Extracellular Hsp90α and Hsp70 Increase Activation of MMP-2 in Breast Cancer Migration and Invasion

A Dissertation

Submitted by

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Abstract

The goal of this thesis was to gain a better understanding of extracellular Heat Shock Protein 90α (Hsp90α) and its role in breast cancer migration and invasion through the activation of Matrix Metalloproteinase-2 (MMP-2). This work, which was begun by Brenda Eustace, a previous graduate student in the Jay Lab, initially identified Hsp90α outside of fibrosarcoma cells and demonstrated that Hsp90α functions to increase the invasiveness of cancer cells by influencing MMP-2 activation. I expanded upon this work by elucidating an export mechanism of Hsp90α from breast cancer cells, investigating how Hsp90α affects MMP-2 activation, and testing the ability of a cell-impermeable Hsp90α inhibitor to reduce breast cancer migration and invasion, both *in vitro* and *in vivo*.

In chapter 2 of this thesis, I address the mode of export of Hsp90α. Hsp90α was found to have two different isoforms, one of which contained an alternative start site and putative signal sequence, indicating that it could be exported through the canonical signal sequence pathway. However, we demonstrated that Hsp90α is not exported through the canonical signal sequence pathway or in an isoform specific manner. Hsp90α is instead exported from breast cancer cells via exosomes.

Chapter 3 explores the role of extracellular Hsp90α in the activation of MMP-2 and in breast cancer cell migration and invasion. I demonstrated that Hsp90α interacts with MMP-2, along with the co-chaperones Hsp70, Hop, Hsp40, and p23, both *in vitro* and in cancer cell conditioned media. This was the first time that all four of these co-chaperones were demonstrated to be present together outside of cancer cells. I showed that Hsp90α, in conjunction with these co-chaperones, was capable of assisting in the

activation of MMP-2 *in vitro*. Also, when Hsp70 was inhibited, the activation of exogenously added MMP-2 in conditioned media was reduced, indicating the importance of Hsp70 in MMP-2 activation. I used wound healing and invasion assays to demonstrate that inhibition of Hsp90α or Hsp70 significantly reduced the ability of breast cancer cells to migrate or invade.

In the appendix, I address the role of Hsp90α and MMP-2 activation in breast cancer cell invasion and metastasis. In order to specifically target extracellular Hsp90α, I tested an Hsp90α function-inhibiting antibody for its ability to reduce cancer cell invasion. I tested the antibody in an *in vitro* invasion assay, where I observed a 40% reduction in invasion. In addition, I tested this antibody for its ability to inhibit metastasis in an *in vivo* breast-to-bone metastasis model, for which the data was inconclusive. The appendix also includes a paper that contains part of my work described in chapter 2 and contributions from Jessica McCready, Ph.D.

This thesis demonstrates that Hsp90α is exported from breast cancer cells via exosomes and describes one function of extracellular Hsp90α. In addition, this dissertation describes a novel mechanism for MMP-2 activation that is independent of MT1-MMP, the enzyme traditionally associated with MMP-2 activation. I also began testing an Hsp90α function-inhibiting antibody for its ability to specifically inhibit extracellular Hsp90α and reduce cancer cell migration and invasion. I demonstrated that the inhibitor is capable of causing a significant reduction in cancer cell invasion *in vitro* and warrants further study both in *in vitro* and *in vivo* models.

Acknowledgements

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Abbreviations

ADAM:	A disintegrin and metalloprotease
ATP:	Adenosine triphosphate
BFA:	Brefeldin A
BRCA1/2:	Breast Cancer Gene 1 or 2
CHIP:	C-terminus of Hsc70-interacting protein
DMEM:	Dulbecco's modified Eagle's medium
EGFR:	Epithelial Growth Factor Receptor
ER:	Endoplasmic Reticulum
FALI:	Fluorphore Assisted Light Inactivation
FBS:	Fetal Bovine Serum
FITC:	Fluorescein isothiocyanate
GFP:	Green Fluorescent Protein
GRP94:	Glucose-Regulated Protein 94
HBSS:	Hanks Buffered Salt Solution
HER-2:	Human Epithelial Growth Factor 3 (Also called ErbB2)
Hop:	Hsp70/Hsp90 organizing protein
Hsp40:	Heat Shock Protein 40
Hsp70:	Heat Shock Protein 70
Hsp90α:	Heat Shock Protein 90α
Hsp90AA1-1:	Heat Shock Protein 90α splice variant with 10-exons
Hsp90AA1-2:	Heat Shock Protein 90α splice variant with 12-exons
HSE:	Heat Shock Elements
HSF1:	Heat Shock Factor 1
IgG:	Immunoglobulin G
IP:	Immunoprecipitate
LD:	Linker Domain
MMP:	Matrix Metalloproteinase
MMP-2:	Matrix Metalloproteinase-2
MT1-MMP:	Membrane Type 1 Matrix Metalloproteinase
MVB:	Multivesicular Body
NOD-SCID	Non-obese diabetic-severe compromised immune deficiency
PBS:	Phosphate Buffered Saline
PCR:	Polymerase Chain Reaction
PKC:	Protein Kinase C
PS:	Phosphatidylserine
ROCK:	Rho-associated coiled-coil-forming protein kinase
RPA:	RNA Protection Assay
RT-PCR:	Real time Polymerase Chain Reaction
SDS-PAGE:	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SHR:	Steroid Hormone Receptor
siRNA:	Small interfering Ribonucleic acid
TGF-β:	Transforming Growth factor Beta
TIMP-2	Tissue Inhibitor of Matrix Metalloproteinase 2
TKI:	Tyrosine Kinase Inhibitors

tPA:	Tissue Plasminogen activator
TPR:	Tetratricopeptide repeat
TRAP1:	TNF Receptor Associated Protein 1
VEGF:	Vascular Endothelial Growth Factor

CHAPTER 1

INTRODUCTION

BREAST CANCER

Overview. Breast cancer is second only to lung cancer in cancer-related deaths and accounts for the majority of new cases of cancer in women (1). Early detection and an ever-growing number of treatments has increased the 5-year survival rate for women diagnosed with breast cancer from 79% in 1986 to 90% in 1995, but mortality due to disease still remains a large problem (1,2). The majority of breast-cancer-related deaths are the result of metastases and even though there have been many improvements in treatments for advanced breast cancer, the median survival time of women with metastatic disease is only 18-24 months (3).

Breast cancer risk factors include early menarche, late menopause, nulliparity, and positive family history. Five to ten percent of breast cancer cases are considered hereditary. Known familial genes include BRCA1, BRCA2, and BRCAX, which account for the majority of the cases, while CHEK2, ATM, NBS1, RAD51, BRIP1, and PALB2 are a few of the genes that contribute to the remaining familial cases (4). Mutations in these genes can cause a reduction of tumor suppression, a break-down of the DNA and chromosomal repair processes, and disruption of proper signaling for cell growth and apoptosis (4). The remaining cases have no known familial bases and have been attributed to a wide range of factors including lifestyle, diet, exercise, and previous exposure to chemicals or radiation (5,6).

The “hallmarks of cancer,” as first described by Hanahan and Weinburg in 2001, are the six stages cancer progresses through to obtain malignant growth. The stages include immortality, abnormal growth regulation, self-sufficient growth, evasion of

apoptosis, sustained angiogenesis, and invasion and metastasis (7). Various mutations in the cell genome, interactions with surrounding cells, viral infections, and environmental factors enable cancer cells to acquire each of these traits (7,8). Sometimes, one mutation can enable a tumor cell to acquire several of these traits at once, but often a number of mutations must occur for cells to progress through each stage. In addition, these steps can occur in any number of orders, leading to the immense variability in tumors, their rate of formation and dissemination, and the treatments that successfully inhibit cancer progression. Each of these different stages represents an area where new chemotherapies can be developed to reduce or prevent the formation of malignant tumors.

In order to gain a better understanding of the genomic changes that occur in breast cancer cells, various genomic microarray technologies have been developed. Numerous analyses, performed on both primary tumors and metastases, have demonstrated the existence of distinct gene expression patterns that are indicative of tumor biology and behavior (9,10). Recently, higher resolution techniques, such as array-based comparative genomic hybridization (aCGH), have been developed (10). aCGH is used to analyze tumor genomes in greater detail in order to confirm the existence of gene expression patterns among different tumor types (10). This technology has demonstrated that certain genetic alterations, such as the amplification of protein tyrosine kinases (PTKs) and human epidermal growth factor receptor 2 (HER-2), can be highly predictive of the response of a tumor to targeted therapies, leading to more effective treatment of tumors and the development of new therapeutic regimens (10). Currently, targeted therapies inhibit a number of different proteins and cellular processes, including HER-2, vascular endothelial growth factor (VEGF), Heat Shock Protein 90 (Hsp90), epithelial growth

factor receptor (EGFR, also called HER-1), c-Raf, B-Raf, MEK1-2, the endocrine system, the proteasome, and the Ras/Raf/ERK pathway (2,11). However, only a few of these have been shown to be effective in the Phase 3 Clinical Trials (11). Further research is being performed to identify other targeted therapies for breast cancer treatment.

Breast Cancer Treatment. The management of breast cancer is based on several factors, including the age of the patient, the stage of the cancer when diagnosed, and the dependency of the tumor on hormones such as estrogen. Treatment regimens commonly include breast conserving surgery or mastectomy and radiation, followed or replaced by systemic treatments, which include chemotherapy, targeted therapy, and hormone therapy (12,13). The primary chemotherapies used to treat early and locally advanced breast cancer are taxanes and anthracyclins. Both of these drugs inhibit cell growth by preventing cell division. Taxanes, such as paclitaxel and docetaxil, interfere with microtubules, preventing chromosome migration during mitosis. Anthracyclins, such as doxorubicin, interfere with DNA synthesis by intercalating between base pairs, inhibiting topoisomerase II, or creating oxidative damage (3). For estrogen-receptor-positive tumors, tamoxifen, an estrogen receptor antagonist, and ovarian ablation are the preferred treatments for pre-menopausal women. Inhibition of the estrogen receptor is considered to be the most important advance in targeted breast cancer therapy; the addition of tamoxifen to anti-cancer treatments reduced the cancer death rate by 9.2% over 15 years (2,12). In post-menopausal women, aromatase inhibitors, which are better tolerated than tamoxifen, are the preferred method of treatment. Aromatase inhibitors can only inhibit the conversion of the hormone androgen to estrogen, but cannot prevent the ovaries from

making estrogen, rendering aromatase inhibitors less effective in pre-menopausal women (12). Increasingly, chemotherapy treatments are used in combination with one or more other therapies, such as hormone blocking treatments or targeted therapies. The use of combinatorial therapies has contributed to the decreased rate of recurrence and increased life expectancy in breast cancer patients. There are increased risks of side effects when combining various treatments, such as increased cardiotoxicity, but in general, combinatorial treatments appear to be an efficacious way to target tumor growth and progression (2,3,14).

Treatments that specifically target overexpressed or mutated proteins have become an area of intense interest in breast cancer research and are beginning to show success in the clinic. HER-2, an orphan receptor that can form heterodimers with other members of the HER family, has been found to upregulated or amplified in approximately 20% of all invasive breast cancers. HER-2 upregulation can cause cell-cycle progression, proliferation, angiogenesis, and inhibition of apoptosis (2,15). Trastuzumab, a monoclonal antibody that binds to the extracellular domain of HER2, inhibits HER-2 function by blocking intracellular autophosphorylation. This targeted therapy has enjoyed a good deal of success and is now the standard of care for tumors that are HER-2 positive (14). Other targeted therapies include the VEGF-inhibiting antibody bevacizumab (Avastin®), tyrosine kinase inhibitors (TKIs) that inhibit EGFR (HER-1), geldanamycin-based Hsp90 inhibitors, proteasome inhibitors, c-Raf and B-Raf inhibitors, and inhibitors of the Ras/Raf/ERK pathway (2,11). However, it should be noted that bevacizumab, a VEGF-inhibiting drug which is used to reduce angiogenesis in tumors, has recently been shown to increase cancer metastasis resulting in increased

morbidity and mortality cancer patients treated with the drug than non-treated patients resulting in its use as a breast cancer inhibitor largely being discontinued (16-18). Bevacizumab aside, many of these inhibitors are also used to help reduce metastatic cancers and will be discussed in further detail below.

Breast Cancer Metastasis. The majority of breast cancer related deaths are caused by metastases and not the primary tumor itself. It has been found that 20-30% of breast cancer patients develop metastases (2). Once breast cancer spreads from the primary site it becomes incurable. Treatment is primarily palliative and focuses on prolonging progression-free survival and the reduction of disease symptoms (3). Even though there have been a number of advancements in cancer treatments, the average survival time of women with metastatic breast cancer is 18-24 months and only one-fifth of patients will be alive 5 years after the initial diagnosis of metastasis (3). The lack of curative treatments for metastatic breast cancer highlights the need for additional anti-metastatic drugs, particularly ones that can inhibit the growth and spread of metastatic tumors.

One of the first places that breast cancer cells disseminate to is the lymph nodes. Lymph fluid circulates through the breast tissue and any breast cancer cells that have detached from the primary tumor can use the circulating fluid as a mechanism to travel into the lymph nodes and establish secondary tumors (19). Although this process does not require migration and invasion to establish a tumor at the secondary site, lymph node status is still considered the best prognostic factor for predicting the survival of a breast cancer patient (20). Originally, it was thought that lymph node tumors were part of the chronology of breast cancer progression; with the formation of a primary breast tumor occurring first, the cancer cells forming secondary tumors in the lymph nodes second,

and finally, metastases occurring in distant sites (21). Even though this chronology has been shown to not be entirely accurate, lymph node status in cancer is still an important indicator of breast cancer aggressiveness. The identification of lymph-node involvement results in patients undergoing more aggressive treatment, including radiotherapy, excision of lymph nodes, and use of adjuvant therapies (20,22).

Once breast cancer has spread to distant sites it is most commonly found in the lung, bone, brain, and liver. It is not fully understood why breast cancer cells preferentially colonize these organs, but it has been hypothesized that these sites create protective niches for disseminated breast cancer cells, enabling then to establish metastases (8,23). The treatments for metastatic breast cancer are similar to those used in primary breast cancer therapy and include chemotherapy, hormonal manipulation, and targeted therapies. However, at this stage the focus of treatment shifts from eradicating the tumor to slowing tumor growth and progression (2). Targeted treatments, such as the HER-2 inhibitor trastuzumab, have become key in increasing the survival rates of patients with metastatic disease. As mentioned above, trastuzumab binds to the extracellular domain of HER-2 preventing its intracellular autophosphorylation (15). Trastuzumab has also been shown to have indirect anti-tumor activity by stimulating the immune system and activating natural killer cell-mediated cell lysis (24). It also has been shown to augment the activity of various chemotherapies. When used in combination with chemotherapies, trastuzumab has been shown to increase median patient survival (25) and when used as an adjuvant therapy, reduces the rate of tumor recurrence by half in women with HER-2 positive tumors (24). Even with its high specificity, trastuzumab has numerous side effects including flu-like symptoms and cardiotoxicity, which are

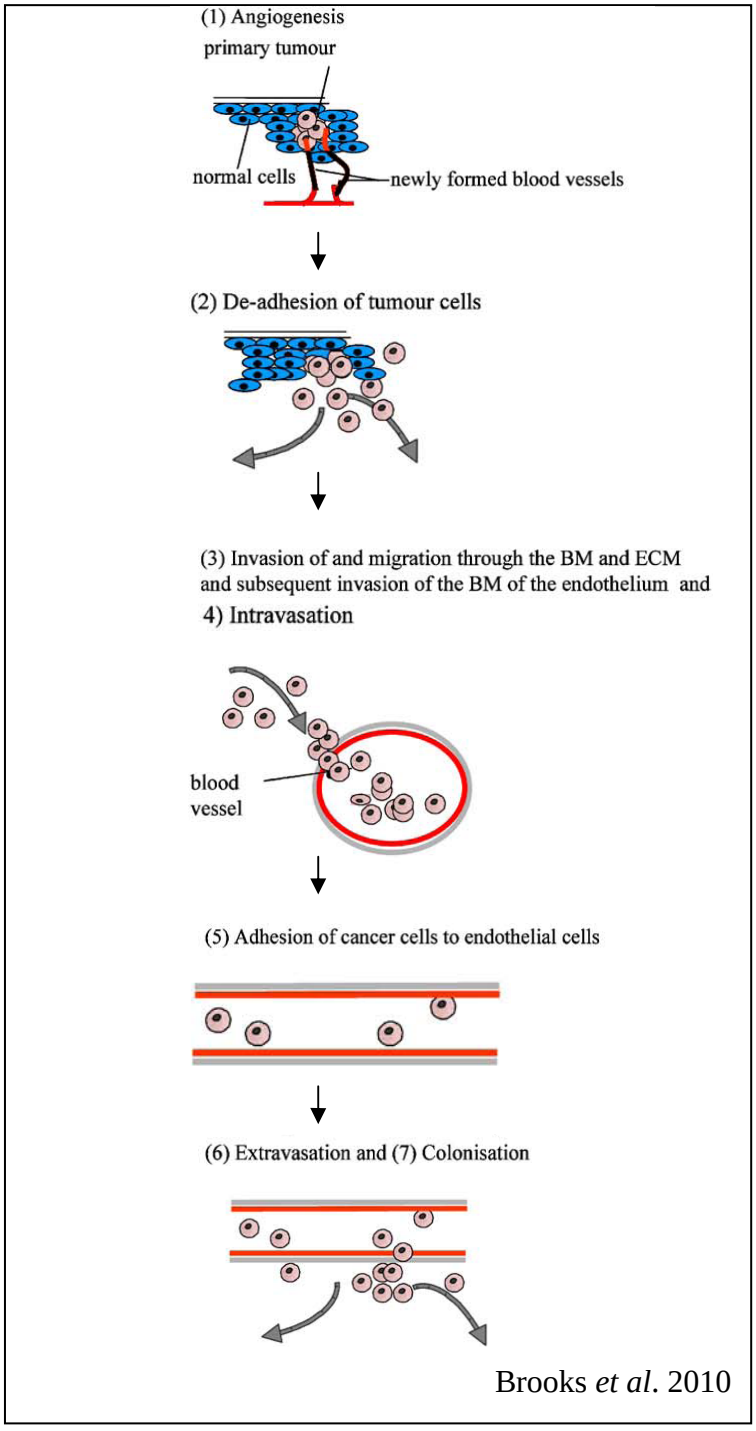
exacerbated when combined with other chemotherapies (24). Unfortunately, almost all tumors eventually develop resistance to trastuzumab, either through constitutive HER-2 autophosphorylation due to HER-1 activation or by a cleavage of HER-2 that leaves only the constitutively active intracellular domain (referred to as p95). However, once resistance has developed, tumors can be treated with small molecule HER-2 inhibitors such as lapatinib, which is a specific tyrosine kinase inhibitor (TKI) of both HER-1 and HER-2, and also can inactivate p95. Lapatinib functions by binding to the ATP binding pocket of the kinase (15,24). HER-1 (or EGFR) is itself amplified or upregulated in a large percentage of breast cancers and can also be inhibited by a number of TKIs including lapatinib, gefitinib, and erlotinib. One benefit to TKIs is their small size which, unlike the larger function-inhibiting antibodies, enables them to cross the blood-brain barrier making them useful in the treatment of brain metastases (14,15). Another targeted therapy is bevacizumab, which reduces cancer progression by inhibiting angiogenesis. Unfortunately this treatment has been shown to increase cancer cell invasion resulting in a reduction of overall survival (OS) in human trials (18). Therefore use of this treatment as a cancer therapeutic has been suspended. Even so, treatments that target various metastatic processes, such as trastuzumab, help delay the metastatic process and have greatly increased the survival time of patients with metastatic disease (2).

As mentioned above, another goal of metastatic treatments is the reduction of disease-related symptoms. This is accomplished through the use of palliative treatments, chosen based on the site of metastasis, to relieve specific symptoms (26). One such class of drugs is the bisphosphonates, which inhibit tumor-induced osteoclast-mediated bone resorption in bone metastasis and can relieve metastatic bone pain, preventing 40-50% of

expected skeletal morbidity in patients with bone involvement (26). While these drugs treat symptoms of metastatic breast cancer, they do not target the process of metastasis itself. In fact, there are no drugs that target metastasis and it is a long-term goal of this thesis to identify potential targets for such drugs. Below is a detailed description of the complex mechanisms that lead to metastasis.

Metastasis. Metastasis, the ability to migrate and invade, enables cancer cells to move away from the primary tumor and establish satellite colonies in other areas of the body (7). It is these secondary tumors that cause the majority of cancer deaths and not the primary tumors themselves (2). The high rate of mortality caused by metastasis makes the study of migration and invasion of crucial importance. The ability of cells to migrate away from the primary tumor and colonize other parts of the body is still not well understood (8). The process of metastasis can be broken down into seven steps. These include angiogenesis, escape from the primary tumor, invasion into the surrounding basement membrane, intravasation into blood vessels, adhesion to endothelial cells, extravasation from blood vessels, and finally, growth of a secondary tumor (8). (Figure 1.1) Each of these steps can be affected by factors such as genetic mutations or other changes in the genome that alter the expression of proteins necessary for these processes. These proteins include growth factors and signaling molecules, such as transforming growth factor beta (TGF-β), MAP kinases, and Wnt; adhesion molecules, such as selectins, integrins and immunoglobulins; and proteases, such as the matrix metalloproteinases (MMPs) and cathepsins (8). Often the same proteins are important in multiple steps. For example, proteases, such as the MMPs, are important in angiogenesis, invasion into the basement membrane, intravasation, and extravasation (27,28). It has

also become evident that the interaction between cancer cells and the surrounding stromal cells are vital to each of the



Brooks *et al*. 2010

Figure 1.1 The seven steps of metastasis. 1) The primary tumor recruits the formation of new blood vessels. 2) Tumor cells detach from the primary tumor. 3) Tumor cells invade through the basement membrane and extracellular matrix of surrounding cells. 4) Cells intravasate into circulation. 5) Cells attach to the endothelial cells of the circulation. 6) Cells extravasate from the circulation. 7) Cancer cells form a tumor colony at the new location. Figure from Brooks *et al*. 2010. (8)

steps leading to metastasis (29). Further study of the interactions, proteins, and signaling cascades that lead to metastasis is vital in increasing our understanding of cancer metastasis and in the development of successful anti-metastatic treatments. In this thesis I further explore one such mechanism, the extracellular Hsp90α-assisted activation of MMP-2, a process found to be important in cancer migration and invasion. The next section details how extracellular Hsp90α was identified to have a role in cancer invasion.

FALI-Based Extracellular Protein Screen

Currently, there is only a basic comprehension of the processes involved in the different steps of cancer metastasis. In order to develop successful anti-metastatic treatments, a more detailed understanding of these processes needs to be achieved. One way in which to do this is to study the cell proteome (the complement of proteins expressed by the cell). In order to take advantage of this method, the Jay Lab developed a functional proteomic screen to identify extracellular proteins that are important in cancer cell invasion, a key step in metastasis. The Jay Lab utilized a technique developed in-house called fluorophore-assisted light inactivation (FALI) to knockdown specific surface proteins and determine their importance in the invasion process (30). FALI is a high-throughput technology that uses singlet oxygen species generated by the activation

of the fluorophore derivative fluorescein to damage specific proteins (31). In this method, antibodies are labeled with fluorescein, incubated with their target proteins, and subjected to 490-nm wavelength light. When fluorescein is excited by 490-nm light it generates singlet oxygen species which inactivate any proteins within a 40Å radius (31). Fluorescein has a relatively high quantum yield of 0.92-0.93 indicating that the majority of the photons that are absorbed are emitted as fluorescence. This leaves a relatively small number of photons to undergo photochemistry, so even though a reduction in protein function was obtained with FALI using fluorescein, there are likely other fluorescent compounds with a lower quantum yield (i.e. a higher photochemical effect) that would provide better results (32). HT-1080 fibrosarcoma cells, which are a highly invasive cancer cell line, were chosen to detect proteins important in cancer invasion. In this screen, two libraries were analyzed: a mouse monoclonal antibody library that was raised against HT-1080 fibrosarcoma cells and a recombinant phage display antibody library that was prepared from spleen mRNA from mice immunized with HT-1080 cells (30). The antibodies of both libraries were labeled with a derivative of fluorescein called fluorescein isothiocyanate (FITC) and the FITC-labeled antibodies were incubated with HT-1080 fibrosarcoma cells. The cells were subjected to FALI and assayed for invasiveness. The FALI-labeled antibodies that caused a loss in invasion were then used to identify the target proteins through immunoprecipitation and mass spectrometry (30). These screens focused on surface proteins because of their accessibility to the FITC-labeled antibodies and because this subset of the proteome provides more easily druggable targets due to drugs not needing to be cell-permeant. One extracellular protein that was identified in both the monoclonal and the phage display library was heat shock

protein 90 alpha (Hsp90α) (30). This finding led to the investigation of extracellular Hsp90α and its role in cancer invasion (30). At this time, Hsp90α did not have a known role in cancer invasion, so Hsp90α was tested for interactions with proteins that have known roles in invasion. One such family of proteins is the matrix metalloproteinases (MMPs) (28). Eustace *et al.* found that Hsp90α co-immunoprecipitated with a member of the MMP family, MMP-2 (30). They demonstrated that inhibition of Hsp90α reduced the amount of activated MMP-2 outside of cancer cells. Also, inhibition of extracellular Hsp90α reduced cancer invasion and this effect could be reversed with the addition of activated MMP-2 (30). These results implicated Hsp90α in MMP-2 activation and led to the studies addressed in this thesis.

Hsp90

Background. Hsp90 is a highly conserved, abundant cytosolic protein. In eukaryotic cells it is present in two isoforms, Hsp90α, the inducible form, and Hsp90β, the constitutive form. There are also two paralogues in higher eukaryotes: Glucose-Regulated Protein 94 (GRP94), which is present in the endoplasmic reticulum and Tumor necrosis factor Receptor-Associated Protein 1 (TRAP1), which resides in the mitochondria (33). Hsp90 has been shown to be involved in the folding and stabilization of over 280 different proteins (hereafter referred to as "client proteins") of which many are important in cell growth and signaling (34). These client proteins include p53, annexin II, HER3, VEGFR1/2, and MAPK6. Hsp90 has been shown to assist in protein folding, signal transduction, inhibition of protein aggregation, refolding of denatured

proteins, facilitating degradation of proteins, assisting in the transportation of proteins, and innate and adaptive immune activity (35,36). Hsp90 interacts with its many client proteins with the assistance of over 20 additional chaperone proteins (co-chaperones) (37). Unlike other heat shock proteins, Hsp90 has not been found to recognize a specific structural motif in its client proteins, but it is thought that the large number of co-chaperones known to associate with Hsp90α assist in its client recognition (33). In addition, Hsp90 is a dynamic protein with intrinsic flexibility, giving it the ability to form various transient complexes and conformations which further assists in its association with its many client proteins (37).

