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Correlation of Metastasis-Related Gene Expression with Metastatic Potential in Human Prostate Carcinoma Cells Implanted in Nude Mice Using an *in Situ* Messenger RNA Hybridization Technique

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The purpose of this study was to determine whether the expression level of several metastasis-regulating genes correlates with the metastatic potential of human prostate cancer cells implanted into the prostate of nude mice. The steady-state mRNA expression levels for epidermal growth factor receptor (EGFR; growth), basic fibroblast growth factor (bFGF) and interleukin (IL)-8 (angiogenesis), 72-kd and 92-kd type IV collagenase (invasion), E-cadherin (adhesion), and multidrug resistance (mdr-1; drug resistance) were measured by Northern blot and colorimetric in situ hybridization techniques in human PC-3M cells and selected cell variants with different metastatic potentials. Highly metastatic cells growing in culture constitutively and uniformly expressed higher levels of bFGF, IL-8, type IV collagenase, and mdr-1 mRNA transcripts than parental PC-3M cells or low metastatic cells, which displayed a heterogeneous pattern of gene expression. Human prostate cancer cells implanted in nude mice at an ectopic site (subcutaneous) expressed lower levels of EGFR, mdr-1, bFGF, IL-8, and collagenase type IV than those implanted in an orthotopic site (prostate), indicating that the expression of these genes was dependent on the organ environment. Highly metastatic cells growing in the prostate expressed higher levels of EGFR, bFGF, type IV collagenase, and mdr-1 mRNA than low metastatic parental cells in the same site. These data demonstrate a direct correlation between the expression of several metastasis-related genes and the metastatic potential of human prostate cancer cells in nude mice and suggest that multiparametric in situ hybridization analyses can be used to identify the metastatic potential of individual patients' prostate cancers. (Am J Pathol 1997, 150:1571–1582)

Prostate cancer is the leading cause of cancer death in men in North America. Most deaths from this cancer are due to metastases that are resistant to therapy.[1] The incidence of prostate cancer continues to rise, and over 317,100 newly diagnosed cases and 41,400 cancer-related deaths are now reported annually.[2] Improvements in the diagnosis and screening of prostate cancer have identified an increasing number of patients with lesions confined to the prostate, which has led to a rise in radical surgeries. Whether radical prostatectomy or other forms of local therapy have improved overall survival, however, is not clear because of the unpredictable biological potential of prostate cancer.

The prognosis and choice of therapy for most patients with prostate cancer are based on the clinical stage, serum prostate specific antigen (PSA), and histological grade (Gleason score) of the tu-

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mor.[3,4] The strongest predictive factors for distant metastasis and lymph-node-positive disease are a serum PSA >20 ng/ml and a Gleason score >8.[5] Although the majority of newly diagnosed prostate cancers do not present with these prognostic factors, metastasis may still develop in a significant number of patients with clinically localized disease treated with radical prostatectomy.[6] There is thus an urgent need to identify new molecular markers of prostate cancer metastasis.

To identify these markers on a rational basis requires an understanding of the process of metastasis, which is highly selective and consists of a series of sequential, interrelated steps that include growth, vascularization, invasion, survival in the circulation, adhesion, extravasation, and proliferation at the distant site. The establishment of metastases requires the successful completion of each step and is dependent on both the intrinsic properties of tumor cells and the response of the host microenvironment.[1,7] Numerous reports have demonstrated that in prostate cancer as well as in other tumor types, the metastatic potential of tumor cells directly correlates with the expression level of several genes, including epidermal growth factor receptor (EGFR),[8,9] basic fibroblast growth factor (bFGF),[10,11] interleukin-8 (IL-8),[12,13] type IV collagenase,[14–16] and multidrug resistance (*mdr*-1)[17] and inversely correlates with E-cadherin expression.[18,19] Most of these studies, however, focused on single factors and concluded that, although the expression of a given gene is necessary, it is in itself insufficient for metastasis. Because discrete steps in the pathogenesis of metastasis are regulated by specific and independent genes,[20–23] the identification of tumor cells capable of producing metastases requires multiparametric analyses.

We have developed a rapid colorimetric *in situ* hybridization (ISH) technique for detecting the expression of specific genes that regulate the fundamental steps of metastasis. This ISH uses oligonucleotide probes to detect specific mRNA transcripts in cultured cells, frozen tissues, and formalin-fixed, paraffin-embedded specimens.[24,25] In the present study, we used this technique to identify the concurrent expression of several metastasis-related genes in human prostate carcinoma cells implanted in nude mice and to determine whether it correlates with metastatic potential. We used Northern blot analyses as well as the newly developed ISH technique to detect mRNA transcripts for EGFR (growth), bFGF and IL-8 (angiogenesis), type IV collagenase (invasion), E-cadherin (adhesion), and *mdr*-1 (drug resistance) expression in the human PC-3 cell line and selected variants with different metastatic potential. The expression of these genes correlated with the ability of human prostate carcinoma cells to metastasize, and the level of gene expression in the tumor cells was dependent on the organ microenvironment.

