Hsp27 Regulates Epithelial Mesenchymal Transition, Metastasis, and Circulating Tumor Cells in Prostate Cancer

Masaki Shiota1, Jennifer L. Bishop1, Ka Mun Nip1, Anousheh Zardan1, Ario Takeuchi1, Thomas Cordonnier1, Eliana Beraldi1, Jenny Bazov1, Ladan Fazli1, Kim Chi1,3, Martin Gleave1,2, and Amina Zoubeidi1,2

Abstract

Defining the mechanisms underlying metastatic progression of prostate cancer may lead to insights into how to decrease morbidity and mortality in this disease. An important determinant of metastasis is epithelial-to-mesenchymal transition (EMT), and the mechanisms that control the process of EMT in cancer cells are still emerging. Here, we report that the molecular chaperone Hsp27 (HSPB1) drives EMT in prostate cancer, whereas its attenuation reverses EMT and decreases cell migration, invasion, and matrix metalloprotease activity. Mechanistically, silencing Hsp27 decreased IL-6–dependent STAT3 phosphorylation, nuclear translocation, and STAT3 binding to the Twist promoter, suggesting that Hsp27 is required for IL-6–mediated EMT via modulation of STAT3/Twist signaling. We observed a correlation between Hsp27 and Twist in patients with prostate cancer, with Hsp27 and Twist expression each elevated in high-grade prostate cancer tumors. Hsp27 inhibition by OGX-427, an antisense therapy currently in phase II trials, reduced tumor metastasis in a murine model of prostate cancer. More importantly, OGX-427 treatment decreased the number of circulating tumor cells in patients with metastatic castration-resistant prostate cancer in a phase I clinical trial. Overall, this study defines Hsp27 as a critical regulator of IL-6–dependent and IL-6–independent EMT, validating this chaperone as a therapeutic target to treat metastatic prostate cancer. Cancer Res; 73(10); 3109–19. ©2013 AACR.

Introduction

Prostate cancer is the most common cancer and the second leading cause of cancer-related death in males in North America. Although early detection and treatment of localized Prostate cancer has improved outcomes, many men still die of metastatic disease that occurs when patients fail androgen therapies and progress to castration-resistant prostate cancer (CRPC; ref. 1). CRPC progression is a complex process whereby cancer cells acquire the ability to survive and proliferate in the absence of androgens. Metastasis of CRPC cells requires a process called epithelial-to-mesenchymal transition (EMT), which endows malignant cells with enhanced migratory and survival attributes that facilitate cancer establishment in new sites (2). Understanding molecular mechanisms that enable prostate cancer cell dissemination, in particular characterizing EMT effectors, will yield new insights into mechanisms of metastasis and may provide novel therapeutic targets that can prevent lethal disease.

During EMT, malignant epithelial cells acquire mesenchymal characteristics with defined morphology, protein expression, and gene signatures. The loss of epithelial cell markers, including E-cadherin, concomitant with gain of mesenchymal markers like N-cadherin, vimentin, and fibronectin, are hallmarks of EMT. EMT can be initiated by a wide variety of signals in the tumor environment, activating transcription factors Twist and Snail and leading to repression of E-cadherin (2). In particular, the interleukin (IL)-6/STAT3 signaling pathway is a well-characterized inducer of EMT in many cancers, including head and neck (3), non–small lung (4), and breast (5). Importantly, IL-6 overexpression in patients with prostate cancer has been implicated in the development of CRPC and high levels of IL-6 correlate with tumor burden and metastasis (6), however, whether IL-6 signaling plays a role in EMT induction in prostate cancer remains unknown.

In addition to IL-6, elevated expression of the heat shock protein Hsp27 occurs in prostate cancer and is associated with CRPC progression (7). Hsp27 is an ATP-independent molecular chaperone that is highly induced in response to cellular stresses, including exposure to mitogens, inflammatory cytokines, growth factors, hormones, oxidative stress, and antican- cer agents (8–10). Not surprisingly, therefore, Hsp27 has been identified as a critical mediator in cancer progression, preventing apoptosis in transformed cells (7, 11, 12). In addition, Hsp27 enhances migration and invasion in breast cancer cells (13), mediates EMT in lung cancer cells (14), and has been
implicated as an inducer of EMT during kidney fibrosis (15). Like IL-6, Hsp27 interacts closely with STAT3; for example, there is a direct interaction between Hsp27 and STAT3 in breast and prostate cancer cells (7, 16). Importantly, the interaction between Hsp27 and STAT3 in prostate cancer cells is required for Hsp27-mediated protection from apoptosis during androgen ablation (7).