Hsp90 is a homodimer with three domains (Figure 1.2). The N-terminal domain is where ATP, most Hsp90 inhibitors, and the co-chaperone p23, bind. The N-terminal domain is connected to the middle domain by a charged linker that has been implicated both in Hsp90 function and in contributing to the high degree of flexibility of Hsp90. The middle domain is important for client recognition and for the binding of some co-chaperones. Finally, the C-domain, where most of the co-chaperones and client proteins bind, is where Hsp90 dimerizes and the MEEVD pentapeptide domain is present (38,39). The MEEVD domain has been shown to be important in interdomain communication and peptide binding. This domain also functions as a tetratricopeptide (TPR) binding site by forming a carboxylate clamp with the positively charged residues of TPRs. TPRs, which are present on many of the Hsp90-associated co-chaperones, are composed of three tetratricopeptide repeats. TPRs are degenerate, 34-amino acid repeated motifs that form amphipathic α-helices that provide a binding site for target proteins. TPR domains have been shown to be important in mediating protein-protein interactions (38,40,41). The

three domains of Hsp90α, in conjunction with the linker domain, make Hsp90 a highly flexible molecular chaperone (33,37,39). (Figure 1.2)

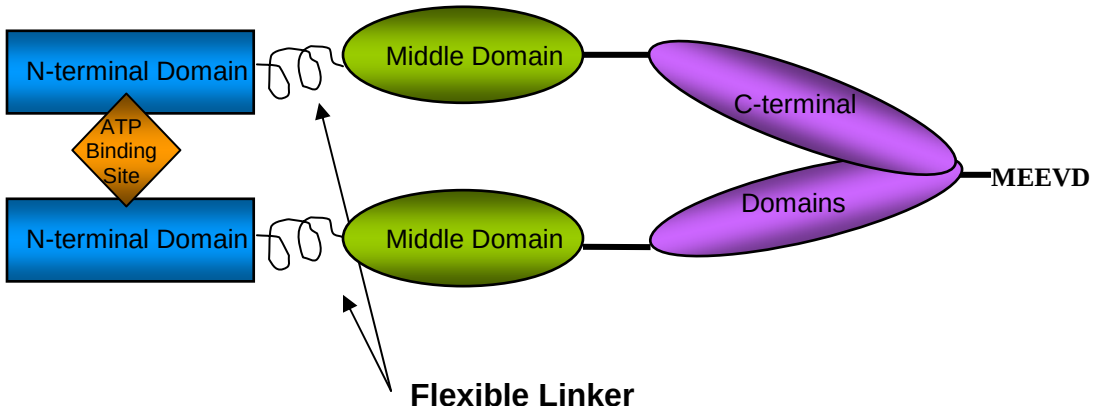


Figure 1.2 The domain structure of Hsp90. The N-terminus contains the ATP binding site and is the primary target of Hsp90 inhibitors. This domain is connected to the middle domain by a flexible linker domain. The middle domain is the binding site for some co-chaperones and is involved in the recruitment of client proteins. The client proteins bind to the C-terminal domain along with most of the co-chaperones. The C-terminus is where Hsp90 dimerizes and the MEEVD domain is located.

As mentioned above, intracellular Hsp90 has been found to function with a wide range of co-chaperone proteins. The most well-defined group, which includes Hop/Sti1, Cyp40, FKBP, and CHIP, contain the aforementioned TPR domain. Other co-chaperones include Hsp70, p23, Cdc37, Aha1, and Cpr6 (42-44). During client folding or activation, Hsp90 goes through a series of conformational changes (42). The most-well-studied of these conformational changes occurs during the activation of the progesterone and glucocorticoid steroid hormone receptors (SHR). The activation of the SHRs will be discussed in more detail in the following section. Briefly, the minimal components required for this process are Hsp70 and Hsp40 to recruit the SHR, Hop to enable complex

formation, and ATP to induce Hsp90 conformation change, enabling the binding of p23 (Figure 1.3). In this activation complex, ATP is hydrolyzed to ADP enabling the folding of the steroid hormone receptor, which is then released from the complex (45,46).

The role of ATP in intracellular Hsp90 function has been hotly contested, but in general it is agreed that ATP is needed for most intracellular Hsp90 functions (46,47). ATP generally binds to Hsp90α upon the formation of an intermediate complex, which usually involves Hsp70, Hop, Hsp40, and the client protein. Upon ATP binding, Hsp90α undergoes a conformational change enabling the binding of p23. When ATP binds to Hsp90α a lid-like segment of the protein folds over the bound ATP, locking the protein in an active ATPase conformation (46). ATP is hydrolyzed to ADP, enabling client protein folding, and then the ADP molecule is then released from the Hsp90 complex. Although it has been demonstrated that Hsp90 is able to access all of the necessary conformations for its chaperoning function without the presence of ATP, it appears that ATP may greatly increase the efficiency of the chaperoning function of Hsp90α (47,48). The ability of Hsp90α to cycle through all of its conformation changes without ATP raises the possibility of Hsp90α functioning without ATP both intracellularly and extracellularly. In this thesis I describe an ATP-independent function of extracellular Hsp90α.

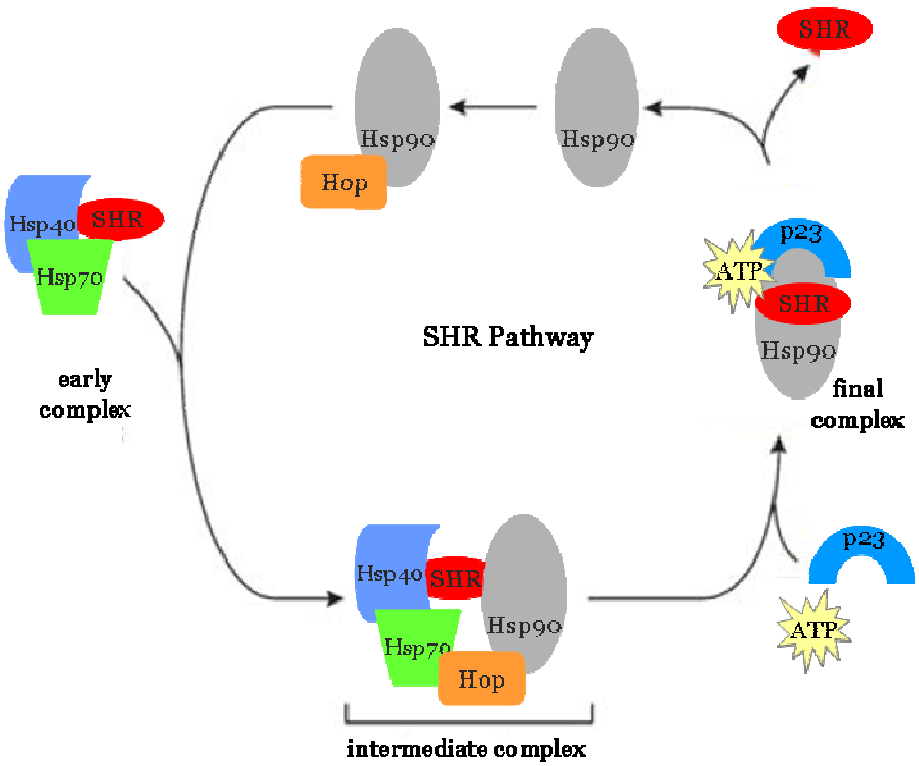


Figure 1.3. Steroid hormone activation pathway. The progression of the Hsp90α-mediated activation pathway for steroid hormone receptors. Adapted from papers from the Toft laboratory (45,49-51).

Hsp90α, the inducible form of Hsp90, is primarily transcriptionally regulated by heat shock factor 1 (HSF1), a transcription factor that is normally bound with Hsp90 and Hsp70 during non-stress situations. During cell stress, HSF1 is released from the heat shock proteins, allowing it to trimerize, enter the nucleus, and bind to heat shock elements (HSEs) in the promoter regions of heat shock protein genes as well as in a large number of non-heat shock protein genes (52). Hsp90α transcription has also been shown to be regulated by Nf-κB, NF-IL6β, STAT1, and STAT3 during immune responses (37). In addition, Hsp90α is post-transcriptionally regulated by its numerous co-chaperones, client proteins, and by various post-transcriptional modifications such as phosphorylation, acetylation, and ubiquitination (37,53). The important role Hsp90α has

in maintaining cellular homeostasis during cellular stress makes it an important player in cancer survival. Hsp90α is upregulated in many cancers and has been found to promote cell survival, growth, and metastasis. In addition, Hsp90α assists in the survival of cancer cells in what would otherwise be an intolerable environment due to hypoxia, a lack of growth factors, and cellular mutations (43,54). Obtaining a better understanding of Hsp90α regulation and function will enable the identification of more effective inhibitors and improve cancer treatments.

Extracellular Hsp90α. As noted previously, Hsp90α has a number of intracellular roles in both normal and oncogenic cells that have been extensively studied, but its extracellular role is not well known. Hsp90α has also been found to be present outside of many cell types and its extracellular roles continue to be elucidated. Hsp90α was first identified outside of fibrosarcoma cells as a tumor-specific antigen that was only recently definitively identified as Hsp90α (55). The Jay Lab identified Hsp90α outside of fibrosarcoma cells in 2004 and demonstrated that its inhibition could reduce *in vitro* cancer cell invasion (30). They also showed that inhibition of Hsp90α reduced MMP-2 activation and addition of activated MMP-2 to cancer cells rescued the effect rendered by inhibiting Hsp90α. These results strongly implicated a relationship between extracellular Hsp90α and MMP-2 activation in cancer invasion (30). Since that initial identification, Hsp90α has been shown to have a number of other roles in cancer invasion. Sidera *et al*. demonstrated that Hsp90α affects cancer cell invasion by increasing heregulin-induced activation of extracellular HER-2 (56). Chen *et al*. showed that Hsp90α increased invasion by inducing the activity of the ERK, PI3K/Akt, and NF-κB pathways and by binding to CD91 and Neu (57). In 2004, Lei *et al*. demonstrated that Hsp90α was also

present in rat endothelial cells (58). Since then, Hsp90α has been detected outside of neurons, dermal fibroblasts, macrophages, epithelial cells, vascular smooth muscle cells, and numerous cancer cells (59,60) Extracellular Hsp90α has been shown to participate in neuronal migration, wound healing, and viral and bacterial infections, demonstrating that it has a number of roles in non-cancerous tissues (59,60). Even with its various roles in normal tissues, the possibility of specifically targeting extracellular Hsp90α to reduce cancer metastasis is being explored. One additional goal of inhibiting extracellular Hsp90α is the reduction of side effects normally associated with intracellular Hsp90α inhibition, including the release of the HSF1 transcription factor and the resultant upregulation of Hsp90 and Hsp70. However, due to the identified roles of extracellular Hsp90α in normal cells, additional side effects, including immune suppression and reduced wound healing and tissue repair may be observed.

When I initiated this thesis it was not clear how Hsp90α got outside of the cell. Various modes of export for Hsp90α have been proposed. In 2004, Dr. Didier Picard noted the existence of two Hsp90α isoforms. One of these isoforms contained two extra exons at the N-terminus that included an alternate start site and a putative signal sequence, suggesting it could be responsible for the isoform-specific export of Hsp90α from the cell (61). If this were so, it would be possible to develop an isoform-specific inhibitor that would target the extra exons and inhibit only the extracellular form of Hsp90α.

Alternatively, Hsp90α could be exported via other non-canonical pathways. These include lysosomal secretion, export mediated by plasma membrane-resident transporters, export through the release of exosomes from multivesicular bodies, and

export by exovesicles via membrane blebbing (62). It has been demonstrated in a number of other cell lines, including immune and cancer cells, that Hsp90α and a number of its co-chaperones are present in exosomes, making exosomes a candidate for the export of Hsp90α in breast cancer cells (63,64). Wang *et al*. demonstrated that Hsp90α secretion is regulated by both the C-terminal MEEVD domain and the phosphorylation status of Thr-90, suggesting that even though Hsp90α is exported by a non-canonical pathway, it is still highly regulated (65). Further elucidation of the mechanism and regulation of Hsp90α secretion from cancer cells is key in understanding the role of Hsp90α in cancer metastasis and in developing treatments to reduce metastasis. In McCready *et al*., 2010, (appendix) we demonstrated that Hsp90α is exported via exosomes in breast cancer and glioblastoma cells and that these exosomes contribute to the motile phenotype of the cancer cells (66).

Hsp90 Inhibitors. The important role Hsp90 plays in cancer progression makes it an ideal candidate for the development of targeted anti-cancer therapeutics. The first Hsp90 inhibitor, geldanamycin, was initially identified as an anti-bacterial compound. This compound was subsequently determined to have anti-tumor properties by Kaken Chemicals in 1980 (67). Later, another natural compound, radicocol, was identified to also have Hsp90 function-inhibiting properties. Geldanamycin and radicocol act as nucleotide mimetics and bind with high affinity to the ATP-binding site on the N-terminus of Hsp90 (Figure 1.4). The binding of these drugs to the ATP-site prevents Hsp90 from cycling between its ATP- and ADP-bound conformations and also increases the recruitment of E3 ubiquitin ligases resulting in an increase in the degradation of Hsp90 and its client proteins (54). Currently, there are 13 known Hsp90 inhibitors in

clinical trials and the majority of them are based on one of these two natural compounds (34,37). The two most studied inhibitors are 17-AAG, the first analogue prototype of geldanamycin, and 17-DMAG, a more soluble and bioavailable derivative of geldanamycin (68). Even though there has been encouraging results and considerable progress in the clinical trials for Hsp90 inhibitors, there are currently no approved drugs targeting Hsp90 (34).

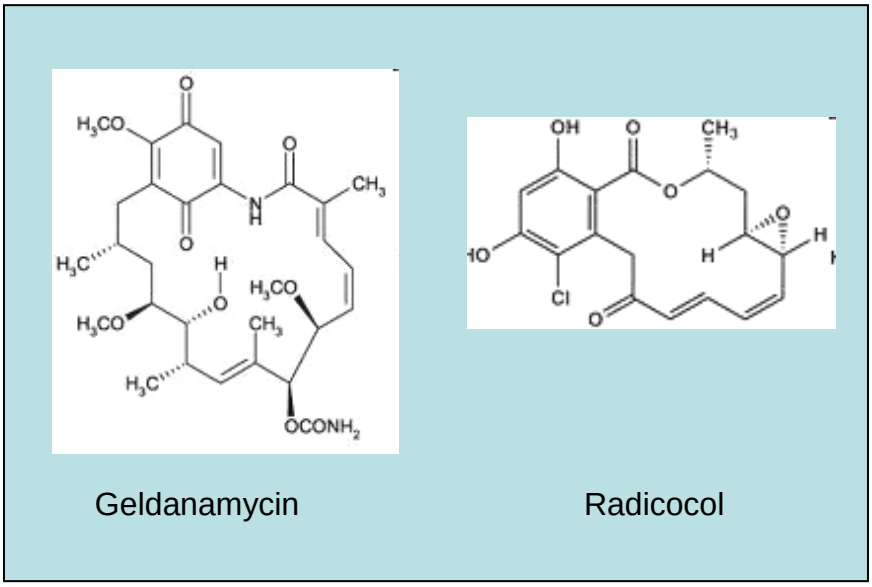


Figure 1.4 Structures of geldanamycin and radicocol. Each compound contains a ring-structure similar to ATP allowing it to tightly bind in the ATP binding site of Hsp90. Adapted from Soga *et al*. 1998 and geldanamycin.info. (69)

Unfortunately, Hsp90-inhibiting drugs have been plagued by unwanted side effects. Geldanamycin, which has significant anti-tumor properties *in vitro*, ended up being highly toxic when tested in animals and therefore was unable to proceed to human trials. 17-AAG, a derivative of Geldanamycin was found to have a much lower toxicity profile, but was still shown to cause liver toxicity, nausea, and fatigue in some patients

(70,71). These side effects have been attributed to multiple non-specific effects of the Hsp90α-inhibitors. On a molecular level, one of the side effects of inhibiting Hsp90 is the upregulation of Hsp70 and other heat shock proteins, which causes a reduction in the effectiveness of Hsp90 inhibitors. This is due to Hsp90 inhibition causing activation of the heat shock response which results in the release of heat shock factor 1 (HSF1), the previously mentioned transcription factor that promotes the upregulation of heat shock genes (35). Hence, the inhibition of Hsp90 leads to an increase in cell stress, the release of HSF1, the upregulation of heat shock genes, and the increase of Hsp70 and other stress-related proteins. Our findings that extracellular Hsp90α acts in MMP-2 activation and cancer migration and invasion suggest that specifically blocking extracellular Hsp90α may be useful to reduce invasiveness without the side effects of intracellular Hsp90α inhibition. In addressing this mechanism of extracellular Hsp90α function, we speculated that other co-chaperones, such as Hsp70, may be involved. In chapter 3 of this thesis, I demonstrate a role for Hsp70 in the Hsp90α-assisted activation of MMP-2 and in breast cancer migration and invasion. A description of Hsp70 follows.

Hsp70. Heat Shock Protein 70 (Hsp70) is one of the key co-chaperones of the Hsp90α activation complex. The Hsp70 family consists of eight different members, of which Hsc70, the constitutive form, and Hsp72, the stress-induced form, are the most common cytosolic isoforms (72). Structurally, Hsp70 consists of three domains: the highly conserved N-terminal nucleotide binding domain, the protein peptide substrate-binding domain, and the less conserved C-terminal domain, which contains an EEVD sequence that, similar to Hsp90, allows for interaction with TPR-containing co-chaperones such as Hop and Cpr6 (72,73). Intracellular Hsp70 is dependent on ATP for its function and its

hydrolysis of ATP is regulated by several co-chaperone proteins including Hsp40, Bag1, and Hop. Hsp70 participates in a number of activities in the cell including protein folding, aggregation prevention, membrane translocation of client proteins, and the control of regulatory protein activity (72,74). Hsp70 upregulation can result in an increase in cell growth and an increase in resistance to apoptosis, making it a key player in many cancers. Elevated Hsp70 levels have been observed in a number of tumors, and these increased levels are usually correlated with poor patient prognosis (75,76). Hsp70 inhibitors, which are discussed below, could significantly improve the prognosis of patients with tumors expressing high levels of Hsp70.

Extracellular Hsp70. Like Hsp90α, Hsp70 has been identified outside of a number of different cell types including glial cells, immune cells, and cancer cells (77,78). In immune cells, it is able to initiate both innate and adaptive immune responses and has been shown to function by presenting antigenic peptides to immune effector cells (79,80). The role of extracellular Hsp70 in immune cells is well documented, and a number of surface receptors on immune cells, including the scavenger receptors LOX-1 and CD-94, have been shown to bind Hsp70, enabling them to induce a pro-inflammatory response (80). Additionally, it has been demonstrated that endothelial and epithelial cells have Hsp70 receptors, further supporting a role for extracellular Hsp70 in non-immune cells (80). Hsp70 has been shown to be exported via exosomes in immune cells and in breast cancer and glioblastoma cells (66,78). The mechanisms that regulate its secretion in breast cancer cells are currently unknown but its presence in the extracellular media of cancer cells makes it a candidate to interact with Hsp90α and assist in enhancing breast

cancer invasion. The role Hsp70 plays in cancer progression also makes it a good target for anti-metastatic therapies, which are discussed next.

Hsp70 Inhibitors. Even though Hsp70 inhibitors have become an area of intense research, few successful inhibitors for Hsp70 have been identified (72). Hsp70-inhibiting compounds that have been identified include an immunosuppressive agent 15-deoxyspergualin, a large, lipophilic compound known as apoptozole, and a small molecule called pifthrin-μ (72). These compounds are only just beginning to be characterized and currently, none of them have entered clinical trials (72). There are, however, a number of other compounds that are found to affect Hsp70 expression or function in a non-specific manner. Aromatase inhibitors have been shown to decrease Hsp70 activity along with inhibiting estrogen production (81). Actinomycin D, an inhibitor of DNA-dependent RNA synthesis, has been shown to reduce the induction of Hsp70 normally seen with Hsp90 inhibitors (82). Methylene Blue, a DNA and RNA stain, has been shown to inhibit Hsp70, but it also is a selective inhibitor of guanylate cyclase, which affects the cGMP signaling pathway (83,84). Methylene blue also directly inhibits both constitutive and inducible forms of nitric oxide synthase (NOS) by oxidation of the ferrous iron bound to the enzyme (85,86). Due to these off-target effects, Methylene Blue is only useful for *in vitro* studies of Hsp70. Further development of Hsp70-inhibiting compounds would be highly beneficial for anti-cancer therapies, particularly in combinatorial treatments.

Other Co-Chaperones. Hsp90 functions with the assistance of a number of different co-chaperones. The four co-chaperones that are thought to compose the core of most

Hsp90-activation complexes are Hsp70, Hop, Hsp40, and p23 (45,51). Some additional co-chaperones include Aha1, Cdc37, CHIP, and Hsp60. Hsp70, which was discussed in detail above, is crucial to Hsp90 activity and participates in many other chaperoning activities apart from Hsp90. Hop was first identified as a linker protein for Hsp90 and Hsp70, but it has since been found to be important for modulating the activities of these two proteins as well. Hop contains nine tetratricopeptide (TPR) repeats enabling it to bind to a wide variety of proteins, likely assisting Hsp90 and Hsp70 in binding to their many client proteins (87). Hop has also been shown to be involved in other Hsp90-independent processes, including functioning as a receptor for prion proteins (88,89).

Hsp40, or DnaJ, proteins are a family of protein orthologs that can be separated into three classes based on structural differences characterized by the presence of a Gly/Phe-rich region and cysteine repeats. All Hsp40 proteins contain a "J" domain, which is a conserved 70-amino acid sequence that assists Hsp40 in binding to Hsp70 and in regulating the ATPase activity of Hsp70. Hsp40 has been shown to be important in protein folding and unfolding, translation, translocation, and degradation (90,91). Hsp40 functions primarily by regulating the ATPase activity of other chaperone proteins, particularly Hsp70 (91). There have been multiple reports that implicate Hsp40 in cancer progression, but interestingly, the majority of them show that Hsp40 primarily reduces cancer growth, raising the question as to how a protein that reduces cancer growth is essential to the function of Hsp70 and Hsp90, two proteins that have a well-documented role in cancer progression (91-93).

P23 has primarily been studied in respect to Hsp90. It interacts with a variety of Hsp90 co-chaperones and modulates Hsp90 activity. With the assistance of Hsp90, it has

been found to regulate estrogen receptor signal transduction and prostaglandin synthesis (94,95). There is also evidence that p23 may have functions entirely separate from Hsp90 (95). In addition, each of these co-chaperones has been demonstrated to be present extracellularly by other investigators. Extracellular Hop has been reported outside of A172 glioblastoma cells where it increases cell proliferation by inducing signaling through cell surface prion proteins and the ERK pathway (96). Hsp40 has been found to associate with Hsp70 to reduce radiation-induced effects in pancreatic and colon carcinoma cell lines (97). Lastly, p23 was identified outside of melanoma cells in a screen to identify structures at the surface of cancer cells (98). The functions of these proteins have been extensively studied as part of the Hsp90α-activation complex, which is described below.

Mechanism of Hsp90α-mediated activation of client proteins. As mentioned previously, the Hsp90-co-chaperone mediated activation of the progesterone or glucocorticoid hormone receptors is the most-studied Hsp90-client protein interaction. Toft and colleagues have identified the co-chaperones required in each step of the *in vitro* activation of these two proteins (Figure 1.3). The progesterone receptor first binds to Hsp40, while the glucocorticoid receptor first binds to Hsp70 (45,99). Hsp70 then binds to Hsp40 in an ATP-dependent fashion, assisted by the "J" domain of Hsp40. The J domain stimulates the ATPase activity of Hsp70, which results in a tight association of Hsp70 and the client protein (100). Hop, with the assistance of its many TPR domains, binds simultaneously to Hsp70 and Hsp90, creating an intermediate complex (101). Once this complex has formed, ATP binds to the N-terminal domain of Hsp90, enabling p23 to bind to the N-terminus, promoting the dissociation of Hsp40, Hsp70, and Hop

from the complex (46). This new complex then allows the client protein to transition to an active conformation that is able to bind hormones. As demonstrated by the difference between these two receptors, Hsp90 and its co-chaperones interact differently with every client protein. Each client likely requires its own complement of co-chaperones that participate in a unique fashion in the activation of that client protein. The large number of associated co-chaperones, along with the inherent flexibility of the protein, contributes to Hsp90's large and varied number of client proteins. In order to elucidate how Hsp90 interacts with all of these different clients, more research is required. Chapter 3 of this thesis focuses on the interaction of Hsp90α with Matrix Metalloproteinase-2. A description of Matrix Metalloproteinases follows.

Matrix Metalloproteinases

Overview. In humans, the matrix metalloproteinase (MMP) family includes at least 25 different members (102). While most are either membrane bound or present in the extracellular milieu, some MMPs have been identified inside the cell, including during the beginning stages of apoptosis (102,103). Taken together, the members of the entire MMP family are able to degrade nearly all of the components of the extracellular matrix. MMPs are zinc-dependent endopeptidases that, at a minimum, consist of the following three conserved domains: the pre-domain that contains a signal sequence that directs the export of the protein from the cell; the pro-domain that keeps the protein in an inactive form; and the zinc-containing domain that includes three conserved histidines that ligate the active zinc to the protein. In addition, most MMPs include a fourth domain that

assists in substrate recognition (Figure 1.5) (104-106). MMPs also contain a non-active zinc, which is thought to help maintain the tertiary structure and active sites of the protein (107). Most MMPs contain two Ca^{2+} ions that have been shown to be important in protein stabilization in MMP-1 (108) and in mediating signal transduction in MMP-2 (109,110).

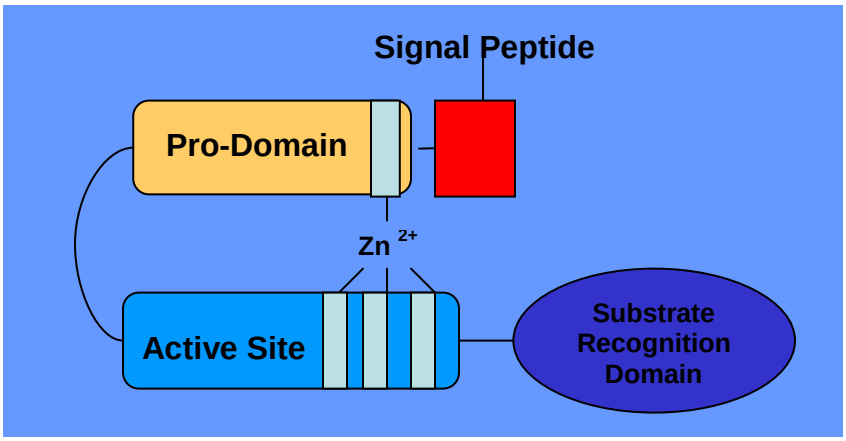


Figure 1.5. General structure of matrix metalloproteinases.

MMP Regulation. Due to its many extracellular roles, MMP function is carefully controlled through multiple forms of regulation. The transcriptional regulation of MMPs varies by cell type, but TNF-α and IL-1 are commonly found to upregulate transcription, while TGF-β and retinoids down regulate transcription (28). Cells carefully control when and where they release MMPs into the extracellular environment. One way this is accomplished is by anchoring MMPs to various membrane proteins such as αvβ3 integrin and CD44, enabling efficient modulation of MMP concentrations when it is needed (104). In yet another mechanism of control, MMPs are translated as inactive proteins, or zymogens, and must therefore be activated in order to function enzymatically. For

activation to occur, the interaction between a conserved thiol of a cysteine residue in the pro-domain and the zinc ion of the catalytic site must be perturbed. There are three primary mechanisms for this activation. The first is direct cleavage of the pro-domain, which is usually performed by either Furin or a Membrane Type-1 (MT1)-MMP/Tissue Inhibitor of Metalloproteinases (TIMP) complex. Second is by reduction of the free thiol on the pro-domain. Third is by allosteric disruption of the zymogen, which is a potential mechanism for Hsp90α to activate MMP-2 (28,104). Finally, MMPs are regulated through enzyme inactivation and degradation (104,106). The primary function of MMPs was initially believed to be the degradation of various components of the extracellular matrix. But additional research has shown that this is actually only a small part of the many functions MMPs perform. They have been found to act on cytokines, chemokines, apoptotic ligands, angiogenic factors, and more (28,104). These new functions have implicated MMPs in the modulation of cell growth, motility, and migration. A few MMPs are present constitutively in cells, but most are only upregulated in certain circumstances, such as during reproductive processes, tissue repair, and remodeling (28).

