Selection of Highly Metastatic Variants of Different Human Prostatic Carcinomas Using Orthotopic Implantation in Nude Mice[1]

Curtis A. Pettaway,[2] Sen Pathak,
Graham Greene, Edilberto Ramirez,
Michael R. Wilson, Jerald J. Killion, and
Isaiah J. Fidler

Departments of Urology [C. A. P., G. G., E. R.] and Cell Biology [S. P., M. R. W., J. J. K., I. J. F.], The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

ABSTRACT

The purpose of this study was to determine whether the implantation of human prostate cancer cells into the prostates of nude mice and their subsequent growth there can be used to select variants with increasing metastatic potential. PC-3M and LNCaP cells were injected into the prostates of athymic mice. Tumors from the prostate or lymph nodes were harvested, and cells were reinjected into the prostate. This cycle was repeated three to five times to yield cell lines PC-3M-Pro4, PC-3M-LN4, LNCaP-Pro3-5, and LNCaP-LN3-4. Parental and variant cells were injected into the prostates of nude mice. PC-3M-LN4 cells produced enhanced regional lymph node and distant organ metastasis as compared to PC-3M-Pro4 or PC-3M cells. After i.v. or intracardiac inoculation, PC-3M-LN4 cells produced a higher incidence of lung metastasis and bone metastasis, respectively, than PC-3M or PC-3M-Pro4 cells. Subsequent to implantation into the prostate, LNCaP-LN3 cells produced a higher incidence of regional lymph node metastases than LNCaP-Pro5 or LNCaP cells. After intrasplenic implantation, LNCaP-LN3 cells also yielded experimental liver metastases. The metastatic LNCaP-LN3 cells exhibited clonal karyotypic abnormalities, were less sensitive to androgen (in vitro and in vivo), and produced high levels of prostate-specific antigen. Collectively, the data show that the orthotopic implantation of human prostate cancer cell lines in nude mice is a relevant model with which to study the biology of prostate cancer metastasis and to select variant cell lines with enhanced metastatic potential.

INTRODUCTION

The incidence and mortality of prostate cancer are a major health problem in the United States (1). Despite an increased awareness of the disease, which has led to earlier detection and treatment of the primary tumor, many patients still die of metastases that are resistant to conventional cytotoxic therapies, including hormonal ablation (2–4). Improvement in the therapy of prostate cancer metastases now depends on improving our understanding the biology of this disease.

The development of relevant in vivo models has made it possible to show that the outcome of metastasis is dependent on the interaction of metastatic cells with host factors (5–7). Studies from our laboratory and others have shown that human tumors implanted into the orthotopic organ environment can grow and produce distant metastases; in contrast, those implanted into ectopic organs rarely metastasize (8–17). We also reported that human prostate cancer cells implanted into the prostates of athymic nude mice were more tumorigenic and metastatic, whereas the same cells implanted s.c. were not (15, 16). Co-inoculating human prostate cancer cells with fibroblasts derived from prostate, bone, or urogenital sinus and injecting prostate cancer cells admixed with growth factor-enriched extracellular matrix promoted the local growth of human prostate cancer cells but not distant metastasis (18, 19). These data confirmed the important role of organ factors in the growth of human prostate cancer cells.

The isolation of cell populations (from heterogeneous human tumors) that differ from the parent neoplasm in metastatic capacity provide a powerful tool with which to study those intrinsic properties that distinguish metastatic from nonmetastatic cells (8–10, 13). In the present study, we show that orthotopic implantation of two heterogeneous human prostate cancer lines, PC-3M (20) and LNCaP (21), into nude mice results in rapid local growth and production of distant metastases. Cells isolated from metastases were more likely to metastasize to different organ sites, including the bone. The metastatic variant cells had clonal karyotypic markers and unique properties. The availability of these cells should facilitate detailed analysis of determinants of human prostate cancer metastasis.

MATERIALS AND METHODS

Animals

Male athymic BALB/c nude mice 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 and used at 8 weeks of age according to institutional and national guidelines as described previously (15, 16).
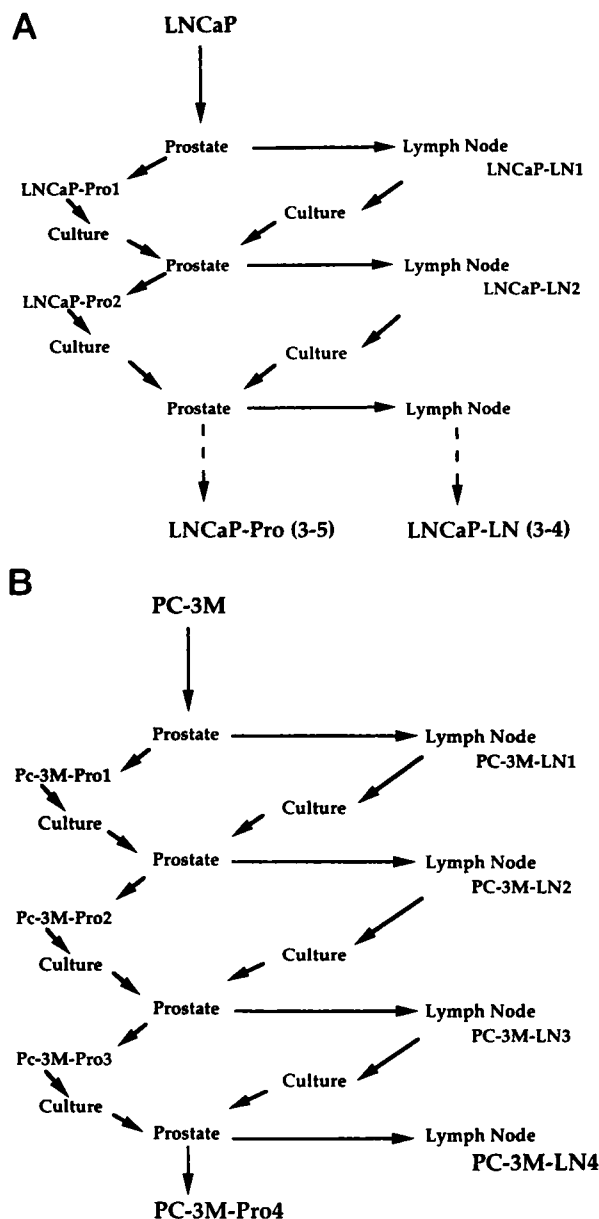
Fig. 1 *In vivo* selection of LNCaP and PC-3M variant lines. *A,* LNCaP selection. Two × 10⁶ cultured LNCaP cells were injected into the prostates of athymic mice. Tumor cells harvested from the prostate and lymph nodes were expanded in culture and then injected into the prostates of nude mice. Cells were again harvested from the prostates of nude mice to generate prostate-selected lines (LNCaP Pro3-Pro5) and lymph node-selected lines (LNCaP LN3-LN4). *B,* PC-3M. Two × 10⁵ cultured PC-3M cells were injected into the prostate of nude mice. *In vivo* recycling of tumor cells from prostate and lymph nodes resulted in the isolation of prostate (PC-3M-Pro4) and lymph node (PC-3M-LN4) variant lines.

Prostate Cancer Cell Lines and Culture Conditions

The LNCaP-FGC (LNCaP) human prostate cancer cell line was obtained from American Type Culture Collection (Rockville, MD; Ref. 21). The PC-3M cell line was isolated in our laboratory from liver metastases produced in nude mice subse-

quent to intrasplenic injection of the androgen-insensitive PC-3 human prostate carcinoma cell line (20, 23). The cell lines were maintained in RPMI 1640 (Celox Corp., Hopkins, MN), supplemented with 10% FBS,[3] sodium pyruvate, nonessential amino acids, L-glutamine, a 2-fold vitamin solution (Life Technologies, Inc., Grand Island, NY), and penicillin-streptomycin (Flow Laboratories, Rockville, MD).

