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14. ABSTRACT In general, the role of eHsp90 in tumor progression has not been well characterized Our work provides novel mechanistic insights into the EMT-promoting activity of eHsp90. Our preliminary findings indicate that eHsp90 is a newly identified regulator of the oncogenic polycomb regulator EZH2. eHsp90 was found to induce EZH2 expression and to direct its recruitment to the promoters of target genes. In this latter capacity, eHsp90 promoted EZH2's methyltransferase activity, enabling the repression of tumor suppressor genes such as the EMT gatekeeper E-cadherin. Moreover, xenograft studies demonstrate that an eHsp90-EZH2 axis is essential for eHsp90-mediated tumor invasion. Therefore, our findings implicate an eHsp90-EZH2 oncogenic pathway that contributes to early invasive events in prostate cancer.					
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INTRODUCTION:

The purpose of this proposal is to elucidate the mechanism by which extracellular Hsp90 (eHsp90) promotes an epithelial to mesenchymal transition (EMT) in prostate cancer (PCa). It is widely believed that EMT activation facilitates a switch towards aggressive behavior. EMT is tightly linked with tumor invasion, a requisite for metastatic spread, and a major cause of PCa lethality. The proposal hypothesis is focused upon the functional cooperation between eHsp90 and the polycomb protein EZH2. EZH2 is upregulated in many solid tumors, including PCa, and is correlated with poor prognosis. We have demonstrated that eHsp90 upregulates EZH2 and initiates EMT events. eHsp90 also induces expression of HDAC1 and HDAC2, histone deacetylases known to serve as cofactors for EZH2 and inducers of EMT. This proposal seeks to clarify the epigenetic mechanism of eHsp90 action, towards the goal of establishing eHsp90 as a therapeutic target in PCa. The objectives of this proposal are to delineate the relationship among eHsp90, EZH2, and HDAC1/2 within the context of EMT events, evaluate the functional implications of this pathway in preclinical models, and elucidate the clinical implications of expression of these proteins in human tumors. These collective pursuits will allow a refined assessment of the utility of eHsp90 as a potential biomarker for progression, as well as provide evidence for the feasibility of therapeutically targeting eHsp90 as a means to restore epigenetic homeostasis and suppress PCa aggressiveness.

BODY:

Task 1: Pre-Award Phase (months 1-4)

- 1a. Obtain Institutional IACUC approval for metastasis model
Completed
- 1b. Obtain IRB approval for patient enrollment
Completed

Task 2: Evaluate eHsp90-mediated recruitment of epigenetic proteins to the E-cadherin promoter and EMT target genes (months 1-12)

- 2a. Chromatin immunoprecipitation (ChIP) analysis of E-cadherin promoter to evaluate recruitment of HDAC1, HDAC2, EZH2, H3K27me3, and H3K27Ac using parental control ARCaPE and ARCaPE-eHsp90 cells.
Partially completed. See Nolan SBUR poster (Fig 5, Appendix materials). The HDAC analysis will be performed later this year to be included in a subsequent paper.
- 2b. Analysis of eHsp90 regulated recruitment of epigenetic proteins to EMT gene promoters using model in 2a, untreated and NPGA (eHsp90 targeting agent), followed by EMT ChIP array.
Partially completed. Analysis of EZH2, H3K27me3 and H3K27Ac at a subset of EMT promoters (Zeb and Snail) was performed in LacZ and eHsp90 derivatives of ARCaPE-eHsp90 (Fig. 1).
- 2c. Create a stable derivative of ARCaPE-eHsp90 with SNAIL shRNA and characterize E-cadherin expression.
Completed.

Tasks 2d-2e have been recently initiated and will be reported in the next annual report.

Tasks 2f-2g have not yet been initiated, but are expected to be started during the next 6-9 months.

Task 2 Summary

The data generated from several of the completed tasks outlined in this section have been compiled into a manuscript, a draft of which is in progress, with a target submission date of April 2014. This published paper will be included with the subsequent progress report. In summary, we found that eHsp90 promotes recruitment of EZH2 to the E-cadherin promoter. This was correlated with increased EZH2 repressive activity (H3K27me3) at the E-cadherin promoter, and decreased acetylation (H3K27Ac). These trends were reversed with the eHsp90 inhibitor NPGA. We have generated ARCaPE-eHsp90 with stable suppression of Snail and determined that this is sufficient to restore E-cadherin expression. We will subsequently evaluate whether Snail plays a major role in the recruitment of EZH2 and/or HDAC1/2 to the E-cadherin promoter in our model. Our initial analysis of EZH2 to other EMT effectors indicated that its eHsp90-dependent regulation at E-cadherin may be distinct from that at other promoters. This is shown in Fig. 1. Unlike the trend for the E-cadherin promoter, eHsp90 does not appear to affect EZH2 recruitment to either the Zeb1 or Snail promoter. This finding is supported by the fact that EZH2's suppressive methyl mark was also not changed by eHsp90 at the Zeb promoter, while a modest effect was seen at the Snail promoter. In contrast, eHsp90 elicited a dramatic increase in histone acetylation at both of these promoters, indicating that eHsp90 promotes Zeb1 and Snail promoter methylation and concomitant activation of these target genes. It is not clear whether this may involve an EZH2-independent pathway. The latter is supported by the fact that suppression of EZH2 activity (via mutants or small molecule targeting) is unable to reduce Zeb1 protein expression (Fig. 1C, 1D). In contrast, Snail protein expression appears to exhibit a greater dependence upon EZH2 activity.