Epithelial-to-mesenchymal transition is a definitive feature of aggressive metastatic prostate cancer. Increased expression of IL-6 and Hsp27, which are known to induce EMT in other cancer models, strongly correlate with CRPC, prostate cancer tumor burden, and poor patient prognosis. However, it is unknown whether Hsp27 regulates EMT in prostate cancer cells downstream of IL-6, or if targeting Hsp27 is a viable strategy to control metastasis in CRPC. Herein, we show that Hsp27 overexpression drives EMT in prostate cancer cells and is required for STAT3-mediated IL-6 induction of EMT. Moreover, inhibition of Hsp27 reverses EMT in vitro, prevents tumor metastasis in a murine prostate cancer model and, importantly, reduces numbers of circulating tumor cells (CTC) in patients with CRPC.

Materials and Methods

Antibodies and reagents

OligofectAMINE, lipofectin, FBS, and ZO-1 antibody were from Invitrogen-Life Technologies, Inc.; IL-6 was from Research Diagnostic Inc.; and Dual-Luciferase Reporter Assay System was from Promega. Total Hsp27/pHsp27 antibodies were from Assay Designs; Total STAT3, pSTAT3Y705, and pGSK3β antibodies were from Cell Signaling Technology; protein-G sepharose, Twist antibody was from Santa Cruz Biotechnology Inc.; E-cadherin, vimentin, and fibronectin antibodies were from BD Biosciences; and Lamin B1 and antiactin were from Abcam and Sigma, respectively.

Cell culture and transfection

LNCaP were provided by Dr. Leland W.K. Chung (1992, MDACC, Houston, TX), tested and authenticated by whole-genome and whole-transcriptome sequencing (Illumina Genome Analyzer IIx, July 2009). ARCaP and ARCaPM [ref. 17; genome and whole-transcriptome sequencing (Illumina B1 and antiactin were from Abcam and Sigma, respectively. Technology; protein-G sepharose, Twist antibody was from

Western blot analysis and immunoprecipitation

Total proteins were extracted as described previously (12) or fractionated using the Celllytic NuCLEAR Extraction Kit (Sigma) according to manufacturer’s protocol. For immunoprecipitation, proteins were extracted with immunoprecipitation lysis/wash buffer [25 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, and 1% NP-40, pH 7.4], precleared with protein-G sepharose for 1 hour, and 500 μg protein was immunoprecipitated overnight at 4°C with 2 μg of anti-STAT3, or immunoglobulin G (IgG) as a control. Immune complexes were recovered with protein-G sepharose for 2 hours and washed with IP lysis/wash buffer 3 times after Western blotting.

Quantitative PCR

Total RNA was extracted from cells using TRIzol reagent (Invitrogen) and 2 μg was reversed transcribed using random hexamers (Applied Biosystems) and 20U of Moloney murine leukemia virus reverse transcriptase (Invitrogen). Quantitative real time PCR (qRT-PCR) amplification of cDNA was carried out using the following: Twist1 (Hs00361186_m1), E-cadherin (Hs01023894_m1), vimentin (Hs00185584_m1), fibronectin (Hs01549976_m1), Hsp27 (Hs03044127_g1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Hs03929097_g1) on the ABI PRISM 7900 HT Sequence Detection System with TaqMan Gene Expression Master Mix (Applied Biosystems). Target gene expression was normalized to GAPDH levels. Results are representative of at least 3 independent experiments, with each sample being run in triplicate.

Cell migration assays

Transwell assay. Cells (5 × 10⁴) in serum-free media were seeded in the top chamber and 20% FBS was used as the chemoattractant in the bottom chamber (BD Biosciences). Migrated cells were fixed in 100% methanol and stained using 0.5% crystal violet. Quantification of the migrated cells was done by counting 4 field of view. Imaging and densitometry was conducted using a LI-COR odyssey imaging system. Triplicate filters were used and the experiments were repeated 3 times.

Wound healing assay. Scratch wounds were made in confluent cell monolayers using a pipette tip. Monolayers were washed twice with serum-free media and the cells were cultured in serum-free media with or without 50 ng/mL of IL-6. Cell migration was recorded in 6 different microscopic fields. The percentage of wound healing was calculated by the equation; (percent wound healing) = average of [(gap area: 0 hour) – (gap area: 24 hours)]/(gap area: 0 hours).

