

Viable Circulating Metastatic Cells Produced in Orthotopic but not Ectopic Prostate Cancer Models¹

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ABSTRACT

Elucidation of the mechanisms of hormone-independent metastatic prostate cancer remains a significant and highly relevant challenge. We report here that hormone-refractory human prostate carcinoma growing orthotopically efficiently deliver viable metastatic cells in the host circulation. This is in contrast to the ectopic tumors of the same lineage, which do not deliver live cells into the circulation. To investigate the malignant potential of viable circulating carcinoma cells, we developed a novel dual-color orthotopic coimplantation model of human prostate cancer metastasis in nude mice. This model is comprised of coinjection of an equivalent mixture of isolated and cultured circulating green fluorescent protein-expressing clones and parental red fluorescent protein-expressing human prostate carcinoma cells. In the dual-color model, the selected green fluorescent protein-labeled viable circulating cells have an increased metastatic propensity relative to the red fluorescent protein-labeled parental cells. The identification and isolation of highly malignant viable circulating human prostate carcinoma cells from orthotopic but not ectopic models will enable important new insights into the metastatic process including the role of the tumor microenvironment.

INTRODUCTION

The role of the host microenvironment in tumor metastasis is poorly understood. However, the orthotopic organ is known to have an enabling effect on metastasis. For example, many human and animal tumors transplanted into nude mice metastasize only if placed in the orthotopic organ (1–6). When human cancer cells derived from metastatic tumors are injected into ectopic sites in nude mice, most do not metastasize (7, 8). Several orthotopic models of human cancer metastasis have been developed (9–15). The orthotopic model of human cancer metastasis in nude mice has also enabled *in vivo* selection of highly and poorly metastatic variants (6, 13–15).

Experimental evidence indicates that enhancement of metastatic capability of human prostate cancer cells transplanted orthotopically is associated with differential expression of several metastasis-associated genes that have been implicated in critical processes of metastasis (16). These data suggest the presence of distinct clonal populations in the orthotopic.

In the current study, we have implanted highly metastatic human prostate cancer at orthotopic and ectopic sites to determine their ability to deliver viable malignant cells into the circulation, an important requirement for metastasis to many distant sites. Using orthotopic and subcutaneous models of hormone-independent human prostate cancer in nude mice, marked by GFP⁴ and RFP, we demonstrate that viable circulating clones are produced only in the orthotopic

setting. Upon fluorescence-based separation and culture of the viable circulating cancer cells, we demonstrate their increased metastatic potential. The current study shows the critical role played by the orthotopic microenvironment in enabling the primary tumor to produce viable circulating metastatic cells.

MATERIALS AND METHODS

Cell Culture. Cell lines used in this study have been described previously (12). Except where noted, cell lines were grown in RPMI 1640 supplemented with 10% fetal bovine serum and gentamicin (Life Technologies, Inc.) to 70–80% confluence as described previously (17–19).

Fluorescent Orthotopic Models of Human Prostate Cancer Metastasis in Nude Mice. We used a PC-3 fluorescent orthotopic model of human prostate cancer based on surgical orthotopic implantation of GFP- or RFP-expressing prostate cancer tissue in the prostate of nude mice (12). Similarly to the parental PC-3-derived tumors, GFP- and RFP-expressing fluorescent orthotopic xenografts exhibit highly aggressive metastatic behavior, in contrast to tumors derived from the same lineages but growing *s.c.* The orthotopic tumors recapitulate to a significant degree the clinical pattern of metastatic spread of advanced clinical prostate cancer (12). Five mice were used in each *in vivo* experimental setting. Preliminary experiments established that GFP-expressing and RFP-expressing parental cells are similarly efficient in tumorigenic and metastatic potential and ability to generate clones that survive in the circulation.

