Stromal Cells Promote Angiogenesis and Growth of Human Prostate Tumors in a Differential Reactive Stroma (DRS) Xenograft Model

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ABSTRACT

Reactive stroma has been reported in many cancers, including breast, colon, and prostate. Although changes in stromal cell phenotype and extracellular matrix have been reported, specific mechanisms of how reactive stroma affects tumor progression are not understood. To address the role of stromal cells in differential regulation of tumor incidence, growth rate, and angiogenesis, LNCaP xenograft tumors were constructed in nude mice with five different human prostate stromal cell lines as well as GeneSwitch-3T3 cells engineered to express lacZ under mifepristone regulation. Alone, LNCaP prostate carcinoma cells were essentially non-tumorigenic, whereas combinations of LNCaP cells with three different human prostate stromal cell lines (L/S tumors) resulted in a tumor incidence (50–63%) similar to that of control LNCaP plus Matrigel (L/M) tumors over a 9-week period. In contrast, LNCaP combinations with two other human prostate stromal cell lines were nontumorigenic, illustrating that stromal cell effects are differential. L/S tumors exhibited well-developed blood vessels at 2 weeks, whereas control L/M tumors were avascular at 2 weeks and exhibited blood lakes in lieu of extensive vessels at later time points. Xenografts constructed under three-way conditions (LNCaP, Matrigel, and stromal cells; L/M/S tumors) exhibited a 100% tumor incidence and showed rapid blood vessel formation as early as day 7 with mature vessels formed by day 10. L/M/S tumors exhibited a 10.3-fold increase in microvessel density, and the corresponding hemoglobin/tumor weight ratio was increased 2-fold relative to L/M control tumors at day 10. L/M/S tumor wet weight and volume increased by 1.6- and 2.4-fold, respectively, by day 21, compared with control L/M tumors. L/M/S tumors made with LNCaP cells plus GeneSwitch-3T3-pGene/lacZ stromal cells showed similar results. Mifepristone-regulated gene expression was observed in stromal cells immediately adjacent to clusters of carcinoma cells and in vessel walls in a mural cell (pericyte) position. This study shows that regulation of angiogenesis is one mechanism through which stromal cells affect LNCaP tumor incidence and growth rate. This regulation may be mediated through direct recruitment and interactions of stromal cells with endothelial cells. Furthermore, this study describes for the first time a model system with regulated transgene expression in the stromal compartment of an experimental carcinoma. These findings point to the stromal compartment as a potential source of new prognostic markers and therapeutic targets and show the utility of the carcinoma-stromal xenograft model system in dissecting specific mechanisms of reactive stroma.

INTRODUCTION

In many human cancers, the stromal microenvironment adjacent to carcinoma cells is fundamentally different from the stroma of normal adult tissues. Studies of human breast, colon, and prostate cancer specimens have identified activated stromal cell phenotypes, modified ECM composition, and increased microvessel density in the reactive stroma of these tumors (1–4). Moreover, the reactive stroma associated with these cancers exhibits biological markers consistent with stroma at sites of wound repair (5). Many of the biological processes in wound repair, including stromal cell activation to the myofibroblast phenotype, stimulation of collagen type I deposition, and induction of angiogenesis, are observed in reactive stroma during cancer progression. Because remodeling of the stromal compartment in wound repair is associated with promoting new tissue growth (formation of granulation tissue and re-epithelialization; Ref. 6), it follows that reactive stroma in cancer might be expected to promote tumorigenesis (7, 8). Accordingly, it is important to better understand specific mechanisms and signaling pathways in reactive stroma of cancer to exploit this compartment for better prognostics and potential therapeutics.

Recent studies have addressed the role of stroma in promoting tumorigenesis using experimental carcinoma models. Elenbaas et al. (9) have shown that combining transformed human mammary epithelium (derived from normal glands) with normal human mammary fibroblasts elevated the incidence of tumor formation to 100% as compared with a 52% incidence in the control (epithelium only). Moreover, these studies showed that coinjection of normal fibroblasts with transformed mammary epithelium reduced the tumor latency period from 52 to 20 days. Similarly, another study showed that the coinoculation of carcinoma-associated fibroblasts with initiated, but not tumorigenic, prostate epithelium resulted in increased tumor incidence and growth (10). Together, these data show that when all other parameters are constant, the stromal microenvironment in cancer plays a critical role in determining the incidence and rate of tumorigenesis.

The specific mechanisms and pathways through which reactive stroma regulates carcinoma tumorigenesis are not fully understood. It is known that in human prostate cancer, markers of reactive stroma are observed quite early in tumor development. The activation of stromal cells to the myofibroblast phenotype and stimulated expression of specific ECM components were observed in the stroma immediately adjacent to precancerous PIN lesions in human surgical specimens. These stromal regions adjacent to PIN have also been reported to exhibit elevated microvessel density relative to normal prostate stroma (11–13). Accordingly, reactive stroma is likely to function early in tumorigenesis, possibly during precancerous stages.