Materials and Methods

PC-3 Human Prostate Carcinoma Cell Line and Selected Metastatic Variants

The PC-3 human prostate cancer cell line was obtained from the American Type Culture Collection, Rockville, MD.[26] The PC-3M cell line was derived from a liver metastasis produced by the parental PC-3 cells growing in the spleen of a nude mouse.[27] PC-3M cells were implanted orthotopically into the prostate of nude mice, and after four cycles of *in vivo* selection, metastatic variants were isolated based on their ability to grow within the prostate (PC-3M-Pro4) and produce lymph node metastases (PC-3M-LN4). The PC-3M-Pro4 cell line grew faster in the prostate and produced larger tumors than the PC-3M or PC-3M-LN4 cell lines. The highly metastatic PC-3M-LN4 cell line produced the smallest prostate tumors and was associated with largest regional lymph node metastases and highest incidence of bone metastases.[28]

In Vitro *Culture Conditions*

All tumor cell lines were maintained as monolayer cultures in RPMI-1640 (Celox Corp., Hopkins, MN) supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, L-glutamine, a twofold vitamin solution (GIBCO, Grand Island, NY), and penicillin-streptomycin (Flow Laboratories, Rockville, MD). Cell cultures were maintained on plastic and incubated in 5% CO_2/95% air at 37°C. Cultures were free of *Mycoplasma* and the following 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).

Animals and Production of Tumors

Male athymic BALB/c nude mice were obtained from the Animal Production Area of the NCI-Frederick Cancer Research Facility (Frederick, MD). The mice

Table 1. *Sequence of Oligonucleotide Probes*

Probe	Sequence 5′–3′ (GC content)	Working dilution	Reference
EGFR	GGA′GCG′CTG′CCC′CGG′CCG′TCC′CGG (87.5%)	1:1000	24,31
bFGF	CGG′GAA′GGC′GCC′GCT′GCC′GCC′ (85.7%)	1:200	11,32
IL-8	CTC′CAC′AAC′CCT′CTG′CAC′CC (65.0%)	1:200	12
Type IV collagenase			
72-kd	TGG′GCT′ACG′GCG′CGG′CGG′CGT′GGC (85.2%)	1:200	34
92-kd	CCG′GTC′CAC′CTC′GCT′GGC′GCT′CCG′GU (77.0%)	1:200	
E-cadherin (mixture)	TCC′AGC′GGG′CTG′GAG′TCT′GAA′CTG (62.5%)	1:200	35
	GAC′GCC′GGC′GGC′CCC′TTC′ACA′GTC′ (75.0%)	1:200	
mdr-1 (mixture)	CAG′ACA′GCA′GCT′GAC′AGT′CCA′AGA′AGA′GGA′CT (53.1%)	1:200	36
	GCA′TTC′TGG′ATG′GTG′GAC′AGG′CGG′TGA′G′ (60.7%)	1:200	
Poly $d(T)_{20}$		1:1000	

were maintained according to institutional guidelines 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 Department of Agriculture, Department of Health and Human Services, and the National Institutes of Health. The mice were used when they were 8 to 10 weeks old. To produce tumors, each of the cell lines growing in culture was harvested by a brief trypsinization, and 2×10^5 cells were implanted into the subcutis or dorsal prostate of nude mice.[29,30] The mice were killed after 3 to 4 weeks, and tumors weighing 500 to 1000 mg were used for the study.

Oligonucleotide Probes

Specific antisense oligonucleotide DNA probes were designed complementary to the mRNA transcripts based on published reports of the cDNA sequence (Table 1).[24,31–36] The specificity of the oligonucleotide sequence was initially determined by a GenEMBL database search using the Genetics Computer Group sequence analysis program (GCG, Madison, WI) based on the FastA algorithm[37]; these showed 100% homology with the target gene and minimal homology with nonspecific mammalian gene sequences. The specificity of each of the sequences was also confirmed by Northern blot analysis. Inconclusive Northern blot analysis for the bFGF sequence was resolved by ISH analysis using the appropriate positive and negative cell controls. A poly $d(T)_{20}$ oligonucleotide was used to verify the integrity and lack of degradation of mRNA in each sample. All DNA probes were synthesized with six biotin molecules (hyperbiotinylated) at the 3′ end via direct coupling using standard phosphoramidite chemistry (Research Genetics, Huntsville, AL).[38] The lyophilized probes were reconstituted to a stock solution at 1 μg/μl in 10 mmol/L Tris (pH 7.6) and 1 mmol/L EDTA. The stock solution was diluted with probe diluent (Research Genetics) immediately before use (Table 1).

Preparation of Samples for in Situ *Hybridization*

Cells were plated onto sterilized ProbeOn Plus slides[24] (Fisher Scientific, Pittsburgh, PA) and allowed to grow to 30% confluence over a period of 3 days. The cultures were washed with cold phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde in PBS for 20 minutes. The cultures were then rinsed with RNAse-free PBS and stored at 4°C when the procedure could not be finished on the same day. Fixed slides were treated with 1% Triton X-100, rinsed with Tris buffer, treated with 0.2 N HCl for 3 minutes at 99°C, and then hybridized as described below.

