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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 findings demonstrate that eHsp90-dependent upregulation of EZH2 is important not only for E-cadherin repression, but also for upregulated expression of several EMT effectors, including Snail, also a known repressor of E-cadherin. Blockade of eHsp90 via NPGA restored E-cadherin expression in several aggressive prostate cancer cell lines, indicating that these cell types are inherently reliant upon eHsp90 action for at least a subset of their malignant properties. Our findings validate the ability of eHsp90 to exert dynamic epigenetic reprogramming at select target genes to enforce EMT events. Our collective findings indicate that HDAC1/2, Snail, and EZH2 cooperate to modulate E-cadherin expression. From a clinical standpoint, it is imperative to understand these molecular relationships towards the goal of dampening EMT activation. While inhibition of EZH2 and HDAC activity may be suitable for restoration of eHsp90-repressed targets (i.e. E-cadherin), it is also essential to clarify their effects upon chromatin remodeling at sites of eHsp90-activated targets such as Snail and Zeb.

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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

Tasks 1a and 1b were completed.

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

- 2a: Partially completed. ChIP analysis of EZH2, H3K27me3 and H3K27Ac at the E-cadherin promoter was performed in ARCaPE-LacZ and matched ARCaPE-eHsp90 cells. Attempts to evaluate recruitment of HDAC1 and HDAC2 have not been successful, but several complementary approaches are being evaluated (see Fig. 1 and summary).
- 2b: Partially completed. Analysis of eHsp90 regulated recruitment of epigenetic proteins to EMT gene promoters (see summary).
- 2c: Completed. Create a stable derivative of ARCaPE-eHsp90 with Snail shRNA and characterize E-cadherin expression (Fig. 2A, B)
- 2d: Initiated. Determine effect of Snail suppression upon recruitment of epigenetic proteins to the E-cadherin promoter (Fig. 2C).
- 2e: Initiated. Determine how epigenomic therapeutics modulate eHsp90 mediated EMT transcription and expression in ARCaPE-eHsp90 model (Fig. 2D).
- 2f: Initiated. Evaluate how epigenomic agents modulate recruitment of Snail, HDAC1/2, EZH2 to the promoters of EMT target genes. See summary, efforts focused on E-cadherin regulation.
- 2g: Initiated. Evaluate ability of NPGA, MS-275, and GSK343 (EZH2 targeting) to modulate EMT events and recruitment of Snail, HDAC1/2, and EZH2 to EMT promoters in aggressive prostate cancer lines (Fig. 3A-D, Fig. 4A-C).

Task 2 Summary

The technique of chromatin immunoprecipitation (ChIP) is technically challenging, requiring exquisite validation for each antibody. Although we have been successful performing ChIP with antibodies against both EZH2 and H3K27me3, we have not yet been successful with antibodies against HDAC1 and HDAC2, the latter to answer the question of whether eHsp90 co-recruits EZH2 and HDACs to elicit E-cadherin suppression. In addition to trying different ChIP-validated antibodies, we will also utilize an alternative approach of employing cell models wherein either HDAC1 or HDAC2 is stably suppresed. These shRNA models for HDAC1/2 suppression have recently been generated (**Fig. 1**). We will additionally utilize the broad-based HDAC inhibitor MS-275 (which also restores E-cad expression) to evaluate whether HDACs influence EZH2 recruitment to the E-cad promoter within the context of eHsp90 signaling. In Task 2b, we proposed to evaluate the role of eHsp90 in recruiting relevant epigenomic proteins to EMT effectors. Although we initially proposed to evaluate this by specific ChIP approaches followed by an EMT gene array, we have instead worked to optimize ChIP-seq approaches, given the likelihood of unbiased and genome-wide information that may be obtained by this approach. Recent pilot experiments indicate steady technical progress. Relevant to Task 2d, we successfully suppressed Snail expression and demonstrated concomitant re-expression of E-cadherin (**Fig. 2A**). We further validated that Snail plays an important role in the disrupted epithelial morphology elicited by eHsp90 (**Fig 2B**).

Surprisingly, our initial results indicate that loss of Snail expression does not have a dramatic effect on the ability of eHsp90 to recruit EZH2 to the E-cadherin promoter (**Fig. 2C**). Although this finding indicates that Snail may not be required for EZH2-mediated E-cadherin suppression, an alternate plausible explanation is that Snail may be required for early recruitment, but is less essential for sustained suppression. To evaluate early chromatin remodeling events at the E-cadherin promoter, Hsp90 protein will be added to Snail suppressed cells. This approach should answer the question of whether Snail plays a role in EZH2 recruitment, and whether it may play a differential role in initiation vs maintenance events. In light of a recent report, it is also possible that EZH2 may tether Snail to the E-cadherin promoter. It was also shown that Snail may form distinct protein complexes at the E-cadherin promoter, either with EZH2 or components of the PRC1 complex. See appendix for additional information on proposed study design. Interestingly, we also found that EZH2 was required for eHsp90's upregulated expression of Snail (**Fig. 2D**). This indicates that EZH2 either possesses an activating function at the Snail promoter, or that EZH2 may be suppressing a repressor of Snail, leading to its de-repression. This finding will warrant further study.