Gelatin Zymography for MMP activity

Cells were incubated in DMEM overnight and conditioned media was collected and concentrated 20× using Amicon centricron-10 concentrators (Amicon). Protein lysates were resolved on 8% non-denaturing polyacrylamide gel containing 1 mg/mL gelatin (Bio-Rad). Gels were incubated in 2.5% Triton X-100 for 1 hour, developed at 37°C overnight in activation buffer (50 mmol/L Tris-HCl, pH 7.6, 50 mmol/L NaCl, and 5 mmol/L CaCl₂) stained with Coomassie Blue for 1 hour, and destained to define signal. Imaging and densitometry was conducted using a LI-COR odyssey imaging system.

Luciferase assay

LNCaP cells (2.5 × 10⁴) were plated in 6-well plates and transfected with Twist or with STAT3-Luc using lipofectin. The reporter plasmids (Twist1-Luc – 969, −745, −451, −186, and −145) were recovered with protein-G sepharose for 2 hours and washed with IP lysis/wash buffer 3 times after Western blotting.
ASO or OGX-427. Scr ASO or OGX-427 of 20 mg/kg was injected into tail veins of 6- to 8-week-old male athymic nude mice. Immunohistochemical staining was conducted according to manufacturer’s protocol. LNCaPsh-Control and LNCaPsh-Hsp27 cells treated with 50 ng/mL of IL-6 for 30 minutes were cross-linked with paraformaldehyde and digested with micrococcal nuclease to achieve a DNA smear of 200—1,000 bp. ChIP assay was conducted using the SimpleChIP Enzymatic Chromatin IP Kit (Agarose Beads) according to the manufacturer’s protocol (Cell Signaling Technology). PCR conditions were 30 cycles of 95°C, 60°C, and 72°C for 30 seconds. qRT-PCR was conducted with 2 μL of DNA extraction, with the following primers: Twist promoter 5′-GCCAGTGTGTGATGG-3′ (forward) and 5′-CGTGACGCGGAAGTTGG-3′ (reverse) and FastStart Universal SYBR Green Master (ROX; Roche Applied Science) on the ABI PRISM 7900 HT Sequence Detection System (19). Results are representative of at least 3 independent experiments.

Prostate tumor immunohistochemistry

A total of 106 prostate cancer specimens were obtained from the Vancouver Prostate Centre Tissue Bank. Two tissue microarrays (TMA) were manually constructed (Beecher Instruments) by punching duplicate cores of 1 mm for each sample. All the specimens were from radical prostatectomy except 12 CRPC samples obtained from transurethral resection of prostate. Immunohistochemical staining was conducted as previously described (12) using the Ventana Discover XT Autostainer (Ventana Medical System) with enzylabeled biotin streptavidin system and a solvent-resistant DAB Map kit by using 1/150 concentration of Twist1 and pSTAT3 (Cell Signaling Technology) and 1/25 concentrations of HSP27 (Sigma) antibodies. TMAs were scored as follows: 0 = no staining, 1 = faint or focal stain, 2 = convincing intensity in a minority of cells, and 3 = convincing intensity in a majority of cells. The overall percentage of cancer cells showing staining (0%–100%) was also indicated. Scoring was conducted at 200×.

In vivo tumor metastasis model

PC-3M-luc (C6) cells (2 × 106; Caliper Life Sciences) were injected into tail veins of 6- to 8-week-old male athymic nude mice (Harlan Sprague-Dawley, Inc.). After 24 hours, mice were randomly divided into 2 groups, n = 10, for treatment with Scr ASO or OGX-427. Scr ASO or OGX-427 of 20 mg/kg was injected intraperitoneally once daily for 7 days followed by 3 weekly treatments thereafter. On weeks 2, 4, 6, 8, and 10 mice were injected intraperitoneally with 150 mg/kg α-luciferin (Caliper Life Sciences), anesthetized with isoflurane, and imaged in the supine position exactly 11 and 15 minutes after injection in an IVIS200 Imaging System (Caliper Life Sciences). Data were acquired and analyzed using Living Image software version 3.0 (Caliper Life Sciences). Animal procedures were done according to the guidelines of the Canadian Council on Animal Care.

