ATF3 Suppresses Metastasis of Bladder Cancer by Regulating Gelsolin-Mediated Remodeling of the Actin Cytoskeleton

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Abstract
Bladder cancer is associated with high recurrence and mortality rates due to metastasis. The elucidation of metastasis suppressors may offer therapeutic opportunities if their mechanisms of action can be elucidated and tractably exploited. In this study, we investigated the clinical and functional significance of the transcription factor activating transcription factor 3 (ATF3) in bladder cancer metastasis. Gene expression analysis revealed that decreased ATF3 was associated with bladder cancer progression and reduced survival of patients with bladder cancer. Correspondingly, ATF3 overexpression in highly metastatic bladder cancer cells decreased migration in vitro and experimental metastasis in vivo. Conversely, ATF3 silencing increased the migration of bladder cancer cells with limited metastatic capability in the absence of any effect on proliferation. In keeping with their increased motility, metastatic bladder cancer cells had increased numbers of actin filaments. Moreover, ATF3 expression correlated with expression of the actin filament severing protein gelsolin (GSN). Mechanistic studies revealed that ATF3 upregulated GSN, whereas ATF3 silencing reduced GSN levels, concomitant with ATF3 expression correlated with expression of the actin filament severing protein gelsolin (GSN). Mechanistic studies revealed that ATF3 upregulated GSN, whereas ATF3 silencing reduced GSN levels, concomitant with alterations in the actin cytoskeleton. We identified six ATF3 regulatory elements in the first intron of the GSN gene confirmed by chromatin immunoprecipitation analysis. Critically, GSN expression reversed the metastatic capacity of bladder cancer cells with diminished levels of ATF3. Taken together, our results indicate that ATF3 suppresses metastasis of bladder cancer cells, at least in part through the upregulation of GSN-mediated actin remodeling. These findings suggest ATF3 coupled with GSN as prognostic markers for bladder cancer metastasis.

Introduction
Despite good prognosis for bladder cancer with an early diagnosis, recurrence is common after surgical intervention and nearly half of all patients with bladder cancer harbor occult metastases that are associated with reduced survival (1). Hence, progression to metastasis marks a significant detrimental step in the disease. Although different markers are associated with the progression of disease, including depth of invasion, stage, and grade, there is a lack of definitive clinical prognostic markers and limited understanding of the cellular mechanism of disease progression (2, 3).
Metastasis involves detachment of tumor cells from the primary tumor, and the acquisition of migratory and invasive capability is the key step in the tumor cell metastasis cascade (3, 4). An important regulatory mechanism in this step involves the regulation of cell motility and adhesion. These processes are critically dependent on a dynamic actin cytoskeleton (4). Gelsolin (GSN) is an actin-binding protein that controls the rate of actin-based cell migration. Increasing evidence suggests a role for GSN as a tumor suppressor (5). Moreover, decreased expression of GSN has been observed in many human cancers, including breast, gastric, and prostate, as well as bladder cancer (6). GSN expression has been associated with tumor staging and overall survival of bladder tumors (7). The loss of GSN expression in invasive tumors compared with nonmuscle-invasive (NMIBC) lesions is consistent with a tumor suppressor role for GSN. It is currently unclear whether dysregulated GSN expression mediates malignant transformation in different cancer cells and tissues, because no major mutations, gross rearrangements, or deletions within the GSN gene have been

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

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identified in malignancies. Moreover, the mechanism of how GSN expression is reduced in cancers remains to be determined. It has been documented that the activating transcription factor 1 (ATF1) modulates the GSN promoter activity in breast cancer (8). Here, we identify a role for another ATF family member, ATF3, in regulating GSN expression in the context of bladder cancer.

The ATF family consists of a large group of basic-region leucine zipper (bZip) transcription factors (9). ATF3 is a bZip DNA-binding domain protein that forms homodimers and various heterodimers with other bZip proteins; it can function as a transcriptional activator or repressor. ATF3 can regulate innate immune responses by suppression of the innate cytokine storms induced by infectious stimuli (10–13). ATF3 is a stress-activated regulator of p53 protein function and stabilizes the tumor suppressor, thereby augmenting functions by direct interaction (14). Interestingly, ATF3 plays different roles in cancer development, depending on the cell type and context (15, 16). In normal tissues, ATF3 may promote both apoptosis and cell proliferation (17, 18), whereas it has been identified as either an oncogene or a tumor suppressor in neoplasms, depending on tumor entity and grade (15, 19, 20). In prostate and breast cancer, ATF3 appears to promote metastasis (8, 21, 22). In contrast, loss of ATF3 function results in tumor suppression in colorectal cancer (23). Hence, the precise role of ATF3 in cancer initiation and progression is unclear.