Relevant to Tasks 2e and 2g, we are evaluating the effects of NPGA, MS-275, HDAC1/2, and GSK343 (EZH2 inhibitor) upon EMT events within the ARCaPE model, and also upon additional prostate cancer cell lines. We demonstrate that EZH2 targeting restores E-cadherin in ARCaPM (**Fig 3A, 3B**), concomitant with increased E-cadherin transcription (**Fig. 3C**). From our expanded analysis, we also show that GSK343 targeting restores E-cadherin expression in aggressive DU145 and M12 lines (**Fig. 3D**). Moreover, NPGA similarly induced E-cadherin expression in both DU145 and M12, while suppressing EZH2 (**Fig 4A**). To demonstrate a more direct connection between eHsp90 and EZH2, we show that NPGA treatment reduces EZH2 recruitment to the E-cadherin promoter in DU145 (**Fig. 4B**). In further support of the relation between eHsp90 and EZH2, we show that eHsp90 targeting in ARCaPM, a cell line with elevated EZH2 expression and high basal H3K27me3 levels, dramatically reduces global H3K27me3 expression (**Fig. 4C**). Ongoing experiments will further characterize how EZH2 and HDAC1/2 and cooperate to elicit eHsp90-mediated E-cadherin suppression and EMT activation at the epigenetic level.

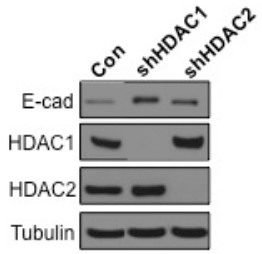

Fig. 1. HDAC1/2 suppression restores E-cadherin expression. ARCaPE-eHsp90 cells were stably transfected with either a nonspecific shRNA (Con) or shRNA to HDAC1 or HDAC2 and E-cadherin was analyzed by immunoblot.

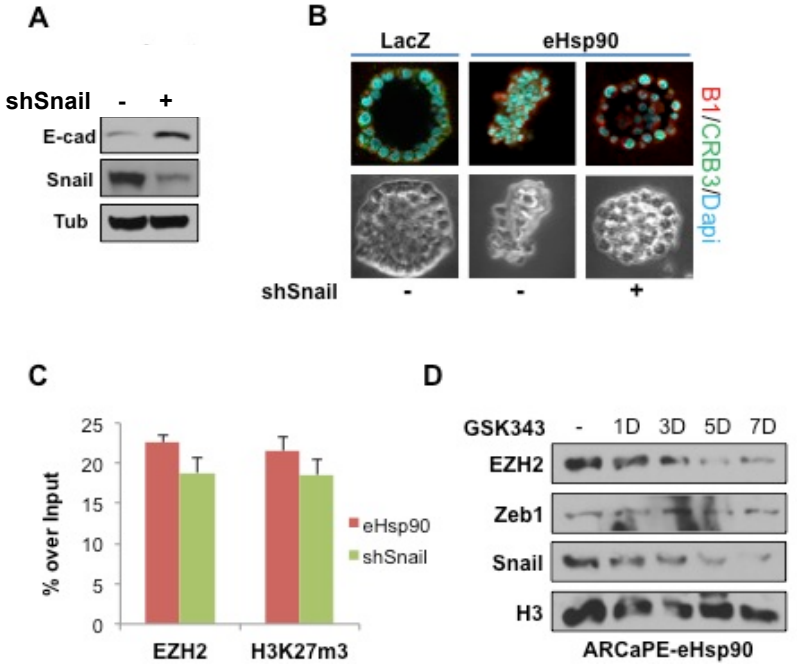

Fig. 2. Evaluation of the relationship between EZH2 and Snail within the context of eHsp90-mediated EMT. A) Snail was stably suppressed in ARCaPE-eHsp90 and indicated proteins evaluated by immunoblot. **B)** 3D imaging of ARCaPE control (LacZ) and ARCaPE-eHsp90 (eHsp90) plated in Matrigel for 14 days. Confocal microscopy (top) and phase contrast images (bottom) confirm that eHsp90-mediated Snail expression is a primary effector of the disrupted epithelial program. **C)** ChIP analysis of EZH2 and H3K27m3 recruitment to the E-cadherin promoter in ARCaPE-eHsp90 cells following Snail suppression. **D)** Evaluation of effects of EZH2 targeting upon EMT effectors.

