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Caveolin-1 Mediates Testosterone-stimulated Survival/Clonal Growth and Promotes Metastatic Activities in Prostate Cancer Cells[1]

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ABSTRACT

Previously, we demonstrated that up-regulation of caveolin-1 (cav-1) was associated with prostate cancer metastasis, biochemical recurrence after radical prostatectomy, and androgen insensitivity. The objective of this study was to characterize the regulation of cav-1 by testosterone (T) and to test the effects of cav-1 on prostate cancer cell survival/clonal growth and metastatic activities. Our results demonstrated that T up-regulated cav-1 protein levels in part through transcriptional regulation and significantly enhanced survival of prostate cancer cell lines ABAC3 and LNCaP after serum starvation (>40% and >60% increased viability, respectively) and in an extended clonogenic assay (approximately 4-fold and 6-fold increase in colonies, respectively). Importantly, antisense cav-1 inhibited the survival effects of T in these assay systems. Modest but not high levels of adenoviral vector-mediated cav-1 expression alone also significantly increased viability (>40%) and clonal growth (10-fold increase in colonies) after serum starvation. Analysis of spontaneous metastasis in stably transfected antisense cav-1 mouse prostate cancer cell clones demonstrated reduction of spontaneous lymph node metastasis incidence (13%), spontaneous lymph node metastasis volume (46%), and experimental lung metastasis incidence (40%) compared with vector control cell clones. Surgical castration further reduced spontaneous lymph node metastasis incidence and volume (18% and 28%, respectively) in antisense cancer cell clones, but not in vector control clones. Our studies demonstrate that cav-1 is a downstream effector of T-mediated prostate cancer cell survival/clonal growth and that modest levels of cav-1 can independently promote prostate cancer cell survival/clonal growth and metastatic activities.

INTRODUCTION

Prostate cancer is a continued threat to the lives of tens of thousands of United States men, despite efforts to control this disease through screening of asymptomatic men and the aggressive use of surgery and irradiation therapy for presumed localized disease (1). Unfortunately, many men continue to present with advanced prostate cancer or recur from localized therapy, and there are no curative therapies available for androgen-resistant metastatic disease. Although prostate cancer was shown to be initially responsive to androgen ablation more than 50 years ago (2), there is only minimal understanding at the mechanistic level with regard to the ultimate hormone-resistant state of prostate cancer that is responsible for the exceedingly high mortality rate. Previously, we reported that cav-1[3] levels were elevated in metastatic mouse and human prostate cancer (3) and that cav-1 positivity had independent prognostic value for cancer recurrence after radical prostatectomy (4). Additional studies demonstrated that suppression of cav-1 levels led to reestablishment of androgen sensitivity *in vitro* and *in vivo* and that enforced cav-1 expression could convert androgen-sensitive prostate cancer cells to androgen-insensitive cells (5). Other reports have shown that cav-1 is up-regulated in multidrug-resistant cancer cells, and in some cases, this up-regulation is independent of P-glycoprotein (6–8). More recently cav-1 was shown to suppress c-myc-induced apoptosis in Rat1A and LNCaP cells (9).

cav-1 is the principal component of caveolae, subinvaginations of the plasma membrane and *trans*-Golgi network that have been implicated in sphingolipid-cholesterol transport and signal transduction pathways (reviewed in Refs. 10–13). Under some conditions, cav-1 has been shown to suppress growth of specific cell lines *in vitro* and *in vivo* (8, 14–17), and it has been suggested that cav-1 functions as a tumor suppressor gene (18). However, specific genetic analysis of cav-1 did not support this contention (19).[4] Recent studies have indicated that some genes can manifest seemingly opposing functional activities in a context-dependent fashion. One example is the *bcl-2* gene that can demonstrate pro- or antiapoptotic activities depending on its level of expression (20). These opposing functions may be related to separate bcl-2 protein domains that have been shown to independently mediate growth arrest or survival depending on cell context (21, 22). Additional examples are the *Cox-1* and *Cox-2* genes that have been shown to be up-regulated in numerous human malignancies, but overexpression of these genes can suppress growth and induce apoptosis *in vitro* (23, 24). Recent studies suggest that the growth-suppressive effects of Cox-1 are not related to its enzymatic activities within the prostaglandin synthesis pathway (24). Overall, these results indicate the need to clearly define the regulation, biological activities, and mechanism(s) of action for these multipotential genes within the context of malignant progression. In this report, we demonstrate that cav-1 is a downstream effector of T-mediated survival activities and that modest but not high levels of cav-1 can promote both cell survival and metastatic activities in mouse and human prostate cancer cells.

MATERIALS AND METHODS

Cell Lines and Cell Culture. The various metastatic mouse prostate cancer cell lines were generated from tumors initiated by retroviral transduction of the *ras* and *myc* oncogenes into fetal prostate tissues from p53 homozygous mutants using the mouse prostate reconstitution model (25). The 148-1PA cell line was established from a primary carcinoma, and 148-1LMD was established from a lung metastasis from the same mouse. The ABAC3 and ABAC5 clonal cell lines were derived from 148-1LMD by introduction of an antisense mouse cav-1 cDNA as described previously (5). Similarly, antisense clone BACS4 was derived from 151-2LMC, a lung metastatic clone from a different mouse (5, 25). ABH11, ABH14, and BHS3 are empty vector clones derived from either 148-1LMD or 151-2LMC and used as controls (5). The mouse cell lines were grown in DMEM with 10% FCS. The human prostate cancer cell line LNCaP was obtained from the American Type Culture Collection, grown

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[3] The abbreviations used are: cav-1, caveolin-1; T, testosterone; SFM, serum-free medium; AR, androgen receptor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SFMT, serum-free medium plus testosterone; RSV, Rous sarcoma virus; MOI, multiplicity of infection.

[4] C. Ren, L. Garza, Y. Yuan, W. Tian, and T. C. Thompson, unpublished data.

in RPMI 1640 with 10% FCS, and used at passage 30–60. The appropriate media without serum but with 0.1% BSA were used as SFM. All cells were routinely grown at 37°C with 5% CO_2.