Circulating tumor cell isolation

Patients with CRPC enrolled in OGX-427 phase I of OGX-427 receiving doses from 400 mg to 1,000 mg i.v. on a 21-day schedule had whole-blood collected for CTC analyses at screening, after OGX-427 initiation, and on day 1 of every cycle. CTC enumeration was conducted as previously described (12). Blood samples were drawn into 10 mL evacuated blood draw tubes (CellSave; Veridex LLC), maintained at room temperature, and processed within 96 hours of collection on the CellSearch System (Veridex LLC) as per manufacturer’s instructions. CTC were defined as nucleated cells lacking CD45 and expressing cytokeratin as per manufacturer’s instructions.

Results

Hsp27 promotes epithelial-to-mesenchymal transition

Clinically, Hsp27 is highly expressed in many cancers (20), including breast (21), prostate (22), where it is associated with aggressive tumor behavior, metastasis, and poor prognosis (23, 24) and its overexpression enhances migration and invasion (25). However, a defining role for Hsp27 in EMT in prostate cancer remained unexplored. To evaluate the relationship between Hsp27 and EMT, Hsp27 was overexpressed in RWPE-2 cells, a normal epithelial prostate cell line transformed with K-Ras, and in the epithelial prostate cancer cell line, ARCaP (17, 18, 26). Hsp27 overexpression in both lines caused decreases in E-cadherin expression (Fig. 1A) and decreases in vimentin and fibronectin (Fig. 1B), whereas its knockdown using siRNA in the prostate cancer cell line DU145, lung cancer cell line A549, and breast cancer cell line MCF-7, led to increased E-cadherin (Fig. 1C) and decreased vimentin and fibronectin (Fig. 1D) expression. These observations suggested that Hsp27 could regulate EMT in different cancers and in prostate cells. As elevated expression of Hsp27 is associated with poor prognosis of metastatic prostate cancer (22), Hsp27 was stably overexpressed in the human androgen receptor (AR)-positive prostate cancer cell line, and LNCaP and EMT was examined. Hsp27 overexpression in LNCaP cells induced changes in cell morphology, protein, and mRNA expression characteristic of EMT. Examination of cells by phase contrast and immunofluorescent microscopy showed a shift from an epithelial, cobblestone-like morphology in mock-treated (mock) cells to an elongated and spindled fibroblastic morphology in Hsp27-overexpressing (Hsp27) cells (Fig. 2A). This effect was accompanied by decrease in cell–cell contact and a loss of E-cadherin and the tight junction protein, ZO-1 (Fig. 2A and B). Hsp27 cells also showed increased...
expression of the mesenchymal markers phospho-GSK-3β, vimentin, fibronectin, Twist, and N-cadherin at both protein and mRNA levels (Fig. 2B). Moreover, consistent with previous studies (27), we found that the expression of Hsp27 increased cell migration (Fig. 2C) and matrix metalloproteinase (MMP) activity (Fig. 2D), 2 important factors in tumor migration and metastasis (28).

**Silencing Hsp27 leads to mesenchymal-to-epithelial in prostate cancer cells**

To effectively relate Hsp27 expression levels to specific cellular behaviors, we developed a LNCaP cell line with stable reduction (up to 95%) of Hsp27 expression using Hsp27 targeting shRNA (sh-Hsp27). sh-Hsp27 cells displayed a clear morphologic transition to an enhanced epithelial or cobblestone-like pattern, with well-organized cell contact and polarity (Fig. 3A). These changes were accompanied with increased E-cadherin and decreased phospho-GSK-3β, vimentin, fibronectin, and Twist at protein and mRNA levels (Fig. 3A and B). Hsp27 knockdown cells also exhibited reduced cell invasion (Supplementary Fig. S1), migration, and MMP activity compared with controls (Fig. 3C and D). To control for nonspecific changes in EMT status potentially induced by stable Hsp27 knockdown, similar experiments were conducted in LNCaP cells treated with siRNA targeting Hsp27. LNCaP cells treated with Hsp27 siRNA also showed induction of markers of epithelial, as opposed to mesenchymal, differentiation; including higher levels of E-cadherin and lower levels of vimentin, fibronectin, and Twist, compared with control siRNA (siCtr; Supplementary Fig. S2A and SB). Hsp27 siRNA also reduced cell invasion and MMP activity (Supplementary Fig. S3A and SB) in LNCaP cells. Taken together, these data suggest that Hsp27 is a positive regulator of EMT.

