeneous malignant neoplasms [47,48]. The isolation of clonal populations of cells that differ in their metastatic capacity has supported the hypothesis of metastatic heterogeneity [7–10,48]. Depending on the neoplasm, the selection process is gradual, requiring several cycles of *in vivo* selection [12,16,19]. This cyclic procedure was originally used to isolate the B16-F10 metastatic cell line from the wild-type B16 murine melanoma cell line [47] and, subsequently, human renal cell carcinoma [13], colon carcinoma [11,12], transitional cell carcinoma [15,16], and prostate carcinoma [18,19]. We implanted pancreatic cancer cells into the spleen (experimental liver metastasis) or pancreas (spontaneous liver metastasis) and harvested metastatic lesions. The recovered cells were expanded in culture to repeat the process. After several cycles, the behavior of the cells was compared with that of the parental cells. The metastatic potential of the pancreatic cancer cells in nude mice was determined by two assays. The first involved the implantation of the cells into the pancreas and a count of spontaneous lymph nodes and liver metastasis. The second measured to ability of the pancreatic cancer cells to grow in the liver parenchyma subsequent to intrasplenic implantation (experimental metastasis). In general, there was good agreement in the results of the two assays.

Earlier data from our laboratory established that the implantation site of metastatic human tumor cells determines whether they will produce distant metastasis. Specifically, the growth of tumors in orthotopic organs facilitates production of metastasis, whereas growth in ectopic organs does not [7–10]. Indeed, human pancreatic cancer cells implanted into the pancreas (orthotopic) or subcutis (ectopic) of severe combined immunodeficient mice produced tumors that differed in growth rate and status of differentiation [49]. These and other biologic differences may be due to differential interaction of tumor cells with organ-specific factors [50].

The intrasplenic injection of human pancreatic carcinoma cells into nude mice produced experimental metastases in

the liver and in the lung [51,52]. Cells injected into the spleen reach the liver parenchyma within minutes [11]. Thus, the intrasplenic implantation of cells measures their ability to grow in the liver parenchyma but fails to measure cell detachment from the primary pancreatic tumor followed by invasion of host stroma [11,12]. This limitation stimulated the development of an orthotopic model for human pancreatic cancer that used tumor fragments, sutured onto the pancreas of nude mice [22,51,52]. The use of tumor fragments avoids the issue of neoplastic heterogeneity [53] and, thus, the orthotopic inoculation of human pancreatic cancer cell suspensions is mandatory for reproducible studies of the biology of pancreatic cancer metastasis. In this study, we undertook a series of intrapancreatic and intrasplenic implantation experiments with cell suspensions of a human pancreatic cancer cell line to select and isolate cell lines with higher tumorigenicity and increased metastatic potential. Intrasplenic and intrapancreatic implantation demonstrated a significant increase in the incidence of primary tumors (pancreas, spleen) and liver metastasis, as well as in median number of liver metastases for L3.6sl and L3.6pl cells over FG cells. Moreover, the incidence and median number of liver metastases were higher after either intrapancreatic or intrasplenic injection of L3.6pl as compared with L3.6sl cells, suggesting that the repeated pancreas-to-liver selection procedure isolated cells with higher potential for metastasis. More than a century ago, Paget [54] proposed that the outcome of metastasis depended on the interaction of specific tumor cells with a favorable organ environment. The present data support the 'seed and soil' hypothesis [54]. After IV injection of the more heterogeneous FG cells, a significantly greater differential favoring lung over liver metastases was produced than after the IV injection of pancreatic cancer cells selected for their ability to produce liver metastases.

The increased metastatic potential of the L3.6pl and L3.6sl variants correlated with expression of several metastasis-regulating genes [55]. First, E-cadherin is a cell surface glycoprotein involved in calcium-dependent homotypic cell-to-cell adhesion [56]. E-cadherin is localized at the epithelial junction complex and is responsible for the organization, maintenance, and morphogenesis of epithelial tissues [57–60]. Reduced levels of E-cadherin are associated with a decrease in cellular/tissue differentiation and increased grade in different epithelial neoplasms [61–65], and transfection of E-cadherin–encoding cDNA into invasive cancer cells has been shown to inhibit motility and invasion [66].

