

ADAM15 Supports Prostate Cancer Metastasis by Modulating Tumor Cell–Endothelial Cell Interaction

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Abstract

Using human tumor and cDNA microarray technology, we have recently shown that the ADAM15 disintegrin is significantly overexpressed during the metastatic progression of human prostate cancer. In the current study, we used lentiviral-based short hairpin RNA (shRNA) technology to down-regulate ADAM15 in the metastatic prostate cancer cell line, PC-3. ADAM15 down-regulation dramatically attenuated many of the malignant characteristics of PC-3 cells *in vitro* and prevented the s.c. growth of PC-3 cells in severe combined immunodeficient (SCID) mice. By inhibiting the expression of ADAM15 in PC-3 cells, we showed decreased cell migration and adhesion to specific extracellular matrix proteins. This was accompanied by a reduction in the cleavage of N-cadherin by ADAM15 at the cell surface. Fluorescence-activated cell sorting analysis revealed reduced cell surface expression of the metastasis-associated proteins α_v integrin and CD44. Furthermore, matrix metalloproteinase 9 secretion and activity were abrogated in response to ADAM15 reduction. In an *in vitro* model of vascular invasion, loss of ADAM15 reduced PC-3 adhesion to, and migration through, vascular endothelial cell monolayers. Using an SCID mouse model of human prostate cancer metastasis, we found that the loss of ADAM15 significantly attenuated the metastatic spread of PC-3 cells to bone. Taken together, these data strongly support a functional role for ADAM15 in prostate tumor cell interaction with vascular endothelium and the metastatic progression of human prostate cancer. [Cancer Res 2008;68(4):1092–9]

Introduction

The dissemination of localized prostate cancer to distant tissues such as the bone, lung, and liver represents a prominent health care burden in the aging adult male population (1). The underlying mechanisms that promote and support the metastatic spread of prostate cancer remain vague. It is clear that fundamental processes, such as cellular detachment, suppression of apoptosis, vascular intravasation, and angiogenesis are essential to the spread of cancer cells and their growth at distant sites (2). One family of proteins supporting malignant progression is the zinc-binding matrix metalloproteinases (MMP) that degrade components of the extracellular matrix, such as collagen, laminin, and fibronectin (3). However, the MMPs are not the only metalloproteinases implicated in human tumorigenesis.

The less studied ADAM (a disintegrin and metalloproteinase) family of membrane metalloproteinases may also support tumor progression by modulating key physiologic cell surface proteins such as membrane-anchored growth factors and their complementary receptors, cell adhesion molecules, and integrins (4). The ADAM family is composed of 40 members, of which 13 members are catalytically active. These catalytically active members contain a metalloproteinase domain that is implicated in growth factor shedding and extracellular matrix (ECM) degradation. ADAM17 has been reported to process pro-tumor necrosis factor (TNF)- α , TNF receptors, interleukin-6 receptor, and amphiregulin (5, 6), and ADAM9, ADAM10, ADAM12, and ADAM17 are believed to cleave the epidermal growth factor receptor (EGFR) ligand, HB-EGF, leading to the transactivation of EGFR (7). ADAM family members have also been shown to cleave different cell adhesion molecules such as N-cadherin (8). Expression of N-cadherin in human tumor cells is usually associated with malignant progression (9). Cell surface N-cadherin supports heterotypic interaction between migrating cancer cells and other cells of the tumor microenvironment such as fibroblasts and endothelial cells to support cancer cell migration, invasion, and metastasis.

Another physiologic feature of ADAMs uses the disintegrin domain to mediate integrin-ECM interactions. Several ADAM family members, including ADAM15, have been shown to bind to specific integrin molecules, suggesting roles in cellular adhesion and tumor cell-ECM interactions (10). Although the ADAMs have been the focus of rigorous research in many biological processes, such as oocyte fertilization, neurogenesis, myogenesis, and growth factor shedding (11, 12), they have only recently been studied in the context of human tumorigenesis (13–15). For example, ADAM9 expression has been associated with the incident of low-grade prostate cancer formation in mice (16); however, the precise role of the ADAMs in malignant processes remains largely unknown.

A number of proteases, including members of the ADAM family, are thought to play regulatory roles in the processes of angiogenesis and neovascularization. ADAMs have been implicated in the processing of several key signaling components of inflammation and angiogenesis as well as adhesion molecules that comprise endothelial adherens junctions, including vascular endothelial (VE)-cadherin, PCAM-1, and integrins (17). A novel role for ADAM15 in endothelial adhesion was suggested by the observation that ADAM15 colocalized in endothelial cell junctions with VE-cadherin (18). Additionally, Horiuchi and colleagues (19) provided a compelling argument for the role of ADAM15 in pathologic neovascularization using a model of prematurity of retinopathy in mice. Further supporting ADAM15 function in endothelial regulation is the evidence that ADAM15 binds the endothelial integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$ via an RGD motif contained within its disintegrin domain (20, 21). Taken together, these studies suggest that ADAM15 is a potential but unexplored component of tumor cell–endothelial cell interaction that may play a role in tumor angiogenesis and/or metastasis.

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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Although a role for ADAM15 in tumor vasculature may be emerging, very little is known concerning the contribution of ADAM15 to tumor initiation and progression. Located on chromosome 1q21, the gene encoding ADAM15 maps to a region of documented amplification associated with the metastatic progression of human cancers, including prostate, breast, ovarian, colon, and melanoma (22–24). We have recently completed the first comprehensive expression profile of ADAM15 in human prostate cancer by using both cDNA microarray and multitumor array technology. This study showed significant increases of ADAM15 expression in multiple adenocarcinomas and a highly significant correlation with the metastatic progression of human prostate cancer (25). Based on previous work characterizing the catalytic metalloproteinase and disintegrin domains of ADAM15, we anticipated that ADAM15 may serve multiple functions in the metastatic progression of prostate tumors through disruption or degradation of the extracellular matrix, via its disintegrin domain, as well as proteolytic processing of key membrane-bound molecules via the metalloproteinase domain.