**IL-6 induces EMT in an Hsp27-dependent manner**

IL-6 is elevated in biopsies and sera of patients with metastatic prostate cancer (6, 29) and its overexpression in the breast cancer cells induces EMT via STAT3-Twist (5). Therefore, we sought to determine whether IL-6 induces EMT in prostate cancer cells and the involvement of Hsp27 in this pathway. As in MCF-7 cells (5), we found that LNCaP cells exposed to IL-6 showed decreased levels of E-cadherin and increased levels of fibronectin and Twist as early as 6 hours after treatment (Fig. 4A). Concomitant with these changes, IL-6 treatment of LNCaP cells increased expression of Hsp27 (Fig. 4A). In addition, STAT3 and Twist transcriptional...
activities were increased by IL-6 treatment (Fig. 4B). Taken together, these data suggest that this pathway is active in LNCaP cells and that IL-6 induces EMT in LNCaP cells most likely via STAT3 and Twist transcription, with potential involvement of Hsp27.

Next, we set out to define the role of Hsp27 in IL-6-induced EMT by analyzing epithelial and mesenchymal markers as well as Twist activity in IL-6 stimulated Hsp27 stable knockdown cells. IL-6 treatment of LNCaP cells induced a morphologic change to a fibroblastic phenotype in control (sh-Ctr), but not in Hsp27 knockdown (sh-Hsp27), cells (Fig. 4C). This effect on cell morphology correlated with changes in EMT markers. In control cells, IL-6 treatment increased Twist and vimentin within 6 hours; by 24 hours, E-cadherin levels decreased and fibronectin levels increased (Fig. 4D). In contrast, in Hsp27 knockdown cells, E-cadherin levels decreased over time with IL-6 treatment, and levels of Twist, vimentin, and fibronectin protein and mRNA expression were markedly reduced almost to undetectable levels, compared with control cells in both the presence and absence of IL-6 (Fig. 4D). In addition, knockdown of Hsp27 in LNCaP cells prevented IL-6 induced wound healing (Fig. 4E). These data suggest that Hsp27 is required for both IL-6–dependent and IL-6–independent EMT in prostate cancer cells.

**Hsp27 expression is required for STAT3 binding to the Twist promoter**

To further dissect mechanisms by which Hsp27 regulates IL-6–mediated EMT responses, we examined effects of Hsp27 knockdown on IL-6 pathway activity. We observed that in control cells (sh-Ctr), IL-6 induced phosphorylation of STAT3 on both tyrosine and serine residues, as well as Hsp27, in a time-dependent manner (Fig. 5A). In contrast, stable Hsp27 knockdown (sh-Hsp27) drastically decreased IL-6–induced STAT3 phosphorylation (Fig. 5A). Decreased STAT3 phosphorylation correlated with reduced IL-6 induced STAT3 nuclear translocation in Hsp27 knockdown cells (Fig. 5B). ChIP assays were conducted to investigate the effect of Hsp27 knockdown on STAT3 binding to the Twist promoter (19). Using primers that covered the proximal 3 STAT3-binding sites in the Twist promoter (19), we found that IL-6 induced STAT3 binding to the Twist promoter DNA in control, but not Hsp27 knockdown, LNCaP cells (Fig. 5C). Finally, we monitored the effect of Hsp27 knockdown on IL-6–induced Twist transcriptional activity using a luciferase transactivation assay. Twist transcriptional activity was analyzed using serial truncations of the human Twist promoter as described by Cheng and colleagues (19) in the presence or absence of IL-6. We found that for each Twist truncation assessed, IL-6 induced more than a 2-fold increase of promoter activation in control-transfected cells, whereas Hsp27 knockdown almost completely abrogated IL-6–induced Twist transcriptional activity (Fig. 5D left panel). Conversely, Hsp27 overexpression (Hsp27 WT) enhanced IL-6–induced Twist transcriptional activity compared with control (Mock) cells (Fig. 5D right panel). Taken together, our data provide evidence that Hsp27 is required for STAT3 binding to the Twist promoter and activating Twist transcription in the presence of IL-6.

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**Figure 2.** Hsp27 induces EMT in LNCaP cells. A, morphology of LNCaP cells stably transfected with control vector (mock) or Hsp27 (Hsp27) was examined by phase contrast microscopy. Immunofluorescence shows expression of E-cadherin, ZO-1, and vimentin [green, target; blue, 4', 6-diamidino-2-phenylindole (DAPI)]. B, total protein or RNA was analyzed by Western blotting or quantitative PCR. Equal protein loading was confirmed by actin expression and EMT marker mRNA expression was normalized to GAPDH. C, cells were plated in transwell chambers for 48 hours and cells located in bottom chamber were counted. D, MMP activity was assessed by gelatin zymography.
Hsp27 and Twist are associated with aggressive human prostate cancer