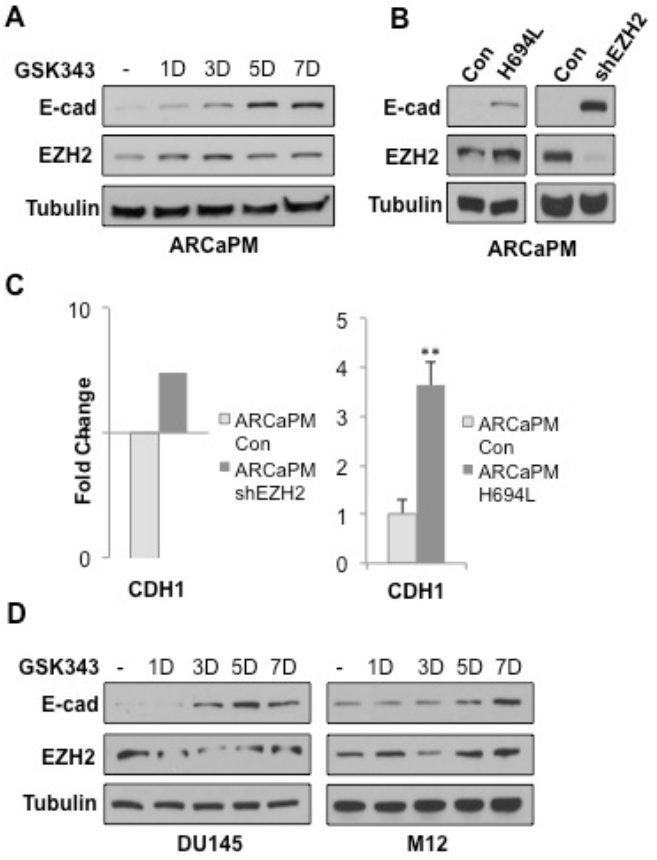

Fig. 3. Role of EZH2 in E-cadherin suppression in a panel of aggressive cell lines. A) EZH2 targeting via pharmacological means restores E-cadherin expression in ARCaPM. **B)** EZH2 targeting via transduction of a dominant mutant (H694L) or shRNA suppression similarly restores E-cadherin expression. **C)** Demonstration that EZH2 targeting transcriptionally increases E-cadherin expression in ARCaPM. **D)** GSK343 targeting similarly increases E-cadherin in DU145 and M12 cells.

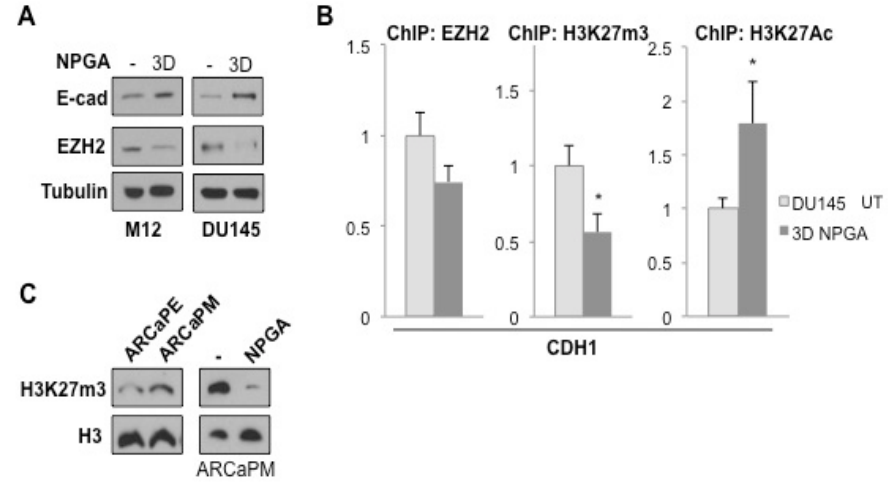

Fig. 4. Evaluation of the relation between eHsp90 and EZH2. A) NPGA treatment restores E-cadherin and inversely suppresses EZH2 protein in additional cell lines. **B)** NPGA elicits modest changes in EZH2 recruitment at the E-cadherin promoter. **C)** (Left panel) Global histone levels of the EZH2-directed H3K27m3 mark in ARCaPE relative to mesenchymal ARCaPM counterpart cells. (Right panel) Effects of NPGA upon global histone H3K27m3 expression in ARCaPM.

Data generated from several of the completed tasks outlined in this section has been compiled into a manuscript. This MS was submitted to Molecular Cancer Research. However, we encountered a challenging reviewer and the MS was not accepted. We are now in the process of revising the MS and will resubmit to the Journal of Biological Chemistry in December 2014.

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

- 3a: Completed. (multi-parameter FACS of eHsp90 hi/low in cells)
- 3b: Completed
- 3c: Initiated (Fig. 5). Implant mice with ARCaPM and monitor BLI values. See summary
- 3d-f: Not yet initiated

Task 3 Summary

As mentioned in our prior report, we were unable to observe micrometastatic lesions of ARCaPE-eHsp90 using the intracardiac delivery method. These results indicate that although eHsp90 was able to initiate tumor invasion, it may not be sufficient to promote metastasis in a lowly metastatic cell line. We have therefore turned our attention towards answering the question of whether eHsp90 is critical for sustaining the metastatic potential of its mesenchymal counterpart ARCaPM (task 3f). We stably transfected ARCaPM with a luciferase lentivirus to enable the noninvasive imaging of tumors. ARCaPM-luc was implanted orthotopically, and we demonstrate the *in vivo* visualization of tumor growth (**Fig. 5**). We are presently conducting pilot experiments to evaluate whether the primary tumor will give rise to micrometastases. We have also created a similar model with the metastatic M12 cells (not shown), and we will test the metastatic potential of M12 in tandem. We will then focus our efforts upon Task 3f, wherein we will evaluate whether eHsp90 blockade impairs tumorigenic and/or metastatic growth, and whether this correlates with a reversal of EMT events.

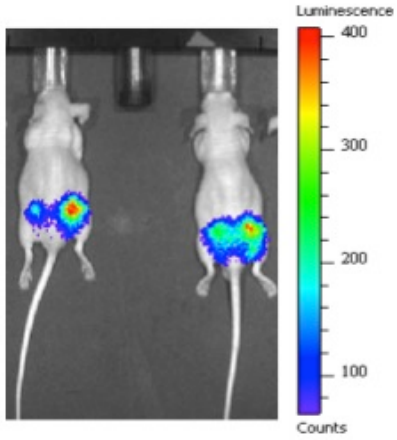

Fig. 5. Initial evaluation of noninvasive tumor imaging with ARCaPM-Luciferase cells. ARCaPM were stably transduced with a lentiviral-luciferase construct. Cells were implanted orthotopically and imaged after 56 days following a luciferin injection and Xenogen photon capture.

