Chromosome 17-mediated Dormancy of AT6.1 Prostate Cancer Micrometastases 1

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ABSTRACT

To improve the diagnosis and treatment of cancer, an increased understanding of the molecular and cellular changes that regulate metastatic ability is required. We have recently demonstrated a prostate cancer metastasis-suppressor activity encoded by a discontinuous ~70-cM region of human chromosome. The presence of this region suppresses the spontaneous metastatic ability of AT6.1 rat prostatic cancer cells by greater than 30-fold (M. A. Chekmareva et al., Prostate, 33: 271–280, 1997). Interestingly, a number of potentially important genes which have been mapped to human chromosome 17, including TP53, NM23, and BRCA1, are not retained (M. A. Chekmareva et al., cited above) or are not expressed in these microcell hybrids (B. A. Yoshida et al., In Vivo, in press), which suggests the presence of a novel metastasis-suppressor gene(s) or novel function of a known gene(s) encoded by this region(s). We hypothesize that identification of the “step” in the metastatic cascade that is inhibited by the presence of the ~70-cM metastasis-suppressor region will facilitate the identification of candidate metastasis-suppressor genes.

For a cancer cell to metastasize, it must escape from the primary tumor, enter the circulation, arrest in the microcirculation, extravasate into a tissue compartment, and grow. This suppression of spontaneous microscopic lung metastases could be due to the inhibition of a number of steps within this cascade. Results of the current study demonstrate that AT6.1 cells containing the ~70-cM region (AT6.1-17-4 cells) escape from the primary tumor and arrest in the lung but are growth-inhibited unless the metastasis-suppressor region is lost. This growth inhibition seems to result from an effect of one or more genes at the metastatic site and not from a circulating angiogenesis inhibitor. Our findings suggest that the ~70-cM region of human chromosome 17 may encode a gene(s) that regulates the “dormancy” of AT6.1-17-4 micrometastases.

INTRODUCTION

Presently, prostate cancer accounts for 29% of all the cancers diagnosed in American males. It is estimated that in 1998, 39,200 men will die from this disease, making it the second leading cause of cancer death in American men and the sixth cause of death overall (1). Aggressive screening programs starting at age 50 have been suggested to diagnose the disease when it is still confined to the prostate and, therefore, curable by local therapy (2, 3). Even in the absence of such mass screening, we are still faced with the problem of how to predict accurately the clinical course of individual lesions that have been diagnosed. In fact, it has been suggested that 80% of histologically localized lesions diagnosed are clinically unimportant (i.e., not life-altering or life-threatening; Refs. 3 and 4). Of the patients who clinically significant disease, only one-half will have cancers confined to the prostate, whereas the remaining patients will have micrometastatic disease (i.e., incurable cancer) at the time of initial diagnosis (2, 3). As an additional complication, a number of ultrasensitive methods to detect disseminated prostate cancer cells have been reported (5, 6). The clinical importance of such cells is a topic of considerable debate. There is, thus, a critical need for markers that will improve the ability to assess the malignant potential of both histological lesions and disseminated prostate cancer cells.

There is growing evidence that the loss of metastasis-suppressor gene function is an important event during malignant progression (7, 8). Because these genes should, by definition, specifically affect metastatic ability, they are particularly attractive for marker development. In an effort to functionally identify such genes, several laboratories recently reported the localization of metastasis-suppressor activities on human chromosomes 8, 10, 11 and 17 (9–12). These studies have used highly metastatic Dunning rat prostate cancer cells as the recipient for the microcell transfer of human chromosomes. This work led to the identification of the novel gene KAI1, which maps to the p11.2 region of human chromosome 11 (13). In subsequent studies, an additional gene encoded by the same region, CD44s, was determined also to have metastasis-suppressor activity (14). A correlation between decreased expression of KAI1 and CD44s proteins and increased metastatic potential of clinical prostate cancer has been demonstrated recently (13).

We have demonstrated recently (15) a metastasis-suppressor activity encoded by a ~70-cM region of human chromosome 17 consisting of three conserved region(s); (D17S932→D17S935, D17S930→D17S797, and D17S944→qter). In addition to STS-based PCR mapping, fluorescence in situ hybridization (FISH) analyses were used to demonstrate that there was a single copy of the ~70-cM region with no obvious rearrangements between human and rat chromosomes present in the AT6.1-17-4 microcell hybrids (15). In vivo studies showed that the presence of this portion of chromosome 17 in the AT6.1-17 microcell hybrids resulted in a greater than 30-fold suppression of metastatic ability as compared with that of the AT6.1 parental and control cell lines (15). Interestingly, a number of potentially important genes, including TP53, NM23, and BRCA1, that have been mapped to human chromosome 17 are not retained (15) or are not expressed in these microcell hybrids (7), which suggests the presence of a novel metastasis-suppressor gene(s) or novel function of a known gene(s) encoded by this region(s).

