Functional Analysis of Secreted Caveolin-1 in Mouse Models of Prostate Cancer Progression

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Abstract

Previously, we reported that caveolin-1 (cav-1) is overexpressed in metastatic prostate cancer and that virulent prostate cancer cells secrete biologically active cav-1. We also showed that cav-1 expression leads to prosurvival activities through maintenance of activated Akt and that cav-1 is taken up by other cav-1–negative tumor cells and/or endothelial cells, leading to stimulation of angiogenic activities through PI-3-K-Akt-eNOS signaling. To analyze the functional consequences of cav-1 overexpression on the development and progression of prostate cancer in vivo, we generated PBcav-1 transgenic mice. Adult male PBcav-1 mice showed significantly increased prostatic wet weight and higher incidence of epithelial hyperplasia compared with nontransgenic littermates. Increased immunostaining for cav-1, proliferative cell nuclear antigen, P-Akt, and reduced nuclear p27Kip1 staining occurred in PBcav-1 hyperplastic prostatic lesions. PBcav-1 mice showed increased resistance to castration-induced prostatic regression and elevated serum cav-1 levels compared with nontransgenic littersmates. Intraprostatic injection of androgen-sensitive, cav-1–secreting RM-9 mouse prostate cancer cells resulted in tumors that were larger in PBcav-1 mice than in nontransgenic littersmates (P = 0.04). Tail vein inoculation of RM-9 cells produced significantly more experimental lung metastases in PBcav-1 males than in nontransgenic littersmates (P = 0.001), and in cav-1+/− mice than in cav-1−/− mice (P = 0.041). Combination treatment with surgical castration and systemic cav-1 antibody dramatically reduced the number of experimental metastases. These experimental data suggest a causal association of secreted cav-1 and prostate cancer growth and progression. (Mol Cancer Res 2009;7(9):1446–55)

Introduction

Caveolin-1 (cav-1) is a major structural component of caveolae, which are specialized plasma membrane invaginations that are involved in multiple cellular processes such as molecular transport, cell adhesion, and signal transduction (1, 2). Although under some conditions cav-1 may suppress tumorigenesis (3), cav-1 is associated with and contributes to malignant progression through various mechanisms (4-8). A high level of expression of intracellular cav-1 is associated with metastatic progression of human prostate cancer (9, 10) and other malignancies, including lung (11), renal (12), and esophageal squamous cell cancers (13).

It has been shown that virulent prostate cancer cell lines secrete biologically active cav-1 protein in vitro, and cav-1 promotes prostate cancer cell viability and clonal growth (14-16). The cancer-promoting effects of secreted cav-1 include antiapoptotic activities similar to those observed following enforced expression of cav-1 within the cells (14, 17). In addition to demonstrating cav-1–mediated autocrine activities, we recently showed that recombinant cav-1 protein is taken up by prostate cancer cells and endothelial cells in vitro and that recombinant cav-1 increases angiogenic activities in vitro and in vivo by activating Akt and/or NOS-mediated signaling (18). We also documented significantly higher serum cav-1 levels in men with prostate cancer than in men with benign prostatic hyperplasia (18) and also in patients with elevated risk of cancer recurrence after radical prostatectomy (19).

Expression and secretion of cav-1 by prostate cancer cells is a unique concept in malignant progression. The autocrine and paracrine activities of cav-1 mediated through activation of Akt and/or NOS signaling may lead to pervasive engagement of the local tumor microenvironment, involving but not limited to the proangiogenic activities we previously showed.

One of the goals of this project was to identify specific phenotypic alterations that result from cav-1 overexpression in prostate cancer, to do that, we generated transgenic mice with targeted overexpression of cav-1 in the prostate using the short rat probasin (PB) promoter, yielding PBcav-1 mice.
Immunostaining analysis showed that cav-1 overexpression resulted in prostatic epithelial hyperplasia accompanied by increased levels of P-Akt. PBcav-1 prostatic tissues showed reduced androgen sensitivity under castration-induced regression but not to exogenous testosterone stimulation in vivo. We found not only those prostatic epithelial cells derived from PBcav-1 mouse possessed the capacity to secrete cav-1 but also that serum cav-1 levels in PBcav-1 male mice were higher than the nontransgenic littermates. Lastly, cav-1-expressing, androgen-sensitive mouse prostate cancer cell line, RM-9, was injected intraprostatically to PBcav-1 mice and their nontransgenic littermates to establish orthotopic tumors, or i.v. to was injected intraprostatically to PBcav-1 mice and their nontransgenic littermates. Cav-1 expression in the ventral and dorsolateral prostatic lobes of PBcav-1 mice (2.0- and 1.5-fold, respectively) than in the nontransgenic littermates was significantly greater than that of their nontransgenic littermates (Supplementary Fig. S1A; Fig. 1A). Quantitative reverse transcription-PCR analysis of cav-1 RNA revealed higher cav-1 RNA expression in the prostates with the dorsolateral lobe having the highest expression but not to exogenous testosterone stimulation in vivo.