Monolayer cultures were maintained on plastic and incubated in a mixture of 5% CO_2 and 95% air at 37°C. For *in vivo* injection, cells were harvested from culture flasks after a brief trypsinization, as described previously (15). Only single-cell suspensions of >90% viability (trypan blue exclusion) were used for injections.

Tumor Cell Injection Techniques

Orthotopic Injection. Male nude mice were anesthetized with methoxyflurane, and a lower midline incision was created. Tumor cell suspensions (20–40 μl) were injected into the dorsal prostatic lobes using a 30-gauge needle, a 1-ml disposable syringe, and a calibrated push button-controlled dispensing device (Hamilton Syringe Company, Reno, NV). The abdominal wound was closed in one layer with wound clips (Autoclip; Clay Adams, Parsippany, NJ; Refs. 15 and 16).

Intrasplenic Inoculation. LNCaP cells were injected into the spleen by methods described in detail previously (24). A small left abdominal flank incision was created, and the spleen was exteriorized. Tumor cells (2 × 10⁶ cells in 200 μl HBSS) were injected into the spleen using a 30-gauge needle.

Intracardiac Injection. Mice were anesthetized with methoxyflurane and placed in the supine position. An upper midline vertical incision was created, and the liver was retracted to visualize the base of the heart. Tumor cell suspensions (5 × 10⁵ cells in 50 μl HBSS) were injected into the base of the heart through the diaphragm using a 30-gauge needle.

i.v. Injection. Tumor cell suspensions (LNCaP, 2 × 10⁶ cells in 200 μl HBSS; PC-3M, 5 × 10⁵ cells in 200 μl HBSS) were injected into the lateral tail vein of unanesthetized mice using a 27-gauge needle.

s.c. Injection. LNCaP cells (2 × 10⁶ cells in 20 μl HBSS) were injected s.c. on the left lateral chest wall in close proximity to the axilla using a Hamilton syringe and a 30-gauge needle.

Necropsy Procedures and Histological Studies

Prostatic, s.c., and Intrasplenic Implantation. Mice were killed by cervical dislocation. Tissues were excised, weighed, fixed in 10% formalin, paraffin-embedded, sectioned, and stained with H&E.

Intracardiac Implantation. All macroscopic metastases, as well as mandibular and femoral bones, were fixed in 10%

---

[3] The abbreviations used are: FBS, fetal bovine serum; PSA, prostate-specific antigen; CSS, charcoal-stripped serum; DHT, dihydrotestosterone; LN, lymph node.

Table 1 Tumorigenicity and production of metastasis by LNCaP cells and selected variants subsequent to orthotopic implantation in athymic mice[a]

LNCaP cell line	Tumorigenicity	Prostate weight (g) mean ± SD	Paraaortic lymph node metastasis	Survival (days)
Parental	24/43	1.2 ± 1.0	12/43	100
Pro2	9/10	1.6 ± 1.1	6/10	80
Pro3	8/8	1.3 ± 1.0	6/8	60
Pro5	10/17	3.3 ± 1.7[b]	2/17	60
LN2	25/30	1.2 ± 0.9	21/30	80
LN3	19/19[c]	1.2 ± 0.9	13/19[d]	60
LN4	5/6	1.0 ± 0.8	4/6[e]	60

[a] Mice underwent prostatic injection of 2 × 10⁶ LNCaP parental or selected variant cells and were killed and necropsied when moribund. Tumorigenicity and metastasis were confirmed by histology.

[b] $P < 0.05$, as compared to all other groups.

[c] $P < 0.001$, as compared to LNCaP; $P = 0.002$, as compared to LNCaP-Pro5.

[d] $P < 0.003$, as compared to LNCaP; $P < 0.001$, as compared to LNCaP-Pro5.

[e] $P = 0.021$, as compared to LNCaP-Pro5.

formalin, and after decalcification (bones only), processed as above for histology.

i.v. Injection. Sites of metastasis were assessed subsequent to necropsy. Lungs (the most common site) were resected, washed in saline, and placed in Bouin's fixative. Lung metastases were counted using a dissecting microscope 24 to 48 h later. Lungs were then processed as above for histology.

In Vivo Selection of Metastatic Variants from LNCaP and PC-3M Prostate Cancer Lines

LNCaP and PC-3M cells were injected into the prostate of nude mice, and the resultant prostate tumor and regional lymph node metastases (obtained after necropsy) were harvested by aseptic techniques, dissociated mechanically using a wire mesh sieve, and placed into culture. To inhibit fibroblast overgrowth of LNCaP cultures, a selective medium consisting of the α modification of MEM with the addition of cholera toxin (500 ng/ml), epidermal growth factor (1 ng/ml; Sigma Chemical Co., St. Louis, MO), 10% FBS, sodium pyruvate, nonessential amino acids, L-glutamine, and a 2-fold vitamin solution (Life Technologies, Inc.) was used (25). Cells were grown in this medium for two passages and then maintained in RPMI 1640 with 10% FBS. Cytogenetic analysis was used to confirm the human origin of the cells. The lines were designated LNCaP-Pro1 (prostate cycle 1), PC-3M-Pro1, LNCaP-LN1 (lymph node cycle 1), and PC-3M-LN1 (Fig. 1). The two variant lines of LNCaP and two variant lines of PC-3M were harvested within three to five *in vitro* passages and injected into the prostate of athymic nude mice. Cells that grew in the prostate were used for sequential isolation and reinjection into the prostate to yield lines LNCaP Pro1-Pro5 and PC-3M Pro1-Pro4. Cells from lymph node metastases were isolated and reinjected into the prostate to produce subsequent lymph node metastases. These lines were designated LNCaP-LN1-LN4 and PC-3M-LN1-LN-4.

Bilateral Scrotal Orchiectomy

Forty-eight to 72 h after injection of prostate cancer cells, mice underwent either a bilateral orchiectomy or sham procedure via a transscrotal approach.

Measurement of PSA

LNCaP cells were plated in RPMI 1640 medium containing 10% FBS for 48–72 h, and the cultures were washed with warm HBSS; then new medium was added. Culture supernatants were collected 24–48 h later and frozen at −20°C. PSA measurements were made on thawed supernatants using a commercially available mouse antihuman PSA monoclonal antibody (Tandem-E PSA Immunoenzymatic Assay; Hybritech, San Diego, CA). The results were expressed as ng/ml of PSA/10⁶ cells. The limit of sensitivity of this assay was 0.2 ng/ml. For measurement of the serum PSA level, blood was obtained from anesthetized mice by cardiac puncture. Individual samples were clotted in glass tubes. The sera were frozen at −20°C. Serum PSA levels were measured after thawing using the above assay. Results were expressed as ng/ml PSA/gram of prostate tissue.

Chromosome Studies of LNCaP and *in Vivo*-derived Cell Lines: Karyotype Analysis

Confluent cultures of LNCaP parental cells and selected variants were treated with Colcemid (final concentration, 0.01 mg/ml), washed with HBSS, and trypsinized as described previously (26). Single-cell suspensions were centrifuged, and the pellet was exposed to a hypotonic solution (0.06 M KCl). After centrifugation, the cells were fixed in a mixture of methanol and acetic acid (3:1 by volume), washed twice with the fixative, and dropped on glass slides for air-drying preparations. Optimally aged slides were processed for G-banding induction following a routine laboratory procedure (26).