MTT Assay. Subconfluent cells were trypsinized, collected by centrifugation, and washed once with SFM. A single-cell suspension was then seeded at low cell density (~200 cells/well of a 96-well plate) in SFM alone or with T (Sigma Chemical Co., St. Louis, MO; SFMT). After 3 days, viability of the cells was determined by incubation with 0.05 mg/ml MTT (Sigma Chemical Co.) at 37°C for a time period ranging from 2 h to overnight. The viability (viable cells:total cells) was determined by counting blue-stained (viable) cells and total cells microscopically and expressed as relative cell viability by normalization to control (control = 1; in cav-1 induction experiments, T = 0 nM was used as control; in virus infection experiments, uninfected was used as control). Previous experiments demonstrated that under conditions of growth/survival factor depletion and low cell density, proliferation was minimal, and therefore the activities monitored by this MTT assay represent predominately cell viability (5). The viability data are representative of at least three independent triplicate experiments. Error bars show SDs of a triplicate experiment.

ATPLite Assay. ABAC3 (1×10^4 cell/well) and LNCaP (1×10^5 cells/well) cells were seeded in SFM or SFM plus various concentrations of T in 12-well plates. After 3 days, floating cells and trypsin-detached cells were combined and counted with a Coulter Particle Counter (Coulter Corp., Miami, FL). One thousand cells were seeded into each well of a 96-well black culture plate (Packard Instrument Co., Meriden, CT). Cell viability was determined with a luminescent ATP detection kit, Packard ATPLite-M, according to the manufacturer's directions. Light units generated by ATP in each sample were normalized to control (T = 0 nM) and expressed as the relative ATP level. The ATPLite assay was also performed on LNCaP cells infected with an adenoviral vector expressing human sense cav-1 or with control RSV adenoviral vector in 6-well plates as described below. After 2 days in the complete medium postinfection, cells were subjected to growth/survival factor depletion for 3 days in SFM and then collected for ATP determination.

Clonogenic Assay. Cells were suspended at low density in SFM or SFMT (T = 10 nM for ABAC3 cells and 5 nM for LNCaP cells) in 96-well plates as described for the MTT assay. After 3 days, the medium was removed carefully, and the cells were trypsinized and reseeded in 10-cm plates at a density of 10^3 cells/plate with complete medium. After 10–15 days, colonies were stained with 0.05 mg/ml MTT in the culture medium for 30 min, and the number of colonies was counted using Advanced Colony Counting software after capturing the image of each plate with a NucleoVision image analysis system (NucleoTech, Hayward, CA). Adenoviral vector-infected cells were grown in complete medium for 2 days after infection and then subjected to low cell density growth/survival factor depletion for 3 days. Cells were then seeded into 10-cm plates at a density of 10^3 cells/plate in complete medium. Colonies were counted as described above after 3 weeks.

Induction of cav-1 Protein by T. Cells were seeded at a density similar to that used in the viability assay (2.0×10^5 cells/15-cm plate) in SFM or SFMT with varying concentrations of T. After 2 days, cells were scraped from plates and collected by centrifugation. The cell pellets were washed once with PBS and then lysed with TNES lysis buffer [50 mM Tris (pH 7.5), 2 mM EDTA, 100 mM NaCl, 1% NP40, 20 μg/ml aprotinin, 20 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride] on ice for 45 min. Proteins were separated on a 12% SDS- polyacrylamide gel and then transferred electrophoretically onto a nitrocellulose membrane. cav-1 and AR were detected with purified polyclonal cav-1 antibody (SC894) and polyclonal AR antibody (SC-826; Santa Cruz Biotechnology, Santa Cruz, CA). A β-actin monoclonal antibody (A5441; Sigma Chemical Co.) was used to detect β-actin for loading control. All Western blots shown are representative of at least three independent experiments.

Luciferase Assay for the Mouse cav-1 Promoter Reporter. A 721-bp mouse cav-1 promoter sequence was subcloned into the luciferase reporter vector, pGL3-basic (Promega Corp., Madison, WI), to generate a mouse cav-1 promoter-controlled luciferase reporter vector, pGL3-mcav-1-luc (9). One μg of pGL3-mcav-1-luc or pGL3-basic was cotransfected with 0.25 μg of pCMV-β-gal into ABAC3 cells (per well of a 6-well plate) using LipofectAMINE Plus (Life Technologies, Inc., Grand Island, NY) according to the manufacturer's protocol. Three h after lipofection, fresh medium was added, and the FCS concentration was brought to 10%. Twenty-four h later, the cells were trypsinized and washed once with SFM, and a single cell suspension was seeded in SFM or SFMT (T = 20 nM) at low density (2×10^5 cells/10-cm plate). Cells were collected after 24 h, lysed in 50 μl of LucLite substrate buffer (Packard) for 15 min at room temperature, and then diluted to the desired volume with PBS containing 1 mM Mg^{2+} and 1 mM Ca^{2+}. Luciferase assays were performed using the Packard LucLite kit (Packard), and luciferase activities were measured on a TopCount luminescence counter (Packard). β-Galactosidase activity was measured as an internal control for the transfection efficiency using a β-galactosidase assay kit (Promega). Tfx-50 reagent (Promega) was used for the transfection of LNCaP cells. Two μg of pGL3-mcav-1-luc or its control vector, pGL3-basic, were cotransfected with 0.25 μg of pCMV-β-gal into LNCaP cells using 2:1 charge ratio of Tfx reagent:DNA. One h after transfection, 2 ml of fresh SFM or SFMT were added to each well (final concentration of T = 10 nM). The androgen antagonist casodex (1 μM) was also added to the selected SFMT wells. Cells were harvested, and cell lysates were prepared 48 h after transfection. The reporter activity was expressed as relative luciferase activity (light units) by normalization to β-galactosidase activity. The data reported are representative of at least three independent experiments.