Results

Cav-1 Expression in PBcav-1 Transgenic Mice

To generate transgenic mice with prostate-specific expression of cav-1, we constructed a vector expressing mouse cav-1 cDNA under the transcriptional regulation of the androgen-responsive short PB promoter (Fig. 1A) that restricts gene expression to the mouse prostate (20, 21). Transgenic mice were generated by pronuclear injection of linearized vector DNA, and three independent founder lines (#8489, #8041, and #8647) were identified by PCR using transgene-specific primers. Preliminary comparative expression analysis of those three transgenic mouse lines using reverse transcription-PCR showed that all three expressed the transgene in the prostate at various levels of specificity (data not shown). Because the PBcav-1 transgenic mouse line #8489 showed the most robust prostate-specific expression of transgene, it was chosen for analysis in the experiments described here.

To analyze the specificity of transgene expression, RNA was isolated from the ventral, dorsolateral, and anterior prostate lobes as well as from multiple mouse tissues and subjected to RT-PCR analysis with the transgene-specific primers P1 and P2 (Supplementary S1A; Fig. 1A). Quantitative reverse transcription-PCR analysis of cav-1 RNA revealed higher cav-1 RNA expression in the ventral and dorsolateral prostatic lobes of PBcav-1 mice (2.0- and 1.5-fold, respectively) than in the nontransgenic mice (Fig. 1B) but relatively low to no detectable levels of cav-1 transgene expressions in the testes, seminal vesicles, spleen, kidney, and liver (Supplementary Fig. S1A).

Cav-1 protein levels in prostatic tissues were analyzed by Western blotting. Cav-1 protein was detected in all lobes of the prostates with the dorsolateral lobe having the highest and anterior lobe the lowest cav-1 levels in the nontransgenic animals. Invariably, the cav-1 level in each lobe was significantly higher in the transgenic mice than in the nontransgenic littermates, although the largest transgenic induction of cav-1 was seen in the ventral lobe (Fig. 1C). The levels of total cav-1 protein in other organs of the PBcav-1 male mice were comparable with those in their nontransgenic counterparts (Supplementary Fig. S1B), confirming the prostate-specific expression of the cav-1 transgene.

Cav-1 Overexpression in Prostatic Tissue Induces Prostatic Hyperplasia

To study the effect of cav-1 overexpression on mouse prostate development, histopathologic and immunohistochemical analysis was done on samples obtained at necropsy from the group of 11- to 13-month-old PBcav-1 transgenic (n = 28) and the nontransgenic littermates (wild-type; n = 29). The wet weight of the whole prostate glands from PBcav-1 transgenic mice was significantly greater than that of their nontransgenic littermates (Fig. 2A). The prostate glands of the transgenic mice also displayed a higher incidence of epithelial hyperplasia than the nontransgenic littermates did (43% versus 6.8%). More strikingly, 18% of the PBcav-1 transgenic mouse prostates were characterized by the presence of focal dysplastic glandular epithelium with disorganized multicellular layers, intraprostatic space formation, and nuclear atypia (Fig. 1D) in addition to the hyperplasia. Focal stromal hyperplasia was also seen in 7% of the transgenic mouse prostates (Fig. 1D). Interestingly, these lesions were focal and restricted to the ventral and dorsolateral lobes and they were not observed in the anterior lobe of the transgenic prostates. The epithelial lesions in the PBcav-1 mice showed strongly positive cytoplasmic staining for both cav-1 and P-Akt proteins (Fig. 1E). In the lesion-free regions of the transgenic mouse prostates and in the nontransgenic mouse prostate tissues, the glandular epithelium was negative or minimally positive for cav-1 and P-Akt staining (data not shown; Fig. 1E). Cav-1 was expressed abundantly in the stromal cells of all prostate tissues. However, in the PBcav-1 prostate tissues, some stromal cells that surround the epithelial lesions tended to have reduced cav-1 expression. The prostatic epithelium also showed increased proliferating cell nuclear antigen (PCNA) staining (Fig. 2B) and reduced nuclear p27kip1 staining in PBcav-1 mice with hyperplastic lesions compared with the normal epithelium of prostate tissues in the nontransgenic mice (Fig. 2C and D), although no significant difference was found in immunostaining between the lesion-free PBcav-1 mouse prostates and that in the nontransgenic mouse prostates (data not shown).