Chromosomes in 15 to 20 G-banded spreads from each sample were counted to determine modal number, and 5–10 metaphase plates were photographed. Five complete karyotypes for each cell line were prepared using the Genetiscan (PSI, Houston, TX) for the possible identification of structural and numerical abnormalities.

In Vitro Growth of LNCaP, PC-3M, and *In Vivo*-selected Lines: Kinetics

LNCaP, PC-3M parental, and *in vivo*-selected variant cells were harvested from monolayer cultures by a brief trypsiniza-
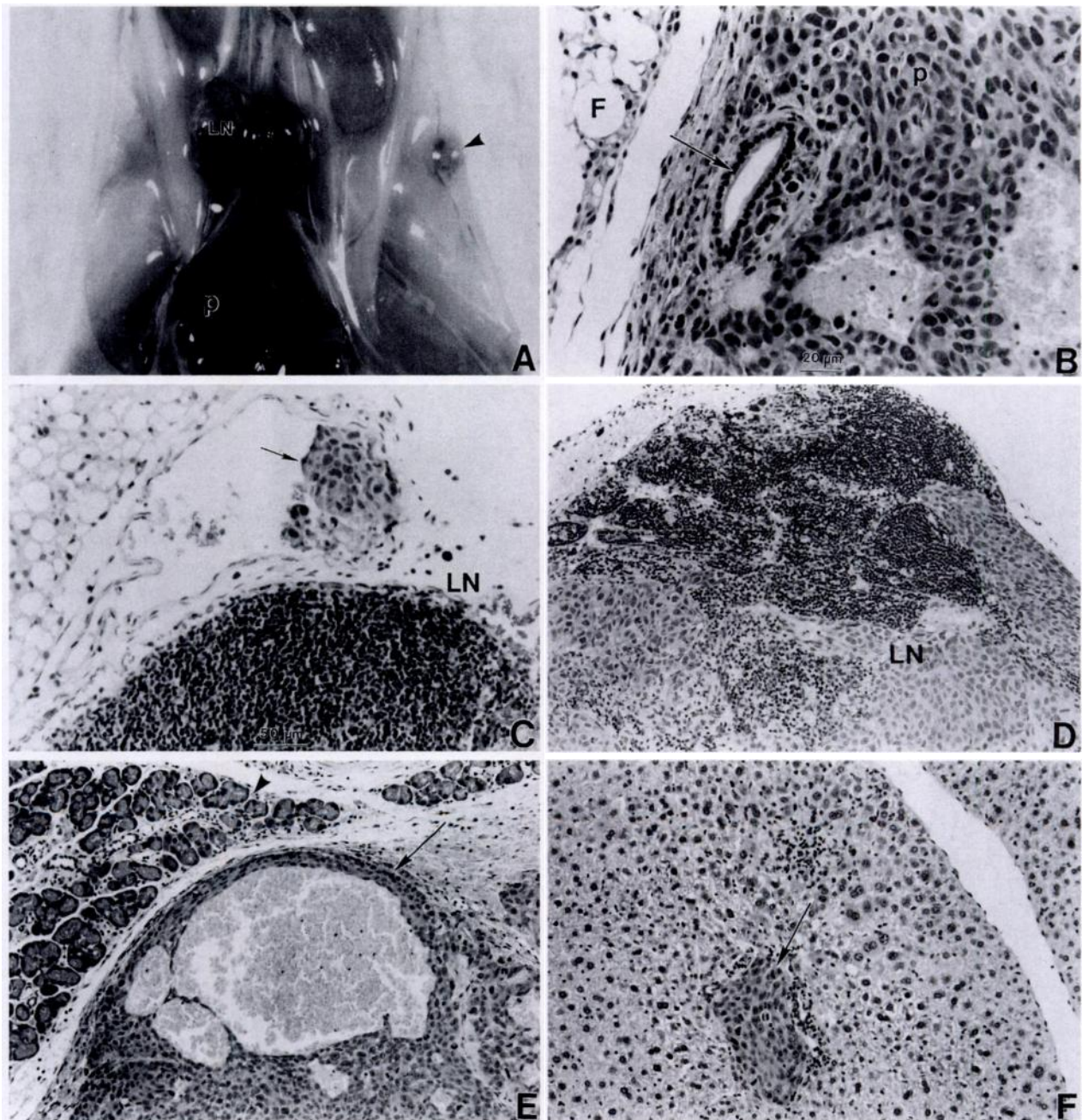
Fig. 2 Gross and histological appearance of LNCaP-LN3 prostate tumor and metastases. *A*, LNCaP-LN3 prostate tumor (*p*) and grossly enlarged regional lymph node metastases (*LN*) at 60 days after orthotopic implantation. *Arrowhead*, an inguinal lymph node metastasis. *B*, histological appearance of LNCaP-LN3 prostate tumor (*p*) replacing the normal gland (*arrow*); *bar*, 20 μm; *F*, periprostatic fat. *C*, LNCaP-LN3 micrometastasis (*arrow*) in lymphatic vessel adjacent to a regional lymph node (*LN*) 28 days after orthotopic implantation. *D*, LNCaP-LN3 lymph node metastasis replacing the lymph node (*LN*) at 60 days after injection. *E*, LNCaP-LN3 tumor (*arrow*) in the tail of pancreas (*arrowhead*) 90 days after intrasplenic injection. *F*, LNCaP-LN3 metastasis (*arrow*) invading the liver parenchyma 90 days after intrasplenic injection. *C-F: bar*, 50 μm.

tion. The cells were washed in cold HBSS and plated at sparse conditions (30 cells/mm²) in RPMI 1640 containing 10% FBS for 48 h. Cells were counted then and at 24–48 h intervals until confluent. *In vitro* doubling times were calculated from curves describing log phase growth.

Inhibition of LNCaP Growth in Androgen-depleted Media

LNCaP parental and variant cells selected *in vivo* (LNCaP-Pro3, Pro5, and LN3) were seeded into 30-mm culture dishes in RPMI 1640 containing 10% FBS for 48 h; then the cultures were washed with warm HBSS, and the medium was changed to

Table 2 Inhibition of LNCaP tumors by orchiectomy[a]

LNCaP cell line	Orchiectomy	Tumorigenicity	Prostate weight (g) mean ± SD	Range
Parental	−	4/9	0.29 ± 0.09	0.27–0.42
	+	0/5		
Pro5	−	5/10	4.60 ± 1.00[b]	2.9–4.3
	+	2/9	0.06 ± 0.03	0.04–0.08
LN3	−	6/6	0.71 ± 0.50[c]	0.09–1.4
	+	6/9	0.21 ± 0.32	0.03–0.9

[a] Bilateral scrotal orchiectomy or sham procedure performed 48 h after injection of LNCaP or *in vivo*-selected cell lines (2 × 10⁶ cells/mouse). LNCaP parental mice were necropsied at 90 days. LNCaP-Pro5 and LN3 mice were necropsied at 60 days.

[b] $P = 0.004$, LNCaP-Pro5 control significantly greater than castrate.

[c] $P = 0.07$, LNCaP-LN3 control not significantly greater than castrate.

phenol red-free RPMI 1640 with either 5% FBS, 5% CSS, or 5% CSS with various concentrations of DHT (1×10^{-8}–1×10^{11} M). Cells counts were obtained 5 days later. The percentage of growth inhibition was calculated as:

$$\% \text{ growth inhibition} = 1 - \frac{\text{Mean number of cells in CSS}}{\text{Mean number of cells in FBS}} \times 100$$

Statistical Analysis

The Fisher's exact test was used to test for differences among proportions. The two-sample independent *t* test was used to test for differences between two groups. When the sample involved more than two groups, the one-way ANOVA was used. Whenever the null hypothesis of equal means was rejected, Tukey's multiple comparison test was used to examine the difference between all possible pairs of means (27).