The development of spontaneous microscopic metastases consists of a cascade of distinct, well-established steps (16). Specifically, a metastatic cancer cell must escape from the primary tumor, enter the circulation, arrest in the microcirculation, invade into a tissue compartment, and grow. The suppression of spontaneous microscopic lung metastases could be due to the inhibition of a number of steps within this cascade. We hypothesize that the identification of the “step” of the metastatic cascade inhibited by the ~70-cM metastasis-suppressor region may facilitate the functional identification of the putative metastasis-suppressor genes encoded by the region. To this
end, experiments were designed to determine whether metastasis suppression was the result of: (a) secretion of a metastasis-suppressing factor; (b) decreased numbers and/or viability of tumor cells colonizing the lung; or (c) inhibition of the growth of micrometastases in the lung. The results of the studies demonstrated that chromosome 17-mediated metastasis suppression of spontaneous macroscopic metastases is caused by the expression of genes that inhibit the growth of micrometastases at the secondary site.

**MATERIALS AND METHODS**

**Cell Lines**

The AT6.1, AT6.1-Neo, AT6.1-17-4, and AT6.1-17-4-ßgal cell lines used in this study were established as described previously (12, 15). Briefly, the AT6.1 cell line is a highly metastatic, anaplastic, androgen-independent rat prostatic cancer cell (11). The AT6.1-17-4 cell line, which contains the ~70-cM metastasis suppressor region of chromosome 17, was established by microcell transfer of human chromosome 17 with an integrated neomycin resistance gene into the AT6.1 cells (15). The AT6.1-17-4 cell line was established by transfection of AT6.1 cells with the pZIPneo plasmid followed by selection in standard cell growth media containing 500 µg/ml G418 (15). The AT6.1-17-4-ßgal cell line was established by transducing AT6.1 cells with the MFG lacz retroviral vector (17). The AT6.1-17-4-ßgal cell line exhibits stable expression of the ß-galactosidase reporter gene and maintains the highly metastatic phenotype of parental AT6.1 cells (17).

**ß-Galactosidase Labeling of the AT6.1-17-4 Cells**

AT6.1-17-4 cells were transduced with MFG lacz retroviral vector as described previously (17). Specifically, the retroviral producer line MB4 CRIP, which contains the ß-galactosidase reporter construct, was allowed to condition its growth medium for 72 h. The conditioned medium was then isolated, and 20 µg/ml DEAE dextran (Sigma) was added for use in the transduction of AT6.1-17-4 cells (17). After a 24-h incubation, the conditioned medium was replaced with standard cell growth medium, and AT6.1-17-4 cells expressing the ß-galactosidase enzyme were isolated by fluorescence-activated cell sorting (FACS) using the FluoroReporter Lacz Flow Cytometry Kit according to the instructions of the manufacturer (Molecular Probes, Inc.). Clonal AT6.1-17-4-ßgal cell lines were subsequently established by limiting dilution cloning. The stability of ß-galactosidase expression in these cell lines was determined by direct staining for ß-galactosidase activity over several passages (17). To ensure that the clonal cell lines used for subsequent experiments had the same in vitro and in vivo characteristics as the parental AT6.1-17-4 cells, the following series of characterization studies was conducted: (a) the region of chromosome 17 retained in cell lines that showed stable expression of ß-galactosidase in ~95% of cells was determined by STS-based PCR mapping using markers present in the ~70-cM region of chromosome 17 retained in parental AT6.1-17-4 cells (15); (b) AT6.1-17-4-ßgal clonal cell lines that retained all of the markers within the minimal metastasis-suppressor region (15) and showed stable ß-galactosidase expression were tested for metastatic ability in spontaneous metastasis assays. Specifically, 2 × 10⁵ cells were injected s.c. into the flank of 4–6-week-old male C57/SCID mice (Taconic Lab Animals and Services) intraperitoneally (15). Three animals were sacrificed at each time point, and a xenograft was excised and weighed. The lungs were removed and examined for macrometastases, and ß-galactosidase activity as described previously (17). To determine whether the AT6.1-17-4 cells, which retain the ~70-cM metastasis-suppressor region, secrete a circulating factor that can inhibit the metastatic ability of AT6.1 cells, mice were injected coherently with 1 × 10⁵ AT6.1-17-4-ßgal cells into the left flank and 1 × 10⁵ AT6.1-17-4 cells into the right flank. As controls, one group of animals received a single s.c. injection of 1 × 10⁵ AT6.1-17-4-ßgal and another received a single s.c. injection of 1 × 10⁵ AT6.1-17-4 cells. Approximately 50 days postsurgery, the animals were killed, and the lungs were excised and weighed.

