Hsp27 regulates epithelial mesenchymal transition, metastasis and circulating tumor cells in prostate cancer

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ABSTRACT
Defining the mechanisms underlying metastatic progression of prostate cancer (PCa) 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 PCa while 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 PCa patients, with Hsp27 and Twist expression each elevated in high grade PCa tumors. Hsp27 inhibition by OGX-427, an antisense therapy currently in Phase II trials, reduced tumor metastasis in a murine model of PCa. More importantly, OGX-427 treatment decreased the number of circulating tumor cells in patients with metastatic castration-resistant PCa in a Phase I clinical trial. Overall, this study defines Hsp27 as a critical regulator of IL-6 dependent and independent EMT, validating this chaperone as a therapeutic target to treat metastatic PCa.
INTRODUCTION

Prostate Cancer (PCa) is the most common cancer and the second leading cause of cancer death in males in North America. While early detection and treatment of localized PCa has improved outcomes, many men still die of metastatic disease that occurs when patients fail anti-androgen therapies and progress to castration resistant prostate cancer (CRPC) (1). CRPC progression is a complex process whereby cancer cells acquire the ability to survive and proliferate in the absence of testicular 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 PCa 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 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 PCa patients 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 PCa remains unknown.

In addition to IL-6, elevated expression of the heat shock protein Hsp27 occurs in PCa 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 anti-cancer 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 PCa cells (7, 16). Importantly, the interaction between Hsp27 and STAT3 in PCa cells is required for Hsp27 mediated protection from apoptosis during androgen ablation (7).

EMT is a definitive feature of aggressive metastatic PCa. Increased expression of IL-6 and Hsp27, which are known to induce EMT in other cancer models, strongly correlate with CRPC, PCa tumor burden and poor patient prognosis. However, it is unknown whether Hsp27 regulates EMT in PCa 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 PCa 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 PCa model and, importantly, reduces numbers of circulating tumor cells in patients with CRPC.
MATERIALS AND METHODS

Antibodies and reagents

OligofectAMINE, lipofectin, and fetal bovine serum (FBS) and ZO-1 antibody were from Invitrogen-Life Technologies, Inc.; IL-6 from Research Diagnostic Inc.; Dual-Luciferase Reporter Assay System from Promega. Total Hsp27/pHsp27 antibodies were from Assay Designs; Total STAT3, pSTAT3<sup>Y705</sup> and pSTAT3<sup>S727</sup> and pGSK3β antibodies from Cell Signaling Technology; protein-G sepharose, Twist antibody from Santa Cruz Biotechnology Inc.; E-cadherin, vimentin and fibronectin antibodies from BD Bioscinces; Lamin B1 from Abcam and anti-actin from and Sigma.

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<sub>E</sub> and ARCaP<sub>M</sub> (17) (derived from ARCaP cells and developed in the laboratory of Dr. Chung (18)) and LNCaP cells were maintained in RPMI+5% FBS. LNCaP were stably transfected with shRNA Hs p27 (Santa Cruz), selected using puromycin (Invitrogen-Life Technologies, Inc.) and maintained in DMEM+5% FBS. For siRNA transfection, cells were treated with Hsp27 siRNA or Scramble siRNA using OligofectAMINE as described previously (12). 24 hours after the second transfection, cells were serum starved overnight and stimulated with IL-6 as indicated.

Western blot analysis and immunoprecipitation

Total proteins were extracted as described previously (12) or fractionated using CelLytic<sup>™</sup> NuCLEAR<sup>™</sup> Extraction Kit (Sigma) according to manufacturer’s protocol. For immunoprecipitation, proteins were extracted with IP lysis/wash buffer (25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA and 1% NP-40, pH 7.4), pre-cleared with protein-G sepharose for 1 hour and 500µg 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 three times prior to western blotting.
qPCR

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). qRT-PCR amplification of cDNA was performed using the following: Twist1 (Hs00361186_m1), E-cadherin (Hs01023894_m1), vimentin (Hs00185584_m1), fibronectin (Hs01549976_m1), Hsp27 (Hs03044127_g1) and GAPDH (Hs03929097_g1) on 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 three independent experiments, with each sample being run in triplicate.