Reduced Androgen Sensitivity in PBcav-1 Prostatic Tissues

PBcav-1 adult male mice and their nontransgenic littermates were subjected to surgical castration, surgical castration plus testosterone pellet implantation, or sham operations. Twenty-one days following these procedures, animals were euthanized, and the whole prostatic tissues were evaluated. Analysis of whole prostatic wet weights showed resistance to castration-induced regression in PBcav-1 mouse prostate tissues, compared with that in the nontransgenic littermates (P = 0.04; Fig. 3A). However, PBcav-1 prostatic tissues showed no significant increase in responsiveness to testosterone stimulation, compared with those from the nontransgenic littermates (P = 0.58; Fig. 3B).
It has been reported that human and mouse prostate cancer cells overexpress and secrete cav-1 protein (14-16) and that patients with prostate cancer show relatively high levels of serum cav-1 (18). To test the secretory potential of the mouse prostate gland, we performed 24-hour organ cultures of prostates collected from PBcav-1 mice and nontransgenic littermates. Western blotting analysis of the organ-culture medium from PBcav-1 mice prostates revealed considerably higher cav-1 protein secretion compared with those of nontransgenic mice prostates (Fig. 4A). We further measured serum cav-1 in PBcav-1 mice, in their nontransgenic littermates, and in cav-1−/− and cav-1+/− (22) adult male mice. Serum cav-1 levels were significantly higher in PBcav-1 transgenic male mice than in the nontransgenic mice, and as expected, serum cav-1 levels were lower in cav-1−/− adult male mice than they were in genetically matched cav-1+/− adult male mice (Fig. 4B).

Greater RM-9 Experimental Metastasis and Orthotopic Prostate Tumor Growth in PBcav-1 Mice

To test the effect of secreted cav-1 on prostate cancer cell growth and metastasis formation in vivo, the androgen-sensitive, cav-1–secreting RM-9 mouse prostate cancer cells (23, 24) were injected into adult male PBcav-1 mice and their nontransgenic littermates through two different routes: intraprostatically (dorsolateral prostate) and i.v. (tail vein). Direct dorsolateral prostate injection of RM-9 cells formed significantly larger orthotopic prostate tumors in PBcav-1 mice than in their nontransgenic littermates after 21 days (Fig. 5A and B). The result is consistent with those from our previous study that showed RM-9 cells formed significantly larger orthotopic tumors in cav-1+/− than in cav-1−/− male mice (18).

For experimental metastasis model, RM-9 cells were injected into the tail vein of PBcav-1 mice and their nontransgenic littermates. After 21 days, the mice were euthanized, and the lung tumor nodules on the lung surface were
counted, and also the sizes of the tumor nodules were measured to evaluate the lung tumor burden. Both the numbers and sizes of the tumor nodules in the PBcav-1 mice were significantly greater than they were in the nontransgenic hosts (Fig. 5A, C, and D). Representative pictures of the lung tumor nodules from both PBcav-1 and their nontransgenic littermates are shown in Fig. 5C. PCNA and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) immunostaining on the lung tumor nodules from PBcav-1 mice, and their nontransgenic littermates were done to compare the proliferative and apoptotic activities. Although PCNA immunostaining was relatively higher in the RM-9 metastatic nodules from PBcav-1 mice, the difference from that in nontransgenic littermates was not statistically significant (data not shown). However, positivity of TUNEL immunostaining was significantly less in the RM-9 lung tumor nodules from PBcav-1 mice than that in the nontransgenic littermates (Fig. 5E).

Up-Regulation of Cav-1, P-Akt, and Vascular Endothelial Growth Factor in PBcav-1 Lung Tumor Nodules

To study molecular pathways associated with reduced apoptosis in lung tumor nodules from PBcav-1 mice, we analyzed the expression of cav-1 and its downstream protein target P-Akt, which is involved in cell proliferation and apoptosis (25-27), and that of the Akt-regulated vascular endothelial growth factor (VEGF; refs. 28-30) using Western blotting. Both normal lung tissue taken from lungs that harbored RM-9 lung tumor nodules and RM-9 lung tumor nodules from PBcav-1 mice and their nontransgenic littermates were microdissected, lysed, and analyzed by immunoblotting.