Isolation of Viable Circulating Metastatic Human Prostate Carcinoma Cells. Blood samples (0.5–1.0 ml) were obtained by cardiac puncture from individual tumor-bearing animals using heparin as anticoagulant and processed immediately. Sample preparation procedure for isolation of the circulating prostate carcinoma cells is similar to the experimental protocol adopted for preparation of peripheral blood or bone marrow cells. Erythrocyte-free nucleated cell fractions were obtained using the standard Ficoll gradient purification protocol or RBC lysis by ammonium chloride solution according to the protocol supplied by the manufacturer (Stemcell Technologies, Inc., Vancouver, Canada). The erythrocyte-free nucleated cell fraction was subjected to FACS analysis and fluorescent cell sorting to isolate GFP-expressing cells using previously described protocols (20). Purified circulating human prostate carcinoma cells were immediately expanded in culture for biological assays. A fluorescence microscopy analysis (see below) confirmed that the resulting cell cultures contained only fluorescent cells.

Fluorescence Microscopy. Images were collected on a DeltaVision Deconvolution Restoration Microscope system running SoftWoRx v.2.5 (Applied Precision Inc., Issaquah, WA). The system is mounted on an Olympus IX70 inverted microscope. With a $\times 20$ objective and an auxiliary magnification of $\times 1.5$, single-color fluorescent images and differential interference contrast images were captured. Images were ultimately processed in Adobe Photoshop v5.5.

Statistical Analysis. Statistical analysis of the number of circulating PC-3 cells and the weight of the primary tumors was made using a two-tailed paired Student's *t* test to evaluate the statistical significance of the difference between the means. Statistical analysis of the incidence of metastasis was performed using Fisher's exact test. The significance of the differences in the numbers of metastases was evaluated using the Mann-Whitney ranked sum test (21).

RESULTS AND DISCUSSION

Human Prostate Carcinomas Growing in the Mouse Prostate Produce Viable Circulating Clones. PC-3-GFP-orthotopic xenografts exhibited a highly aggressive metastatic phenotype in contrast

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⁴ The abbreviations used are: GFP, green fluorescent protein; RFP, red fluorescent protein; FACS, fluorescence-activated cell-sorting.