It is clear that the ability to model reactive stroma in an animal system would aid in advancing our understanding of specific mechanisms (14). Currently, there is no promoter with which to target gene expression exclusively to the stromal compartment of a specific tissue in transgenic animals. Accordingly, xenografts of LNCaP human prostate carcinoma cells have been used to address tumorigenesis of an androgen sensitive prostate carcinoma in many previous studies (15–17). The purpose of the present study was to assess the differential effects of several human prostate stromal cell lines initiated by our laboratory on early tumorigenesis events in LNCaP xenografts in a...
nude mouse model system. Specifically, tumor incidence, tumor growth, and angiogenesis were evaluated under different xenograft conditions. We report here that LNCaP tumor incidence and rate of tumor growth was dependent on the stromal cell conditions used, and that tumor-promoting stromal cells were associated with early and elevated angiogenesis. Furthermore, these studies show the development of a modular DRS LNCaP xenograft model system that exhibits a 100% tumor incidence, tumor homogeneity, early angiogenesis, and regulated transgene expression in the stromal cell population of the tumor.

MATERIALS AND METHODS

Cell Lines. LNCaP human prostate carcinoma cells were purchased from American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 (Life Technologies, Inc., Rockville, MD) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 100 units/ml penicillin, and 100 μg/ml streptomycin (Sigma Chemical Co., St. Louis, MO).

Human prostate stromal cell lines were established according to our protocol published previously (18, 19). Briefly, fresh tissue cores were obtained from the Baylor College of Medicine Prostate Specialized Program of Research Excellence Pathology and Tissue Microarray Core. Tissues originated from organ donors or patients undergoing radical prostatectomy. Unless noted, tissue cores were isolated from regions of normal prostate, based on histopathological criteria. The cores were diced into 1-mm cubes, washed with HBSS, and put into 24-well tissue culture plates containing BFs medium: DMEM (Life Technologies, Inc., Rockville, MD) supplemented with 5% fetal bovine serum, 5% Nu Serum (Collaborative Research, Bedford, MA); 0.5 μg/ml testosterone, 5 μg/ml insulin, 100 units/ml penicillin, and 100 μg/ml streptomycin (Sigma, St. Louis, MO). The explants were incubated at 37°C with 5% CO2, and culture medium was changed every 48 h. Stromal cells migrated out of the tissue and attached to the culture dish. After the cells reached confluence, the remaining tissue was removed, and the cells were passaged using standard trypsin/EDTA procedures. The following stromal cell lines were generated: HTS-40C (transition zone of a 40-year-old), HPS-40F (peripheral zone of a 40-year-old), HPS-44D (peripheral zone of a 44-year-old), HTS-2T (transition zone of a 16-year-old organ donor), and HTS-TZ1A (transition zone of benign prostatic hyperplasia specimen). Standard immunocytochemistry procedures were used to evaluate cell phenotype. Cytokeratin expression was not detected with the AE1/AE3 pooled monoclonal antibody (Boehringer Mannheim, Indianapolis, IN), and all cell lines were 100% vimentin positive (sc-7557; Santa Cruz Biotechnology, Santa Cruz, CA). Expression of sm α-actin (clone 1A4; Sigma) varied among stromal cell lines, from no expression (HTS-TZ1A) to approximately 50% of the population (HTS–40C) to nearly 100% (HTS-2T). Cultures at passages 5–15 were used for experiments.

The GeneSwitch System (Invitrogen, Carlsbad, CA), a mifepristone-regulated expression system for mammalian cells, was used to create a stromal cell line with regulated expression of the lacZ gene. The GeneSwitch-3T3 fibroblast vector, which expresses the GeneSwitch regulatory protein from the pSwitch vector, was cultured in DMEM supplemented with 10% fetal bovine serum and 50 μg/ml hygromycin B (Invitrogen, Carlsbad, CA). This cell line was transfected with the pGENE/V5-HaslacZ vector (Invitrogen) using FuGENE 6 transfection reagent according to manufacturer’s recommendations (Roche, Indianapolis, IN). Stably transfected cells were selected with 300 μg/ml Zeocin (Invitrogen). Zeocin-resistant clones were treated with 100 μM mifepristone (Sigma) and screened for β-gal expression using standard in vitro X-gal staining procedures. The GeneSwitch-3T3-pGeneSwitch-lacZ cell line used in this study represents a pooled population of stably transfected cells.

Animals. Athymic NCI-nu/nu male homozgyous nude mice, 6–8 weeks of age, were purchased from Charles River Laboratories (Wilmington, MA). All experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Preparation of LNCaP Xenografts. Xenograft tumors were generated under different stromal conditions: one-way xenografts (LNCaP cells, stromal cells, or Matrigel/ECM only); two-way xenografts (LNCaP + Matrigel, LNCaP + stromal cells, or stromal cells + Matrigel); and three-way xenografts (LNCaP cells + stromal cells + Matrigel). LNCaP cells and stromal cells were harvested from culture plates by trypsinization, resuspended in growth medium, and counted with a hemacytometer. Cells were used immediately or frozen in aliquots for modular xenograft experiments. For frozen aliquots, cells were pelleted at ~950 × g for 50 s in a clinical centrifuge (IEC model CL with rotor 809, setting 5), resuspended in filtered freezing medium (culture medium + 10% DMSO), and transferred to cryogenic vials (Corning Inc., Corning, NY). Cells were frozen using a StrataColder Cryo preservation module (Sstartagene, La Jolla, CA) and stored at −80°C. Matrigel ECM (Becton Dickinson, Bedford, MA) was stored in 0.5-ml aliquots at −20°C and then thawed on ice at 4°C for 3 h before use.