There were no significant differences between the levels of cav-1, P-Akt, total Akt, and VEGF immunostaining in the PBcav-1 mice and those in their nontransgenic littermates for the normal lung tissues (Fig. 6A and C). However, in the lung tumor nodules isolated from PBcav-1 mice, the expression levels of cav-1, P-Akt, and VEGF were significantly up-regulated relative to the tumor lung nodules from nontransgenic littermates (Fig. 6B and C). We also analyzed serum VEGF levels in metastasis-bearing PBcav-1 mice and their nontransgenic littermates. Serum levels of VEGF in PBcav-1 mice bearing RM-9 lung metastases were significantly higher than in the tumor-bearing nontransgenic littermates or in nontumor bearing PBcav-1 mice. However, no difference was found between the nontumor bearing mice of both types (Fig. 6D).

To detect any association between relatively high-serum VEGF levels and the number of lung metastases in PBcav-1 mice and their nontransgenic littermates, we performed regression analysis. A significant correlation between serum VEGF level and the number of experimental lung metastases was observed in PBcav-1 mice (correlation coefficient, 0.639; \( P = 0.0268 \)) but not in the control mice (data not shown). These data suggest that high serum level of VEGF is not due exclusively to the host associated effect but rather reflects an interaction between the host and the presence of metastatic lung tumor nodules.

Suppression of Experimental Metastasis by Combined Androgen Deprivation and Cav-1 Ab Treatment

To analyze the effects of testicular androgens and/or serum cav-1 on experimental metastastic activities, surgical castration and i.p. cav-1 Ab were administered to cohorts of four different genotypes of adult male mice—PBcav-1, nontransgenic littermates, cav-1\(^{-/-}\), and cav-1\(^{+/+}\) (20). These four cohorts, which showed various serum cav-1 levels (Fig. 4B), were injected with RM-9 prostate cancer cells via tail vein. Two days after the tumor cell injections, the mice were randomized and treated with either sham + HBSS, castration + HBSS, rabbit IgG, cav-1 Ab, or castration + cav-1 Ab (Fig. 7A). The treatments were given i.p. every other day for 21 days. At the end of the time course, the number of lung tumor nodules were scored and compared among the treatment groups for each mouse genotype. Control rabbit IgG treatment produced similar results as the control sham + HBSS treatment (\( P = 0.31–0.68 \)) in all four groups.
mouse genotypes. Compared with sham + HBSS treatment, surgical castration resulted in significant suppression in the number of lung tumor nodules in all four mouse genotypes ($P = 0.0002$). Interestingly, the cohort of $cav-1^{-/-}$ mice showed the greatest reduction in the lung tumor nodules by castration treatment (85% reduction from sham + HBSS) among the four genotypes. As expected, the highest numbers of lung tumor nodules were found in PBcav-1 mice (five times more than $cav-1^{-/-}$ mice), and this group benefited most from cav-1 Ab treatment (83% reduction in sham + HBSS). Although, extensive lung tumor nodule formation occurred in the PBcav-1 sham + HBSS control treatment group [number of lung tumor nodules ($n$) = 20.3], the PBcav-1 cav-1 Ab treatment group showed a significantly reduced number of lung tumor nodules ($n = 3.4$), almost to the level found in $cav-1^{-/-}$ Ab treatment group ($n = 2.2$). Generally, suppression of lung metastases by cav-1 Ab treatment was much more extensive ($P < 0.0001$) than suppression by castration treatment in all the genotypes of the mice except in the cohort of $cav-1^{-/-}$ mice (Fig. 7B). However, the difference in actual number of tumor lung nodules between castration and cav-1 Ab treatment for $cav-1^{-/-}$ mice is marginal and not statistically significant ($n = 0.6$ versus $2.2; P = 0.06$). The combination of androgen deprivation by castration and cav-1 Ab treatment resulted in suppression of experimental metastasis similar to the suppression produced by cav-1 Ab treatment alone in all four genotype cohorts.