To specifically elucidate the role of ADAM15 in prostate cancer progression, we developed a stable short hairpin RNA (shRNA)-mediated knockdown of ADAM15 in the highly malignant prostate cancer line PC-3. We have established that the reduction of ADAM15 expression abolishes the malignant characteristics of this cell line *in vitro* and *in vivo* and is associated with alterations of metastatic-associated cell surface proteins. To our knowledge, this study is the first to provide functional evidence that ADAM15 plays an important role in the metastatic progression of human prostate cancer.

Materials and Methods

Cell lines and cell cultures. LNCaP and PC-3^{Luc} (26) cells were maintained in RPMI (Bio Whittaker) with either 8% or 5% fetal bovine serum (HyClone), respectively, and supplemented with 2 mmol/L L-glutamine (Life Technologies), 100 units/mL penicillin (Life Technologies), 100 µg/mL streptomycin (Life Technologies), and 0.25 µg/mL Fungizone (Life Technologies). Cells were incubated at 37°C and subcultured weekly.

Generation of shADAM15 PC-3 and ADAM15-overexpressing LNCaP cell lines. ADAM15-specific knockdown in PC-3^{Luc} cells and the vector control that features a scrambled sequence that is designed to control for off-target effects were generated as described previously (27). The forward and complementary targeting sequences for ADAM15 were 5'-AACCCAGCTGTCCACCTCGAA-3' and 5'-TTCGAGGGTGACAGCTGGGTT-3'. The shRNA cassette also featured a TTCAAGAGA loop situated between the sense and reverse complementary targeting sequences and a TTTT terminator at the 3' end.

To generate ADAM15-overexpressing LNCaP cells, ADAM15 cDNA was tagged with hemagglutinin at the COOH terminus and transfected into LNCaP cells lines as described previously (25).

Protein isolation and Western blotting. Cells were harvested by mechanical disruption with cell scrapers followed by gentle centrifugation. Cell pellets were then lysed in appropriate volumes of lysis buffer [50 mmol/L Tris (pH 8), 120 mmol/L NaCl, 5% NP40, 1 mmol/L EGTA, 100 µg/mL phenylmethylsulfonyl fluoride, 50 µg/mL aprotinin, 50 µg/mL leupeptin, and 1.0 mmol/L sodium orthovanadate] for 1 h on ice. Tissues were briefly washed in PBS and homogenized in lysis buffer on ice for 20 s with a Tissue Tearor. Cellular debris was then pelleted by centrifugation, and supernatants were collected and quantitated using a Bradford protein assay (Bio-Rad). Equal amounts of protein were then separated on precast Tris-glycine SDS-polyacrylamide gels (Novex) and transferred to Hybond nitrocellulose membrane (Amersham Pharmacia Biotech). Membranes were then blocked, probed, and developed as previously described (28). Primary antibodies were obtained as follows: actin, luciferase, GFP, and N-cadherin (Sigma);

ADAM10, ADAM15, and α_v integrin (Chemicon); and ADAM17 and CD44 (R&D Systems).

***In vivo* tumorigenesis assay.** PC3^{Luc} cells were trypsinized, washed twice with PBS, collected, and resuspended in PBS; the viability of collected cells was tested by staining with trypan blue. To establish PC3^{Luc} tumor xenografts in mice, 6-week-old C57BL/6 severe combined immunodeficient (SCID) mice were injected s.c. in the right flank with 1×10^7 vector or shADAM15 PC3^{Luc} cells in 100 µL of PBS. Tumor volume was monitored weekly by external measurements with a caliper and calculated as $V = (L^2 \times l) / 2$, where L and l represent the smaller and the larger tumor diameter. The observations were ended for ethical reasons after 8 weeks due to large tumor burden in vector control mice. Animals were maintained under specific pathogen-free conditions with *ad libitum* food and water in the University of Michigan animal housing facilities. At this time, animals were euthanized by CO₂ inhalation followed by induction of a bilateral pneumothorax and tumors were immediately frozen in dry ice or fixed in formalin.

***In vitro* migration assay.** Cell migration was evaluated by using the scratch wound assay; vector or shADAM15 PC-3^{Luc} cells were cultured for 2 to 3 days to form a tight cell monolayer. Cells were then serum starved for 16 h. Following the serum starvation, the cell monolayer was wounded with a 10 µL plastic pipette tip. The remaining cells were washed twice with culture medium to remove cell debris and incubated at 37°C with normal serum-containing culture medium. At the indicated times, migrating cells at the wound front were photographed using a Nikon inverted microscope (Thornwood). The cleared area at each time point was measured as a percentage of the cleared area at time 0 h using NIH Image J software.

Cell adhesion assay. Collagen I, collagen IV, fibronectin, vitronectin, laminin, or bovine serum albumin-coated 96-well plates were obtained from Chemicon and adhesion was performed per manufacturer's instructions. Briefly, cells were detached using a cell dissociation buffer (Sigma), washed, and plated at 2.5×10^4 per well in culture medium. Adhesion was allowed to occur for 1 h. Nonadherent cells were removed by gentle washing with warm PBS. Cells were fixed, stained, and solubilized for absorbance reading at 550 nm using a VersaMax microplate reader (Molecular Devices) and Softmax Pro Software.

Fluorescence-activated cell sorting analysis. Cell surface integrin and CD44 receptor expression was monitored by fluorescence-activated cell sorting (FACS). Briefly, cells were detached using cell dissociation buffer (Sigma) and resuspended in PBS. After washing twice with PBS, cells were incubated with primary antibodies (α_v and CD44) for 1 h on ice. Cells were washed twice with PBS and incubated with Phar Red-conjugated secondary antibodies (Molecular Probes) for 1 h at 4°C. Cells were washed twice with PBS and fixed in 7.5% formaldehyde for 5 min at 4°C. Cells were then washed twice and resuspended in PBS and analyzed using a FACSvantage SE three-laser High-Speed Cell Sorter (BD Biosciences) with CellQuest Pro Software. Isotype IgG and secondary antibodies alone were used as controls.