RESULTS

Selection and Characterization of Metastatic Variants from the LNCaP Line. The cumulative data on tumorigenicity and production of metastasis are shown in Table 1. Mice injected with LNCaP parental cells were killed 100 days after the orthotopic implantation. In 24 of 43 (56%) injected mice, we found LNCaP prostate tumors, and 12 of 43 mice (28%) had microscopic metastases in the regional lymph nodes. No discernible differences in the tumorigenic and metastatic properties were noted between LNCaP-Pro1 and LNCaP-LN1 cells and parental LNCaP cells (data not shown). However, LNCaP-Pro2 and LNCaP-LN2 cells were highly tumorigenic and produced regional lymph node metastases in 60–70% of the mice (Table 1).

The increased tumorigenic and metastatic potential was reproduced when we studied the LNCaP-Pro3 and LNCaP-LN3 cells. Tumors formed in the prostates of all injected mice (Table 1) and regional lymph node metastases were again found in the majority of the mice. The growth of the prostate tumors was enhanced as well. By day 60 of these experiments, the prostate tumors were large and caused morbidity. The subsequent necropsy revealed mean prostatic tumor weights of approximately 1 g (Table 1), which is considerably larger than the median weight of the normal mouse prostate (50–60 mg). The differ-

ence in the size of local prostate tumors was evident as early as 28 days after the intraprostatic injection, when median prostate weight ($n = 3$) was 350 mg for LNCaP-Pro3 tumors, 131 mg for LNCaP-LN3, and 50 mg for LNCaP parental cells (data not shown). At this time (28 days after implantation), micrometastases were detected in the lymph node of a mouse implanted with LNCaP-LN3 cells (Fig. 2C). By day 60, the mice were moribund and were necropsied. Prostate tumors and lymph node metastases were prominent in mice injected with LNCaP-LN3 cells (Fig. 2, *A*, *B*, and *D*). The histological appearance of the primary tumors and metastases was similar and consisted of a highly vascular, poorly differentiated carcinoma with necrotic zones (Fig. 2, *B* and *D*).

Continued selection for variants that grew in the prostate (and do not produce a high incidence of metastasis) resulted in the isolation of line LNCaP-Pro5, which produced larger prostate tumors but a low incidence of lymph node metastasis (Table 1). This difference suggests that growth in the prostate gland *per se* does not predict metastasis. The final selection for lymph node metastases yielded LNCaP-LN4 cells, which were highly tumorigenic and metastatic.

Growth of LNCaP Cells at Ectopic Organs. We next injected the LNCaP parental and selected variant cells into the subcutis, spleen, and venous circulation of different nude mice. The i.v. injection of the LNCaP cells (parental and variants) did not produce any gross or microscopic lesions in the lungs or other visceral organs in mice killed 90 days after the injection. When parental LNCaP cells were injected s.c. ($n = 10$), only one s.c. tumor resulted; LNCaP-LN3 cells ($n = 10$) produced two s.c. tumors. These tumors attained a large size (1.5–2.0 g) but did not produce axillary lymph node metastasis.

The intrasplenic injection of LNCaP-Pro3, Pro5, and LN3 cells produced local tumors in the spleen and tail of the pancreas in 2 of 5, 1 of 7, and 6 of 10 mice, respectively (Fig. 2*E*). Experimental liver metastases occurred only in mice injected intrasplenically with LNCaP-LN3 cells (4 of 10 mice; Fig. 2*F*). LNCaP parental cells did not produce any tumors in the spleen or pancreas.

Inhibition of LNCaP Tumors by Orchiectomy. Since growth of LNCaP cells is sensitive to androgen stimulation (21, 22), we next determined whether orchiectomy would affect the production of orthotopic tumors. Mice underwent bilateral scrotal orchiectomy or sham orchiectomy 48 h after the intraprostatic implantation of LNCaP parental, Pro5, or LN3 cells. The mice were necropsied 60 days (Pro5 and LN3) or 90 days (LNCaP parental) later. Orchiectomy resulted in the total suppression of LNCaP tumor formation and decreased the incidence and weight of LNCaP-Pro5 tumors (Table 2). In fact, Pro5 tumors in the castrated mice weighed only 1% (mean weight ratio castrate:control, 0.06 g:4.60 g) of that of the tumors in intact mice. In contrast, orchiectomy did not decrease the tumorigenicity of LNCaP-LN3 cells. The mean prostate weight of castrated mice with tumors was affected but not as much as for mice injected with LNCaP-Pro5 cells (mean weight ratio castrate:control, 0.271 g:0.71 g × 100 = 30%).

Serum PSA Level in Athymic Mice. PSA was undetectable in the serum of normal male nude mice, mice with PC-3M tumors, and mice injected with LNCaP cells that were tumor free. PSA was detected in the sera of all mice with LNCaP tumors, regardless of the growth site (prostate, spleen, and

Table 3 Tumorigenicity and production of regional and distant abdominal metastasis by orthotopic injection of PC-3M and selected variants in athymic mice[a]

Cell line	Tumorigenicity	Prostate weight (g) mean ± SD	Incidence of regional lymph node metastasis	Mean weight of regional lymph nodes (mg)	Distant metastasis
PC-3M	9/10	0.4 ± 0.2	9/10	25 ± 20	3/10
PC-3M-Pro4	9/9	1.7 ± 0.7[b]	9/9	27 ± 24	4/9
PC-3M-LN4	7/9	0.8 ± 0.5	7/9	114 ± 60[c]	7/9[d]

[a] Orthotopic implantation of 2.0 × 10⁵ cells. Mice were necropsied when moribund, and metastases were confirmed by histology.
[b] $P < 0.05$, significantly greater than PC-3M and PC-3M-LN4.
[c] $P < 0.05$, significantly greater than PC-3M-Pro4 and PC-3M.
[d] $P = 0.025$, significantly greater than PC-3M.

subcutis). The serum PSA level directly correlated with tumor burden (data not shown). At the time of necropsy, tumors of various sizes (0.08–6.0 g) were discovered in the prostate. Even in those mice whose tumors weighed less than 0.2 g, were nonpalpable, and were not yet clinically detectable, the detectable serum PSA level revealed that tumor was present. Serum PSA in mice implanted with metastatic variant cells was twice that in mice bearing variants selected for growth in the prostate when normalized for tumor weight [experiment 1: LNCaP-Pro3, 33 ± 6.0 ng/ml/g tissue; LNCaP-LN3, 62 ± 17 ng/ml/g tissue ($P = 0.001$); experiment 2: LNCaP-Pro5, 13 ± 3.5 ng/ml/g tissue; LNCaP-LN4, 26 ± 5 ng/ml/g tissue ($P = 0.002$)].