Task 4: Utilize FACS to evaluate the expression of eHsp90 in primary tumor tissue

- 4a: Completed
- 4b: Completed
- 4c: Initiated. Perform FACS for eHsp90 hi/low tumor subpopulations and isolate RNA.

Task 4 Summary

During the course of our studies, we found that a restricted subpopulation of tumor cells from patient samples exhibit surface Hsp90. Given that this minor population is on average, approximately 5% of the whole population, we have elected not to co-sort for accessory markers. Instead, efforts are focused upon the isolation of $eHsp90^{hi}$ and $eHsp90^{low}$ followed by analysis of relevant EMT-related transcripts. We have had challenges procuring sufficient tissue and in optimizing parameters for isolation. These issues have been largely resolved and we expect to analyze at least a dozen samples in the coming months.

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

- 5a: Not yet initiated
- 5b: Initiated. 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.
- 5c-e: Not yet initiated, see Appendix.

Task 5 Summary

Our efforts in the last funding period have focused on MS resubmission, molecular studies in cell-based models (relevant primarily to Task 2), and initiation of the in vivo analysis (Task 3). Therefore, most of the experimentation associated with Task 5 has not yet been initiated. See Appendix for amended Statement of Work.

KEY RESEARCH ACCOMPLISHMENTS:

- We determined that suppression of either HDAC1 or HDAC2 was sufficient to restore E-cadherin expression, thereby antagonizing the suppression elicited by eHsp90. Efforts are underway to discern whether these HDACs promote EZH2 recruitment to the E-cadherin promoter.
- Snail is a major effector of both eHsp90-mediated suppression of E-cadherin and loss of epithelial polarity. Despite this, Snail suppression had minimal effect upon EZH2 recruitment to the E-cadherin promoter. We now hypothesize that EZH2 may recruit Snail, and that Snail modulates recruitment of Polycomb complex 1 (PRC1) components to govern E-cadherin expression (see Appendix). We also found that EZH2 is critical for Snail expression, which will be subsequently explored.
- We evaluated the epigenetic effects of eHsp90 and EZH2 upon E-cadherin in an expanded cohort of aggressive prostate cancer cell lines. EZH2 suppression, either by pharmacologic targeting (GSK343), shRNA suppression, or introduction of a dominant inactive mutant (H694L), restored E-cadherin expression at the transcriptional level in ARCaPM. EZH2 targeting also restored E-cadherin expression in DU145 and M12 cells.
- We found that NPGA treatment of prostate cell lines such as DU145 restored E-cadherin protein expression. This restoration was accompanied by a modest reduction of EZH2 recruitment, and loss of the associated EZH2-directed inhibitory H3K27m3 mark. We also noted a robust increase in histone acetylation (H3K27m3). These expression and epigenetic remodeling trends inversely mirrored effects elicited by eHsp90 exposure, thereby validating eHsp90's epigenetic remodeling function.
- We generated 2 luciferase-transduced derivatives of the aggressive prostate cancer cell lines ARCaPM and M12. ARCaPM-Luc was orthotopically implanted to validate our ability to noninvasively visualize tumor growth via luciferase monitoring. These pilot experiments are ongoing, and mice will be evaluated for metastatic lesions. A similar set of pilot experiments will be initiated for M12-Luc derived tumors. Subsequently, we will evaluate whether eHsp90 targeting reduces tumorigenesis and/or metastasis, and whether phenotypic changes may be linked to modulation of EMT events.

REPORTABLE OUTCOMES:

Presentations

2014 Society for Basic Urological Research (SBUR) Fall Symposium, Dallas, TX
Michael W Hance, Krystal Nolan, and **Jennifer S Isaacs**
Tumor secreted Hsp90 disrupts epithelial polarity via ERK dependent signaling
(Selected for oral presentation)

2014 Society for Basic Urological Research (SBUR) Fall Symposium, Dallas, TX
Krystal Nolan, Michael Hance, and **Jennifer S Isaacs**
Tumor secreted Hsp90 supports a stem-like cell subpopulation in prostate cancer
Note: This represents an extension of our current work (see appendix).

Development of cell lines

- ARCaPM with stable luciferase expression plasmid
- M12 with stable luciferase expression plasmid
- ARCaPE-eHsp90 with Snail shRNA
- ARCaPE-eHsp90 with ERK2 shRNA
- ARCaPE-eHsp90 with HDAC1 shRNA
- ARCaPE-eHsp90 with HDAC2 shRNA

Funding applied for based on work supported by this award

- Several findings from this DOD proposal were expanded and included within the RO1 application 'Role of extracellular Hsp90 in epithelial cell polarity and invasion', submitted in October 2013. This proposal was recently funded by the NCI Tumor Cell Biology (TCB) study section. Current plans are to expand our current findings in preparation for an RO1 grant submission either June or October 2015.

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 findings demonstrate that eHsp90-dependent upregulation of EZH2 is important not only for E-cadherin repression, but also for upregulated expression of several EMT effectors, including Snail, also a known repressor of E-cadherin. Blockade of eHsp90 via NPGA restored E-cadherin expression in several aggressive prostate cancer cell lines, indicating that these cell types are inherently reliant upon eHsp90 action for at least a subset of their malignant properties. The ability of NPGA treatment to elicit epigenetic effects at the E-cadherin promoter inverse to those mediated by enforced eHsp90 expression validates the ability of eHsp90 to exert dynamic epigenetic reprogramming at select target genes. However, it should be noted that some context dependent differences were evident. Whereas in some cell types, E-cadherin expression accompanied chromatin remodeling events favoring EZH2 eviction, in other instances, E-cadherin expression was more dependent upon changes in promoter acetylation. Further molecular studies in an expanded cohort of cell models is ongoing.