Substrate gel electrophoresis. MMP9 secretion and activity were assessed using gelatin zymography. Vector or shADAM15 PC-3^{Luc} cells were grown up to subconfluence and then serum starved in serum-depleted medium for 0, 24, and 48 h. At each time point, conditioned medium was collected by spinning the samples at 2,000 rpm for 15 min to pellet any cell debris. The supernatant was collected and 3 mL of it were concentrated using Amicon Ultra concentrators (Millipore) according to manufacturer's directions. Equal volume of the retentate was loaded on a gelatin zymogram and developed according to manufacturer's protocol (Invitrogen).

Transendothelial interaction and migration. The endothelial-epithelial interactions assay was performed as previously described (29). Briefly, human microvascular endothelial cells (HDMEC) or human umbilical vein endothelial cells (HUVEC) were grown to full confluence in six-well dishes. Vector or shADAM15 PC-3^{Luc} epithelial cells (2×10^5) were added in 1.8 mL of 50:50 mixture of the EGM-2/RPMI 1640 culture medium in triplicate. The unbound fraction were collected at 0, 5, and 20 min and cells were counted. Data were calculated as the percent of bound epithelial cell fraction by subtracting the unbound fraction from the total cells (at the zero time point calculated for each cell line).

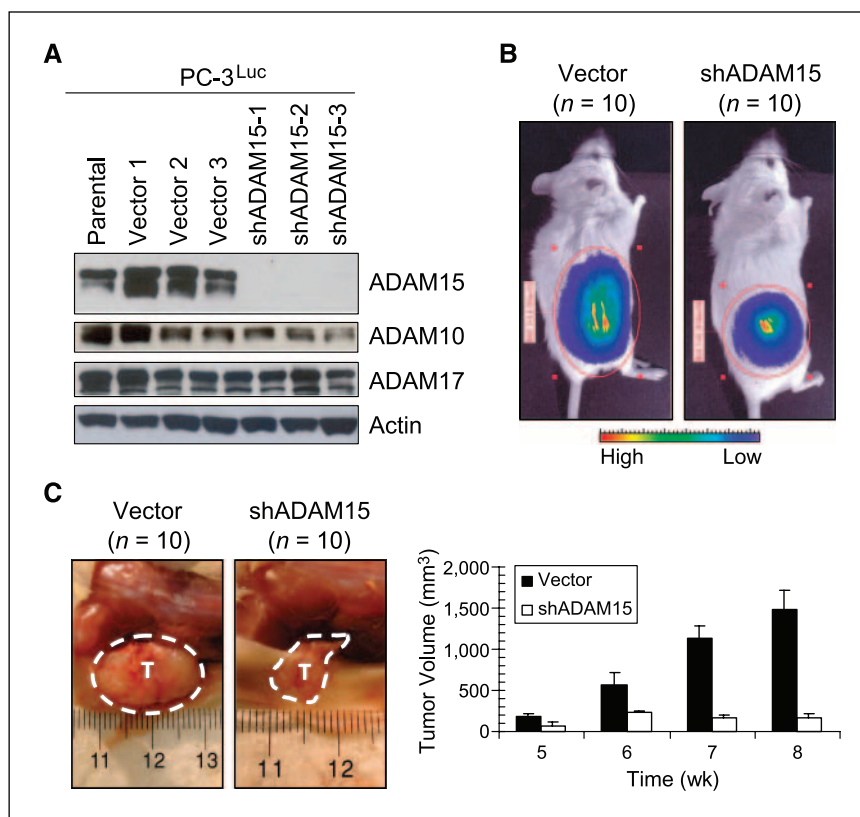


Figure 1. Knockdown of ADAM15 reduces PC-3 tumorigenicity *in vivo*. *A*, three separate shRNA ADAM15 PC-3^{Luc} cell lines were established (shADAM15-1-3). Actin was used as loading control. To control for off-target effects, ADAM10 and ADAM17 were also examined. *B*, vector or shADAM15 PC-3^{Luc} cells injected s.c. into male SCID mice were monitored weekly via bioluminescent imaging. The color scale indicates the intensity of photon emissions. *C*, gross examination of tumors at 8 wk revealed significantly larger vector PC-3^{Luc} tumors (7) compared with shADAM15 PC-3^{Luc} tumors. Tumor volume was monitored by caliper measurements weekly for weeks 5 to 8. Two independent experiments were performed with five animals per cell line that were injected on the right flank and the average tumor volumes were plotted; bars, SE.

The transendothelial migration (TEM) assay was performed as previously described (30). Briefly, HUVECs were plated on 3- μ m transwells to confluency. Vector or shADAM15 PC-3^{Luc} cells (3.8×10^4) were added onto the confluent HUVEC monolayer for 4 and 8 h. Nonadherent PC-3^{Luc} cells and endothelial cells were removed by Q-tip swab from the top filter. The transwell filter was stained with crystal violet, solubilized in 10% acetic acid, and quantified by fluorometric analysis using a VersaMax microplate reader (Molecular Devices) and Softmax Pro Software.

N-cadherin proteolysis. For the *in vitro* proteolysis of N-cadherin, ADAM15 was isolated through immunoprecipitation using a hemagglutinin-specific antibody (Millipore) from LNCaP whole-cell lysates. Similarly, N-cadherin was isolated through immunoprecipitation using an N-cadherin-specific antibody (Sigma) from whole-cell lysates of N-cadherin-expressing PC-3^{Luc} cells. Isolated ADAM15 and N-cadherin were resuspended in equal amounts of ice-cold PBS (calcium- and magnesium-free, Life Technologies), then mixed together at a 1:1 (v/v) ratio and incubated for the designated time points at 37°C. At the end of each time point, the reaction was stopped with the addition of reducing loading buffer and boiling. Samples were then loaded on SDS-PAGE for protein analysis. Levels of soluble N-cadherin in conditioned medium were monitored via N-cadherin immunoprecipitation from dilute conditioned medium samples generated using the MMP9 zymography protocol. Isotype IgG was also used for the immunoprecipitation control.