In the present study, we provide evidence that reduced ATF3 expression correlates with advanced stage of bladder cancer, specifically for metastasis. We show that downregulation of ATF3 stimulates bladder cancer cell migration in vitro and cell metastasis in vivo. In addition, ATF3 shows a positive correlation with GSN expression in the same tumors, and we show that the transcription factor ATF3 directly regulates the GSN gene through 6 putative binding sites, coincident with cell cytoskeletal depolymerization and migration of bladder cancer cells. Our data reveal that ATF3 regulates cell migration through the actin cytoskeleton via a GSN-mediated mechanism. Both analyses of data from the Oncomine database and our own data indicate that low ATF3 and GSN expression are strongly correlated with patient survival. Therefore, we propose that ATF3 and GSN could serve as prognostic markers for metastatic disease in bladder cancer.

Materials and Methods

Clinical samples

This study included samples from 64 cases of bladder cancer, obtained directly from cystectomy patients in Xinhua Hospital, Shanghai Jiaotong University School of Medicine (Shanghai, China), between 2005 and 2007. Five years of follow-up data were available for the patients. Tissue samples from primary tumors and matched adjacent non-neoplastic bladder tissues were collected and prepared for tissue microarray (TMA). Fresh-frozen tissues from 20 patients with bladder cancer and matched benign bladder tissue were also collected and immediately snap-frozen for RNA and protein analyses. All experimental procedures were approved by the Institutional Review Board of the Xinhua Hospital. Written informed consent was obtained for all patient samples used in this study.

Cell lines and culture conditions

Human bladder cancer cell lines T24 and 5637, and human normal uroepithelial cell line SV-HUC-1 were purchased from the American Type Culture Collection and cultured as described (http://www.atcc.org/). These cell lines were used within 6 months of resurrection of the original cultures. T24-L, a more metastatic variant of T24, was created in our laboratory (Supplementary Fig. S1). Primary mouse embryonic fibroblasts (MEF) were isolated from day 12.5 wild-type C57BL/6 or ATF3 knockout embryos. The identity of all cells was authenticated in October 2011 with short tandem repeat (STR) analysis using the AmpF/STR Identifiler Kit from Applied Biosystems.

Establishment of metastasis cell subline (T24-L)

Using a protocol approved by the Xinhua Hospital Institutional Review Board, T24-L, a more metastatic variant of the T24 cell line, was created by reiterated serial injections as described previously (24, 25). In brief, 1 × 10^6 T24 cells were injected into mice via the lateral tail vein. After 16 weeks, identifiable lung lesions were isolated, tumor cells grown in tissue culture, and injected intravenously into mice. This process of injection and isolation was repeated 3 times, and the resulting cell line was designated as T24-L. The proliferation and metastatic characteristics of bladder cancer cell sublines (T24 and T24-L) were examined in vivo and in vitro (Supplementary Fig. S1).

Cell motility and wound healing assay

Cells were plated at 10% confluence in 24-well plates and fluorescently labeled with 8 mmol/L Cell Tracker Green CMFDA (Invitrogen). Cell movement was imaged by phase contrast using a motorized staged environment-controlled Zeiss microscope (Zeiss) by collecting a 30-minute interval time-lapse series for 24 hours. Centroids of fluorescent cells were computed with Imaris (Bitplane) using the built-in "spots" function, and tracks of individual cells were generated with "autoregressive motion" tracking algorithms as described previously (26). When cells were plated at confluence in 24-well plates, a wound was made by scraping the confluent monolayer with a p200 pipette tip, then the cells were grown in medium supplemented with 1% FBS. The migration of the cells was digitally recorded at 0, 6, 12, 24, and 48 hours using a MicroFire camera fitted to a Zeiss Inverted Phase Microscope (Zeiss). The area of the uncovered wound gap was measured using Image J software. All experiments were done in triplicate.