Our collective findings indicate that HDAC1/2, Snail, and EZH2 cooperate to modulate E-cadherin expression. Ongoing and planned experiments will further unravel how eHsp90 orchestrates these molecular effectors to suppress E-cadherin in a concerted fashion. As indicated, future experiments will also begin to dissect the epigenomic regulatory mechanism for eHsp90-mediated activation of EMT effectors such as Snail. An unexpected finding from our study is that EZH2 activity is required for Snail expression. Further work is required to understand this important aspect of regulatory control. From a clinical standpoint, it is imperative to understand these molecular relationships towards the goal of dampening EMT activation. While HDAC inhibitors and EZH2 targeting may be suitable for modulation of E-cadherin expression, it is also essential to understand how these epigenetic modifiers may remodel the promoters of prototypic EMT activators, such as Snail and Zeb.

APPENDICES:

Request to amend Statement of Work

The aims of the initial proposal were based upon our preliminary findings placed within the framework of conceptual knowledge aggregated from published reports at that time. Our overarching goal was to define how eHsp90 signaling elicited changes at the epigenetic level to promote prostate tumor progression. Stated metrics included suppression of E-cadherin, increased expression of EMT effectors, tumor growth and metastasis. The original proposal aimed to define the molecular and functional relationships between eHsp90-initiated EZH2 signaling and participation of additional relevant repressor proteins, such as HDAC1 and HDAC2 and Snail, with the goal of utilizing epigenomic therapeutics to reverse EMT events.

Based upon our recent findings, we request to amend the SOW in research directions more aligned with our accumulating data and hypotheses. We propose that these new directions will be effective in illuminating mechanistic and functional aspects relevant to the molecular action of eHsp90 in tumor progression. As elaborated below, one new aspect of the work includes an expanded analysis of eHsp90-driven events. It is well known from published reports that, addition to EMT, the loss of E-cadherin and upregulation of EZH2 is highly associated with the maintenance of a cancer stem-like population. Cancer stem cells are implicated in resistance to androgen deprivation therapy and chemotherapy and are essential for tumor repopulation. We therefore evaluated whether eHsp90-EZH2 signaling plays a role in development of a cancer stem-like population. As shown in the accompanying poster, we have demonstrated that in addition to eliciting EMT events, eHsp90-EZH2 signaling also promotes a de-differentiated phenotype. We believe that it will be clinically informative to include this expanded functional metric within the proposed molecular and pharmacologic analyses.

A focus of the aims in Task 2 is to further elaborate the relations among eHsp90, EZH2, Snail and HDAC. Our current findings indicate these inter-relations are quite a bit more complex and interdependent than originally anticipated. Thus, our modified SOW is crafted to include the creation of additional cell models that will enable elucidation of this multi-tiered level of regulation. Although not in the original proposal, we had demonstrated in the appendix of the last progress report that eHsp90 sustains ERK activation, an event critical for EZH2 expression and for its recruitment to the E-cadherin promoter. We have also found that eHsp90-ERK signaling is also required for expression of Snail. Moreover, we show herein that eHsp90-EZH2 expression is also required for Snail expression. Hence, it appears that an eHsp90-ERK-EZH2 axis also sustains Snail expression. (Also note that an eHsp90-ERK-EZH2-Snail axis sustains the cancer stem-like population). We have also found that blockade of eHsp90 or EZH2 function similarly reduces expression of HDAC1 and HDAC2. Given these interdependent relations, it is currently impossible to clearly define key molecular mechanisms of action within the context of eHsp90-dependent epigenetic remodeling. In light of this development, we propose to focus on subaims relevant to an expansion of Task 2, while de-emphasizing the correlative experiments associated with Task 5, which has less relevance to the maturing project and is less likely to be mechanistically informative.

Modified SOW

In addition to ongoing subaims, we propose a modification of the remaining work as outlined below.

Task 1: Generate requisite cell models to evaluate the key effectors of eHsp90-mediated E-cadherin repression.

- 1a: Generate an EZH2-expressing derivative of ARCaPE-eHsp90. This model will minimize the ability either eHsp90 or ERK blockade to diminish EZH2 expression.
- 1b: Generate a Snail-expressing derivative of ARCaPE-eHsp90. This model will minimize the ability either eHsp90 or ERK blockade to diminish Snail expression. However, it is possible that eHsp90-ERK targeting may reduce EZH2 expression, which may be a required component of Snail expression. Should this be observed, we will still be able to better understand the higher order relationships.
- 1c: Generate a derivative of ARCaPE-eHsp90 wherein EZH2 function is suppressed but Snail expression enforced. This model will further clarify the interdependence of these proteins within the context of eHsp90 signaling.

- 1d: Evaluate the respective effects of these pharmacologic and molecular manipulations upon expression of E-cadherin, EZH2, Snail and HDAC1/2.

Task 2: Determine the epigenetic mechanism of eHsp90-mediated E-cadherin suppression.