For modular three-way xenograft experiments, frozen aliquots of cells (16 × 106 LNCaP cells and 4 × 106 stromal cells) were thawed in a 37°C water bath for 1–2 min, transferred to separate 15-ml conical tubes, washed with Serum and culture medium, and pelleted as described above. LNCaP cells were resuspended in 10 ml of culture medium, whereas stromal cells were resuspended in 3 ml of culture medium. Then, cells were combined in one tube and pelleted again. The supernatant was aspirated to the 300-μl mark, and cells were gently resuspended in the remaining medium. Before combining with Matrigel, cells were incubated on ice for 5 min. A prechilled pipette was used to transfer cells to the tube containing 0.5 ml of Matrigel (on ice). Cells were mixed with the Matrigel and then drawn into a prechilled pipette 1-ml syringe with a 20-gauge needle. Using a 25-gauge needle, 100 μl of cell suspension (2 × 106 LNCaP cells and 0.5 × 106 stromal cells, 4:1 ratio) were injected s.c. in each lateral flank. Three animals (6 total injection sites) were used for each experiment set.

For LNCaP/pstromal cell two-way xenograft experiments, 16 × 106 LNCaP cells and 8 × 106 stromal cells were washed, combined, and resuspended as described above. The cells were mixed with 500 μl of PBS instead of Matrigel, and 100 μl of cell suspension (2 × 106 LNCaP cells and 1 × 106 stromal cells, 2:1 ratio) were injected s.c. in each lateral flank. For LNCaP/Matrigel two-way xenograft experiments, 16 × 106 LNCaP cells were washed twice with 10 ml of culture medium, resuspended in 300 μl of medium, incubated on ice, mixed with 0.5 ml of Matrigel, and injected s.c., as described above. For one-way xenograft experiments, 16 × 106 LNCaP cells or 8 × 106 stromal cells were washed, resuspended in 300 μl of medium, mixed with 500 μl of PBS, and injected s.c. as described above.

To induce expression of the lacZ reporter gene in GeneSwitch-3T3-pGeneSwitch-lacZ cells, animals received mifepristone (RU 486; Sigma) at 0.3 mg/kg administered as 100-μl i.p. injections. Animal dosage of mifepristone was based on protocols shown previously to induce gene expression in vivo (20, 21). Treatment began at the time of experimental setup and was repeated every 48 h. Mifepristone was dissolved in ethanol (2 mg/ml) and then diluted in sesame oil (Sigma) to the appropriate concentration. For vehicle control, animals were treated with ethanol diluted in sesame oil.

Processing of Tumors. Animals were monitored daily, and tumors were harvested at time points ranging from 4 days to 9 weeks, depending on the parameter being evaluated. Tumor volume was not allowed to exceed ~150 mm3. Tumors visible with the naked eye were surgically removed and dissected away from surrounding skin and fascia. Tumors were measured in three dimensions with calipers, and tumor volume (mm3) was calculated with the formula: \( V = 0.52 \times width \times height \times length \) (22). Tumors were then transferred to preweighed tubes on ice. Tissues were reweighed, and tumor weight (g) was determined. Tissues were fixed in 4% paraformaldehyde overnight at 4°C and then washed 3× with PBS and processed for histology. Tissues were embedded in paraffin, and 5-μm sections were cut and mounted onto ProbeOn Plus slides (Fisher Scientific, Pittsburgh, PA). Sections were stained with H&E and Masson’s Trichrome (Sigma) for histological analysis.

Immunohistochemistry and In Situ Hybridization. Antibodies used were: sm α-actin, mouse monoclonal 1A4 (Sigma); antimouse CD31/PECAM-1, rat monoclonal MEC13.3 (BD PharMingen, San Diego, CA); biotin-conjugated Universal Secondary (Research Genetics, Huntsville, AL) used for sm-α-actin; and biotin-conjugated goat antirat IgG (BD PharMingen) used for CD31. Specificity of each primary antibody has been evaluated and published previously. No significant staining was observed if sections were incubated with secondary antibody only.

Immunostaining was performed with the MicroProbe Staining System (FisherBiotech, Pittsburgh, PA). Reagents formulated for use with capillary action systems were purchased from Research Genetics (Huntsville, AL) and used according to the manufacturer’s protocol. Tissues were deparaffinized...
using Auto Dewaxer and cleared with Auto Alcohol. Brigati’s Iodine and Auto Prep were used to improve tissue antigenicity. Then, sections were washed with Universal Buffer. For sm α-actin staining, sections were incubated in 20 μg/ml goat-antimouse Fab fragment (Jackson ImmunoResearch, West Grove, PA) for 30 min at 37°C to block host immunoglobulins. For CD31 staining, antigen retrieval was required; tissues were incubated in 0.1% trypsin (Zymed, South San Francisco, CA) for 10 min at 37°C. After these treatments, sections were washed with Universal Buffer, incubated in Protein Blocker, and washed again. Antibodies were diluted in Primary Antibody Diluent and used at the following conditions: sm α-actin, 1:200 for 8 min at 50°C; and CD31, 1:50 overnight at 4°C. Tissues were washed with Universal Buffer and incubated in secondary antibody: Universal Secondary undiluted for 4 min at 50°C, and antirat IgG 1:100 for 45 min at 37°C. Tissues were washed in Universal Buffer, treated with Auto Blocker to inhibit endogenous peroxidase activity, and washed again. For detection, sections were incubated in streptavidin horseradish peroxidase (sm α-actin) or RTU Vectastain Elite ABC reagent (Vector Laboratories, Burlingame, CA; CD31), washed in Universal Buffer and then incubated in stable diaminobenzidine tetrahydrochloride twice for 3 min each at 50°C. Tissues were counterstained with Auto Hematoxylin for 30 s.