Increased expression of Hsp27 is found in prostate cancer and is associated with poor prognosis and metastasis (23, 24). However, correlation between increased Hsp27 and Twist expression in human tumors has not been reported. Using IHC, we observed a direct correlation between Gleason score and Hsp27 and Twist-staining intensity; Gleason 4 or 5 cancers had significantly higher Hsp27 and Twist staining than Gleason 3 cancers (Fig. 6A and B) and Hsp27 and Twist staining positively correlated across all Gleason grades (Pearson coefficient = 0.218). In addition, we observed increased pSTAT3 staining in Gleason 4 versus 3 tumors (Fig. 6A). Quantification of positive versus negative pSTAT3 signal in our TMAs showed that 83.33% of Gleason 3 tumors were positive for pSTAT3 staining, whereas 98.31% of Gleason 4 tumors were positive for pSTAT3. In addition, there was a positive correlation between pSTAT3 and Hsp27 (Pearson coefficient = 0.114) or Twist expression (Pearson coefficient = 0.112) across all Gleason grades. These results suggest a direct relationship between Hsp27 in Twist signaling in aggressive human prostate cancer.

Hsp27 is required for metastasis in a mouse model of prostate cancer

Our data suggested that Hsp27 expression is a feature of aggressive human prostate cancer tumors and EMT in prostate cancer cell lines. Therefore, we wanted to determine whether Hsp27 knockdown affects metastasis in a mouse model of highly metastatic prostate cancer using PC-3M cells. In vitro, Hsp27 knockdown reduced migration of PC-3M cells (Supplementary Fig. S4). In vivo, after systemic delivery of luciferase expressing PC-3M cells, athymic nude mice were treated with either control scrambled antisense oligonucleotide (Scr ASO) or ASO targeting Hsp27 (OGX-427, OncoGeneX Pharmaceuticals), and tumor spread was monitored over 10 weeks using a bioluminescent imaging system. We found a drastic reduction in luminescent signal, indicating the presence of tumor cells, throughout the entire body of mice treated with OGX-427 compared with Scr ASO at 10 weeks after tumor injection (Fig. 7A). Quantification of luminescence at 6, 8, and 10 weeks after injection showed a significant reduction in signal at each time point in mice treated with OGX-427 compared with Scr ASO (Fig. 7B). These results indicate that Hsp27 enhances dissemination of prostate cancer tumor cells in vivo and further link increased Hsp27 expression with cancer aggressiveness and metastatic capacity.

OGX-427 treatment decreases circulating tumor cells in patients with mCRPC

Decreases in CTC counts in patients with prostate cancer undergoing treatment is associated with improved survival (30). To provide clinical relevance to our work, we evaluated the effects of OGX-427 on CTC counts in patients with prostate cancer. In a phase 1 study of OGX-427, 19 patients with...
metastatic CRPC were treated with doses of OGX-427 ranging from 400 to 1,000 mg and blood was collected from 17 evaluable patients for CTC analyses (CTC at baseline and at least 1 posttreatment time point). The median baseline CTC count was 40 CTC/7.5 mL blood (range 4–938), which decreased to a median of 15 CTC/7.5 mL (range 0–446) as a best CTC decline while on therapy. Mean CTC count was 152/7.5 mL (SD ≈ 239) at baseline, which decreased to 66/7.5 mL (SD ≈ 118) as a best CTC decline on therapy (t test ≈ 0.088, 2-tailed). The median best percentage decline in CTC from baseline by patient was 58% (range /C0 43%–100%). One patient had a decline in CTC from 39 CTC/7.5 mL at baseline to 0 CTC/7.5 mL by the fifth treatment cycle (OGX-427 dose ≈ 400 mg; summary, Supplementary Table S1). These CTC data provide the first clinical evidence of anticancer activity after Hsp27 inhibition, and are compatible with our preclinical data supporting a role for Hsp27 in EMT and dissemination of tumor cells to metastatic sites.