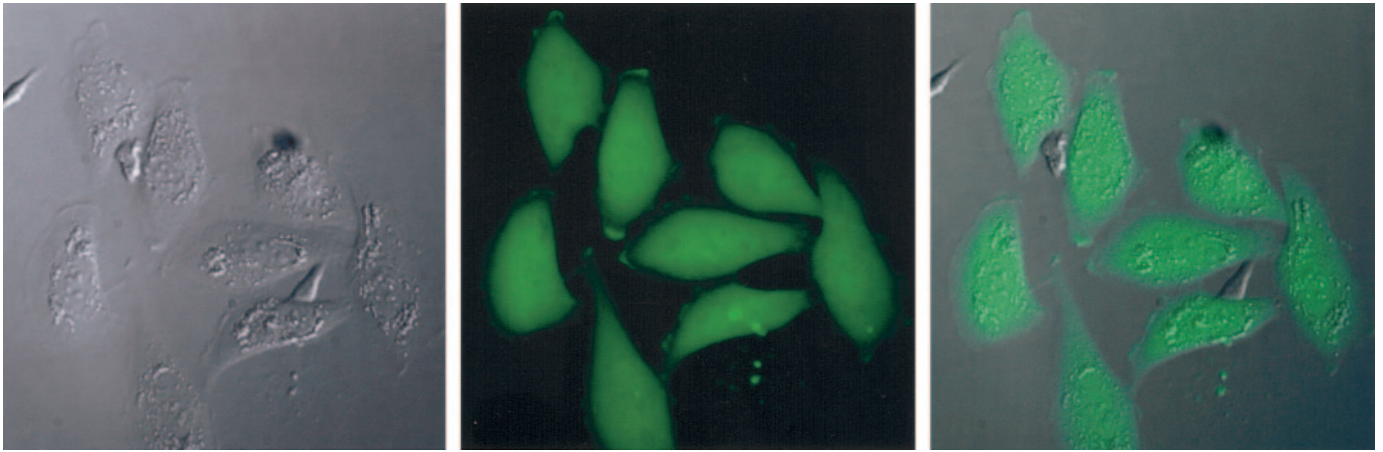
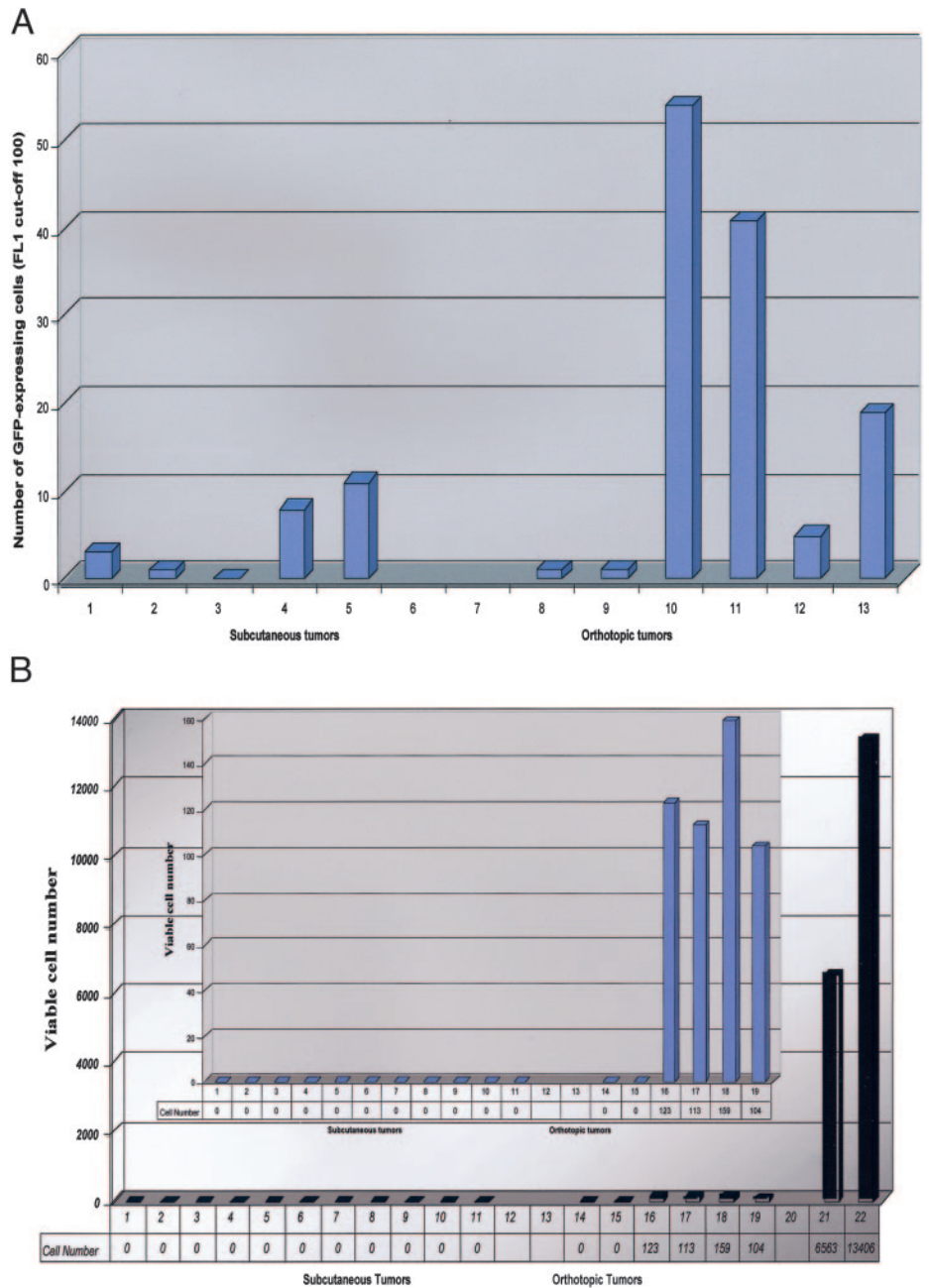


Fig. 1. Fluorescent microscopy of a culture of circulating PC-3-GFP cells growing *in vitro* after separation from the blood of nude mice bearing orthotopic xenografts by fluorescent cell sorting. A, differential interference contrast image; B, fluorescent image; C, merge.