- 2a: Perform ChIP studies for EZH2, H3K27m3, H3K27Ac, and Snail with above models, within the context of -/+ eHsp90 blockade (NPGA) or ERK inhibition (UO126 or ERK2 suppression).
- 2b: Perform ChIP studies for EZH2, H3K27m3, H3K27Ac, and Snail with newly generated HDAC1 vs HDAC2 KD and also compare with MS275 treated cells.
- 2c: Re-evaluate the relation between EZH2 and Snail at the E-cadherin promoter. Although a prior report indicated that Snail recruits EZH2 to the E-cadherin promoter, our results herein indicate that this is not a major mechanism for eHsp90-mediated E-cadherin repression. A very recent report demonstrated an inverse order of events, specifically that EZH2 was required for Snail recruitment. We will therefore evaluate whether EZH2 blockade or suppression functions to regulate Snail recruitment within the context of eHsp90 action.
- 2d: Evaluate whether PRC1 components may be modulated by an eHsp90-EZH2-Snail axis. This same paper also demonstrated that Snail forms distinct protein complexes with epigenetic effectors. While a Snail-EZH2 complex was identified, a distinct Snail-Ring1B complex (PRC1 component) was also identified. Hence, we will also evaluate how Ring1B recruitment to E-cadherin is affected by eHsp90-mediated effects upon EZH2 and Snail. Utilization of the models generated in Task 1 will be invaluable in dissecting out the regulatory mechanism of Ring1B recruitment.
- 2e: Utilize biochemical approaches to validate the formation of differential epigenetic complexes. Several reports, including the aforementioned paper, have demonstrated the use of size exclusion chromatography followed by immunoblotting of fractions to obtain a preliminary understanding of co-segregation profiles indicative of co-associating proteins. Our preliminary data indicate that eHsp90 alters the elution profile of several relevant proteins. We will utilize the stated models and pharmacologic agents to evaluate the elution patters for EZH2, Snail, HDAC1/2, and Ring1b. Future co-immunoprecipitation experiments can be employed to validate observed trends.

Task 3: Evaluate of the role of eHsp90-regulated molecular effectors in maintenance of stem-like population.

- 3a: Utilize the cell models generated in Task 1 to further define how eHsp90-ERK-EZH2-Snail signaling supports a cancer stem-like population. As indicated in the accompanying poster, stemness will be determined by use of flow cytometry utilizing either the dye exclusion assay or the ALDHA1 assay. In addition, qPCR will be performed for prototypic markers of stemness.
- 3b: Determine whether E-cadherin loss is a requisite for generation of the cancer stem-like population. We have generated E-cadherin suppressed cells in LacZ and can easily evaluate this possibility. This finding will reveal whether eHsp90-mediated suppression of E-cadherin is a shared property of EMT activation and maintenance of a cancer stem-like population.
- 3c: Evaluate the ability of flow sorted stem-like populations to form multi-generational prostaspheres, a functional assay for self renewal. This property is highly associated with tumor repopulation activity.

Meeting Presentations:

(2014) Society for Basic Urological Research (SBUR) Fall Symposium, Dallas, TX
Krystal Nolan, Michael Hance, and **Jennifer S Isaacs**
Tumor secreted Hsp90 supports a stem-like cell subpopulation in prostate cancer

Background: Prostate cancer (PCa) is one of the most commonly diagnosed cancers and the second leading cause of cancer deaths in men. The progression of benign, localized PCa to metastatic PCa is a complex process. The epithelial to mesenchymal transition (EMT) genetic program is implicated as a key facilitator of aggressive disease. We recently demonstrated that tumor-secreted Extraellular Heat Shock Protein 90 (eHsp90) initiates EMT events in vitro, and promotes tumorigenesis and invasion in vivo. These activities of eHsp90 are dependent upon EZH2, an epigenetic repressor also implicated in the development of cancer stem-like cells, a cell population linked with tumor recurrence and lethality. We herein sought to establish further mechanistic insights into the role of eHsp90 in the transition from indolent to aggressive disease. As such, we investigated whether an eHsp90-EZH2 pathway may support the development of cancer stem-like cells.

Methods: We developed and utilized a lentiviral-based approach to enforce eHsp90 secretion in a model of localized PCa. Evidence for a stem-like cancer cell population in transduced cells was assessed by a variety of methods, including flow cytometry, immunoblotting, qRT-PCR, and functional approaches such as prostasphere formation. Moreover, the role of EZH2 was determined through use of pharmacological inhibitors.

Results: We herein demonstrate that eHsp90 increases the population of PCa stem-like cells in a pathway dependent upon EZH2 activity. This was confirmed by increased mRNA expression of a number of characteristic “stem-like” markers, as well as increased ALDH1A1 activity as determined by ALDEFLUOR assays. Furthermore, increased eHsp90 secretion leads to increased ABC transporter activity, a known hallmark for cancer stem-like cells.

Conclusions: We recently demonstrated that eHsp90 elicits EMT events, thereby offering a mechanistic basis for its role in tumor progression. Our current findings are the first to demonstrate eHsp90’s ability to support a stem-like cell population . These data support the notion that eHsp90-mediated stem cell generation may be a major contributor to PCa development, and may potentially confound therapy. Further characterization of this pathway is expected to advance our understanding of key mechanisms driving aggressive disease.

