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Report

Dual-Color-Coded Imaging of Viable Circulating Prostate Carcinoma Cells Reveals Genetic Exchange Between Tumor Cells In Vivo, Contributing to Highly Metastatic Phenotypes

Gennadi V. Glinsky[1,†,*]
Anna B. Glinskii[1]
Olga Berezovskaya[1]
Brian A. Smith[2]
Ping Jiang[3]
Xiao-Ming Li[3]
Meng Yang[3]
Robert M. Hoffman[3,†]

[1]Ordway Research Institute Cancer Center; Center for Medical Science; Albany, New York USA

[2]Sidney Kimmel Cancer Center; San Diego, California USA

[3]AntiCancer, Inc.; San Diego, California USA

[†]These authors share senior authorship.

*Correspondence to: Gennadi V. Glinsky; Translational and Functional Genomics Laboratory; Ordway Cancer Center; Ordway Research Institute, Inc., and Division of Urology; Albany Medical College; Center for Medical Science; 150 New Scotland Avenue; Albany, New York 12208 USA; Tel.: 518.641.6975; Fax: 518.641.6303; Email: gglinsky@ordwayresearch.org

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KEY WORDS

xenograft models, GFP, RFP, prostate cancer, orthotopic-implantation, nude mice, circulating cancer cells, lateral gene transfer

ABBREVIATIONS

FBS	fetal bovine serum
s.c.	sub-cutaneous
SOI	surgical orthotopic implantation
GFP	green fluorescent protein
RFP	red fluorescent protein
DIC	differential interference contrast

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ABSTRACT

Color-coded imaging analysis revealed yellow fluorescent metastasis precursor cells that were readily recognized in the blood of tumor-bearing mice after mixtures of red fluorescent protein (RFP)- and green fluorescent protein (GFP)-expressing PC-3 human prostate carcinoma cells were co-implanted in the nude mouse prostate. The yellow fluorescent cells were purified from the blood of nude mice to 99% homogeneity by FACS, expanded in culture, and reimplanted in the prostate of nude mice. The yellow fluorescent phenotype was heritable and stably maintained by tumor cells for many generations in vitro and in vivo. In the animals implanted with the yellow-fluorescing cells, 100% developed aggressive metastatic cancer. Lung metastases were demonstrated in 100% of the animals as early as four weeks after injection of the yellow-fluorescing cells in the mouse prostate. In contrast, when the GFP- and RFP-expressing parental cells were inoculated into the mouse prostate separately, none of the animals developed lung metastasis. All animals had almost exclusively yellow fluorescent cells in the blood and bone marrow. These results are consistent with the idea that spontaneous genetic exchange between tumor cells in vivo contributes to genomic instability and creation of highly metastatic cells.

INTRODUCTION

Elucidation of cellular, molecular, and genetic mechanisms of metastatic human prostate cancer remains one of the most significant and challenging problems. Development and characterization of reliable and clinically relevant in vivo models of human prostate cancer metastasis is an essential component of continuous progress toward understanding of pathobiology of prostate cancer metastasis. Discovery of the metastasis-promoting effect of orthotopic implantation of human cancer cells had a major impact on development of several orthotopic models of human cancer metastasis in nude mice.[1-7] One of the most significant advances enabled by implementation of orthotopic models of human cancer metastasis was the in vivo selection from parental cells of highly and poorly metastatic cell variants.[8-10]

Using fluorescent orthotopic models of human prostate cancer metastasis in nude mice,[7] we demonstrated the critical role played by the orthotopic microenvironment in enabling the primary tumors to produce viable circulating metastatic cells.[11,12] Upon fluorescence-based isolation and culture of the viable circulating human prostate carcinoma cells, we demonstrated their high metastatic potential after reimplantation into the mouse prostate.[11,12]

In the current study, we employed the dual-color fluorescent model of human prostate cancer metastasis in nude mice to characterize distinct populations of viable circulating metastatic cells. In the dual-color model, when the selected GFP-labeled viable circulating carcinoma cells were coinjected with RFP-labeled parental cells into the mouse prostate, yellow fluorescent carcinoma cells were readily detectable in the blood of tumor-bearing mice. Upon fluorescence-based separation and culture of viable yellow fluorescent carcinoma cells, we demonstrated that the yellow fluorescence phenotype is stable and heritable for many generations in vitro and in vivo. After reimplantation of the yellow fluorescent prostate carcinoma cells into the mouse prostate, 100% of the animals developed highly metastatic tumors which maintained the yellow fluorescence. These results are consistent with the idea that the orthotopic microenvironment enables spontaneous genetic exchange between tumor cells in vivo contributing to genomic instability of tumors and creation of viable circulating highly metastatic cells.