Discussion

Epithelial-to-mesenchymal transition is a critical component of prostate cancer progression, facilitating development of lethal metastatic CRPC. Using Hsp27 overexpressing, as well knockdown prostate cancer cell lines, we showed that Hsp27 is a critical mediator of IL-6–dependent and -independent EMT. Investigation into the mechanism of Hsp27 function in IL-6 induced EMT showed that Hsp27 mediates STAT3 phosphor-

lation, nuclear translocation and STAT3 binding to the Twist promoter. These data were supported by immunohistochemistry showing increased Hsp27 and Twist staining in high Gleason grade tumors from patients with prostate cancer. We have also shown a role for Hsp27 in promoting prostate cancer metastasis in vivo, as tumor cell dissemination was significantly suppressed in mice treated with the Hsp27 inhibitor, OGX-427. More importantly, targeting Hsp27 in men with metastatic CRPC using OGX-427 led to reduced CTC counts,
providing the first clinical evidence of anticancer metastatic activity for this drug in humans. Collectively, our results highlight Hsp27 as a key modulator of prostate cancer cell plasticity, driving EMT and endowing prostate cancer cells with metastatic potential.

High levels of Hsp27 have been reported in many cancers including breast (21), ovarian (31), glial (32), and prostate (33, 34), and it has been implicated in EMT in breast cancer (35), lung cancer (14), and kidney fibrosis (15). Our data show that Hsp27 is also required for EMT in prostate cancer cell lines and can drive EMT independently of exogenous factors like IL-6. This may be related to its function in actin rearrangement, cytoskeleton organization, and cell migration (27). Our results showing that Hsp27 silencing increased expression of the tight junction protein ZO-1 and prevented cell migration, further support these reports. Such effects on Hsp27-deficient cells may be dependent not on IL-6, but the ability of Hsp27 to interact with STAT3. Multiple reports have shown direct interactions between Hsp27 and STAT3 in the absence of IL-6 (7, 16). Furthermore, Hsp27 overexpression enhances cell migration via the modulation of Fak-dependent actin organization and STAT3-dependent MMP-2 expression (25). Factors that facilitate Hsp27 and STAT3 interactions to promote IL-6 independent EMT are under further investigation.

We also identified a role for Hsp27 downstream of IL-6, a key regulator of EMT with clinical relevance to aggressive carcinoma. In prostate cancer, IL-6 mediates the development and progression of CRPC (6, 36) and elevated IL-6 levels in sera of patients with prostate cancer correlate with tumor burden and metastases (6, 29). In normal prostate cell lines, ectopic expression of IL-6 induces cell transformation, EMT, tumor formation, and tumor progression to metastasis (37). In breast cancer cell lines, IL-6 overexpression leads to a reduction in E-cadherin, increases of vimentin, fibronectin, and Twist, as

Figure 5. Hsp27 is required for IL-6/STAT3/Twist pathway activity. A, serum-starved LNCaP sh-Ctr or sh-Hsp27 cells were treated with ± 50 ng/mL of IL-6 for 0 to 60 minutes. Proteins were analyzed by Western blot, and actin was used as a loading control. B, serum-starved cells were treated with ± 50 ng/mL of IL-6 for 30 minutes. Protein from nuclear and cytoplasmic fractions was analyzed by Western blot. Lamin B1 and actin were used as loading controls and fraction purity. C, ChIP assays were conducted on nuclear extracts from LNCaP cells treated with ± 50 ng/mL of IL-6 for 6 hours before lysis. The luciferase activity of Twist-Luc alone was set as 1.
Gleason grade 4 invasion in human prostate cancer cell lines (40, 41). Our TGF-β reports showing knockdown of Hsp27 inhibits VEGF and β6–dependent EMT. These results are similar to previous in vitro metastatic disease. Accordingly, Hsp27 was also required for well, mediating effects of EMT inducers like IL-6 during Hsp27 may modulate the Twist pathway in human cancer as high Gleason grade prostate cancer tumors suggest that vented STAT3 binding to the Twist promoter. Our data from STAT3 phosphorylation and nuclear translocation, and pre-ular, we found that Hsp27 knockdown decreased IL-6 induced STAT3/Twist-induced EMT in prostate cancer cells. In partic-

Figure 6. Hsp27 expression is associated with aggressive human prostate cancer. A, representative sections of prostate cancer tumors from radical prostatectomy specimens with Gleason scores of 3 or 4 were stained for IHC specific for Hsp27 Twist and pSTAT3. B, Hsp27 and Twist staining intensity was quantified in 2 cores each of 30 Gleason grade 3, 27 grade 4, and 10 grade 5; radical prostatectomy prostate cancer tumors and ANOVA statistical analysis was used to show significance. Error bars represent SD. C, pSTAT3 positivity was quantified in 2 cores each of 30 Gleason grade 3 and 27 grade 4 tumors. Fisher’s test was used to analyze statistical significance of positive versus negative staining and risk associated with positive staining and increased Gleason grade. D, Pearson’s coefficient was calculated to analyze correlation between Hsp27, Twist, and pSTAT3 or Twist and pSTAT3 across Gleason grades.

well as aberrant activation of STAT3 (5). Here, we show that exogenous IL-6 treatment increased Hsp27, fibronectin, and Twist expression and decreased E-cadherin expression in LNCaP cells, indicating that IL-6 also induces EMT in prostate cancer.