Tissue sections (4 μm) of formalin-fixed, paraffin-embedded specimens were mounted on silane-treated ProbeOn slides. The slides were placed in the microprobe slide holder, dewaxed, and rehydrated with Autodewaxer and Autoalcohol (Research Genetics), followed by enzymatic digestion with pepsin,[35] and then hybridized as described below.

In Situ *mRNA Hybridization*

ISH was performed as described previously[24,25,39,40] with minor modifications. ISH was carried out by using the microprobe manual staining system (Fisher Scientific).[41,42] Hybridization of the probes was carried out for 45 minutes at 45°C, and the samples were then washed three times for 2 minutes each time with 2X standard saline citrate (SSC) at 45°C. RNAse-free water was used to make up Tris buffer and 2X SSC solutions. The samples were then incubated with alkaline phosphatase-labeled avidin for 30 minutes at 45°C, rinsed in 50 mmol/L Tris buffer (pH 7.6), rinsed with alkaline phosphatase

enhancer for 1 minute, and incubated with a chromogen substrate for 15 minutes at 45°C. Additional incubation with fresh chromogen substrate was done when it was necessary to enhance a weak reaction. A positive enzymatic reaction in this assay stained red. Known positive controls were used in each hybridization reaction. Controls for endogenous alkaline phosphatase included treatment of the sample in the absence of the biotinylated probe and use of chromogen alone.

Image Analysis to Quantify Intensity of Color Reaction

Stained sections were examined in a Zeiss photomicroscope (Carl Zeiss, Thornwood, NY) equipped with a three-chip-charged coupled device (CCD) color camera (model DXC-960 MD, Sony Corp., Tokyo, Japan). The images were analyzed using the Optimas software (Optimas Corp., Bothell, WA). The slides were prescreened by one of the investigators to determine the range in staining intensity of the slides to be analyzed. Images covering the range of staining intensities were captured electronically, a color bar (montage) was created, and a threshold value was set in the red, green, and blue modes of the color camera. All subsequent images were quantified based on this threshold. The integrated optical density of the selected fields was determined based on its equivalence to the mean log inverse gray value multiplied by the area of the field. The samples were not counterstained, so the optical density was due solely to the product of the ISH reaction. Three different fields in each sample were quantified to derive an average value. Due to the limitations of enzymatic reactions in tissue sections, the intensity of staining does not correlate linearly with mRNA quantity. To determine differences in mRNA expression among different samples, the intensity of staining was determined by comparison with the integrated optical density of poly $d(T)_{20}$, which was set at 100.

Northern Blot Analysis

Polyadenylated mRNA was extracted from cultured cells or tissues using the FastTrack mRNA isolation kit (Invitrogen Co., San Diego, CA). The mRNA was electrophoresed on a 1% denaturing agarose gel containing formaldehyde, electrotransferred at 0.6 A to a GeneScreen nylon membrane (DuPont Co., Boston, MA), and UV cross-linked with 120,000 $\mu J/cm^2$ using a UV Stratalinker 1800 (Stratagene, La Jolla, CA). Hybridizations were performed as described previously.[43] Nylon filters were washed at 65°C with 30 mmol/L sodium citrate (pH 7.2) and 0.1% sodium dodecyl sulfate (w/v).

cDNA Probes

The cDNA probes used in this analysis were as follows: 1) a 1.3-kb *Pst*I cDNA fragment corresponding to rat glyceraldehyde phosphate dehydrogenase (GAPDH),[442] a 3.8-kb *Xho*I cDNA fragment of human EGFR (kindly provided by Dr. F. Kern, Washington, DC),[313] a 1.4-kb *Eco*RI cDNA fragment of bovine bFGF,[324] a 0.5-kb *Eco*RI cDNA fragment of human IL-8 (kindly provided by Dr. Matsushima, Kanazawa, Japan),[335] a 1.4-kb *Eco*RI cDNA fragment of human *mdr*-1,[366] 0.6-kb *Eco*RI-*Ava*I cDNA fragment of mouse E-cadherin (kindly provided by Dr. M. Takeichi, Kyoto, Japan),[45] and[7] a 1.1-kb *Eco*RI cDNA fragment of human type IV collagenase (kindly provided by Dr. W. G. Stetler-Stevenson, Bethesda, MD). Each cDNA fragment was purified by agarose gel electrophoresis, recovered by using GeneClean (BIO 101, La Jolla, CA), and radiolabeled with the random primer technique using [α-^{32}P]deoxyribonucleotide triphosphates.[46]

Densitometric Quantitation

Expression of the mRNA was quantified by densitometry of autoradiograms using the ImageQuant software program (Molecular Dynamics, Sunnyvale, CA), with each sample measurement calculated from the ratio of the average areas between the specific mRNA transcripts and the 1.3-kb GAPDH mRNA transcript in the linear range of the film.