MATERIALS AND METHODS

Cell culture. Cell lines used in this study were previously described.[7,11,12] Except where noted, cell lines were grown in RPMI1640 supplemented with 10% FBS and gentamycin (Gibco BRL) to 70-80% confluence and continuously maintained in fresh complete media, supplemented with 10% FBS.[11-15]

Fluorescent orthotopic models of human prostate cancer metastasis in nude nice. We utilized a PC-3 fluorescent orthotopic model of human prostate cancer based on surgical orthotopic implantation (SOI) of green fluorescent protein (GFP)- or red fluorescent protein (RFP)-expressing prostate cancer tissue in the prostate of nude mice.[7,11,12] Similar 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 subcutaneously. The orthotopic tumors recapitulate to a significant degree the clinical pattern of metastatic spread of advanced clinical prostate cancer.[7] Previous experiments established that GFP-expressing and RFP-expressing parental cells are equipotent in tumorigenic and metastatic potential in vivo and ability to generate viable circulating cells.[7,11]

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 immediately processed as described.[11,12] Briefly, erythrocyte-free nucleated cell fractions were obtained using the standard ficol gradient purification protocol or red blood cells 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, RFP-expressing, or double positive yellow cells using previously described protocols.[16] Purified circulating human prostate carcinoma cells were immediately expanded in culture for biological assays and microarray analysis. Fluorescent microscopy analysis (see below) confirmed that resulting cell cultures contained at least 98% fluorescent cells.

Anoikis assay. Cells were harvested by 5-min digestion with 0.25% trypsin/0.02% EDTA (Irvine Scientific, Santa Ana, CA, USA), washed and resuspended in serum free medium. Cells at a concentration of 1.7×10^5 cells/ well in 1 ml of serum-free medium were plated in 24-well ultra-low attachment polystyrene plates (Corning Inc., Corning, NY, USA) and incubated at 37°C and 5% CO_2 overnight. Viability of cell cultures subjected to anoikis assays was >95% in the trypan-blue dye-exclusion test. Each measurement was carried out in quadruplicate and repeated at least twice to ensure reproducibility.

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 I x 70 inverted microscope. Single-color or double-color fluorescent images and DIC images were captured with a 20 x objective and an auxiliary magnification of 1.5 x. Images were ultimately processed in Adobe Photoshop v5.5.

Confirmation of the presence and expression of the GFP and RFP genes. The presence of the RFP and GFP genes and corresponding mRNAs was confirmed by direct sequencing of the respective PCR products derived from genomic DNA and cDNA isolated from the yellow flourescent PC cells (>99% purity by FACS analysis). Genomic DNA and RNA were isolated from cultured cells using DNeasy and Rneasy kits (Qiagen). First strand cDNA synthesis was performed using SuperScriptII RT (Invitrogen) and oligo(dT). 30 ng of cDNA and 200 ng of genomic DNA were utilized as templates. PCR product derived from the GFP gene was amplified and isolated using a two-step nested PCR protocol with the following sets of primers: Step 1 (product size 197 bp): TCGTGACCACCCTGACCTAC (F1), CAGCTCGATGCGGTTCAC (R1); Step 2 (product size 156 bp): TCGTGACCACCCTGACCTAC (F2), CCTCGGCGCGGGTCTTGTAG (R2). After the first step the PCR product was purified using a QIAquick PCR purification kit and utilized as a template for nested PCR. The PCR protocol for GFP was performed using Jump Start Ready Mix REDTag DNA polymerase (Sigma) following thermal cycling parameters: 94°C (5 min); 37 cycles: 94°C (30 sec); 55°C (30 sec); 72°C (30 sec); hold: 72°C (7 min). PCR product (product size 348 bp) derived from the RFP gene was amplified and isolated using the following set of primers: TACGGCTCCAAGGTGTACGT (F); CATGTAGATGGACTTGAACTCCAC (R). The PCR protocol for RFP was performed using HotStarTag DNA polymerase (Qiagen) and the following thermal cycling parameters: 95°C (15 min); 37 cycles: 94°C (30 sec); 60°C (30 sec); 72°C (30 sec); hold: 72°C (7 min). Final PCR products were isolated using the QIAquick PCR purification kit (Qiagen) and subjected to sequence analysis. Control genomic DNA and RNA were isolated from nontransfected parental PC-3 cells and corresponding single-tagged PC-3 cell lines. Sequence alignments to the published mRNA sequences of GFP and RFP genes were performed with the Sequencher software (Gene Code Corp., Ann Arbor, MI) using the Clean Data Alignment method with two consecutive mismatches allowed, 90% minimum match percentage, and 90% minimum overlap.

Statistical analysis. Statistical analysis of the number of circulating PC 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 metastasis was evaluated using the Mann-Whitney ranked sum test.[17]

RESULTS AND DISCUSSION

Yellow fluorescent cells were readily recognized in blood of mice with orthotopic tumors after mixtures of red fluorescent protein (RFP)- and green fluorescent protein (GFP)-expressing PC-3 human prostate carcinoma cells were implanted in the nude mouse prostate and they metastasized (Fig. 1).

Viable circulating cancer cells were isolated 2–3 weeks after cell implantation. Approximately 15% of the fluorescent cells isolated from blood were yellow with the remaining divided between the RFP and GFP expressors. The yellow-fluorescing cells were isolated to 99% purity by fluorescent sorting (Fig. 1) and cultured in vitro. The pure population was shown to contain both the RFP and GFP genes and RFP and GFP mRNA by direct sequencing of the corresponding PCR-amplified products. These data thus demonstrate that the yellow-fluorescing cells are dual-positive with respect to the parental cells, since they contain both the RFP and GFP genes and express corresponding mRNAs and gene products.