**Genomic DNA Preparation and Molecular Analysis of Cell Lines and Lung Metastases**

Genomic DNA was isolated from lung metastases or cultured cells as described previously (15). The regions of human chromosome 17 retained by the AT6.1-17-4-ßgal cell line and AT6.1-17-4 metastasis-derived cell lines were determined by PCR mapping of STS markers as described (15). Total human genomic DNA (Sigma) and genomic DNA isolated from parental AT6.1-17-4 cells were used as positive controls. AT6.1 parental genomic DNA was used as a negative control. STS and ß-actin primers (XAH20 and XAH21R17), which amplify both human and rodent actin sequences, were purchased from Research Genetics. Amplification conditions were as described previously (15) or as per the manufacturer. PCR products were fractionated by electrophoresis through a 3% 3:1 agarose gel (Genetex Laboratories) in 1× Tris-borate EDTA buffer and visualized by ethidium bromide staining. A 123-bp DNA Ladder (Life Technologies) was used as a molecular weight marker.

**In Vivo Studies of Growth and Metastasis**

Spontaneous metastasis assays were performed as described previously (15). Briefly, 4–6-week-old male C57/SCID mice (Taconic Lab Animals and Services) were s.c. injected in the flank with 2 × 10⁵ cells. Approximately 43 days postsurgery, animals were killed and the occasional metastases observed in the lungs of animals bearing AT6.1-17-4 tumors, and macroscopic metastases from the AT6.1 cells and AT6.1 Neo6 were excised and used for the preparation of genomic DNA and the development of LM cell lines. To rule out the possibility that metastasis suppression was mediated by NK cell activity, C57/SCID-beige mice (Taconic Lab Animals and Services) were s.c. injected in the flank with 2 × 10⁵ AT6.1 and AT6.1-17-4 cells as described for spontaneous metastasis assays. This double-mutant mouse model carries the SCID mutation that results in a lack of both T and B lymphocytes as well as the beige mutation that results in cytotoxic T-cell and macrophage defects as well as selective impairment of NK cell function.³ To determine whether AT6.1-17-4 cells, which retain the ~70-cM metastasis-suppressor region, secrete a circulating factor that can inhibit the metastatic ability of AT6.1 cells, mice were injected coherently with 1 × 10⁵ AT6.1-17-4-ßgal cells into the left flank and 1 × 10⁵ AT6.1-17-4 cells into the right flank. As controls, one group of animals received a single s.c. injection of 1 × 10⁵ AT6.1-17-4-ßgal and another received a single s.c. injection of 1 × 10⁵ AT6.1-17-4 cells. Approximately 50 days postsurgery, the animals were killed, and the lungs were excised and rinsed with PBS and fixed with PBS containing 1% glutaraldehyde. To detect both macroscopic and microscopic metastases, lungs were stained for ß-galactosidase activity as described previously (17).

**Cell Culture Conditions**

AT6.1 and AT6.1-17-4-ßgal cell lines were grown in standard RPMI 1640 with l-glutamine (Cell Gro) containing 8% FCS (Life Technologies), penicillin (100 units/ml)/streptomycin (100 µg/ml), and 250 nm dexamethasone (Sigma Chemical). The AT6.1 Neo, AT6.1-17-4, and AT6.1-17-4-ßgal cell lines were grown in the standard RPMI 1640 containing G418 (500 µg/ml; Life Technologies). Incubation conditions were standard: 37°C in 95% air and 5% CO₂.

staining; (b) clongenic viability assays; and (c) a direct bioassay for i.p. tumor formation.