Selection and Characterization of Metastatic Variants from the PC-3M Line. The PC-3M cell line was originally selected in our laboratory for enhanced tumorigenicity (20). Hence, these cells were tumorigenic in 9 of 10 injected mice and produced both regional as well as distant metastases at approximately 40 days. The PC-3M-Pro4 and PC-3M-LN4 cells produced rapidly growing prostate tumors, with mice becoming moribund after 25–29 days. Subsequently, necropsy revealed that the mean weight of prostate tumors was 2–5-fold greater than PC-3M tumor-bearing mice (Table 3). The increased tumorigenicity of the selected variants was also demonstrated by the findings that the implantation of 1–2 × 10⁴ PC-3M, PC-3M-Pro4, and PC-3M-LN4 cells produced tumors in 1 of 10, 8 of 9, and 3 of 10 mice, respectively. PC-3M and the selected variants readily formed lymph node metastases (Table 3). The weight of the involved regional lymph nodes was significantly higher for PC-3M-LN4 cells, and this was independent of the weight of the primary tumor. PC-3M-LN4 cells also produced a higher incidence of distant metastases (distant lymph nodes and visceral sites). The histology of the PC-3M tumors (parental or selected variants) in the prostate or metastatic sites was similar and consisted of poorly differentiated carcinomas growing as sheets of cells, as described previously (15, 16).

Hematogenous Metastasis of the PC-3M Cells. Mice ($n = 20$) were injected i.v. with PC-3M, Pro4, and LN4 cells. The mice were killed after 90 days. Metastases were found only in the lungs of 4 of 19 surviving mice injected with PC-3M cells, 9 of 18 surviving mice injected with PC-3M-Pro4 cells, and 12 of 20 mice injected with PC-3M-LN4 cells.

In the next set of experiments, we injected the PC-3M, Pro4, and LN4 cells into the heart of nude mice (transdiaphragmatic injection). The mice were killed when moribund or on day 45 after the injection. Tumors formed in the thorax (lung,

Table 4 Tumorigenicity and production of metastasis by PC-3M cells and selected variants subsequent to intracardiac implantation in athymic mice[a]

Cell line	Intrathoracic growth[b]	Intraabdominal growth[c]	Bone metastasis	
			Mandible	Femur
PC-3M	10/14	1/14	3/14	0/14
PC-3M-Pro4	6/9	0/9	2/9	0/9
PC-3M-LN4	5/10	3/10	6/10[d]	2/10

[a] Athymic mice were injected with 5 × 10⁵ viable cells. The mice were necropsied when moribund or by 45 days after injection. Sites of macroscopic tumors were noted and confirmed by histology.
[b] Lung, pleural surface, and mediastinal lymph nodes.
[c] Viscera and retroperitoneal lymph nodes.
[d] $P = 0.067$, not significantly greater than PC-3M.

mediastinal lymph nodes, and pleura), abdomen (liver, retroperitoneal lymph nodes, and psoas muscle), and bones (Table 4). Intrathoracic growth was identical among the three cell lines and was the primary cause of morbidity. The metastasis of PC-3M-LN4 cells to abdominal sites and bone was enhanced. Gross bone metastases were found in the mandible and distal femur (Fig. 3a). There was no gross evidence of metastasis to the vertebral column. Histological examination of bone metastases revealed destruction of bone by the tumor cells (Fig. 3b).

In Vitro Growth of LNCaP, PC-3M Parental Cells, and Selected Variants. The LNCaP-LN3 cells had a longer doubling time (34 h) than either LNCaP parental (28 h) or LNCaP-Pro5 (26 h) cells. PC-3M and in vivo-derived lines (PC-3M-Pro4 and LN4) had a similar doubling time of 27–29 h. The in vitro growth of the LNCaP (parental and selected variant) cells was inhibited in medium containing CSS and hence depleted of androgens (22), as compared with medium containing FBS (Fig. 4). Specifically, the growth of LNCaP parental cells and that of Pro3 and Pro5 variants was significantly inhibited in medium containing CSS as compared with FBS or medium supplemented with CSS and DHT. The growth of the metastatic LN3 line was less influenced by removal of androgen from the serum (Fig. 4). The growth of PC-3M cells was unaffected by removal of androgens.

In Vitro Production of PSA. Although the absolute PSA levels varied with test batches, the LNCaP-LN3 cells (120 ± 24 ng/ml medium/10⁶ cells) consistently produced a higher PSA level (normalized for cell number) than LNCaP parental (21 ± 3 ng/ml medium/10⁶ cells) or LNCaP-Pro5 (8 ± 2 ng/ml me-
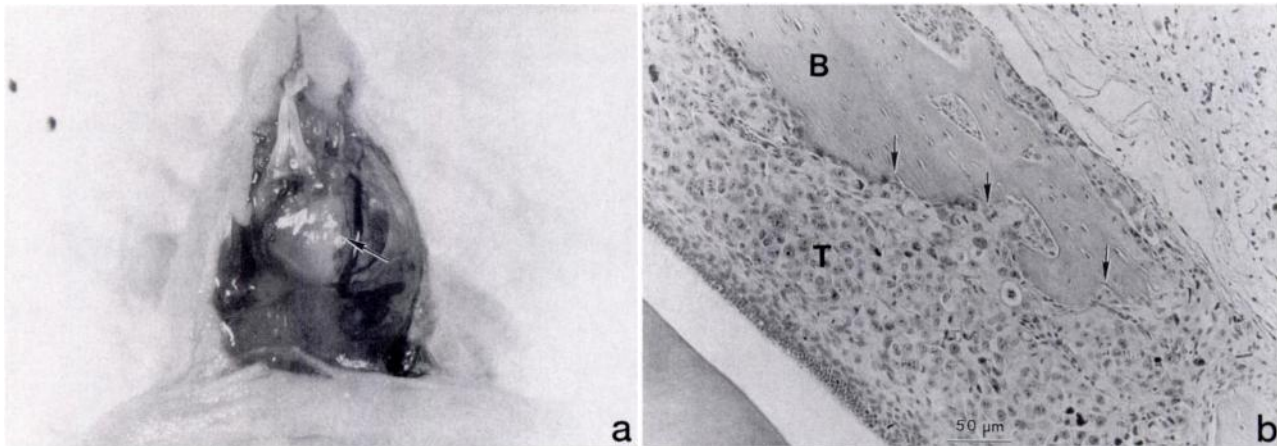
Fig. 3 Gross and microscopic appearance of PC-3M-LN4 bone metastases. *a,* PC-3M-LN4 metastasis (*arrow*) to the left side of the mandible. *b,* histological appearance of PC-3M-LN4 metastases (*T*) to mandibular bone (*B*). Note destruction of bone (*arrows*). *Bar,* 50 μm.
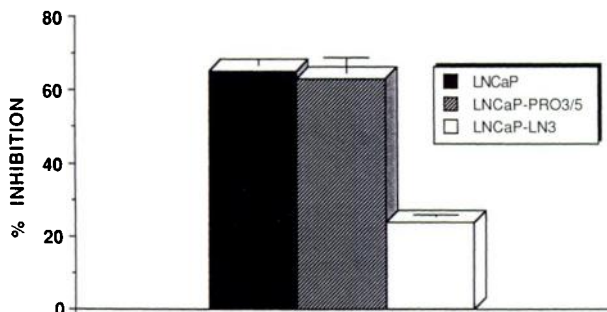

Fig. 4 Inhibition of LNCaP cell growth in androgen-depleted medium. LNCaP parental and selected variants were cultured in RPMI 1640 supplemented with 5% FBS (2 × 10⁴ cells/well) for 48 h. The medium was replaced with RPMI 1640 supplemented with 5% FBS, 5% CSS, or 5% CSS plus DHT. The number of viable cells was determined 5 days later. The results of LNCaP-Pro3 and LNCaP-Pro5 were similar and thus were combined. The percentage of inhibition in androgen-depleted medium was: LNCaP, 65 ± 1.7%; LNCaP-Pro3/5, 64 ± 4.6%; and LNCaP-LN3, 23 ± 1.5% ($P < 0.05$ less than LNCaP or LNCaP-Pro5). *Columns,* means of three independent experiments; *bars,* SD.

dium/10⁶ cells). PSA expression in the culture supernatants of PC-3M (or selected variants) was below the limit of detection (≤0.2 ng/ml).