These trends are being investigated. We will subsequently evaluate how epigenomic therapeutics affect EZH2 recruitment to other targets. Although we originally proposed an experimental strategy consisting of ChIP followed by use of an EMT array, RNA-seq approaches have recently been implemented at our institute and we therefore plan to take advantage of this more unbiased and powerful approach. We are currently optimizing our protocols for EZH2 ChIP-seq. We are also using two additional approaches to inhibit EZH2 function. First, we have substituted DZNep for the newer, more powerful and more selective small molecule inhibitor GSK126. Second, we have transfected cells with a dominant mutant version of EZH2, leading to abrogation of all EZH2 methyltransferase function. We have also applied more selective methodologies for suppression of HDAC activity. Although MS-275 inhibits HDAC1 and HDAC2, this drug also targets several other HDAC proteins. Thus, to achieve enhanced specificity, we have created clonal populations wherein either HDAC1 or HDAC2 have been suppressed by shRNA approaches. Data generated from tasks associated with analysis of HDAC

recruitment and EMT events will be compiled into a subsequent paper.

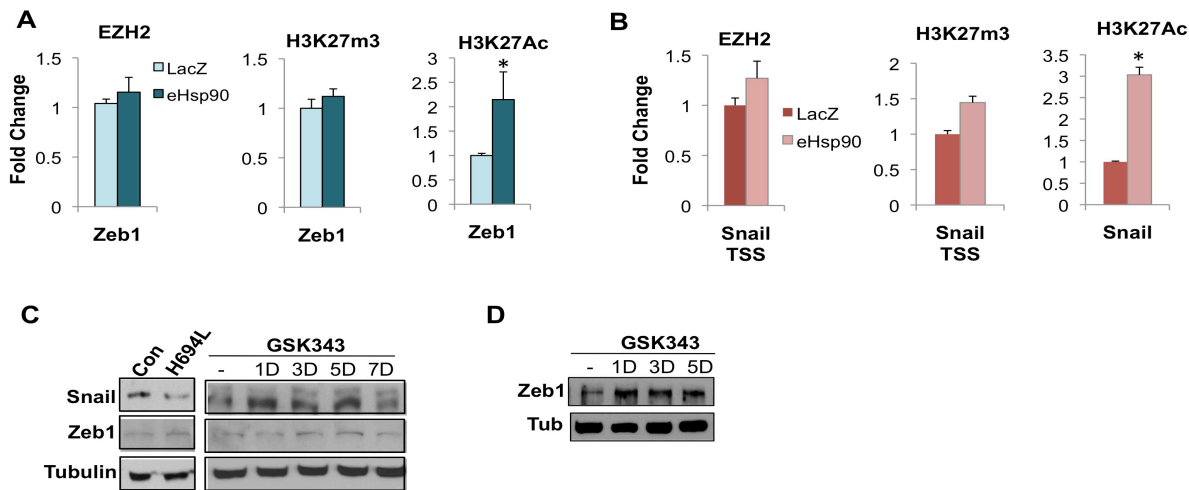


Fig. 1. EZH2 regulation of EMT transcription factors. **A)** EZH2 ChIP-qPCR evaluation at the Zeb1 promoter is shown. Evaluation of its mark, H3K27m3, and the corresponding acetylation, H3K27Ac, is shown. **B)** Similar ChIP-qPCR results are shown at the Snail promoter site. **C)** Snail and Zeb1 immunoblot analysis in ARCaPE-eHsp90 cells (Con) or within the context of EZH2 inhibition (via EZH2 mutant H694L) or via treatment with the EZH2 small molecule inhibitor GSK343. **D)** Independent experiment demonstrating effects of GSK343 upon ZEB1 protein expression.

Task 3: Evaluate the metastatic potential of ARCaPE-eHsp90 and sorted ARCaPM-eHsp90^{hi/low} cells via intracardiac delivery and noninvasive BLI monitoring. (months 10-20)

3a. Optimize multiparameter FACS analysis of eHsp90, E-cadherin, EpCAM, N-cadherin, LRP1, and CD49f using intact cells (ARCaPE, ARCaPE-eHsp90 and ARCaPM). In addition to respective isotype matched negative control antibodies, utilize cell lines indicative of normal prostate epithelium (provided by Dr. Simon Hayward) as relative negative control tissue.
Initiated.

3b. Implant mice (14/group) with ARCaPE and ARCaPE-eHsp90 and monitor BLI values and morbidity for 6-8 weeks. Completed. (Fig. 6 Nolan et al., SBUR poster in Appendix).

3c-3f – Not yet initiated, but expected to be initiated over the next 6-9 months.

Task 3 Summary

In our pilot experiments, we were unable to observe micrometastatic lesions derived from ARCaPE-eHsp90. This indicates that eHsp90 may support early events that contribute to metastasis. To further evaluate this, we evaluated growth of ARCaPE-LacZ and ARCaPE-eHsp90 in the subrenal model. We found that the eHsp90 derivative grew significantly better and we observed areas of frank invasion. Implantation of ARCaPE-eHsp90 with the mutant EZH2 reversed this invasion, indicating that EZH2 was required for eHsp90's invasive activity. Subsequent work will focus on evaluation of these tissues and therapeutic targeting in the ARCaPM model, the latter as proposed.

Task 4: Utilize FACS to evaluate the expression of eHsp90 in primary tumor tissue (months 20-32)

4a. Confirm infrastructure for coordination of clinical studies. Communicate with urological oncologist, urological pathologist, study coordinator, and pathology lab.
Completed.

4b. Consent patients and accrue clinical samples for: 1) radical prostatectomy (tissue and serum, 2) radical cytoprostatectomy (tissue and serum), 3) age matched cancer free volunteers (serum)
Initiated.