In vivo bladder cancer experimental metastasis assay

To examine the metastatic ability of the bladder cancer cells expressing ATF3 in animals, 1.0 × 10^5 cells in 0.2 mL of PBS were injected into the lateral tail vein of 6-week-old SCID mice obtained from the Shanghai Laboratory Animal Center of China. Each tumor cell subline was injected into 15 mice. Mice were euthanized at 8 weeks. Genomic DNA was extracted from 5 mouse lungs of each group and 12p real time PCR was performed for DNA PCR metastasis assay as described previously (25). Metastatic lesions were gross examined for metastasis in 10 mouse lungs of each group and the number of visible metastases was evaluated as explained previously (27). Animal...
experiments were performed with the approval of the Institutional Committee of Shanghai Jiaotong University School of Medicine for Animal Research.

**Immunohistochemical staining**

Standard immunohistochemical procedures were carried out using anti-ATF3 polyclonal antibody (sc-188; Santa Cruz Biotechnology) and anti-GSN monoclonal antibody (G4896; Sigma) in human bladder cancer and normal tissues. For negative controls, the primary antibody was omitted and replaced by negative immunoglobulin G (IgG; Supplementary Fig. S2A). The specimens were digitized via whole-slide scanning with the Aperio T2 scanner (Aperio Technologies) and were independently scored by 2 pathologists and automated Aperio systems. Agreement between pathologist and automated scores was measured and good consistency was achieved (Supplementary Fig. S2B). Staining intensity (0, no staining; 1, weak; 2, moderate; and 3, intense) and the proportion of stained cells (0, no staining; 1, <10%; 2, 11%–33%; 3, 34%–66%; and 4, >67%) were semiquantitively determined. The immunoreactive score was obtained by summing these 2 scores (negative, 0; weak, 1–4; moderate, 5–8; strong, 9–12).

**Statistical analysis**

Unless otherwise stated, all statistics were conducted with the GraphPad Prism software and displayed as the mean and SEM. If not otherwise stated, the statistical significance of the difference was assessed using Student t test, and the one-way ANOVA with Tukey posttest was conducted for multiple comparisons. Correlation between variables was evaluated by Spearman rank correlation coefficients. Survival analysis was carried out using the Kaplan–Meier method and log-rank test.

**Additional materials and methods**

A detailed description of additional materials and methods can be found in the Supplementary Data.

**Results**

**Expression of ATF3 is inversely associated with tumor stage in bladder cancer**

To examine the potential clinical relevance of ATF3 to cancer progression, we investigated its expression using a human bladder TMA (Fig. 1A and Supplementary Fig. S3A). Immunohistochemical staining revealed weak ATF3 expression in invasive bladder cancer cells as compared with benign urothelial cells and carcinoma in situ (Fig. 1A). Next, we investigated whether ATF3 expression is correlated with key clinical parameters in human bladder cancer. ATF3 expression was correlated with disease state, compared with normal, being lower in low grade, and further downregulated in high-grade tumors (Fig. 1B and Supplementary Table S1). Muscle-invasive stage disease is associated with a higher incidence of metastasis than NMI disease, which is consistent with our finding that ATF3-positive staining was significantly reduced in muscle-invasive compared with NMI (Fig. 1C). We also found that compared with invasive tumor cells, adjacent normal bladder tissue and tumor stromal compartments retained a strong expression of ATF3 (Supplementary Fig. S3B). It has been reported that elevated ATF3 expression in the stromal compartment may be tumor promoting (28), however, there was no significant correlation between ATF3 expression in the stromal cells and tumor stage in our study (Supplementary Fig. S3C). We further confirmed that mRNA and protein levels of ATF3 were significantly reduced in the tumor tissues as compared with matched adjacent normal tissue (Fig. 1D). As reported previously, a useful method to uncover genes associated with the aggressive phenotype, is to use available lineage-related human cell lines with defined metastatic capabilities such as T24 cells in the mouse experimental lung metastasis model (29, 30). We have generated the highly metastatic T24-L cell lines using in vivo selection (Supplementary Fig. S1). We observed a trend toward decreased ATF3 levels that paralleled the increase in metastatic behavior reported for bladder cancer cell lines: HUC-1 (immortalized uroepithelial cell), 5637 (primary bladder carcinoma cell), T24 (modestly metastatic), and T24-L (highly metastatic) using experimental metastasis models (Fig. 1E). Taken together, these data strongly indicate that suppression of ATF3 expression may play a critical role in bladder cancer progression.