Cell migration assays

**Transwell assay:** 5×10^4 cells in serum-free media were seeded in the upper 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 migrated cells was done by counting 4 region of the filter under the microscope. 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 50ng/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 h]- [gap area: 24 h])/[gap area: 0 h]).

Gelatin Zymography for MMP activity

Cells were incubated in DMEM overnight and conditioned media was collected and concentrated 20x using Amicon centricon-10 concentrators (Amicon). Protein lysates were resolved on 8% nondenaturing polyacrylamide gel containing 1 mg/ml gelatin (Bio-Rad). Gels were incubated in 2.5% Triton X-100 for 1 h, developed at 37°C overnight in activation buffer (50 mM Tris-HCl, pH 7.6, 50 mM NaCl, and 5 mM CaCl2), stained with Coomassie Blue for 1 hour and distained to define signal. Imaging and densitometry was performed using a LI-COR odyssey imaging system.
Luciferase Assay

LNCaP cells (2.5 x 10⁵) were plated in six-well plates and transfected with Twist or with STAT3-Luc using lipofectin. The reporter plasmids (Twist1-Luc –969, –745, –451, –186 and –105) containing various lengths of the promoter of the wild-type Twist gene were gifted from Dr. Wang LH (Mount Sinai School of Medicine, New York, NY) (19). Total plasmid DNA was normalized to 0.5 µg per well by the addition of a control plasmid. At 24 h post-transfection, cells were incubated with 50 ng/mL of IL-6 for 6 h and luciferase activities were measured using a Dual-Luciferase Reporter Assay System (Promega) and a microplate luminometer (EG&G Berthold). The Firefly luciferase activities were corrected by the corresponding Renilla luciferase activities. Results are representative of at least three independent experiments. All experiments were carried out in triplicate.

Chromatin immunoprecipitation assay (ChIP assay)

ChIP assay was performed according to manufacturer’s protocol. LNCaPsh-Control and LNCaPsh-Hsp27 cells treated with 50 ng/mL of IL-6 for 30 min were cross-linked with paraformaldehyde and digested with micrococcal nuclease to achieve a DNA smear of 200–1000 bp. ChIP assay was performed using SimpleChIP™ Enzymatic Chromatin IP Kit (Agarose Beads) according to the manufacturer’s protocol (Cell Signaling Technology). PCR conditions were 30 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s. qRT-PCR was performed with 2 µL of DNA extraction, with the following primers: Twist promoter (19) 5’-GCCAGGTCGTTTTTGAATGG-3’ (forward) and 5’-CGTGCAGGCGGAAAGTTTGG-3’ (reverse) and FastStart Universal SYBR Green Master (ROX) (Roche Applied Science) on the ABI PRISM 7900 HT Sequence Detection System. Results are representative of at least three independent experiments.

Prostate tumor immunohistochemistry

106 prostate cancer specimens were obtained from the Vancouver Prostate Centre Tissue Bank. 2 tissue microarrays were manually constructed (Beecher Instruments, MD, USA) by punching duplicate cores of 1mm for each sample. All the specimens were from radical prostatectomy except 12 CRPC samples obtained from transurethral resection of Prostate (TURP). Immunohistochemical staining was conducted as previously described (20) using the Ventana
Discover XT™ autostainer (Ventana Medical System, Tuscan, Arizona) with enzyme labeled biotin streptavidin system and solvent resistant DAB Map kit by using 1/150 concentration of TWIST-1 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, 3=convincing intensity in a majority of cells. The overall percentage of cancer cells showing staining (0-100%) was also indicated. Scoring was performed at 200x.

**In vivo tumor metastasis model**

2x10^6 PC-3M-luc (C6) cells (Caliper Life Sciences, Hopkinton, MA) were injected into tail veins of 6 to 8-wk-old male athymic nude mice (Harlan Sprague-Dawley, Inc., Indianapolis, IN). After 24 hours, mice were randomly divided into two groups, n=10, for treatment with Scr ASO or OGX-427. 20 mg/kg of Scr ASO or OGX-427 was injected i.p. once daily for 7 days followed by three weekly treatments thereafter. On weeks 2, 4, 6, 8 and 10 mice were injected i.p. with 150 mg/kg D-luciferin (Caliper Life Sciences), anesthetized with isoflurane, and imaged in the supine position exactly 11 and 15 min 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 (CTC) isolation**