4c. Perform multi-parameter FACS with 40 microdissected matched benign and cancer tissue, and for 35 non-matched benign cytoprostatectomy tissue specimens, for eHsp90^{hi/low} populations and respective populations

of CD49f, E-cadherin, EpCAM, N-cadherin, LRP1. Following analysis, sort a subset (20 specimens) of matched eHsp90^{hi}/low populations and lyse cells in trizol. Analyze FACS data. Initiated. See pilot data Fig. 5 in Hance et al., AACR poster in Appendix.

Task 4 Summary

In our studies, we found that a restricted subpopulation of tumor cells from patient samples exhibit surface Hsp90. This population is on average, approximately 5% of the whole population. Given this limitation, we have opted not to co-sort for other markers, which would further diminish cell numbers. Instead, we are collecting the eHsp90^{hi} and eHsp90^{low} populations in an unbiased manner and subsequently analyzing the indicated transcripts for evidence of EZH2 expression and EMT activation.

Task 5: Utilize qRT-PCR and ELISA to evaluate the co-expression of eHsp90 with EMT and epigenetic markers (months 30-36)

5a. Optimize qRT-PCR for TMPRSS2-ERG fusion gene using VCaP cells as a positive control.

Not yet initiated

5b. Utilizing FACS sorted eHsp90^{hi/low} patient material in 4e, perform qRT-PCR for EMT genes (EMT array), HDAC1/2, EZH2, and for TMPRSS2-ERG fusion gene. Analyze data within and among samples and correlate with clinical information.

Initiated.

5c-5e: Not yet initiated.

Task 5 Summary

Most of these studies have not been initiated. However, we have been able to demonstrate that we can analyze transcripts from RNA derived from sorted cancer cells from patient specimens (Hance et al, J. Biol Chem 2012). Early data indicates that tumor cells with higher surface expression of eHsp90 may exhibit a more aggressive EMT profile.

KEY RESEARCH ACCOMPLISHMENTS:

- eHsp90 promotes recruitment of EZH2 to the E-cadherin promoter. EZH2 was recruited, along with deposition of the repressive H3K27me3 mark, and reduction of the active H3K27Ac (acetyl) mark. This indicates that eHsp90 regulates EZH2's repressive function, providing a mechanistic explanation for the ability of eHsp90 to suppress E-cadherin and promote cell motility.
- We unexpectedly found that EZH2 recruitment to the promoters of other EMT target genes may occur via a pathway distinct from that typified by recruitment to E-cadherin. In support of this, eHsp90 did not promote EZH2 recruitment, or deposition of, H3K27me3 at the Zeb1 or Snail1 promoter. However, we observed an eHsp90-dependent increase of H3K27Ac, indicating that HDAC1/2 may be evicted from these sites coincident with expression of these transcripts. Experiments are underway to further evaluate these trends.
- eHsp90 is not sufficient to promote metastatic spread in ARCaPE, a model for early PCa. This indicates that eHsp90 may regulate early invasive events and that it may collaborate with subsequent genetic insults or signaling pathways.
- We generated a unique subrenal xenograft model that enabled the demonstration of eHsp90 as a factor capable of promoting localized invasion and tumor growth. The invasive activity of eHsp90 within this model may indicate that the tumor microenvironment participates in eHsp90's effects. This pro-invasive activity of eHsp90 was dependent upon EZH2 activity, validated via implantation of tumor cells with a dominant negative EZH2 mutant. Tumor tissue from the noninvasive EZH2 mutant demonstrated restoration of E-cadherin protein, compatible with our molecular findings in our cell-based models.
- We demonstrate genetic trends with eHsp90-sorted tumor cells from patient specimens. Accordingly, mRNA derived from eHsp90^{hi} populations has a profile resembling that of an aggressive and pro-EMT phenotype, relative to tumor matched RNA from the eHsp90^{low} population. Some of these findings have been recently published (Hance et al. J. Biol Chem 2012).

REPORTABLE OUTCOMES:

Development of cell lines, tissue or serum repositories

- ARCaPE-eHsp90 created with stable expression of mutant (inactive) EZH2
- ARCaPE-eHsp90 created with shRNA to suppress HDAC1
- ARCaPE-eHsp90 created with shRNA to suppress HDAC2
- ARCaPE-eHsp90 created with shRNA to suppress Snail1

Infomatics and animal models

- Generation of a new animal model consisting of implantation of ARCaPE-LacZ and ARCaPE-eHsp90 into the mouse kidney. This model clearly demonstrates the ability of eHsp90 to promote both tumor growth and invasion, and is an ideal system for evaluation of events associated with early invasive events.
- Resultant tissue from this animal model will be analyzed by RNA-seq, thereby allowing an analysis of eHsp90-mediated genetic changes leading to prostate tumor invasion. Inclusion of dominant negative EZH2 cells will further enable dissection of the eHsp90-EZH2 pathway in tumor progression.
- EZH2 ChIP-seq approaches will further enable an informatic analysis of the mechanistic basis for EZH2-mediated tumor promotion.

Funding applied for based on work supported by this award

- An RO1 application 'Role of extracellular Hsp90 in epithelial cell polarity and invasion' was submitted to the Tumor Cell Biology (TCB) study section, for the October review cycle. Several findings from the DOD proposal were expanded and included within this grant application. The proposal is currently recommended for funding (8%).

CONCLUSION:

In general, the role of eHsp90 in tumor progression has not been well characterized. Our work provides novel mechanistic insights into the EMT-promoting activity of eHsp90. Our preliminary findings indicate that eHsp90 is a newly identified regulator of the oncogenic polycomb regulator EZH2. eHsp90 was found to induce EZH2 expression and to direct its recruitment to the promoters of target genes. In this latter capacity, eHsp90 promoted EZH2's methyltransferase activity, enabling the repression of tumor suppressor genes such as the EMT gatekeeper E-cadherin. Moreover, xenograft studies demonstrate that an eHsp90-EZH2 axis is essential for eHsp90-mediated tumor invasion. Therefore, our findings implicate an eHsp90-EZH2 oncogenic pathway that contributes to early invasive events in prostate cancer. Future planned experiments will elucidate how HDAC1/2 proteins function as accessory agents to regulate EZH2 function and orchestrate EMT events and tumor progression.