Adenoviral Vector-mediated Sense and Antisense Human cav-1 Expression. Recombinant adenoviral vectors containing sense (AdScav-1) or antisense (AdAScav-1) human cav-1 cDNA or control AdRSV without a cDNA were generated as described previously (5, 9). LNCaP cells were seeded at a density of 5.0×10^5 cells/well in 6-well plates. After overnight incubation, the medium was replaced with 1 ml of SFM, and adenoviral vector at different MOIs was added. After 3 h, the medium was removed and replaced with complete culture medium. After 48 h, the cells were trypsinized for MTT assay and for the preparation of protein lysates. For ATPLite and clonogenic assays, the culture medium was replaced with SFM 48 h after infection, and the cells were subjected to growth/survival factor depletion for 3 days before each assay. Expression of cav-1 was also confirmed in adenoviral vector-infected cells by immunostaining with cav-1 antibody as described previously (4).

A double infection with AdAScav-1 was adopted to minimize endogenous cav-1. ABAC3 cells were seeded at 1.0×10^5/well in a 6-well plate and grown overnight. The next day (day 1), cells were infected with the adenoviral-vector at the indicated MOI. A second infection was performed on day 3 (MOI calculations adjusted for increased cell number), followed by another 2-day growth period in complete medium. On day 5, the cells were trypsinized, washed with SFM, and seeded in SFM or SFMT (T = 10 nM) at low cell density as described above for examination of cav-1 protein expression and viability. For the clonogenic assay, SFM- or SFMT-treated cells were detached from a 96-well plate and seeded into 10-cm plates for colony counting as described above.

***In Vivo* Metastasis Analyses.** A panel of mouse stable antisense cav-1 clones (ABAC3, ABAC5, and BACS4) and control vector clones (ABH11, ABH14, and BHS3) established from high cav-1-expressing lung metastatic cell lines [148-1LMD or 151-2LMC (3)] were used for orthotopic injection or tail vein injection into syngeneic 129/SV mice as described previously (26). Each cell clone was injected into eight or nine animals. Orthotopic tumors were established by injection of 5,000 cells, a cell number sufficient to establish a 100% tumor take (26), into the dorsolateral prostate. In some experiments, animals were surgically castrated or received sham surgery 3 days after orthotopic inoculation as described previously (5). Two weeks after orthotopic inoculation, animals were euthanized, the tumor was excised carefully, and the wet weight was recorded. The pelvic and retroperitoneal lymph nodes were excised, placed in formalin, embedded in paraffin, cut into 4–5-μm sections, and stained with H&E for histological examination. The extent of metastasis was assessed quantitatively on the stained slides via computer-assisted image analysis (5). An experimental metastasis assay consisted of the tail vein injection of 50,000 cells. Mice were euthanized after 14 days, and the lungs were weighed and fixed in Bouin's fixative, and visible lung metastases were counted with the aid of a dissecting microscope at ×10 magnification.

All mice were maintained in facilities accredited by the American Association for Accreditation of Laboratory Animal Care, and all experiments were conducted in accordance with the principles and procedures outlined in the NIH Guide for the Care and Use of Laboratory Animals.

Statistical Analysis. Statistical analyses were performed with Statview 5.0 (SAS Institute, Inc, Cary, NC). Significance was determined by ANOVA with Fisher's protected least significant difference.

RESULTS

T Significantly Enhances Cell Survival of Androgen-sensitive Prostate Cancer Cells under Conditions of Growth/Survival Factor Depletion. We initially tested the capacity of T to stimulate viability of both mouse and human prostate cancer cells *in vitro* using both the MTT assay and the ATPLite assay in SFM with various concentrations of T. As expected, T significantly enhanced cell survival of androgen-sensitive prostate cancer cells after growth/survival factor depletion. In both the MTT assay and the ATPLite assay, maximum viability was observed at 10 nM T for ABAC3 mouse prostate cancer cells with a >40% increase in viable cells compared with SFM ($T = 0$ nM; $p < 0.0001$; Fig. 1, *A* and *B*). For the human prostate cancer cell line LNCaP, maximum protection was observed at 5 nM T with a >60% ($P < 0.0001$) increase in viability (Fig. 1, *A* and *B*). To analyze the long-term effects of T-stimulated viability, we extended the viability assay to a modified clonogenic assay (see "Materials and Methods"). The results revealed that long-term T stimulation resulted in a ~4-fold increase in colonies for ABAC3 and ~6-fold increase in colonies for LNCaP (Fig. 1*C*).