MMP activity is traditionally assayed using a technique called zymography. In this method, an SDS-PAGE gel that has been co-polymerized with a proteinase substrate, such as gelatin, is used to electrophoretically run a sample containing the MMP of interest. Any active MMPs will degrade the substrate in the gel. The gel is than stained with Coomassie Brilliant Blue, destained, and the areas where the substrate has been degraded appear as white bands. However, bands appear in the zymograms that represent both the pro- and active form of the MMP being assayed. This result is due to the reducing environment of the SDS-PAGE exposing the active site of pro-MMPs, resulting

in enzymatic activity without cleavage of the pro-domain. This enables the pro-MMP to degrade the substrate, resulting in the appearance of a band at the molecular weight of the pro-molecule (111). In addition to zymography, there are other methods that are used to assay the presence of pro and/or active MMPs. These include gelatinase and FITC-casein assays and SDS-PAGE followed by western blotting with antibodies directed toward the pro and active forms of MMPs (112).

MMPs in Cancer. The role of MMPs in cancer has been known for over 30 years (113). Since this initial identification in 1979, MMPs have been shown to be upregulated in many cancers, to be an indicator of poor prognosis, and to be a sign of metastasis (28). MMPs play multiple roles in cancer progression. Their ability to degrade the extracellular matrix enables cancer cells to break down barriers and invade other tissues leading to the formation of metastases (114). MMPs can also assist cancer cells by releasing various growth-promoting factors from the extracellular environment that can inhibit apoptosis and recruit the growth of new blood vessels to the tumor (105). Due to their crucial role in cancer progression, MMP inhibitors have been an area of intense research. *In vitro* inhibition of MMPs led to a notable decrease in cancer progression, prompting the development of several synthetic metalloproteinase inhibitors. These compounds were tested in *in vivo* models and found to significantly reduce cancer progression and metastasis (115). Two of these, Batimastat and Marmistat, quickly moved on to clinical trials, but for unknown reasons, performed poorly in human trials. One hypothesis for this poor performance is that the inhibitors were not specific enough and targeted not only MMPs but other related proteins such as ADAMs, causing severe side effects (28,104,115). Inhibitors that target specific MMPs are currently under

investigation and, if validated, could enhance current anti-metastatic treatments. Our studies focus on MMP-2 and our understanding of this protease is summarized next.

MMP-2. It has been recently demonstrated that increased levels of MMP-2 is an indicator of breast cancer metastasis, making MMP-2 a particularly relevant target for breast cancer therapeutics (116). Along with MMP-9, MMP-2 is a member of the gelatinase family of MMPs. Gelatinases perform the final cleavage of fibrillar collagens after the initial cleavage by collagenases, which has led to the hypothesis that they play a key role in cancer invasion (106). It has also been demonstrated that MMP-2 can break down adhesion molecules such as E-cadherin and CD166 leading to an increase in metastasis. Similar to the other MMPs, MMP-2 must be activated in order to perform its functions. The most-well-studied mode of MMP-2 activation is via an MT1-MMP and TIMP-2 complex that binds and cleaves the pro-domain of MMP-2 (117). MMP-2 has also been observed to be activated independently of MT1-MMP or TIMP-2, indicating that other forms of activation exist (118). In this thesis, we describe another mechanism of MMP-2 activation involving extracellular Hsp90α and a co-chaperone complex. One way to test potential Hsp90α inhibitors and their ability to reduce metastasis is through a number of mouse models, which are discussed next.

Mouse Models of Metastasis

Before a promising compound can enter clinical trials, it is usually tested in an *in vivo* model. The process of metastasis is difficult to simulate in an animal model, nonetheless a number of animal models of cancer metastasis have been developed. The

majority of these models are either xenograft-based or involve a genetically engineered strain of mice (119). The xenograft-based models use a number of different mouse or human cancer cell lines to produce the tumors. In breast cancer models the majority of metastases form in the lymph nodes or lungs, but some metastases also develop in the bone, brain, and liver, the three other common locations of breast cancer metastasis in humans (120). In order to more closely recapitulate what is occurring in human tumors many of these models involve human cancer cells and, if possible, transplanted human tissue on which the primary tumors cells can form metastases. An example of such a model was developed by Dr. Charlotte Kuperwasser and colleagues (119). In this model, NOD-SCID mice are implanted with a human bone core obtained from hip replacement surgery. Over several months, the bone is allowed to engraft into the mouse's circulation. Once the bone core is established, fluorescently labeled human breast cancer cells are injected into the mammary fat pad of the mouse where they develop a primary tumor. The tumor is allowed to grow and metastasize to other areas of the mouse. The human bone core is then examined for metastases by imaging the mouse in a live animal imager and looking for the presence of fluorescent human breast cancer cells (Figure 1.6) (119). Various inhibitors can be tested in this model to determine their ability to reduce or prevent cancer metastasis.

The benefit of this model is its ability to closely recapitulate the metastatic process that occurs when a primary human tumor metastasizes to bone, albeit the whole process is taking place in a mouse. This model provides a novel way to test the efficacy of anti-metastatic drugs. In addition, the effectiveness of the inhibitors and the progression of the primary tumor and metastases can be monitored *in vivo* using live

animal imaging technology. Once the mouse has been sacrificed, it can be further examined for metastases and micro-metastases to determine the sites of cancer metastasis and if there is any pattern to the location of these tumors. The data can then be examined to determine if the amount or size of metastases are reduced by various drug treatments (119). Cancer metastasis poses the biggest challenge and greatest opportunity in cancer research. As mentioned previously, currently, we only have palliative treatments for metastatic cancer and the need for further research into cancer metastasis in order to develop better treatments is of utmost importance.

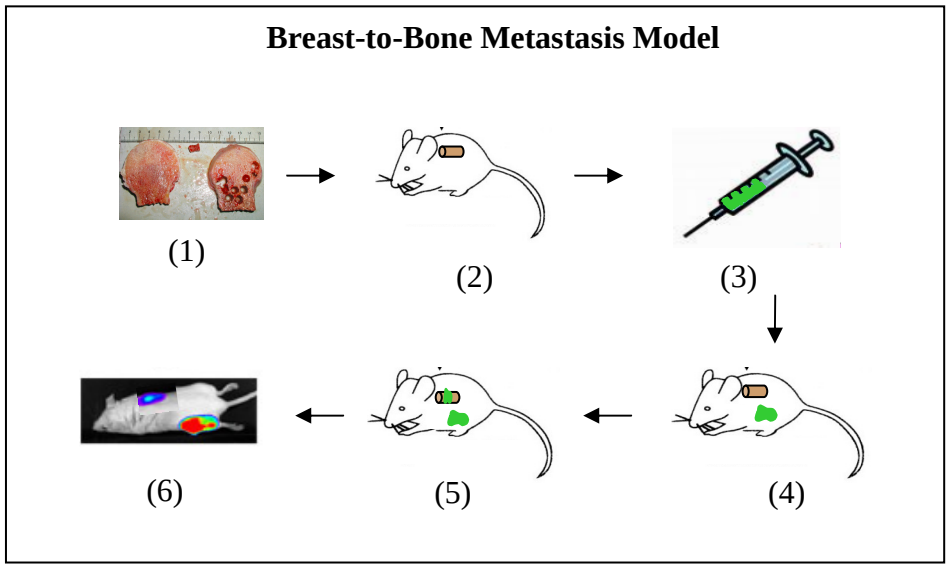


Figure 1.6 Breast-to-bone metastasis model. (1) Human bone cores are obtained from hip replacements surgery. (2) Mice are implanted with human bone cores. (3) Human breast cancer cells are labeled with GFP and luciferase. (4) Mice are injected with the labeled breast cancer cells in the fourth mammary fat pad three months later. (5) Primary and metastatic tumors form. (6) Tumors are imaged using a Xenogen live animal imager.

Contributions of this thesis

In this thesis, I addressed the role of extracellular Hsp90α and Hsp70 in MMP-2 activation and their role in breast cancer cell migration and invasion. I investigated the mode of export of Hsp90α from breast cancer cells and demonstrated that Hsp90α assists in MMP-2 activation in a co-chaperone-mediated fashion (chapter 2). I showed that inhibition of one of the co-chaperones, Hsp70, decreased MMP-2 activation and cancer cell migration and invasion (chapter 3). I also tested a function-blocking Hsp90α antibody as a potential cell impermeable anti-metastatic drug in *in vitro* invasion assays and in preliminary *in vivo* experiments using a murine breast-to-bone metastasis model (chapter 3 and appendix).

After the identification of Hsp90α outside of fibrosarcoma cells by Eustace *et al.* (30), it was noted that two isoforms of Hsp90α exist and that one contains two additional N-terminal exons (61). I hypothesized that these extra exons, which contain a putative canonical signal sequence, targeted the isoform outside of the cell (61) and tested this theory in chapter 2. In this thesis I demonstrated that Hsp90α was not exported by classical secretion mechanisms. I determined that an additional isoform did exist, but that it was not preferentially exported outside of the cell. Instead, together with Dr. Jessica McCready, I showed that Hsp90α was exported from cells via exosomes. We also demonstrated the existence of other client proteins for extracellular Hsp90α, including several pro-proteins. One of these pro-proteins was plasminogen, whose Hsp90α-mediated activation to plasmin plays a role in the migration of several cancer types including breast cancer (66).

In chapter 3, we confirmed the findings by Eustace *et al.* that three co-chaperones, Hop, Hsp40, and p23 were present outside of the cell (121) and demonstrated that one additional co-chaperone, Hsp70, is also outside of the cell. We demonstrated that these co-chaperones interact with Hsp90α and MMP-2 both *in vitro* and in conditioned medium collected from breast cancer cells and that the presence of these co-chaperones increases the interaction of Hsp90α and MMP-2. We found that Hsp90α and these four co-chaperones were sufficient to stimulate activation of MMP-2 *in vitro* and that this activation was found to be ATP independent. In addition, the conditioned media, which contains all of the chaperone proteins listed above, was able to activate exogenously added MMP-2. Also, the inhibition of Hsp70, one of the identified co-chaperones, significantly reduced this activation. The questions that remain to be discussed are whether there are other components present outside of the cell that enhance MMP-2 activation and how extracellular Hsp90α and Hsp70 function without ATP. In this chapter, I also tested the role of Hsp90α-mediated activation of MMP-2 in breast cancer migration and invasion. Using wound healing assays, I demonstrated that inhibition or depletion of Hsp90α or Hsp70 decreased breast cancer cell migration. I also showed that Hsp90α or Hsp70 inhibition caused a significant decrease in breast cancer invasion.

In the appendix, I tested an Hsp90α function-inhibiting antibody identified by Li *et al.*, and demonstrated that it reduced cancer migration and invasion (122). I also used a mouse breast-to-bone invasion assay developed by Dr. Charlotte Kuperwasser to test the above antibody to determine if it would cause a reduction in cancer cell metastasis *in vivo*. Due to low sample numbers, I was unable to draw any conclusions from these experiments. It has yet to be determined if extracellular Hsp90α inhibitors are a viable

treatment option for reducing cancer metastasis and if intracellular Hsp90α levels change when extracellular Hsp90α is inhibited.

CHAPTER 2

HSP90α IS EXPORTED VIA EXOSOMES AND NOT IN A SPLICE VARIANT SPECIFIC MANNER

(J. Sims contributed Figure 1, 5, 6, and 7 and J. McCready contributed figures 2, 3, 4, 6, and 7)

ABSTRACT

Extracellular Hsp90α was identified in fibrosarcoma cells by Eustace *et al*. in 2004, demonstrating a new role for Hsp90α in cancer invasion. It is not known how Hsp90α is exported from cancer cells. Hsp90α does not contain a signal sequence, however, the existence of an additional Hsp90α splice variant that contains two extra N-terminal exons with an alternate start site and a putative signal sequence has been demonstrated. In 2004, Picard hypothesized that this splice variant was responsible for the export of Hsp90α through its putative signal sequence. In this chapter, with the assistance of Dr. Jessica McCready, I verified that both of the splice variants are present in breast cancer cells, but showed that Hsp90α was not exported in an isoform-specific fashion. We also used Brefeldin A, an inhibitor that prevents transport of proteins from the endoplasmic reticulum to the Golgi Network, to verify that Hsp90α was not exported through the canonical signal sequence pathway. We instead found that, similar to extracellular Hsp70, Hsp90α was exported from cancer cells via exosomes. These experiments demonstrate that Hsp90α is exported from cancer cells through exosomes in an isoform independent manner. These results provide a new anti-metastatic target for drug development.

INTRODUCTION

Metastasis is responsible for over 90% of breast cancer related deaths (2,3). The major contributing factor to this high statistic is that current metastatic breast cancer treatments are only palliative and not curative (3). The lack of successful metastatic treatments emphasizes the need to identify new pathways and proteins that are required

for cancer metastasis. Gaining a better understanding of the processes involved in metastasis could lead to the identification of novel targets and enable the development of new drugs that can prevent or cure cancer metastasis.

In order to better understand metastasis and the proteins important in its processes, the Jay Lab performed a screen to identify surface proteins that are essential for cancer invasion, a critical early step in metastasis (8). They used a technique developed in-house called Fluorophore Assisted Light Inactivation (FALI) to inactivate specific surface proteins and test for their importance in cancer invasion (31). One extracellular protein identified in the screen was Heat Shock Protein 90 alpha (Hsp90α) (30). Hsp90α was first identified extracellularly as a tumor-specific antigen in fibrosarcoma cells in 1986 by Ullrich *et al.* (55). Hsp90α has since been identified outside of numerous cell types including neurons, dermal fibroblasts, macrophages, epithelial cells, vascular smooth muscle cells, and various cancer cells (59,60). Extracellular Hsp90α has been best characterized outside of immune cells, but it continues to be implicated in additional extracellular functions in other cell types (123). Other heat shock proteins, such as Hsp70, have also been identified extracellularly, further highlighting the importance of heat shock proteins outside of the cell (60,77,124). Initially, it was thought heat shock proteins were present outside of the cell due to cellular leakage or lysis, and that their presence functioned as a signal to the immune system of cellular disease or dysfunction (125). However, as heat shock proteins were found outside of more cell types, it was hypothesized that they were being exported in a controlled and regulated manner.

Shortly after Eustace *et al*. identified Hsp90α outside of fibrosarcoma cells, it was suggested that Hsp90α may be exported in an isoform-specific manner. Didier Picard noted that two Hsp90α isoforms had been identified and that one of them contained two extra N-terminal exons which included a putative signal sequence in the second exon (61). He proposed that the isoform with the extra exons was preferentially exported from cells, while the isoform without the extra exons remained intracellular. Soon after, Chen *et al.* genetically confirmed the existence of the two Hsp90α splice variants and noted that the larger splice variant also contained a putative start site and canonical signal sequence in the first extra exon (126). Chen *et al.* denoted the Hsp90α splice variant with 10 exons, the classical number, Hsp90AA1-1, and the variant with two additional exons, Hsp90AA1-2 (126). They will be referred to by these names for the remainder of this thesis. Export through the canonical signal sequence pathway via an N-terminal signal sequence is the most well characterized mechanism of export. The presence of a putative signal sequence in the extra exons of Hsp90AA1-2 suggests that mode of export for this isoform. There are, however, several non-traditional modes of export that the cell could be using to target Hsp90α outside. These include lysosomal secretion, plasma membrane resident transporters, exosomes and multivesicular bodies, and exovesicles and membrane blebbing (127). Hsp70 has been shown to be exported from immune cells via exosomes, making this form of export a possibility for extracellular Hsp90α as well (60,78).

In this chapter we verified the presence of the two Hsp90α splice variants in multiple cancer and non-cancerous cell lines through PCR cloning, RNase Protection Assay, and quantitative RT-PCR. We then tested to see if Hsp90AA1-2, with its putative

signal sequence, was preferentially exported from cancer cells. We tested this by first knocking down the extra exon isoform, but found that this did not significantly alter the amount of Hsp90α outside of the cell. We further tested this conclusion by tagging both isoforms with GFP and found that they were both detected outside of the cell. These results suggested that the Hsp90AA1-2 isoform is not preferentially exported from the cell. To verify that Hsp90α was not exported via a canonical signal sequence, we treated the cells with Brefeldin A (BFA), a drug known to inhibit export through the canonical signal sequence pathway by preventing the transport of proteins from the ER to the Golgi Network (128,129). We demonstrated that BFA did not inhibit the export of Hsp90α; however, it did inhibit the export of MMP-2, a protein known to have a canonical signal sequence. From these data we concluded that Hsp90α was exported through a non-canonical signal sequence pathway. Since Hsp70 has been shown to be exported via exosomes in immune cells (78,130), we hypothesized that Hsp90α is exported in the same way. Indeed, we found Hsp90α present in the exosomes of several different cancer cell lines including HT-1080, MDA-MB-231, MCF-7, and SUM1315.

MATERIALS AND METHODS

Antibodies and Reagents. All antibodies were purchased from Assay Designs, MI, except for anti-MMP-2, which was purchased from R&D, MN. Brefeldin A was purchased from Sigma, MO. Unless noted, all other reagents were purchased from Sigma, MO.

Cell Culture. MDA-MB-231, HT-1080, MCF-7, and HS27 cells were obtained from ATCC and maintained in DMEM supplemented with 10% FBS, 1% non-essential amino acids, and 1% penicillin-streptomycin. SUM1315 cells were a kind gift from Dr. Charlotte Kuperwasser and were maintained in Ham's F12 media supplemented with 5% FBS, 5 μg/mL insulin, 10 ng/ml epidermal growth factor (EGF) and 1% penicillin-streptomycin. All cells were grown at 37ºC under 7.5% CO_2.

RT-PCR and Cloning. RNA was collected from HT-1080, MDA-MB-231, HS27, and SUM1315 cell lines with TRIzol (Invitrogen, CA) and 2 μg RNA was reverse transcribed into cDNA using Superscript III (Invitrogen, CA) as directed in the product manual. The following primers were used to amplify the two extra exons and the whole Hsp90α gene with and without the additional exons. Extra Exons: forward 5'CAAAGAGCGGAGGAAGAGC-3' and reverse 5'TCAACTGGGCAATTTCTGC-3'; Hsp90AA1-1: forward 5'GGCTTCAGCTAGTGGGGTCT-3' and reverse 5'AAACAAGCCCTGTGGAGAGA-3'; Hsp90AA1-2: forward 5'TTCTCTGGCATCTGATGGTG-3' and reverse-5'CTACTTCTTCCATGCGTGATGTG-3'. These genes were amplified using PCR and ligated into the CT-GFP Fusion TOPO Vector (Invitrogen, CA) following the manufacturer's instructions. The vector was purified and amplified in chemically competent *E. coli*. The vector was then collected and purified using a Midi-Prep Kit (Qiagen, CA) and transfected into each of the above cell lines using Lipofectamine (Invitrogen, CA).

RNase Protection Assay. The RNA Protection Assay was performed as described in (131). In brief, three mRNA constructs were created, (one to show the presence of the two isoforms, and two others to function as positive controls), by cloning the corresponding cDNA into a pcDNA3.1 vector. The amplified cDNA was purified and converted to RNA and labeled with ^{32}P. The probes were then purified and the presence of the label verified. RNA from MDA-MB-231, HT-1080, MCF-7, and HS27 cells was added to the labeled probes and allowed to hybridize. The sample was treated with RNase to digest any single-stranded RNA. The samples were then run on a 4.75% acrylamide 8M urea gel. The gel was transferred to Whatman paper and dried. The dried gel was exposed to film with an intensifying screen at -70ºC overnight and visualized.

Quantitative Real Time PCR. RNA was collected from HT-1080, MDA-MB-231, HS27, and SUM1315 cell lines with TRIzol (Invitrogen, CA) and 2 μg RNA was reverse transcribed into cDNA using Superscript III (Invitrogen, CA) as directed in the product manual. Real time PCR was performed at the Tufts University Center for Neuroscience Research using the Stratagene real time cycler. Primer sequences were as follows: HSP90AA1-1 forward 5'-GGCAGAGGCTGATAAGAACG-3' and reverse 5'CCCAGACCAAGTTTGATCATCC-3'; HSP90AA1-2 forward 5'-CATCTGATGGTGTCTGGATCC-3' and reverse 5'-AATGGCTGCAGATCCTTGTAG-3'. Samples were analyzed using the 2-ΔΔCT method (132) with GAPDH as the positive control (66).

Brefeldin A Treatment. MDA-MB-231 cells were treated with 10 μg/mL Brefeldin A (BFA), (Sigma, MO) or vehicle control for 16 hours. Conditioned media was collected and subjected to SDS-PAGE followed by Western blotting as detailed below. The blots were then probed with MMP-2, Hsp90α, or β-actin antibody. β-actin protein should not be present in conditioned media samples from intact, alive cells and serves as a control for intracellular proteins found in conditioned media from lysed cells.

RNAi Treatment. MDA-MB-231 cells were transfected with either scrambled siRNA (non-targeting) or 100 nM siRNA directed against the HSP90AA1-2 (sense 5'-GTTAACTGGTACCAAGAAA-dTdT-3') isoform using Oligofectamine (Invitrogen, CA). RNA was extracted as indicated above.

Preparation of Cell Lysates and Conditioned Media for Western Blot Analysis. To prepare lysates, cells were lysed in 750 μl lysis buffer (20mM Tris pH 7.5, 1% Triton X-100, 3 μl/ml protease inhibitor cocktail (Sigma, MO)), incubated for 30 minutes on ice and spun down at 4000 RPM (3300 g) on a table top centrifuge for 4 minutes. The supernatant was then collected and a Bicinchoninic Acid (BCA) protein assay was performed (Pierce, IL). To prepare conditioned media, 3 million cells were plated in a T-150 flask. Twenty-four hours after plating, cells were washed twice with HBSS (Gibco, CA) and re-fed with 15 mL serum-free DMEM with 1% non-essential amino acids. The conditioned media were collected 48 hours later and concentrated by centrifugation in centrifugal filters (Millipore, MA). Protein quantification was determined by BCA assay

(Pierce, IL). The samples were run on an SDS-PAGE on a blank gel, transferred to a nitrocellulose membrane, and blotted with antibodies against the indicated proteins.

Exosome Preparation. Exosomes were isolated from HT-1080, MDA-MB-231, MCF-7, and HS27 cells as described in (133). Briefly, $6x10^6$ cells were plated in a 100 mm dish and incubated overnight. Cells were washed twice with serum-free media and refed with 5 ml of serum-free media and incubated overnight. The culture media was centrifuged at 1800 RPM (300xg) for 5 minutes to pellet cells. Supernatant was filtered through a 0.2 μM filter into ultra-centrifuge tubes. Filtered supernatant was centrifuged at 38,000 RPM (110,000xg) for 1 hour using the Ti70 Rotor ultra-centrifuge. Supernatant was removed and the pellet was gently washed with 5 ml PBS and centrifuged again at 38,000 RPM (110,000xg) for 1 hour. Supernatant was removed and the pellet was resuspended in 60 μl of PBS. Samples were mixed with 4x Sample Buffer and run on an SDS-PAGE gel and blotted for Hsp90α.

RESULTS

Hsp90α has been identified outside of numerous cells types, and recently has been shown to have an important role in cancer progression (30,59,60). Currently, it is not known how Hsp90α is exported from cancer cells. There are two transcripts for Hsp90α in the Ensembl.org database, suggesting the presence of multiple Hsp90α splice variants. One transcript, Hsp90AA1-1, contains the traditional 10 exons, while the other, Hsp90AA1-2, contains an additional two exons at the N-terminus. The existence of these

splice variants has been demonstrated and verified by several investigators, further supporting that they are transcribed genes and are not just experimental artifacts (61,126). In 2004, Picard noted that the two extra exons contained an alternative start site followed by a putative signal sequence (61). He hypothesized that the longer splice variant was responsible for the export of Hsp90α due to the signal sequence, while the other isoform, which does not have a signal sequence, remained inside the cell.

To test this hypothesis, we examined whether both splice variants were transcribed by cancer cells. In order to demonstrate the presence of these isoforms in cancer cells we isolated RNA from several cell lines—including MDA-MB-231, MCF-7, HT-1080, and SUM1315—and generated cDNA. We then designed primers to clone out Hsp90AA1-1, Hsp90AA1-2, and the two extra exons from the cDNA (Figure 1A). We demonstrated that the extra exons were transcribed in four different cell lines (Figure 1B-D). We also tested for the presence of the extra exons in a normal breast cell line, HS27, and found that the exons were indeed present, indicating that not only were the extra exons produced in cancer cells, but they were also produced by non-cancerous cells. These results showed that the tested cell lines transcribed the larger 12-exon splice variant, but they did not definitely demonstrate that both of the isoforms were being transcribed.

Figure 1

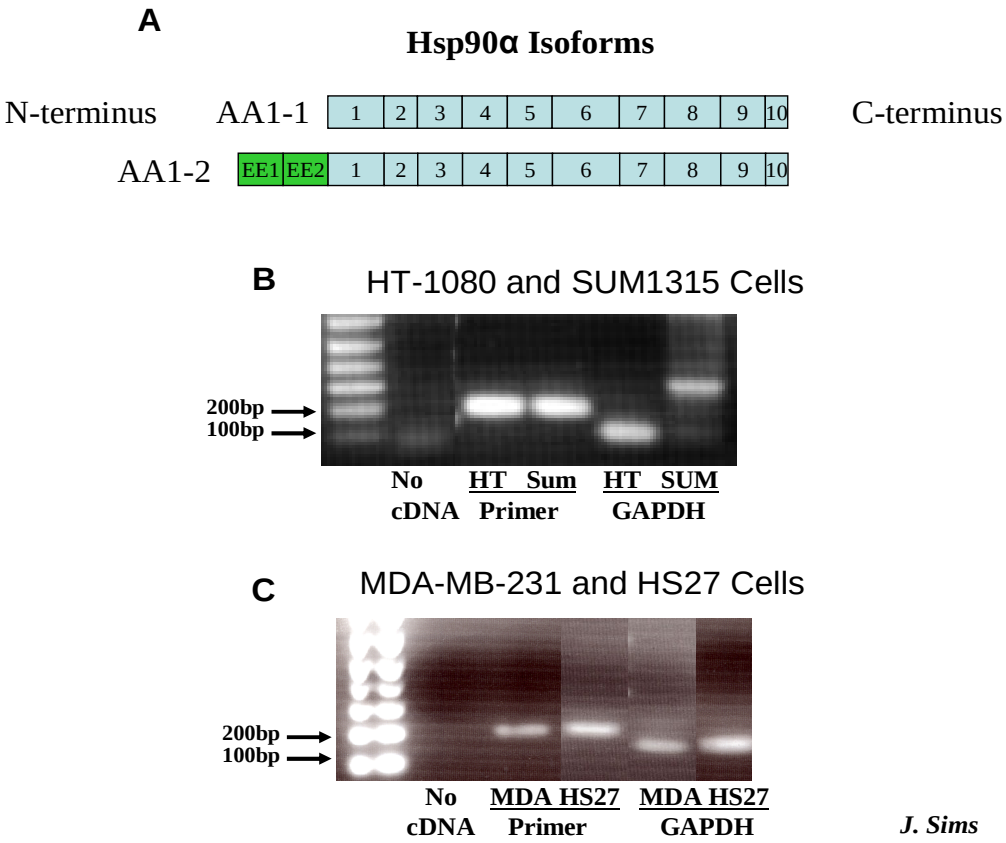


Figure 1-Two extra exons of Hsp90AA1-2 present in non-cancerous and breast cancer cells. Primers were designed for the N-terminal extra exons of Hsp90AA1-2. RNA was collected from the indicated cell lines and converted to cDNA. PCR was used to show the presence of the two extra exons in four different cell lines. (A) Depiction of the two Hsp90α splice variants. (B) A PCR demonstrating the presence of the two extra exons in HT-1080 and SUM1315 cells. (C) A PCR showing that the two extra exons are present in MDA-MB-231 cells and in the breast cell line HS27.