Intracardiac metastasis model. Six- to seven-week-old C57BL/6 SCID mice were anesthetized with 1.75% isoflurane/air anesthesia. Cells (2×10^5) in a volume of 100 μ L of sterile PBS were injected into the left ventricle using a 25-gauge needle. The animals were then monitored daily. Mice were imaged using the University of Michigan Small Animal Imaging Resource facility. Images were collected on a cooled CCD IVIS system with 50-mm lens (Xenogen Corp.). The LivingImage software was used for analysis of all animals (Xenogen). Before imaging, each mouse received an i.p. injection containing 100 μ L of a solution containing 40 mg/mL Luciferin dissolved in PBS. These mice were imaged at the indicated time intervals to assess the growth and metastasis of the luciferase-positive cells. At the

termination of each experiment, mice were anesthetized, sacrificed, and a full necropsy was carried out. Organs of interest were dissected out, weighed, and used for histologic analysis.

Results

Increased expression of ADAM15 correlates with prostate cancer progression. Members of the ADAM family have been suggested to play a role in human angiogenesis and cancer progression. We have focused on one member of this family, ADAM15, which we determined was transcriptionally and translationally up-regulated during the progression of several types of adenocarcinoma, including metastatic prostate cancer (25). To further evaluate ADAM15 expression in human prostate cancer, we examined the Oncomine database³ to search published cancer cDNA expression arrays. Supplementary Table S1 is a compilation of six independent and published cDNA microarray studies that revealed significantly increased ADAM15 expression during prostate cancer progression.

shRNA-mediated knockdown of ADAM15 reduces PC-3 tumorigenicity. To assess the role of ADAM15 in prostate cancer progression, we inhibited the expression of ADAM15 using a shRNA-mediated approach in the malignant prostate cancer cell line, PC-3. Three stable ADAM15 shRNA-mediated knockdown lines, shADAM15-1, 2, 3 (Fig. 1A), were established using a lentiviral construct to transduce the shRNA into the luciferase-tagged PC-3 cells (PC-3^{Luc}). The shRNA construct eliminated both the precursor and mature forms of ADAM15 protein in the PC-3^{Luc} shADAM15

³ <http://www.oncomine.com>

cells compared with the parental and vector control cells. Off-target effects of the shRNA on other genes, including relatives of ADAM15, ADAM10, and ADAM17, were assessed and were shown to be unaltered in the ADAM15 knockdown lines confirming the specificity of the shRNA for ADAM15.

To investigate whether the loss of ADAM15 reduced the tumorigenic capacity of PC-3 cells, vector or shADAM15 PC-3^{Luc} cells were injected s.c. into the flanks of male SCID mice and monitored for 8 weeks in live animals using bioluminescent imaging and caliper measurements (Fig. 1B and C). Vector control cells exhibited rapid growth as measured by bioluminescence and tumor volume. In stark contrast, the shADAM15 PC-3^{Luc} cells had a significantly reduced tumor growth rate and its tumor volume plateaued between 5 and 6 weeks. At 8 weeks postinjection, mice were euthanized and tumors were extracted for final tumor volume measurements. We found that the vector control mice had an ~6-fold increase in tumor volume compared with the shADAM15 PC-3^{Luc} cell line (Fig. 1B and C). H&E histology and anti-luciferase immunohistochemistry verified the presence of prostate-derived PC-3^{Luc} cells (Supplementary Fig. S1A and B).

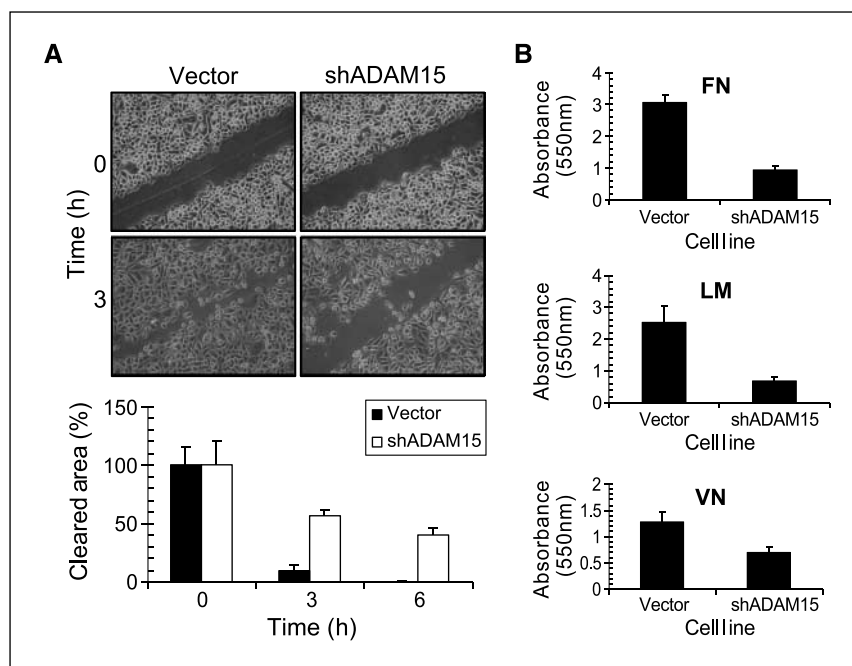
Reduction of ADAM15 in PC-3 cells attenuates cell migration and adhesion. Cellular migration is a characteristic of metastatic tumors. To assess whether ADAM15 down-regulation in PC-3^{Luc} cells reduces cell migration, we performed a wound-healing migration assay (31). Vector or shADAM15 PC-3^{Luc} cell monolayers were abraded with a pipette tip, stimulated with serum-containing growth medium, and monitored over 24 h. Microscopic examination at 3 and 6 h revealed a significant delay in the wound closure rate of shADAM15 PC-3^{Luc} cells compared with the vector control cell line that had closed the wound channel by 3 h (Fig. 2A). This finding was corroborated by use of a colloidal gold assay, which showed a decrease in random motility of the shADAM15 PC-3^{Luc} cells compared with the vector control PC-3^{Luc} cells (Supplementary Fig. S2). Interaction between cell adhesion molecules (CAM) and their complementary ECM targets support cell migration. ADAM15 has been shown to interact with several integrins, which,

in turn, bind their complementary ECM substrates to mediate cellular adhesion and migration (20, 21). To assess the role of ADAM15 in cell adhesion to ECM substrates, vector or shADAM15 PC-3^{Luc} cells were plated on plastic coated with collagen I, collagen IV, fibronectin, laminin, and vitronectin. The shADAM15 cell line had a significant reduction in their ability to adhere to fibronectin, laminin, and vitronectin compared with vector control (Fig. 2B). Both vector and shADAM15 cells adhered equally unto collagen I and IV (data not shown).