In situ hybridization to specifically label human cells was performed with the MicroProbe Staining System according to a procedure published previously (23). All reagents were purchased from Research Genetics and used according to the manufacturer’s protocol. Tissue sections were deparaffinized in xylene and then rehydrated in 100, 95, and 70% ethanol, followed by double-distilled H₂O. Slides were incubated in Auto Blocker for 1 min at room temperature and washed three times with Universal Buffer. Tissues were treated with pepsin for 1 min at 105°C, and then Ahu1/Ahu2 probe was added (prediluted and used at 1 μg/ml). Tissues were incubated in Ahu1/Ahu2 probe for 5 min at 105°C and then at 45°C for 1.5 h. Sections were washed with Post Hybe Wash for 5 min at 45°C and incubated in streptavidin horseradish peroxidase for 10 min at 50°C. After incubation in Probe Lock for 10 s at room temperature, tissues were treated with stable diaminobenzidine tetrahydrochloride, rinsed with Auto Wash, and counterstained with Auto Hematoxylin as described above.

**Microvessel Density Analysis**. Analysis was performed according to standard procedures (24). Tissue sections were stained for CD31 as described above. Sections were scanned at ×200, and areas with the highest vascular density were identified. Vessels in three high-power fields (×400) were counted by two independent observers, one of whom was blinded to experimental conditions. The average vessel count was determined for each specimen. Six tumors from each condition were analyzed, and counts were combined for statistical analysis by unpaired t test.

**Hemoglobin Assay**. Hemoglobin content was measured using Drabkin’s method (25). Whole tumors were homogenized in 50 μl of double-distilled H₂O using disposable pellet pestles for microtubes (Fisher Scientific). Hemogenates were incubated in 0.5 ml of Drabkin’s Solution (Sigma) for 15 min at room temperature. Samples were centrifuged to pellet cell debris. The supernatants were transferred to cuvettes, and the absorbance was measured at 540 nm. Drabkin’s Solution was used as a blank. The absorption, which is proportional to the total hemoglobin concentration, was divided by tumor weight. Six tumors from each condition were assayed, and measurements were combined for statistical analysis by unpaired t test.

**Whole-Mount β-gal Staining**. Whole tumors were stained according to standard procedures. Briefly, tumors were harvested at day 10 and fixed with 2% paraformaldehyde/0.2% glutaraldehyde (Polysciences, Inc., Warrington, PA) in PBS for 30 min at 4°C on a platform rocker. Tissues were washed three times for 30 min each at room temperature with rocking in PBS for 30 min at 4°C on a platform rocker. Tissues were washed with Universal Buffer, incubated in Protein Blocker, and washed again. Antibodies were diluted in Primary Antibody Diluent and used at the following conditions: β-galactosidase, 1:200 for 8 min at 50°C; and Alu1/Alu2 probe was added (prediluted and used at 1 μg/ml). Tissues were incubated in Alu1/Alu2 probe for 5 min at 105°C and then at 45°C for 4.5 h. Sections were washed with Post Hybe Wash for 5 min at 45°C and incubated in streptavidin horseradish peroxidase for 10 min at 50°C. After incubation in Probe Lock for 10 s at room temperature, tissues were treated with stable diaminobenzidine tetrahydrochloride, rinsed with Auto Wash, and counterstained with Auto Hematoxylin as described above.

**Statistical Analysis**. Incidence of tumor formation between two experimental conditions was analyzed by Fisher’s exact test, whereas multiple conditions were compared with the χ² test. Tumor weight, tumor volume, microvessel density, and hemoglobin content were each evaluated with an unpaired t test. Analysis was performed with GraphPad Prism for Macintosh version 3.0 (GraphPad Software, San Diego, CA). P < 0.05 was considered statistically significant.

**RESULTS**

Stromal Cells Differentially Promote LNCaP Tumorigenesis. To investigate the role of the stromal microenvironment in prostate cancer, we examined the effect of human prostate stromal cell lines on LNCaP tumorigenesis under several xenograft conditions. LNCaP human prostate carcinoma cells were combined with stromal components and then injected s.c. in athymic nude mice. Stromal components included different human prostate stromal cell lines and stromal cell-derived ECM (Matrigel; Refs. 26 and 27). For one-way control xenografts, LNCaP cells or human prostate stromal cells were individually injected with buffer. For two-way xenografts, LNCaP cells were injected with either Matrigel as a control or with prostate stromal cells in buffer. For three-way xenografts, LNCaP cells were injected with both Matrigel and prostate stromal cells in buffer. Five different human prostate stromal cell lines were evaluated. As described in “Materials and Methods,” the human prostate stromal cell lines were cytokeratin negative and vimentin positive, with varied expression of sm α-actin. Thus, the stromal cell lines represented a mix of fibroblasts and myofibroblasts, which are the cell phenotypes observed in human prostate cancer reactive stroma.