**ATF3 attenuates the motility of bladder cancer cells**

Given the correlation of ATF3 expression with clinically relevant features, we investigated ATF3 as a potential regulator of bladder cancer cell metastasis. Migration and invasion are critical steps in the initial progression of cancer that facilitate metastasis. As reduced ATF3 levels paralleled the increase in migratory behavior reported for these cells models, we overexpressed ATF3 in the highly invasive and metastatic T24-L cell (Fig. 2A, i). There was no significant difference in cell proliferation (Fig. 2A, ii). However, overexpression of ATF3 inhibited migration of T24-L cells by 36.8% (Fig. 2A, iii and iv). Conversely, knockdown of ATF3 enhanced migration by 43.4% without affecting proliferation (Fig. 2B, i–iv). We also evaluated motility by quantitative analysis of individual cell movements over 24 hours. The trajectories of the cells showed a random orientation but with more extended trajectories detected for T24-L compared with T24, both when the cells were restrained by ATF3 overexpression, or freed by knocking down the protein levels (Fig. 2C).

We further examined the role of ATF3 in cell motility and found that overexpressing ATF3 in T24-L cells inhibited serum-induced directional migration (Fig. 2D) and cell invasion (Fig. 2E) compared with a control plasmid by about 65% and 60%, respectively. Consistent with this, ATF3 knockdown significantly increased T24 migration and invasion by more than 60% relative to control (Fig. 2D and E). Consistent with these findings for the bladder carcinoma cell lines, a marked difference in cell migration between wild-type and ATF3-null MEFs (Fig. 2F) was detected. These data show that ATF3 regulates cell motility in vitro.

**The effect of ATF3 on migration is independent of Src, ILK, and p38**

To address the intermediate mechanism by which ATF3 suppresses bladder cancer progression and metastasis, we...
selected genes associated with cell migration and evaluated the expression pattern in our metastatic potential model. First, we found p-Src was downregulated in parallel with ATF3 expression in T24-L cells compared with the parental line (Fig. 3A). However, both cell lines had comparable strong expression of Cav-1. Although tyrosine kinase c-Src has been shown to promote cell growth and migration in various human cancers, one recent study showed that high Cav-1 and low active Src were associated with bladder cancer metastasis (31). In contrast to ATF3, proteins previously reported as promigratory such as FAK, ILK, and p38 mitogen-activated protein kinase (MAPK) have similar tendencies to increase, suggesting that inhibition activities of these proteins would interfere with tumor cell migration. To test this, we conducted migration
altering cell shape and accelerating retraction of bladder cancer cell lines increased the levels of actin stress. With this, overexpression of ATF3 in T24-L cells reduced F-actin and CapG expression. Interestingly, GSN, but not CapG, was profoundly altered by ATF3 status. This indicated that GSN may be a downstream target of ATF3, whereas the other related migration genes mentioned earlier could be upstream factors.

ATF3 regulates the expression of the actin-modifying protein GSN

To identify the mechanism by which ATF3 controls cell motility, we investigated the effect of ATF3 on the actin cytoskeleton. Polymerization and depolymerization of filamentous (F) actin controls cytoskeletal reorganization, leading to morphologic changes critical to cell motility. Staining of the F-actin within wild-type and atf3−/−MEFs showed cells ablated for ATF3 had significantly more actin stress fibers compared with wild-type MEFs (Fig. 4A). Consistent with this, overexpression of ATF3 in T24-L cells reduced F-actin staining (Fig. 4B, i). In contrast, knockdown of ATF3 in bladder cancer cell lines increased the levels of actin stress fibers (Fig. 4B, ii).