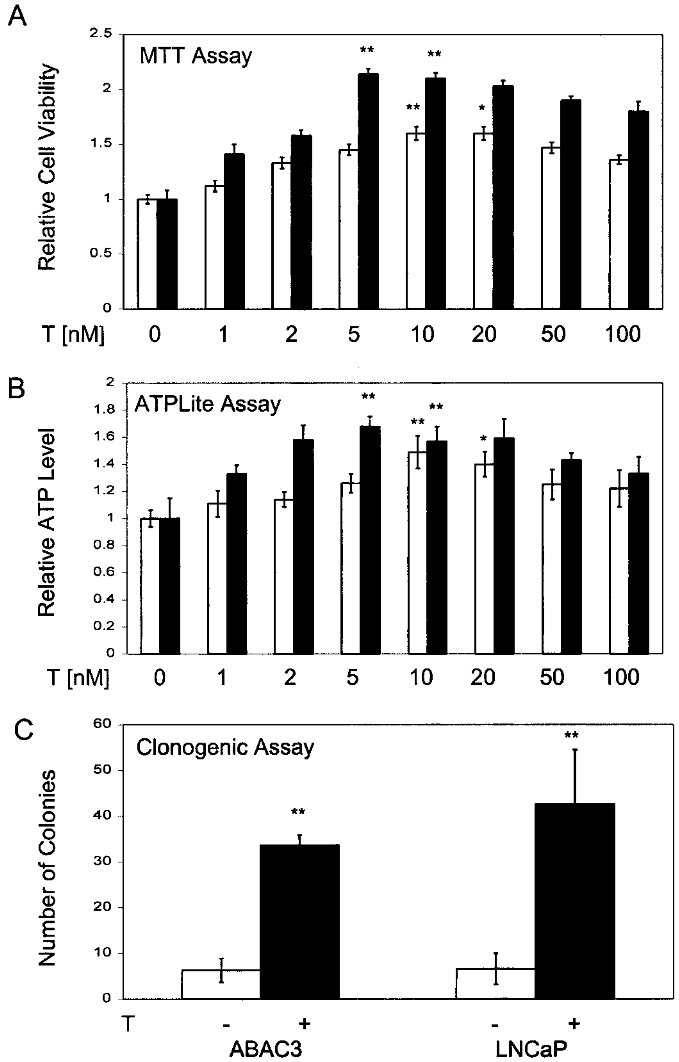

Fig. 1. T significantly enhances cell survival of androgen-sensitive prostate cancer cells under conditions of growth/survival factor depletion. *A*, ABAC3 (□) and LNCaP (■) cells were seeded at low density (200 cells/well in a 96-well plate) and incubated for 3 days in SFM or SFMT with various concentration of T. Cells were stained with MTT, and then living and dead cells were counted microscopically. Viability was expressed as a fraction of viable cells (viable cells:total cells) and normalized to control (control = 1; $T = 0$ nM was used as control). *B*, ABAC3 (□) and LNCaP (■) cells were seeded in SFM or in SFMT with various concentrations of T in 12-well plates (1×10^4 cells/well for ABAC3 and 1×10^5 cells/well for LNCaP) for 3 days. ATP levels were used as an indicator of cell viability. Light units generated by ATP in each sample were normalized by that of the control ($T = 0$) and expressed as the relative ATP level. *C*, ABAC3 and LNCaP cells were pretreated with SFM (□) or SFMT (■; $T = 20$ nM for ABAC3 and 5 nM for LNCaP) in 96-well plates for 3 days, trypsinized, and reseeded in 10-cm plates with complete media for 10–15 days, and then colonies were stained and counted. *Error bar*, SD. *, $P < 0.05$. **, $P < 0.0001$ compared with $T = 0$.

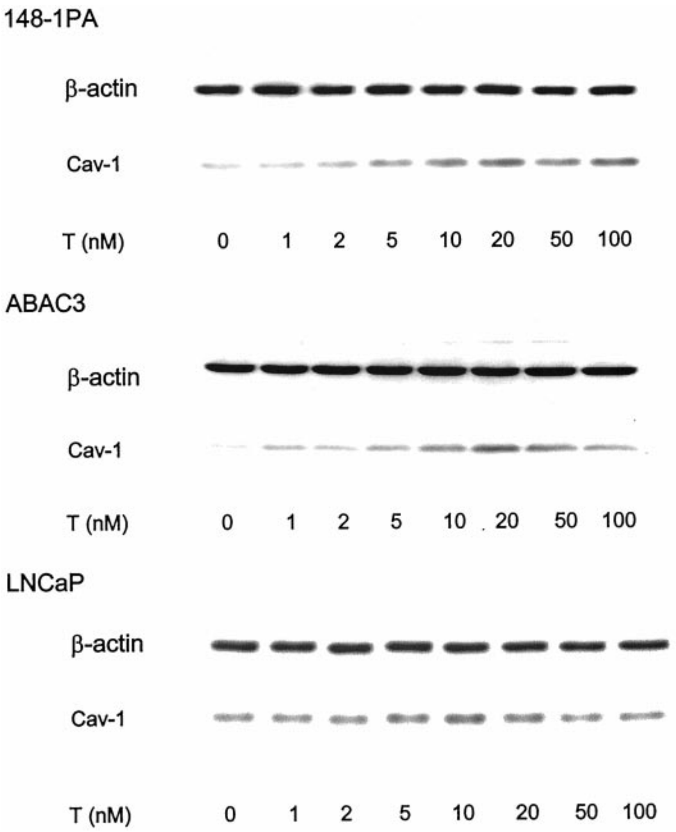

Fig. 2. Dose-dependent induction of cav-1 protein by T. Cells were seeded at low density (2.0×10^5 cells/15-cm plate) and grown in SFM or SFMT with the indicated concentrations of T. Cell lysates were prepared after 2 days, and cav-1 and β-actin were detected on Western blots.

Dose-dependent Up-Regulation of cav-1 Protein by T. To analyze the relationship between T levels and cav-1 expression, we determined the dose-dependent effect of T on cav-1 protein expression in mouse and human prostate cancer cell lines. 148-1PA and ABAC3 cells showed maximal induction of cav-1 protein at 20 nM T (Fig. 2). LNCaP cells demonstrated slightly higher sensitivity to T with maximal induction of cav-1 at 5–10 nM T (Fig. 2). These results are in agreement with and extend the findings of a previous report demonstrating increased cav-1 protein levels after T treatment *in vitro* (27).

Transcriptional Activation of Cav-1 Promoter by T. To determine whether up-regulation of cav-1 by T occurs at the level of transcriptional regulation, we used a luciferase reporter vector under the transcriptional control of the mouse cav-1 promoter, pGL3-mcav-1-luc. The relative activity of the cav-1 promoter was increased more than 2-fold ($P < 0.0001$) by T in ABAC3 cells and approximately 2-fold ($P < 0.0001$) by T in LNCaP cells (Fig. 3). This activity could be blocked by the addition of 1 μM casodex, a direct AR antagonist (Fig. 3), indicating that the up-regulation of cav-1 by T is mediated by the AR.