In order to verify that both of the splice variants were being transcribed, we performed an RNA Protection Assay (RPA). In an RPA assay, labeled RNA probes are created for the sequences of interest. RNA is collected from the cells in question and the probe is hybridized to the collected RNA, forming a double-stranded RNA molecule. The remaining single-stranded RNA is degraded using a single strand-specific RNase.

The remaining hybridized RNA is purified and run on an SDS-PAGE and visualized (134). We chose to use an RPA for detecting the presence of the splice variants because of its sensitivity in measuring low levels of RNA (134).

For this assay, we designed three probes in order to demonstrate the presence of both of the Hsp90α splice variants. The first two probes served as markers to show where the two isoforms should be located on the final blot (Figure 2A and 2B). The third experimental probe spanned the end of the second extra exon to the beginning of the first exon of the shorter isoform (Figure 2A). If the smaller variant, Hsp90AA1-1, is present in the cells, only the part of the probe that covers the first exon will be hybridized and protected from the RNase digestion, resulting in the shorter RNA strand as demonstrated by the second marker probe. If the larger, 12-exon variant, Hsp90AA1-2, is present the whole probe will bind to the RNA molecule and a larger RNA strand will be protected, corresponding to the size of the larger marker probe.

PCR and cloning techniques were used to construct the RNA probes. The probes were labeled with ^{32}P and incubated with mRNA collected from MDA-MB-231, HT-1080, MCF-7, or HS27 cells. The probes were hybridized to their corresponding mRNA and the samples were subject to an RNase that degraded any single-stranded RNA. The samples were purified and run on an SDS-PAGE gel. The gel was dried and the presence of any radioactivity was visualized on film. In figure 2C, lane 1 shows the probe without RNase treatment. The next two lanes show the two marker probes that indicate the location of the two isoforms if they are present in the test mRNA. In the last four lanes of the gel, one can see evidence of Hsp90AA1-1, the 10-exon isoform, in three of the four different cell lines, but we were unable to detect Hsp90AA1-2, the 12-exon isoform. We

did not observe either isoform in the normal breast cell line HS27, but we did observe the smaller splice variant in the three cancerous cell lines (Figure 2C). This is most likely due to the Hsp90AA1-2 variant being present in extremely small quantities making it difficult to detect even with a sensitive RPA assay.

Figure 2

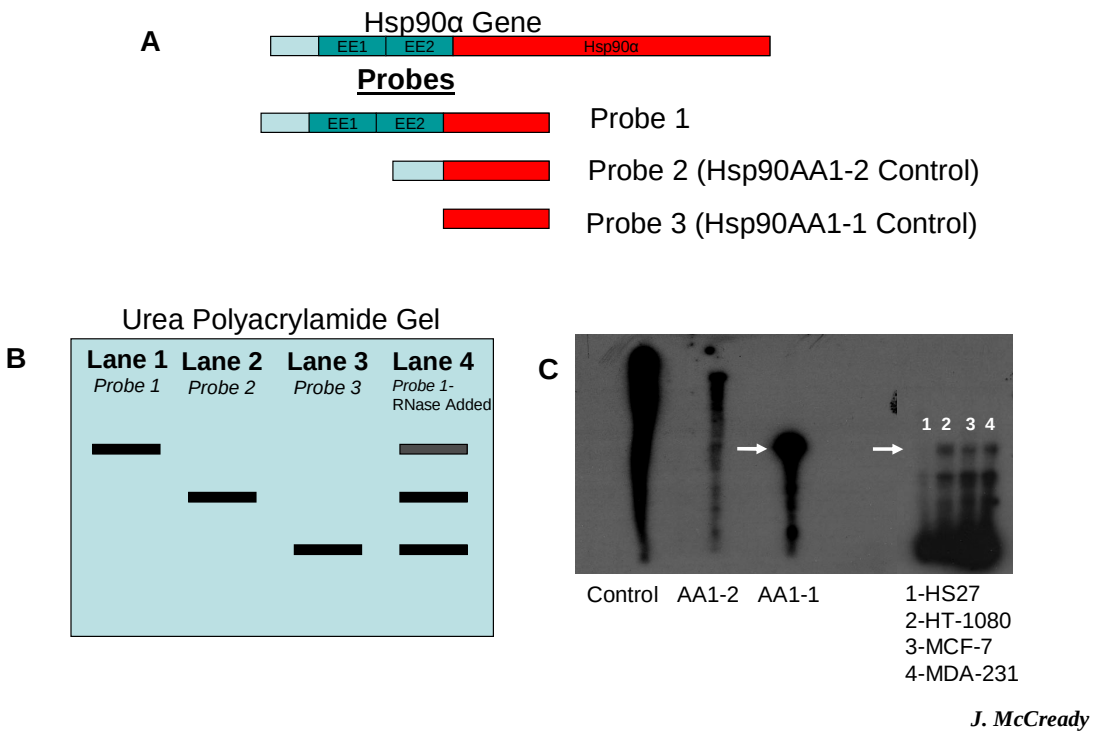


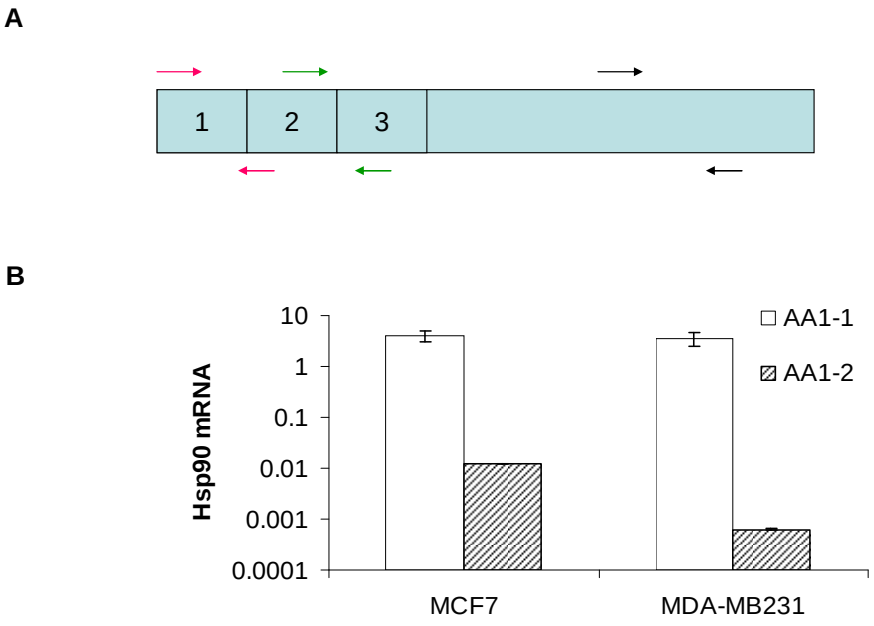
Figure 2-RPA demonstrates presence of the smaller Hsp90α splice variant. An RNA Protection Assay was used to show that both of the splice variants were being transcribed in four different cell lines. (A) A diagram of the three primers designed for the RPA. The first primer will show which of the isoforms are present, the second is a control marker for the larger splice variant, and the third primer shows the location of the smaller splice variant. (B) A mock diagram of the location of each marker primer and the experimental primer if both of the splice variants are present. (C) The resultant blot from the RPA experiment. Lanes 1, 2, and 3 are the markers to show the size of the primers. The last four lanes represent the RNA from the four tested cell lines: HS27, HT-1080, MCF-7, and MDA-MB-231. The smaller splice variant, Hsp90AA1-1, was observed in the three cancer cell lines, but not in the regular breast cell line.

Even though we could not detect the Hsp90AA1-2 isoform in the RPA, in Figure 1 we demonstrated that the larger isoform was present in the cancer cells. Therefore, we surmised that we were unable to detect the larger isoform in the RPA because it was present in very small quantities. Radioactive RPAs are only capable of detecting mRNA at levels between 0.1 and 1 pg per sample (135). This indicates that the 12-exon splice variant must be present at levels below this threshold.

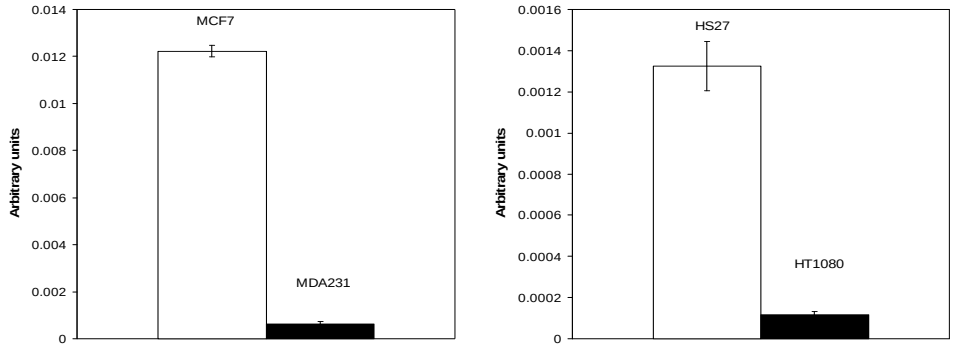
In order to verify in a single experiment that both of the Hsp90α isoforms are present in cancer cells, and confirm that the two isoforms are indeed present in disparate amounts from each other, we performed quantitative RT-PCR. We designed three sets of primers for this assay. The first primer set spanned the first and second extra exon of the larger, Hsp90AA1-2. The second spanned the second extra exon and the first exon of the shorter Hsp90AA1-1 isoform. The third covered a sequence further down in the body of the Hsp90α protein (Figure 3A). These three primers enabled us to verify that each splice variant was being transcribed and to determine the relative amounts of the larger and smaller Hsp90α isoforms in each of the tested cell lines. Figure 3B shows that both of the splice variants are present, but there are two orders of magnitude less of the larger Hsp90AA1-2 isoform in MCF-7 cell line and three orders of magnitude less in the MDA-MB-231 cell line. These results show that even though the amount is small, there is significantly more of the larger Hsp90AA1-2 isoform in the less invasive MCF-7 cell line than the MDA-MB-231 cells. This finding does not support the hypothesis that the 12-exon isoform contributes to increased cancer invasiveness. We further examined the relative amounts of the splice variants in two other cell lines: the invasive HT-1080 and the non-cancerous HS27 cells. Again, the above trend was observed and we saw much

less of the 12-exon isoform in the invasive HT-1080 cells than in the normal cell line. These results further indicate that both of the Hsp90α splice variants are transcribed by the cancer cells, but that the 12-exon isoform is present in much lower quantities. In addition, the 12-exon isoform does not appear to contribute to the invasive phenotype of the observed cancer cells, which is inconsistent with our original hypothesis.

Figure 3



C



D

Cell Line	AA1-2 transcript	AA1-1 transcript
HS27	1	70
HT1080	1	243
MCF7	1	16
MDA231	1	457

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Figure 3-Hsp90AA1-1 is present in much higher quantities than Hsp90AA1-2. Quantitative RT-PCR was performed for both splice variants to verify transcription and to show the relative amounts of the two isoforms in four different cell types. (A) A diagram of the Hsp90α primers designed for the RT-PCR experiment. (B) A graph showing the relative amounts of the two splice variants in MCF-7 and MDA-MB-231 cells. (C) Graphs showing the relative amount of the larger, Hsp90AA1-2 splice variant in four cell lines. (D) A table showing the relative amount of the shorter Hsp90AA1-1 in relation to the longer Hsp90AA1-2.

Thus far, we have demonstrated that both of the Hsp90α splice variants are present in several cancerous cell lines and in a regular breast cell line, and that the 10-exon isoform is by far the predominant isoform present in these cell lines. We have also shown that there is significantly less of the larger, Hsp90AA1-2 isoform present in the more invasive cancer cell lines suggesting that this isoform is not responsible for the invasive effects of Hsp90α. However, we have not definitively demonstrated that the

mode of export of Hsp90α is not through the canonical signal sequence pathway. In order to test whether a signal sequence in Hsp90AA1-2 is targeting Hsp90α outside the cell, we designed siRNA primers that were targeted to the two extra exons of Hsp90α. We used those siRNA constructs to specifically knock down the Hsp90AA1-2 isoform and determine if that isoform was necessary for export (Figure 4). We obtained between a 57% and 83% knock down of the extra exon in the MDA-MB-231 and MCF-7 cells, respectively. We then collected lysate and conditioned media of the siRNA or control treated cells and examined the levels of Hsp90α. There was no change in the protein levels of Hsp90α in either the lysate or conditioned media (Figure 4B). This result indicates that the export of Hsp90α is not due to a signal sequence in the extra exons because knocking down the Hsp90AA1-2 isoform did not reduce the amount of extracellular Hsp90α. Also, the lack of reduction of Hsp90α in the lysate or the conditioned media further confirmed the above results that the extra exon form is not a major component of either the intracellular or extracellular pool of Hsp90α.

Figure 4

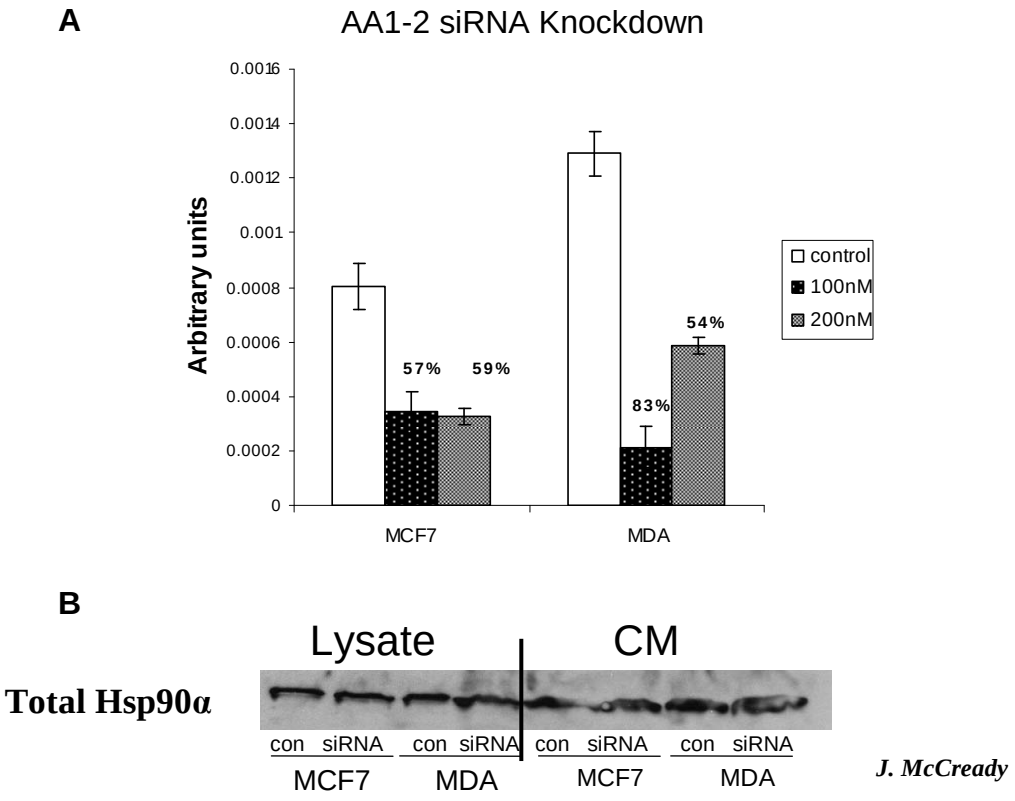

Figure 4-Knockdown of Hsp90AA1-2 does not reduce extracellular Hsp90α levels. To further test if the extra exon isoform is responsible for the export of Hsp90α, we knocked down the larger splice variant using siRNA. (A) A graph of the relative knock down obtained in MCF-7 and MDA-MB-231 cells. The knock down in each cell line was at least 54%. (B) Lysate and conditioned media were collected from each cell type. These samples were run on an SDS-PAGE and blotted for Hsp90α. There was no change in the amount of Hsp90α in either the lysate or conditioned media of the knock down cells compared to the control cells.

To confirm that this splice variant was not preferentially exported to the extracellular space via a canonical signal sequence, we used a C-terminal-labeled GFP TOPO vector. We first isolated the two extra exons from HT-1080 cells and cloned them into the GFP vector. We then transfected the vector back into the HT-1080 cells. We collected lysate and conditioned media from the transfected cells and blotted for GFP.

Interestingly, we only detected the two extra exons in the extracellular media of the cells, but not in the lysate. If the extra exons contained an export signal, we would have expected to see a small amount of GFP in the lysate, with the majority of it in the conditioned media. Instead, we detected GFP in the lysate and conditioned media in cells transfected with the vector alone. This indicated to us that the GFP itself was being targeted non-specifically from the cells. Upon a literature search, we found that this phenomenon had been observed by other groups as well (136). Tanudji *et al*. demonstrated that an improperly folded form of GFP was secreted from certain cancer types that had GFP overexpressed in them (136). They also demonstrated that when used as a tag to a properly folded protein, the GFP was not targeted for export, indicating that the results of the experiments were still viable (136).

These initial results called into question our hypothesis that the extra exons of Hsp90α contained a canonical signal sequence and targeted Hsp90α outside of the cell. To further test our hypothesis, we cloned the entire 10- and 12-exon Hsp90α splice variants into the GFP vector and transfected them back into HT-1080 cells. We collected lysate and conditioned media from these cells and ran the samples on an SDS-PAGE. We blotted for both Hsp90α and GFP. GFP was detected at two different sizes, 28 kD, the normal size of GFP, and 118 kD, the size of Hsp90α plus GFP. As can be seen in Figure 5B, GFP was seen outside the cell in the positive control and in both the vectors with the 10- or 12-exon Hsp90α isoform. These results further confirm the results obtained in Figure 5A that both isoforms are being targeted outside of the cell and that GFP alone is targeted outside of the cell. Interestingly, in the blot for Hsp90α, one can see that the amount of extracellular Hsp90α dramatically increases in the samples that

have been transfected, showing that when under stress, such as that caused by the transfection process, extracellular Hsp90α is upregulated. This observation mimics what we see in cancer cells, which also being under stress, upregulate extracellular Hsp90α (27,28).

Figure 5

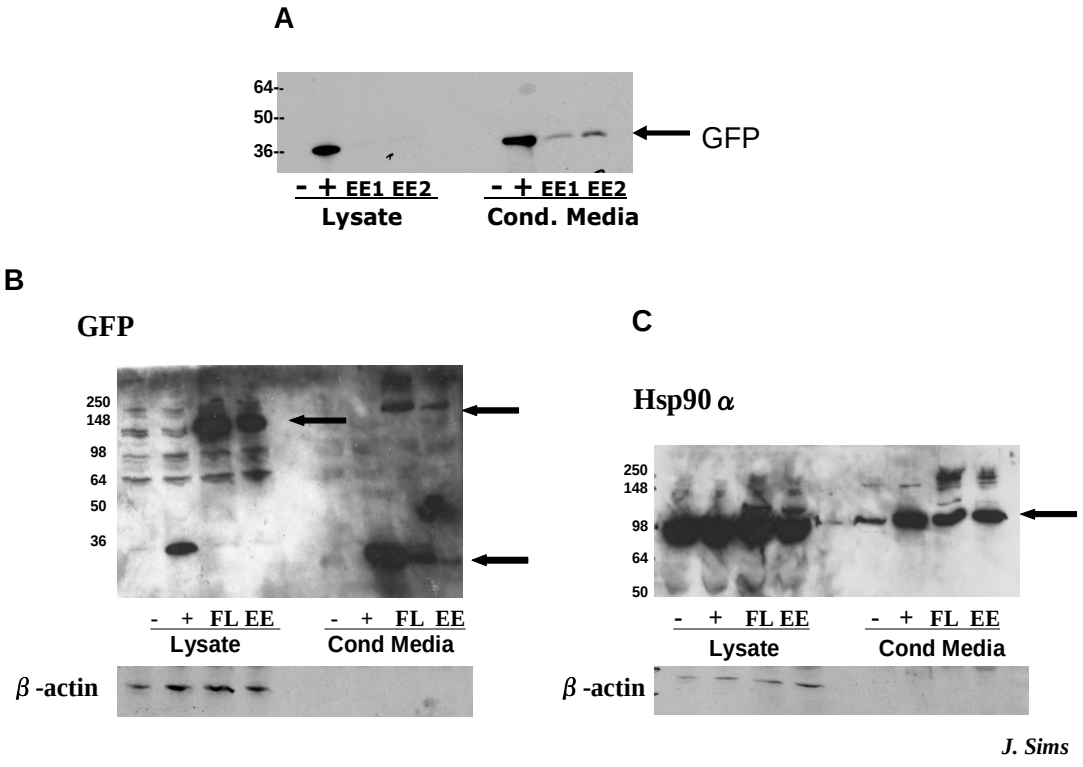

Figure 5-GFP-Tagged AA1-2 isoform is not preferentially exported from cells. The two extra exons, Hsp90AA1-1 and Hsp90AA1-2 were each cloned into a CT-GFP-TOPO vector. (A) The GFP vector with just the two extra exons was transfected into HT-1080 cells and the lysate and conditioned media was collected. The samples were subject to SDS-PAGE and blotted for GFP. (B and C) The GFP vectors with the two splice variants were transfected into HT-1080 cells and lysate and conditioned media were collected from each condition. The samples were run on an SDS-PAGE and blotted with Hsp90α and GFP antibodies. GFP was found in both the lysate and conditioned media of the positive control and in both the Hsp90AA1-1 and Hsp90AA1-2 transfected cells.

Finally, to verify that Hsp90α is not exported via a signal sequence, we treated HT-1080 cells with BFA, a compound that inhibits the canonical signal sequence pathway by inhibiting the transport of proteins from the ER to the Golgi Network. For this experiment, we used MMP-2 as a positive control because it contains a signal sequence and is known to be exported via the canonical signal sequence pathway. As can be seen in Figure 6, when the cells are treated with BFA, MMP-2 is no longer present in the conditioned media, indicating that the signal sequence pathway has been inhibited. However, Hsp90α is still detected in the conditioned media, demonstrating that Hsp90α is exported via a different export pathway. In addition, the amount of extracellular Hsp90α is significantly increased when the cells are treated with BFA, indicating that additional extracellular Hsp90α is exported when cells undergo stress. This result, combined with the above RT-PCR, siRNA, and GFP-tagging results, indicates that the extra exons are not responsible for transporting Hsp90α outside of the cell and that Hsp90α is not exported via the canonical signal sequence pathway, but is instead exported via a non-classical pathway.

Figure 6

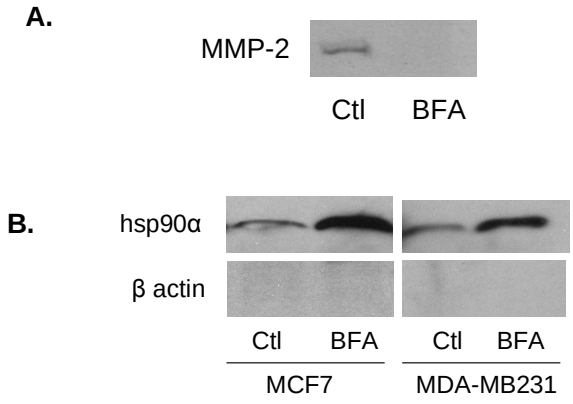

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Figure 6-Hsp90α is not exported via the canonical signal sequence pathway. HT-1080 cells were treated with BFA for 16 hours. Lysate and conditioned media of control and BFA treated cells were collected and subject to SDS-PAGE. (A) Samples were blotted with MMP-2 antibody. MMP-2 was detected in the conditioned media of the control, but not in the BFA treated conditioned media. (B) Samples were blotted with Hsp90α antibody. Hsp90α was detected in the conditioned media of both the control and BFA treated cells.

We next investigated which non-classical export pathway is responsible for the export of Hsp90α. Nickel described four non-classical pathways for protein export (127): lysosomal secretion, export mediated by plasma membrane-resident transporters, export through the release of exosomes from multivesicular bodies, and export by exovesicles via membrane blebbing. In order to determine if any of these pathways are responsible for the export of Hsp90α, we searched the literature for any precedent for the export of heat shock proteins. In 1999, Thery *et al*. demonstrated that Hsp70, another heat shock protein, was transported outside of the cells via exosomes (29). We hypothesized that Hsp90α is transported outside of breast cancer cells in a similar manner. Exosomes were isolated from MDA-MB-231 and MCF-7 cells according to the protocol described by Thery *et al*. (27). Hsp90α was detected via western blotting in the exosome preparations, indicating that Hsp90α is exported from cancer cells via exosomes (Figure 7).

Figure 7

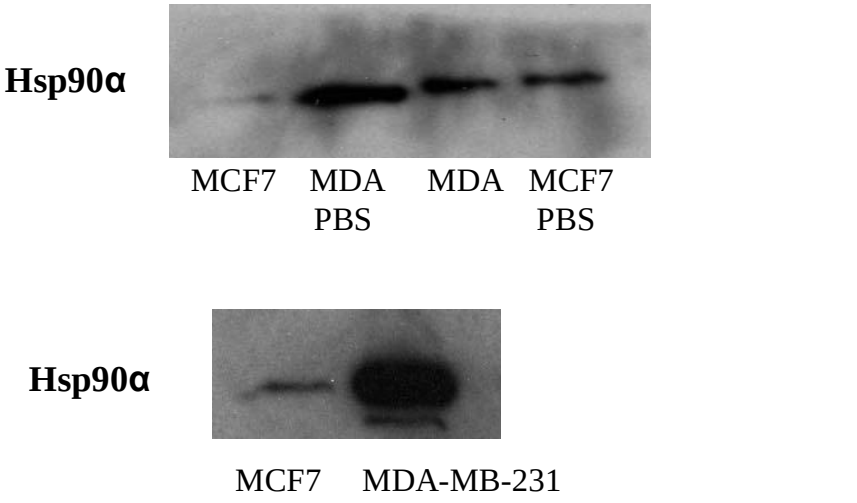

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Figure 7-Hsp90α present in exosomes collected from breast cancer cells. Exosomes were collected from MCF-7 and MDA-MB-231 cells. The exosomes were run on an SDS-PAGE and blotted for Hsp90α. Hsp90α was observed in the exosomes of both cell types.

The results of these experiments led us to conclude that Hsp90α is not exported in an isoform-specific manner. The Hsp90AA1-2 isoform is not preferentially exported and inhibiting the canonical signal sequence pathway does not reduce the export of Hsp90α. The Hsp90AA1-2 isoform is not the primary isoform in the cytosol or extracellular medium and knocking down that isoform does not affect Hsp90α levels. Currently, we do not know the function of the Hsp90AA1-2 isoform, but it appears to be downregulated in invasive cancer cells, indicating a potential anti-metastatic role. Lastly, Hsp90α is exported from cancer cells via the release of exosomes from multivesicular bodies.