The yellow-fluorescing cells were cultured in vitro and then reimplanted in the nude mouse prostate and compared for metastatic potential with the parental RFP- and GFP-expressing PC-3 cells. In the animals implanted with the yellow-fluorescing cells, 100% developed aggressive metastatic cancer. Lung metastases were demonstrated in 100% of the animals as early as four weeks after injection of the yellow-fluorescing cells in the mouse prostate (Table 1). All animals had exclusively yellow fluorescent cells in the blood and bone marrow (Fig. 3). In contrast, when the GFP- and RFP-expressing parental cells were inoculated into the mouse prostate separately, none of the animals developed lung metastasis (Table 1). The GFP- and RFP-expressing parental cells when injected orthotopically were less efficient in producing viable circulating carcinoma cells compared to the PC-3-GFP-13 and PC-3-RFP-44 variants selected from the circulation (Table 1). The GFP- and RFP-expressing parental cells when injected orthotopically as a mixture did not produce a detectable level of yellow cells (Table 1). In contrast, when a mixture containing viable circulating metastatic carcinoma cells and non-selected parental cells were coinjected into mouse prostate, we consistently detected and isolated yellow cells from blood of tumor-bearing mice (Fig. 1 and Table 1).

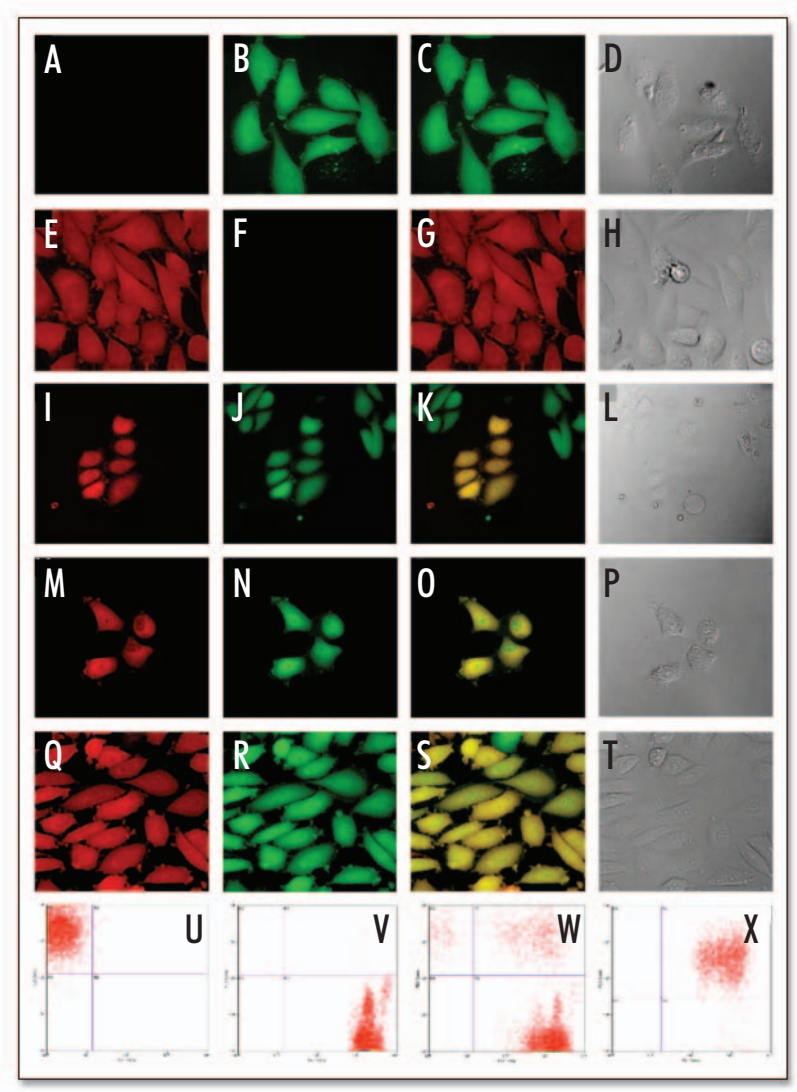

Figure 1. Visualization, isolation, and characterization of the viable circulating yellow-fluorescing human prostate carcinoma cells, identified in blood of mice co-implanted orthotopically with preselected GFP-expressing viable circulating carcinoma cells and nonselected RFP-expressing parental carcinoma cells, as described earlier (11,12). (A–D) Fluorescence microscopy of a culture of blood-borne PC-3-GFP-13 cells cultured in vitro, after isolation from the blood of nude mice bearing orthotopic xenografts by fluorescent-cell sorting. (E–H) Fluorescence microscopy of a culture of parental PC-3-RFP cells. (I–L) Fluorescence microscopy of a representative culture of viable circulating human prostate carcinoma cells isolated from blood of mice co-implanted orthotopically with preselected GFP-expressing viable circulating carcinoma cells and nonselected RFP-expressing parental carcinoma cells. (M–P) Fluorescence microscopy of a representative culture of yellow-fluorescing human prostate carcinoma cells after isolation from the blood of nude mice co-implanted orthotopically with preselected GFP-expressing viable circulating carcinoma cells and nonselected RFP-expressing parental carcinoma cells. A representative early passage culture is shown. (Q–T) Fluorescence microscopy of a representative culture of yellow-fluorescing human prostate carcinoma cells continuously maintained and expanded in vitro after isolation from the blood of nude mice co-implanted orthotopically with preselected GFP-expressing viable circulating carcinoma cells and nonselected RFP-expressing parental carcinoma cells. A representative late passage sub- confluent culture is shown. (U–X) Representative FACS analyses of the cultured cell variants exhibiting distinct fluorescent phenotypes: parental PC-3-RFP (U); selected viable circulating PC-3-GFP-13 cells (V); viable circulating prostate carcinoma cells isolated from the blood of nude mice co-implanted orthotopically with preselected GFP-expressing viable circulating carcinoma cells and nonselected RFP-expressing parental carcinoma cells (W); yellow-fluorescing viable circulating prostate carcinoma cells isolated by fluorescent cell sorting from the blood of nude mice co-implanted orthotopically with preselected GFP-expressing viable circulating carcinoma cells and nonselected RFP-expressing parental carcinoma cells (X). A, E, I, M and Q, red fluorescence channel; B, F, J, N and R, green fluorescence channel; C, G, K, O and S, merged fluorescence images; D, H, L, P and T, DIC images.