N-cadherin proteolysis is reduced in shADAM15 PC-3 cells. Tumor cell migration, invasion, and metastasis require continuous membrane interactions with the surrounding microenvironment through surface adhesion molecules (i.e., N-cadherin) onto and from the underlying stroma. Members of the ADAM family have been shown to mediate proteolysis of several CAMs, including N-cadherin, to support cell migration. Because PC-3 cells express high levels of N-cadherin but very little, if any, E-cadherin, we assessed the catalytic activity of ADAM15 in full-length N-cadherin processing into the soluble 90 kDa fragment. We first looked at the levels of solubilized N-cadherin in the conditioned medium of vector and shADAM15 PC-3^{Luc} cells. Soluble N-cadherin was dramatically decreased in the shADAM15 PC-3^{Luc} cells (Fig. 3A). To directly test whether ADAM15 cleaves N-cadherin, we coin-cubated purified ADAM15 and N-cadherin to allow for ADAM15-mediated proteolysis over time and found that ADAM15 processed N-cadherin into a soluble fragment by 6 h (Fig. 3B).

Attenuation of ADAM15 levels decrease CD44 and MMP9 expression. Integrins and CD44 receptors have also been shown to play an important role in facilitating cell migration and adhesion (32, 33). To assess whether these cell surface receptors are affected by reduced ADAM15 expression, we performed FACS and Western blot analysis on vector and shADAM15 PC-3^{Luc} cells. FACS analysis showed that the α_v integrin and the CD44 receptor are significantly down-regulated at the cell surface in response to ADAM15 knockdown (Fig. 4A). Unlike cell surface integrins, whose expression is difficult to assess by Western blot analysis, CD44 protein expression

Figure 2. Knockdown of ADAM15 attenuates PC-3^{Luc} cell migration and adhesion *in vitro*. **A**, monolayers of vector or shADAM15 PC-3^{Luc} cells were abraded and then monitored at 0, 3, and 6 h for wound channel closure. The cleared area was measured and plotted as the percentage of the original time point (0 h); bars, SD. Magnifications, $\times 200$. **B**, vector or shADAM15 PC-3^{Luc} cells were plated on fibronectin (FN)-, laminin (LM)-, or vitronectin (VN)-coated plastic dishes and adhesion was measured by absorbance at 550 nm. Columns, mean values of three independent experiments measured in sextuplets; bars, SD.



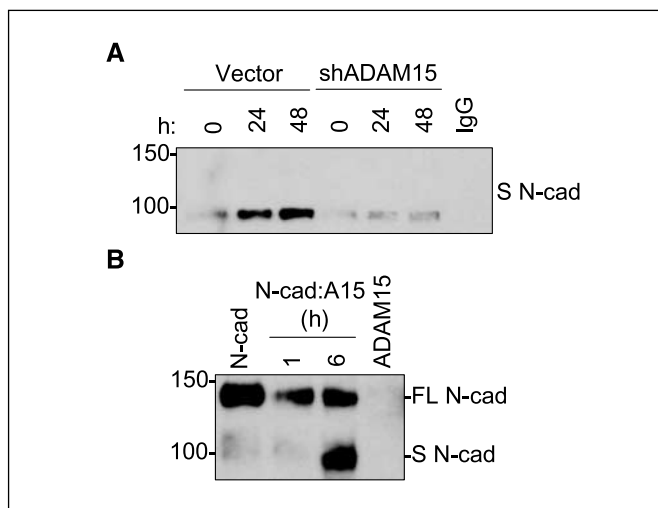


Figure 3. ADAM15 mediates N-cadherin proteolysis. *A*, vector and shADAM15 PC-3^{Luc} cells were serum starved for 0, 24, and 48 h and soluble N-cadherin (S N-cad) was assessed in the conditioned medium. *B*, ADAM15 was purified and incubated together with purified N-cadherin for 1 or 6 h. Purified N-cadherin was used as a size control and purified ADAM15 was used as a control for nonspecific binding of the soluble N-cadherin fragment. ADAM15 mediated the processing of the 130 kDa full-length N-cadherin (FL N-cad) into 90 kDa soluble N-cadherin.

was nearly depleted in shADAM15 PC-3^{Luc} cells as well as in the shADAM15-derived tumors, shown in Fig. 1, compared with vector control (Fig. 4B). This finding is supported by a previous study showing a correlation between the tumorigenicity of prostate cancer cell lines and CD44 expression (34). Due to CD44 regulation of MMP9 secretion and activity (35), we evaluated the levels of MMP9 in vector and shADAM15 PC-3^{Luc} cells using gelatin zymography and found dramatic reduction in both MMP9 secretion and activity in response to ADAM15 loss (Fig. 4C). To rule out the involvement of ADAM15 in gelatin degradation, purified wild-type ADAM15 was analyzed for gelatinase activity using gelatin zymography. Wild-type ADAM15 did not digest the gelatin substrate (data not shown).

ADAM15 supports epithelial-endothelial interaction and transmigration. For cancer cells to metastasize, they must interact with the surrounding microvasculature and intravasate through the

vascular endothelium to gain access to the bloodstream. To assess the potential role of ADAM15 in tumor cell-endothelial interaction, we performed a tumor cell-endothelial cell adhesion assay. Vector or shADAM15 PC-3^{Luc} cells were plated on two types of primary human endothelial cell (HUVEC or HDMEC) monolayers for 0, 5, and 20 min, and bound cells were calculated as described in Materials and Methods. We observed that shADAM15 PC-3^{Luc} cells had a significantly reduced ability to adhere to the endothelial cell monolayers compared with the vector control (Fig. 5A). Both vector and shADAM15 epithelial cells attached more effectively on HUVEC monolayer than the HDMEC monolayer. To assess whether ADAM15 reduction would also affect epithelial cell intravasation *in vitro*, we performed a transendothelial migration (TEM) assay. Vector or shADAM15 PC-3^{Luc} cells were seeded on top of a confluent HUVEC monolayer in a transwell chamber and stimulated for 4 or 8 h. Nearly 3-fold fewer shADAM15 PC-3^{Luc} cells were found to have migrated through the endothelial monolayer at the 8 h time point compared with the vector control (Fig. 5B).