(2014) Society for Basic Urological Research (SBUR) Fall Symposium, Dallas, TX
Michael W Hance, Krystal Nolan, and **Jennifer S Isaacs**
Tumor secreted Hsp90 disrupts epithelial polarity via ERK dependent signaling

Background: Prostate cancer (PCa) is a leading cause of male cancer related death. Early stage localized PCa is usually responsive to treatment, but advanced metastatic disease is correlated with poor prognosis. The epithelial to mesenchymal transition (EMT) genetic program is considered a major factor contributing to cancer cell dissemination. A hallmark of EMT is the disruption of junctional complexes and loss of cellular polarity, collaborative events that support cell motility and invasion. Our goal is to understand these early events that subsequently contribute to aggressive prostate cancer. Targeting the onset of pathogenic EMT is complicated by poor knowledge of the key factors initiating this process. Reports demonstrate that tumor secreted extracellular Hsp90 (eHsp90) promotes cell motility, invasion, and metastasis in a number of cancers, and is detected in the plasma of cancer patients, including PCa. Although this suggests a potential causative role for eHsp90 in cancer progression, the mechanisms involved remain unknown. We recently reported that eHsp90 initiates EMT events, thereby offering a mechanism for its support of tumor metastasis. The goal of this current study is to further define how eHsp90 coordinates changes in cell polarity to support tumor invasion and metastasis.

METHODS: To evaluate the impact of eHsp90 upon PCa cell polarity, a lentiviral approach was utilized to enforce the secretion of eHsp90 in a noninvasive epithelial cell line. Conversely, pharmacologic approaches were used to block eHsp90 function in eHsp90-expressing metastatic PCa cells. In addition, shRNA approaches were utilized to identify key functional drivers of this program. Expression of master EMT effectors was determined by qRT-PCR and immunoblot analysis, while microscopy of 2D and 3D models were used to assess morphological changes and to evaluate the integrity of epithelial polarity.

RESULTS: We report that eHsp90 significantly alters the expression and/or spatial localization of key proteins that maintain junctional complexes and enforce epithelial morphology. We further show that eHsp90 activates ERK, and that this eHsp90-ERK axis is critical for the deregulation of epithelial polarity. Moreover, eHsp90 blockade suppressed ERK activity and modestly restored cell polarity in metastatic cells.

CONCLUSIONS: We now highlight a novel role for eHsp90 as a central regulator of PCa cell polarity via an ERK signaling pathway. We therefore uncover a complex interplay between eHsp90-directed EMT effectors and deregulated epithelial cell polarity. An understanding of this pathway is expected to provide insight into role of eHsp90 in PCa progression. Furthermore, our results indicate that blockade of eHsp90 activity may be an approach to restore cell polarity in aggressive cell types, thereby dampening their metastatic potential.

Tumor Secreted Hsp90 Supports a Stem-like Subpopulation in Prostate Cancer

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INTRODUCTION

Identification of the mechanisms and effectors involved in PCa progression may improve therapeutic opportunities. The epithelial to mesenchymal transition (EMT) serves as one route for metastatic initiation due to the pathway's characteristic ability to increase cancer cell motility and invasion. EMT is also associated with increased cellular plasticity, evidenced by the development of cancer 'stem-like' cells. This cell population is capable of tumor regrowth, and is associated with tumor recurrence and treatment resistance. The Polycomb methyltransferase EZH2, which is overexpressed in PCa, supports both EMT and stem-like processes. We recently identified extracellular Heat Shock Protein 90 (eHsp90) as an initiator of EMT, an activity dependent upon an eHsp90-directed ERK-EZH2 axis. Herein, we evaluate the role of this signaling axis within the context of cellular plasticity and stemness. Our findings support the premise that eHsp90 regulates critical EMT effectors that participate in development of a cancer stem-like population. Hence, eHsp90 augments central pathways that govern cellular plasticity and cooperate in tumor progression.

eHsp90 sustains anoikis resistance

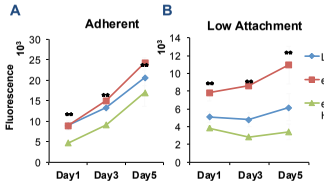

Figure 1

A) CellTiter analysis of adherent cells. ARCaPE-eHsp90 exhibits increased proliferation over ARCaPE-LacZ at 3 and 5 days and ARCaPE-eHsp90 H694L at 1, 3 and 5 days. ** = P<0.01.

B) CellTiter analysis of non-adherent cells. ARCaPE-eHsp90 exhibits anoikis resistance relative to ARCaPE-LacZ and ARCaPE-eHsp90 H694L. ** = P<0.01.

eHsp90 increases in EZH2 and Snail expression

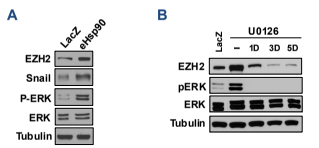

Figure 2

A) ARCaPE-eHsp90 promotes phosphorylation of ERK and upregulates EZH2 and Snail. Loss of ERK signaling reduces EZH2 and Snail expression.

B) Treatment of ARCaPE-eHsp90 with U0126 reduces EZH2 protein expression.