The yellow-fluorescing cells were of similar size and morphology as the parental RFP- and GFP-expressing PC-3 cells (Fig. 1). The yellow fluorescent cells are predominantly mononuclear and contain a similar DNA content as the RFP and GFP parental cell types. These data suggest that a limited amount of gene transfer between the parental cells gave rise to the yellow fluorescent cells with at least the GFP or RFP gene transferred.

Despite the similarity between cell size, the number of polynuclear cells, and DNA content, the yellow-fluorescing cells were significantly more metastatic than the parental cell types, suggesting that the lateral gene transfer might contribute to their increased metastatic potential.

Several observations support the notion that the yellow-fluorescing cancer cells have a high level of genomic and phenotypic plasticity. Metastatic lesions in the lung of mice with orthotopic yellow-fluorescing primary tumors derived after inoculation of the yellow-fluorescing prostate cancer cells often have a multi-color appearance (Fig. 2B). Sub-cutaneous implantation of the yellow-fluorescing cancer cells produces rapidly growing nonmetastatic multi-color tumors (data not shown). These data provide striking visual demonstration of continuous phenotypic changes in tumors derived from the yellow-fluorescing cancer cells during adaptation to growth conditions in different micro-environments.

Survival in lymph or blood is an essential prerequisite for metastasis of carcinoma cells and formation of distant metastatic lesions. Recently, we demonstrated that the metastatic human prostate carcinoma cells selected for survival in the circulation have increased resistance to anoikis, which is apoptosis induced by cell detachment.[12] We therefore tested whether prostate carcinoma cells while adopting to survive in the nonadherent state would yield more dual-positive yellow fluorescent cells. We found that coculture of GFP- and RFP-expressing human prostate carcinoma cells in a non-adherent state consistently produce a 5–10 fold higher yield of dual-positive yellow fluorescent cells compared to adherent culture (Fig. 4). These data suggest that highly metastatic yellow fluorescent cells might be generated during metastatic dissemination while adopting to survive in a non-adherent state and/or hostile microenvironment of distant organs. Consistent with this hypothesis, we found that lymph node metastases of human prostate carcinoma are greatly enriched for dual-positive yellow fluorescent cells compared to the parental orthotopic primary prostate tumors (Fig. 5).

The identification of the yellow-flourescence cells was made possible by the use of the different fluorescent protein markers in the parental cells. Thus, after mixed injection in the mouse prostate, the yellow fluorescent cells that metastasized to the circulation were

Table 1 **Malignant potential of GFP- and RFP-labeled and yellow fluorescent PC-3 human prostate carcinoma cell variants**

Cell Line	Method of Orthotopic Implantation	Primary Tumors	Lymph Node Metastasis	Lung Metastasis	Viable Cells in Blood	Yellow Cells in Blood	Viable Cells in BM Metastasis
PC-3-GFP	SOI	5/5	5/5	0/5	5/5	0/5	5/5
PC-3-GFP	SOI	6/6	6/6	0/6	4/6	0/6	ND
PC-3-GFP-13	Cell Injection	3/3	3/3	0/3	3/3	0/5	1/3
PC-3-RFP	Cell Injection	4/4	3/4	0/4	3/4	0/5	1/4
PC-3-RFP-44	Cell Injection	5/5	5/5	0/5	2/5	0/5	ND
PC-3-GFP-13	Cell Injection	5/5	5/5	0/5	2/5	0/5	ND
PC-3-GFP-13 + PC-3-RFP	Cell Injection	4/4	4/4	ND	2/4	2/2	1/4
PC-3-Yellow	Cell Injection	4/4	4/4	4/4	4/4	4/4	3/4
PC-3-GFP + PC-3-RFP	Cell Injection	5/5	5/5	0/5	1/5	0/1	1/5
PC-3-RFP-44 + PC-3-GFP	Cell Injection	5/5	5/5	4/5	4/5	4/4	4/5
PC-3-GFP	S.C. cell injection	7/7	0/7	0/7	0/7	NA	ND