Fig. 2. Identification of circulating human prostate carcinoma cells. A, nucleated cells were recovered from the blood of nude mice bearing orthotopic PC-3-GFP tumors or s.c. tumors and subjected to FACS analysis. Bar graphs represent a green fluorescent cell count (per 10,000 events) in blood of nude mice bearing s.c. (left) or orthotopic (right) PC-3-GFP tumors. B, the estimated number of viable human prostate carcinoma cells recovered from 1 ml of blood of tumor-bearing mice with s.c. (left) or orthotopic (right) tumors. Inset bar graph shows a subset of data at the scale 0–160 viable cells. As shown in the figure, we were unable to recover viable human prostate carcinoma cells from the blood of mice with s.c. tumors. In contrast, viable human prostate carcinoma cells were successfully recovered and expanded from the blood of 75% of mice with orthotopically implanted tumors in the prostate. Blood samples from both groups of mice were obtained simultaneously at day 30 after tumor inoculation by cardiac puncture and processed immediately according to a nucleated-cell isolation protocol. After isolation, cells were cultivated *in vitro* and monitored as described in “Materials and Methods.” In each experiment, viable cell number was counted using a trypan blue dye exclusion assay during the passage of the first subconfluent cultures of recovered blood-borne cells and extrapolated to time 0 of the experiment.



