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

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Running title: ATF3 suppresses the metastasis of bladder cancer

Keywords: Bladder cancer; ATF3; Metastasis; Gelsolin; Cytoskeleton

Disclosure of Potential Conflicts of Interest: None of the authors have any financial conflict of interest that might be construed to influence the results or interpretation of the article.

Word count: 4238

Total number of figures 6

Precis: Mechanistic findings identify a transcription factor that suppresses metastasis of bladder cancer cells, suggesting new markers and strategies to define and address aggressive bladder tumors.
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 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 \textit{in vitro} and experimental metastasis \textit{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 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 bladder cancer patients harbor occult metastases that are associated with reduced survival (1). Hence, progression to metastasis marks a significant detrimental step in the disease. While 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 to non-muscle-invasive 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 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), while 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 demonstrate that the transcription factor ATF3 directly regulates the GSN gene through six 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, between 2005 and 2007. 5 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. 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 (ATCC) and cultured as described (http://www.atcc.org/). These cell lines were used within 6 months of resuscitation of the original cultures. T24-L, a more metastatic variant of T24, was created in our laboratory (Supplementary Fig. S1). Primary mouse embryonic fibroblasts (MEFs) 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 reiterative serial injections as described previously (24, 25). In brief, $1 \times 10^6$ T24 cells were injected into mouse via the lateral tail vein. After 16 weeks, identifiable lung lesions were isolated, tumor cells grown in tissue culture, and injected intravenously into new mice. This process of injection and isolation was repeated three 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 \textit{in vivo} and \textit{in vitro} (Supplementary Fig. S1).

Immunohistochemical staining

Standard immunohistochemical procedures were carried out using anti-ATF3 polyclonal antibody (sc-188, Santa Cruz) 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 IgG (Supplementary Fig. S2A). The specimens were digitized via whole-slide scanning with the Aperio T2 scanner (Aperio Technologies) and were independently scored by two 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, intense).
>67%) were semi-quantitatively determined. The immunoreactive score was obtained by summing these two scores (Negative, 0; Weak, 1-4; Moderate, 5-8; Strong, 9-12).

Statistical analysis

Unless otherwise stated, all statistics were performed 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's t-test, and the one-way ANOVA with Tukey's post test was performed for multiple comparisons. Correlation between variables was evaluated by Spearman's 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 Information section.

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 tissue microarray (TMA) (Fig. 1A; Supplementary, Fig. S3A). Immunohistochemical staining revealed weak ATF3 expression in invasive bladder cancer cells compared to 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 to normal, being lower in low grade, and further downregulated in high grade tumors (Fig. 1B; Supplementary Table S1). Muscle-invasive-stage (MI) disease is associated with a higher incidence of metastasis.
than non-muscle-invasive (NMI) disease, which is consistent with our finding that ATF3-positive staining was significantly reduced in MI 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 (26), 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 compared to 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, (27, 28). 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, iv). Conversely, knockdown of ATF3 enhanced migration by 43.4% without affecting proliferation (Fig. 2B-i, ii, iii, 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 to 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 to a control plasmid by about 65% and 60%, respectively. Consistent with this, ATF3 knockdown significantly increased T24 migration and invasion by over 60% relative to control (Fig. 2D, 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 demonstrate 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. Firstly, we found p-Src was down regulated 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. While tyrosine kinase c-Src has been demonstrated 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 (29). In contrast to ATF3, proteins previously reported as pro-migratory such as FAK, ILK and p38 MAPK have similar tendencies to increase, suggesting that inhibition activities of these proteins would interfere with tumor cell migration. To test this, we performed migration assays using chemical inhibitors against p38 and Src and siRNA targeting ILK. In T24-L cells treated with p38 inhibitor SB203580 or siILK, migration was markedly inhibited. In contrast, Src inhibitor Dasatinib enhanced wound healing in T24 cells (Fig 3B). Despite this, overexpression or knockdown of ATF3 had no effect on the expression level of these proteins (Fig 3C). To examine whether ATF3 expression affects actin serving protein GSN as other ATF family member such as ATF1 (8), we also investigated levels of GSN 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, while the other related migration genes mentioned above 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 to 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 (30). 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 tissue microarrays from cancer patients with various stages of bladder disease were examined immunohistochemically. Loss or reduction of GSN expression in muscle-invasive compared with non-muscle-invasive bladder cancer was observed (Fig. 4C). Moreover, there was a statistically significant direct positive correlation between ATF3 and GSN expression in the same tumors ($r=0.71$, $P<0.01$ in MI cases, Fig. 4D; and $r=0.29$, $P<0.05$ in all cases, Supplementary Table S2). Consistent with the tissue arrays and the cell line model of progression, ATF3 positively regulated GSN gene in T24-L or T24 cells (Fig. 4E). In addition, GSN expression was significantly attenuated in ATF3 knockout MEFs (Fig. 4F).