Statistical Analysis

The significance of ISH data was determined using Student's *t*-test (two-tailed).[47]

Results

Constitutive Expression of the Metastasis-Related Genes in PC-3 Cells Growing in Culture

In the first set of experiments, we used ISH analysis to examine the constitutive expression of several metastasis-related genes in PC-3 cells and selected variants growing in culture. The integrity of the mRNA in the samples was verified by using a poly d(T) probe. All samples had an intense histochemical

Table 2. *mRNA Expression Level of Metastasis-Related Genes in Cultured Cells (*in Situ *Hybridization)*

Cells	Metastatic potential†	mRNA expression index*						
							Collagenase	
		EGFR	bFGF	IL-8	*mdr*-1	E-cadherin	72-kd	92-kd
PC-3M	Low	100	52	48	60	62	36	0‡
PC-3M-Pro4	Low	90	38	54	48	64	44§	0‡
PC-3M-LN4	High	94	62	68§	62	54	54§	66§

*The intensity of the cytoplasmic staining was quantitated by an image analyzer and compared with the maximal intensity of poly d(T) staining in each sample defined as 100.
†Spontaneous metastasis to regional lymph nodes and bone after orthotopic implantation in nude mice.
‡No mRNA detected. The presence of cells was confirmed by hematoxylin and eosin staining.
§$P < 0.001$ compared with PC-3M.

reaction, indicating that the mRNA was not degraded. Next, we examined the expression level of the EGFR, bFGF, IL-8, type IV collagenase, E-cadherin, and *mdr*-1 genes. For each probe, the intensity of cytoplasmic staining was quantified by an image analyzer and compared with the intensity of staining with the poly $d(T)_{20}$ probe taken to be the maximal reaction and assigned a numerical value of 100. Representative examples for the ISH analysis are shown in Figure 1, and the results of all analyses are recorded in Table 2.

Highly metastatic PC-3M-LN4 cells exhibited stronger homogeneous staining with bFGF, IL-8, *mdr*-1, and type IV collagenase probes than did the parental PC-3M cells. There was no significant difference in staining intensity for E-cadherin between the cell lines. Expression of EGFR was uniformly intense in all cell lines. The 92-kd type IV collagenase probe did not react with the low metastatic PC-3M and PC-3M-Pro4 cells. Parental PC-3 cells exhibited a pattern of staining similar to that of PC-3M for all probes tested (data not shown).

Inducible Gene Expression and Effect of Organ Microenvironment

In the second set of experiments, we examined whether the differential gene expression seen in tissue culture between PC-3 and selected metastatic variants was maintained *in vivo*. Each cell line was orthotopically implanted into the prostate of nude mice, and the resulting tumors (0.5 to 1 g) were harvested for ISH analysis. The integrity of mRNA was confirmed by poly d(T) staining, and ISH analysis was performed with the same probes used on cultured cells. Within the prostate microenvironment, the highly metastatic PC-3M-LN4 cells continued to exhibit higher levels of bFGF, IL-8, and type IV collagenase than the lower metastatic PC-3M cells (Figure 2). The ISH technique identified intratumor heterogeneity for expression of type IV collagenase (72-kd) and bFGF with increased intensity of staining occurring at the tumor margin (Figure 2, arrow). The 92-kd type IV collagenase probe did not prove useful in the ISH analysis of formalin-fixed, paraffin-embedded specimens.

Next, we evaluated the effect of the organ microenvironment on expression of metastasis-related genes by implanting the highly metastatic PC-3M-LN4 cells into the subcutis and prostate of nude mice. Tumors were harvested and processed for ISH. Spontaneous metastases in the lymph nodes regional to the prostatic tumors were also evaluated. PC-3M-LN4 tumors grown in the prostate exhibited higher levels of EGFR, bFGF, IL-8, type IV collagenase, and *mdr*-1 than those grown in the subcutis (Figure 3). Within the lymph node metastases, only the staining intensity for type IV collagenase and bFGF was higher than that in the prostatic tumors. Representative examples of ISH are shown in Figure

Table 3. *mRNA Expression Level of Metastasis-Related Genes in PC-3M-LN4 Tumors Growing in Nude Mice (*in Situ *Hybridization)*

Site of growth	mRNA expression index*					
	EGFR	bFGF	IL-8	*mdr*-1	E-cadherin	Collagenase
Skin	60	70	42	36	64	48
Prostate	78	74	64†	66†	60	64†
Lymph node	78	92	56	60	66	66

*The intensity of the cytoplasmic staining was quantitated by an image analyzer and compared with the maximal intensity of poly d(T) staining in each sample defined as 100.
†$P < 0.05$; prostate *versus* skin.