**Discussion**

Here, we report generation and characterization of PBcav-1 transgenic mice, which express prostate-specific cav-1 under the transcriptional regulation of the short PB promoter. The transgenic mice expressed relatively high levels of cav-1 in prostatic tissues, which was associated with the development of prostatic epithelial hyperplasia. Overexpression of cav-1 led to stabilization of P-Akt, reduced nuclear p27Kip1 staining, and increased PCNA staining in the hyperplastic prostatic epithelia of male PBcav-1 animals (Figs. 1 and 2). This up-regulation of P-Akt and reduction in nuclear p27Kip1 is consistent with our previous data demonstrating a novel mechanism of Akt stabilization mediated through cav-1 binding to and inhibition of protein phosphatase 2A (17, 31). It is of interest to note that these cav-1–mediated activities are associated with epithelial hyperplasia and epithelial atypia but not with prostate cancer and thus the data suggest additional oncogenic pathways are required for the transformation of prostatic epithelium.

We further characterized the responses of the prostatic tissues to androgen deprivation and stimulation through the use of surgical castration and exogenous testosterone stimulation in vivo. The results indicated that prostatic
tissues of PBcav-1 transgenic mice were refractory to castration-induced regression but were not overly sensitive to the stimulatory effects of exogenous testosterone relative to those in their nontransgenic littermates (Fig. 2). Although these data are consistent with increased levels of P-Akt, further studies are necessary to test whether stabilization of P-Akt by cav-1 underlies these aberrant responses to hormonal manipulation.

Recently, we reported that secreted cav-1 protein is taken up by prostate cancer cells and cancer-associated endothelial cells and through this process stimulates Akt-mediated angiogenic activities (18). To better understand the biological and potential clinical significance of prostate cancer cell–derived secreted cav-1, we tested the capacity of PBcav-1 mouse prostatic tissues to secrete cav-1 and to produce relatively high serum cav-1 levels. What we found was that PBcav-1 transgenic mice prostate indeed secreted more cav-1 and had higher serum cav-1 levels than their nontransgenic littermates (Fig. 4). These data led us to reason that the relatively high serum cav-1 level derived from PBcav-1 mouse prostatic tissues could stimulate the growth of local prostate tumors and the development of distant prostate cancer metastases.

As postulated, PBcav-1 mice produced significantly larger tumors from direct dorsolateral prostate injection of RM-9 mouse prostate cancer cells compared with their nontransgenic littermates after 21 days. Given the prosurvival and proangiogenic activities of cav-1 within the context of prostate cancer and other malignancies (4-8), it is interesting to speculate whether cav-1 secretion from premalignant cells contributes to a “field effect.” Further studies may reveal a role for cav-1 expression in the development of hyperplastic and/or premalignant prostatic lesions, including the establishment of a tumor-promoting environment.

The results of our experimental metastasis analyses using RM-9 cells also showed that host-associated factors promoted the growth of experimental lung metastases in PBcav-1 mice compared with that in the nontransgenic littermates (Fig. 5). Lower apoptotic activity and higher P-Akt levels in the PBcav-1 mice compared with those in the nontransgenic littermates (Fig. 5E and 6E–C) are consistent with the uptake of serum cav-1 and with cav-1–mediated stabilization of P-Akt levels in metastatic cells. Although a statistically significant increase in tumor-associated angiogenesis was not documented in metastases in the PBcav-1 mice compared with those in the nontransgenic littermates, we did observe greater VEGF expression in the metastases and greater serum VEGF levels in the tumor-bearing PBcav-1 mice than in the tumor-bearing nontransgenic littermates or in the mice of the both types that were not exposed to tumor (Fig. 6D).

It is well-established that various tumor growth factors are expressed and secreted under the regulation of intracellular PI3-K-Akt signaling (25-27, 32, 33). In cancer cells, activated

FIGURE 5. Elevated levels of cav-1 in prostate and serum stimulate prostate cancer progression in PBcav-1 mice. A. Diagram of orthotopic prostate or experimental metastasis experiment (tail vein injection) using mouse prostate cancer cell line RM-9 in PBcav-1 and nontransgenic male mice. B. Increased orthotopic RM-9 prostate tumor weight in PBcav-1 mice (TG) compared with nontransgenic littermates (WT). C. Representative micrographs of RM-9 lung metastases in nontransgenic littermates and PBcav-1 mice. D. Increased number and size of RM-9 metastatic lung nodules in PBcav-1 mice compared with nontransgenic littermates. E. Histologic analysis of experimental lung metastatic lesions in nontransgenic and PBcav-1 mice. Top, representative example of TUNEL staining. TUNEL-positive staining (AI, apoptotic index) is reduced in metastases of PBcav-1 mice compared with the metastases in nontransgenic animals. Differences in PCNA or CD31 (MVD)-positive staining were not significant between these two groups of mice (data not shown).
Akt has been reported to be a positive regulator of tumor angiogenesis factors such as VEGF (28-30). It is important to note that higher than normal expression of VEGF may have clinical significance in prostate cancer (34). We hypothesize that cav-1-mediated increases in the level of intracellular P-Akt trigger enhanced expression of this angiogenic factor. Increased levels of intracellular cav-1 in the metastatic RM-9 nodules that developed in PBcav-1 mice may be due to increased uptake of circulating serum cav-1 compared with RM-9 nodules that develop in nontransgenic littermates. Further studies may reveal important associations between cav-1 overexpression, stabilization of P-Akt, and VEGF overexpression and secretion.