For tumor incidence studies, the growth period ranged from 2 to 9 weeks, and tumors were harvested at different time points for histological analysis. A tumor was defined as a palpable mass that contained nodules of LNCaP carcinoma cells upon histological examination. Inoculation of nude mice with LNCaP cells alone (one-way control xenografts) resulted in a low incidence of tumor formation (8%; Fig. 1, Column a), which is consistent with previous reports (28, 29). After 9 weeks, the human prostate stromal cell lines did not form palpable masses when injected alone (Fig. 1, Columns b–d) or when injected with Matrigel (data not shown). Thus, under one-way xenograft conditions, the LNCaP carcinoma cells were only weakly tumorigenic, whereas the human prostate stromal cells were nontumorigenic.

Similar to previous studies that established standard LNCaP xenograft procedures (15, 30), coinjection of LNCaP cells with Matrigel (Fig. 1, Column e) increased tumor incidence to 63% (P < 0.001, Fisher’s exact test). Throughout this report, standard LNCaP/Matrigel xenografts (L/M) were used as a positive control by which to examine the effect of stromal cells on LNCaP tumorigenesis. Coinjection of

![Fig. 1. Stromal conditions regulate incidence of tumor formation in LNCaP xenografts.](image-url)

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<th>Condition</th>
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<td>LNCaP</td>
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<td>LNCaP + Matrigel</td>
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**Table 1. Stromal conditions regulate incidence of tumor formation in LNCaP xenografts.** Columns: a, LNCaP only (n = 24); b, HTS-2T only (n = 5); c, HPS-40F only (n = 5); d, HTS-2T only (n = 5); e, LNCaP + Matrigel (n = 24); f, LNCaP + HTS-40C (n = 24); g, LNCaP + HPS-40F (n = 12); h, LNCaP + HPS-TZ1 (n = 12); i, LNCaP + HTS-2T (n = 12); j, LNCaP + HPS-44D (n = 6); k, LNCaP + Matrigel + HTS-40C (n = 16); l, LNCaP + Matrigel + HTS-2T (n = 24); m, LNCaP + Matrigel + GeneSwitch-3T3-pGenE (n = 6); n, LNCaP + Matrigel + GeneSwitch-3T3-pGenE/ALCZ (n = 18).
human prostate stromal cells with LNCaP cells in two-way xenograft experiments (L/S) differentially regulated the incidence of LNCaP tumor formation. Three independent prostate stromal cell lines (HTS-40C, HPS-40F, and HPS-TZ1A) promoted growth of LNCaP tumors in at least 50% of injection sites (Fig. 1, Columns f–h), whereas two other stromal cell lines (HTS-2T and HPS-44D) did not support LNCaP tumorigenesis (0% tumor incidence at 9 weeks after inoculation; Fig. 1, Columns i–j). Compared with injection of LNCaP cells alone, the increase in tumor incidence attributable to coinjection of LNCaP cells with HTS-40C, HPS-40F, or HPS-TZ1A stromal cells was statistically significant (P = 0.003, 0.009, 0.003, respectively, Fisher’s exact test). Moreover, there was no significant difference in the incidence of LNCaP tumor formation with these stromal cell lines as compared with Matrigel. In addition, there were no obvious differences in the rate of tumor growth between L/M and tumorigenic L/S xenografts.

Combining LNCaP cells with both Matrigel and human prostate stromal cells (three-way L/M/S xenografts) further elevated LNCaP tumor incidence to 100% (Fig. 1, Columns k–l). Relative to tumor incidence in two-way xenografts, the increased tumor incidence in three-way conditions was statistically significant (P < 0.001, χ² test). Interestingly, HTS-2T normal human prostate stromal cells promoted LNCaP tumor growth in three-way xenografts but not in two-way xenografts (P < 0.001, Fisher’s exact test). These data show that the stromal microenvironment, including both the stromal cells and the ECM, is a critical regulator of LNCaP tumorigenesis.

Analysis of Two-way Xenograft Tumors. Histological examination revealed dramatic differences in the morphology of control 2-way
LNCaP/Matrigel (L/M) tumors as compared with two-way LNCaP/stromal cell (L/S) tumors. At early time points (2 weeks), control L/M tumors consisted of small clusters of LNCaP cells embedded in Matrigel (Fig. 2A). Invasion of mouse host stromal cells into the Matrigel was also observed (Fig. 2A, asterisk). Similar to observations by Gao et al. (31), the stromal cells did not form a collar of smooth muscle around the clusters of carcinoma cells. In contrast to L/M tumors, the histological appearance of early L/S tumors was more typical of the epithelial-stromal architecture observed in human prostate carcinoma. Masson’s trichrome staining showed that nodules of LNCaP cells were surrounded by a stroma rich in collagen fibers and blood vessels (Fig. 2C). Thus, the stroma in two-way L/S tumors exhibited features more consistent with human prostate cancer reactive stroma, which we have described previously (4). Additionally, the LNCaP nodules were more extensive in two-way L/S tumors relative to two-way control L/M tumors. These data suggest that human prostate stromal cells may confer a growth/survival advantage to LNCaP cells and thus enhance LNCaP tumorigenesis.

At later time points (6 weeks), both control L/M tumors and L/S tumors resembled poorly differentiated carcinomas composed of sheets of LNCaP cells with little intervening stroma (Fig. 2, E and G). However, although connective tissue septa in L/S tumors contained blood vessels (Fig. 2G, arrows), control L/M tumors appeared to lack normal vasculature. Rather, the L/M tumors were hemorrhagic, and large pools of RBCs, previously termed “blood lakes” (32), were observed frequently (Fig. 2E, asterisk).