CRPC Patients enrolled in OGX-427 phase I of OGX-427 receiving doses from 400 mg to 1000 mg IV on a 21 day schedule had whole blood collected for CTC analyses at screening, prior to OGX-427 initiation, and on day 1 of every cycle. CTC enumeration was performed as previously described (20). Blood samples were drawn into 10-mL evacuated blood draw tubes (CellSave; Veridex LLC), maintained at room temperature, and processed within 96 h 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 (21), including breast (22), prostate (23), where it is associated with aggressive tumor behavior, metastasis and poor prognosis (24, 25) and its overexpression enhances migration and invasion (26). However a defining role for Hsp27 in EMT in PCa 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 PCa cell line, ARCaPE (17, 27) (18). Hsp27 overexpression in both lines caused decreases in E-cadherin expression (Fig. 1A) and increases in vimentin and fibronectin (Fig.1B), while its knockdown using siRNA in the PCa 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. Since elevated expression of Hsp27 is associated with poor prognosis of metastatic PCa (23), Hsp27 was stably overexpressed in the human androgen receptor (AR) positive PCa cell line, 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 decreases 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 (28), we found that the expression of Hsp27 increased cell migration (Fig. 2C) and MMP activity (Fig. 2D), two important factors in tumor migration and metastasis (29).

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

In order 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 morphological 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-3B, vimentin, fibronectin and Twist at protein and mRNA levels (Fig. 3A and B). Hsp27 knockdown cells also exhibited reduced cell invasion (Supplemental Fig. 1), migration, and MMP activity compared to controls (Fig. 3C and D). In order to control for non-specific changes in EMT status potentially induced by stable Hsp27 knockdown, similar experiments were performed 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 to control siRNA (siCtr) (Supplemental Fig. 2A and B). Hsp27 siRNA also reduced cell invasion and MMP activity (Supplemental Fig. 3A and B) 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 PCa (6, 30) 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 PCa 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 post-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 morphological 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 increased 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 to 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 independent EMT in PCa 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-stimulated 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 performed to investigate the effect of Hsp27 knockdown on STAT3 binding to the Twist promoter (19). Using primers that covered the proximal three 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). Lastly, 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 et al. (19) in the presence or absence of IL-6. We found that for each Twist truncation assessed, IL-6 induced more than a two-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 to 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.
Hsp27 and Twist are associated with aggressive human PCa cancer.

Increased expression of Hsp27 is found in PCa and is associated with poor prognosis and metastasis (24, 25). 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’s Coefficient=0.218). In addition, we observed increased pSTAT3 staining in Gleason 4 vs. 3 tumors (Fig. 6A). Quantification of positive vs. negative pSTAT3 signal in our TMAs showed that 83.33% of Gleason 3 tumors were positive for pSTAT3 staining, while 98.31% of Gleason 4 tumors were positive for pSTAT3. In addition, there was a positive correlation between pSTAT3 and Hsp27 (Pearson’s Coefficient=0.114) or Twist expression (Pearson’s Coefficient=0.112) across all Gleason Grades. These results suggest a direct relationship between Hsp27 in Twist signaling in aggressive human PCa.

Hsp27 is required for metastasis in a mouse model of PCa.

Our data suggested that Hsp27 expression is a feature of aggressive human PCa tumors and EMT in PCa cell lines. Therefore, we wanted to determine whether Hsp27 knockdown affects metastasis in a mouse model of highly metastatic PCa using PC-3M cells. In vitro, Hsp27 knockdown reduced migration of PC-3M cells (Supplemental Fig. 4). In vivo, after systemic delivery of luciferase expressing PC-3M cells, athymic nude mice were treated with either control scrambled anti-sense 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 to Scr ASO at 10 weeks after tumor injection (Fig. 7A). Quantification of luminescence at 6, 8 and 10 weeks post-injection showed a significant reduction in signal at each time point in mice treated with OGX-427 compared to Scr ASO (Fig. 7B). These results indicate that Hsp27 enhances dissemination of PCa 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 circulating tumour cell (CTC) counts in PCa patients undergoing treatment is associated with improved survival (31). To provide clinical relevance to our work, we evaluated the effects of OGX-427 on CTC counts in PCa patients. In a phase I study of OGX-427, nineteen patients with metastatic CRPC were treated with doses of OGX-427 ranging from 400 to 1000 mg and blood was collected from 17 evaluable patients for CTC analyses (CTC at baseline and at least 1 post-treatment time point). The median baseline CTC count was 40 CTC/7.5 ml blood (range 4 to 938), which decreased to a median of 15 CTC/7.5 ml (range 0 to 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 -43% to 100%). One patient had a decline in CTC from 39 CTC/7.5 ml at baseline to 0 CTC/7.5 ml by the 5th treatment cycle (OGX-427 dose = 400 mg) (summary, supplemental table 1). These CTC data provide the first clinical evidence of anti-cancer activity after Hsp27 inhibition, and are compatible with our pre-clinical data supporting a role for Hsp27 in EMT and dissemination of tumor cells to metastatic sites.
DISCUSSION