In our previous analysis of serum cav-1 levels, we found significantly higher serum cav-1 levels in men with prostate cancer than in normal men and men with benign hyperplasia (18, 19), raising the possibility that serum cav-1 may also affect the metastatic potential of human prostate cancer. Together with the possibility of a field effect mediated by secretion of cav-1 in hyperplastic and/or premalignant prostatic tissues, the presence of substantial levels of serum cav-1 in the absence of malignancy may be considered a possible risk factor for the development and progression of prostate cancer, and further studies in this area are warranted.

The results of our experimental metastasis analyses specifically showed that the secretion of cav-1 into the circulation promoted the growth of experimental lung metastases in PBcav-1 mice (Fig. 7). Although RM-9 cells are cav-1–secreting cells, our results clearly show that host-derived serum cav-1 contributes to metastatic activities once the cells are established at an attached site in the experimental metastasis model system. The PBcav-1 cohort generated two to five times more lung metastases than the other three genotype cohorts, but after the systemic cav-1 Ab treatments, the number of the lung nodules was brought down close to the cav-1−/− cohort level. Surgical castration treatment also resulted in significant reduction in the number of tumor lung nodules in all genotype cohorts. However, the data showed that in general, cav-1 Ab treatment was more effective than surgical castration in suppression of experimental metastasis (Fig. 7). Combination treatment with surgical castration plus cav-1 Ab was remarkably effective in suppressing metastasis, but the suppression achieved was not statistically different than the effects of cav-1 Ab treatment alone (P = 0.69–0.89 except in case of cav-1−/−). But because actual number of metastasis was very low in both treatment cases, it is conceivable that further studies using additional models would reveal potential therapeutic advantages associated with this.
combination approach. Overall, these data suggest the possible convergence of cav-1 expression and secretion, and androgen-receptor signaling. This notion is consistent with our initial report regarding cav-1 expression in prostate cancer (35), which defined an important relationship between cav-1 expression, androgen receptor expression, and androgen sensitivity in prostate cancer cells.

In summary, we have generated a novel transgenic mouse, PBcav-1, and showed that cav-1 overexpression leads to epithelial prostatic hyperplasia accompanied by increased levels of P-Akt, reduced nuclear p27Kip1, and increased serum cav-1 levels. We exploited the induction of secreted cav-1 without the complications of active malignancy to test the effects of localized cav-1 secretion and serum cav-1 on orthotopic prostate cancer growth and experimental metastasis, respectively. We showed that RM-9 cells formed significantly larger orthotopic prostate tumors in PBcav-1 mice than in their nontransgenic littersmates. Using this model of androgen-sensitive metastatic prostate cancer, we also tested the effects of surgical castration and/or cav-1 Ab treatment on the development of experimental lung metastases. The data showed that host-derived cav-1 is causally associated with experimental metastasis and validated systemic cav-1 Ab treatment as a potential therapy for prostate cancer. Further experiments should test the feasibility of targeting systemic cav-1 protein as well as combination treatment of cav-1 Ab and antiandrogens as novel therapeutic strategies.

Materials and Methods

Generation and Genotyping of Cav-1-Expressing Transgenic Mice

Plasmid carrying PBcav-1 transgene was generated using our previously constructed MMTVcav-1 plasmid containing mouse mammary tumor virus promoter, rabbit β-globin splice sequences (part of exon I, intron I, and exon II) 687-bp mouse cav-1 cDNA (35), and bGH-polyadenylation sequence (36). The mouse mammary tumor virus promoter was excised by BamHI and replaced by a short rat PB promoter fragment (−426/+28) that was lifted from SK-PB/SV40Tag plasmid by SalI digestion (20). To make the vector and promoter DNA compatible for ligation, GA and TC base fill-ins were done on BamHI and SalI fragments, respectively. Recombinant PBcav-1 plasmid DNA was purified, and the integrity of the PBcav-1 transgene was confirmed by sequencing. The resulting PBcav-1 plasmid was linearized by KpnI and NotI, and the 2.2-kb PBcav-1 transgene fragment, devoid of vector sequences, was microinjected into fertilized C57B6 mouse embryos.