As indicated, we recommended several changes to improve ongoing and/or future work. These included: 1) Genetic suppression of HDAC1 and HDAC2 to evaluate their specific contribution to the eHsp90-EZH2 axis in EMT regulation; 2) Targeting EZH2 via the small molecule GSK126 or via transduction of cells with a dominant negative EZH2 construct; 3) Use of RNA-seq approaches rather than the gene arrays initially proposed; 4) Use of a subrenal animal model system that enables tumor recovery and evidence of eHsp90-mediated tumor invasion.

APPENDICES:

(2013) Society for Basic Urological Research (SBUR) Fall Symposium, Nashville, TN

Michael W Hance, Krystal Nolan, Isla Garraway, Omar Franco, Simon Hayward, and Jennifer S. Isaacs

Extracellular Hsp90 suppression of miR-200 promotes disruption of cell polarity

Note: This work is an extension of the current work in that EZH2 also modulates EMT events via its suppression of the epithelial microRNA miR-200.

INTRODUCTION: Prostate Cancer (PCa) is a disease with the highest incidence and the second highest lethality in men. Although early cancer detection and treatment is often curative, subsequent metastatic spread of tumor cells renders the disease incurable. This highlights the urgent need to identify factors that contribute to metastasis and subsequent lethality. Activation of the epithelial to mesenchymal transition (EMT) genetic program confers metastatic potential in preclinical models and correlates with poor prognosis.

OBJECTIVES: Our goal is to reduce the metastatic potential of PCa. Although targeting EMT is a promising approach to improve treatment outcome, a caveat is that the key mediators driving this process remain elusive. Multiple reports indicate that extracellular Hsp90 (eHsp90) promotes cell motility, invasion, and metastasis in a number of cancer cell types, and eHsp90 has been detected from the plasma of metastatic PCa patients, suggesting a potential causative role in PCa. Our objective was to investigate whether eHsp90 may regulate EMT events and modulate the metastatic potential of PCa.

METHODS: eHsp90 expression was evaluated in a panel of matched pairs of PCa cell lines comprised of less aggressive and more aggressive derivatives. Further, eHsp90 activity was modulated (either induced or inhibited) in PCa cell lines and protein and genetic changes monitored by immunoblot and qRT-PCR, respectively. Microscopy was used to assess morphological changes, as well as changes in protein localization concomitant with EMT events.

RESULTS: First, we report a consistent trend in that more aggressive PCa tumor cell lines secrete higher levels of eHsp90. Second, blockade of eHsp90 attenuates the activation of several critical mediators of cell motility and effectively blocks PCa migration. Third, eHsp90 profoundly alters cellular morphology towards a mesenchymal phenotype. Lastly, eHsp90 upregulates several of the key effector genes associated with the EMT phenotype.

CONCLUSIONS: We now highlight a novel role for eHsp90 as a central regulator of EMT events in PCa. We submit that therapeutic targeting of eHsp90 in PCa may have the potential to suppress EMT activation and prevent the dissemination and subsequent metastatic propensity of tumor cells. The translation of these findings is expected to have significant clinical ramifications.

(2013) Keystone Symposia: Epigenetic Marks and Cancer Drugs, Santa Fe, NM

Krystal Nolan, Michael W Hance, and Jennifer S Isaacs

Extracellular Hsp90 dependent regulation of E-cadherin via coordinate EZH2 recruitment and HDAC1/2 activity in prostate cancer

Prostate cancer (PCa) is a leading cause of cancer deaths among men, primarily due to metastases to bone. While the metastatic process involves complex cellular processes, the epithelial to mesenchymal transition (EMT) is considered to be a key initiating factor. Loss of the cell-cell adhesion gatekeeper E-cadherin (E-cad) is an early initiating event of EMT that promotes cell motility and tumor dissemination. Potential tumor-derived secreted factors driving E-cad loss remain poorly defined in PCa. The intracellular chaperone Heat shock protein 90 (Hsp90) is preferentially secreted by tumor cells, a trend validated by its increased expression in plasma from cancer-afflicted patients. Although extracellular Hsp90 (eHsp90) has been shown to support tumor metastasis, a mechanism for this action has been unknown. We recently discovered that eHsp90 promotes PCa tumor cell motility, E-cad suppression, and EMT events, thereby offering a mechanism for its tumorigenic activity. We demonstrate that eHsp90-mediated EMT events are regulated in part by epigenetic factors, such as the polycomb methyltransferase EZH2 and histone deacetylases (HDACs). More aggressive PCa cell lines demonstrate elevated EZH2 and HDAC1/2 expression, concordant with their increased eHsp90 levels and reduced expression of E-cad. Moreover, expression of EZH2 and HDAC1/2 was regulated by direct modulation of eHsp90 activity. Treatment of PCa cells with an inhibitor of eHsp90, EZH2, or HDAC1/2 restored E-cad expression and promoted an epithelial phenotype. We further demonstrate that eHsp90 modulates EZH2 recruitment to the E-cad promoter, a critical event that regulates E-cad expression. These results indicate that eHsp90 possesses a novel role as a regulator of key epigenetic factors crucial for the expression

of E-cad. Current research is ongoing to delineate how eHsp90 coordinately regulates EZH2 and HDAC1/2 to promote E-cad suppression and EMT events in PCa.