DISCUSSION

Hsp90α has been increasingly implicated in many processes of cancer progression, resulting in the development of Hsp90α inhibitors with the goal of obtaining more successful cancer treatments. One side effect of intracellular Hsp90α inhibitors is the upregulation of HSF1, the transcription factor for Hsp90α, Hsp70, and other heat shock related genes (35,137). Inhibiting only the extracellular form of Hsp90α would facilitate the reduction of extracellular Hsp90α function and the subsequent reduction of metastasis without the upregulation of the heat shock genes, likely resulting in a reduction of negative side effects. Here we demonstrate that two Hsp90α splice variants are transcribed in multiple cancer cell lines and the shorter variant, Hsp90AA1-1 is produced in much larger quantities, than the longer Hsp90AA1-2. We show that the splice variants are not responsible for the export of Hsp90α which was demonstrated to be exported via exosomes instead of by the classical canonical signal sequence pathway. These results indicate that the best way to inhibit extracellular Hsp90α is by treating cells with a cell-impermeable Hsp90α inhibitor.

As noted above, we demonstrated the presence of a 12-exon alternative splice variant for Hsp90α in several cancer lines and one non-cancerous cell line. These results indicated that Hsp90AA1-2 is widely transcribed. This variant, however, is produced in extremely small quantities compared to the 10-exon variant. Even though we initially proposed that the Hsp90AA1-2 splice variant was responsible for the extracellular Hsp90α found in cancer cells, thereby implying it would be upregulated in cancer cells, we actually found that this variant is present in smaller amounts in invasive cancer cells

than in less-invasive or non-cancerous cells. This suggests that this isoform may have anti-metastatic qualities, and is hence downregulated in cancer cells. However, we would need to test a larger sample size of both normal and cancerous cell lines in order to verify this conclusion. This isoform could function in a dominant negative fashion, with the two extra exons inhibiting the binding of ATP or preventing the N-terminal dimerization that occurs when ATP binds to Hsp90α (42). In order to determine if this variant has such a role, it would need to be upregulated in cancer cells and tested for a change in invasiveness. This isoform could have other roles in cell function as well, which could be elucidated through upregulation and siRNA treatment. Prior to these experiments we would need to first verify that both transcripts are not only transcribed but also translated by producing an antibody that specifically binds the two extra exons of the Hsp90AA1-2 variant and verifying that the corresponding 12-exon splice variant protein is indeed present in the cancer cells.

In this chapter, we demonstrated that Hsp90α is exported through a non-classical pathway. An increasing number of proteins have been found to be exported through these alternative pathways, but the reason for these additional modes of export is still unknown (127). It was not surprising to find the extracellular Hsp90α observed in cancer cells to be exported via exosomes. At the time of these experiments, Hsp70 had been shown to be exported from immune cells by exosomes, making this a likely export mechanism for Hsp90α (124). However, we did not eliminate the possibility of Hsp90α also being exported through other non-canonical pathways. (This possibility will be discussed in more depth in chapter 4). After the conclusion of these experiments, additional investigators have also shown that Hsp90α is present in exosomes in both

cancerous and normal cell types, further supporting our data (66,130). Nevertheless, it is unclear how Hsp90α is targeted to the multivesicular body (MVB), the intracellular site of exosome formation. Wang *et al*. demonstrated that the C-terminal MEEVD domain and a phosphorylation site at Thr-90 play an important role in the export of Hsp90α (65). This phosphorylation site could target Hsp90α to the MVB and exosomal pathway (138). Hsp90α has also been shown to have a number of potential ubiquitination sites and to be ubiquitinated by the E3 ligase, CHIP. Hsp90α could also be targeted for non-degradative ubiquitination, another method for targeting proteins to the MVB (138,139).

In this chapter we demonstrated through cloning, RPA, and quantitative RT-PCR, that two splice variants of Hsp90α, Hsp90AA1-1 and Hsp90AA1-2, are transcribed by breast cancer cells. The first, Hsp90AA1-1, contains the traditional 10 exons and is present in much larger amounts than the second splice variant, Hsp90AA1-2. The second variant contains an additional 2 exons at the N-terminus, and was present at a very low abundance in all cells examined. Using cloning and siRNA, we determined that even though the extra exons contained a putative signal sequence, this isoform was not preferentially exported from cancer cells. We also determined that Hsp90α is not exported through the canonical signal sequence pathway but is instead exported via exosomes. Further elucidation of this export pathway will assist in the development of new anti-Hsp90α drugs that target the extracellular form of the protein, potentially reducing cancer metastasis with a reduction in side effects.

The experiments described in this chapter led to a publication in BMC Cancer in 2010 entitled *Secretion of extracellular hsp90alpha via exosomes increases cancer cell motility: a role for plasminogen activation* by J. McCready, J. Sims, and D. Jay. In this

manuscript we demonstrate that Hsp90α is not secreted from breast cancer cells through the canonical signal sequence pathway or in an isoform-specific manner, but is instead exported via exosomes. We also show that adding additional exosomes or recombinant Hsp90α to cancer cells causes the cells to become more motile as can seen by a change in both cell shape and in the rate the cells are able to migrate after a scratch wound. We also implicate Hsp90α in the activation of plasminogen by demonstrating that Hsp90α co-immunoprecipitates with tissue plasminogen activator and that inhibition of Hsp90α causes a decrease in the amount of plasmin (activated plasminogen) observed in cancer cell conditioned media. The full manuscript detailing these experiments has been included in the appendix of this thesis.

CHAPTER 3

EXTRACELLULAR HEAT SHOCK PROTEIN (HSP)70 AND HSP90α ASSIST IN MATRIX METALLOPROTEINASE-2 ACTIVATION AND BREAST CANCER CELL MIGRATION AND INVASION

Jessica D. Sims[1], Jessica McCready[2], Daniel G. Jay[1]

ABSTRACT

Breast cancer is second only to lung cancer in cancer-related deaths in women, and the majority of these deaths are caused by metastases. Obtaining a better understanding of migration and invasion, two early steps in metastasis, is critical for the development of treatments that inhibit breast cancer metastasis. In a functional proteomic screen for proteins required for invasion, extracellular heat shock protein 90 alpha (Hsp90α) was identified and shown to activate matrix metalloproteinase 2 (MMP-2). The mechanism of MMP-2 activation by Hsp90α is unknown. Intracellular Hsp90α commonly functions with a complex of co-chaperones, leading to our hypothesis that Hsp90α functions similarly outside of the cell. In this study, we show that a complex of co-chaperones outside of breast cancer cells assists Hsp90α mediated activation of MMP-2. We demonstrate that the co-chaperones Hsp70, Hop, Hsp40, and p23 are present outside of breast cancer cells and co-immunoprecipitate with Hsp90α *in vitro* and in breast cancer conditioned media. These co-chaperones also increase the association of Hsp90α and MMP-2 *in vitro*. This co-chaperone complex enhances Hsp90α-mediated activation of MMP-2 *in vitro,* while inhibition of Hsp70 in conditioned media reduces this activation and decreases cancer cell migration and invasion. Together, these findings support a model in which MMP-2 activation by an extracellular co-chaperone complex mediated by Hsp90α increases breast cancer cell migration and invasion. Our studies provide insight into a novel pathway for MMP-2 activation and suggest Hsp70 as an additional extracellular target for anti-metastatic drug development.

INTRODUCTION

Breast cancer is the most commonly diagnosed cancer among women and is second only to lung cancer in cancer-related deaths (1). There are a variety of treatments for primary breast tumors ranging from chemotherapies to tumor resection, but once breast cancer has metastasized, there is a poor rate of patient survival (140). There are currently no therapies that limit metastasis. Obtaining a better understanding of the factors and mechanisms that regulate breast cancer cell migration and invasion is crucial to the development of treatments that limit breast cancer metastasis. Extracellular proteins are thought to play an important role in regulating cancer cell migration and invasion, (8,23) and due to their accessibility, provide good targets for drug development. To explore this role, our lab conducted a functional proteomic screen for extracellular proteins that are essential for invasion in fibrosarcoma cells. One protein that was identified in the screen was extracellular heat shock protein 90α (Hsp90α).

Hsp90α is a highly conserved and abundant protein, constituting about 1% of the total intracellular protein (47,141). In the cytoplasm, Hsp90α has over 200 interacting proteins (hereafter referred to as "client proteins") (56) and it commonly functions in concert with various co-chaperones including Hsp70, Hsp40, Hop, Hip, and p23. These proteins form a complex that binds to client proteins and assists in their folding or activation. Briefly, Hsp70 and Hsp40 form a complex with the client protein. This complex binds to Hsp90α using Hop as a scaffold. ATP binds to Hsp90α, initiating a conformation change that enables p23 to bind to Hsp90α and causes Hsp70, Hsp40, and

Hop to disassociate from the complex. This facilitates the folding and activation of the client protein, which is then released from the complex (47,142).

Many studies have focused on the intracellular role of Hsp90α in tumorigenesis, (143-145) but recently its extracellular role in migration and invasion is beginning to be elucidated. Hsp90α is exported from cancer cells via exosomes and contributes to breast cancer cell migration (66). We, and others, have demonstrated that extracellular Hsp90α enhances breast cancer cell invasiveness through MMP-2 activation (30) as well as other client proteins including ErbB2 and plasmin (56,66). The mechanism of how Hsp90α acts in the activation of these client proteins is not known. We focus here on MMP-2 due to its well established role in invasion and migration (28,106).

MMP-2 is a zinc-dependent endopeptidase and a member of the metalloproteinase family, which degrades various components of the extracellular matrix (27,104). MMP-2 has been shown to be important in development and cell motility, and has a well-documented role in cancer metastasis (28,106). MMP-2 is secreted from the cell as an inactive zymogen and, once outside, it is activated through cleavage of its pro-domain (106). MMP-2 is thought to be primarily activated by the membrane type 1 matrix metalloproteinase (MT1-MMP) and tissue inhibitors of metalloproteinases (TIMPs), but has also been shown to be activated in the absence of MT1-MMP, suggesting the presence of alternative modes of activation such as auto-activation (118).

Based on its intracellular function, we hypothesized that extracellular Hsp90α enhances breast cancer migration and invasion by activating MMP-2 with the assistance of the co-chaperones, Hsp70, Hop, Hsp40, and p23. In this paper, we demonstrate that these co-chaperones are secreted from breast cancer cells and that they physically interact

with Hsp90α, both as recombinant proteins and in breast cancer cell conditioned media. Additionally, the presence of these co-chaperones increases the interaction of Hsp90α and MMP-2 *in vitro*. We show that Hsp90α, Hsp70, Hsp40, Hop, and p23 by themselves do not cleave and activate pro-MMP-2, but enhance auto-activation. This set of co-chaperones is similar to the minimal complex needed for the activation of intracellular proteins such as the steroid hormone receptor (51). We also provide evidence that Hsp70, as part of the above complex, is important in the activation of MMP-2 in cancer cell conditioned media. The inhibition of extracellular Hsp70 reduces MMP-2 activation and decreases cancer cell migration and invasion, implicating this co-chaperone as an additional extracellular therapeutic target.

MATERIALS AND METHODS

Antibodies and Reagents. All antibodies were purchased from Assay Designs (MI), except for anti-MMP-2, which was purchased from R&D (MN), and anti-fibronectin which was purchased from BD (NJ). All recombinant proteins were purchased from Assay Designs except for pro-MMP-2, which was purchased from EMD (NJ). Batimastat was purchased from Tocris Biosciences (MO) and 17-AAG was purchased from R&D (MN). Unless noted, all other reagents were purchased from Sigma (MO).

Cell Culture. MDA-MB-231 and HT-1080 cells were obtained from ATCC and MDA-MB-231-s4175 cells were a kind gift from Dr. Joan Massagué. Cells were maintained in DMEM supplemented with 10% FBS, 1% non-essential amino acids, and 1% penicillin-

streptomycin. SUM1315 cells were a kind gift from Dr. Charlotte Kuperwasser and were maintained in Hams F12 media supplemented with 5% FBS, 5 μg/mL insulin, 10 ng/ml epidermal growth factor (EGF) and 1% penicillin-streptomycin. All cells were grown at 37ºC under 7.5% CO_2.

Preparation of Cell Lysates and Conditioned Media. To prepare lysates, cells were lysed in 750 μl lysis buffer (20mM Tris pH 7.5, 1% Triton X-100, 3 μl/ml protease inhibitor cocktail (Sigma)), incubated for 30 minutes on ice and spun down at 4000 RPM (3300 g) on a table top centrifuge for 4 minutes. The supernatant was then collected and a Bicinchoninic Acid (BCA) protein assay was performed (Pierce, IL). To prepare conditioned media, 3 million cells were plated in a T-150 flask. Twenty-four hours after plating, cells were washed twice with HBSS (Gibco) and re-fed with 15 mL serum free DMEM with 1% non-essential amino acids. The conditioned media were collected 48 hours later and concentrated by centrifugation in centrifugal filters (Millipore, MA). Protein quantification was determined by BCA assay.

Co-Immunoprecipitation and Western Blot Analysis. Recombinant proteins or conditioned media were pre-cleared with washed Protein G beads (Sigma) for 1 hour, spun down to pellet the beads, and the supernatant was collected. Antibody was added and incubated for 3 hours at 4ºC with constant rotation. Protein G beads that had been pre-cleared for 3 hours with 1 mg/ml BSA and then washed 5 times with PBS, were added to the protein-antibody mixture and incubated for 1 hour at 4ºC on a rotator. The beads were washed 5 times with lysis buffer, and the protein was eluted by boiling for 5

minutes in 4X sample buffer. The samples were run via SDS-PAGE on a blank gel, transferred to a nitrocellulose membrane, and blocked with antibodies against Hsp90α.

Zymography/MMP-2 Activation. Pro-MMP-2 was activated by incubating recombinant MMP-2 (0.4 μg) in 4-Aminophenylmercuric Acetate (APMA) (1 mM in 0.1M Tris-HCl) for 2 hours at 37ºC. For *in vitro* Hsp90α activation of MMP-2, the indicated recombinant proteins and ATP or ATPγS were incubated in activation buffer (25mM Tris pH7.5, 50mM KCl, 5mM $MgCl_2$, and 20mM $NaMoO_4$ (146)) for 30 minutes at 30ºC. The samples were boiled for 5 minutes in 4X non-reducing sample buffer and run on a 10% polyacrylamide gel containing gelatin (4 mg/ml) (147,148). The gel was renatured in 2.5% Triton X-100 for 40 minutes at room temperature, washed in digestion buffer (0.1M Tris pH 7.5, 50mM $CaCl_2$ and 1% NaN_3) for 30 minutes and then incubated in fresh digestion buffer at 37ºC for 18 hours. For activation of MMP-2 in conditioned media, conditioned media and untreated media were collected and concentrated. Aliquots of PBS, untreated media, and conditioned media (30 μl) were treated with the indicated inhibitors and then pro-MMP-2 (0.75 μg) was added. Samples were incubated with gentle agitation at room temperature for 5 minutes, boiled for 5 minutes in 4X reducing sample buffer and run on an SDS-PAGE gel, transferred to a nitrocellulose membrane, and blocked with antibodies against MMP-2. Densitometry was performed to determine the relative amount of active MMP-2 in both the zymograms and SDS-PAGE gels.

FITC-Casein Assay. A FITC-Casein Assay (Sigma, MO) was performed according to the manufacturer's directions except for the substitution of the incubation buffer for buffer (A): 50 mM Tris–HCl, pH 7.5, 0.1 M NaCl, 10 mM $CaCl_2$, and 0.1% Brij-35 (149). In brief, pro-MMP-2 was activated with APMA for 2 hours as described above. Pro-MMP-2 was combined with the indicated recombinant proteins in activation buffer for 30 minutes at 30ºC. The samples were then added to the indicated amount of buffer (A) and FITC-Casein substrate and allowed to incubate at 37ºC in the dark for 1 hour. The reaction was then stopped with 150ul of 0.6N Trichloroacetic acid (TCA). The samples were centrifuged on a table top centrifuge for 10 minutes at 10,000 g. 2ul of the supernatant was added to 200ul of assay buffer in each well of black 96-well plate and read on a plate reader (excitation wavelength of 485nm and an emission wavelength of 535nm).

Invasion Assay. 2.5 million MDA-MB-231 or SUM1315 cells were plated on five T-75 flasks and incubated for 48 hours. Then $2x10^4$ MDA-MB-231 or SUM1315 cells were labeled with cell tracker orange (CMTMR, Invitrogen), treated with either 10 μg/ml 17AAG, 30 μM Vehicle (water), or 30 μM Methylene Blue, and added to each well of a porous membrane (pore size 8μm (Neuroprobe, Gaithersburg, MD)) with a basement membrane barrier (0.3 μg/μl Matrigel (BD Biosciences, Bedford, MA)). The cells were incubated for 24 hours at 37 °C under 7.5% CO_2. The number of cells that had invaded into the matrigel and migrated through the pores of the membrane was quantified for each well with a fluorescence plate reader (excitation 544 nm, emission 590 nm).

Depletion of Hsp90α and Hsp70 from conditioned media. Conditioned media was collected as described above and pre-cleared with Protein G beads for 1 hour. Antibodies against Hsp90α or Hsp70 were added to the pre-cleared conditioned media and incubated for 3 hours. Afterwards, Protein G beads were added to the conditioned media-antibody mixture and rotated at 4ºC overnight. The beads were then spun down and the depleted supernatant was collected.

Wound Healing. MDA-MB-231-s4175 cells were plated ($1.5x10^5$/per chamber) in an 8-well chamber slide. The cells were incubated for 18 hours to allow formation of a monolayer. A wound was created in the monolayer with a 200 µl pipette tip. Cells were washed once with DMEM to remove any detached cells and pictures were taken of the wounds (0 hour time point). Serum-free DMEM, untreated conditioned medium obtained from MDA-231-s4175 cells, Hsp90α-depleted, Hsp70-depleted, or fibronectin-depleted (serves as a depletion control) conditioned media, or inhibitor-treated conditioned media was added to the wounded cells. The cells were incubated at 37ºC under 7.5% CO_2. Images were taken to determine the amount of cell movement from the wound edge at 16 hours (16 hour time point). Wound closure was quantified by measuring the area of the wound before and after the 16-hour incubation period.

Cell Viability Assay. Plated 20,000 MDA-MB-231 cells per well in a 96-well plate for 24 hours before addition of increasing volumes of 17-AAG, SPS-771, or Rabbit IgG that was incubated for 24 hours. Cells were subjected to a Celltiter 96 Aqueous Non-

radioactive Cell Proliferation Assay (Promega, WI). The number of viable cells was calculated as previously described (150).

Results

Hsp90α, MMP-2, and the co-chaperones Hsp70, Hop, Hsp40, and p23 are present in breast cancer cell conditioned media.

Intracellular Hsp90α forms an activation complex with various co-chaperones including Hsp70, Hop, Hsp40, p23, and Hip (151). This complex carries out the conformational changes necessary to fold or activate the client protein. We proposed that extracellular Hsp90α is functioning in a similar manner to intracellular Hsp90α; therefore we tested for the presence of the above co-chaperones in breast cancer cell conditioned media. We probed the extracellular media of the invasive breast cancer cell line, MDA-MB-231, for these co-chaperones. We observed Hsp70, Hop, Hsp40, and p23, but not Hip, in the conditioned media (Fig. 1). In addition to not finding Hip, the intracellular protein β-tubulin was not detected (Fig.1), which indicates that the co-chaperones in the media were not the result of intracellular leakage from lysed or dying cells. All the co-chaperones were less abundant in conditioned media than in cell lysate. Hsp90α, Hsp70, and Hop were three times more abundant in the lysate than in the conditioned media, while MMP-2, Hsp40, and p23 were, respectively, 8, 6, and 10 times more abundant in the lysate than the conditioned media as determined by densitometry. These results, which demonstrate the presence of co-chaperones outside of invasive cancer cells, confirm and extend our previously published data that co-chaperones are present in

conditioned media of fibrosarcoma cells (121), suggesting a general role for extracellular co-chaperones in cancer progression.

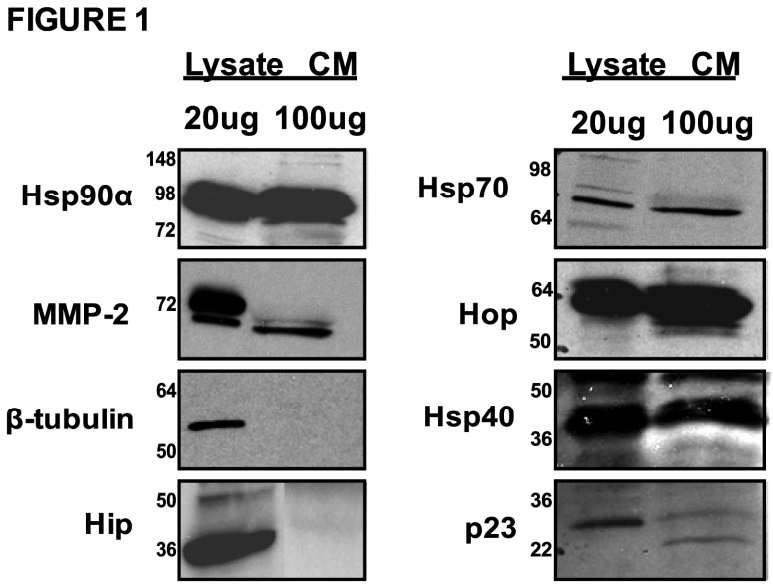


Figure 1. Hsp90α, MMP-2 and several co-chaperones are present in the conditioned media of breast cancer cells. Lysate and conditioned media were collected from MDA-MB-231 cells. 20 μg of protein was loaded into each lysate lane and 80 μg of protein was loaded into each conditioned media lane and immunoblotted for the Hsp90α, MMP-2, Hsp70, Hop, Hsp40, p23, Hip, and β-tubulin. The first six proteins were found to be present in both the cell lysate and in the conditioned media, while Hip was only found in the lysate. β-tubulin was used as a negative control. The molecular weight markers are indicated on the left.

Hsp90α interacts with Hsp70, Hop, Hsp40, p23, and MMP-2 both *in vitro* and in conditioned media.

To determine whether these extracellular co-chaperones can interact with Hsp90α, *in vitro* interaction was tested by co-immunoprecipitation with recombinant proteins and antibodies against Hsp70, Hop, Hsp40, p23, and MMP-2. Hsp90α was found to co-immunoprecipitate with each of these recombinant proteins (Fig. 2a). To determine if

extracellular co-chaperones interact with Hsp90α *in culture*, interaction was tested in conditioned media from MDA-MB-231 breast cancer cells. Confirming our previous findings (30) and our *in vitro* results, Hsp90α interacted with MMP-2 in MDA-MB-231 conditioned media. Hsp90α also interacted with Hsp70, Hop, Hsp40, and p23 (Fig. 2b). To further ensure that the observed interaction was specific, co-immunoprecipitation with Hsp90α was tested for fibronectin, a protein detected outside of MDA-MB-231 cells that is not known to interact with Hsp90α. As can be seen in Fig. 2c, fibronectin did not co-immunoprecipitate with Hsp90α. Together, these findings show that Hsp90α forms a complex with Hsp70, Hop, Hsp40, and p23, in addition to MMP-2, both *in vitro* and in breast cancer cell media (47,142).

FIGURE 2

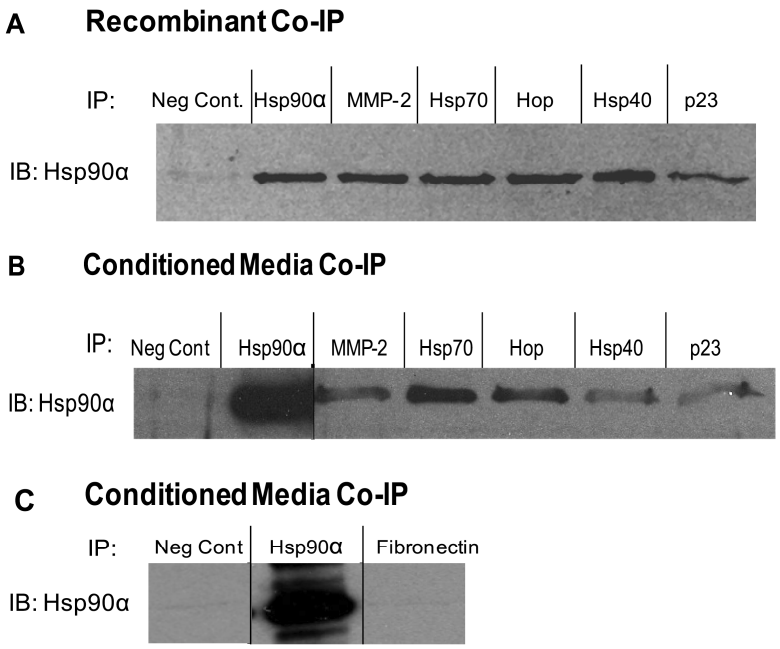


Figure 2. Hsp90α, MMP-2, Hsp70, Hop, Hsp40, and p23 interact *in vitro* and outside of the cell. (a) 1 μg Hsp90α, 0.5 μg MMP-2, 1 μg Hsp70, 0.25μg Hop, 0.1 μg Hsp40, and 0.25 μg p23 recombinant proteins were combined and the indicated IP antibody was added to the proteins. The protein complexes were pulled down using Protein G beads. The immunoprecipitate was then run on an SDS-PAGE and blotted for Hsp90α. (b and c) Conditioned media was collected from MDA-MB-231 cells and the indicated IP antibody was added to equal volumes of conditioned media. The resulting complex was then pulled down with Protein G beads and run on an SDS-PAGE and blotted for Hsp90α.

Hsp70, Hop, Hsp40, and p23 increase the interaction between Hsp90α and MMP-2, enhancing MMP-2 activation.

Intracellular Hsp90α relies on co-chaperones to recruit its client proteins and to assist in the formation of an activation complex (152). Consequently, the interaction between Hsp90α and its client proteins increases in the presence of these co-chaperones (47). To test if the co-chaperones Hsp70, Hop, Hsp40, and p23 enhance the interaction between Hsp90α and MMP-2, we performed co-immunoprecipitations with Hsp90α and

MMP-2 either alone or in the presence of the four co-chaperones that we previously determined were outside of the cell. Figure 3 shows that addition of the co-chaperones increased the interaction between Hsp90α and MMP-2 by 2.9 fold (Fig. 3, $p<0.01$). We observed a small amount of non-specific binding, which was accounted for in the densitometry calculations.