EMT is a critical component of PCa progression, facilitating development of lethal metastatic CRPC. Using Hsp27 overexpressing, as well knockdown PCa 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 phosphorylation, 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 PCa patients. We also demonstrated a role for Hsp27 in promoting PCa 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 circulating tumor cell counts, providing the first clinical evidence of anti-cancer metastatic activity for this drug in humans. Collectively, our results highlight Hsp27 as a key modulator of PCa cell plasticity, driving EMT and endowing PCa cells with metastatic potential.

High levels of Hsp27 have been reported in many cancers including breast (22), ovarian (32), glial (33) and prostate (34, 35), and it has been implicated in EMT in breast cancer (36), lung cancer (14) and kidney fibrosis (15). Our data show that Hsp27 is also required for EMT in PCa 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 (28). 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 (26). 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 PCa, IL-6 mediates the development and progression of CRPC (6, 37) and elevated IL-6 levels in sera of PCa patients correlate with tumor burden and metastases (6, 30). In normal prostate cell lines, ectopic expression of IL-6 induces
cell transformation, EMT, tumor formation and tumor progression to metastasis (38). In breast cancer cell lines, IL-6 overexpression leads to a reduction in E-cadherin, increases of vimentin, fibronectin and Twist, as 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 PCa.

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, 39). Recent evidence highlighted the importance of the IL-6/STAT3 pathway in PCa stem cells, identifying epigenetic modifications of key genes that interact with STAT3 to promote invasiveness (40). Interestingly, Hsp27 binds STAT3 in breast (16) and PCa cells (7), and in vivo, Hsp27 overexpression in the LNCaP xenograft mouse model confers hormone resistance post-castration via the activation of STAT3 (7). In this study, we showed that Hsp27 is required for IL-6/STAT3/Twist induced EMT in PCa 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 PCa 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 PCa cell lines (41, 42). Our data therefore suggest that Hsp27 promotes IL-6 signaling in tumor microenvironments to induce EMT, highlighting an important mechanism in PCa progression and metastasis. The dramatic effect on reduction of circulating tumor cell number in CRPC patients 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 (20) 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 (36). While it remains unknown how NF-κB signaling mediates EMT in PCa, reports have shown that NF-κB is activated downstream of Egr-1 in LNCaP and PC-3 PCa cells (43) and Egr-1 induces EMT in breast cancer (44).

In summary, Hsp27 is a component of several pathways known to induce EMT in PCa. 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.
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CONFLICTS OF INTEREST
The University of British Columbia has submitted patent applications on OGX-427, listing Dr Gleave as inventor. This IP has been licensed to OncoGenex Technologies, a Vancouver-based company that Dr. Gleave has founding shares in.
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FIGURE LEGENDS:

**Figure 1: Hsp27 modulates EMT in cancer cell lines.**  
*A-B*) Hsp27 was overexpressed in RWPE-2 and ARCaP E, Mock was used as a control.  
*A*) Total proteins were examined by Western Blot, vinculin was used as a loading control.  
*B*) Target genes were evaluated by qPCR of total RNA and normalized to GAPDH.  
*C-D*) DU145, A549 and MCF-7 cells were treated with Hsp27 or control siRNA.  
*C*) Total proteins were extracted and examined by western blot, vinculin was used as a loading control.  
*D*) Target genes were evaluated by qRT-PCR of total RNA and normalized to GAPDH.