The presence of metastatic lesions was confirmed by microscopic examination. The number of mice with viable cancer cells in the blood and bone marrow represents the number of successful attempts / total number of attempts to isolate and establish in culture viable fluorescent carcinoma cells. ND, not done; SOI, surgical orthotopic implantation. PC-3-GFP-13; PC-3-RFP-44; PC-3-Yellow are viable circulating variants of the PC-3 human prostate carcinoma cell line recovered from blood of nude mice bearing orthotopic xenografts derived from corresponding parental PC-3-GFP or PC-3-RFP cells.[11,12] PC-3-Yellow cells were recovered from blood of mice with dual-color orthotopic xenografts.

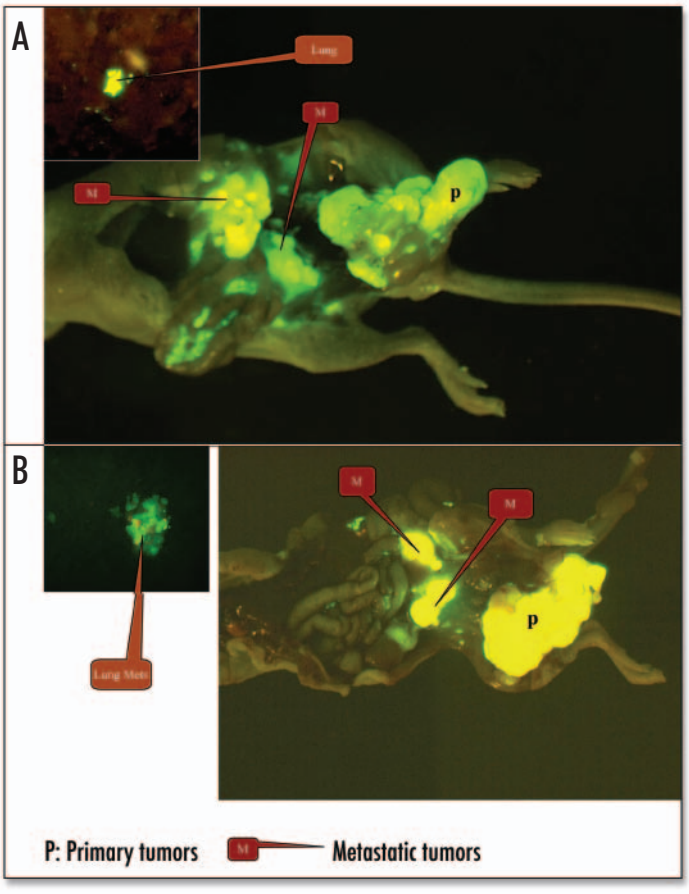

Figure 2. Yellow-fluorescing viable circulating human prostate carcinoma cells manifested a highly metastatic phenotype in an orthotopic model of human prostate cancer metastasis in nude mice. Representative macroscopic and microscopic (A and B; inserts) fluorescence images of the primary and metastatic tumors four weeks after orthotopic inoculation of cancer cells are shown. (A) Preselected viable circulating PC-3-RFP or PC-3-GFP variants and nonselected parental cells were injected either separately (single-color orthotopic model) or simultaneously at a 1:1 ratio (dual-color orthotopic model is shown) in the prostate of nude mice (five animals per group). The animals were monitored for tumor growth and metastasis. The images were taken four weeks after tumor cell inoculation. (B) Yellow fluorescent PC-3 human prostate carcinoma cells were isolated from the blood of prostate tumor-bearing mice with dual-color orthotopic xenografts, expanded in culture, and reimplanted in the mouse prostate. Note that after orthotopic implantation of yellow fluorescent PC-3 cells 100% of the animals developed metastatic prostate cancer, including lung metastases (insert). The images were taken four weeks after tumor cell inoculation. All mice developed rapidly growing primary tumors and distant metastasis. Mice with yellow-fluorescing cells developed lung metastases at the time of observation (B, insert and Table 1).

readily identified and distinguished from the parental RFP- and GFP-expressing cells, which also metastasized into the circulation. The data suggest that lateral gene transfer between cancer cells may contribute to the genetic heterogeneity observed in cancer cells and to their degree of malignancy. Indeed, the cells capable of genetic exchange by lateral gene transfer might be the cells in the population most variable genetically and thereby most capable of producing high metastatic variants.