Antisense cav-1 Significantly Inhibits the Effects of T on Cell Survival and Clonal Growth. Because T enhanced survival and induced cav-1 expression, we asked whether survival activities in-

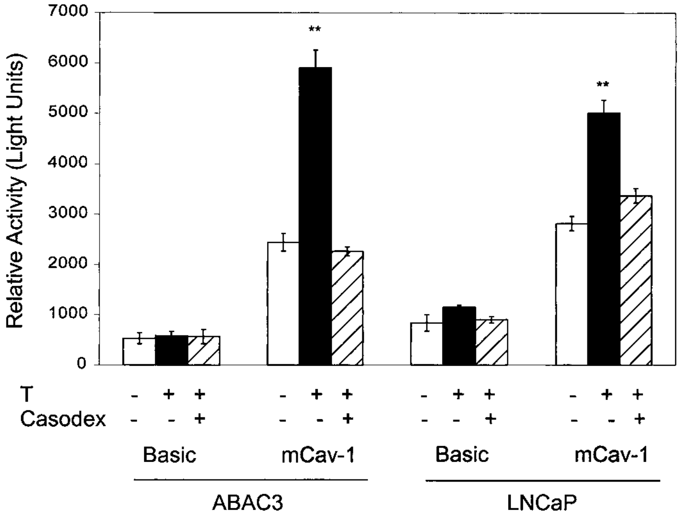

Fig. 3. Transcriptional up-regulation of cav-1 by T. The 721-bp mouse cav-1 promoter luciferase reporter (*mCav-1*) or its control pGL3-basic luciferase reporter (*Basic*) was cotransfected with pCMV-β-gal into ABAC3 or LNCaP cells. After 48 h of treatment in SFM (☐), SFMT (■; T = 20 nM for ABAC3 cells and 10 nM for LNCaP cells), or SFMT with 1 μM casodex (▨) cell lysates were prepared, and reporter activities were determined. *Error bar,* SD. **, $P < 0.0001$ compared with SFM.

duced by T were a consequence of the up-regulation of cav-1. To address this question, we infected ABAC3 cells with AdAScav-1 and then simulated them with T. The results in Fig. 4*A* show that antisense cav-1 abrogated T-stimulated cav-1 up-regulation (compare *AS25* with *Un* or *R25* and *AS50* with *Un* or *R50*). To demonstrate that the suppression of cav-1 up-regulation by T was a specific consequence of direct cav-1 gene antisense suppression rather than the result of a general toxic and/or nonspecific effect on cell viability, we also evaluated the expression of a known androgen-responsive gene, AR (28). The up-regulation of AR by T was not altered in the antisense cav-1-treated cells, confirming the cav-1 specificity of the antisense suppression (Fig. 4*A*). As a consequence of the suppression of cav-1 up-regulation by T, the increased viability stimulated by T was significantly reduced (Fig. 4*B*). At a MOI of 25 with the control AdRSV vector (*R25*), relative cell viability in SFMT was increased to 1.42 relative to SFM ($P = 0.0007$), whereas with antisense cav-1 (*AS25*), relative survival was only 1.1 in SFMT ($P = 0.3214$). Similarly, at a MOI = 50, T-stimulated survival was reduced from 1.5 (*R50*, $P = 0.0003$) to 1.07 (*AS50*, $P = 0.5208$). In clonogenic assays (Fig. 4*C*), T stimulated ~2–3-fold increase colonies in uninfected and RSV controls ($P < 0.0001$), whereas in antisense cav-1-infected, SFMT-treated cells, the colony number was increased by 41% in AS25 ($P = 0.09954$) and 22% in AS50 ($P = 0.3256$). The results of these experiments, together with those described above, demonstrate that cav-1 is a downstream effector of T that is responsible in part for the survival/clonal growth stimulated by T in prostate cancer cells under these experimental conditions.

Cav-1 Expression Promotes Survival and Clonal Growth. To determine whether cav-1 can promote survival in human prostate cancer cells in the absence of T, we infected LNCaP cells with AdScav-1. Dose-dependent expression of human cav-1 was achieved with increasing MOI (Fig. 5*A*). cav-1 expression increased the relative viability of LNCaP cells after growth/survival factor depletion. Interestingly, the maximum survival protection of cav-1 (>40%; $P < 0.0001$) was observed at a MOI = 10 (Fig. 5, *B* and *C*), which corresponded with a moderate level of cav-1 protein (Fig. 5*A*). Viability protection was observed with AdScav-1 at MOI 5–50, but not at MOI = 100 or 200 (Fig. 5, *B* and *C*). These data clearly suggest that cav-1 can induce survival activities when it is expressed at moderate levels; however, when it is expressed at high levels, cav-1 may be toxic to the cells. To determine the long-term effect of cav-1 expression on survival/clonal growth, we also performed a clonogenic assay on infected cells. A significant difference between the effects of AdScav-1 and control vector AdRSV was observed in this extended assay, with ~8–10-fold more colonies for the cav-1 group in a 3-week period (Fig. 5*D*). To confirm expression of cav-1 in the LNCaP cells, we performed immunohistochemical staining that revealed an absence of cav-1 in uninfected LNCaP cells (Fig. 5*E*) but readily detectable expression of cav-1 in LNCaP cells infected with AdScav-1 (Fig. 5*F*).

Reduced cav-1 Expression in Metastatic Mouse Prostate Cancer Cells Results in Suppression of Metastasis *in Vivo*. To test the effects of cav-1 expression on metastatic activities *in vivo*, we analyzed spontaneous (lymph node metastasis from orthotopic tumors) and experimental (tail vein-injected cells) metastasis in a panel of high cav-1-expressing, lung metastasis-derived mouse prostate cancer cell