To examine the vasculature in two-way xenograft tumors, we performed immunohistochemistry with an antibody to CD31 (PECAM-1), an endothelial cell marker. CD31 immunostaining was rarely detected in control L/M tumors at 2 weeks (Fig. 2B), which is consistent with the lack of blood vessels observed by H&E or Masson’s Trichrome stain. In contrast, numerous CD31-positive vessels were present in the stromal compartment of early (2 weeks) L/S tumors (Fig. 2D). Moreover, sm α-actin-positive mural cells (pericytes or smooth muscle cells) surrounded these blood vessels, indicating that vessels were mature (data not shown; Ref. 33). Although some CD31-positive vessels were observed in late-stage L/M tumors (Fig. 2F, arrow), most RBCs appeared to be in blood lakes, which were CD31 negative (Fig. 2F, asterisk). Late-stage L/S tumors were well vascularized, and vessels were positive for both CD31 (data not shown) and sm α-actin (Fig. 2H). Analysis of two-way xenograft tumors suggests that stromal cells influence LNCaP tumor development and vascularization, particularly during early tumorigenesis.

Analysis of Three-Way Xenograft Tumors. Comparison of two-way control L/M tumors with three-way L/M/S tumors allowed a more direct evaluation of the role of stromal cells in LNCaP tumorigenesis. As shown above, coinjection of stromal cells with LNCaP cells and Matrigel significantly increased tumor incidence relative to two-way control L/M tumors (Fig. 3A; $P < 0.001$, unpaired $t$ test). Moreover, at this same time point, the three-way tumors exhibited a 47% increase in wet weight compared with two-way control L/M tumors at day 10; mean tumor weight of L/M ($n = 6$) and L/M/S ($n = 6$) tumors at day 10; mean tumor weight of L/M ($n = 4$) and three-way L/M/S tumors ($n = 4$) tumors at day 10; mean tumor volume of L/M ($n = 4$) and L/M/S ($n = 10$) tumors at day 21. Asterisk, statistically significant increase in weight or volume of three-way L/M/S tumors compared with two-way L/M tumors at the same time point ($P < 0.01$). All three-way tumors were generated with HTS-2T cells. Bars, SE.

The stromal cells were frequently adjacent to clusters of LNCaP cells, similar to the histological architecture of human prostate cancer. These stromal cells were sm α-actin positive, which is consistent with the myofibroblast phenotype (Fig. 5B, arrowheads). In addition, staining with Masson’s trichrome showed that stromal cells adjacent to carcinoma cells and blood vessels were producing collagen matrix (Fig. 4C, arrow).

By day 21, three-way L/M/S tumors consisted of sheets of LNCaP cells with intervening stroma, typical of poorly differentiated human prostatic carcinoma (Fig. 4E). The wet weight (Fig. 3A) of three-way L/M/S tumors was significantly higher than two-way control L/M tumors at the day 21 time point (64% increase; $P < 0.001$, unpaired $t$ test). Moreover, at this same time point, the three-way tumors exhibited a 2.4-fold increase in tumor volume compared with two-way L/M tumors (Fig. 3B; $P < 0.001$, unpaired $t$ test). Histological analysis of L/M tumors at day 21 revealed considerable heterogeneity. Sheets or large clusters of LNCaP cells were observed in some regions (Fig. 4G), whereas other areas of the two-way L/M tumors exhibited small clusters of LNCaP cells, similar to day 10 (Fig. 4F). It was noted that regions with increased LNCaP cell density contained blood vessels (Fig. 4G, arrow), although some blood lakes had also formed (Fig. 4G, asterisk). Neither the average wet weight (Fig. 3A) nor the volume (Fig. 3B) of two-way control L/M tumors exhibited statistically significant differences between days 10 and 21 ($P = 0.2$ and 0.8, respectively, unpaired $t$ test). Together, these data show that stromal cells increase LNCaP tumor incidence and accelerate the rate of LNCaP tumor growth.

Angiogenesis in Three-Way L/M/S Tumors. To investigate the vasculature in early three-way L/M/S tumors, we examined tissues harvested 4, 7, and 10 days after injection. Blood vessels were not observed in H&E-stained tissue sections at day 4. However, vessels were typically observed in day 7 tumors, and tumors were well
vascularized by day 10 with no evidence of blood lakes. Vessels were lined with CD31-positive endothelial cells (Fig. 5A) and surrounded by sm α-actin-positive mural cells (Fig. 5B, arrows). Thus, vascularization of early three-way L/M/S tumors was attributable to the development of mature blood vessels, not blood lakes.