GSN protein binds to and severs large actin filaments, altering cell shape and accelerating retraction of filopodia during motility (32). As another member of the ATF family had previously been reported to regulate the levels of GSN, we sought to see whether ATF3 might be affecting the state of actin via regulation of GSN. The levels of GSN in TMAa from patients with cancer with various stages of bladder disease were examined immunohistochemically. Loss or reduction of GSN expression in muscle-invasive compared with NMI bladder cancer was observed (Fig. 4C). Moreover, we examined whether the effects of ATF3 on cytoskeletal changes and cell migration are via its regulation of GSN and found that elevated GSN expression significantly inhibited monolayer wound closure within 24 hours in T24-L cells (Fig. 5A). T24-L cells overexpressing GSN were devoid of most large actin protofilaments and had a shorter average filament length and a decreased total amount of filamentous actin compared with control (Fig. 5B). Moreover, silencing GSN in T24-L cells with stable overexpression of ATF3 enhanced cell migration, reversing the consequence of ATF3 overexpression (Fig. 5C). Conversely, overexpression of GSN in T24 cells with stable ATF3 knockdown inhibited migration (Fig. 5D). Taken together, these findings show that the effects of ATF3 on cell motility are GSN dependent and play an important role in cell migration.

The role of ATF3 in cytoskeleton remodeling and cell migration is GSN dependent

We examined whether GSN-mediated cytoskeleton remodeling mainly through depolymerization. Taken together, these findings show that the effects of ATF3 are dependent on GSN. The increased levels of GSN may prevent rearrangement of the actin cytoskeleton, thus accounting for the attenuated migratory capability noted on altered ATF3 expression.

ATF3 and GSN are prognostic biomarkers for metastasis

To extend our findings in vivo, we next examined the role of ATF3 in metastatic colonization. A low incidence and few lung tumor nodules were found in mice inoculated intravenously with the parental T24 cell line (Fig. 6A), similar to previous observations (29, 30, 33). However, ATF3 knockdown in the T24 cell line increased lung tumor burden as compared with the T24 vector cells (Fig. 6A and B). Overexpressing ATF3 in the T24-L cell line reduced lung tumor burden as compared with the control stable cells. Thus, our data clearly establish that ATF3 plays an important role in suppressing experimental bladder cancer metastasis in vivo. To further support the concept that ATF3 and its target gene GSN contribute to bladder cancer metastasis, we investigated the relationship between ATF3 and GSN in patient samples from 2 bladder cancer studies in the Oncorine database, Lee and colleagues and Sanchez-Carbayo and colleagues (34, 35). We found that expression of both ATF3 and GSN are higher in normal samples and lower in superficial and invasive tumors (Fig. 6C). The data of Sanchez-Carbayo and colleagues showed a positive correlation between ATF3 and GSN mRNA expression in the same
**A**

(i) T24-L

(ii) Number of cells (10^3)

(iii) 0 h 12 h 24 h

(iv) Mock pCtrl pATF3

**B**

(i) T24

(ii) Number of cells (10^3)

(iii) 0 h 12 h 24 h

(iv) Mock shCtrl shATF3

**C**

(i) T24-L

(ii) Migration speed

(iii) T24

(iv) Mock shCtrl shATF3

**D**

(i) T24-L

(ii) Migration speed

**E**

(i) T24-L

(ii) Invasion

**F**

(i) MEFs

(ii) Relative wound closure

(iii) Average speed

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Figure 3. Identification of ATF3-regulated genes associated with migration. A, immunoblot analysis of phosphorylated and total protein, as indicated, was conducted to determine expression pattern in selected bladder cancer cell lines. B, serum-induced chemotaxis assays were carried out in T24-L cells after 72 hours transfection of ILK siRNA or 24 hours treatment of 20 μmol/L SB203580 (p38 inhibitor), or in T24 cells after 24-hour treatment of 0.5 μmol/L dasatinib (Src inhibitor). Representative images of stained cells are shown. The quantification is presented as mean ± SEM (n = 3; *, P < 0.05).