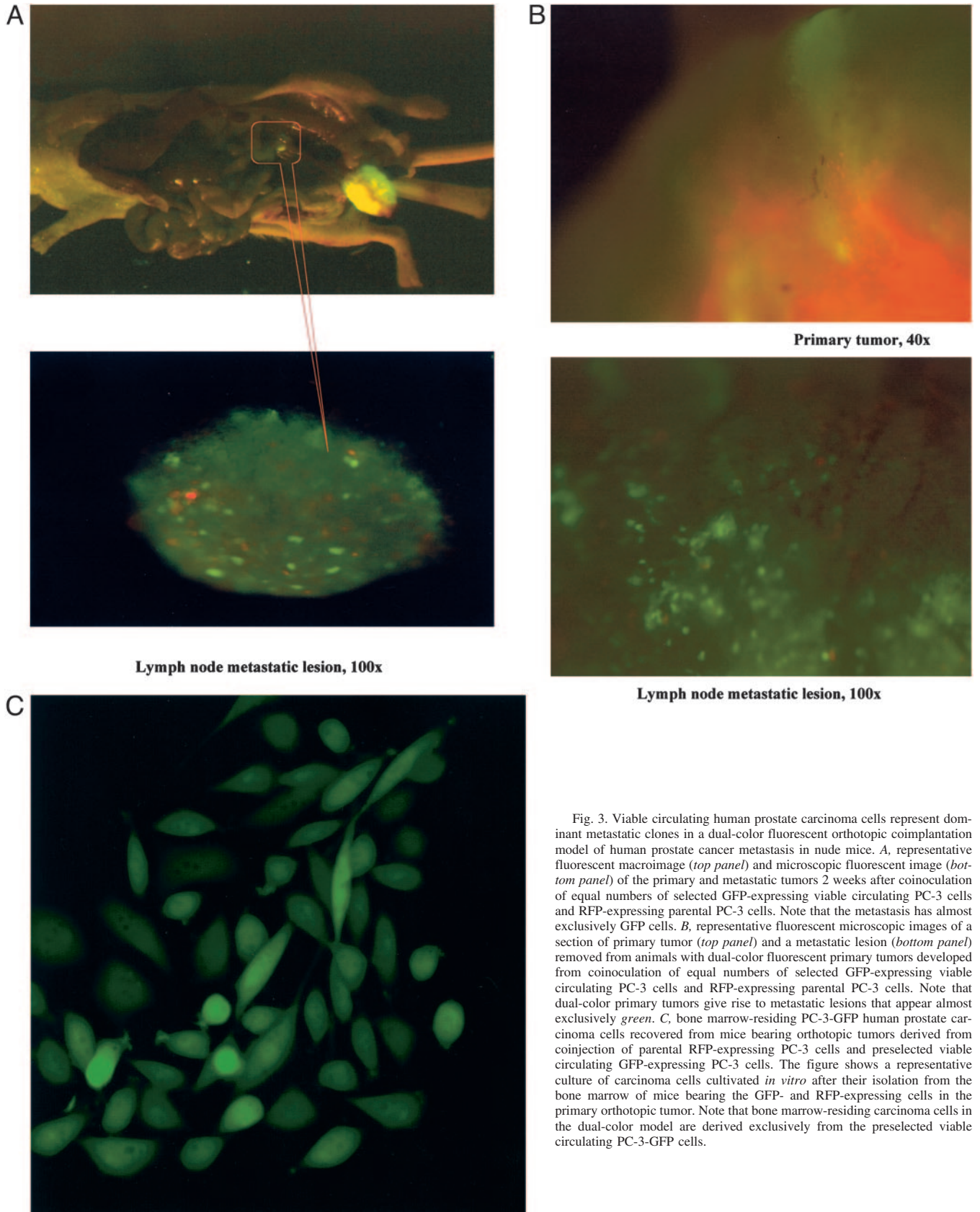


Fig. 3. Viable circulating human prostate carcinoma cells represent dominant metastatic clones in a dual-color fluorescent orthotopic coimplantation model of human prostate cancer metastasis in nude mice. *A*, representative fluorescent macroimage (*top panel*) and microscopic fluorescent image (*bottom panel*) of the primary and metastatic tumors 2 weeks after coinoculation of equal numbers of selected GFP-expressing viable circulating PC-3 cells and RFP-expressing parental PC-3 cells. Note that the metastasis has almost exclusively GFP cells. *B*, representative fluorescent microscopic images of a section of primary tumor (*top panel*) and a metastatic lesion (*bottom panel*) removed from animals with dual-color fluorescent primary tumors developed from coinoculation of equal numbers of selected GFP-expressing viable circulating PC-3 cells and RFP-expressing parental PC-3 cells. Note that dual-color primary tumors give rise to metastatic lesions that appear almost exclusively green. *C*, bone marrow-residing PC-3-GFP human prostate carcinoma cells recovered from mice bearing orthotopic tumors derived from coinjection of parental RFP-expressing PC-3 cells and preselected viable circulating GFP-expressing PC-3 cells. The figure shows a representative culture of carcinoma cells cultivated *in vitro* after their isolation from the bone marrow of mice bearing the GFP- and RFP-expressing cells in the primary orthotopic tumor. Note that bone marrow-residing carcinoma cells in the dual-color model are derived exclusively from the preselected viable circulating PC-3-GFP cells.

to tumors growing *s.c.* Extensive local invasive growth and distant metastases of orthotopic PC-3 tumors are readily detectable in 100% of the animals (data not shown). As reported previously (12), the PC-3-GFP orthotopic tumors recapitulate to a significant degree the clinical pattern of metastases of advanced clinical prostate cancer. In contrast, *s.c.* tumors derived from the same cells grew only locally and rarely produce metastasis.

To test whether increased invasiveness of orthotopic tumors is sufficient to provide a detectable circulatory load of carcinoma cells, we isolated rare circulating human prostate carcinoma cells in nude mice bearing orthotopic or *s.c.* human PC-3-GFP tumors (Fig. 1). FACS analysis of nucleated cells recovered from the blood of tumor-bearing animals showed that mice with orthotopic PC-3-GFP tumors have a higher circulatory load of cells with high green fluorescence (Fig. 2A). These PC-3-GFP cells are capable of surviving circulatory stress because viable cultures of these cells can be grown and expanded *in vitro* (Figs. 1 and 2B). We successfully recovered and expanded in culture viable blood-borne carcinoma cells from 75% of mice with orthotopic PC-3-GFP tumors (Fig. 2B). In contrast, we failed to recover viable circulating PC-3-GFP cells from mice bearing *s.c.* tumors in multiple independent experiments (Fig. 2B).