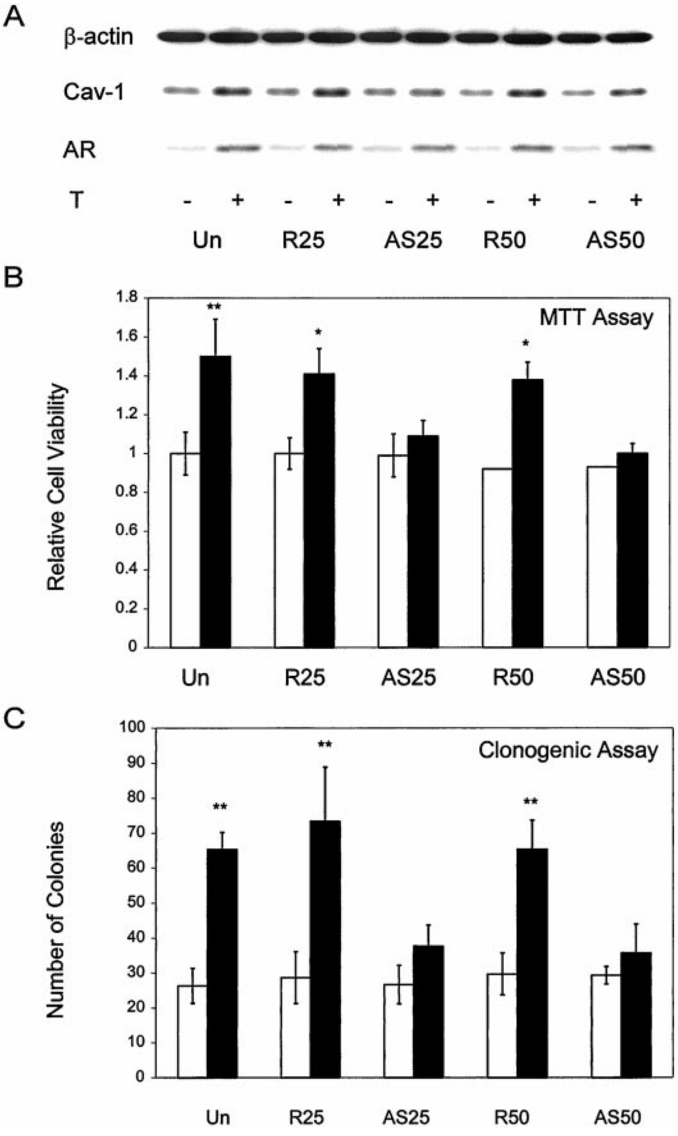

Fig. 4. Antisense cav-1 significantly inhibits survival activities mediated by T. ABAC3 cells were double-infected with adenoviral vectors at a MOI of 25 or 50 and then split into SFM or SFMT (T = 20 nM). *Un,* uninfected; *R,* control vector AdRSV; *AS,* AdAScav-1. *A,* the expression of cav-1 and AR protein was determined by Western blotting after 2 days. *B,* viability of cells as determined by the MTT assay after 3 days in SFM (☐) or SFMT (■). *C,* after 3 days in SFM (☐) or in SFMT (■), cells were trypsinized and reseeded in 10-cm plates for the clonogenic assay. *Error bar,* SD. *, $P < 0.05$. **, $P < 0.0001$ (compared with SFM).