Purified transgene DNA was injected into the pronucleus of a fertilized C57BL6/J embryo according to the standard technique used in the Transgenic Core facility at Baylor College of Medicine.

The founder transgenic mice were screened for transgene presence and transmission by using PCR with forward 5′-CCATGTTGCTGCTTCTC-3′ and reverse 5′-ATCGTAGACACAAGCGGTA-3′ primers using the following conditions: 95°C × 2 min, 94°C × 30 s, 55°C × 30 s, and 72°C × 30 s, for 35 cycles.

Mice were housed under specific pathogen-free conditions in facilities accredited by the American Association for Accreditation of Laboratory Animal Care. Three to 13-mo-old mice were used throughout this study unless otherwise indicated in the text. All experiments were conducted in accordance with the principles and procedures outlined in the NIH’s Guide for the Care and Use of Laboratory Animals.

RNA Isolation and Analysis

Total RNA was isolated from microdissected mouse tissues by using a RiboPure RNA purification kit from Ambion. The
cDNA was generated using 1.6 μg of total RNA, and RT reactions were carried out using a High Capacity cDNA Archive kit (Applied Biosystems) according to the manufacturer’s protocol. Reverse transcription-PCR was done as previously described (37) using PBcav-1 transgene-specific primers P1: 5′-CTTTGTCAGTGGTCCAGATA-3′ and P2: 5′-CGTCTCGTTGAG-ATGTCT-3′. Quantitative real-time PCR analysis of cav-1 expression was done using the Universal PCR Master Mix and TaqMan Gene expression assay for mouse cav-1 (Applied Biosystems).

Analysis of Androgen Sensitivity in PBcav-1 Prostatic Tissues
PBcav-1 adult male mice and their nontransgenic littersmates were subjected to surgical castration, surgical castration plus implantation of testosterone propionate in silastic tubing pellets, or sham operations as previously described (35). Three weeks following these procedures, animals were euthanized, and whole prostatic tissues were evaluated. Wet weights of the whole prostates from PBcav-1 mice and their nontransgenic littersmates were compared for each experimental condition.

Animal Models
Orthotopic Prostate Tumors. Subconfluent mouse RM-9 prostate cancer cells were prepared and injected to PBcav-1 and nontransgenic mice as described previously (22). Cells (5 × 10⁶) were injected directly into dorsolateral prostates of the mice, and after 21 d, the tumors were excised and weighed.

Experimental Metastasis. Cells (5 × 10⁶) of RM-9 were injected via tail vein as described previously (22). After 21 d, mice were euthanized, and serum and tumor lung nodules were collected. The number and size on the long axis of lung tumor nodules were measured with help of dissecting microscope. Lung tumor nodules that were 2 to 3 mm long on the longest axis and normal lung tissues were microdissected and snap frozen in liquid nitrogen and stored at −80°C. For histologic examinations, the lung tissues of interest were snap frozen for cryostat sectioning or were fixed in 10% phosphate-buffered formaldehyde, 1 mmol/L EGTA, 1% sodium deoxycholate, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na₂VO₃, and 1 μg/mL of leupeptin. The lysates were centrifuged for 10 min at 20,000 × g and 4°C to remove any debris and insoluble material. The resulting samples were stored at −80°C, and protein concentrations were determined using a protein assay kit (Bio-Rad).

Suppression of Experimental Metastasis
Four different genotypes of adult male mice-PBcav-1, nontransgenic littersmates, cav-1−/−, and cav-1+/+ (20) were injected with RM-9 prostate cancer cells via tail vein as described above. Each cohort of four genotypically different mice were randomly divided into five treatment groups: sham + HBSS, castration + HBSS, rabbit IgG, cav-1 Ab, and castration + cav-1 Ab. Mice that were included in the castration treatment groups underwent surgical castration 1 wk before the tumor cell injection. Mice that were included in the Ab treatment groups were treated as appropriate with 100 μL of HBSS (control), 10 μg/100 μL rabbit IgG (Santa Cruz Biotechnology), or 10 μg/100 μL cav-1 Ab (Santa Cruz Biotechnology) every other day for 21 d as previously described (13). At the end of the time course mice were euthanized, and the number of lung tumor nodules were counted with the aid of a dissecting microscope as described previously (13).