Viable Circulating Human Prostate Carcinoma Cells Are More Malignant than Parental Cells. To determine whether the viable circulating human prostate carcinoma cells are a highly malignant subpopulation, we implanted them orthotopically and monitored them for tumor growth and metastasis. Development of highly aggressive metastatic prostate tumors was documented in 100% of animals, with distant metastases appearing as early as 2–3 weeks after orthotopic inoculation of cultured cancer cells isolated from circulation (data not shown).

We then compared the metastatic propensity of GFP-expressing cells isolated from the circulation of mice with orthotopic tumors and subsequently co-implanted orthotopically with an equivalent number of the parental RFP-expressing population of cancer cells. The metastatic lesions in all five mice bearing dual-color orthotopic xenografts appeared to contain almost exclusively GFP-expressing human prostate carcinoma cells (Fig. 3, A and B) when analyzed between 2 and 4 weeks after coimplantation. FACS analysis showed that, on average, PC-3-GFP cells represent 85.9% ($n = 4$; range, 83.2–87.8; SD = 1.99) of the circulating population of human prostate carcinoma cells recovered from mice bearing dual-color orthotopic tumors. Experiments with the single-color fluorescent orthotopic models showed that parental GFP- and RFP-expressing human prostate carcinoma cells are similarly efficient in tumorigenic and metastatic potential *in vivo* and ability to generate viable circulating cells. Furthermore, the weights of mouse prostates bearing primary tumors derived from either single-color or dual-color models were similar (data not shown) and statistically indistinguishable. The pattern of anatomical distribution of distant metastasis in animals bearing orthotopic fluorescent xenografts was similar to that reported previously (12). The incidence of macroscopic metastasis was 100% in all experimental groups bearing orthotopic tumors. The number of macroscopic metastatic lesions did not differ significantly between various experimental groups (data not shown).

Bone marrow-residing GFP- or RFP-expressing human prostate carcinoma cells were successfully recovered and expanded in culture from all animals bearing single-color GFP- or RFP-expressing orthotopic xenografts, which produce viable prostate carcinoma cells in the blood. Bone marrow-residing human prostate carcinoma cells isolated from mice bearing dual-color orthotopic xenografts were represented exclusively by preselected viable circulating PC-3-GFP cells, but not parental PC-3-RFP cells (Fig. 3C).

The data from the dual-color coimplantation experiments are con-

sistent with the notion that viable circulating human prostate carcinoma cells derived from primary orthotopic tumors have an increased metastatic propensity.

In conclusion, we demonstrated that hormone-refractory human prostate carcinomas growing orthotopically produce viable circulating metastatic cells. Using a dual-color tumor model *in vivo*, we showed that viable circulating human prostate carcinoma cells have an increased metastatic propensity and, therefore, can be defined as a precursor of metastatic lesions.

This study shows the critical role played by the orthotopic microenvironment in enabling the primary tumor to produce viable circulating metastatic cells. These experiments suggest that the viable circulating tumor cells released from the orthotopic site are the metastatic precursors. The present study also demonstrates that the viable circulating tumor cells produced from the orthotopic tumors have increased malignant potential compared with the parental cells in the primary orthotopic tumor. This is clearly shown in experiments where the selected circulating tumor cells and parental tumor cells are co-implanted, resulting in metastases that are almost exclusively from the selected circulating tumor cells at the time of analysis. These findings explain why orthotopic tumors producing viable circulating carcinoma cells frequently metastasize, and *s.c.* tumors very infrequently metastasize. Future studies will investigate genetic and other mechanisms that underlie the increased malignancy of the circulating cells as well as their mechanism of production from orthotopic tumors in contrast to *s.c.* tumors.

The identification and isolation of highly malignant circulating human prostate carcinoma cells from orthotopic but not ectopic models will enable important new insights into the metastatic process including the role of the tumor microenvironment.

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