Loss of ADAM15 reduced the metastatic spread of PC-3 cells *in vivo*. We have shown that the targeted knockdown of ADAM15 reduces PC-3 cellular motility, ECM binding, endothelial binding, transendothelial migration, and growth *in vivo*. Coupled to the fact that ADAM15 significantly correlates with the metastatic progression of human prostate cancer, these findings strongly support a functional role for ADAM15 in prostate cancer metastasis. To examine this possibility, we injected vector or shADAM15 PC-3^{Luc} cells into the left ventricle of male SCID mice to establish systemic metastasis (26). The growth of metastatic lesions was monitored by bioluminescent imaging over a 6-week time course in live animals. Bioimaging 24 h postinjection showed equal distribution of both cell lines inside the mice (data not shown). Over the 6-week time course, mice injected with vector control PC-3^{Luc} cells exhibited an increasing accumulation of bioluminescence at disseminated sites indicative of increased metastatic development compared with the shADAM15 PC-3^{Luc} cells, which exhibited weak luminescent signals at distant sites (Fig. 6A and B). Animals injected with vector control developed secondary metastasis in the lungs (*arrowhead*), tibia (*arrow*), and spine (Fig. 6A and C). Animals injected with shADAM15 PC-3^{Luc} cells developed few to no metastatic lesions. Tissues exhibiting highly elevated bioluminescence were dissected and prepared for histologic analysis. H&E histology and anti-luciferase immunohistochemistry verified the

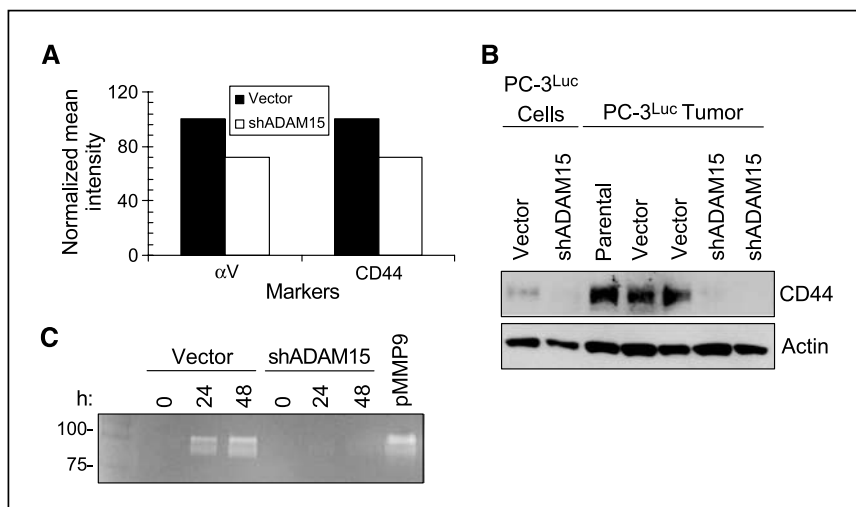
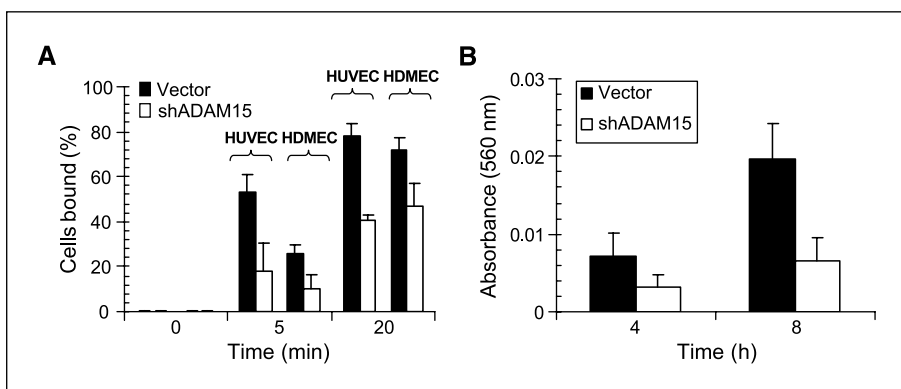


Figure 4. Loss of ADAM15 disrupts cell surface receptor levels. The α_v and CD44 cell surface receptors were assessed via FACS (A) or Western blot (B) analysis in vector and shADAM15 PC-3^{Luc} cells and tumor lysates. C, vector and shADAM15 PC-3^{Luc} cells were serum starved for 0, 24, and 48 h and conditioned medium was analyzed for MMP9 activity using a gelatin zymogram. Purified MMP9 (pMMP9) was used as a positive control.

Figure 5. The loss of ADAM15 reduces PC-3-endothelial interaction and transendothelial migration. *A*, vector or shADAM15 PC-3^{Luc} cells were incubated on top of confluent HUVEC or HDMEC monolayers. The number of bound cells was determined at 0, 5, and 20 min. *B*, transendothelial migration of vector or shADAM15 PC-3^{Luc} cells through a confluent endothelial monolayer grown on a 3- μ m transwell. The relative migration rate was monitored at 4 and 8 h via colorimetric analysis. *Columns*, mean values of independent experiments measured in duplicates and repeated thrice; *bars*, SE.