(2013) Society for Basic Urological Research (SBUR) Fall Symposium, Nashville, TN
Krystal Nolan, Michael W. Hance, Omar Franco, Simon Hayward, and Jennifer S Isaacs
Extracellular Hsp90 mediates E-cadherin suppression via EZH2 activity

Background: Metastatic prostate cancer is the second leading cause of cancer deaths in men. While the metastatic process is complex, the epithelial to mesenchymal transition (EMT) program is considered a key initiating factor. Recently, extracellular Heat Shock Protein 90 (eHsp90) was shown to support tumor metastasis in preclinical models and has been detected in the plasma from cancer-afflicted patients. Despite this potential clinical significance for eHsp90 function, the pathway by which eHsp90 promotes tumorigenesis is not well defined. We recently demonstrated that eHsp90 is sufficient to initiate EMT events in prostate cancer cells. We herein explored the mechanism for eHsp90-mediated suppression of E-cadherin (E-cad), a primary gatekeeper that restrains EMT events. As epigenetic modifiers such as EZH2, can suppress E-cad, we evaluated whether eHsp90 elicited the upregulation and subsequent recruitment of EZH2 to the E-cad promoter to mediate its suppression.

Method: We have developed a genetic model to direct the secretion of eHsp90. This secreted eHsp90 is competent to initiate EMT events in transduced cells. By use of various inhibitors, we have determined a signaling axis that is required for eHsp90-mediated EMT. Several techniques were utilized to determine signaling and transcriptional events, including Western blotting, PCR, and chromatin immunoprecipitation (ChIP).

Results: We demonstrate that the ability of eHsp90 to suppress E-cadherin and promote cell motility is dependent upon its upregulation of EZH2 and subsequent recruitment to the E-cad promoter. We further demonstrate that eHsp90 robustly activates ERK, which is an upstream effector for EZH2 expression. Pharmacologic blockade of ERK resulted in a loss of eHsp90-mediated EZH2 expression and diminished EZH2 recruitment to the E-cad promoter. Moreover, blockade of eHsp90, ERK, or EZH2 similarly restored E-cad expression and epithelial cellular morphology. Finally, we demonstrate that eHsp90 is sufficient to promote tumor growth and invasive properties.

(2013) American Association for Cancer Research: Tumor Invasion and Metastasis, San Diego, CA
Michael W Hance, Krystal Nolan, Udhayakumar Gopal, Agnieszka Jezierska-Drutel, Carola Neumann, Omar E Franco, Simon Hayward, Haibo Liu, Isla Garraway, and Jennifer S Isaacs
Secreted Hsp90 is a novel potentiator of EMT events and tumor growth

Prostate Cancer (PCa) is the most commonly diagnosed malignancy in men, responsible for the second highest lethality. Although early cancer detection and treatment is often curative, subsequent metastatic spread of tumor cells renders the disease incurable. Activation of the epithelial to mesenchymal transition (EMT) genetic program is considered a key event contributing to metastatic potential and subsequent lethality. Although targeting EMT may be a promising approach for improving treatment outcome, the main, potentially druggable, targets driving this pathway in PCa remain poorly defined. Multiple reports indicate that extracellular Hsp90 (eHsp90) promotes cell motility, invasion, and metastasis in a number of cancer cell types. Intriguingly, eHsp90 has been preferentially detected in the serum or plasma from aggressive cancers. Its presence in the serum of metastatic PCa patients implicates a potential causative role in PCa. Therefore, our objective was to investigate whether eHsp90 may influence EMT events, thereby influencing the metastatic potential of PCa. We now report that paired sets of differentially aggressive PCa tumor cell lines exhibited higher expression of eHsp90. Functionally, exposure of PCa cells to eHsp90 elicited a significant increase in cell motility. Inversely, blockade of eHsp90 attenuated the activation of several pro-motility effectors, concomitant with the significant suppression of cell migration. These events were associated with a profound alteration of cellular morphology towards a mesenchymal phenotype. Changes in cell phenotype were coupled with a loss and redistribution of the EMT suppressive gatekeeper protein E-cadherin. eHsp90 was also found to impact on the localization of other junctional proteins known to maintain a cuboidal epithelial phenotype. Finally, eHsp90 was found to transcriptionally upregulate several of the established transcriptional factors known to initiate EMT events. Confirmation of the EMT inducing ability of eHsp90 was determined by focused quantitative real time EMT PCR arrays. Our results therefore highlight a novel role for eHsp90 as a central regulator of EMT events in

PCa. Our findings further support the premise that therapeutic targeting of eHsp90 may have the potential to suppress EMT activation and diminish the dissemination and metastatic propensity of PCa tumor cells.

(2013) Society for Basic Urologic Research (SBUR) Fall Symposium, Nashville, TN

Jennifer S. Isaacs

Novel cancer secreted factors driving fusion-independent EZH2 upregulation

Note: I was an invited speaker at this conference. The majority of the discussed data was based upon findings generated from this award.

Secreted Hsp90 Promotes EMT in Prostate Cancer via a Polycomb Dependent Pathway

Krystal D. Nolan¹, Michael W. Hance¹, Omar E. Franco², Simon W. Hayward², and Jennifer S. Isaacs¹
¹Department of Cell and Molecular Pharmacology, Hollings Cancer Center, Medical University of South Carolina, USA
²Department of Urology, Vanderbilt University, USA

INTRODUCTION

Prostate cancer (Pca) is a leading cause of cancer deaths in men, primarily due to the metastatic progression to bone. Activation of epithelial to mesenchymal transition (EMT) events plays a major role in tumor metastasis. Loss of key epithelial proteins, including the cell-cell adhesion gatekeeper E-cadherin, are initiating events of EMT, cell motility and tumor dissemination. Determination of tumor specific factors that may promote EMT in Pca has remained poorly defined.