Karyotype of LNCaP Cells. The LNCaP cell lines exhibited seven marker chromosomes, characteristic of chromosomal rearrangements, denoted by the letter "M" (Fig. 5). A unique marker present only in the LNCaP-LN3 cells was denoted by "m1" (Fig. 5, *inset*). The chromosome number ranged from 71 to 97 in all cell lines, with a peak of 88 chromosomes in the parental LNCaP, 89 chromosomes in LN1 and LN2, and 92 chromosomes in LN3 cells. All markers except M5 were present in two copies. M5 was present as a single copy. Tentative identification of the common markers are: M1, del(1p); M2, del(2)(p22); M3, del(6)(p21); M4, del(10)(q24); M5, der(12)(13)(p+); M6, t(1;15)p22;p23; M7, 5(6;16)(p21;q22). There were two copies of the X chromosome and one or two copies of the Y chromosome in the parental LNCaP line.

The LN1 cell line contained two populations with respect to marker M6. A large majority of the metaphases had two copies of M6, whereas a small proportion of cells showed one normal copy of M6, with the other copy modified as M6⁺ (additional chromosome element; Fig. 5, *inset*). In the LN2 line, all of the cells exhibited one copy of M6 marker and the modified homologue as M6⁺. In the LN3 line, these markers were present in every metaphase plate in addition to a unique marker, m1. Marker m1 (a modified chromosome 14) was present only in the LN3 line and absent in all other lines, including parental cells. The LN lines with the M6⁺ marker had only one copy of the intact X chromosome. This finding, together with G-banding characteristics, suggested that a portion of the X chromosome was translocated to M6. Subsequent analysis by fluorescence *in situ* hybridization using an X chromosome-specific probe confirmed this hypothesis (data not shown).

DISCUSSION

The isolation of populations of cells that differ from the parent neoplasm in metastatic capacity has supported the hypothesis that not all of the cells in a primary tumor can successfully disseminate (8–10, 13). In this procedure, metastatic cells are selected *in vivo*; tumor cells are implanted into orthotopic organs of nude mice and metastatic lesions are harvested. The cells that are recovered can be expanded in culture or used immediately to repeat the process. The cycle is repeated several times, and the behavior of the cycled cells is compared with that of the cells of the parent tumor. This procedure was originally used to isolate the B16-F10 line from the wild-type B16 melanoma (28). It has also been successfully used to produce tumor cell lines with increased metastatic capacity from tumors growing in nude mice (9, 13).

We undertook a series of orthotopic implantation experiments to select and isolate human prostate cancer cells with increased metastatic potential from two heterogeneous prostate cancer cell lines. The *in vivo* cycling of cells (prostate to lymph node to prostate) tends to enrich for cells with a growth advantage and also for cells capable of producing metastasis (9, 13).

Fig. 5 G-banding of LNCaP parental and variant lines; LNCaP marker chromosomes M1–M7. As shown in the *inset,* lymph node metastatic lines exhibited karyotypic abnormalities. For LNCaP-LN1, a subset of cells exhibit modified marker 6 (M6$^+$). For LNCaP-LN2, M6$^+$ was found in all cells. For LNCaP-LN3, M6$^+$ was found in all cells, along with a new marker, m1.

In different neoplasms, this enrichment is gradual and requires several cycles of selection (9, 13). The LNCaP tumor was not an exception. Growth in the prostate *per se* was not associated with nor correlated with an increased incidence of metastasis. For example, the LNCaP-Pro5 cells (selected four times for rapid growth in the prostate) produced large prostatic lesions (3.3 ± 1.7 g) but a low incidence of metastasis (2 of 17 mice). In contrast, the selection of cells that produced lymph node metastases, *i.e.,* LNCaP-LN3, resulted in smaller prostate tumors but enhanced metastatic propensity. The lymph node-selected cells (PC-3M-LN4) also produced a higher incidence of rapidly growing lymph node metastases but not enhanced growth in the prostate. These data suggest that the lymph node tumors contained a higher proportion of metastatic cells than the tumors in the prostate.

The growth of human tumors in nude mice is enhanced by specific factors in the organ environment (8, 10–12, 18). Indeed, the implantation of the LNCaP cells into the subcutis, spleen, and venous circulation failed to produce tumors. The highly metastatic LN3 cells produced experimental liver metastases. Whether this was associated with expression of specific growth factor receptors (6) is still unclear.

More than a century ago, Paget (29) proposed that the outcome of metastasis was dependent on the interaction of specific tumor cells with a favorable organ environment. The present data support this "seed and soil" hypothesis (29). The i.v. injection of PC-3M cells produced lung metastases, whereas that of LNCaP did not. The *in vivo*-selected PC-3M variants produced an even higher incidence of lung metastasis but not lymph node metastasis, suggesting an increased metastatic propensity to a specific site rather than all organ sites. Intracardiac implantation of PC-3M cells and variant lines was performed using a modification of methods published previously (30). Undoubtedly, transdiaphragmatic intracardiac injection (left ventricle) led to inadvertent tumor spill within the thoracic cavity, resulting in intrathoracic growth of tumor cells (lung, surface, and pleura). A high incidence of distant bone metastasis, however, was found with the highly metastatic PC-3M-LN4 cells. In this tumor system, then, the increased ability of cells to produce lymph node metastases was also correlated with growth in the bone.

Several reports have described the development of *in vivo* models to study prostate cancer bone metastasis (31–34). PC-3 cells selected *in vitro* for enhanced invasiveness produced bone lesions in SCID mice subsequent to i.v. injection (33), directly correlating with the level of type IV collagenase production (35). We have reported that human prostate cancer lines are heterogeneous in regard to invasive properties. PC-3M cells

invaded through laminin- and fibronectin-coated filters in the modified Boyden chamber assay, secreted type IV collagenase *in vitro*, and were metastatic to regional lymph nodes, whereas LNCaP cells produced no gelatinase activity, were noninvasive *in vitro*, and were nonmetastatic *in vivo* unless the mice were allowed to survive >120 days (15). Preliminary data (not shown) suggest that the PC-3M-LN4 line produces more gelatinase activity than either parental- or prostate-selected lines. However, additional factors that may modulate the incidence of bone metastases, including organ-specific adhesion to bone marrow endothelium by tumor cells and soluble growth factors produced by bone stromal cells (34, 36), await elucidation.

Chromosome analysis of LNCaP parental and *in vivo*-selected lines revealed clonal abnormalities in the lymph node-selected lines. These data, like those from the Dunning rat prostate carcinoma (37), confirm that metastases may have a clonal origin (38). LNCaP cells selected *in vivo* for androgen-independent growth were more metastatic and exhibited a clonal karyotype (32). In our study, the selection of highly metastatic LNCaP cells was also associated with decreased growth requirements for androgen. Whether this independence was due to alterations in androgen receptor structure, number, binding, or functional activity (39–41) is still to be determined.

In patients with metastatic prostate cancer, the inherent heterogeneity of metastases for androgen sensitivity is responsible for the failure of hormonal ablation to cure the disease. The experimental data confirm this inherent heterogeneity specifically for androgen requirements (prior to hormonal manipulation) and that clonal selection is a mechanism of androgen-independent prostate cancer progression (42).