FIGURE 3

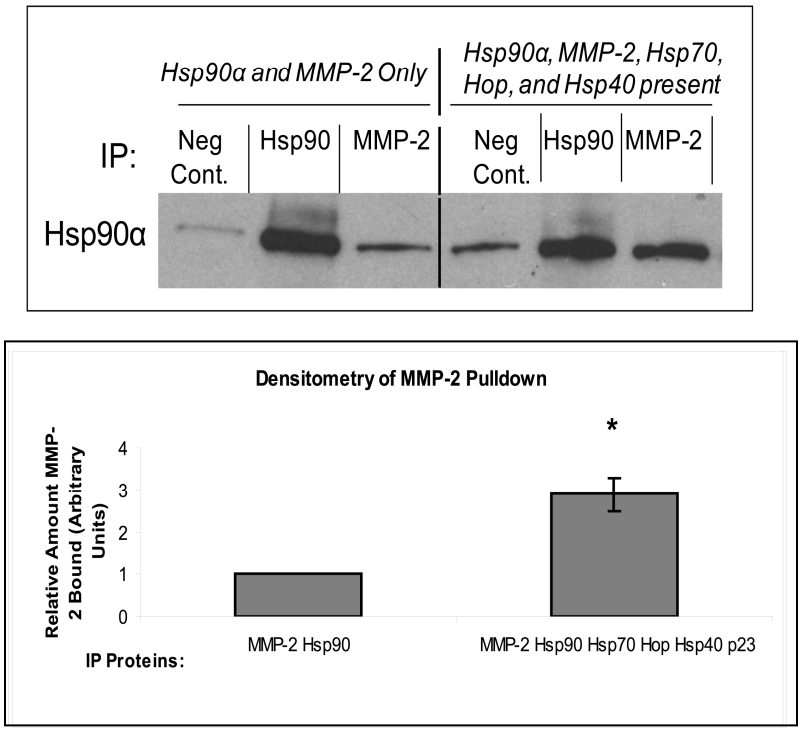


Figure 3. The presence of Hsp70, Hop, Hsp40, and p23 increase the interaction between Hsp90α and MMP-2. Using recombinant proteins, two co-immunoprecipitations were performed, one with Hsp90α and MMP-2 alone and one with Hsp90α, MMP-2, Hsp70, Hop, Hsp40, and p23 present using the same quantities as in Fig. 2. Hsp90α and MMP-2 primary antibody were used to pull down Hsp90α and then the resulting proteins were run on an SDS-PAGE and blotted for Hsp90α. The bands were then subject to densitometry to determine the relative interaction between Hsp90α and MMP-2 with and without the presence of the co-chaperones. Experiments were repeated three times. P-value <0.001

Previous studies on Hsp90α and MMP-2 activation were preformed with conditioned media, which contains many proteins including active MMP-2. It was not known if Hsp90α alone could activate MMP-2 *in vitro*. In the following experiments, MMP-2 activity was assessed by measuring the relative amount of digestion seen in a gelatin zymogram. When MMP-2 is activated in an *in vitro* environment three bands are observed: the 72kDa band is pro-MMP-2; the 64kDa band is an intermediate, active form that is generated during *in vitro* activation; and the 62kDa band represents the normal sized active MMP-2. Activation of MMP-2 in a completely *in vitro* environment, such as the above experiment, results in an active, 64kDa, isoform, as can be seen in Fig. 4a (118,153,154). We then investigated if pro-MMP-2 can be activated by Hsp90α, Hsp70, Hop, Hsp40, and p23 *in vitro*. When these proteins were added to pro-MMP-2, no activation was observed (Fig. 4). However, when we combined this mixture with a very small amount of activated MMP-2 (4pg), cleavage and activation of pro-MMP-2 occurred. We observed a significant increase in the generation of the 64kDa band (33% $p<0.05$) when Hsp90α and the co-chaperones were present, compared with MMP-2 alone (Fig. 4a). To show that Hsp90α and the individual co-chaperones are important in this activation, we removed Hsp90α, Hsp70, or Hsp40 from the complex and in each instance saw a significant decrease in the amount of activated MMP-2 (Supplemental Figure 1b). We also used a FITC-Casein assay to quantitatively measure the activation of MMP-2. We found the level of activation to be very similar (38%) to that detected in the zymogram (Supplemental Figure 1c).

These findings demonstrate that the added co-chaperones can assist in MMP-2 activation *in vitro* and suggests a role for the complex in facilitating active MMP-2

cleavage of pro-MMP-2. The amount of activation observed is modest (33%), perhaps because there are additional components in the extracellular media that further increase the efficiency of the MMP-2 activation *in vivo.* For example, while we observed *in vitro* activation of MMP-2 in the absence of ATP, ATP could potentially increase the rate of MMP-2 activation as it does for the intracellular client proteins of Hsp90α. Therefore, we tested if ATP could enhance activation by combining the Hsp90α-co-chaperone complex proteins as described above, with 100μM ATP, 100μM ATPγS or no ATP and measuring the amount of MMP-2 activity. We found that neither ATP nor ATPγS increased MMP-2 activity and that ATPγS decreased MMP-2 activity (Fig. 4b). These results indicate that ATP does not enhance Hsp90α-mediated MMP-2 activation. This supports our hypothesis that extracellular Hsp90α functions with co-chaperones to assist in the activation of MMP-2 and can do so independently of ATP.

FIGURE 4

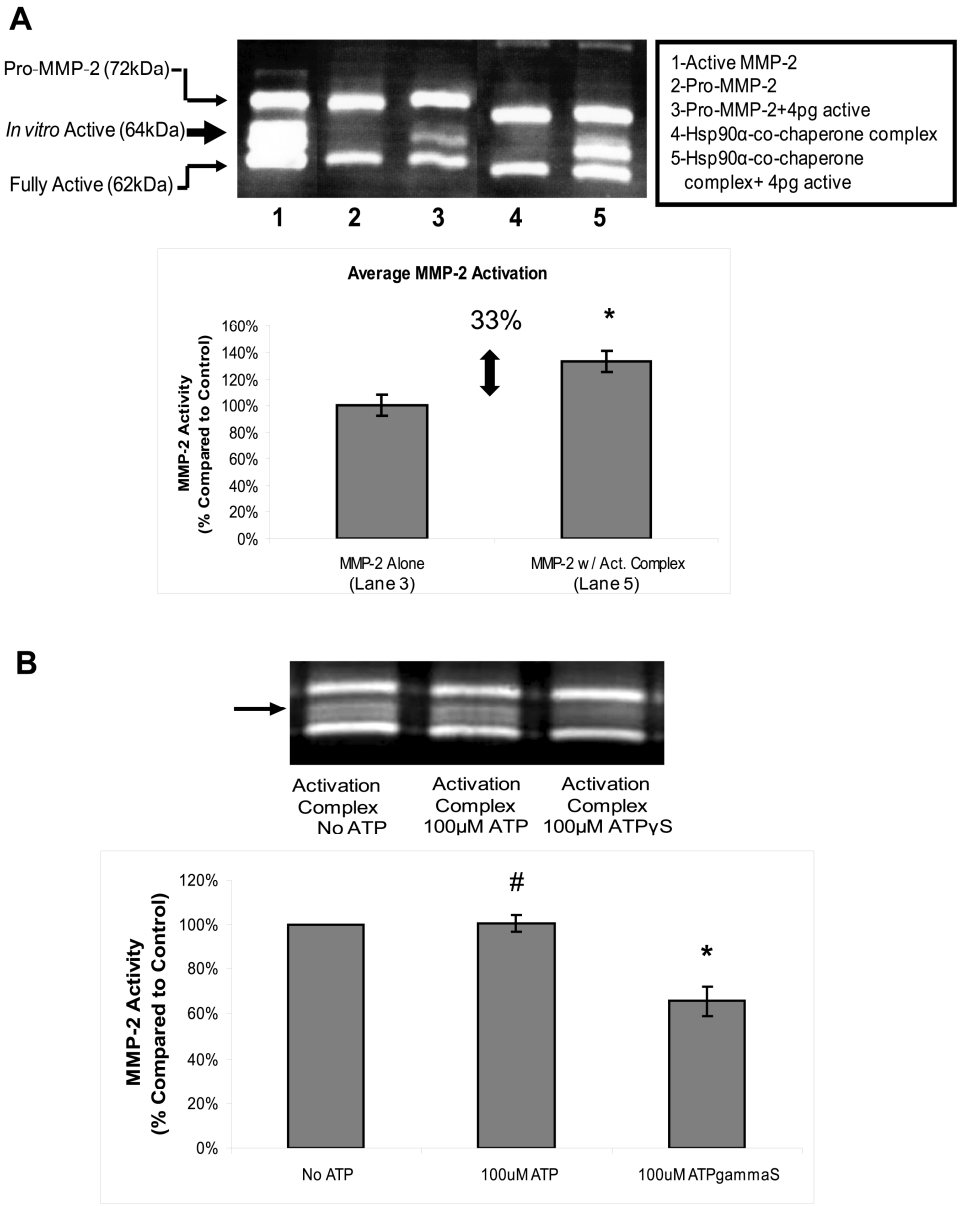


Figure 4. Hsp90α and co-chaperones Hsp70, Hop, Hsp40, and p23 are sufficient to activate MMP-2 *in vitro*. (a) 0.5 μg Pro-MMP-2 was incubated with and without 4 pg of activated MMP-2 and was incubated with 1 μg Hsp90α, 1 μg Hsp70, 0.25 μg Hop, 0.1 μg Hsp40, and 0.25 μg p23 recombinant proteins as indicated in the figure at 30ºC for 30 minutes. 0.5ug Pro-MMP-2 was activated in 1mM APMA at 37ºC for 2 hours (b) The same amounts of protein as above were incubated with 100 μM ATP, 100 μM ATPγS, or alone at 30ºC for 30 minutes. The proteins were then added to a non-reducing sample buffer and then run on a gelatin containing SDS-PAGE. The gels were renatured for 40 minutes, digested for 18 hours and stained with Coomassie Brilliant Blue. Hsp90α Chaperone Complex = Hsp90α, MMP-2, Hsp70, Hop, Hsp40, and p23. Experiments were repeated three times. (a) P-value<0.05 (b) #P-value>0.3, *P-value<0.05

Inhibiting Hsp70 reduces MMP-2 activation.

We next investigated whether the endogenous Hsp90α and co-chaperones present in the extracellular media of breast cancer cells can activate MMP-2. First, we demonstrated that the conditioned media collected from MDA-MB-231 cells contain the necessary components to activate MMP-2. In brief, recombinant pro-MMP-2 was added to PBS, untreated media, or MDA-MB-231 conditioned media. Then the amount of active MMP-2 was measured by SDS-PAGE and western blotted for MMP-2. Western blotting is a more accurate way of assessing the amount of active MMP-2 than zymography when comparing levels of active MMP-2 from samples which may contain TIMPs, which can interfere with MMP-2 activity (155). When cellular components are present, as in the conditioned media experiments in Fig. 5a, MMP-2 is processed to the fully active 62kDa band and the middle 64kDa band is usually not seen (156). As shown in Fig 5a, the MMP-2 added to the conditioned media has 3.5-fold ($P<0.01$) greater activity than MMP-2 added to PBS and 2.5-fold ($P<0.05$) greater activity than MMP-2 added to untreated media. The amount of active MMP-2 is much greater than the amount of MMP-2 present in the conditioned media (conditioned media alone, lane 1), so the increase in MMP-2 activity is not the result of endogenous MMP-2. The amount of MMP-2 in the control lane is significantly less than that shown in figure one due to a lower exposure time for the film and the use of less conditioned media in the blot.

We then tested if Hsp70, a component of the co-chaperone complex, contributed to the observed MMP-2 activation by using an Hsp70 inhibitor, Methylene Blue (83). When conditioned media was subject to Methylene Blue treatment, MMP-2 activation

was reduced by 53% ($p<0.01$) (Fig. 5b). These findings show that Methylene Blue can reduce MMP-2 activation in the conditioned media, suggesting a role for Hsp70 in MMP-2 activation in breast cancer cells. It is possible that other proteases could influence MMP-2 activation, but the significant decrease in MMP-2 activation when Hsp70 was inhibited suggests that the Hsp90α-activation complex plays an important role in this activation.

FIGURE 5

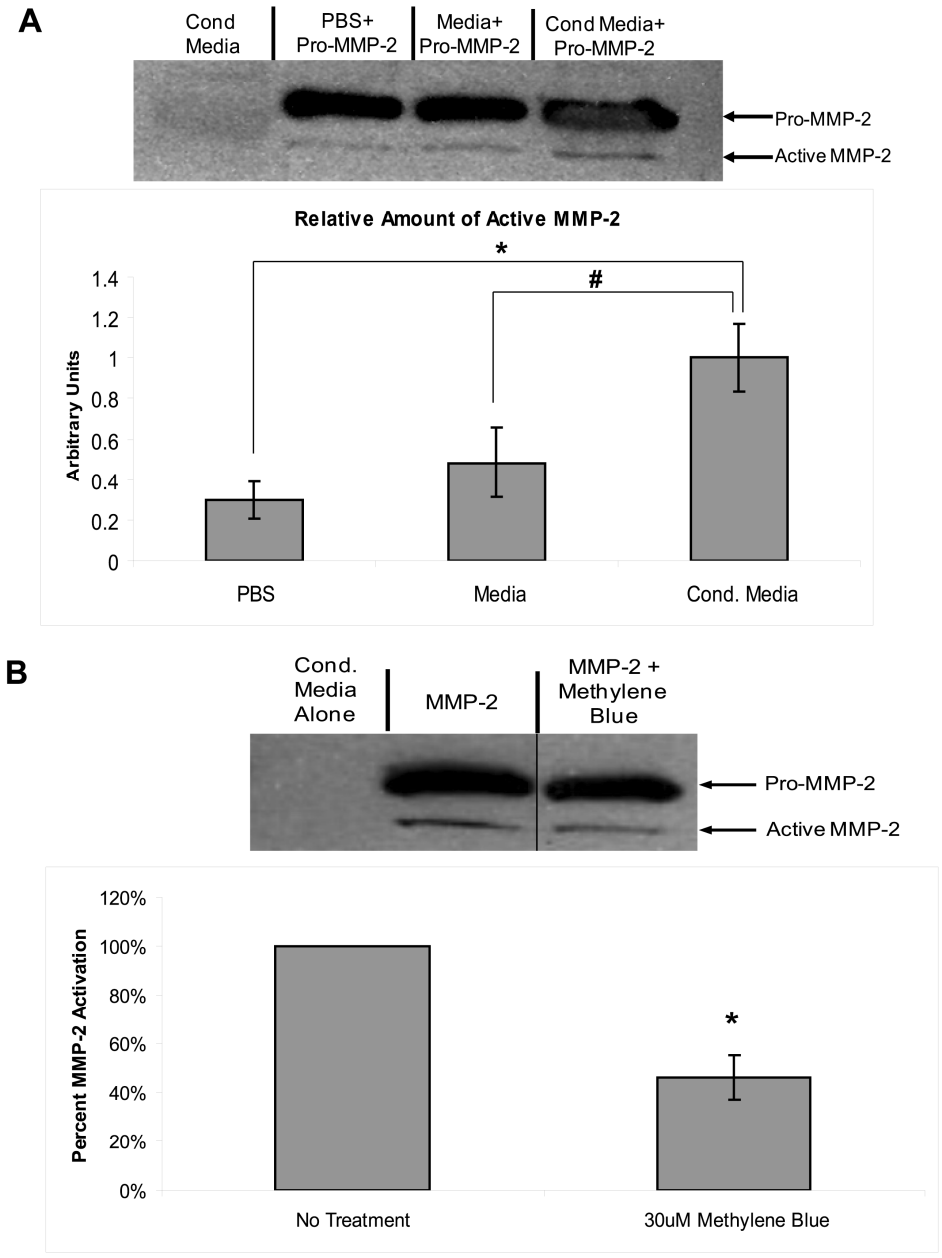


Figure 5. Conditioned media from cancer cells contains necessary components to activate MMP-2. (a) Conditioned media was collected from MDA-MB-231 cells, samples were incubated with and without 0.75 µg pro-MMP-2 for 5 minutes and the relative amount of active MMP-2 was analyzed via SDS-PAGE. (b) Conditioned media was collected and then treated with 30 µM Methylene Blue. 0.75 µg recombinant pro-MMP-2 was added to treated and untreated conditioned media. Samples were incubated at room temperature for 5 minutes and the relative amount of active MMP-2 was analyzed via SDS-PAGE. The difference in overall band intensity was taken into account in the densitometry calculations. Experiments were repeated three times. (* P-value<0.01, #P-value<0.05)

Extracellular Hsp90α and Hsp70 increase breast cancer cell migration.

We next investigated the ability of the Hsp90α activation complex, specifically Hsp70, to increase breast cancer cell migration using a wound healing assay. MMPs are central in cancer cell invasion due to their ability to digest components of the extracellular matrix, allowing cancer cells to intravasate into the bloodstream (157). MMPs have also been implicated in cancer cell migration. For instance, MMP-2 can cleave the adhesive contacts and cellular networks that cells use to adhere to their basement membrane, facilitating cell migration (106,158,159). Therefore, we hypothesized that extracellular Hsp90α and Hsp70 increase cancer cell migration. To test this, we performed wound healing assays with MDA-231-s4175 cells, a derivative of the MDA-MB-231 cell line. MDA-MB-231 cells move slowly in *in vitro* wound healing assays and are thus not suited for this assay, whereas the s4175 derivative of the MDA-MB-231 cell line is highly motile in this assay. The MDA-MB-231 s4175 cell line was selected for its ability to metastasize to the lungs in a mouse model by Massagué *et al*. (160) Prior to the wound healing assays we verified that all of the co-chaperones identified in the original MDA-MB-231 line were also present in this sub-line (Supplemental Figure 2). MMP-2 and all the co-chaperones examined are found at higher levels in the conditioned media of s4175 cells compared with media from the parent line, consistent with the high invasiveness of these cells. Wounds were created in monolayers of MDA-231-s4175 cells and incubated with either untreated media or conditioned media collected from other MDA-231-s4175 cells. Wound healing was measured after 16 hours. Cells that had been fed conditioned media migrated 16%

($p<0.01$) further than control cells that were fed with normal media (Fig. 6a and b). Therefore additional extracellular proteins present in conditioned media significantly increased the rate of cell migration. When we immunodepleted either Hsp90α or Hsp70 (Fig. 6c) the observed increase in migration from the conditioned media disappeared; the wound size did not significantly differ from the wound size of the control media-treated cells. Roles for Hsp90α and Hsp70 in conditioned media are further supported using inhibitors of these chaperones. When 17AAG or Methylene Blue (83) was added to the conditioned media there was a similar reduction in wound healing. To verify that the reduction in migration is due to the effect of Hsp90α on MMP-2 activation, and not Hsp90α affecting some other factor, we compared undepleted conditioned media treated with 40nM Batimastat (a pan-MMP inhibitor) with Hsp90α-depleted conditioned media treated with 40nM Batimastat to wounded cells. There was no significant difference in the reduction in migration between the two conditions, indicating that the effect of Hsp90α depletion and MMP inhibition is not additive (Fig. 6). A proliferation assay was performed to verify that the above conditions did not alter the cells' rate of proliferation (data not shown).

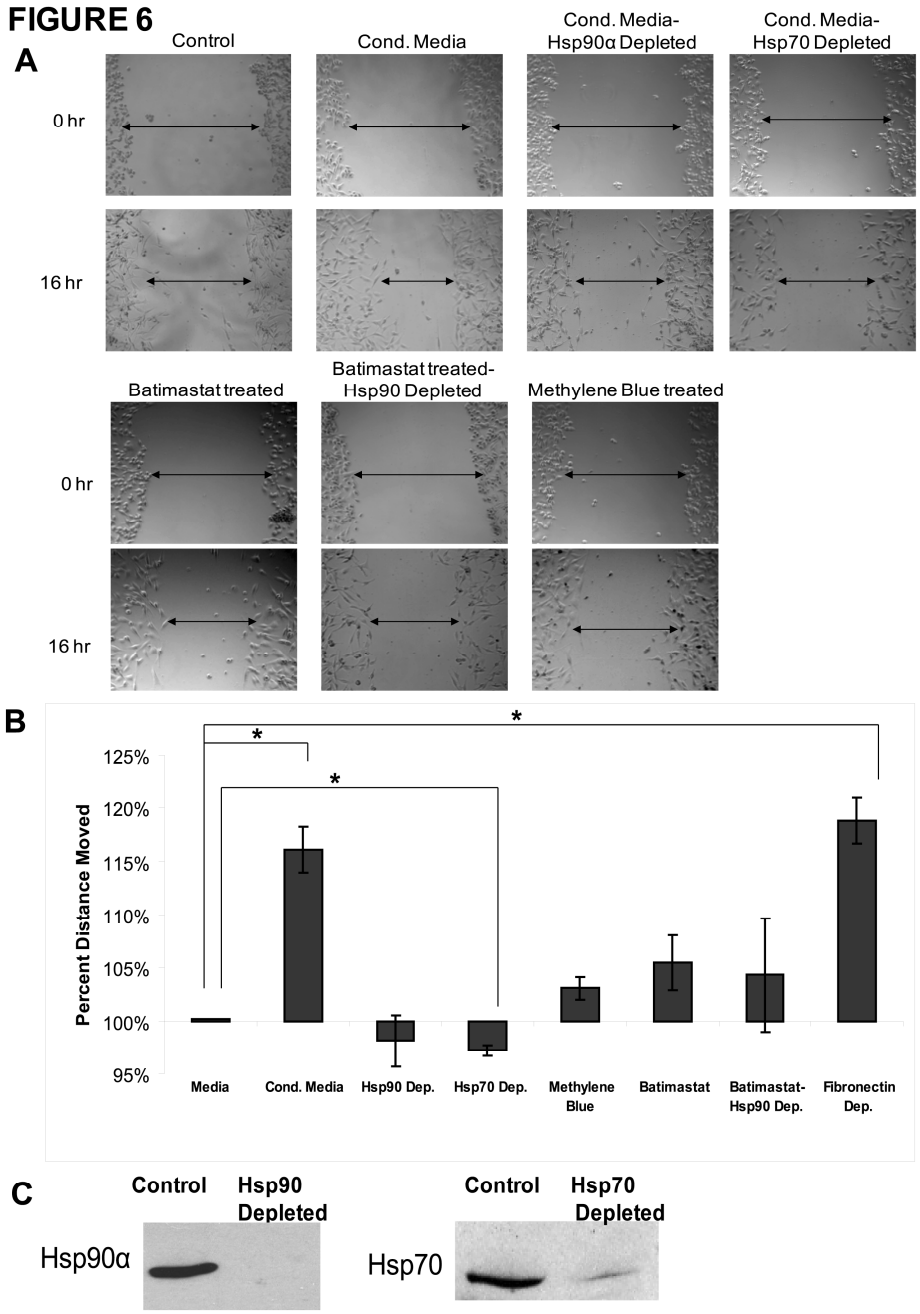


Figure 6. Hsp90α and its co-chaperone complex assist in wound healing. MDA-MB-231-s4175 cells were plated in an 8-well chamber slide and allowed to grow to confluency. A wound was created in the monolayer and either media, untreated conditioned media, Hsp90α depleted conditioned media, Hsp70 depleted conditioned media, 40 nM Batimastat (an MMP inhibitor) treated conditioned media, or 30 μM Methylene Blue (an Hsp70 inhibitor) treated conditioned media were added to the cells. The cells were incubated for 16 hours, and then wound closure was observed. (a) Representative pictures of wounded cells at 0 hours and 16 hours. (b) Graph of relative movement of cells. (c) SDS-PAGE showing Hsp90α and Hsp70 depletion. Experiment was repeated three times. (*P-value<0.01)

Inhibiting Hsp70 reduces breast cancer cell invasion.

Tumor cell invasion through the basement membrane and extracellular matrix that surrounds cancer cells is a key step in cancer cell metastasis and requires several cellular processes including migration and extracellular matrix remodeling (7,8). As Hsp70 increases Hsp90α-mediated MMP-2 activation and cell migration, we hypothesized that inhibition of Hsp70 would reduce cell invasion. Cells treated with 30ug/ml of Methylene Blue saw a 50% decrease in invasion ($p<0.05$). Cells treated with vehicle showed no decrease in invasion (Fig 7). Methylene Blue is cell-permeable, and can therefore also affect intracellular Hsp70. When cells in our invasion assay were treated with 40nM Batimastat, we observed a similar decrease in invasion as seen with the Hsp70 or Hsp90α inhibitors. Combining Batimastat with the Hsp70 or Hsp90α inhibitors, we saw additive effects on invasion. These findings suggest that while Hsp70 and Hsp90α affect migration primarily via MMP-2 activation, they may have other roles in invasion. This is consistent with recent literature demonstrating that other proteins required for invasiveness are activated by Hsp70 and Hsp90α (56,66). The difference between migration and invasion is not surprising as invasion requires additional cellular processes than migration. Taken together, these findings indicate that Hsp90α and Hsp70 assist in MMP-2 activation, which increases breast cancer cell migration and contributes to breast cancer invasion.

FIGURE 7

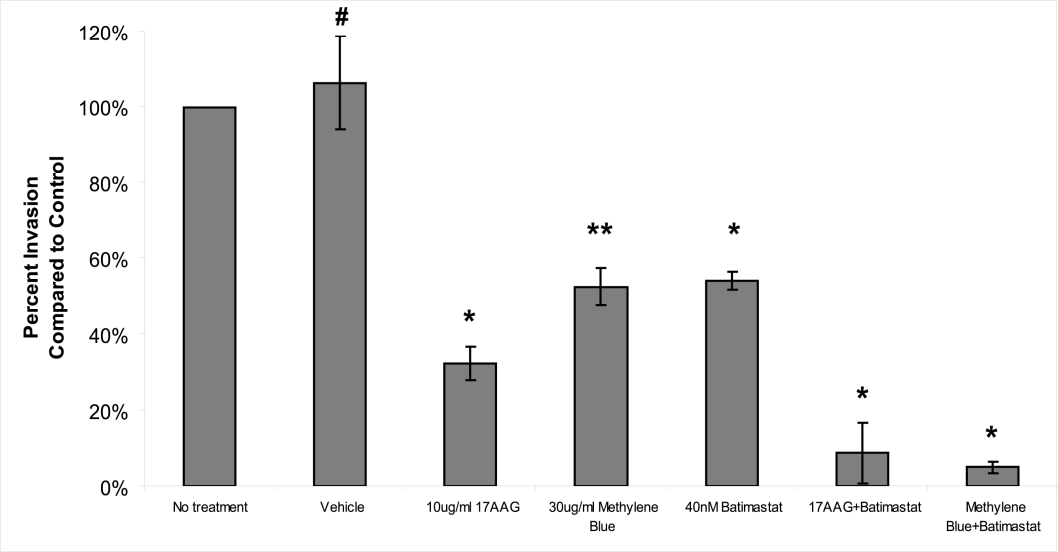


Figure 7. Inhibiting Hsp70 reduces breast cancer cell invasion *in vitro*. We performed invasion assays on MDA-MB-231 breast cancer cells to test the Hsp90α inhibiting compound 17AAG, the Hsp70 inhibiting compound Methylene Blue and the pan-MMP inhibitor Batimastat in a Chemotex invasion assay. We treated cells with 10 μg/ml 17AAG, 30 μg/ml water (vehicle), 30 μg/ml Methylene Blue or 40 nM Batimastat as indicated in the figure. Experiments were repeated three times. (*P<0.01,**P<0.05, #P>0.05)

Discussion

Extracellular Hsp90α has been implicated in activating a number of proteins important for disease- relevant processes (59). While the mechanism of Hsp90α activation of intracellular client proteins is well understood, this is not yet known for extracellular Hsp90α. Here we showed that extracellular Hsp90α binds to these co-chaperones both *in vitro* and in the conditioned media of MDA-MB-231 breast cancer cells. Moreover, the presence of these co-chaperones increases MMP-2 binding to Hsp90α and assists in MMP-2 activation *in vitro* and in conditioned media. Depletion or

inhibition of Hsp70 significantly decreases MMP-2 activation, migration and invasion. Together, these findings implicate Hsp70 as part of a co-chaperone complex that acts with Hsp90α in MMP-2 activation and contributes to breast cancer migration and invasion.