**Direct β-Galactosidase Staining.** To assess the number of AT6.1-Tβgal or AT6.1-17-4-Tβgal cells in the circulation, 200–300-μl aliquots of blood were isolated from tumor-bearing animals and hemolysed by the addition of three volumes of a hypotonic solution of 5 mM Tris (pH 7.5) and 135 mM NaCl, followed by centrifugation at 37°C for 15 min. Intact cells (i.e., WBCs, tumor cells, and so forth) were collected by centrifugation at 1000 x g for 10 min. The cell pellet was washed twice with PBS and once with Solution A (1× = 10 mM sodium phosphate (pH 7.3), 150 mM NaCl, and 1 mM MgCl₂), resuspended in Solution A containing 1% glutaraldehyde, and allowed to stand at room temperature for 15 min. Cells were then collected, washed, and resuspended in Solution A containing 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆·3H₂O, and 1 mg/ml X-Gal. The cell suspension was allowed to incubate at 37°C for 2–18 h after which, cells were collected on a microscope slide via cytospin and fixed with 95% ethanol. The cells were counterstained with nuclear fast red and dehydrated by consecutive washings with 80, 95, and 100% ethanol and finally xylene. β-galactosidase-staining cells were detected by microscopic evaluation. As a control for this protocol, samples of blood were “spiked” with increasing numbers (10–2000 cells) of AT6.1-Tβgal cells to generate a standard curve for detection. Analogous mixtures of AT6.1 parental and AT6.1-Tβgal cells were assayed to demonstrate the specificity of β-galactosidase staining.

**Direct Bioassays for i.p. Tumor Formation.** A direct bioassay for circulating tumor cells was performed as another measure of viability. In this assay 300 μl of blood was taken from mice bearing ≥1 cm³ AT6.1-Tβgal or AT6.1-17-4-Tβgal tumors and injected i.p. into naive mice. Approximately 90 days post injection the animals were sacrificed and examined for i.p. tumors. As a control, blood from mice bearing ≥ 1 cm³ MatLyLu tumors, a highly metastatic Dunning variant known to produce i.p. tumors in this type of assay, was similarly injected into another set of naïve animals.

**Clonogenic Assays for Culturing Cell Viability.** Blood was hemolysed as described for the direct β-galactosidase staining using sterile technique. Surviving cells were resuspended in standard cell growth media and plated on 60-mm² tissue culture dishes. As a control, 200–μl aliquots of blood from naïve mice were spiked with fixed numbers (i.e., 10–200 cells) of AT6.1-Tβgal cells that had been processed as described for the direct-staining method. The surviving cells were resuspended in standard growth media, plated on 60-mm² tissue culture dishes, and grown under standard tissue culture conditions.

**Establishment of Metastasis-derived Cell Lines**

Individual macroscopic metastases were excised and treated with collagenase (2 mg/ml; Worthington Biochemical; 225 units/mg) in MEM containing 10% FCS. The tissue was incubated at 37°C overnight with gentle agitation. The cells were then collected by centrifugation at 200 x g for 10 min, resuspended in growth media, and transferred to 12-well tissue culture plates. As a control, 200-μl aliquots of blood from mice bearing AT6.1-Tβgal cells, which stably express the β-galactosidase reporter gene, were injected either colaterally or as a mixture with AT6.1-17-4 cells. We hypothesized that if AT6.1-17-4 tumors secrete a factor that could suppress the metastasis of AT6.1-Tβgal cells, the colateratial injection of the cells would allow for detection of a systemic activity, whereas injection of a cell mixture would allow a local effect to be observed. Thus, animals were separated into experimental groups, the first of which received colateral injections of 1 x 10⁵ AT6.1-17-4 cells and 1 x 10⁶ AT6.1-Tβgal cells in the right and left flanks, respectively. Mice in the second group were injected in the right flank with a mixture of 0.5 x 10⁵ AT6.1-17-4 and 0.5 x 10⁶ AT6.1-17-4 cells. As controls, a group of animals was injected singly with either AT6.1-Tβgal cells or with AT6.1-17-4 cells. Approximately 43 days postinjection, the animals were killed, the lungs were excised, and the macroscopic and microscopic metastases were visualized by either fixation with Bouin’s solution or direct staining for β-galactosidase activity. Animals that were injected with AT6.1-17-4 cells either colaterally or as a mixture with AT6.1-Tβgal had approximately the same number of macroscopic β-galactosidase-positive metastases present in their lungs (i.e., 70–80 metastases) at the experimental end point (Table 1). This number was not significantly different from the number of macroscopic metastases in the lungs of control animals injected singly with AT6.1-Tβgal cells (i.e., 90–100 metastases per lung). Importantly, the number of metastases in each of these experimental groups was ≥5-fold greater than in control animals injected singly with AT6.1-17-4 cells. These data suggest that AT6.1-17-4 does not secrete local or systemic factors that can suppress the dissemination of metastatic AT6.1-Tβgal cells.

To exclude the possibility that observed metastasis suppression is the result of NK cell functions, CB17/SCID-beige mice were used in a spontaneous metastasis assay. Two groups of mice (3 animals each) were injected s.c. in the flank with 2 x 10⁵ AT6.1 or AT6.1-17-4 cells, respectively. Lungs of animals injected with parental AT6.1 cells demonstrated a high number of macroscopic metastases (i.e., 150–200), whereas lungs of animals injected with AT6.1-17-4 cells demonstrated a greatly suppressed number of metastases (i.e., 0–1; Table 1). The results of this experiment with the relatively small number of animals were similar to our previous experiments using SCID mice, demonstrating that suppression by the ~70-cM region is not due to an NK-mediated mechanism (12, 15).