PSA is a tissue-specific glycoprotein produced by human prostatic epithelial cells. It is central for prostate cancer detection, staging, and for monitoring the response to therapy (43). PSA expression in the tissues and serum is heterogeneous. The serum PSA levels in patients (and animal models of human prostate cancer) are dependent on prostate cancer volume, androgen status, and several other factors (43–46). In the present studies, serum PSA appeared, whereas LNCaP tumors were still nonpalpable, and the levels correlated with tumor weight. A novel observation was that per gram of prostate tissue, mice injected with LNCaP lymph node variants (LNCaP-LN3 and LNCaP-LN4) produced higher serum PSA levels. Even under *in vitro* conditions, LNCaP-LN3 cells produced up to 10-fold higher PSA than did parental cells. LNCaP cells selected for androgen insensitivity in nude mice also produced higher levels of PSA, in this case because of up-regulation of the PSA message (40). Since the promoter of the PSA gene is regulated by androgen (46), the mechanism by which androgen-independent LNCaP cells produced higher levels of PSA protein remains unclear. One possibility is that the LNCaP-LN3 cells produce more prostate-specific autocrine factor, a nonandrogen protein that increases the steady-state level of PSA transcript and protein (47). Recently, PSA was shown to function as a local protease within the seminal fluid and to be capable of cleaving insulin growth factor binding protein 3 to free insulin-like growth factor 1 (a mitogen for prostate cancer cells; Refs. 48 and 49). Whether higher PSA production by metastatic prostate cancer cells could provide a mechanism for enhanced growth needs further study.

In summary, our study shows that the implantation of human prostate cancer cells into the prostates of nude mice promotes their growth and metastasis. Selection of cells with increased metastatic potential is feasible in this animal model. Cells that are capable of giving rise to lymph node metastasis may not be capable of growing in the bones, demonstrating the validity of the seed and soil hypothesis. Moreover, the pattern of metastasis in nude mice of the selected PC-3M and LNCaP cells was very similar to that reported for the original patients (21, 23). The availability of human prostate cell lines with defined metastatic propensity in nude mice can allow for definition of the molecular determinants of human prostate cancer metastasis and for the design of relevant models to test novel therapeutic agents against prostate cancer metastasis (50).

ACKNOWLEDGMENTS

REFERENCES

1. Wingo, P. A., Tong, T., and Bolden, S. Cancer statistics. Cancer (Phila.), *45:* 8–30, 1995.

2. Catalona, W. J., Richie, J. P., Ahmann, F. R., Hudson, M. A., Scardino, P. T., Flanigan, R. C., de Kernion, J. B., Ratliff, T. L., Kavoussi, L., Dalkin, B. L., Waters, W. B., MacFarlane, M. T., and Southwick, P. C. Comparison of digital rectal examination and serum prostate specific antigen in the early detection of prostate cancer: results of a multicenter clinical trial of 6,630 men. J. Urol., *151:* 1283–1290, 1995.

3. Catalona, W. J., Smith, D. S., Ratliff, T. L., and Basler, J. W. Detection of organ confined prostate cancer is increased through prostate-specific antigen based screening. J. Am. Med. Assoc., *270:* 948–954, 1993.

4. Eisenberger, M. A. Chemotherapy and palliation in prostate cancer. *In:* D. A. S. Crawford (ed.), Cancer of the Prostate, pp., 369-396. New York: Marcel Dekker, 1993.

5. Fidler, I. J. Critical factors in the biology of human cancer metastasis. Cancer Res., *50:* 6130–6138, 1990.

6. Ware, J. L. Growth factors and their receptors as determinants in the proliferation and metastasis of human prostate cancer. Cancer Metastasis Rev., *12:* 287–301, 1993.

7. Fidler, I. J. The nude mouse model for studies of human cancer metastasis. *In:* V. Schirrmacher and R. Schwartz-Abliez (eds.), pp. 11-17. Berlin: Springer-Verlag, 1989.

8. Giavazzi, R., Campbell, D. E., Jessup, J. M., Cleary, K., and Fidler, I. J. Metastatic behavior of tumor cells isolated from primary and metastatic human colorectal carcinomas implanted into different sites in nude mice. Cancer Res., *46:* 1928-1933, 1986.

9. Morikawa, K., Walker, S. M., Jessup, J. M., and Fidler, I. J. *In vivo* selection of highly metastatic cells from surgical specimens of different primary human colon carcinomas implanted into nude mice. Cancer Res., *48:* 1943-1948, 1988.

10. Naito, S., von Eschenbach, A. C., Giavazzi, R., and Fidler, I. J. Growth and metastasis of tumor cells isolated from a renal cell carcinoma implanted into different organs of nude mice. Cancer Res., *46:* 4109-4115, 1986.

11. Price, J. E., Polyzos, A., Zhang, R. D., and Daniels, L. M. Tumorigenicity and metastasis of human breast carcinoma lines in nude mice. Cancer Res., *50:* 717-721, 1990.

12. Ahlering, T. E., Dubeau, L., and Jones, P. A. A new *in vivo* model to study invasion and metastasis of human bladder carcinoma. Cancer Res., *47:* 6660–6665, 1985.

13. Dinney, C. P. N., Fishbeck, R., Singh, R. K., Eve, B., Pathak, S., Brown, N., Xie, B., Fan, D., Bucana, C. D., Fidler, I. J., and Killion, J. J. Isolation and characterization of metastatic variants from human transitional cell carcinoma passaged by orthotopic implantation in athymic nude mice. J. Urol., *154:* 1532-1538, 1995.

14. McLemore, T. L., Liu, M. C., Blacker, P. C., Gregg, M., Alley, M. C., Abbott, B. J., Shoemaker, R. H., Bohlman, M. E., Litterst, C. C., Hubbard, W. C., Brennan, R. H., McMahon, J. B., Fine, D. L., Eggleston, J. C., Mayo, J. G., and Boyd, M. R. Novel intrapulmonary model for orthotopic propagation of human lung cancers in athymic nude mice. Cancer Res., *47:* 5132-5140, 1987.

15. Stephenson, R. A., Dinney, C. P. N., Gohji, K., Ordonez, N. G., Killion, J. J., and Fidler, I. J. Metastatic model for human prostate cancer using orthotopic implantation in nude mice. J. Natl. Cancer Inst., *84:* 951-957, 1992.

16. Pettaway, C. A., Stephenson, R. A., and Fidler, I. J. Development of orthotopic models of metastatic human prostate cancer. Cancer Bull. (Houst.), *45:* 424-429, 1993.

17. Fu, X., Herrera, H., and Hoffman, R. M. Orthotopic growth and metastasis of human prostate carcinoma in nude mice after transplantation of histologically intact tissue. Int. J. Cancer, *52:* 987-990, 1992.

18. Gleave, M., Hsieh, J. T., Gao, C., von Eschenbach, A. C., and Chung, L. W. K. Acceleration of human prostate cancer growth *in vivo* by factors produced by prostate and bone fibroblasts. Cancer Res., *51:* 3753-3761, 1991.

19. Pretlow, T. G., Delmoro, C. M., Dilley, G. G., Spadafora, C. G., and Pretlow, T. P. Transplantation of human prostatic carcinoma into nude mice in Matrigel. Cancer Res., *51:* 3814-3817, 1991.

20. Koslowski, J. M., Fidler, I. J., Campbell, D., Xu, Z., Kaighn, M. E., and Hart, I. R. Metastatic behavior of human tumor cell lines grown in the nude mouse. Cancer Res., *44:* 3522-3529, 1984.