Recently, Song *et al*. (2010) showed that extracellular Hsp90α can stabilize MMP-2, a process important for angiogenesis (161). We did not observe the same MMP-2 degradation products as Song *et al.* indicating the identification of a different mechanism than the one described by their group (Supplemental Figure 3). The different cell types and processes addressed in the two studies suggest that there may be multiple mechanisms for MMP-2 activation that may depend on what other proteins are present extracellularly. In this paper we show that extracellular Hsp90α, in conjunction with co-chaperones including Hsp70, assists in MMP-2 activation in an ATP-independent manner. It is well established that intracellular Hsp90α works together with co-chaperones to activate its client proteins, and we provide evidence suggesting that extracellular Hsp90α functions in a similar way. Intracellular Hsp90α has a minimum number of co-chaperones required for the activation of its client proteins *in vitro*: Hsp70, Hop, Hsp40, and p23 (47,142). Here, we demonstrated that these co-chaperones are present outside of MDA-MB-231 breast cancer cells.

Our findings suggest a role for Hsp70 in MMP-2 activation, as well as in the migration and invasion of breast cancer cells. While Methylene Blue is cell permeable and, can thus also inhibit intracellular Hsp70, antibody-based immunodepletion experiments implicate extracellular Hsp70 in these cancer-relevant processes. While extracellular Hsp70 has been identified outside immune cells, where it activates both the

innate and adaptive immune system by interacting with antigen-presenting cells, (60) its role in cancer cells and invasion is not yet clear (123). Thus, our findings implicate a second chaperone, Hsp70, in MMP-2 activation and breast cancer cell migration and invasion. In addition, several of the other co-chaperones that we identified outside of MDA-MB-231 cells were shown to be in HT-1080 fibrosarcoma cell media (121). Similar to our results, this study also did not find Hip in HT-1080 conditioned media; however they also did not find Hsp70. The absence of Hip likely does not impinge on the proposed mechanism because it is not required for the activation of most Hsp90α client proteins (142). We tested for Hsp70 in HT-1080 conditioned media and did observe its presence (data not shown). The difference between these results is likely due to the use of better antibodies in the current study (Cell Cycle. 2010 Dec 1;9(23):4769).

Our studies have focused on MMP-2 in breast cancer cells, but extracellular Hsp90α may be important for other cancers as well (66). Many proteases that are secreted in their inactive form are likely required for invasion and Hsp90α may also activate these proteases. Recently, we identified plasmin as a second client protein for extracellular Hsp90α in breast cancer and glioblastoma conditioned media. Extracellular Hsp90α activates plasmin in conjunction with tissue plasminogen activator and annexin II (66). Hsp90α has been shown to be important in other cancers as well. Extracellular Hsp90α is upregulated in malignant melanomas (162), colorectal cancer (57) and fibrosarcomas (30), and is shown to increase heregulin-induced Her-2 activation and signaling (56). Outside of the cell, Hsp90α has been shown to have a role in eliciting a host immune response against various antigens, (163,164) to activate plasminogen in smooth muscle cells during oxidative stress, (165) and increase wound healing in human

dermal fibroblasts (122). Extracellular Hsp90α is also found in neurons and Schwann cells during development (166) and in TGF-alpha stimulated keratinocytes (63). While the mechanism of extracellular Hsp90α in these additional roles is not known, we speculate that the role we identified for Hsp90α and Hsp70 in MMP-2 activation may have bearing on the role of Hsp90α in these other processes.

Interestingly, we did not find ATP to be necessary for MMP-2 activation, even though it is known to increase the reaction efficiency for intracellular Hsp90α function. This may be due to the fact that all of the conformational changes Hsp90α undergoes in its chaperoning processes can be accessed without the presence of ATP. Furthermore, recent work indicates that ATP is not necessary for Hsp90α to carry out its chaperoning function (167). In addition we demonstrated that ATPγS decreased MMP-2 activity. This may occur because when ATP is bound by Hsp90α it must be hydrolyzed in order to evoke a conformation change to release its client protein. Binding a slowly hydrolysable form of ATP would reduce this transition and hence reduce the amount of MMP-2 activation. ATP has been found to be present outside of the cell in sub-micromolar concentrations as an extracellular ATP gradient (168,169); however, these low concentrations are unlikely to affect the function of extracellular Hsp90α. Our results are consistent with a recent finding that the extracellular Hsp90α stabilization of MMP-2 is ATP-independent (161). In addition, Hsp70, which we demonstrated to be an essential co-chaperone in the Hsp90α-mediated activation of MMP-2, is also known to be an ATPase. But as noted above, we were able to obtain MMP-2 activation without the assistance of ATP. There is little literature on the mechanism of Hsp70 outside of the cell and whether it functions with the assistance of ATP. Hsp70 has a high affinity for

ADP and therefore needs a high concentration of ATP to initiate the ATPase cycle which would be difficult to obtain outside of the cell. In addition, Hsp70 has been found to have a high level of conformational plasticity, especially in the nucleotide binding domain, possibly assisting in an ATP-independent extracellular function (72). Our results, combined with other findings regarding Hsp70 function, suggest the existence of an ATP-free mechanism for extracellular Hsp70. We previously demonstrated that Hsp90α is exported via exosomes, but it is not known how the other co-chaperones are exported (66). Hsp70 has been shown to be exported via exosomes in immune cells so it is possible that Hsp70 and the other co-chaperones are exported in a similar manner in breast cancer cells.

In this paper we identified an Hsp90α-co-chaperone complex that is necessary to activate MMP-2 *in vitro* and in cell culture. We identified the minimum components needed to obtain activation, but it is likely that there are other proteins that make the activation process more efficient. Since Hsp90α has been shown to activate other extracellular proteins (66,122), it would be interesting to determine if this activation complex exists in normal cells as well as cancer cells and if it assists in the activation of other extracellular client proteins. Hsp90α and Hsp70 may be found to chaperone or activate other proteins using a similar mechanism to the activation for MMP-2. As such, these extracellular proteins may be targets for discovering drugs to prevent breast cancer invasion and metastasis.

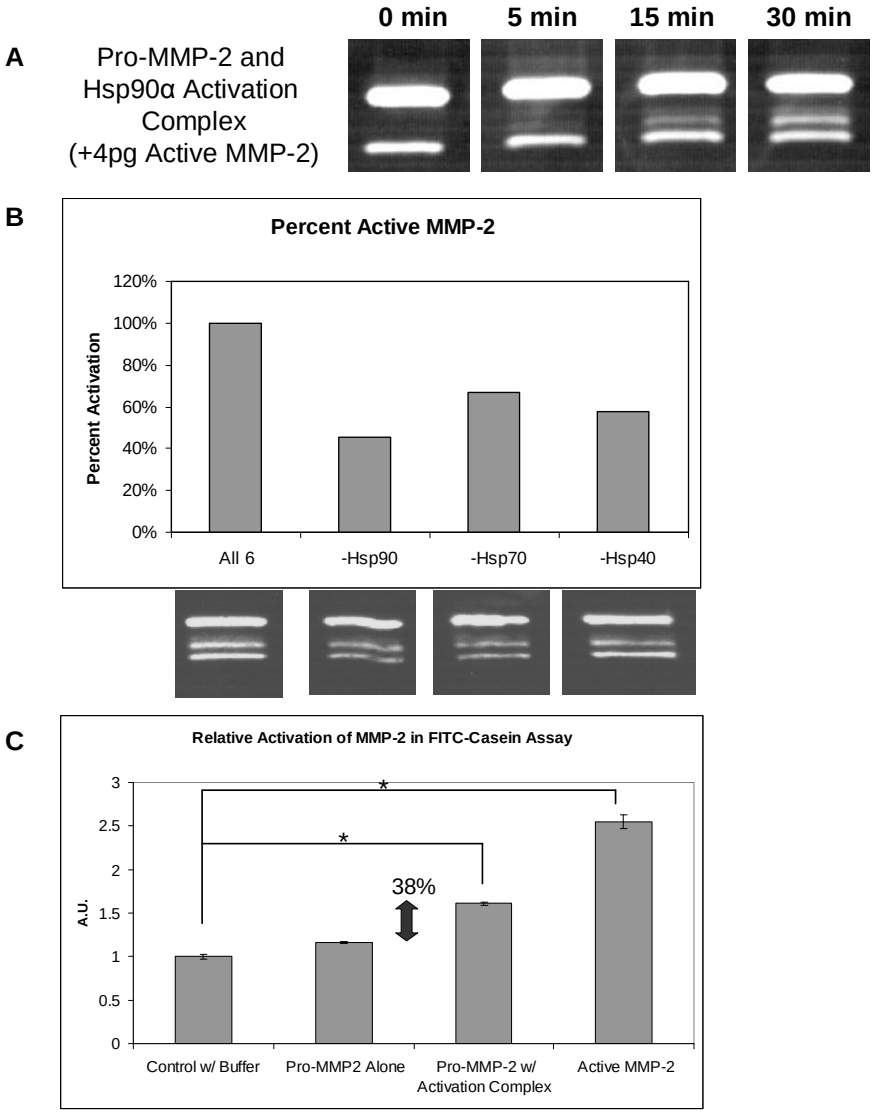
Acknowledgements

We greatly appreciate the gift of MDA-MB-231-s4175 cells from Dr. Joan Massagué (Memorial Sloan-Kettering Cancer Center, New York, New York). We would like to give our thanks to Dr. Michael Forgac, Dr. Gary Sahagian, and Dr. Charlotte Kuperwasser for their helpful discussion.

We would like to dedicate this manuscript in memory of Dr. J. Fred “Paulo” Dice, who made invaluable contributions to this project

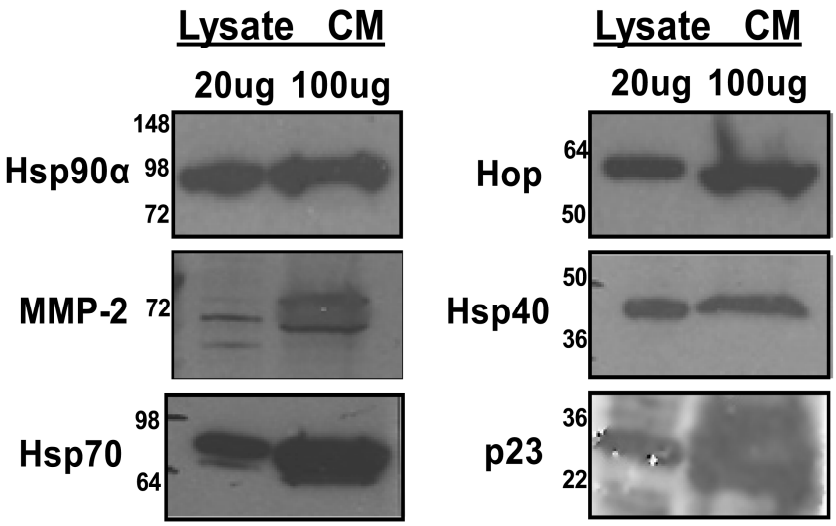
SUPPLEMENTARY FIGURES

SUPPLEMENTAL FIGURE 1



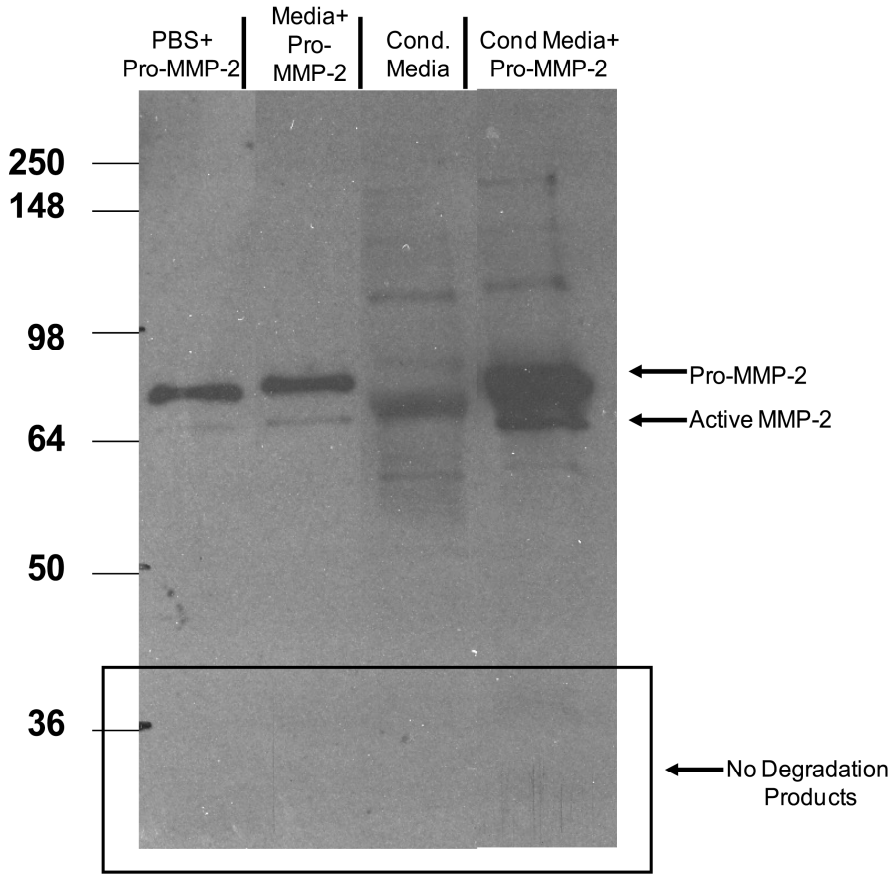
Supplementary Figure 1. (a and b) 0.5 μg Pro-MMP-2 and 4 pg of activated MMP-2 were incubated with 1 μg Hsp90α, 1 μg Hsp70, 0.25 μg Hop, 0.1 μg Hsp40, and 0.25 μg p23 recombinant proteins as indicated in the figure at 30ºC for 0, 5, 15, and 30 minutes. The proteins were then added to a non-reducing sample buffer and run on a gelatin containing SDS-PAGE. The gels were renatured for 40 minutes, digested for 18 hours and stained with Coomassie Brilliant Blue. Hsp90α Chaperone Complex = Hsp90α, MMP-2, Hsp70, Hop, Hsp40, and p23. (c) A FITC-Casein Assay was performed according to the manufactures directions except that the incubation buffer was substituted with 50 mM Tris–HCl, pH 7.5, 0.1 M NaCl, 10 mM $CaCl_2$, and 0.1% Brij-35. Results were normalized to the control. *P-value<0.01.

SUPPLEMENTAL FIGURE 2



Supplementary Figure 2. Lysate and conditioned media were collected from MDA-MB-231(s4175) cells. 20 μg of protein was loaded into each lysate lane and 80 μg of protein was loaded into each conditioned media lane and immunoblotted for the Hsp90α, MMP-2, Hsp70, Hop, Hsp40, and p23. The molecular weight markers are indicated on the left.

SUPPLEMENTAL FIGURE 3



Supplementary Figure 3. PBS, Serum-free media, or conditioned media collected from MDA-MB-231 cells were incubated with and without 0.75 μg pro-MMP-2 for 5 minutes at room temperature and the relative amount of active MMP-2 was analyzed via SDS-PAGE.

CHAPTER 4

DISCUSSION AND FUTURE DIRECTIONS

Summary

Eustace *et al.* previously identified Hsp90α outside of fibrosarcoma cells and demonstrated that Hsp90α increases cancer cell invasion through MMP-2 activation (30). In this thesis, I further investigated the mechanism of Hsp90α export from breast cancer cells and examined the role of extracellular Hsp90α and Hsp70-mediated activation of MMP-2 in breast cancer cell migration and invasion. The overall hypothesis of this dissertation is: extracellular Hsp90α is exported from breast cancer cells via exosomes, where, with the assistance of Hsp70, it increases MMP-2 activation and cancer migration and invasion.

In chapter 2 of this thesis I explore the mechanism of Hsp90α export with the goal of finding new ways of targeting the extracellular form of the protein. I demonstrated that Hsp90α is not exported via the canonical signal sequence pathway or in an isoform specific manner, even though we showed that two isoforms are present in breast cancer cells. In chapter 2 and the appendix, Dr. Jessica McCready and I found that Hsp90α is exported through the multivesicular body (MVB) and exosome pathway.

In chapter 3, I show that Hsp90α, in conjunction with Hsp70, assists in the activation of MMP-2 *in vitro* and in breast cancer cell conditioned media in an ATP-independent manner. Intracellular Hsp90α has been shown to function with the assistance of a host of co-chaperones that aid in its many roles (45,162,170-172). I hypothesized that extracellular Hsp90α operates with the assistance of co-chaperones, similar to its intracellular counterpart (45). I identified four co-chaperones in cancer cell conditioned media and, along with showing that they interact with Hsp90α, I demonstrated that their presence increases the interaction of Hsp90α with the proposed

client protein, MMP-2. In the presence of these co-chaperones, I showed that Hsp90α assists in MMP-2 activation *in vitro* and the depletion or inhibition of Hsp70, one of the four identified co-chaperones, decreases MMP-2 activation.

In chapter 3, I demonstrate that inhibition of Hsp90α or Hsp70 reduces breast cancer cell migration and invasion. The role extracellular Hsp90α plays in both of these processes make it a candidate for anti-metastatic treatments. Inhibitory compounds, such as function-blocking antibodies, are being tested for their ability to specifically inhibit extracellular Hsp90α function. In the appendix, I test a cell-impermeable function-blocking Hsp90α antibody as a potential anti-metastatic drug both in *in vitro* invasion assays and in a breast-to-bone murine metastasis model.

Metastasis causes the majority of cancer-related deaths, and currently, there are no curative treatments for metastasis (2). Determining what proteins are important in the metastatic process will enable the development of more effective inhibitors. In this dissertation I identify several proteins that are important in cancer migration and invasion and provide new targets for anti-metastatic drug development. This thesis explores a new mechanism for MMP-2 activation but also raises a number of questions regarding the function of extracellular Hsp90α and its extracellular co-chaperones. First, how are Hsp70 and the other co-chaperones exported from breast cancer cells and are they secreted individually or as part of an Hsp90α-bound complex? Are other co-chaperones, in addition to the four I identified, needed for efficient activation of MMP-2? Inside the cell, ATP is necessary for the function of Hsp90α (173-175), but chapter 3 of this thesis and the work of other groups have shown that extracellular Hsp90α can function without ATP (161). However, it is not known how extracellular Hsp90α accomplishes this.

Finally, the viability of extracellular Hsp90α inhibitors as a metastatic treatment needs to be further examined. Are the side effects demonstrated by the inhibition of intracellular Hsp90α reduced when extracellular Hsp90α is inhibited? Will extracellular inhibition of Hsp90α, either alone or in combination with other chemotherapies, significantly reduce cancer metastasis?

Extracellular Hsp90α

Hsp90α Splice Variants. Hsp90α was first shown to be present extracellularly in 1986 by Ullrich *et al.* and has since been found outside of many other cell types (55,59,60). However, prior to the initiation of this thesis, it was not known how Hsp90α was exported from cancer cells (61). In 2004, Picard noted the existence of an alternative Hsp90α splice variant with two additional N-terminal exons that contained an alternative start site and a putative signal sequence. He hypothesized that this splice variant was responsible for the export of Hsp90α (61,126). In chapter 2 of this thesis, Dr. McCready and I verified that both splice variants are transcribed by a number of cell lines. Interestingly, we found that the splice variant that contained the two extra exons, Hsp90AA1-2, was present in extremely small quantities in comparison to the other variant, Hsp90AA1-1. We also found that when normal and cancerous cell types were examined, the Hsp90AA1-2 splice variant was more abundant in non-cancerous and less invasive cell types. Consequently, these results did not support our hypothesis that the extra exon-containing isoform was responsible for the export of extracellular Hsp90α. We showed that Hsp90AA1-2 did not cause the export of Hsp90α and that Hsp90α is not

exported through the canonical signal sequence pathway. This was the first time that the two Hsp90α splice variants were shown to be expressed in breast cancer cells and that they were present in varying amounts depending on the invasiveness of the cells. Of note, these experiments demonstrated that both splice variants were transcribed but did not show that they were both translated into their corresponding proteins. Antibodies against the two extra exons would need to be produced in order to verify that the 12-exon splice variant was definitively being translated into its respective protein. If both variants are indeed transcribed into proteins, these findings suggest that the two splice variants may play different roles in cancer progression and could provide new anti-metastatic targets.

In this thesis, we demonstrated the presence of two Hsp90α splice variants in multiple cell lines. However, the role of each variant in cellular function was not elucidated. One way the larger Hsp90AA1-2 isoform may function is by negatively influencing cancer invasiveness. The N-terminus, the location of the two extra exons, is where the ATP binding site is located and where the majority of Hsp90α's conformational changes occur upon the binding of ATP (33,39) (Figure 1.4). The presence of the two extra exons may impede ATP binding or prevent the conformational changes that are necessary for Hsp90α to carry out its chaperoning activities. Hsp90AA1-2 could then function as a dominant negative protein by dimerizing with Hsp90AA1-1 and inhibiting its function. This hypothesis could explain why the extra exon splice variant is down regulated in invasive cancer cell lines. One way to test this hypothesis would be to knockdown the larger, Hsp90AA1-2 variant in less invasive cells and test for a change in invasiveness. The converse could also be performed by

upregulating Hsp90AA1-2 in invasive cancer cells and testing for a reduction in invasiveness. If these experiments prove inconclusive, another option would be to produce an antibody specific for the extra exons in Hsp90AA1-2 and use it to visualize the location of that variant in the cell. The presence of Hsp90AA1-2 in specific locations in the cell could provide a clue as to the role of this isoform. Alternatively, the extra exons could function as another binding site for Hsp90α co-chaperones or client proteins, thus enabling it to perform additional functions. Performing immunoprecipitation with the extra exons and then identification of any bound proteins by mass spectrometry could help identify any proteins that interact with the two extra N-terminal exons of Hsp90AA1-2. Determining the roles of these isoforms would help increase the understanding of the functions of Hsp90α in both normal cellular processes and in cancer progression, potentially providing novel targets for Hsp90α inhibition.

Export of Hsp90α. The experiments in chapter 2 confirmed that Hsp90α was not exported via the canonical signal sequence pathway, but was instead exported via exosomes. These experiments were based upon previous work that demonstrated Hsp70 was exported via the exosome pathway in immune cells (78,130). To our knowledge, this was the first time that Hsp90α had been demonstrated to be exported from breast cancer cells via exosomes. This finding demonstrates that the non-classical mechanism of exosome export, which is used by a variety of normal cells from reticulocytes to immune cells (176,177), also plays an important role in cancer progression. This result is intriguing due to the many potential roles of exosomes. As described below, the exosome pathway can provide cancer cells with a way to communicate with surrounding cells, reminiscent of immune cells (177), and a way in which to export proteins that can

increase proliferation and motility, as seen in epidermal cell wound healing (122). We demonstrated that cancer cells use exosomes to export proteins that participate in cancer migration and invasion (chapter 2 and appendix). Skog *et al*. (2008) demonstrated that cancer cells are able to communicate with other cells by incorporating an mRNA reporter into exosomes and demonstrating that the message was delivered to, and translated by, the recipient cell (177). Targeting cancer cells with exosome-reducing compounds, such as the exosome-inhibitor amiloride (178), would allow us to test if inhibiting exosomes reduces cancer cell communication, reduces the viability of the tumor as a whole, and inhibits migratory and invasive phenotypes. Amiloride is primarily used as an inhibitor of Na^+/H^+ antiporters and thus has numerous other effects on cells such as inhibiting growth and proliferation (179). Due to these effects any results obtained from these experiments could not be definitively attributed to exosomes . These results could provide important clues to the importance and function of exosomes in cancer progression. Research has indicated that exosomes contribute in multiple ways to the complexity of cancer metastasis, and these results would further verify that inhibiting their release could prove to be a powerful tool in better understanding the role of exosomes in both cancerous and normal cells.

Other Modes of Export. In this thesis, we demonstrate that Hsp90α is exported by exosomes; however, we did not eliminate the possibility that Hsp90α is also exported through other non-canonical pathways. Indeed, Triantafilou *et al*. demonstrated that Hsp90α can be exported in a lipid-raft dependent pathway (180) suggesting the existence of other non-classical pathways for Hsp90α export. There are a number of pathways Hsp90α could be exported through (62,127), and the multiple functions that have been

identified for extracellular Hsp90α could contribute to the need for the additional regulatory control that would be provided by multiple export mechanisms (59,60). Depending on its role outside of the cell, Hsp90α could be exported through a number of different pathways. These pathways include membrane flip-flop, (likely with the assistance of phosphatidylserine (PS)), plasma membrane resident transporters, lysosomal secretion, and membrane blebbing (62,127). Before testing these alternate pathways we would first determine the amount of Hsp90α that is exported via exosomes by treating cancer cells with the exosome inhibitor amiloride (178). If the relative level of exosome reduction is not equivalent to the level of decrease in extracellular Hsp90α, Hsp90α is likely exported by additional mechanisms. The above mentioned export mechanisms could be tested as follows. The possibility of Hsp90α being exported via the PS/flip-flop mechanism could be tested by treating cells with the flippase inhibitor Pyridyldithioethanolamine (PDA), and then testing for a reduction in extracellular Hsp90α (181). Export via lysosmal secretion could be tested by overexpressing the Longin Domain of TI-VAMP as described by Proux-Gillardeaux at al. (182). Lastly, the importance of membrane blebbing could be examined by reducing membrane blebbing with the ROCK inhibitor Y27632 (183) and testing for extracellular Hsp90α levels. If Hsp90α is found to also be exported by one or more of these pathways, it would be valuable to determine which, if any, of these pathways are upregulated in cancer progression. Those pathways could then be used as drug targets for the inhibition of Hsp90α export and reduction of cancer progression.

Targeting to the Exosome Pathway. This thesis, and work from other researchers, demonstrates that Hsp90α is exported from breast cancer cells via exosomes, but it does

not show how Hsp90α is targeted to the exosome pathway (66,123,184). There have been multiple hypotheses as to how various proteins are targeted to the multivesicular/exosome pathway. One potential targeting mechanism is through the phosphorylation of specific amino acids, which are then recognized by various proteins in the exosome pathway. In support of this theory, Bao *et al.* demonstrated that threonine phosphorylation by protein kinase C (PKC) targeted EGFR for recycling to the cell surface (185). In addition, Wang *et al*. demonstrated that a phosphorylation site at Threonine-90 (Thr-90) in the N-terminal domain of Hsp90α is important in regulating the export of Hsp90α (65) (Figure 4.1). Hence, the phosphorylation of Thr-90 by PKC, a protein known to associate with Hsp90α (186), could function to target Hsp90α to the multivesicular body (MVB) for export via exosomes. In order to determine if phosphorylation of Thr-90 not only targets Hsp90α outside of the cell, but to the exosomal pathway, a similar mechanism could be used as described in (65). Briefly, Thr-90 would be mutated to alanine in a wild type construct producing a T90A mutant. Exosomes from wild type and T90A mutant cells would be examined for the presence of Hsp90α. If the Thr-90 phosphorylation site is required for exosomal targeting, Hsp90α would no longer be present in the exosomes from the T90A mutant. In addition to this particular site, there are a number of other potential phosphorylation sites on Hsp90α that could also target Hsp90α to exosomes (Figure 4.1). Similar to the above experiment, each of the lysines at these sites could be mutated to glutamine, as demonstrated by Yang *et al*., and the exosomes of those cells examined for the presence of Hsp90α (187). Finally, another way that Hsp90α could be targeted to exosomes is through mono-ubiquitination. It has been shown that mono-ubiquitination and oligo-ubiquitination

participate in targeting proteins to the endosomes and the MVB (138,188,189), making it reasonable to hypothesize that Hsp90α could be targeted to the MVB and exosomes using a similar mechanism. The E3 ubiquitin ligase, CHIP, interacts with Hsp90α and targets Hsp90α substrates for degradation through ubiquitination (190). In addition, CHIP has been shown to ubiquitinate Hsp90α, which has several potential ubiquitination sites, any of which could be used to target Hsp90α to the MVB pathway for export (139) (Figure 4.1). In order to determine if any of these potential ubiquitination sites are important in targeting Hsp90α to the MVB, each of the sites would be individually mutated to arginine to prevent the site from being ubiquitinated (191). Exosomes collected from those cells would then be tested for the presence of Hsp90α. If any of these ubiquitination sites are vital for Hsp90α export, a reduction in extracellular Hsp90α will be observed.