Figure 2. Functional characterization of ATF3 expression related to migration in bladder cancer. T24-L (A) or T24 cells (B) were transfected with pcDNA3.1-ATF3 or shRNA-ATF3, respectively, and monitored by scratching and monitored over 24 hours to determine the rate of wound closure. Representative images of triplicate experiments are shown [A(iii) and B(iii)]. C, time-lapse microscopy was used to track the movements of individual bladder cancer cells with pATF3 (T24-L; i and ii) and with shATF3 (T24; iii and iv), over a time course of up to 24 hours. The average path length that cells traveled was quantified as migration speed (*, P < 0.05). D and E, the metastatic (D) and invasive (E) properties of the T24-L subline transfected with pATF3 and T24 cells with shATF3. Three independent experiments with 3 fields for each were carried out and representative fields are shown (*, P < 0.05). F(i), wound-healing migration assay was conducted in wild-type (WT) MEFs and atf3Δ/Δ (ATF3-KO) MEFs. F(ii), quantification of relative wound closure and average speed (*, P < 0.05). Error bars represent the mean ± SEM (n = 3). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Tumors from invasive cancer cases (r = 0.90; P < 0.01; Fig. 6D). We found very similar results in our TMA immunohistochemical staining of ATF3 and GSN (Fig. 4D and Supplementary Table S2). Moreover, the cohorts of DOD (dead as a result of disease) had a significant decrease in ATF3 expression relative to the NED (no evidence of disease) group, but no difference in the presence of lymph node metastasis (Fig. 6D) was seen. We then evaluated the association between ATF3 expression and survival following radical cystectomy in our own patients with bladder cancer. As tumor recurrence after cystectomy is usually metastatic and in most cases incurable, overall survival is an accurate surrogate for metastasis development. Our data showed that low expression of ATF3 is associated with a poor outcome following radical cystectomy (Fig. 6E, i). Furthermore, patients with both low ATF3 expression plus low GSN expression predicted a higher risk of poor survival (Fig. 6E, ii). Importantly, the result of a multivariate Cox regression analysis shows that the combination of ATF3 and GSN was an independent prognostic marker in bladder cancer (P < 0.001; Supplementary Table S3). Together, our studies confirm the reciprocal role for these 2 proteins in metastasis and strongly support
Figure 4. ATF3 regulates transcription of the actin-modifying protein GSN. A(i), actin cytoskeleton staining with phalloidin in wild-type (WT) and atf3⁻/⁻ (ATF3-KO) MEFs is shown (left, ×400; right, ×600). White arrows show the filamentous (F) actin. A(ii), quantitative results of the actin fluorescence intensity and number of actin filaments per cell are presented as mean ± SEM of triplicate experiments (*, P < 0.05). B, representative immunofluorescence staining for F-actin (red), ATF3 (green) and nuclei [4',6-diamidino-2-phenylindole (DAPI); blue] in stable pATF3-T24-L cells (i) or shATF3-T24 cells (ii) are shown (×600). C, representative immunohistochemistry of GSN expression in NMI and muscle-invasive (MI) bladder cancer (left, ×40; right, ×100). GSN expression levels were significantly decreased in muscle-invasive compared with NMI (P < 0.05; χ² test). D, representative micrographs of ATF3 and GSN from consecutive sections are shown (×200), and the correlation between these 2 molecules in 30 cases of muscle-invasive tissues was analyzed. The intensity of the symbols on the graph indicates the repetition frequency. E, the relative levels of GSN mRNA expression were measured by quantitative PCR, following manipulation of ATF3 expression (*, P < 0.05). F, immunoblot analysis of ATF3 and GSN in WT and ATF3-KO MEFs. G, ATF3 is associated with the GSN regulatory element. G(i), diagram shows the GSN regulatory regions containing or lacking high-affinity ATF3-binding sites (black and white boxes on the map). G(ii), quantitation of relative enrichment of GSN regulatory regions. G(iii), the enrichment of GSN regulatory regions was evaluated by comparing the value of the percentage of immunoprecipitation to the value of the corresponding input on T24 cells, with or without knockdown of ATF3. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
Figure 5. GSN is required for ATF3-mediated regulation of cytoskeleton remodeling. A, overexpression of GSN inhibits wound healing in T24-L cells. A(i), Western blot analysis confirmed the GSN expression in T24-L cells transfected with pcDNA3.1-GSN vector (pGSN). A(ii), representative images of the wound captured at the times indicated are shown. A(iii), quantification of migration was conducted as previously described. Data represented as mean ± SEM, n = 3 (i, P < 0.05; **, P < 0.01). B, representative image of actin cytoskeleton staining with phalloidin in pGSN-T24-L cells. The white arrow indicates F-actin. The intensity of F-actin is quantified on the right (**, P < 0.05). C and D, scratches were made in confluent cultures and wound closure was monitored over 24 hours after transfections of the T24-L or T24 cells in the presence of ATF3-overexpression/GSN-knockdown (C) or ATF3-knockdown/GSN-overexpression (D). Representative images at different time points are shown. E and F, actin staining was carried out in T24-L (E) or T24 cells (F) after transfections, as indicated. Representative images of F-actin staining are shown (left, ×600; right, ×1,000); white arrows show the F-actin. Quantitative results are presented as mean ± SEM (n = 3; *, P < 0.05). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
their use as prognostic biomarkers for recurrence in patients undergoing cystectomy.