Lateral transfer of DNA between bacteria and mammalian cells has been documented in vitro by conjugation.[18] Ingestion of DNA by pregnant mice results in subsequent expression of the genes encoded in the DNA by leucocytes, spleen, liver, and other organs of the fetus.[19] Engulfment of bacteria by mammalian cells can result in expression of the bacterial DNA in the mammalian cells.[20] However, to our knowledge spontaneous lateral gene transfer between cancer cells associated with the metastatic process in vivo has not been reported.

One of the properties of the red fluorescent protein (RFP; DsRed) is the ability to acquire an unstable transitional "green" fluorescence during protein maturation process.[21-24] A genetically engineered mutant form of DsRed protein was described that changes its fluorescence from green to red over time in living cells.[25] Under certain experimental conditions the "greening" effect occurs

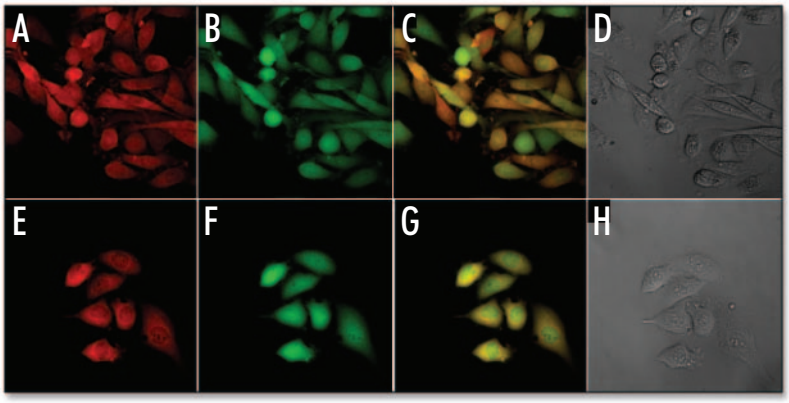

Figure 3. Fluorescence microscopy of representative cultures of bone marrow-residing (A–D) and viable circulating (E–H) human prostate carcinoma cells. The cells were cultured in vitro after isolation from bone marrow or blood of nude mice implanted orthotopically with yellow fluorescent PC-3 cells preselected from blood of mice bearing dual-color orthotopic xenografts. Note that both bone marrow-residing and viable circulating human prostate carcinoma cells are exclusively yellow-fluorescing cells (C, G). (A and E), red fluorescent channel; (B and F), green fluorescent channel; (C, G), merged fluorescence images; (D and H), DIC images.

in live mammalian cells and resultant color changes persists for ~30 h without affecting cell viability.[26] The fluorescence-transition effect was not detectable by either fluorescence microscopy or FACS analysis in the multiple independent in vivo and in vitro control experiments carried out with several stably maintained RFP-expressing PC-3 variants (Table 1). These experiments included viable circulating PC-3-RFP-44 cells that were continuously maintained in vitro for several months, reimplanted into the mouse prostate and recovered again from the circulation of tumor-bearing mice as stable monochromatic RFP- expressing cells with exclusively red fluorescence. Based on these observations, we concluded that the "greening" effect is an unlikely source of artifact contributing to the appearance of the yellow fluorescence phenotype.

In the present study, the yellow fluorescent highly metastatic cancer cells were isolated from the circulation of mice bearing mixtures of RFP- and GFP-expressing orthotopic human prostate carcinoma xenografts. We have recently demonstrated that with the PC-3 prostate carcinoma cell line, tumor cells implanted orthotopically into the prostate produced viable circulating cells with increased metastatic potential.[11,12] In contrast, tumor cells implanted subcutaneously did not. It is possible that the tumor cells underwent lateral gene exchange in the prostate and lymph nodes during the process of metastasis. This phenomenon might contribute to genomic instability of tumor cells and may play an important role in the creation of highly metastatic anoikis-resistant cancer cells that survive in blood and serve as precursors of distant metastatic lesions.[12] To our knowledge, this is the first report documenting spontaneous lateral gene transfer between cancer cells associated with the metastatic process in vivo.