eHsp90 promotes a stem-like population

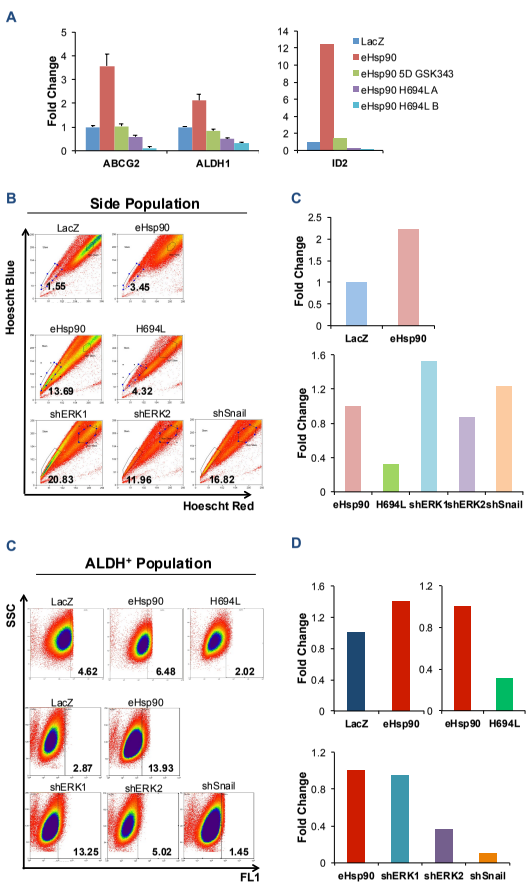

Figure 3

A) ARCaPE-eHsp90 exhibits increased transcriptional expression of ABCG2, ALDH1A1, and Id2, which is reduced upon loss of EZH2 activity via drug treatment (GSK343) or introduction of mutant EZH2 (H694L).

B) eHsp90 increases the side population in ARCaPE (top panels). This effect was primarily dependent upon EZH2, as Snail and ERK2 suppression had little effect (middle and bottom panels).

C) Graphical representation of the fold changes between ARCaPE-LacZ and ARCaPE-eHsp90 side population (top panel) and ARCaPE-eHsp90 and the derivative cell lines (bottom panel).

D) ALDEFLUOR assays demonstrate that eHsp90 increases ALDH1A1 activity, which is diminished by loss of EZH2 activity (top panel), ERK2 or Snail (middle and bottom panels).

E) Graphical representation of the fold changes in ALDH1A1 activity between ARCaPE-LacZ and ARCaPE-eHsp90 and ARCaPE-eHsp90 H694L (top panel) and ARCaPE-eHsp90 and the derivative knockdowns (bottom panel).

Transcription of stem-like markers is enhanced in the side population

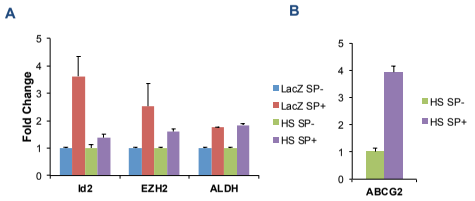

Figure 4

A) Transcriptional expression of Id2, EZH2, and ALDH1A1 are increased in the side population (SP) of ARCaPE-LacZ and ARCaPE-eHsp90 (HS) relative to the non-side population in the respective line.

B) Transcriptional expression of ABCG2 is increased in the side population of ARCaPE-eHsp90 relative to the non-side population of ARCaPE-eHsp90. ABCG2 was not expressed in the ARCaPE-LacZ samples.

eHsp90 increases prostasphere formation

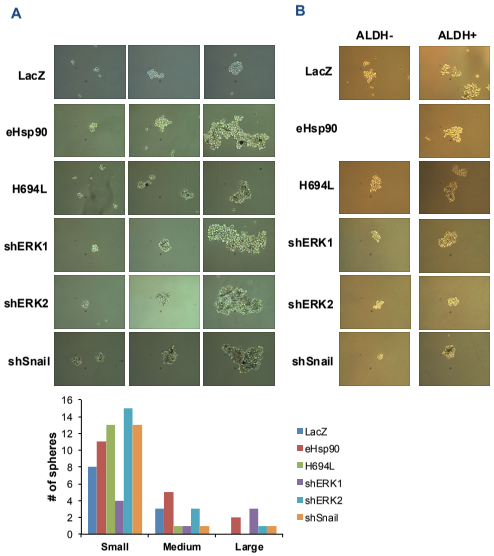

Figure 5

A) Representative images of secondary prostaspheres (P2), highlighting variation in sphere sizes (top panel). Categorical analysis of frequency of sphere sizes reveals that loss of EZH2 activity, ERK2, and Snail severely affects the generation of larger spheres, whereas ERK1 loss promotes larger spheres, perhaps via ERK2 hyperactivation (bottom panel).

B) Representative images of primary prostaspheres generated from the flow sorted ALDH1⁺ population. ALDH1A1 activity correlates with larger spheres, which may be dependent upon EZH2, ERK2, and Snail.

Model for eHsp90 directed development of stem-like subpopulation

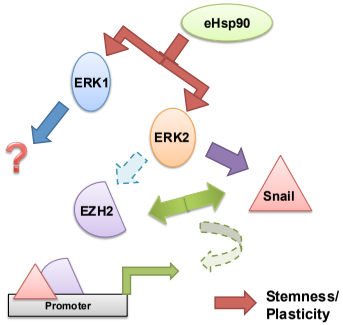

Figure 6

Our current data suggest that ERK1 does not play a critical role in regulating an eHsp90-dependent stem-like phenotype. However, ERK2 is a primary effector, possibly via its upregulation of EZH2 and Snail (not shown). Snail and/or ERK2 may recruit EZH2 to target genes to promote stemness/plasticity.

SUMMARY

- eHsp90 promotes the development of a stem-like subpopulation, supported by an increased side population, ALDH activity, and prostasphere growth.
- EZH2 is a primary driver of eHsp90's maintenance of stem-like characteristics.
- ERK2 and Snail also participate in stem cell development, highlighting functional links between eHsp90-dependent regulation of EMT effectors and cellular plasticity.

FUTURE WORK

- Further characterization of the side population via qRT-PCR, functional assays, and tumorigenic potential.
- Define the mechanism for the functional cooperativity of ERK2, Snail and EZH2 in stem cell maintenance.
- Define the key effectors of eHsp90 action in generation of the stem-like phenotype.

ACKNOWLEDGEMENT

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