**Suppression of macroscopic AT6.1-17-4 lung metastases may also**

| Table I Results of colateral and mixed cell injections of AT6.1-Tβgal and AT6.1-17-4 cells |
|-----------------------------|-------------|-----------------------------|
| Cell line(s)                | Mode of injection | No. of macroscopic metastases/lung |
| AT6.1-Tβgal, AT6.1-17-4    | Colateral injection ² | 82 ± 10                  |
| AT6.1-Tβgal, AT6.1-17-4    | Single injection (cells mixed) ³ | 74 ± 14                  |
| AT6.1-Tβgal                | Single injection ⁴ | 95 ± 20                  |
| AT6.1-17-4                 | Single injection ⁵ | 15 ± 5                   |
| AT6.1-17-4                 | Single injection ⁷ | 184 (150–200) ²          |

³ 5-10 animals per experimental group were used.

² Each mouse in this experimental group received colateral s.c. injections of 1 x 10⁵ AT6.1-Tβgal cells in the right flank and 1 x 10⁵ AT6.1-17-4 cells in the left flank.

³ Each mouse in this experimental group received a s.c. injection of a mixture of 1 x 10⁵ AT6.1-Tβgal cells and 1 x 10⁵ AT6.1-17-4 cells in the flank.

⁴ Each mouse in this experimental group received a s.c. injection of 1 x 10⁵ AT6.1-Tβgal cells in the flank.

⁵ Each mouse in this experimental group received a s.c. injection of 1 x 10⁵ AT6.1-17-4 cells in the flank.

⁷ Three animals per experimental group were used.

⁶ Each mouse in this experimental group received a s.c. injection of 2 x 10⁵ cells in the flank.

* ² Average of three animals; range is given in parentheses.

**RESULTS**

Initial studies were designed to address the possibility that metastasis suppression is the result of decreased dissemination of cells from the primary tumor. This could be mediated by one or more locally or systemically secreted factors. To address these possibilities, AT6.1-Tβgal cells, which stably express the β-galactosidase reporter gene, were injected either colaterally or as a mixture with AT6.1-17-4 cells. To exclude the possibility that observed metastasis suppression is the result of NK cell functions, CB17/SCID-beige mice were used in a spontaneous metastasis assay. Two groups of mice (3 animals each) were injected s.c. in the flank with 2 x 10⁵ AT6.1 or AT6.1-17-4 cells, respectively. Lungs of animals injected with parental AT6.1 cells demonstrated a high number of macroscopic metastases (i.e., 150–200), whereas lungs of animals injected with AT6.1-17-4 cells demonstrated a greatly suppressed number of metastases (i.e., 0–1; Table 1). The results of this experiment with the relatively small number of animals were similar to our previous experiments using SCID mice, demonstrating that suppression by the ~70-cM region is not due to an NK-mediated mechanism (12, 15).

Suppression of macroscopic AT6.1-17-4 lung metastases may also...
be caused by a decrease in the number and/or viability of circulating AT6.1-17-4 cells. These two parameters are highly interrelated. For example, a decrease in the number of detectable cells in the circulation could be the result of a decrease in the ability of AT6.1-17-4 cells to dislodge from the primary tumor or, alternatively, it could be due to the decreased survival of AT6.1-17-4 cells once they have entered the circulation. The net effect of either of these scenarios would be an effective decrease in the number of cells in the circulation that could potentially become entrapped in the lungs. Experiments using AT6.1-Tßgal and AT6.1-17-4-Tßgal cells, which express the ß-galactosidase reporter gene, were designed to compare the number and viability of AT6.1 and AT6.1-17-4 cells in the circulation. Initially a direct staining assay was developed to assess the number of ß-galactosidase-expressing cells in the circulation. Similarly, an assay for the clonogenic ability of tumor cells in the circulation was used as a qualitative measure of cell viability. The control experiments for the direct staining and clonogenic approaches using blood “spiked” with increasing numbers of AT6.1-Tßgal cells demonstrated the ability to detect ~20 cells/200 µl of blood. When the direct staining approach was tested on blood isolated from AT6.1-Tßgal-tumor-bearing animals, a small number of ß-galactosidase-staining cells were detected but the results were inconsistent (data not shown). In the case of the clonogenic assays, 200 µl of blood taken from AT6.1-Tßgal tumor-bearing mice was hemolysed, and, after collection by centrifugation, the surviving cells were plated under standard tissue culture conditions. Several of these experiments showed the presence of one colony-forming cell per 200 µl of blood. Although these experiments demonstrated the presence of circulating AT6.1-Tßgal cells, the absolute number of cells was so low that a suppression of this parameter would be undetectable by this method. Similar results were obtained from a direct bioassay for tumorigenic cells (data not shown). These data suggest that the metastatic ability of AT6.1 cells is not dependent upon a high number of these cells in the circulation.