A number of solid tumors secrete heat shock proteins, including Hsp90. Secreted extracellular Hsp90 (eHsp90) supports metastasis in preclinical models, and eHsp90 levels are elevated in cancer patients. We recently demonstrated that eHsp90 promotes EMT, which may offer insight into eHsp90's metastatic activity. Although we reported that eHsp90 signaling through its receptor LRP1 is essential for ERK activation and subsequent EMT events, the downstream effectors have not been defined.

The enhancer of zeste homolog 2 (EZH2), a component of the epigenetic repressor polycomb complex (PRC2), suppresses E-cadherin and facilitates EMT events. EZH2 is frequently overexpressed in Pca and correlates with poor prognosis. We now demonstrate that an eHsp90-ERK axis upregulates EZH2, and we identify EZH2 as an essential mediator of eHsp90-dependent E-cadherin suppression and tumorigenic activity. These results support a novel paradigm wherein Hsp90 plays a dominant role in regulating EZH2 activity, thus defining eHsp90 as an epigenetic regulator of Pca EMT. Further exploration of this pathway is expected to provide further understanding of the potential significance of the co-existence of eHsp90 and EZH2 expression in prostate cancer.

Model for eHsp90 driven E-cadherin suppression

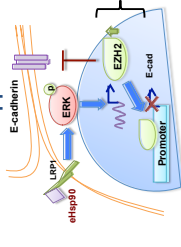


Figure 1

Extracellular Hsp90 (eHsp90) signals through LRP1 to promote ERK activation, which in turn activates EZH2. EZH2 upregulation of EZH2 which is then recruited to the E-cadherin (E-cad) promoter. EZH2 mediates the suppression of E-cad by laying down the repressive H3K27m3 mark. This suppression leads to increased EMT processes and a pro-tumorigenic profile.

eHsp90 promotes suppression of E-cadherin and upregulation of EZH2

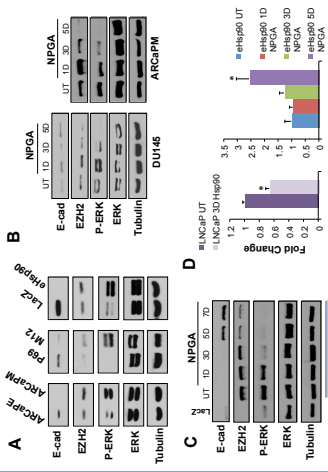


Figure 2

A) E-cadherin is less expressed in the more metastatic derivatives of each of 2 cell lines. Similar trends are found in the more aggressive, less metastatic model (ARCaPE-eHsp90), whereas EZH2 and P-ERK exhibit a corresponding increased expression pattern. B) eHsp90 blockade with non-permeable geldanamycin (NPGA) treatment in DU145 elicited loss of EZH2 and P-ERK in ARCaPE cells. C) E-cadherin is restored in ARCaPE-eHsp90 cells concurrent with P-ERK and EZH2 suppression. D) E-cadherin mRNA (CDH1) levels are decreased in LNCaP cells treated with Hsp90 for 3 days. CDH1 levels are elevated in NPGA treated ARCaPE-eHsp90 cells. Student's t-test was performed and *P < 0.05.

eHsp90 requires ERK for EZH2 upregulation

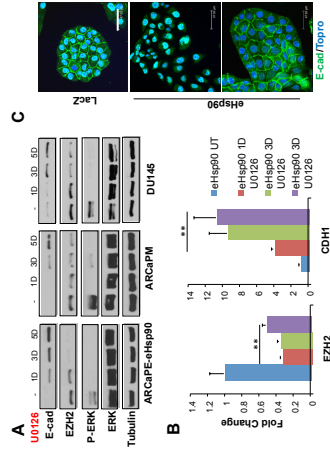


Figure 3

A) U0126 treatments in ARCaPE-eHsp90, ARCaPM, and DU145 cells promote restoration of E-cadherin and concurrent loss of EZH2. B) U0126 treatment in ARCaPE-eHsp90 resulted in loss of EZH2 mRNA and increased E-cadherin mRNA. **P < 0.01. C) E-cadherin localization is restored to the membrane in U0126 treated ARCaPE-eHsp90 cells.

E-cadherin suppression is regulated by EZH2

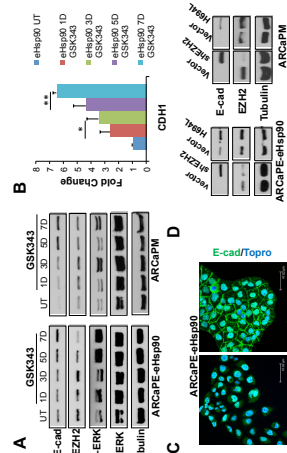


Figure 4

A) EZH2 blockade (GSK343) increased E-cadherin in both ARCaPE-eHsp90 and ARCaPM without suppressing P-ERK. B) GSK343 increased E-cadherin mRNA levels in ARCaPE-eHsp90. *P < 0.05, **P < 0.01. C) EZH2 blockade with DZNep restored E-cadherin to the membrane in ARCaPE-eHsp90 cells. D) Suppression of EZH2 via shRNA or transduction of a point mutant (H694L) leads to increased E-cadherin.