**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=DAPI).  
*B*) Total protein or RNA was analyzed by Western Blotting or qPCR.  
*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 lower chamber were counted.  
*D*) MMP activity was assessed by gelatin zymography.

**Figure 3: Stable knockdown of Hsp27 in LNCaP cells prevents EMT.**  
*A*) The morphology of LNCaP cells transfected with either Hsp27 shRNA (sh-Hsp27) or control shRNA (sh-Ctr) was examined by phase contrast microscopy.  
*Immunofluorescence shows expression of E-cadherin, ZO-1 and vimentin (green=target, blue=DAPI).  
*B*) Total protein or RNA was analyzed by Western Blotting or qPCR.  
*Equal protein loading was confirmed by actin expression and EMT marker mRNA expression was normalized GAPDH.  
*C*) Cells were plated in transwell chambers for 48 hours and cells located in lower chamber were counted.  
*D*) MMP activity was assessed by gelatin zymography.

**Figure 4: Hsp27 is required for IL-6 induced EMT.**  
*A*) LNCaP cells were serum starved overnight and treated -/+ 50ng/ml of IL-6 for 0-24 hours. Total proteins were analyzed by Western Blotting.  
*β-actin was used as a loading control.  
*B*) LNCaP cells were transfected with Twist or STAT3-Luciferase promoter plasmids for 24 hours, then stimulated with IL-6 for 6 hours.  
*Luciferase activity was measured using the Dual-Luciferase Reporter Assay System.  
*C*)
Serum starved LNCaP sh-Ctr or sh-Hsp27 cells were stimulated -/+50ng/ml IL-6 for 24 hours and cell morphology was examined by phase contrast microscopy. D) Serum starved cells were treated -/+ 50ng/ml IL-6 for 0-24 hours. Total protein or RNA was analyzed by Western Blotting or qPCR. Equal protein loading was confirmed by actin expression and EMT marker mRNA expression was normalized to GAPDH. E) Serum starved cells were treated -/+ 50ng/ml IL-6 and percentage of migrating cells in a scratch assay was quantified at 0 and 24 hours post stimulation.

Figure 5: Hsp27 is required for IL-6/STAT3/Twist pathway activity. A) Serum starved LNCaP sh-Ctr or sh-Hsp27 cells were treated -/+ 50 ng/ml IL-6 for 0-60 minutes. Proteins were analyzed by Western Blot, actin was used as a loading control. B) Serum starved cells were treated -/+50 ng/ml IL-6 for 30 min. 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 performed on nuclear extracts from LNCaP cells treated +/-IL-6, IP were performed using 2.0 μg of the indicated antibodies and 20 μL of Protein G agarose. The conventional (left) and quantitative (right) PCR was performed using immunoprecipitated DNAs, soluble chromatin and specific primer pairs for twist. Results of immunoprecipitated samples were corrected for the results of the corresponding soluble chromatin samples. D) LNCaP sh-Ctr and LNCaP sh-Hsp27 cells (left) or LNCaP cells transfected with Mock and Hsp27 WT (right) were co-transfected with 0.5 μg/mL of Twist-Luc plasmids and 0.05 μg/mL of pRL-TK, and treated -/+ 50 ng/ml IL-6 for 6 hours before lysis. The luciferase activity of Twist-Luc –969 alone was set as 1.

Figure 6: Hsp27 expression is associated with aggressive human PCa. A) Representative sections of PCa 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 PCa 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 vs. 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.

**Figure 7: Hsp27 is required for PCa tumor metastasis in vivo.** (A-B) $2 \times 10^6$ PC-3M-luc (C6) cells were injected into tail veins of 6-8-wk-old male athymic nude mice. After randomization, 20 mg/kg of Scr ASO or OGX-427 was injected i.p. once daily for 7 days, followed by three weekly treatments thereafter. **A)** Representative images of mice taken at week 10. **B)** Average bioluminescence between 11 and 15 min is shown mice analyzed at 6, 8 and 10 weeks post-treatment. A representative experiment is shown, n=10, error bars=SD.
Figure 1

A

Mock Hsp27  Mock Hsp27  Hsp27  E-cadherin  Vinculin

RWPE-2  ARCaP

B

<table>
<thead>
<tr>
<th>Relative expression</th>
<th>RW-mock</th>
<th>RW-Hsp27</th>
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<tr>
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<td>Vimentin</td>
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<td></td>
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<tr>
<td>Fibronectin</td>
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</tbody>
</table>