We next examined the hypothesis that a gene(s) encoded by the ~70-cM region of chromosome 17 suppresses the formation of spontaneous macroscopic metastases by inhibiting the growth of AT6.1-17-4 micrometastases in the lung. If this were the case, histological examination of the AT6.1-17-4 lungs, which had few observable macroscopic metastases, would reveal the presence of micrometastases. To test this, three discontinuous H&E sections of representative lungs harvested at the experimental end point from mice bearing AT6.1 or AT6.1-17-4 tumors were examined (Fig. 1). Twenty random fields were examined for the presence or absence of tumor foci. Tumor foci were categorized as being small (<500 cells) or large (>1000 cells) as described in “Materials and Methods.” The percentage of fields with either no foci, small foci, or large tumor foci are shown (Fig. 1A). As would be expected, 91% of the fields examined in sections of AT6.1 lung showed tumor foci, most of which were large. Interestingly, 54% of fields examined from sections of AT6.1-17-4 lung showed the presence of small foci. In addition, we observed what appeared to be “cuffs” of 2–3 layers of cells around lung vessels (data not shown). These data suggested that AT6.1-17-4 cells are able to reach the lungs but are growth-inhibited.

To address this possibility further, AT6.1-17-4-Tßgal cells were used in spontaneous metastasis assays. At the experimental end point, animals were killed, and the lungs were removed and stained for ß-galactosidase expression. The approach allowed the visualization of microscopic AT6.1-17-4-Tßgal surface metastases. A comparison of the results from spontaneous metastasis assays using AT6.1 cells, AT6.1-17-4-Tßgal cells (fixed with Bouin’s solution), and AT6.1-17-4Tßgal cells (stained with Bouin’s solution) is shown in Fig. 1B. Injection s.c. of 2 × 10⁵ AT6.1 parental cells resulted in the formation of a mean number of 97 macroscopic metastases per lung. As expected, the number of macroscopic metastases detected by Bouin’s staining after the s.c. injection of 2 × 10⁵ AT6.1-17-4Tßgal cells was greatly reduced. The use of Bouin’s fixative allowed for the visualization of macroscopic metastases; however, micrometastases were not detected. In contrast, when lungs removed from animals carrying AT6.1-17-4-Tßgal tumors were excised and stained for ß-galactosidase activity, numerous blue-staining microscopic metastases

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**Fig. 1.** Examination of AT6.1 macroscopic metastases and AT6.1-17-4 micrometastases. A, histological examination of lungs. Three discontinuous 5- µm sections of lungs from representative AT6.1 and AT6.1-17-4 tumor-bearing animals were examined by H&E staining for the presence of macro- and micrometastases. Twenty random fields per section were examined for the presence or absence of tumor foci. Tumor foci were categorized as being small (<500 cells) or large (>1000 cells) as described in the “Materials and Methods” section. Examples of unaffected lung and of large and small tumor foci are shown. The percentage of fields with each category of foci detected is shown below the panels. Left, lung tissue with no detectable tumor cells; middle, small tumor focus; right, large tumor focus. B, quantitation of surface macro- and micrometastases. AT6.1-17-4 cells were tagged with the ß-galactosidase reporter gene, enabling the sensitive detection of AT6.1-17-4-Tßgal micrometastases. The numbers of macroscopic and microscopic metastases observed were determined using Bouin’s and X-Gal staining, respectively. At the experimental end point, lungs were removed from animals bearing AT6.1, AT6.1-17-4, and AT6.1-17-4-Tßgal tumors. Left, lung from AT6.1 tumor-bearing animal stained with Bouin’s solution; middle, lung from AT6.1-17-4 tumor-bearing animal stained with Bouin’s solution; right, lung from AT6.1-17-4-Tßgal tumor-bearing animal stained for ß-galactosidase activity. The average numbers of macrometastases or micrometastases and SE are shown below the panels.