We investigated the regulatory role of transcription factor ATF3 in GSN transcription. Bioinformatic analysis predicted that six ATF3 binding motifs exist in the first intron of the $GSN$ gene. Consistent with GSN being a direct target, chromatin immunoprecipitation (ChIP) assay showed binding of endogenous GSN to two distinct regions of the GSN regulatory element harboring ATF3 or ATF/CREB binding sites (Fig. 4G-i and ii); such binding was abolished by ATF3 knockdown (Fig. 4G-i and iii). Hence, ATF3 appears to regulate the levels of GSN via direct transcription control.
The role of ATF3 in cytoskeleton remodeling and cell migration is GSN dependent

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 to 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, this suggests that the effects of ATF3 on cell motility are GSN dependent and play an important role in cell migration.

Next we tested whether GSN mediated the ATF3-dependent effects on the actin cytoskeleton by modulating the levels of GSN. Knockdown of GSN in T24-L cells that overexpressed ATF3 reinstated the levels of actin filaments (Fig. 5E). Conversely, restoration of GSN expression in T24 cells with ATF3 knockdown reduced the levels of actin stress fibers (Fig. 5F). GSN is mainly localized in cytoplasm and nucleus, with some association to actin fibers, very little GSN staining can be seen at the leading edge of the cell. This fits with a greater role for GSN in depolymerization of actin fibers rather than formation of new microfilaments at the leading edge (Supplementary Fig. S4A, B). ATF3 regulated GSN-mediated cytoskeleton remodeling mainly through depolymerization. Taken together, these findings demonstrate 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 was found in mice inoculated intravenously with the parental T24 cell line (Fig. 6A), similar to previous observations (27, 28) (31). However, ATF3 knockdown in the T24 cell line increased lung tumor burden compared to the T24 vector cells (Fig. 6A, B). Overexpressing ATF3 in the T24-L cell line reduced lung tumor burden compared to 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 two bladder cancer studies in the Oncomine database, Sanchez-Carbayo et al. and Lee et al. (32, 33). 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 et al. showed a positive correlation between ATF3 and GSN mRNA expression in the same tumors from invasive cancer cases (r=0.90, P<0.01) (Fig. 6D). We found very similar results in our tissue microarray 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). We then evaluated the association between ATF3 expression and survival following radical cystectomy.
in our own bladder cancer patients. 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 two proteins in metastasis and strongly support their utility 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 biological 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 overexpression 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.

Theodorescu and other groups, including ourselves (34, 35), have previously developed tumorigenic and metastatic isogenic variants of T24 cells (31, 34, 36). T24-L established in this study was confirmed by variation analysis to have an identical origin to the parental T24 line and these two 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 utilized this model to implicate several genes that may be involved in bladder cancer metastasis such as Src, p38 and ILK. (29, 37, 38) 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 TLR (Toll-like receptor) induced ATF3 can be regulated by c-Src (39). Interestingly, p38 MAPK promotes ATF3 induction (40), here p38 is suggested to have the opposite function of inhibition of ATF3. Whether ATF3-mediated cell migration is controlled by these two kinases needs further characterization. Our current data demonstrate 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 (41). Here, we show that ATF3 is protective in advanced urothelial cancer, ATF3 expression 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, 42). 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 biological 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 sever actin stress-filaments (depolymerization) and trigger migration. There appears to be minimal impact on the ability of GSN to form new microfilaments (polymerization), as others have shown in certain cell types (43). It is well established that dysregulated motility is a feature of cancer cell lines (4). Due to its role in cell migration, the actin cytoskeleton is considered a key regulator of tumor metastasis, and its polymerization status is pivotal to its homeostasis.

GSN is a key actin-modifying protein (6, 44). 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 (44). 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 (45). 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 gelsolin 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 (HDACs) and inhibitors of DNA methylation increase GSN levels (46, 47). 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 (48, 49). 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, while 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 biomarker 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 two bladder cancer studies (32, 33) in Oncomine demonstrate 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 utility and are candidate therapeutic targets for bladder cancer.

Acknowledgments

We are grateful to the staff at the Department of Urology, Xinhua Hospital for the collection of samples from related patients, and F. Cribbin for assistance with preparation of the manuscript.