Discussion

Here, we show that the transcription factor ATF3 has a previously unrecognized role in cancer metastases as an actin modifier through control of the actin-binding protein GSN. This has fundamental biologic consequence via regulating the cell cytoskeleton and metastasis development. We show that ATF3 suppresses bladder cancer cell motility and experimental metastasis and affects the state of the cell cytoskeleton. By both in vitro assay with overexpressed or knockdown ATF3 in bladder cancer cells and by assessing endogenous function, we show that ATF3 positively regulates GSN. This regulation also occurred in wild-type mouse-derived cells compared with ATF3-deficient cells. Furthermore, establishment of bladder cancer metastasis in vivo was significantly reduced with over-expression of ATF3 and enhanced metastasis occurred with
downregulated ATF3. Our findings support a role for ATF3 suppression of bladder cancer cell motility and metastasis via a GSN-mediated mechanism.

Several studies, including our own, have previously developed tumorigenic and metastatic isogenic variants of T24 cells (33, 36–38). T24-L established in this study was confirmed by variation analysis to have an identical origin to the parental T24 line and these 2 lines do not differ in their proliferative capacity in vitro. The ability of the T24-L to form lung metastases may provide a useful tool in understanding the mechanisms driving metastasis in bladder cancer. Consistent with previously published data and our own work, we used this model to implicate several genes that may be involved in bladder cancer metastasis, such as Src, p38, and ILK (31, 39, 40). However, ATF3–GSN axis may play an alternative role in mediating bladder cancer metastasis and progression independently of the above-mentioned regulators. Alternatively, c-Src, p38, FAK, and ILK may have roles upstream of ATF3, as a recent publication showed that Toll-like receptor (TLR)-induced ATF3 can be regulated by c-Src (41). Interestingly, p38 MAPK promotes ATF3 induction (42); here p38 is suggested to have the opposite function of inhibition of ATF3. Whether ATF3-mediated cell migration is controlled by these 2 kinases needs further characterization. Our current data show that ATF3 does not directly regulate FAK, ILK, p38, and Src but that GSN is a downstream target.

The precise role of ATF3 in cancer is unclear. ATF3 plays differing roles, either an oncogene or a tumor suppressor, depending on the cell type and context (43). Here, we show that ATF3 is protective in advanced urothelial cancer, ATF3 expression decreases with the stage of bladder cancer, and ATF3 does not directly regulate FAK, ILK, p38, and Src but that GSN is a downstream target.

ATF3expression decreases with the stage of bladder cancer, and ATF3 inhibits the migration and metastatic capability of bladder cancer cells. Although there have been reports of a tumor metastasis suppressor role for ATF3 in cancer, the molecular mechanisms have not been described (19, 21, 44). Our data show that ATF3 is intimately linked to the control of the cell cytoskeleton. The cell cytoskeleton is made up of intermediate filaments, microtubules, and actin filaments. It not only maintains cell morphology, but is also the driving force underlying cell motility for fundamental biologic processes. During migration, old actin filaments are disassembled and new actin filaments are established (actin polymerization and depolymerization). Our data indicate a role for ATF3 in repressing the ability of GSN to seve actin stress filaments (depolymerization) and trigger migration. There seems to be minimal impact on the ability of GSN to form new microfilaments (polymerization), as others have shown in certain cell types (45). It is well established that dysregulated motility is a feature of cancer cell lines (4). Because of its role in cell migration, the actin cytoskeleton is considered as a key regulator of tumor metastasis, and its polymerization status is pivotal to its homeostasis.