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<th>AT6.1</th>
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<td>Bouin’s solution</td>
<td>9%</td>
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Number of Visible Lung Metastases

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<th>AT6.1</th>
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<tr>
<td>AT6.1</td>
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<td>AT6.1-17-4</td>
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were observed. Interestingly, the mean number of AT6.1-17-4-Tßgal micrometastases (i.e., 62 ± 12) detected by this method was comparable to the number of AT6.1-Tßgal macroscopic metastases (i.e., 97 ± 6). These results suggested that AT6.1-17-4 cells are capable of escaping from the primary tumor and arresting in the lungs, but that the presence of the ~70-cM metastasis-suppressor region inhibits their growth in the lungs.

Because of the similarity of our findings to the angiostatin-mediated dormant dormancy reported by Holmgren et al. (18), we investigated the possibility that AT6.1-17-4 primary tumors secrete a substance that suppresses the growth of its own micrometastases. Ten animals were injected s.c. in the flank with 2 × 10⁵ AT6.1-17-4-Tßgal cells. When the primary tumors reached a volume of 1 cm³, the tumors were surgically resected in the experimental group. The remaining five animals served as a control and received a sham surgery. Sixty-six days postinjection, animals were killed, and the lungs were removed and examined for the presence of macroscopic metastases. If the primary tumor secretes a substance which suppresses the growth of its own metastases, we expected to find a significant increase in the number of metastases in animals that had surgically resected tumors. Examination of the lungs from these animals showed no difference in the numbers of metastases in experimental and control groups.

Taken together, the aforementioned results led us to hypothesize that AT6.1-17-4 cells are able to escape from the primary tumor and become entrapped in the lung; however, the microscopic foci are growth-inhibited (Fig. 2). The mechanism of growth inhibition seems to be different than previously reported angiostatin-mediated dormancy, which led us to posit that a gene(s) encoded by the ~70-cM region may be directly suppressing the growth of AT6.1-17-4 micrometastases. If true, that implies that micrometastases would remain "dormant" until the metastasis-suppressor region of chromosome 17 is lost (Fig. 2A). Furthermore, AT6.1-17-4 micrometastases that lose chromosome 17 could then grow into the macroscopic lesions occasioned in our studies.

To test this possibility, genomic DNA was extracted from individual macroscopic (~2 mm) metastases that were excised from lungs harvested from AT6.1-17-4 tumor-bearing animals (Fig. 2B). The retention of 14 STS markers that are present in the ~70-cM metastasis-suppressor region was examined by PCR (Fig. 3). Markers D17S945, D17S799, D17S955, D17S953, D17S798, D17S920, D17S791, D17S970, and D17S784 were lost in all of the samples examined. Other markers (D17S921, D17S839, and D17S783) were retained in 15–17% of the samples examined. Interestingly, two markers—D17S261 and D17S113—were retained in the majority of samples. At this time, we cannot say whether this retention frequency is of any functional significance. Furthermore, by PCR-based analysis, we cannot rule out the possibility that more complex rearrangements involving various segments of chromosome 17 have occurred. The observation that 12 of 14 STS markers examined were lost in >85% of the macroscopic lung metastases examined supports the first assertion of our model; AT6.1-17-4 cells do reach the lung but once in the lung are growth-inhibited unless the ~70-cM metastasis-suppressor region is lost (Fig. 2B).

If AT6.1-17-4 macrometastases arise from micrometastases that have lost the ~70-cM metastasis-suppressor region, we reasoned that AT6.1-17-4 macrometastases would be composed of a mixture of cells that have lost ~70-cM metastasis-suppressor region (shown in gray), and cells that still retain this region (shown in black; Fig. 2C). Furthermore, if the presence of the ~70-cM region induces a state of dormancy, cells containing this metastasis-suppressor region should still be viable (i.e., retain proliferative capacity). Therefore, individual macrometastases could be excised and treated with collagenase to form single-cell suspensions, and cells with the ~70-cM metastasis-suppressor region could be selected by growth in media containing neomycin (Fig. 2C). If the ~70-cM metastasis-suppressor region were still functional, the AT6.1-17-4 macrometastasis-derived cell lines would be suppressed when assayed for spontaneous metastatic ability in SCID mice.

To test these hypotheses, 12 individual AT6.1-17-4 macrometastases were excised and used to establish LM cell lines. These lines were established from individual macroscopic lung metastases and grown for numerous passages in media containing 500 µg of G418/ml. Thus, they represent the mixture of neomycin-resistant cells present in the macroscopic metastases. To ensure that these cells were uniformly
more, the number of macroscopic metastases observed in the lungs of animals bearing AT6.1-17-4-LM tumors was similar to AT6.1-17-4 cells, which are suppressed for metastasis (Table 2). These findings support the model proposed in Fig. 2C and provide evidence that tumor dormancy is mediated by the ~70-cM region of human chromosome 17.