Grant Support

This work was supported by grants from the National Natural Science Foundation of China (81072009, 21035004, 81273247 and 81202009), the Shanghai Municipal Natural Science Foundation (10ZR1420000), the Key Directional Project of Shanghai Science and Technology Commission for Medical Devices (09DZ1907203), the National Basic Research Program of China (2012CB911200), and the Basic Research Project of Shanghai Science and Technology Commission (11JC1408300). This work was also supported by grants from the National Health and Medical Research Council of Australia (606425 and 1006588 to BRG Williams and D Xu) and the Victorian Government's Operational Infrastructure Support Program. D.X. is the recipient of a Longjiang Scholar Award.
References


**Figure Legends**

**Figure 1.** ATF3 is downregulated and negatively correlated with stage in bladder cancer.

A. Representative images of ATF3 expression in benign, carcinoma in situ (CIS) and invasive (INV) bladder cancer are shown (40×). Immunoreactivity score was determined according to a semiquantitative method, as described in Materials and Methods (* P<0.05). B. Representative images of ATF3 expression are shown (200×). ATF3 scores were significantly lower in high grade bladder cancer than in low grade and normal specimens (P<0.05). C. ATF3 was significantly reduced in muscle invasive (MI) compared to non-muscle invasive bladder cancer (NMI) (P<0.05). D. (i) ATF3 protein expression in corresponding human bladder tumor (T) and non-tumorous (N) tissues was analyzed by western blot. (ii, iii) Quantification of western blots and RT-qPCR for ATF3 in matched human tissues was conducted using paired t test (** P<0.01, * P<0.05). E. (i) ATF3 expression in bladder cancer cell lines with different metastatic potential was performed by western blot. (ii) The relative ATF3 mRNA expression of cell lines was standardized to GAPDH (* 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 confirmed by western blot [A(i) and B(i)]. Cell proliferation was determined by cell counting and no significant difference was found in the numbers of pATF3 T24-L cells [A(ii)] or shATF3 T24 cells [B(ii)]. For wound healing assay, transfected cells were wounded 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)]. Cell migration was assessed by measuring relative wound closure and average speed. Data represent mean ± SEM, (n=3) [A(iv) and B(iv)].

C. Time-lapse microscopy was used to track the movements of individual bladder cancer cells with pATF3 (T24-L) (i, ii) and with shATF3 (T24) (iii, iv), over a time course of up to 24 hours. The average path length that cells traveled was quantified as migration speed.

D, 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 three fields for each were performed and representative fields are shown (*P<0.05). F. (i) Wound-healing migration assay was performed in wild-type (WT) MEFs and ATF3<sup>−/−</sup> (ATF3-KO) MEFs. (ii) Quantification of relative wound closure and average speed (*P<0.05). Error bars represent the mean ± SEM (n=3).

**Figure 3.** Identification of ATF3-regulated genes associated with migration.

A. Immunoblot analysis of phosphorylated and total protein, as indicated, was performed 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). C. Immunoblot analysis of the migration-related protein expression, as indicated, in either T24-L cells with ATF3 overexpression or T24 cells with ATF3 knockdown.
**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. (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 (DAPI; blue) in stable pATF3-T24-L cells (i) or shATF3-T24 cells (ii) are shown (600×). **C.** Representative immunohistochemistry of GSN expression in non-muscle invasive (NMI) and muscle invasive bladder cancer (MI) (left, 40×; right, 100×). GSN expression levels were significantly decreased in MI compared to NMI ($P<0.05$, $\chi^2$ test). **D.** Representative micrographs of ATF3 and GSN from consecutive sections are shown (200×), and the correlation between these two molecules in 30 cases of MI 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 qPCR, 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. (i) Diagram shows the GSN regulatory regions containing or lacking high-affinity ATF3-binding sites (black and white boxes on the map). (ii) Quantitation of relative enrichment of GSN regulatory regions. (iii) The enrichment of GSN-regulated 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.
Figure 5. GSN is required for ATF3-mediated regulation of cytoskeleton remodeling. 

**A.** Overexpression of GSN inhibits wound healing in T24-L cells. (i) Western blot confirmed the GSN expression in T24-L cells transfected with pcDNA3.1-GSN vector (pGSN). (ii) Representative images of the wound captured at the times indicated are shown. (iii) Quantification of migration was conducted as previously described. Data represented as mean ± SEM, n=3 (* 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, 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, 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, 1000×); white arrows show the F-actin. Quantitative results are presented as mean ± SEM (n=3, * P<0.05).

Figure 6. Influence of ATF3 and GSN expression on experimental bladder cancer metastases and association with disease-free survival (DFS). 