Invasion of the host stroma and degradation of the blood vessel basement membrane are necessary for metastasis [41–46,67]. The levels of collagenase type IV in human and rodent neoplasms directly correlate with invasion and metastasis, and specific inhibitors of matrix metalloproteinases have been shown to inhibit tumor cell invasion [41–45]. We analyzed the tumors produced by FG cells (low metastatic) by ISH, IHC, and zymography of tumor tissue lysates and medium conditioned by the respective cultures. The metastatic variants expressed higher levels of MMP-9 as compared with the low metastatic FG cells. In contrast, the E-cadherin expression was significantly higher in the FG tumors than in the metastatic tumors. Thus, a decrease in the expression of E-cadherin and an increase in collagenase type IV activity was associated with enhanced tumor invasion and metastasis [37,67–69].

Increased motility is conducive to tumor cell invasion and metastasis [70,71]. Invasive pancreatic cancer cells can produce a dissociation factor that increases cell motility [72]. Similar to our previous study with metastatic human prostate cancer [19], we determined the relative motility of the FG, L3.6sl, and L3.6pl cells by using a modified Boyden chamber coated with fibronectin. This assay revealed that motility *in vitro* directly correlated with production of metastasis.

The progressive growth and metastasis of neoplasms is dependent on angiogenesis, the extent of which is determined by the balance between proangiogenic and antiangiogenic molecules secreted by both tumor and host cells [73–75]. Among the major molecules identified is bFGF, which induces the proliferation, migration, proteolytic activity, and differentiation of endothelial cells [73,76,77]. VEGF/VPF induces the proliferation of endothelial cells, increases the cells' vascular permeability, and induces the production of plasminogen activator by these cells [78–81]. IL-8, a chemoattractant cytokine produced by a variety of tissues and blood cells, attracts and activates neutrophils in inflammatory regions and is angiogenic [82,83]. The levels of MMP-2 and MMP-9 have been associated with active neovascularization because these MMPs can degrade the ECM, an essential step in the process of angiogenesis [84].

The expression of angiogenic molecules differed significantly among the 3 pancreatic cell lines. Both *in vitro* cultured cells and *in vivo* tumors derived from L3.6pl and L3.6sl cells expressed higher levels of VEGF/VPF, bFGF, IL-8, and MMP-9 than did the low metastatic FG cells or tumors. The increased expression level of the angiogenic molecules directly correlated with MVD of the tumor lesions as measured by immunostaining of endothelial cells with antibodies against CD31/PECAM-1. Finally, the biological activity of the angiogenic molecules was proven by their ability to stimulate growth of HUVEC. In good agreement with the IHC data, the conditioned media of all 3 pancreatic tumor cell variants stimulated the growth of HUVECs; however, the conditioned media of L3.6sl and L3.6pl cells stimulated the growth of HUVECs significantly more than the conditioned medium of FG cells.

Increased levels of the epidermal growth factor receptor and its ligand, transforming growth factor, have been associated with increased malignancy of human bladder, breast, and colon cancers [85–88]. The expression level for these genes, however, did not vary among the pancreatic cell lines. The c-*met* oncogene, which encodes the receptor for hepatocyte growth factor, has been shown to promote cell motility and invasiveness [89–92]. We did not find discernible differences in expression of c-*met* among the three lines.

In summary, we report the development of a reproducible orthotopic model to study the biology of human pancreatic

cancer metastasis. We also succeeded in isolating variant lines with enhanced metastatic properties as demonstrated by a battery of assays. The availability of these unique cells and the *in vivo* model should facilitate in-depth studies to understand the interaction of metastatic pancreatic cancer cells with host factors and the design of biologically based new therapies.

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