21. Horoszewicz, J. S., Leong, S. S., Chu, T. M., Wajsman, Z. L., Friedman, M., Papsidero, L., Kim, U., Chai, L. S., Kakati, S., Arya, S. K., and Sandberg, A. A. The LNCaP cell line: a new model for studies on human prostatic carcinoma. *In:* D. S. Coffey (ed.), Models for Prostate Cancer, pp., 115-132. New York: Alan Liss, 1980.

22. Van Steenbrugge, G. J., Groen, M., Vandongen, J. W., Bolt, J., van der Korput, H., Trapman, J., Hasenson, M., and Horoszewicz, J. The human prostatic carcinoma cell line LNCaP and its derivatives. Urol. Res., *17:* 71-77, 1989.

23. Kaighn, M. E., Narayan, K. S., Ohnuki, Y., Lechner, J. F., and Jones, L. W. Establishment and characterization of a human prostate carcinoma cell line (PC-3). Invest. Urol., *17:* 16-23, 1979.

24. Giavazzi, R., Jessup, J. M., Campbell, D. E., Walker, S. M., and Fidler, I. J. Experimental nude mouse model of colorectal cancer liver metastases. J. Natl. Cancer Inst., *77:* 1303-1307, 1986.

25. Peehl, D. M., and Stamey, T. A. Serial propagation of adult human prostatic epithelial cells with cholera toxin. In Vitro, *20:* 981-986, 1984.

26. Pathak, S. Chromosome banding techniques. J. Reprod. Med., *17:* 25-28, 1976.

27. Zar, J. M. Biostatistical analysis, Ed. 2, pp. 162-186. Englewood Cliffs: Prentice Hall, 1984.

28. Fidler, I. J. Selection of successive tumor lines for metastasis. New Biol., *242:* 148-149, 1973.

29. Paget, S. The distribution of secondary growths in cancer of the breast. Lancet, *1:* 571-573, 1889.

30. Arguello, F., Baggs, R. B., and Frantz, C. N. A murine model of experimental metastasis to bone and bone marrow. Cancer Res., *48:* 6876-6881, 1988.

31. Shevrin, D. H., Kukreja, S. C., Ghosh, L., and Lad, T. E. Development of skeletal metastasis by human prostate cancer in athymic nude mice. Clin. Exp. Metastasis, *6:* 401-409, 1988.

32. Thalman, G. N., Anezinis, P. E., Chang, S. M., Zhau, H. E., Kim, E. E., Hopwood, V. L., Pathak, S., von Eschenbach, A. C., and Chung, L. W. K. Androgen-independent cancer progression and bone metastasis in the LNCaP model of human prostate cancer. Cancer Res., *54:* 2577-2581, 1994.

33. Wang, M., and Sterns, M. E. Isolation and characterization OFPC-3 human prostatic sublines which preferentially metastasize to select organs in SCID mice. Differentiation, *48:* 115-125, 1991.

34. Haq, M., Goltzman, D., Tremblay, G., and Brodt, P. Rat prostate adenocarcinoma cells disseminate to bone and adhere preferentially to bone marrow-derived endothelial cells. Cancer Res., *52:* 4613-4619, 1992.

35. Sterns, M. E., and Wang, M. Regulation of kinesin expression and type IV collagenase secretion in invasive human prostate PC-3 tumor sublines. Cancer Res., *51:* 5866-5875, 1991.

36. Chackal-Roy, M., Niemeyer, C., Moore, M., and Zetter, B. R. Stimulation of human prostatic carcinoma cell growth by factors present in human bone marrow. J. Clin. Invest., *84:* 43-50, 1989.

37. Isaacs, J. T., Wake, N., Coffey, D. S., and Sandberg, A. A. Genetic instability coupled to clonal selection as a mechanism for tumor progression in the Dunning R-3327 rat prostatic adenocarcinoma system. Cancer Res., *42:* 2353-2361, 1982.

38. Talmadge, J. E., Wolman, S. R., and Fidler, I. J. Evidence for the clonal origin of spontaneous metastases. Science (Washington DC), *217:* 361-363, 1982.

39. Newmark, J. R., Hardy, D. O., Tonb, D. C., Carter, B. S., Epstein, J. I., Isaacs, W. B., Brown, T. R., and Barrack, E. R. Androgen receptor gene mutations in human prostate cancer. Proc. Natl. Acad. Sci. USA, *89:* 6319-6323, 1992.

40. Wu, H. C., Hsieh, J. T., Gleave, M. E., Brown, N. M., Pathak, S., and Chung, L. W. K. Derivation of androgen-independent human LNCaP prostatic cancer cell sublines: role of bone stromal cells. Int. J. Cancer, *57:* 406-412, 1994.

41. Culig, Z., Hobisch, A., Cronaver, M. V., Cato, A. C. B., Hittmar, A., Radmayr, C., Eberle, J., Bartsch, G., and Klocker, H. Mutant androgen receptor detected in an advanced-stage prostatic carcinoma is activated by adrenal androgens and progesterone. Mol. Endocrinol., *7:* 1541-1550, 1993.

42. Isaacs, J. T., and Kyprianou, N. Development of androgen-independent tumor cells and their implication for the treatment of prostatic cancer. Urol. Res., *15:* 133-138, 1987.

43. Oesterling, J. E. Prostate-specific antigen: a critical assessment of the most useful tumor marker for adenocarcinoma of the prostate. J. Urol., *145:* 907-923, 1991.

44. Babaian, R. J., Troncoso, P., Steelhammer, L. C., Loreta-Trull, L. J., and Ramirez, E. I. Tumor volume and prostate-specific antigen: implications for early detection and defining a window of curability. J. Urol., *154:* 1808-1812, 1995.

45. Gleave, M. E., Hsieh, J. T., Wu, H. C., von Eschenbach, A. C., and Chung, L. W. K. Serum prostate-specific antigen levels in mice bearing human prostate LNCaP tumors are determined by tumor volume and endocrine and growth factors. Cancer Res., *52:* 1598-1605, 1992.

46. Young, C. Y. F., Montgomery, B. T., Andrews, P. E., Qiu, S., Bilhartz, D. L., and Tindall, D. J. Hormonal regulation of prostate-specific antigen messenger RNA in human prostatic adenocarcinoma cell line LNCaP. Cancer Res., *51:* 3748-3752, 1991.

47. Hsieh, J. T., Wu, H. C., Gleave, M. E., von Eschenbach, A. C., and Chung, L. W. K. Autocrine regulation of prostate-specific antigen gene expression in a human prostate cancer (LNCaP) cell line. Cancer Res., *53:* 2852-2857, 1993.

48. Cohen, P., Graves, H. C. B., Peehl, D. M., Kamarei, M., Giuice, L. C., and Rosenfeld, R. G. Prostate-specific antigen (PSA) is an insulin-like growth factor binding protein-3 protease found in seminal plasma. J. Clin. Endocrinol. Metab., *75:* 1046-1053, 1992.

49. Pietrzkowski, Z., Mulholland, G., Gomella, L., Jameson, B. A., Wernicke, D., and Baserga, R. Inhibition of prostatic cancer cell lines by peptide analogues of insulin-like growth factor 1. Cancer Res., *53:* 1102-1106, 1993.

50. Delworth, M. G., Nishioka, K., Pettaway, C. A., Gutman, M., Killion, J. J., von Eschenbach, A. C., and Fidler, I. J. Systemic administration of 4-amidinoindanon-1-(2'-amidino)-hydrazone, a new inhibitor of S-adenosylmethionine decarboxylase, produces cytostasis of human prostate cancer in athymic nude mice. Int. J. Oncol., *6:* 293-299, 1995.