A key regulator of Twist transcription is STAT3, which is constitutively active in many tumors to control the expression of cell survival as well as EMT genes (19, 38). Recent evidence highlighted the importance of the IL-6/STAT3 pathway in prostate cancer stem cells, identifying epigenetic modifications of key genes that interact with STAT3 to promote invasiveness (39). Interestingly, Hsp27 binds STAT3 in breast (16) and prostate cancer cells (7), and in vivo, Hsp27 over-expression in the LNCaP xenograft mouse model confers hormone resistance postcastration via the activation of STAT3 (7). In this study, we showed that Hsp27 is required for IL-6/STAT3/Twist-induced EMT in prostate cancer cells. In particular, we found that Hsp27 knockdown decreased IL-6 induced STAT3 phosphorylation and nuclear translocation, and prevented STAT3 binding to the Twist promoter. Our data from high Gleason grade prostate cancer tumors suggest that Hsp27 may modulate the Twist pathway in human cancer as well, mediating effects of EMT inducers like IL-6 during metastatic disease. Accordingly, Hsp27 was also required for in vitro wound healing, further indicating its importance in IL-6-dependent EMT. These results are similar to previous reports showing knockdown of Hsp27 inhibits VEGF and TGF-β–induced cell migration and STAT3-mediated cell invasion in human prostate cancer cell lines (40, 41). Our data therefore suggest that Hsp27 promotes IL-6 signaling in tumor microenvironments to induce EMT, highlighting an important mechanism in prostate cancer progression and metastasis. The dramatic effect on reduction of CTC number in patients with CRPC treated with the Hsp27 inhibitor, OGX-427, indicate that Hsp27 may indeed drive metastasis in cancers associated with high levels of IL-6.

While it is required for STAT3 binding to Twist downstream of IL-6, Hsp27 also induced EMT and metastasis in vivo, as it did in vitro, independently of this factor. As aforementioned, this could be explained by Hsp27/STAT3 interactions that mediate aspects of cell migration and invasion. In addition, since Hsp27 is a molecular chaperone that stabilizes protein complexes (12) it is possible that Hsp27 stabilizes STAT3 binding with its kinases, explaining the dramatic effect of Hsp27 knockdown on STAT3 phosphorylation in the presence or absence of IL-6. Alternatively, Hsp27 may play a role in other STAT3 independent signal pathways that can promote EMT. For example, Hsp27 maintains a population of breast cancer stem cells that display EMT characteristics by increasing degradation of IκBα, enhancing nuclear translocation of NF-κB, and stabilizing Snail, thereby repressing E-cadherin expression (35). While it remains unknown how NF-κB signaling mediates EMT in prostate cancer, reports have shown that NF-κB is activated downstream of Egr-1 in LNCaP and PC-3 prostate cancer cells (42) and Egr-1 induces EMT in breast cancer (43).

In summary, Hsp27 is a component of several pathways known to induce EMT in prostate cancer. This has widespread...
implications, as EMT is not simply a feature of metastatic cells; it is also a characteristic of cancer stem cells and associated with cancer dormancy and treatment resistance (2). These data provide support for the role of Hsp27 in IL-6 signaling and EMT and as a therapeutic target for CRPC.

Disclosure of Potential Conflicts of Interest
K.N. Chi has a commercial research grant in OncoGeneX Pharmaceuticals Inc. and is a consultant/advisory board member of OncoGenex Technologies Inc. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: A. Zoubeidi
Development of methodology: K. Nip, A. Zardan, J. Bazov, A. Zoubeidi
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Shiota, A. Takeushi, K.N. Chi, L. Fazli
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Shiota, A. Zardan, A. Takeushi, T. Cordonnier, J. Bazov, K.N. Chi, M. Gleave, A. Zoubeidi
Writing, review, and/or revision of the manuscript: M. Shiota, J.L. Bishop, A. Zardan, K.N. Chi, M. Gleave, A. Zoubeidi
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T. Cordonnier, E. Beraldi
Study supervision: A. Zoubeidi

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