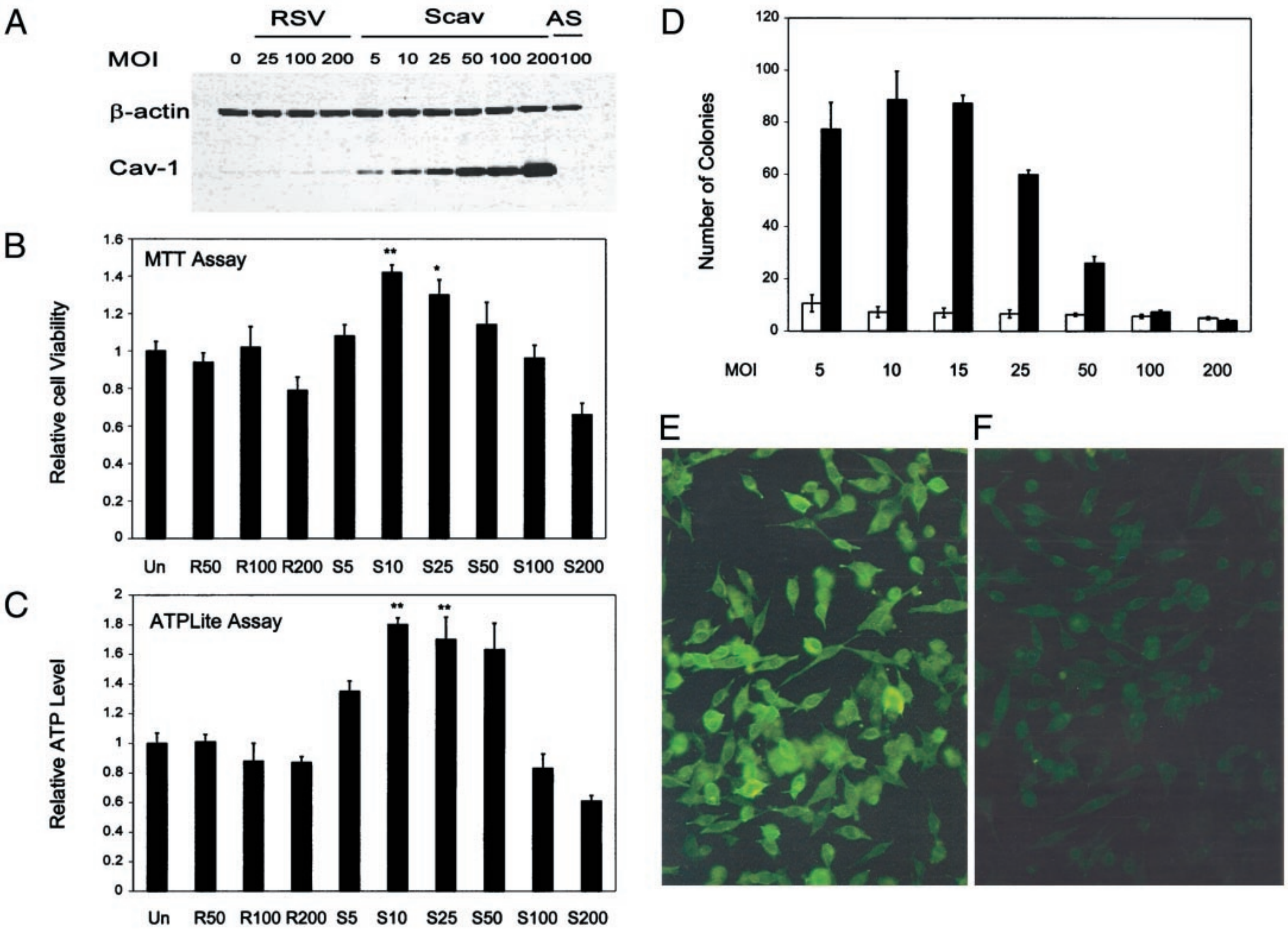

Fig. 5. cav-1 expression alone accounts for a significant component of the survival activities in LNCaP cells. *A*, Western blot showing dose-dependent expression of cav-1 mediated by adenovirus expressing human cav-1. (*RSV*, control vector AdRSV; *Scav*, sense human cav-1 vector AdScav-1; *AS*, antisense human cav-1 vector; the *number after R* or *S*, MOI). A cav-1 dose-dependent viability protection after growth/survival factor depletion was demonstrated by the MTT assay (*B*), the ATPLite assay (*C*), and the clonogenic assay (*D*). *Symbols* in *D*: AdScav-1, ■; vector control AdRSV, □. Immunostaining of cav-1 in AdScav-1-infected LNCaP cells (MOI = 25, *E*) or uninfected LNCaP cells (*F*). *Error bar,* SD. *, $P < 0.05$; **, $P < 0.0001$.

lines stably transfected with antisense cav-1 or control vector (5). The growth of the cell lines as orthotopic tumors was compared with that of vector controls after sham surgery (Fig. 6*A*). The antisense clones were about 10% smaller than the vector clones in the sham-operated animals, but this was not a significant difference ($P = 0.226$). However, a significant (39%; $P < 0.001$) decrease in tumor weight was observed in the antisense clones in castrated animals, but not in the vector clones. In these same animals, the extent of spontaneous lymph node metastasis was evaluated in terms of the number of animals with metastases (incidence) and the relative volume of the metastases as determined by computer-assisted microscopic quantitation (Fig. 6*B*). The antisense clones had less metastatic activity compared with the vector control clones in the sham-operated animals with a 17% decrease in incidence ($P = 0.003$) and a 52% reduction in relative volume ($P < 0.0001$). In castrated animals, there was no difference between the vector control clones and the sham-operated animals; however, the antisense clones had a significantly greater decrease in both incidence and volume of lymph node metastasis than the antisense clones in sham-operated animals (18% and 28%, respectively; $P < 0.001$). To further evaluate metastatic activity, we injected cell clones directly into the tail vein and counted the number of lung metastatic deposits that formed at 2 weeks (Fig. 6*C*). The antisense cav-1 clones had 40% fewer lung metastases as compared with vector control clones ($P < 0.001$).

DISCUSSION

Our previous studies have demonstrated that cav-1 is overexpressed in human and mouse metastatic prostate cancer and that overexpression of cav-1 is an independent predictor for recurrence after radical prostatectomy (3, 4). Subsequently, we demonstrated that cav-1 can protect against androgen withdrawal-induced apoptosis *in vitro* and *in vivo* and that cav-1 can block c-*myc*-induced apoptosis in human prostate cancer cells (5, 9). These studies established a foundation on which to more clearly define a role for cav-1 in prostate cancer progression. In this report, we show that T up-regulates cav-1 expression in prostate cancer cells in part through transcriptional regulation. We further demonstrate that increased cell viability and clonal growth *in vitro* resulting from T treatment is mediated by cav-1 protein and that modest but not high levels of cav-1 alone can independently lead to increased cell viability and clonal growth. Finally, we establish that cav-1 contributes to metastasis *in vivo*.

In the first series of experiments we demonstrated that T induces cav-1 expression in part at the level of transcriptional regulation. We