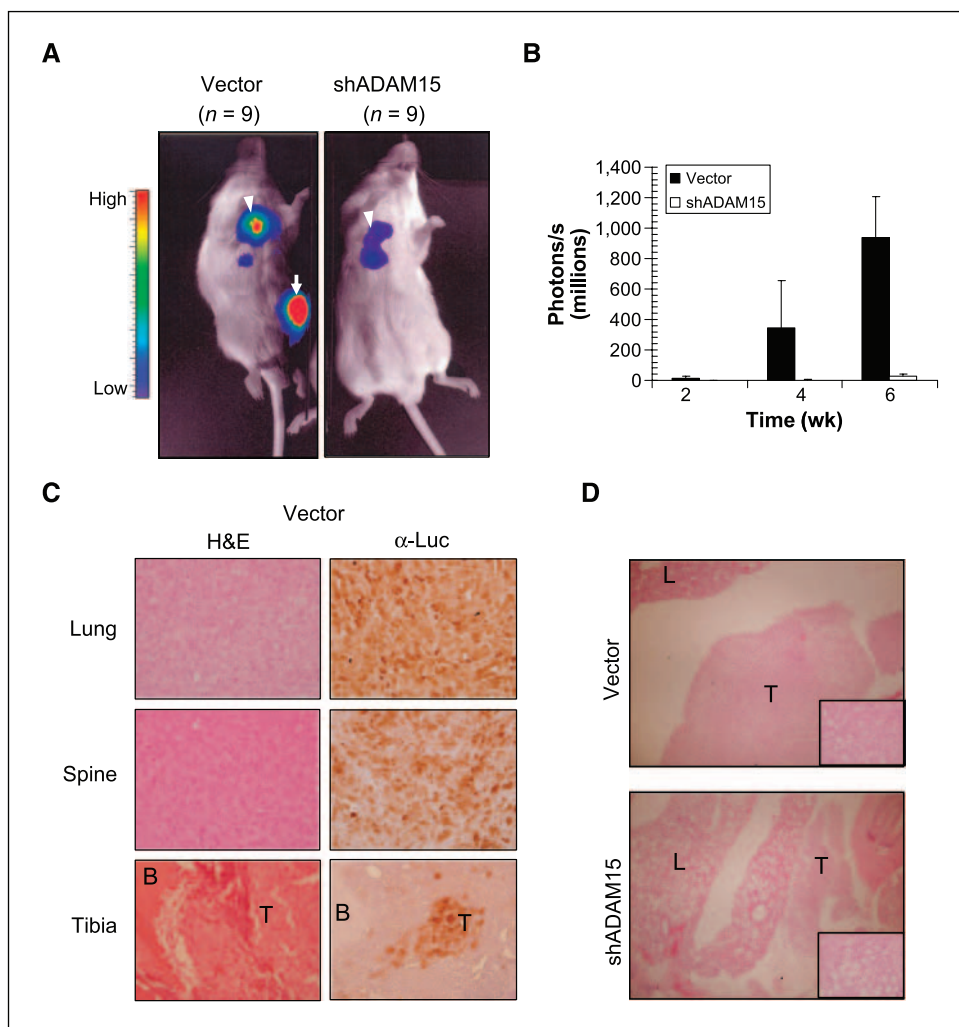
presence of substantial metastatic deposits of vector PC-3^{Luc} cells within the lung, tibia, and spine of vector cell-injected mice (Fig. 6C). Histologic analysis of tissues from the shADAM15 PC-3^{Luc} mice revealed micrometastasis only in the lungs (Fig. 6D).

Discussion

Alterations in the cellular microenvironment contribute significantly to the progression of human prostate cancer to metastatic

disease. In turn, metastatic progression requires multiple steps that involve cell detachment, stromal invasion, and intravasation into the vasculature (2). The MMPs have been shown to catalyze the degradation of the extracellular matrix and are implicated in tumor cell migration, invasion, and angiogenesis. However, MMPs are not the only metalloproteinases thought to support human tumorigenesis. Another, less studied family of metalloproteinases, ADAMs, may also support tumor progression through their ability to affect diverse physiologic cell surface proteins such as

Figure 6. Loss of ADAM15 attenuates PC-3 metastasis in an intracardiac dissemination assay. *A*, vector or shADAM15 PC-3^{Luc} cells were injected into the left ventricle of male SCID mice and metastasis was monitored via bioluminescent imaging for the duration of the 6-wk study. Vector control PC-3^{Luc} cells were visualized in the tibia (*arrow*) and lung (*arrowhead*), whereas the shADAM15 PC-3^{Luc} exhibited very low signals in the thoracic cavity representing a lung micrometastasis (*arrowhead*). *B*, total bioluminescence was graphed for both cohorts. *Columns*, mean values of bioluminescence imaging (photons/s); *bars*, SE. *C*, metastatic tissues representing sites of strong bioluminescence were extracted for histologic evaluation. Lung, tibia, and spine metastasis were observed in vector control sites as indicated by H&E histology and anti-luciferase immunohistochemistry. Magnification, $\times 400$. *B*, bone. *T*, tumor. *D*, shADAM15 PC-3^{Luc} cells formed micrometastatic tumors in the lungs (*L*) compared with the large lung tumors observed in vector control. The inset is a $\times 400$ magnification of the $\times 40$ tumor image in each animal.



membrane-anchored growth factors and their receptors, ectoenzymes, and cell adhesion molecules, including the cadherins (4). These disintegrin-metalloproteinases contain the conserved modular metalloproteinase motif as well as an integrin-binding domain (disintegrin) in the extracellular region of the molecule and a src-homology (SH3) protein binding domain in the cytosolic portion of the protein. The ADAMs have been implicated in normal biological processes, which include oocyte fertilization, neurogenesis, myogenesis, growth factor shedding (11, 12), and have recently been studied in the context of human tumorigenesis (13–15). ADAM9 expression has been associated with the incident of low-grade prostate cancer formation in mice (16). Furthermore, ADAM12 expression was elevated in the stroma adjacent to tumor cells in mouse models of breast, colon, and prostate cancer (36). However, the precise role of these proteinases in human malignant processes remains largely unknown.

We have recently completed the first comprehensive study of ADAM15 expression in human cancer. Using the Oncomine database, we examined the cDNA expression arrays of published studies examining normal tissue versus tumor in a number of adenocarcinomas. Using this database and the associated statistical software, we found that ADAM15 was up-regulated in multiple adenocarcinomas (25). In addition, we have examined the expression of ADAM15 in 23 independent adenocarcinoma cell lines representing melanoma and tumors of the prostate, breast, and colon and in all cases have observed high-level expression of activated ADAM15 protein (data not shown). The chromosomal location of ADAM15 on 1q21, a region of specific and high-level amplification in metastatic prostate cancer, makes this disintegrin a strong candidate in the malignant progression of this disease. The current study suggests that aberrant function of ADAM15 supports tumor growth, endothelial interaction, and metastasis of prostate cancer cells. Previous research from other laboratories indicated functional roles of ADAM15 in vascular endothelial biology with clear implications in endothelial interaction and angiogenesis (19, 37, 38). However, the specific actions of ADAM15 in tumor epithelium and influences on neighboring endothelial cells in the tumor microenvironment have not, until now, been explored.