**A.** Representative macroscopic lung images, with or without metastatic nodes, are shown in the top panel. Black arrows show the lung metastatic lesions. The numbers of tumor-bearing mice and lung metastases are summarized in the bottom panel. **B.** Human 12p genomic DNA was determined by qPCR of homogenized mouse lung samples after six weeks of inoculation, using the indicated cancer cell lines. * P<0.05.  

**C.** There are significant decreases in ATF3 (upper panel) and GSN (bottom panel).
gene expression in human bladder tumor samples based on two independent studies from Oncomine data. Box plots are shown for each study, including normal urothelium (NU), superficial cancer (SUP) and invasive tumor (INV) (**P<0.01). D. Datasets were taken from Sanchez-Carbayo et al. (33) for scatter plot and linear regression analyses of 72 invasive bladder cancer samples on HG-U133A Gene chip array (upper panel). Data for the expression of ATF3 were also plotted as normalized ATF3 mRNA expression against survival status [no evidence of disease (NED) vs dead as a result of disease (DOD), *P=0.042] or lymph node metastases (lower panel). E. (i) Kaplan–Meier survival curve analysis of 64 patients with bladder cancer as determined by immunohistochemistry scoring of ATF3 as low (score ≤4) versus high (score >4) (** P<0.01). (ii) Kaplan–Meier survival curves showing overall survival as determined by either low score of ATF3 or GSN, as well as by low score (≤4) of both ATF3 and GSN (*P<0.05).
Fig 1. A, B, C, D, E

A. Low grade Benign Invasive Cancer

B. Normal Low grade High grade

C. NMI MI

D. (i) ATF3 Protein relative expression (n=20) (ii) ATF3 mRNA relative expression (n=20)

E. HUC-1 5637 T24 T24-L

Metastatic potential

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Research. on October 23, 2017. © 2013 American Association for Cancer Research.
**Fig 2.**

**A**

(i) Western blot analysis of T24-L cells with Mock, pCtrl, and pATF3 treatments showing ATF3 and GAPDH levels.

(ii) Graph showing the number of cells (x10^5) over 3 days for Mock, pCtrl, and pATF3 treatments.

**B**

(i) Western blot analysis of T24 cells with Mock, shCtrl, and shATF3 treatments showing ATF3 and GAPDH levels.

(ii) Graph showing the number of cells (x10^5) over 3 days for Mock, shCtrl, and shATF3 treatments.

**C**

(i) Migration of T24-L cells with Mock, pCtrl, and pATF3 treatments.

(ii) Graph showing migration speed (μm/min) for Mock, pCtrl, and pATF3 treatments.

**D**

(i) Invasion of T24-L and T24 cells with Mock, pCtrl, and pATF3 treatments.

(ii) Graph showing cells per field for Mock, pCtrl, and pATF3 treatments.

**E**

(i) Invasion of T24-L and T24 cells with Mock, shCtrl, and shATF3 treatments.

(ii) Graph showing cells per field for Mock, shCtrl, and shATF3 treatments.

**F**

(i) Migration of MEFs with WT and ATF3-KO treatments over 0h, 6h, and 12h.

(ii) Graph showing relative wound closure and average speed (μm/min) for WT and ATF3-KO MEFs.
**Fig 3. A, B, C**

**A**

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Metastatic potential

**B**

(i) T24-L

DMSO | p38 inhibitor

(ii) siCtrl | silILK

ILK

GAPDH

(iii) T24-L

siCtrl | silILK

(iv) T24

DMSO | src inhibitor

**C**

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**Research.**
Fig 4. A, B, C, D, E, F, G

A. Fluorescence microscopy images of WT and ATF3-KO MEFs showing F-Actin intensity and number of actin filaments/cell.

B. Immunofluorescence images of T24-L and shATF3 cells showing F-Actin, ATF3, and DAPI.

C. Immunohistochemistry images of NMI and MI showing GSN expression.

D. Western blot analysis of GSN, ATF3, and GAPDH in MEFs.

E. qRT-PCR analysis of GSN mRNA expression in T24-L and T24 cells.

F. ChIP-PCR analysis of ATF3 binding to GSN promoter.

G. Schematic representation of GSN promoter showing non-ATF3 and ATF3 binding sites with relative enrichment of IgG, ATF3, and H3K27Ac.
Fig 5. A,B,C,D,E,F
Fig 6. A,B,C,D,E
ATF3 suppresses metastasis of bladder cancer by regulating gelsolin-mediated remodeling of the actin cytoskeleton

Xiangliang Yuan, Liang Yu, Junhua Li, et al.

Cancer Res  Published OnlineFirst March 27, 2013.

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