Microvessel density and hemoglobin content were measured to quantitate the vascularity in control L/M tumors and in L/M/S tumors. Microvessel density analysis was performed on CD31-stained tissue sections. The average microvessel count per ×400 field in three-way tumors at day 10 was $31 \pm 5$ (Fig. 6A, Column b). In contrast, the average microvessel count of early two-way control L/M tumors was $3 \pm 2$ (Fig. 6A, Column a). The 10.3-fold increase in vascularization...
of three-way L/M/S tumors was statistically significant ($P < 0.001$, unpaired $t$ test). In addition, two-way L/M tumors and three-way DRS tumors harvested at day 10 were assayed for hemoglobin content by Drabkin’s method. The concentration of hemoglobin, normalized to tumor weight, in three-way L/M/S tumors was 2-fold higher than in control L/M tumors (Fig. 6B; $P = 0.004$, unpaired $t$ test). These data show that the accelerated LNCaP tumorigenesis observed in three-way xenografts with stromal cells, relative to two-way xenografts with Matrigel only, correlates with elevated microvessel density and hemoglobin content in early tumors. Moreover, these data suggest that stromal cells enhance LNCaP tumorigenesis by promoting angiogenesis.

Regulated Gene Expression in Stromal Cells of Three-Way L/M/S Tumors. To assess the utility of the three-way L/M/S model for regulated stromal gene expression and to determine the localization of stromal cells relative to carcinoma cells and developing blood vessels, we used mouse 3T3 fibroblasts stably transfected with the pSwitch and pGeneswitch vectors. The incidence of tumor formation when LNCaP cells were coinfected with Matrigel and Geneswitch-3T3-pGeneswitch cells was 100% (Fig. 1, Column $n$). In addition, tumor size, morphology, and vascularity at day 10 were similar to three-way tumors generated with LNCaP cells and human prostate stromal cells. Animals were treated with vehicle control or with mifepristone (RU 486) to induce expression of the lacZ reporter gene. No statistically significant differences in weight or volume were observed between vehicle control and mifepristone-treated tumors (data not shown). Tumors harvested from animals treated with vehicle control did not exhibit detectable $\beta$-gal activity in whole tissues (Fig. 7A) or in tissue sections (Fig. 7C). In contrast, mifepristone induced expression of the lacZ gene in LNCaP/3T3-pGeneswitch cells, as shown by the high level of X-gal staining (Fig. 7B). Examination of tissue sections revealed that $\beta$-gal activity was restricted to stromal cells (Fig. 7, D and E). Moreover, positive staining stromal cells were frequently adjacent to clusters of LNCaP cells (Fig. 7D, arrows), which is consistent with the tissue architecture of other three-way L/M/S tumors (Fig. 4D). Significantly, stromal cells expressing $\beta$-gal were also observed surrounding blood vessels in a mural cell (pericyte) position in the vessel wall (Fig. 7E, arrows). To our knowledge, this is the first study to show regulated stromal cell gene expression in an in vivo cancer model. Furthermore, these data suggest a direct interaction between inoculated stromal cells and the developing vasculature.

**DISCUSSION**

Studies of human breast, colon, and prostate cancers suggest that a stromal response occurs as a component of carcinoma progression (1–4). This reactive stroma exhibits activated stromal cell phenotypes and expresses different ECM components as compared with normal tissue stroma. The specific mechanisms of the stromal response and how reactive stroma affects cancer progression are unknown. However, because reactive stroma exhibits characteristics typified by wound repair stroma, it has been suggested that cancer-associated stroma is tumor promoting (4, 5, 7). Data presented in this study confirm that the stromal microenvironment promotes and differentially regulates tumorigenesis of a human prostate cancer xenograft. Furthermore, our data suggest that one mechanism through which stromal cells affect the incidence and rate of tumorigenesis is by stimulating early angiogenesis.

Our data demonstrate that the stromal effect on LNCaP tumorigenesis is differential, in that not all human prostate stromal cell lines were tumor promoting in two-way combinations. Moreover, under two-way xenograft conditions, the incidence of tumor formation did not exceed 63%. The results of the three-way experiments show that a more complete stromal microenvironment was required to reach maximal tumor incidence, accelerated tumor growth, and more ho-
mogeneous tumors. The three-way combinations represented a more complete tumor model in that all three critical components are supplied: tumor cells, stromal cells, and a stromal cell-derived matrix. Of most significance, this three-way mix results in a tumor with stimulated angiogenesis during early tumor formation.

It is clear that the appropriate rate and extent of angiogenesis is a critical component of cancer progression (34–36). The mechanisms through which stromal cells promote angiogenesis in our model are not yet understood; however, stromal cells may affect angiogenesis at several levels. Stromal cells may stimulate host endothelial cell migration into the xenograft as well as promote the subsequent formation of endothelial tubes. Indeed, growth factors such as vascular endothelial growth factor and fibroblast growth factor-2, known to be important for endothelial migration and tube formation, are secreted by stromal cells (37, 38). Moreover, deposition of specific ECM components by stromal cells may regulate endothelial cell response to angiogenic factors (39). It has also been shown that stromal cells directly promote vessel maturation by cell-cell interactions. Endothelial cells recruit adjacent stromal cells as mural cells destined to become pericytes or smooth muscle cells (33). This endothelial cell-mural cell interaction functions to stabilize the developing vessel (33). It has been shown that nascent endothelial cell tubes are unstable and regress in the absence of perivascular stromal cells if vascular endothelial growth factor is withdrawn (40). Accordingly, in addition to stimulating the process of angiogenesis, stromal cells are an integral component of the mature vessel wall and are required for vessel function. Indeed, our data with lacZ-positive stromal cells show a close association of these cells with endothelial tubes, in a mural cell position. It is likely that the human stromal cells are also incorporated into vessel walls, although they were not readily observed in tissue sections stained with a human-specific probe. The chromosome in situ hybridization technique does not label stromal cells with the same intensity as epithelial cells, and stromal cells in vessel walls are attenuated in cross section. Thus, chromosome in situ hybridization may not be sufficient to identify human stromal cells in the vessel wall.