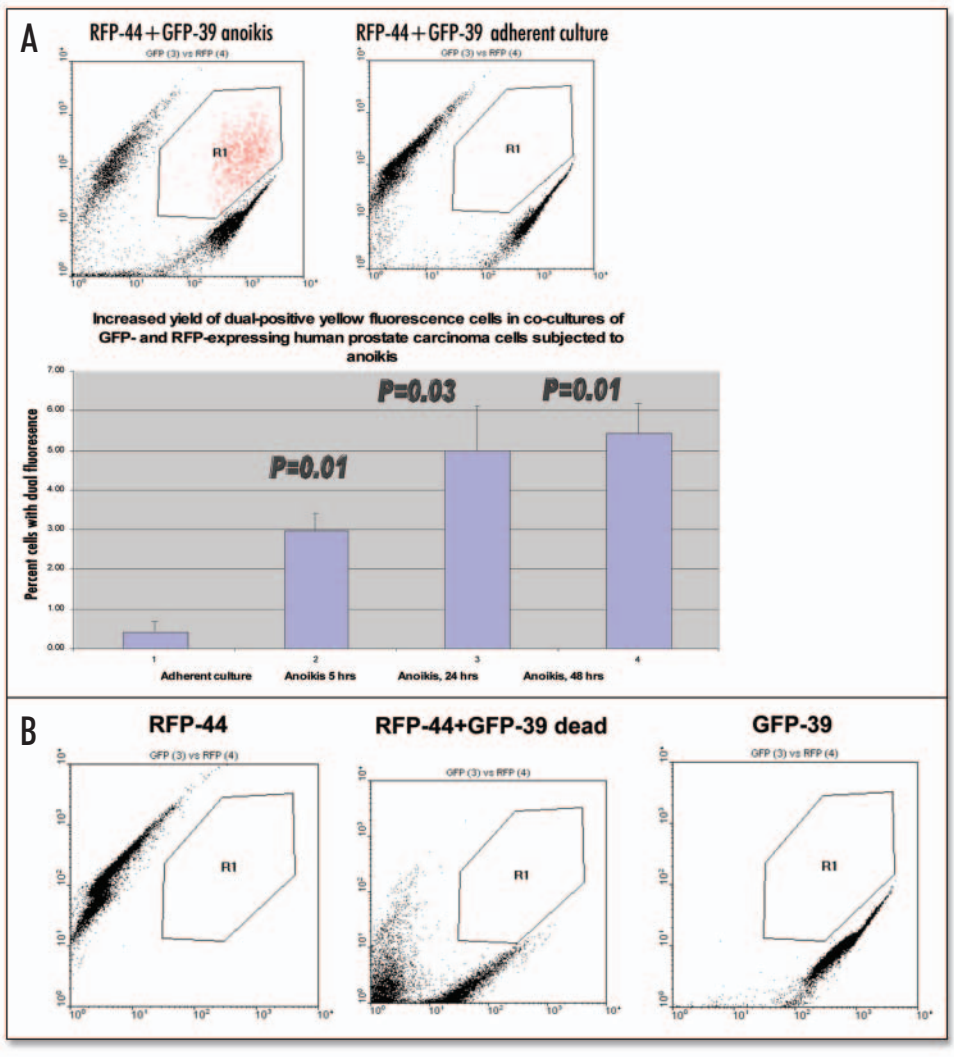

Figure 4. Growth of human prostate carcinoma cells in non-adherent conditions. Non-adherent culture promotes lateral gene exchange as manifested by increased yield of dual-positive yellow fluorescent cells in co-cultures of GFP- and RFP-expressing carcinoma cells. (A) Representative FACS analysis of co-cultures of GFP- and RFP-expressing human prostate carcinoma cells grown in detached conditions (top left) or in adherent cultures (top right). Bottom panel represents a quantitative analysis of yields of dual-positive yellow fluorescent cells in co-cultures growing under the different conditions. (B) Representative FACS analysis of control cultures of RFP-expressing (left), GFP-expressing (right), and adherent co-cultures of both GFP- and RFP-expressing cells (middle) containing ~75% dead cells (determined by the trypan-blue exclusion assay). R1 and R2 gating was established to exclude 99.9% (R1) and 99% (R2) of events detected during FACS analysis of multiple control conditions, including mono-fluorescent and non-fluorescent cells as well as adherent co-cultured GFP- and RFP-expressing cells.