GSN is a key actin-modifying protein (6, 46). GSN knockout cells exhibit a variety of motility and actin defects; GSN fibroblast cells have increased numbers of actin stress fibers, which is consistent with our ATF3-null or low ATF3 expression cells (46). The expression of GSN is decreased in many transformed cell lines and tumor tissues, including bladder cancer, which indicates a role as either a tumor or metastasis suppressor (47). Directly pertinent to this study, GSN has been studied previously as a marker for clinical validation and is associated with bladder cancer progression and clinical outcome (7). A decrease in GSN expression in tumors is not attributable to mutation of the GSN gene (8). Rather, as we propose here, the downregulation of GSN in bladder cancer is likely secondary to reduced expression of ATF3. We show that ATF3 binds directly to the GSN regulatory element. Accordingly, low levels of GSN could be rescued by increased expression of ATF3. Other reports have shown that epigenetic silencing plays a role in the regulation of GSN and both histone deacetylases (HDAC) and inhibitors of DNA methylation increase GSN levels (48, 49). We and others have previously reported that ATF3 interacts with HDAC1 (11, 12). Hence, ATF3 could control GSN epigenetically, in addition to direct transcriptional activation of the GSN regulatory element.

Metastasis suppressor genes alter the signal transduction of cancer cells and are known to play a role in bladder cancer metastasis. GSN is an important metastasis suppressor gene with decreased expression in various human cancers (50, 51). In the present study, we showed downregulation of ATF3 levels paralleled with GSN expression and an increase in metastatic behavior, without the blocking of primary tumor formation. Here, we have evaluated the clinical and functional significance of ATF3 in bladder cancer metastasis for the first time. We found that ATF3 upregulated GSN, whereas knockdown of ATF3 in bladder cancer cells reduced the levels of GSN, concomitant with alterations in the actin cytoskeleton and subsequently leading to migration and invasion. The combination of ATF3 with GSN may represent a novel group of metastasis suppressors in bladder cancer.

It is important to understand the molecular mechanisms underlying bladder cancer metastasis, not only to develop new therapeutic targets against metastasis, but also to find reliable biomarkers for an accurate assessment of patient status or prognosis. Thus, there is a need to identify molecular targets that may predict which superficial bladder tumors will later progress to become invasive. The data from 2 bladder cancer studies (34, 35) in Oncomine show that both expression of both ATF3 and GSN are higher in normal samples and lower in invasive samples, consistent with our data. There are presently few molecular markers to identify localized tumors with high metastatic potential. Thus, ATF3 may be a clinically useful marker. Our results take this idea a step further by showing that ATF3 makes a critical functional contribution to bladder cancer migration and metastasis.

Critically, we show that the ATF3-dependent effects on motility and metastasis of bladder cancer cells are dependent on GSN. Significantly, the altered state of actin in cells low in ATF3 can be rescued by restoring GSN expression. Appropriately, both ATF3 and GSN strongly correlate with bladder cancer metastasis and survival of patients. Hence, ATF3 and GSN may have clinical prognostic use and are candidate therapeutic targets for bladder cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.
Authors' Contributions
Conception and design: X. Yuan, J. Li, A.T. Irving, J.-P. Liu, L. Shen, D. Xu
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Acknowledgments
The authors thank the staff at the Department of Urology, Xinhua Hospital for the collection of samples from related patients and F. Cribbin for assistance in preparation of the article.

References
Correction: ATF3 Suppresses Metastasis of Bladder Cancer by Regulating Gelsolin-Mediated Remodeling of the Actin Cytoskeleton

In this article (Cancer Res 2013;73:3625–37), which appeared in the June 15, 2013, issue of Cancer Research (1), there were erroneous duplications of images in Figs. 3B and 5A. In Fig. 3B (iii), an image that showed the effect of the siILK knockdown was duplicated from Fig. 3B (i). A corrected Fig. 3B is shown below with the intended image of the migration of T24-L cells with siILK knockdown in panel (iii). In Fig. 5A, the authors erroneously duplicated the 12-hour panel at the 24-hour time point in the time series shown in Fig. 5A (ii). A corrected figure is shown below with the intended image of the pGSN 24-hour time point replacing the duplicated panel. There is no change in the legend or text for Fig. 3B and Fig. 5A. These images were intended to provide a visual representation of the accompanying quantitative analysis, and so these errors do not change either the results or the conclusions of the article. The authors regret these errors.

The online version of the article has been corrected and no longer matches the print.

Reference
ATF3 Suppresses Metastasis of Bladder Cancer by Regulating Gelsolin-Mediated Remodeling of the Actin Cytoskeleton

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Cancer Res 2013;73:3625-3637. Published OnlineFirst March 27, 2013.

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