EZH2 is recruited in an ERK dependent manner to E-cadherin promoter

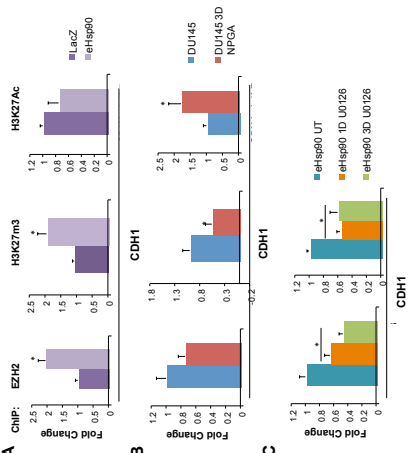


Figure 5

A) EZH2 and its catalytic mark (H3K27m3) are increased at the E-cadherin promoter in ARCaPE-eHsp90 cells compared to LacZ. H3K27Ac is slightly decreased at the E-cadherin promoter. *P < 0.05. B) NPGA diminished both EZH2 and H3K27m3 at the E-cadherin promoter in DU145, while H3K27Ac is increased. *P < 0.05. C) U0126 treatment decreased both EZH2 and H3K27m3 at the E-cadherin promoter in ARCaPE-eHsp90. *P < 0.05.

EZH2 regulates eHsp90 directed tumor growth

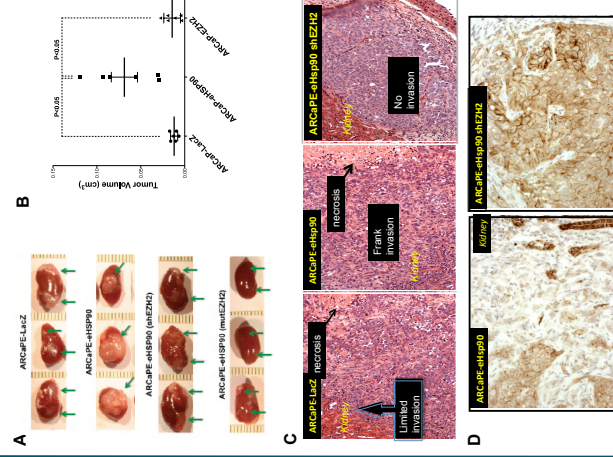


Figure 6

A) ARCaPE-eHsp90 drives increased tumor size in comparison to LacZ, which is abrogated by loss of EZH2 (via shRNA or mutant EZH2). N = 6. B) Tumor volume was quantitated and statistically analyzed via ANOVA. P < 0.05. C) H&E staining of representative tissue demonstrates that ARCaPE-eHsp90 tumors show no invasion in comparison to ARCaPE-eHsp90 cells. D) E-cadherin is restored in vivo in ARCaPE-eHsp90 shEZH2 tumors in comparison to ARCaPE-eHsp90.

CONCLUSION

- ◆ An eHsp90-ERK axis plays a major role in regulating EZH2 expression
- ◆ eHsp90 promotes E-cadherin suppression via recruitment of EZH2, also mediated by ERK activation
- ◆ eHsp90 drives increased tumorigenesis and invasion in vivo
- ◆ EZH2 attenuates eHsp90 tumor growth, invasion, and restores E-cadherin expression in vivo

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Secreted Hsp90 is a novel potentiator of EMT events and tumor growth

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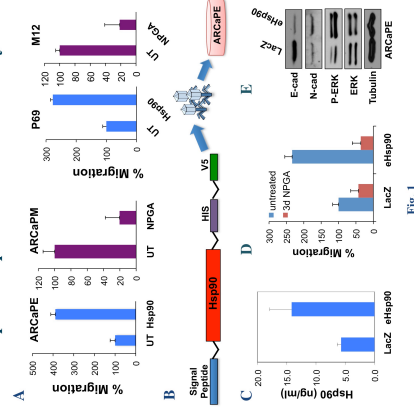
INTRODUCTION

Prostate cancer (Pca) is the second leading cause of cancer related death among males. Lethality is primarily a result of the propensity of tumor cells to metastasize to bone. Our goal was to uncover novel factors involved in early Pca progression.

Activation of the epithelial to mesenchymal transition (EMT) genetic program represents a significant risk factor for cancer progression in Pca and other solid tumors. The maintenance of epithelial morphology, regulated in large part by E-cadherin, suppresses initiation of EMT; inversely, loss of epithelial properties and acquisition of mesenchymal morphology and behavior are hallmarks of the EMT process and are correlated with tumor 90 (Hsp90).

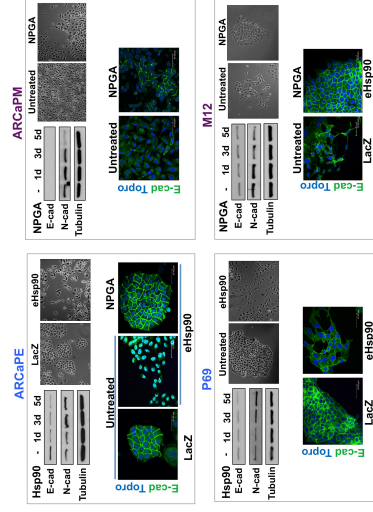
The molecular chaperone heat shock protein 90 (Hsp90) regulates cellular stress responses and signaling. An extracellular population (eHsp90) transduces signaling events through its cell surface receptor LRP1. eHsp90 is essential for cell motility, invasion, and metastatic behavior of several cancer types, although a mechanism for this activity has remained unknown. A clinical role for eHsp90 is supported by its preferential expression in plasma from cancer-afflicted patients. Herein, we demonstrate that eHsp90 is a potent mediator of EMT events in Pca cells, thereby providing a sound mechanistic basis for its role in cancer progression.