To elucidate the function of ADAM15 in prostate cancer, we developed a stable shRNA-mediated ADAM15 knockdown in the highly malignant PC-3 prostate cancer cell line. We showed in this study a dramatic reduction in the malignant capacity of PC-3 cells in the s.c. implantation model. In agreement with a tumorigenic role of ADAM15 in PC-3 cells, we have seen that an exogenous overexpression of ADAM15 increased the malignant potential of the minimally invasive LNCaP cells in this same model (data not shown). Taken together, the tumor cell implantation experiments using two complementary models to alter ADAM15 levels strongly support the role of ADAM15 in the malignant progression of human prostate cancer cells.

ADAM15 is believed to support cellular adhesion and motility in several systems (31, 39). Here, we showed that the loss of ADAM15 in PC-3 cells attenuated the migratory capacity in a wound channel migration assay and decreased PC-3 basal adhesion to fibronectin, laminin, and vitronectin. Additionally, we showed that shRNA knockdown of ADAM15 decreased CD44 and α_v integrin surface expression as well as MMP9 secretion and activity. This may have significant ramifications as α_v -containing integrins are known to bind vitronectin whereas CD44 is considered to encompass laminin and fibronectin binding, respectively (32, 33). Because binding to other substrates, such as collagen I and IV, were unchanged in

response to ADAM15 down-regulation, we believe that these results are specific to the ADAM15 phenotype. It has also been reported that low $\alpha_v\beta_3$ (40, 41) and CD44 (34, 42) levels portend to a less aggressive prostate cancer cell phenotype. The ECM glycoprotein, osteopontin, using its RGD sequence has been shown to interact with the $\alpha_v\beta_3$ integrin to regulate cell surface CD44 and MMP9 (35). ADAM15 is the only member of the ADAM family that has an RGD sequence within its disintegrin domain and has been shown to interact directly with the integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$ (20, 21). Analogous to osteopontin, ADAM15 may regulate CD44, which, in turn, has been shown to regulate MMP9 expression, through this unique sequence. The interaction between ADAM15 and specific integrins or other transmembrane receptors may coordinate the proteolytic activity of ADAM15 directly to induce ECM degradation and cell migration. Alternatively, ADAM15 could potentially activate downstream substrates such as MMP9 in a hierarchical manner to support cancer cell dissemination.

E (epithelial), N (neuronal), and P (placental) cadherins function in maintaining cell-to-cell adhesion through homotypic ligation. The loss of those interactions through a variety of aberrant processes such as cadherin gene mutation, loss of heterozygosity, promoter methylation, or proteolytic cleavage can support cancer cell migration and invasion. In aggressive prostate cancer cells, N-cadherin is highly expressed and has been shown to mediate their adhesion and migration (43). The loss of full-length N-cadherin reduces cell adhesion and may promote cell migration (44). Furthermore, serum levels of soluble N-cadherin are up-regulated in multiple cancers including prostate cancer and correlated directly with an increase in PSA levels (45). Within our current study, we have made the interesting observation that shADAM15 PC-3^{Luc} cells had significantly reduced levels of shed N-cadherin in conditioned medium compared with vector control. These findings were corroborated by the fact that an N-cadherin-expressing melanoma cell line, Mel147, also had reduced soluble N-cadherin levels in its conditioned medium in response to ADAM15 down-regulation (data not shown). In addition, reconstitution of immunoprecipitated ADAM15 and N-cadherin resulted in the cleavage of N-cadherin into the identical 90 kDa fragment observed in the conditioned medium. These observations support two possible scenarios: (a) that ADAM15 cleaves N-cadherin directly or (b) that ADAM15 represents an indirect or upstream event in the shedding of N-cadherin. Regardless of a direct or an indirect role, we believe that N-cadherin solubilization may support the migratory phenotype observed in ADAM15-expressing PC-3 cells by disrupting the homotypic interaction between N-cadherin molecules and increasing N-cadherin cell surface turnover.

The $\alpha_v\beta_3$ integrin has been shown to support endothelial-epithelial adhesion and TEM of tumor cells (29). To assess whether the loss of ADAM15 and the associated reduction of α_v integrin would hinder PC-3 epithelial-endothelial adhesion, we examined the adhesive capacity of PC-3 cells to human endothelial cells and showed that the loss of ADAM15 resulted in a significant reduction in endothelial adhesion. A concomitant reduction in transendothelial migration of shADAM15 PC-3 through HUVEC and HDMEC monolayers not only supports that ADAM15 mediates endothelial adhesion of tumor cells but coordinates the transmigration of tumor cells through endothelial monolayers.

The CD44 receptor has been implicated in prostate cancer adhesion, migration, invasion, and metastasis (34). In this study, we showed that CD44 is down-regulated in cell culture and in tumors in response to the loss of ADAM15. To investigate whether

ADAM15 contributes to *in vivo* prostate cancer metastasis, we used an invasive model of prostate cancer metastasis. We showed that vector PC-3^{Luc}-injected mice developed substantial metastatic lesions in the lung, spine, and tibia. ADAM15 down-regulation within these cells hindered their ability to form distant metastasis. Considering the microarray data, which shows a significant correlation of ADAM15 expression with the metastatic progression of human prostate cancer, results from the mouse metastasis model suggests that ADAM15 may support the metastatic progression of prostate cancer.

The current study, which has its foundations on the clinical correlation of ADAM15 and the metastatic progression of prostate cancer, provides strong experimental support for this disintegrin in

prostate cancer progression. Based on the current findings, we suggest that ADAM15 plays critical regulatory roles in the malignant progression of prostate cancer to metastatic disease.

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