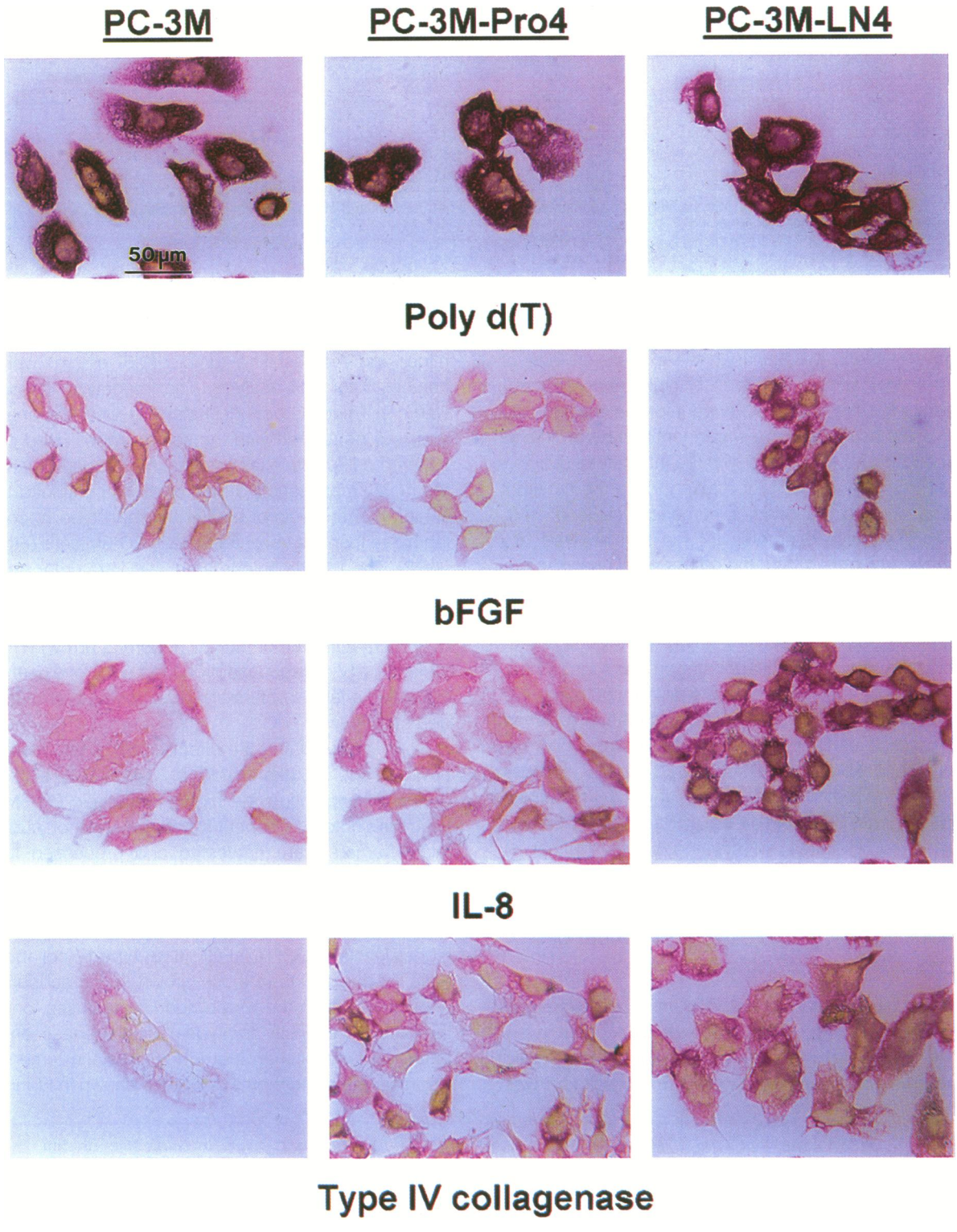