eHsp90 is required for Pca cell motility



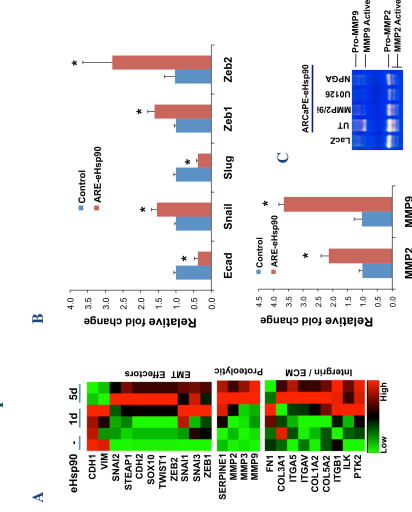
A. A model of matched epithelial (blue) and mesenchymal (purple) counterparts, the latter of which secrete more Hsp90, demonstrates eHsp90-dependent regulation of cell motility. NPGA is a non-permeable small molecule drug targeting eHsp90.
B. Schematic for creation of genetic model for directed Hsp90 secretion.
C. ELISA detection of eHsp90 in control (LacZ) and eHsp90 transduced ARCAPE cells.
D. Cell motility for cells in C, untreated or NPGA treated.
E. Expression of respective proteins in secretion model.

eHsp90 promotes a mesenchymal phenotype



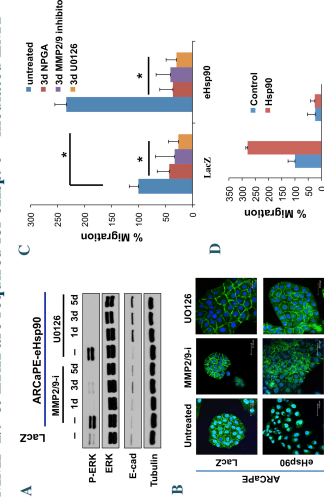
Temporal effects of exogenous addition of recombinant Hsp90 or NPGA treatment (days) upon cadherin expression and localization, and cell morphology.

eHsp90 induces EMT events in ARCAPE cells



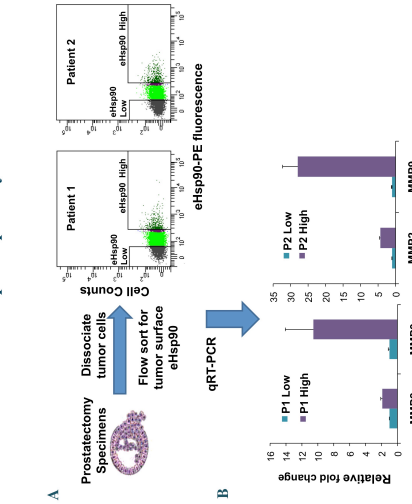
A. qRT-PCR results of an EMT-focused array following addition of exogenous Hsp90 protein.
B. qRT-PCR validation of select targets in ARCAPE-eHsp90 genetic model.
C. Demonstration of eHsp90-mediated activation of MMP-2 and MMP-9 in and ERK-dependent pathway.

MMP-2/9 & ERK are required for eHsp90 – mediated EMT

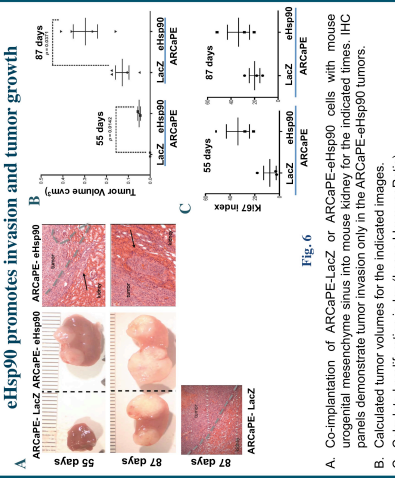


A. Relation among MMP-2/9, ERK and E-cadherin in ARCAPE-eHsp90 relative to LacZ.
B. Targeting ERK or MMP-2/9 restores epithelial morphology.
C. Motility effects of the indicated treatments. Motility is diminished concomitant with restoration of membrane-bound E-cadherin and epithelial morphology.
D. LRP1 is required for eHsp90 motility. LRP1 was stably suppressed via shRNA.

Detection of eHsp90 in primary Pca tumors

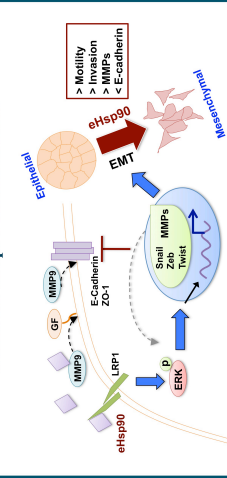


A. Primary prostatectomy tumor specimens were processed for flow cytometry. Intact cells were incubated with PE-labeled Hsp90 antibody and eHsp90 high and low populations were isolated.
B. qRT-PCR analysis was performed on mRNA isolated from the respective cell populations in A. P1 and P2 indicate patient number, Gleason 7 and 9, respectively.



A. Co-implantation of ARCAPE-LacZ or ARCAPE-eHsp90 cells with mouse preputial mesenchyme sinus into mouse kidney for the indicated times. IHC panels demonstrate tumor invasion only in the ARCAPE-eHsp90 tumors.
B. Calculated tumor volumes for the indicated images.
C. Calculated proliferation index (ImageJ Immunofluorescence).

Model for eHsp90-mediated EMT



Tumor-secreted eHsp90 signals via its receptor LRP1 to activate ERK. eHsp90-LRP1-ERK signaling subsequently initiates transcription of pro-EMT transcription factors (Snail/Zeb/Twist), as well as MMPs. MMP activation reinforces sustained ERK activation and E-cadherin suppression. eHsp90 may also directly activate MMPs, which further cleave E-cadherin to block its function. Collectively, eHsp90 secretion initiates a complex and potent cascade to support tumor growth.

SUMMARY

- Tumor secreted Hsp90 is a potent initiator of cell motility and EMT via an LRP1-ERK-MMP dependent pathway.
- Forced secretion of Hsp90 promotes both tumor growth and tumor cell invasion.
- The detection of eHsp90 on the surface of localized prostate tumors, and its association with transcripts supportive of aggressive behavior, implicates a clinical role for eHsp90 in Pca progression.