C

siCtr  siHsp27  siCtr  siHsp27  siCtr  siHsp27

Hsp27  E-cadherin  Vinculin

DU145  A549  MCF-7

D

<table>
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<tr>
<th>Relative expression</th>
<th>DU145-siCtr  DU145-siHsp27</th>
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<tbody>
<tr>
<td>Hsp27</td>
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<td>Fibronectin</td>
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<td>Vimentin</td>
<td></td>
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<tr>
<td>Fibronectin</td>
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</table>
Figure 2

A

Mock Hsp27

E-cadherin

Vimentin

Phase

B

Mock Hsp27

Hsp27

E-cadherin

pGSK3b

Vimentin

Fibronectin

actin

Graphs showing relative expression of N-Cadherin, Vimentin, Fibronectin, Twist, and relative expression of Hsp27, Vimentin, and Fibronectin in Mock and Hsp27 conditions.

C

Graph showing migrating cells (mean) in Transwell assay.

D

Mock Hsp27

Pro-MMP-9

MMP-9

Pro-MMP-2

MMP-2

Hsp27

Graph showing expression of Pro-MMP-9, MMP-9, Pro-MMP-2, MMP-2, and Hsp27 in Mock and Hsp27 conditions.
Figure 3

A

sh-Ctr  sh-Hsp27

Phase

E-cadherin

ZO-1

B

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<th>Protein</th>
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<td>Actin</td>
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C

Migrating Cells (mean Transwell assay)

D

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<tr>
<th>Protein</th>
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Figure 4

A. Western blot analysis showing the expression of Twist1, E-cadherin, Fibronectin, Hsp27, and Actin at different time points: 0, 6, 12, and 24 hours after IL-6 stimulation.

B. Bar graphs showing the STAT3-Luc activity and Twist-Luc activity in cells treated with IL-6 or without IL-6.

C. Cell images showing the control (sh-Ctr) and Hsp27 (sh-Hsp27) conditions.

D. Western blot analysis showing the relative expression of Twist, E-cadherin, Vimentin, Fibronectin, and Actin in LNCaP cells under the control (sh-Ctr) and Hsp27 (sh-Hsp27) conditions.

E. Wound healing assay showing the number of migrating cells (% of wound healing) at different time points: T=0 and T=24H.
Figure 5

A

<table>
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<tr>
<td></td>
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<tr>
<td>0</td>
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<td>10</td>
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<td>30</td>
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<tr>
<td>60</td>
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min (IL-6)

- p-STAT3^Tyr705
- p-STAT3^Ser727
- T-STAT3
- p-Hsp27
- T-Hsp27

B

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<thead>
<tr>
<th>sh-Ctr</th>
<th>sh-Hsp27</th>
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<td>Vehicle IL-6</td>
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<tr>
<td>Nuc</td>
<td>Cyt</td>
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</table>

- STAT3
- Hsp27
- Lam B1
- actin

C

<table>
<thead>
<tr>
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T-Hsp27

Cyt

D

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<th>sh-Hsp27 IL-6 -</th>
<th>sh-Control IL-6 +</th>
<th>sh-Hsp27 IL-6 +</th>
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Twist promoter

IgG

STAT3

Immunoprecipitants/input (%)
Figure 6

A

Gleason Grade 3

Gleason Grade 4

Hsp27

Twist

pSTAT3

B

Hsp27

Twist

Correlation Hsp27

Correlation Twist

C

pSTAT3

D

Correlation HSP27

Correlation Twist

Correlation pSTAT3

Correlation pSTAT3

Twist

pSTAT3

Hsp27

pSTAT3
Figure 7

A

Scr ASO

OGX-427

B

Luciferase Intensity

$\text{Luminescence}$

Counts

$\text{Color Scale}$

$\text{Min} = 42$

$\text{Max} = 115$

Graph:

- Scr ASO
- OGX-427

0 10 20 30 40 50 60 70 80 90 100

6w 8w 10w

*
Hsp27 regulates epithelial mesenchymal transition, metastasis and circulating tumor cells in prostate cancer

Masaki Shiota, Jennifer L. Bishop, KaMun Nip, et al.

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