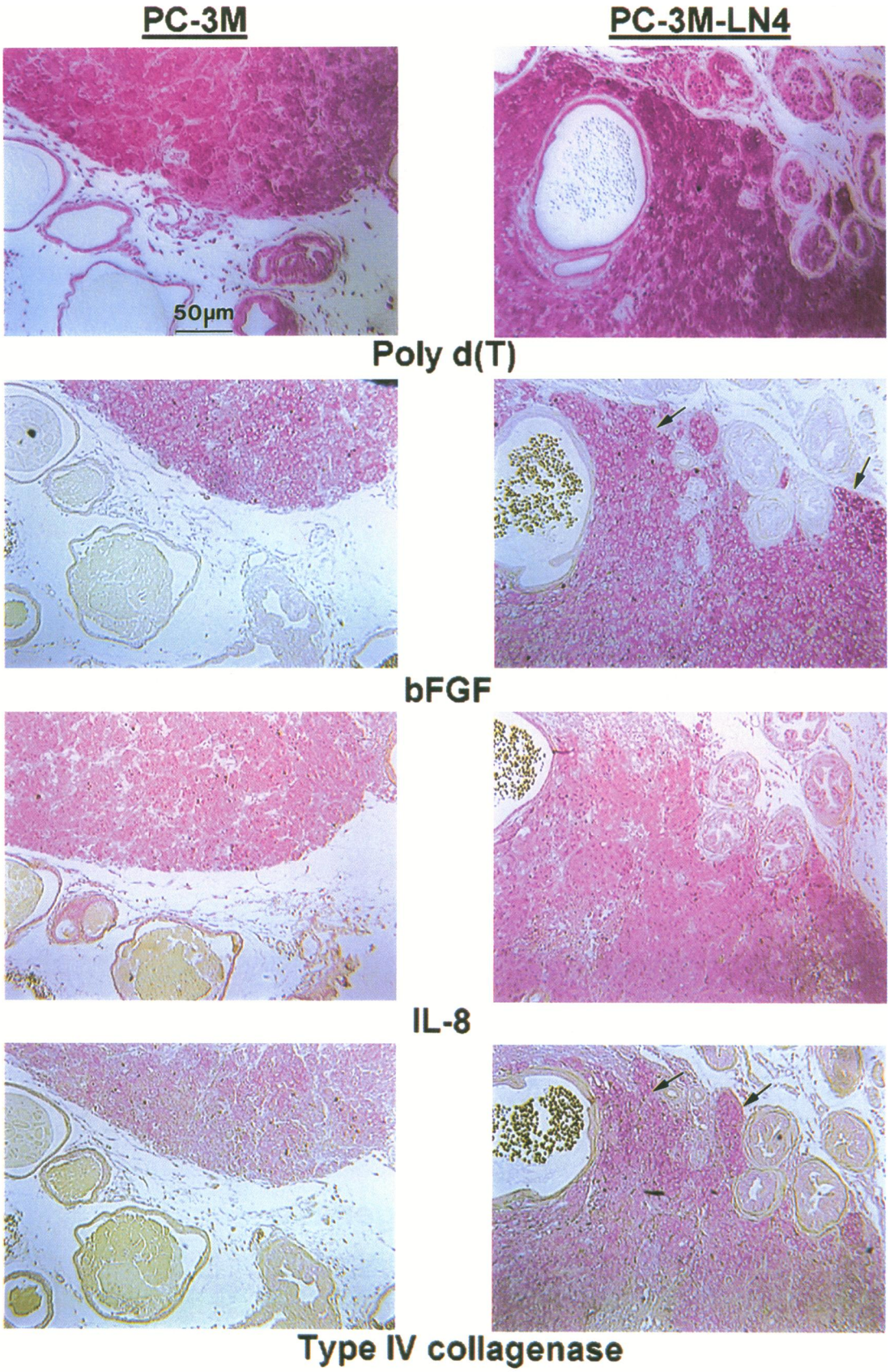