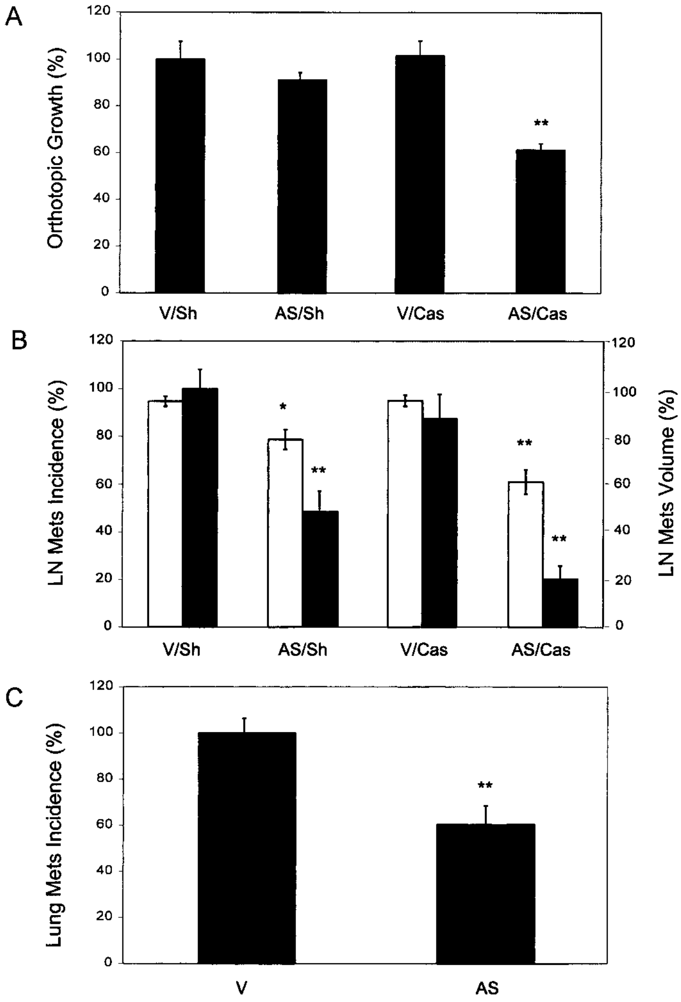

Fig. 6. Antisense cav-1 suppresses prostate cancer metastasis *in vivo. A,* analysis of orthotopic tumor wet weight for vector control clones with sham surgery (*V/Sh*), antisense cav-1 clones with sham surgery (*AS/Sh*), vector control clones with surgical castration (*V/Cas*), and antisense cav-1 clones with surgical castration (*AS/Cas*). Each clone was evaluated in eight or nine animals, and tumor weight was compared with that in the V/Sh group. *B,* the number of animals in each group with microscopic evidence of lymph node metastasis; the incidence for the four groups of animals in *A* is depicted by □. The relative extent of metastatic infiltration of the lymph nodes as measured by computer-assisted analysis was compared with the V/Sh group (■). *C,* number of lung metastatic deposits, counted microscopically, resulting from tail vein inoculation of vector or antisense clones in the experimental metastasis assay. *Error bar,* SE. *, $P < 0.05$; **, $P < 0.0001$.

demonstrated previously that cav-1 is expressed at very low to nondetectable levels in normal prostate epithelium but is expressed focally in prostate cancer, and further increased expression is associated with prostate cancer metastases (3–5). Together, these results suggest that T is responsible, in part, for inducing cav-1 in prostate cancer cells during progression, but it is not yet clear how the cav-1 gene, which is relatively inactive in normal prostate epithelial cells, becomes responsive to T induction. Conceivably, demethylation could play a role, but previous reports have been inconclusive regarding the role of methylation in cav-1 expression in prostate cancer, and additional studies are needed (19).

To study the effects of T and cav-1 expression on prostate cancer cell survival and clonal growth activities *in vitro*, we developed a two-step assay system that mimics specific steps of metastasis *in vivo*. In the first step of this analysis, prostate cancer cells are maintained for 3 days at low density under serum-free conditions, mimicking the reduced growth factor and low density conditions encountered during vascular transit. After this 3-day period, cell viability was analyzed using two independent methods of analysis (the MTT and ATPLite assays). Cells were subsequently seeded into a clonogenic assay, which involved a 2–3-week growth period *in vitro* followed by analysis of colony number. This second step approximates growth at a distal metastatic site and is dependent on continued cell survival. The initial experiments using this assay system demonstrated that T can stimulate cell survival and clonal growth in both mouse and human prostate cancer cells.

Additional experiments in mouse prostate cancer cell lines using adenoviral vector-mediated antisense cav-1 demonstrated that cav-1 induction was responsible, in part, for T-stimulated cell survival/clonal growth *in vitro*. These results are consistent, in general, with the results of our previous studies that demonstrated that elevated cav-1 levels are associated with androgen insensitivity (5). In the absence of T, it is conceivable that other growth factors stimulate cav-1 expression in prostate cancer. Others have shown that polypeptide growth factors can regulate cav-1 expression in NIH-3T3 cells (29). However, to establish a clear correlation between cav-1 expression and androgen-insensitive human prostate cancer, it will be necessary to demonstrate that cav-1 expression is increased in androgen-insensitive disease and to generate experimental support for androgen-independent regulation of cav-1 expression in androgen-insensitive prostate cancer cells. Additional studies in this area are needed.

The substitution of increased cav-1 expression via infection with AdScav-1 demonstrated that modest levels of cav-1 could also maintain viability in the assay systems described above. The results of the clonogenic assay supported and extended the results of the survival analyses, indicating a severalfold increase in the number of colonies in AdScav-1-infected cells compared with that in control Ad-RSV-infected cells in human (LNCaP) prostate cancer cells.

The data presented in this report, together with our previous studies (9), indicate that relatively modest but not high levels of cav-1 expression can lead to increased cell viability consistent with malignant progression. Overall, these results further reconcile previous reports that have shown that high levels of cav-1 can suppress growth in various cell types (8, 14–17). A recent study indicates that although cav-1 is initially down-regulated in colon cancer cells, reexpression of cav-1 is selected for during the development of drug resistance and metastasis (8). At the molecular level, this dichotomy between the role of cav-1 in tumorigenesis and metastasis may be explained in part by specific interactions between phosphorylated cav-1 and downstream signaling molecules (30). Additional studies are required to define the molecular mechanism(s) through which cav-1 specifically promotes survival/clonal growth in prostate cancer cells.

Finally, we generated *in vivo* data that support our *in vitro* studies and demonstrate that experimental reduction of cav-1 expression results in suppression of metastatic activities *in vivo*. Using stable antisense mouse prostate cancer cell clones, our results indicate that both spontaneous and experimental metastatic activities can be significantly reduced by the suppression of cav-1 expression. The results of our castration studies further suggest that the presence of circulating T together with cav-1 can produce synergistic effects that increase metastatic activities. Interestingly, although a reduction of cav-1 levels suppressed metastatic activities it did not suppress primary tumor growth, demonstrating that the effects of cav-1 *in vivo* are metastasis specific in this prostate cancer model.

Our results have demonstrated that T can induce cav-1 expression in part through transcriptional regulation and that cav-1 overexpression is, in part, responsible for T-stimulated survival of mouse prostate cancer cells *in vitro*. Additional studies documented that modest but not high levels of cav-1 can support survival of prostate cancer cells under proapoptotic conditions, *i.e.*, growth/survival factor depletion and low cell density, and promote clonal growth *in vitro*. Finally, analysis of spontaneous and experimental metastasis using stably transfected antisense cav-1 prostate cancer cells confirmed that elevated cav-1 levels contribute to metastasis of prostate cancer cells *in vivo*. Additional studies will be needed to define the molecular mechanism(s) through which cav-1 contributes to prostate cancer metastasis.

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