Mouse Cav-1 and VEGF Levels in Serum Samples
Serum samples were assayed for mouse cav-1 by ELISA, as previously described (18, 19). For the serum VEGF assay, a mouse VEGF ELISA kit was purchased from R&D Systems. The serum samples were diluted (1:2 in the specific diluent) and tested according to the manufacturer’s instructions. The kit recognizes both 164- and 120-amino acid isoforms of mouse VEGF. The minimum detectable concentration is <3 pg/mL.

Preparation of Tissue Extracts and Conditioned Medium
Tissues were homogenized in ice-cold lysis buffer [20 mmol/L Tris (pH 7.5) containing 150 mmol/L NaCl, 1 mmol/L Na₂EDTA, 1 mmol/L EGTA, 1% NP40, 1% sodium deoxycholate, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na₂VO₃, and 1 μg/mL of leupeptin]. The lysates were centrifuged for 10 min at 20,000 × g and 4°C to remove any debris and insoluble material. The resulting samples were stored at −80°C, and protein concentrations were determined using a protein assay kit (Bio-Rad).

For preparation of prostate organ culture-conditioned medium, the prostate tissues from individual mice were collected and cut into ~1-mm pieces and washed thrice with ice-cold PBS. After the organ pieces were incubated in 0.5 mL of serum-free medium for 24 h at 37°C, the medium was collected and centrifuged at 1,000 × g and then at 100,000 × g to minimize debris contamination. The protein in the medium was precipitated by application of trichloroacetic acid and then the precipitate was washed with acetone and redissolved in 30 μL of lysis buffer.

Western Blot Analysis
Protein extract from mouse tissues (50 μg) or 15 μL of the prepared conditioned medium was mixed with 6× loading buffer and separated on 12% SDS-PAGE. The separated proteins were transferred to nitrocellulose membranes and were blotted with specific Ab for 12 to 18 h at 4°C. Horseradish peroxidase–conjugated secondary antibodies and an enhanced chemiluminescence substrate kit (Pierce Biotechnology) were used for detection of specific proteins. Densitometry of the bands was done with the NIH IMAGE software program. For quantitative analyses, the intensity of protein bands was normalized to the intensity of β-actin in the same sample.

Rabbit polyclonal antibodies against cav-1 (sc-894) and VEGF (sc-507) were obtained from Santa Cruz Biotechnology. Monoclonal Ab against PKB/ Akt (clone 55) was purchased from BD Biosciences, and rabbit polyclonal Ab against phospho-Akt (Ser473) was from Cell Signaling Technology. Monoclonal anti-β-actin (clone AC-15) was from Sigma-Aldrich.

Histologic and Immunohistochemical Procedures
Prostate tissues from PBcav-1 transgenic mice and their nontransgenic littersmates or from lung metastases these mice developed were fixed in 10% formalin and processed for routine paraffin embedding and sectioning. Histologic features of the tissues were evaluated on H&E-stained sections. Apoptotic and proliferative activities in the tissues were investigated using the TUNEL technique and PCNA immunostaining, respectively, following the procedure previously developed (38). The tissues were also immunostained with antibodies to cav-1 (Santa Cruz Biotechnology), P-Akt
(Ser473; Cell Signaling), and p27<sup>Kip1</sup> (a generous gift from Dr. Xin Lu, Ludwig Institute for Cancer Research, Oxford Branch, Oxford, United Kingdom) using the avidin-biotin-peroxidase complex procedure and an ABC kit (Vector Laboratories). Immunolabeling was assessed quantitatively via computer-assisted image analysis of 20 to 30 randomly selected microscopic fields at >200 magnification. The data were recorded as apoptotic index, which is the number of apoptotic bodies per 1,000 epithelial cells or the nuclear labeling rate for p27<sup>Kip1</sup> or PCNA staining.

**Statistical Analyses**

All values are expressed as means ± SEM. Statistical analyses were carried out using the two-tailed Student’s unpaired t test and, for more than two groups, by using ANOVA followed by Fisher’s protected least significant difference analysis.

The correlation between serum VEGF level and the number of experimental lung metastases was evaluated with the simple regression analysis. Differences were considered statistically significant when the P value is <0.05. All analyses were done using Statview 5.0 software (SAS Institute).

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**References**

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