Figure 1. *ISH of PC-3M and selected metastatic variants in culture. The highly metastatic PC-3M-LN4 cells exhibited a uniform staining pattern with intense reactivity for bFGF, IL-8, and type IV collagenase as compared with the lower metastatic PC-3M and PC-3M-Pro4 cells. Hybridization of cells with a hyperbiotinylated poly d(T)$_{20}$ oligonucleotide probe confirmed mRNA integrity and lack of degradation.*


PC-3M
PC-3M-LN4
50μm
Poly d(T)
bFGF
IL-8
Type IV collagenase

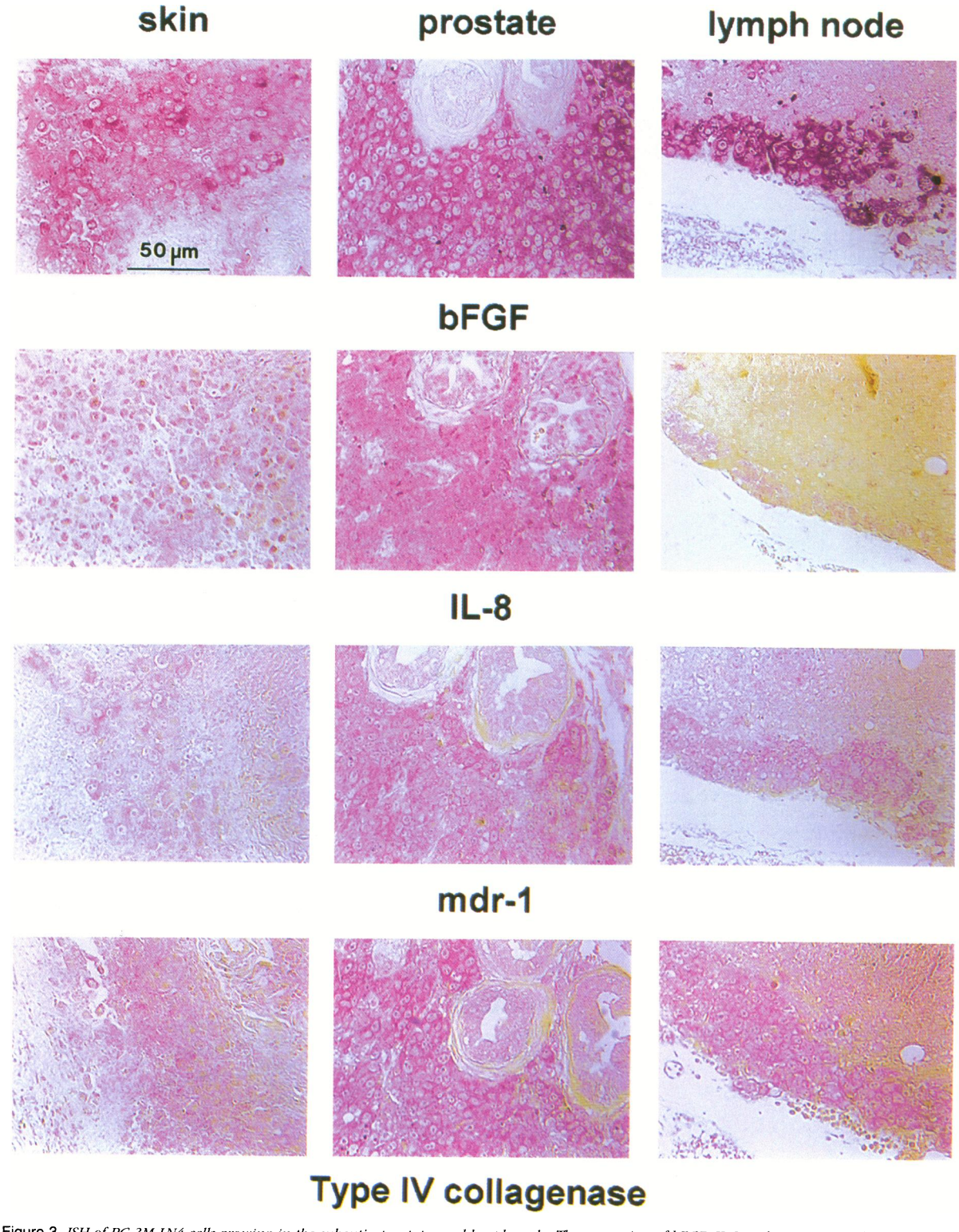


Figure 3. *ISH of PC-3M-LN4 cells growing in the subcutis, prostate, and lymph node. The expression of bFGF, IL-8, mdr-1, type IV collagenase, and EGF-R (not shown) mRNA transcripts was higher in the prostate than in the skin. With the exception of higher bFGF reactivity, the level of gene expression in regional lymph node metastases was similar to that observed in the prostate.*

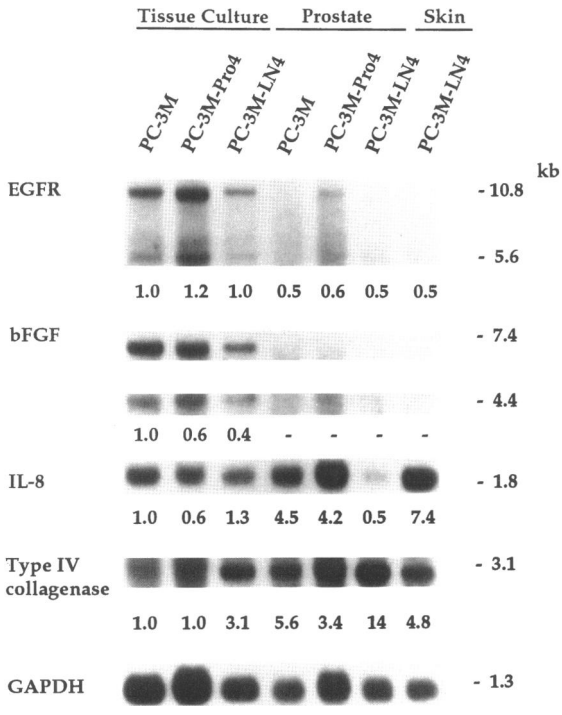


Figure 4. *Northern blot analysis for metastasis-related genes in PC-M cells and selected metastatic variants growing in culture or in nude mice. Polyadenylated mRNA (2 μg/lane) was used to detect the specific mRNA transcripts. A rat GAPDH probe was used as an internal control. For densitometric quantitation, the ratio of the area between the specific transcripts and the 1.3-kb GAPDH was compared in each case with the PC-3M culture cells defined as 0. This is one representative experiment of three.*

3, and results of all analyses are recorded in Table 3. These results clearly emphasize that the organ microenvironment can affect the expression of metastasis-related genes.

Northern Blot Analysis

To confirm the ISH data, we examined the level of EGFR, bFGF, IL-8, *mdr*-1, E-cadherin, and type IV collagenase mRNA transcripts using Northern blot analysis in PC-3M, PC-3M-Pro4, and PC-3M-LN4 cells growing in tissue culture as well as in the prostate or subcutis of nude mice (Figure 4). The mRNA expression level of type IV collagenase closely correlated with ISH results. The highly metastatic PC-3M-LN4 cells expressed over threefold the level of type IV collagenase both in tissue culture and prostate of nude mice as low metastatic PC-3M and PC-3M-Pro4 cells or PC-3M-LN4 cells growing subcutaneously. ISH analysis was more sensitive than Northern blot analysis in detecting differential expression of EGFR and IL-8 in the cultured cell lines. The pattern of bFGF expression as detected by Northern blot analysis was inverse to that described by ISH analysis. We found very weak levels of E-cadherin and *mdr*-1 expression in cells growing in culture (data not shown).