**DISCUSSION**

Despite the significant advances in our understanding of fundamental aspects of cancer, the development of metastatic lesions remains the predominant cause of death for most cancer patients. To improve the diagnosis and treatment of cancer, an increased understanding of the molecular and cellular changes that regulate metastatic ability is required. Identification of such changes may allow for the development of molecular and cellular markers that can be used in combination with pathological staging to predict metastatic ability (3, 7). This is of particular importance in the case of prostate cancer, given the prevalence of histologically localized disease in the male population. To this end, there has been a concerted effort by a number of laboratories to functionally identify genes that regulate metastasis. We have recently demonstrated a metastasis-suppressor activity encoded by a ~70-cM portion of human chromosome 17 consisting of three conserved region(s) (i.e., D17S952-*D17S805, D17S930^D17S797, and D17S944^qter).

As part of our ongoing effort to identify the metastasis-suppressor genes encoded by the ~70-cM region, we hypothesized that identi-
fication of the step in the metastatic cascade inhibited by these genes may facilitate their identification. In the AT6.1 system, metastasis suppression is defined as a reduction in the number of spontaneous macroscopic lung metastases. The presence of the ~70-cM region of chromosome 17 results in a dramatic suppression of macroscopic metastases with ≥30-fold reductions being commonly observed (15). It should be stressed that this suppression is specific; the presence of the ~70-cM region has no effect on the growth rate of the primary tumor. Ongoing candidate gene studies suggest that the observed phenomenon is likely to be mediated by a novel gene or a known gene with a novel function (15).

For a cancer cell to metastasize, it must escape from the primary tumor, enter the circulation, arrest in the microcirculation, extravasate into a tissue compartment, and grow. The observed suppression of the formation of spontaneous macroscopic lung metastases could be due to the inhibition of a number of steps within this cascade. Results from our studies demonstrate that AT6.1-17-4 cells containing the ~70-cM metastasis-suppressor region escape from the primary tumor and arrest in the lung but are growth-inhibited unless the metastasis-suppressor region is lost. This finding in vivo is in agreement with in vitro studies showing that presence of the ~70-cM region had no effect on the invasiveness or motility of these cells.6 In addition, the observed growth suppression is a durable phenomenon up to 66 days, the longest end point examined thus far. The possibility that the ~70-cM region confers a chronic state of dormancy is presently being investigated using longer term assays (i.e., 90, 120, and so forth days). Interestingly, the growth inhibition of AT6.1-17-4 micrometastases seems to result from the effect of gene(s) at the metastatic site and not from a circulating angiogenesis inhibitor. Taken together, our findings suggest that the ~70-cM region of human chromosome 17 may encode a gene(s) that regulates the dormancy of AT6.1-17-4 micrometastases. Although prostate cancer metastasis-suppressor activities encoded by human chromosomes 8, 10, 11, and 17 have previously been reported, to our knowledge, this is the first study to address dormancy as a possible mechanism of chromosome-mediated suppression of AT6.1 macroscopic metastases.

In light of an increased emphasis on the early detection of prostate cancer, insights into genetic factors and or mediators of tumor dormancy could be of considerable importance (18, 19). During the past 5 years, there has been an enormous push to develop ultrasensitive methods to detect prostate cancer cells in the circulation and bone marrow of patients (5, 6). The assumption of such studies is that the presence of prostate cancer cells in the circulation or in the bone marrow will be a valuable prognostic factor for the aggressiveness of this disease. This assumption has yet to be validated and is presently a topic of debate. There is a real possibility that such disseminated cells may stay dormant and will require additional genetic or epigenetic changes to become active (5, 19). The contribution of dormancy to malignancy has been difficult to determine; however, it must be addressed if meaningful information is to be obtained from such studies. Therefore, there is a critical need for improved markers for the malignant potential of both histological lesions and disseminated prostate cancer cells.

The findings presented herein suggest the intriguing possibility that the ~70-cM region of human chromosome 17 encodes a gene(s) that, in effect, renders AT6.1 cells dormant. If this is the case in the progression of human disease, then the finding that the loss of heterozygosity of chromosome 17 is an uncommon event in primary tumors is not unexpected (20). It would be more appropriate to examine metastatic lesions for such losses. Furthermore, the identification of the gene(s) that controls this phenomenon may allow for the development of probes that would predict accurately the clinical significance of disseminated prostate cancer cells. Efforts to identify the gene(s) that is mediating this effect are currently underway (7). Additional in vivo mechanistic studies are also being conducted to identify the events that are controlling the dormancy phenotype.

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