Nonstromal L/M control tumors at early time points showed essentially no vessel development and exhibited blood lakes at later time points. The blood lakes were not lined by CD31-positive endothelial cells or smooth muscle cells. This is consistent with data reported by Wilson and Sinha (41), where LNCaP-Matrigel tumors exhibited “large dilated blood vessels” that were negative for von Willebrand factor, an endothelial marker. Our results suggest that the subsequent formation of blood lakes in two-way L/M tumors functions to compensate for lack of adequate vascularization attributable to the absence of stromal cells in early tumors. In contrast, our

Fig. 7. Regulated expression of lacZ in stromal cells of three-way L/M/S tumors. A and B, tumors generated with LNCaP cells, Matrigel, and GeneSwitch-3T3-pGenelacZ cells were assayed for β-gal activity by whole-mount X-gal staining at day 10. X-gal staining was observed in tumors harvested from animals treated with mifepristone (B), but not in tumors from animals treated with vehicle control (A). Bar, 1 mm. C, β-gal activity was not detected in tissue sections of vehicle control tumors. D and E, tissue sections from mifepristone-treated tumors. β-gal activity was restricted to stromal cells. lacZ-positive cells were observed adjacent to LNCaP clusters (D, arrows) and surrounding blood vessels in a mural cell (pericyte) position (E, arrows). Bar (C–E), 50 μm.
three-way L/M/S tumors exhibited normal-appearing vessels as early as day 7 and were well vascularized with mature vessels by day 10 after inoculation. Accordingly, the pattern and type of vascularization in two-way and three-way LNCaP xenograft tumors appear to be dependent on the presence of stromal cells.

Precancerous PIN lesions in the human prostate gland induce phenotypic changes in the immediate stroma, characterized by activation of stromal cells and stimulation of matrix remodeling (42). Increased microvessel density in the stroma adjacent to PIN lesions has also been reported (11, 12). This suggests that early events in epithelial transformation act to induce reactive stroma, which in turn stimulates angiogenesis required for further progression of the precancerous lesion. Indeed, the preponderance of data now indicate that angiogenic switches occur early in the premalignant stages of most carcinomas (43, 44). Our data suggest that the induction of a reactive stroma in developing cancer with the resulting angiogenic switch is an important and probably required step in successful tumorigenesis.

Although our studies have addressed angiogenesis, stromal cells are likely to provide paracrine-acting growth factors as well as structural and regulatory ECM components that directly affect LNCaP cell survival and growth. Accordingly, the stromal microenvironment likely regulates tumorigenesis through several mechanisms. Indeed, coculture of prostate cancer-associated fibroblasts with transformed prostate epithelial cells resulted in increased proliferation and reduced apoptosis of the epithelial cells (10). Again, these observations suggest that the three-way xenograft approach is a more complete tumor model.

We have termed the experimental approach reported here as the DRS xenograft model. The model has been useful to address the role of the stromal microenvironment in tumorigenesis. Furthermore, we have extended the utility of the model by showing the regulated expression of a transgene in the stromal cells of an experimental carcinoma xenograft. The DRS model system will allow for the direct testing of stromal cell gene products in regulating carcinoma tumorigenesis. This approach will be valuable in dissecting specific mechanisms of stromal-epithelial interactions in prostate cancer. Of particular value was the finding that this system was efficient in regulation of transgene expression. Expression was stimulated in response to mifepristone; however, it was undetectable in vehicle control-treated animals. In addition, the model is based on the use of frozen cell aliquots (epithelial and stromal cells) for establishing the xenograft. The use of frozen cell aliquots as opposed to passaging cells from growing cultures greatly increased throughput and homogeneity of xenograft experiments. Combined with the use of engineered stromal cells, this provides for a modular approach. We anticipate being able to combine carcinoma cells with differentially transfected stromal cells, all derived from an initial pSwitch transfectant cell line. The advancement of regulated stromal gene expression in the DRS xenograft tumor model is significant because promoters do not currently exist for targeting gene expression to the stromal compartment of a transgenic animal in a tissue-specific manner. Moreover, regulatable expression with the GeneSwitch system will allow for expression of a transgene at any time point during tumorigenesis. One potential caveat of the DRS model in its current form is the use of 3T3 cells as an engineered stromal cell source. For the specific questions asked in this study, use of 3T3 cells has been suitable for examination of early events; however, 3T3 cells are tumorigenic and can form sarcomas with time in nude mice. Accordingly, we are establishing GeneSwitch transfectants of prostate stromal cell lines derived from C57/BL6 mice for use in further studies with human carcinoma cells in nude mice and with TRAMP carcinoma cells (45) in syngeneic C57/BL6 hosts.

The studies presented here represent an advancement in the understanding of stroma-regulated events in early tumorigenesis, particularly in the regulation of angiogenesis. Furthermore, these studies have led to the development of a modular and rapid DRS xenograft model system, which will be useful in evaluating the biological function of differential gene expression in the stromal compartment of cancer. It is becoming more clear that the stromal compartment is important to tumor development and progression. It follows that the development of prognostic markers and therapeutic targets in the stromal compartment is a distinct possibility in the future.

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