Significant disparity was observed between the *in vivo* results obtained by Northern blot analysis and ISH for EGFR, bFGF, *mdr*-1, and E-cadherin mRNA transcripts. This may have been in part due to the inability of Northern blot analysis to distinguish infiltrating normal cells from tumors cells in a tissue specimen. In addition, necrotic and apoptotic areas of tumors will both have degraded mRNA. Subcutaneous tumors are usually encapsulated and can be dissected free of the fibrous capsule, whereas prostate tumors are highly invasive and the specimens are likely to include normal mucosa. Because Northern blotting measures the average expression in a tissue, these morphological differences could obscure the results. Northern blot analysis cannot measure intratumor heterogeneity for gene expression, whereas the ISH technique can.

Discussion

The biology of prostate cancer metastasis involves the expression of several genes that control angiogenesis (bFGF and IL-8), invasion (collagenase type IV), adhesion (E-cadherin), growth (EGFR), and resistance to chemotherapy (*mdr*-1).[8,10,13,15,19] To study the expression of any one of these genes in isolation from the others is unlikely to result in an accurate description of the metastatic phenotype. We hypothesized that multiparametric ISH analysis could identify prostate cancer cells with different metastatic potentials. We have recently shown in surgical specimens of human colon carcinoma that multiparametric ISH for the same panel of genes was successful in differentiating low-pathological-stage cancers (Dukes' A and B) from high-stage, metastatic cancers (Dukes' D).[39,40,48] Specifically, the ratio of E-cadherin to type IV collagenase expression on the invasive edge of the tumor predicted which organ-confined (Dukes' B) cancers were likely to recur locally and produce metastasis.[48]

Currently, there are but few established prostate cancer cell lines suitable for study both in tissue culture and in a clinically relevant animal model system. To date, most *in vivo* studies have involved implantation of prostate carcinoma cell lines in the subcutis of rodents. But abundant experimental evidence clearly demonstrating that the organ microenvironment influences the expression of metastasis-related genes[1] has raised serious questions about the suitability of the subcutis as an implanta-

tion site to study the process of prostate cancer metastasis. We have recently shown that orthotopic implantation of prostate cancer cells is critical in producing clinically relevant metastases to regional lymph nodes and bone.[28] PC-3M cells were orthotopically implanted into the prostate of nude mice, and after four cycles of *in vivo* selection, we isolated two variants with different metastatic potentials. (We chose the PC-3 cell line because its *in vitro* and *in vivo* behavior closely paralleled the clinical picture of hormone-refractory prostate cancer.)

The highly metastatic PC-3M-LN4 cell line constitutively exhibited higher levels of bFGF, IL-8, type IV collagenase, and *mdr*-1 gene expression than the lower metastatic PC-3, PC-3M, and PC-3M-Pro4 cell lines (Figure 1). This differential expression was maintained in the prostate of nude mice (Figure 2). There was a trend toward decreased E-cadherin expression in the highly metastatic PC-3M-LN4 cell line. The reactivity of EGFR was uniformly high in all cell lines, both in tissue culture and tumor specimens. Expression of all the genes studied with exception of E-cadherin was higher in the prostate than the skin, which reaffirms the importance of the organ microenvironment in regulating metastasis-related gene expression. These results confirm other reports in the literature that conclude that increased expression of EGFR,[49,50] bFGF,[51] type IV collagenase,[52,53] and *mdr*-1[54] and decreased expression of E-cadherin[55,56] are associated with metastatic potential in prostate cancer.

The ISH technique was more informative than Northern blot analysis in identifying the heterogeneous pattern of gene expression in cultured cells and especially in tumors growing in nude mice. ISH of cells growing in culture revealed a heterogeneous staining pattern in the low metastatic parental PC-3 cells, whereas in cultures of the highly metastatic PC-3M-LN4 cells, it was homogeneous (and intense). Tumors growing in the prostate of nude mice exhibited intratumor heterogeneity for expression of bFGF and type IV collagenase; the staining intensity for these transcripts increased along the periphery of the lesions. This finding agrees with our previous reports that surgical specimens of human carcinomas[57] and human colon carcinomas[40,48] exhibit intratumor heterogeneity for expression of several metastasis-related genes. ISH technique and immunohistochemical staining can also identify whether the signal (mRNA transcript or protein) originates from host or tumor cells. Because Northern blot analysis measures total mRNA from pooled samples, it can identify neither the cellular source of the transcript nor intrasample heterogeneity.

In summary, the present study has demonstrated that the *in vitro* expression level of several metastasis-related genes correlates with the metastatic potential of human prostate cancer cells in nude mice. In addition, the results show that the organ microenvironment regulates the expression of metastasis-related genes in prostate cancer. The ISH technique demonstrated its unique ability to define inter- and intratumor heterogeneity and its potential to identify individual human prostate cancers with metastatic potential. Multivariate ISH analyses are currently underway to correlate patterns of gene expression with high and low Gleason scores in radical prostatectomy specimens. As this analysis can be performed on formalin-fixed, paraffin-embedded tissues, a large study of archival material will help define whether these results will have prognostic significance for disease recurrence and patient survival.

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