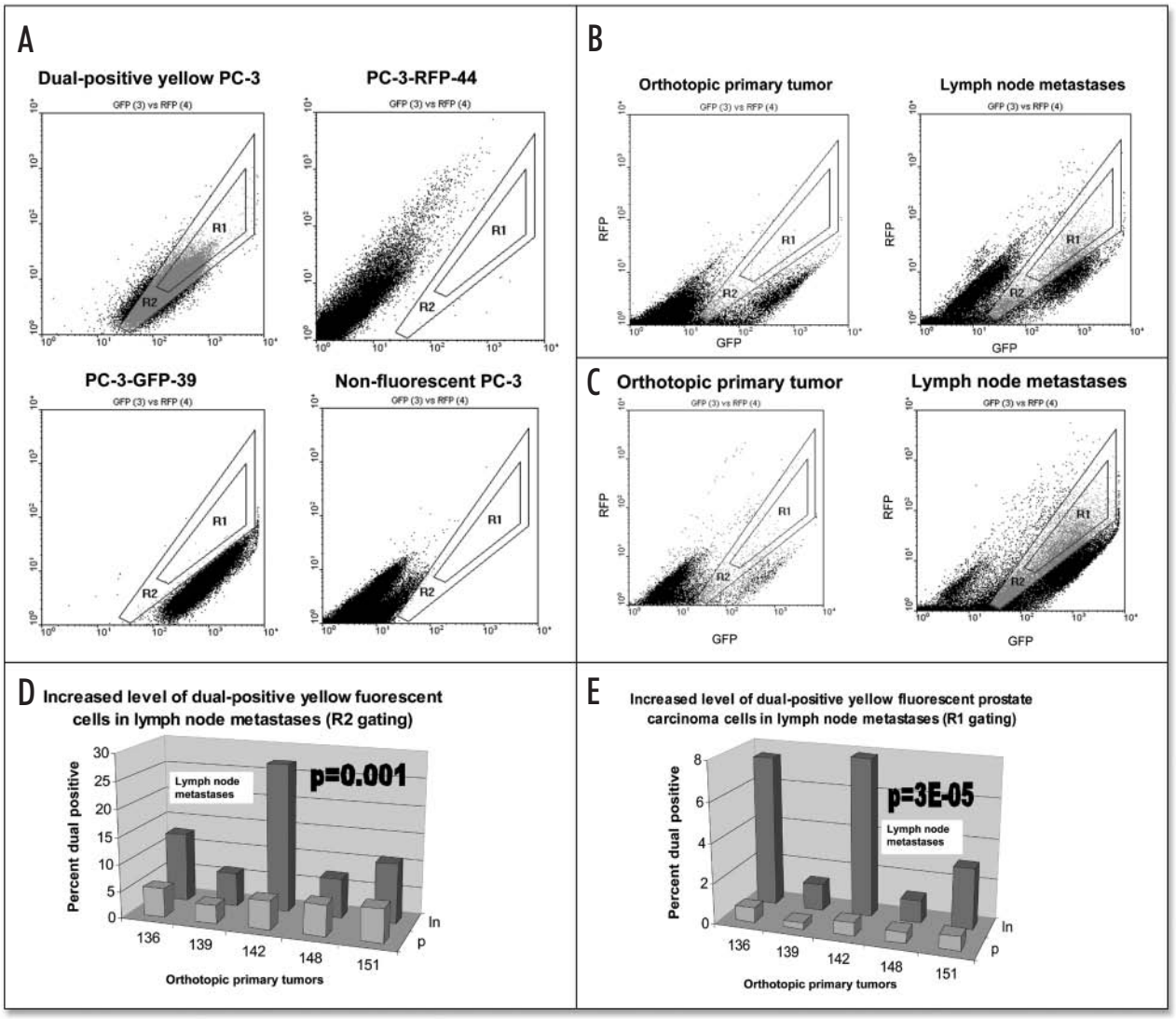

Figure 5. Lateral gene transfer contributes to creation of metastatic cancer cells in vivo. Lymph nodes, which are the most frequent sites of cancer metastases, were identified as sites of predominant accumulation in vivo of "hybrid" metastatic carcinoma cells created during metastatic dissemination of prostate cancer as documented by increased yield of dual-positive yellow fluorescent cells in lymph node metastases compared to the primary tumors in a dual-color orthotopic fluorescent model of human prostate cancer metastasis in nude mice. (A) Representative FACS analysis of primary orthotopic tumors growing in the mouse prostate after orthotopic inoculation of human prostate carcinoma cells with dual-positive yellow fluorescent phenotype expressing both RFP and GFP (top left); RFP-expressing red fluorescent cells (top right); GFP-expressing green fluorescent cells (bottom left); non-fluorescent parental PC-3 cells (bottom right). (B,C) Representative FACS analysis of orthotopic primary tumors (left) and lymph node metastases (right) of individual mice after orthotopic co-implantation of both GFP- and RFP-expressing human prostate carcinoma cells. Note markedly higher yield of dual-positive yellow fluorescent cells in metastases to the lymph nodes compared to the corresponding primary tumors. (D,E) Quantitative analysis of relative yields of dual-positive yellow fluorescent cells in primary and metastatic tumors of individual mice at the 99% (R2 gating) and 99.9% (R1 gating) specificity detection levels (see Materials and Methods and Figure 4B legend). P-values were derived using the two-tailed Student's test.

Exchange of genetic information between human cancer cells was demonstrated in vitro in several studies.[27-30] Horizontal transfer of apoptotic DNA to normal cells resulted in cell cycle arrest and senescence.[28-30] It has been demonstrated that uptake of apoptotic bodies triggers p53-dependent $p21^{Cip1/Waf1}$-mediated cell cycle arrest and senescence and blocks propagation of engulfed DNA in normal cells.[29,30] These data suggest that defects in cell cycle checkpoints are a prerequisite for sustained propagation of genetic material transferred to the recipient cells through this mechanism. Another documented mechanism of horizontal gene transfer is mediated by cell fusion resulting in formation of reprogrammed somatic cell hybrids.[31-34] Cell fusion has been observed in stem cells and may be important for tissue repair and regeneration.[31-34] It has been suggested that cell fusion between normal stem cells and transformed cells might contribute to formation of reprogrammed somatic cell hybrids with a highly metastatic self-renewing phenotype.[35] The progeny of these cells would likely retain a high propensity for lateral gene transfer and maintain a self-renewal potential contributing to development of highly malignant disease. Consistent with this hypothesis, drug-resistant human cancers diagnosed in multiple organs were shown to express a stem cell-like profile of genes.[36] The ability of viruses to induce cell fusion of transformed human cells[37,38] is mediated by viral proteins facilitating the entry of enveloped viruses into the cells. These viral proteins are members of the fusion protein family, which includes both viral and cellular proteins.[37-39] These data raise the possibility that at a certain stage of tumor progression

viruses may propagate the fusion of cancer cells or normal stem cells and cancer cells and contribute to the creation of highly malignant cancer cells.

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