One additional way Hsp90α could be targeted to exosomes is by piggybacking with other proteins (such as client proteins) that are targeted to the exosomes. Hsp90α has been shown to associate with over 200 client proteins, some of which may be exported from cancer cells via exosomes (34). In general Hsp90α requires a complex of chaperones to assist in its binding to other proteins, and its MEEVD domain, located at the C-terminus plays, a key role in this interaction. One way to test if Hsp90α uses client proteins as a way to reach exosomes would be to mutate or remove the MEEVD domain. Without this domain, Hsp90α would be unlikely to bind to other client proteins and it would be found in much lower quantities in exosomes if it is dependent on this mode of export. However, this scenario is unlikely due to data shown by Wang et al. that not only is Hsp90α exported without its MEEVD domain, but that removal of this domain increases export of Hsp90α from the cell (65).

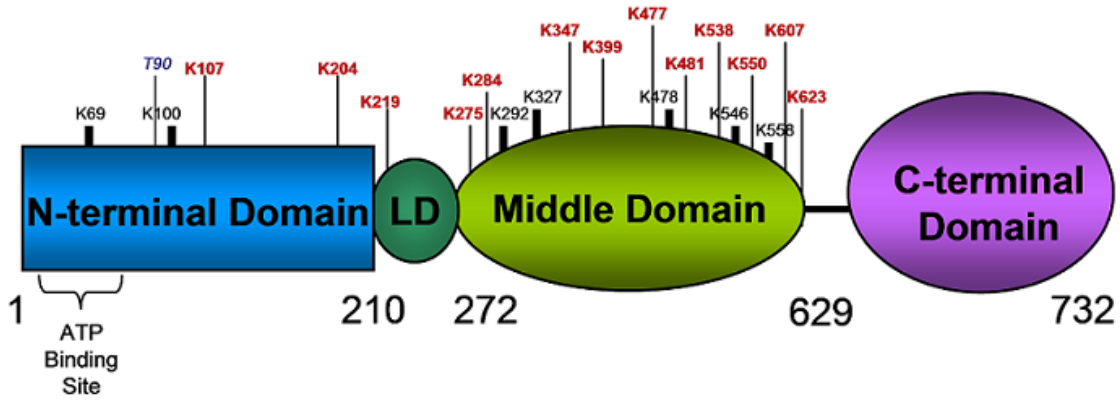


Figure 4.1-Diagram of ubiquitination and phosphorylation sites on Hsp90α. The sites labeled in black represent potential phosphorylation sites (187) and the sites labeled in red represent potential ubiquitination sites (139). The tyrosine labeled in blue has also been implicated in Hsp90α export, though not specifically in targeting Hsp90α to exosomes (65). Figure adapted from (65,139,187)

Hsp90α Co-Chaperones

In addition to the export of Hsp90α, Eustace *et al.*, and our data in chapter 3, indicate that several Hsp90α-associated co-chaperones are present outside of breast cancer cells (121). Each of these co-chaperones has been reported to be outside other cells types, strengthening our finding that all four co-chaperones are present outside of breast cancer cells (96-98). The data presented in chapter 3 is the first time that these four co-chaperones have been shown to be outside of one cell type. The presence of these co-chaperones, which are the minimum required components for the Hsp90α-mediated activation of hormone receptors, suggests that extracellular Hsp90α may be functioning in a similar manner to intracellular Hsp90α and requires the assistance of various co-chaperones to perform its chaperoning functions (45,49). This finding suggests that extracellular Hsp90α may have a number of client proteins outside of the

cell, with each client requiring its own cohort of co-chaperones proteins. In this thesis, two of these client protein were identified, MMP-2 and plasminogen. Both of these proteins are proteinases that are secreted as pro-proteins and require activation once outside of the cell (192,193). One important role Hsp90α may play outside the cell is the activation of various pro-proteins. It would be of benefit to see what other proteins Hsp90α chaperones in order to better understand its extracellular functions.

In this thesis we identified four co-chaperones outside of cancer cells, but there is likely a number of other co-chaperones present extracellularly and the compliment of extracellular co-chaperones likely varies with cell type to suit the extracellular needs of that cells. It would provide valuable information about the function of extracellular Hsp90α to further investigate the presence of other co-chaperones outside of a variety of cell types. The presence of additional co-chaperones would enable Hsp90α to bind to a wide variety of these client proteins. In addition, these co-chaperones represent additional targets for drug development that could inhibit the function of Hsp90α and its activation complex.

Co-Chaperone Export. This thesis did not address how the four co-chaperones are exported from breast cancer cells. The co-chaperones do not have known signal sequences indicating that, like Hsp90α, their export is also occurring through a non-canonical signal sequence pathway. Currently, there is only data indicating a mode of export for Hsp70, which is also exported through exosomes (78,80). There is no data as to how the other co-chaperones, Hop, Hsp40, and p23, get outside of the cell, but it would be logical to conclude that they are being exported in a similar manner to Hsp90α

and Hsp70. Since these co-chaperones are often found in a complex inside the cell it is possible that they are exported as a complex as well. While inside the cytosol, these components could form a complex with Hsp90α, which could then be targeted to the multivesicular body/exosome pathway by phosphorylation or ubiquitination, as discussed above, thus enabling the export of the rest of the complex. Alternatively, each of these co-chaperones could be ubiquitinated or phosphorylated and hence individually targeted to the MVB. To determine the mechanism of co-chaperone export, I would first verify that the co-chaperones are present in exosomes by performing large scale exosome collections and testing for each co-chaperone. If they are detected in exosomes, I would next determine if the co-chaperones are targeted to the exosomes by ubiquitination. First, any potential ubiquitin binding sites on the co-chaperones would be determined. As described by Kundrat *et al*., I would perform *in vitro* ubiquitination reactions on each co-chaperone and identify any modified lysines with tandem mass spectrometry (139). Once potential ubiquitination sites have been identified, each site would be systematically mutated to arginine (191). Cells with the mutated lysines would be tested for the presence of the co-chaperones in the exosomes. If these proteins are not targeted to exosomes by ubiquitination, the possibility of phosphorylation-based targeting could be explored in a similar manner.

The presence of four well-characterized Hsp90α co-chaperones outside of the cell makes it likely that other co-chaperones are also present extracellularly. If this is so, it could expand Hsp90α's functions outside of the cell by enabling it to bind to additional client proteins. To our knowledge, the work described in this thesis is the first time that all of these co-chaperones have been shown to interact extracellularly and for Hsp70 to

assist Hsp90α in an extracellular chaperoning function. These findings could be used to further elucidate the role of extracellular Hsp90α in cancer migration and invasion and to design novel inhibitors of these processes to reduce cancer metastasis.

Hsp90α-mediated activation of MMP-2. In this thesis we describe a novel chaperoning function for extracellular Hsp90α. In chapter 3, we showed that Hsp90α binds to the co-chaperones Hsp70, Hop, Hsp40, and p23, as well as MMP-2, both *in vitro* and in the conditioned media of breast cancer cells. In addition, the interaction of Hsp90α and MMP-2 increased when the above four co-chaperones were present (Figure 3.3). We also demonstrated that Hsp90α, in conjunction with Hsp70, assists in the activation of MMP-2 (51). With the data we obtained from chapter 3 of this thesis, we proposed the following Hsp90α-mediated activation of MMP-2. First, Hsp70 and Hsp40 recruit MMP-2 (45,100). Hop then binds to Hsp90α and provides a scaffold for the binding of the Hsp70-Hsp40-MMP-2 complex (45). P23 binds to the N-terminus of Hsp90α (46), causing a conformational change in MMP-2, allowing access to the catalytic zinc molecule and the cysteine that binds the pro-domain to this zinc. Hsp70 and Hsp40 disassociate from the complex and an active MMP-2 comes in and perturbs the cysteine bond, activating the catalytic zinc. The now active "pro-MMP-2" self-cleaves, removing the pro-domain and obtaining the standard-sized 62kDa active MMP-2. The newly activated MMP-2 is released from the Hsp90α complex (See Figure 4.2).

In this thesis we demonstrated that Hsp70 is indeed important in the Hsp90α-mediated activation of MMP-2, but even though we showed the presence of the other three co-chaperones, we did not definitely demonstrate that they are necessary for the activation process. One way to verify this would be to perform FALI on each of the

components in an *in vitro* MMP-2 activation experiment to determine if inactivating one or more of the proteins caused a reduction in the activation of MMP-2. This experiment would be carried out as described by Beck *et al* (31). This same experiment could also be used to inhibit specific proteins in the conditioned media of breast cancer cells prior to the addition of exogenous pro-MMP-2 to see if a reduction in the activation of the pro-MMP-2 is observed. This would verify that each of these co-chaperones is indeed necessary for the activation of MMP-2 both *in vitro* and in conditioned media.

While these findings are interesting, they are unlikely to be the whole story. Even though I determined that the above co-chaperones are sufficient for MMP-2 activation, it is likely that other co-chaperones and proteins assist in MMP-2 activation. In my *in vitro* experiments for MMP-2 activation, I obtained only a 33% to 38% increase in activation with the presence of Hsp90α and the co-chaperone complex. While significant, the level of activation could be much higher. Identifying other components of this complex could prove informative and provide additional anti-Hsp90α drug targets. One way to determine if other proteins are involved in the complex would be to use co-immunoprecipitation to identify other proteins that bind to each of the members of the activation complex and then mass spectrometry to identify the associated proteins. Any proteins found could be tested to see if they increase the rate of the Hsp90α-mediated activation of MMP-2 *in vitro* or if their inhibition in conditioned media reduces MMP-2 activation.

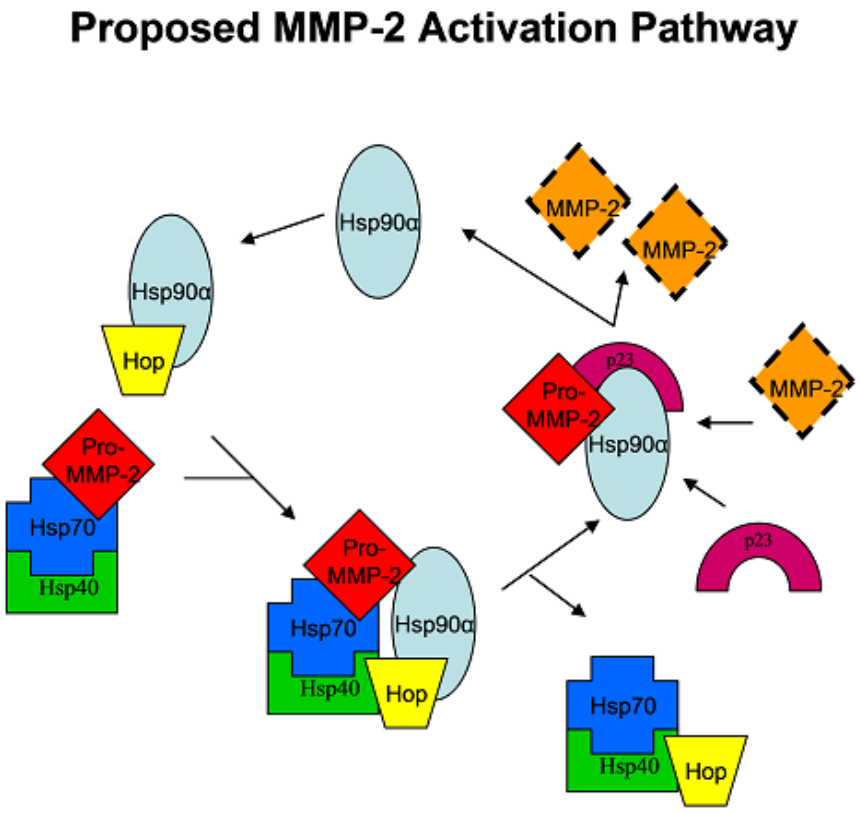


Figure 4.2-Proposed MMP-2 activation pathway. This pathway is modeled after the pathway proposed by Cintron *et al.* for the intracellular activation of the steroid hormone receptor (45).

Extracellular ATP

In this thesis, we also demonstrated that extracellular Hsp90α functions without ATP. This is interesting due to intracellular Hsp90α primarily requiring ATP to perform its chaperoning processes (173-175). In addition, Hsp70 also requires ATP to carry out its chaperone functions (74). ATP levels are extremely low outside of the cell, so it would seem unlikely that Hsp90α or Hsp70 would be able to function with any significant level of activity if they required the assistance of ATP (168,169). The data in chapter 3 that shows extracellular Hsp90α functioning independently of ATP is supported

by findings by Song *et al*. who also demonstrated an ATP-independent role for extracellular Hsp90α in the stabilization of active MMP-2 (161).

However, there are several potential issues with the ATP data obtained in Chapter 3. ATP was added to the MMP-2 activation assay and was shown to not affect MMP-2 activation, but we did not verify that ATP was still present throughout the activation process. All of the ATP could have been rapidly converted to ADP, resulting in us not detecting a change in MMP-2 activation. In order to definitively demonstrate that Hsp90α does not require ATP for the activation of MMP-2 we would need to add an ATP-regenerating system to the activation buffer similar to that described in (47). We would then use an ATP assay kit to demonstrate that ATP is still present at the end of the activation process (194).

To further test the role of ATP in the Hsp90α-mediated activation of MMP-2, we could overexpress both wild type Hsp90α and a mutant form of Hsp90α that is unable to bind ATP, as described in (195). We would then test the ability of both cells to activate exogenous pro-MMP-2. If ATP is not necessary for the activation process, there should be no difference in the level of MMP-2 activation between the wild type cells and the cells with the mutant Hsp90α. However, if there is a reduction in activation, Hsp90α may be dependent upon ATP to activate MMP-2 *in vivo*. If this is the case, Hsp90α may be exported from cells with ATP already bound to its ATP binding site. We could test for this possibility by probing for the presence of ATP in exosomes using SDS-PAGE and western blotting.

There is evidence for Hsp90α being able to function without the presence of ATP (167), however there is no precedent for Hsp70 functioning without ATP. One way

Hsp70 may get around this issue is by being exported from the cell with ATP already bound in its binding site, enabling it to function extracellularly. Similar to Hsp90α, we could use an Hsp70 mutant that is unable to bind ATP to test if ATP is crucial to Hsp70's ability to assist in the activation of MMP-2. An Hsp70 mutant, as described by Milasrski *et al.* (196) could be overexpressed along with its wild type counterpart in cancer cells and the relative amount of activation of MMP-2 measured. If there is no decrease in activation, Hsp70 likely does not need ATP to function. The ability of extracellular Hsp90α and Hsp70 to function independently from ATP is a novel finding. It implies that even though some of the functions of extracellular Hsp90α are similar to its intracellular counterpart, extracellular Hsp90α functions differently in how it chaperones and activates its client proteins. Most Hsp90α-inhibitors function by binding to the ATP site and preventing Hsp90α from interacting with ATP. If ATP binding is not required for Hsp90α function, these drugs may not be as effective extracellularly as they are intracellularly.

Other Extracellular Roles of Hsp90α

Hsp90α has long been identified as an important protein in cancer progression. Its chaperoning abilities help maintain the proper conformation of proteins in what would otherwise be a toxic cell environment (43,54). Even so, the identification of Hsp90α as an important player in migration and invasion is a relatively new development. Several examples of the roles of extracellular Hsp90α in cell migration have been described, including a role in dermal cell wound healing and in the developing nervous system (122,166). But to our knowledge we were the first group to identify two proteins, MMP-

2 and plasminogen, that are activated by Hsp90α and increase cell migration. These results open a new opportunity for the inhibition of cancer migration. Inhibiting the ability of cells to move away from the primary tumor could reduce the formation of cancer metastases and increase the time to progression for cancer patients. In addition to migration, MMP-2 has been shown to be important in increasing cancer growth through the release of growth factors and similar proteins from the extracellular matrix (106,157). Hence, inhibiting MMP-2 activation through Hsp90α inhibition would not only reduce cancer migration, but would help to inhibit the growth of the primary tumor by reducing the growth signals it receives from the extracellular milieu.

Another important part of cancer metastasis is invasion. Eustace *et al.* was first to demonstrate a role for Hsp90α in cancer invasion and implicate the interaction of Hsp90α with MMP-2 in causing this effect (30). Since that finding, Hsp90α has been shown to influence cancer invasion in a number of ways, such as increasing α-V-integrin expression and affecting HER-2 activation and signaling (56,57). The roles that have been identified for extracellular Hsp90α are likely only a small subset of the full complement of Hsp90α client-protein interactions that exist in the extracellular milieu. Hsp90α has over 280 client proteins inside of the cell (34), so it would be reasonable to suppose that it also has a large number outside of the cell. One way to identify new Hsp90α client proteins is through co-immunoprecipitation and mass spectrometry. This method was already employed to identify the interaction between Hsp90α and plasminogen and would likely result in the identification of additional Hsp90α client proteins (66) (appendix). Use of this method in different cell types, both normal and cancerous, would likely yield a wide variety of different client proteins and provide clues

to the different extracellular functions of Hsp90α. Obtaining a better understanding of the proteins that Hsp90α chaperones outside of the cell will help us understand the effects that extracellular Hsp90α inhibitors may have on cells and perhaps assist in the development of more effective and specific inhibitors.

MMP-2 Activation

The first MMP-2 activation mechanism that was identified involved another member of the MMP family, Membrane Type 1 (MT1)-MMP and the associated protein tissue inhibitor of metalloproteinases-2 (TIMP-2) (193,197). TIMP-2 has a unique role in MMP-2 activation in that it possesses a concentration-dependent affect on MMP-2 activation (117). At lower concentrations, TIMP-2 associates with MT1-MMP and assists in the activation of MMP-2. While at higher concentrations, TIMP-2 sequesters MMP-2 from MT1-MMP and prevents its activation. In addition to the MTI-MMP/TIMP-2 mode of activation, Morrison *et al.* demonstrated that MMP-2 could be activated without the presence of MT1-MMP or TIMP-2, demonstrating the existence of alternative modes of MMP-2 activation (118,198). It has also been previously suggested that MMP-2 is capable of self-activation (118), but it was not understood how this occurs or how this process is regulated. In this thesis we propose a new mechanism for MMP-2 activation that is independent of MT1-MMP and involves Hsp90α and a co-chaperone complex that assists MMP-2 in auto-activation with the assistance of an active MMP-2, providing one theory as to how MMP-2 may be self-activating. This is the first detailed mechanism of MMP-2 activation independent of other members of the MMP family. The mechanism also provides a way for MMP-2 self-activation to be regulated. The presence

of Hsp90α and its co-chaperones provides another mechanism for MMP-2 activation which, due to the high level of extracellular Hsp90α in cancer cells, could be upregulated in cancer cells, increasing their invasiveness.

MMP-2 in Cancer Migration and Invasion. The matrix metalloproteinase family, whose primary function was initially thought to be the digestion of the various components of the extracellular matrix, turns out to have key roles in signaling events (102,104,105) and in the release of cell-proliferation factors from the extracellular environment that promote cell proliferation, inhibit apoptosis, and modulate angiogenesis (28). These many roles all contribute to the importance of MMPs throughout cancer progression and not just in cancer metastasis (104,106). The lack of clinical success of pan-MMP inhibitors makes the ability to indirectly inhibit MMP-2 through Hsp90α inhibition an attractive alternative (115). These experiments are the first to demonstrate that inhibition of extracellular Hsp90α reduces cancer migration and invasion through the inhibition of MMP-2. In addition, more evidence is pointing to the importance of the surrounding stromal cells in cancer progression (199,200) and that often MMPs are not upregulated by the tumor cells themselves but by the adjacent stromal cells. Inhibition of extracellular Hsp90α would inhibit the activation of MMP-2 regardless of its cell of origin, reducing cancer migration and invasion induced by both tumor and supporting cells.

In chapter 3 of this thesis, I show that the activity of MMP-2, which has an important role in cancer migration and invasion, is increased by Hsp90α. I found that inhibition of Hsp90α by small molecules, immunodepletion, or function-inhibiting antibodies causes a significant decrease in breast cancer cell migration and invasion. One

shortcoming of these data is that they do not indicate what percentage of MMP-2 is activated by Hsp90α and if this amount varies depending on the level of invasiveness of the cell. One way to test this would be to add exogenous MMP-2 to cell types of varying invasiveness and then treat with either an Hsp90α inhibitor or a pan-MMP inhibitor, such as Batimastat (115). The conditioned media would then be collected and assayed for MMP-2 activity. The relative levels of activity would be compared between the different cell types with the different inhibitor treatments. If Hsp90α is an important player in MMP-2 activation, the reduction in MMP-2 activation with an Hsp90α inhibitor or with the Pan-MMP inhibitor should be similar. If other proteins are playing a larger role in MMP-2 activation, we will not see a significant decrease in MMP-2 activation with Hsp90α-inhibitor treatment. If a larger percentage of MMP-2 is activated by Hsp90α in invasive cancer cells than in non-invasive cells, inhibiting extracellular Hsp90α would provide a way to significantly inhibit only the activation of MMP-2 in cancer cells resulting in the reduction of invasiveness of those cells.

Hsp90 Inhibitors and Combinatorial Therapies

Two relatively successful Hsp90α inhibitors are 17-AAG and 17-DMAG, which are both geldanamycin derivatives (68,70,201). These compounds have been tested in murine models and have been shown to have good efficacy and reduced toxicity compared to geldanamycin, but they still cause significant side effects (71). The discovery of the role of extracellular Hsp90α in cancer migration and invasion provided a new anti-metastatic target. Extracellular Hsp90α can be specifically targeted with cell-impermeable inhibitors which can reduce cancer migration and invasion while leaving

the intracellular Hsp90α intact, likely reducing negative side effects such as the upregulation of the HSF1 transcription factor (35).

A number of cell-impermeable Hsp90α inhibitors have been identified, including small molecules such as GAI-1, a geldanamycin analogue, (Xerion Pharmaceuticals) and Hsp90α function-inhibiting antibodies (122,163). Due to toxicity issues with some small molecule inhibitors, I focused on function-inhibiting antibodies for the inhibition of extracellular Hsp90α in this thesis. The majority of identified Hsp90α function-inhibiting antibodies target the ATP-binding region in the N-terminus of Hsp90α, similar to geldanamycin (33,67,202). The most well-characterized of these antibodies, SPS-771, which also binds to the ATP domain, was identified by Li *et al.* in 2007 (122). This antibody along with 17-AAG, a cell permeable geldanamycin derivative, was used in most of the studies performed in this thesis.

These two drugs are only a small subset of the many Hsp90α inhibiting compounds that have been identified. Many groups are working to develop new libraries of small molecule Hsp90α inhibitors and any of the compounds in these screens that are found to be cell-impermeable would be ideal candidates for further testing in extracellular Hsp90α-inhibition assays. There are also other function-inhibiting antibodies that have been identified that could be tested for their ability to reduce cancer migration and invasion (164). Both small molecule inhibitors and function-inhibiting antibodies are promising candidates for the inhibition of extracellular Hsp90α. Small molecules are much less expensive to produce and due to their small size are able to access areas, such as the brain, that the larger antibodies cannot (14,15). Although antibodies are expensive to produce compared to most small-molecule inhibitors, they often have less side effects

and an increased specificity (15). They also have been found to activate the patient's immune system, which has been shown to have an added anti-tumor benefit (24). The most promising role for extracellular Hsp90α inhibitors is in combinatorial therapies. Increasingly, multiple chemotherapeutic drugs are being used to treat cancer (2,3,14). One role for drugs that target extracellular proteins is in combinatorial therapies. One widely used method is to employ several intracellular chemotherapies to target cell growth and anti-apoptotic pathways while simultaneously targeting an extracellular protein, such as Hsp90α, to reduce tumor dissemination while the primary tumor is being treated. The reduced chance of severe side effects to extracellular drugs would increase the chance of multiple drugs being tolerated resulting in a decrease in both primary tumor size and in tumor metastases.

Murine Models of Cancer Metastasis

Testing metastasis-inhibiting compounds in small animal models is an important step in drug testing prior to the initiation of clinical trials. Mice are commonly used in these *in vivo* tests due to the ease of inducing a number of different tumor types in immune-compromised animals. In the appendix of this thesis, we employ a mouse breast-to-bone metastasis model developed by Dr. Charlotte Kuperwasser (119). This assay uses human breast cancer cells and human bone engrafted in a mouse to simulate the process of human metastasis (119) (Figure 1.2). In these experiments, I performed several preliminary tests on the function-inhibiting Hsp90α antibody, SPS-771. We verified that this antibody was not toxic to the mice and remained at high levels in their blood for up to a week. SPS-771 was then tested for its ability to reduce breast cancer

metastasis to bone using the above mentioned metastasis model. Due to low sample numbers, we were unable to draw any conclusions as to the efficacy of the antibody as an inhibitor of metastasis. These experiments are discussed in further depth in the appendix.

Even though I was unable to make any conclusions from the metastasis model, I did demonstrate that the function-inhibiting antibody had a relatively long half-life in the mouse circulation and had a low rate of toxicity. The exploratory experiments described in the appendix provide a good basis for the commencement of more rigorous testing of the function-inhibiting Hsp90α antibody, SPS-771. In order to do this, the Breast-to-Bone metastasis model should be repeated with a larger number of subjects in each group, which hopefully will enable a conclusion to be drawn from the data and allow one to determine whether or not the antibody reduces cancer metastasis. In addition, performing other metastatic models would be highly beneficial in determining the efficacy of the antibody.

Although there are many benefits to the breast-to-bone metastasis model, there are also several downsides. These include the inability to determine which part of the metastatic process Hsp90α is affecting. This assay only indicates if the inhibitor being tested can reduce overall invasion; it does not show if the inhibitor is important in cell detachment, invasion, migration, intravasation, or extravasation; all of which are important parts of metastasis. Also, due to the complexity of the experiment, if the assay is inconclusive it is difficult to ascertain what may have caused the issues that were encountered. These issues could be caused by incomplete connection of the bone to the mouse circulation, insufficient amount of inhibitor being used, inability of the inhibitor to

reach either the primary tumor or the metastases, or the inhibitor getting sequestered elsewhere in the mice.

Many of the issues with the above metastasis model could be alleviated if a more simplistic model of metastasis was performed. One such model is a model where human breast cancer cells are injected into the mouse tail vein and allowed to metastasize for several weeks with and without drug treatment. Specially selected cell lines, such as the MDA-MB-231-s4175 subline (160), specifically metastasize to certain areas of the mice, such as the lung in this case. These cell lines could be used in metastasis models, such as the one described above, to identify anti-metastatic drugs that prevent or reduce metastasis to certain areas of the body. These treatments could then be used to help treat patients that have developed metastases to those areas. I performed preliminary studies of the MDA-MB-231-s4175 subline and found that, similar to the parent line, Hsp90α and the four co-chaperones were present outside of the cell. Interestingly, the subline was more motile in wound healing assays than the parent line, demonstrating that certain genetic changes caused a different phenotype to arise in the subline. This line would be an excellent candidate for testing the efficacy of extracellular Hsp90α inhibitors in reducing cancer metastasis. This assay would further elucidate the anti-metastatic properties of the Hsp90α function-inhibiting antibody. In addition, these *in vivo* assays could be repeated with the addition of proven chemotherapeutics to see if there is a reduction of tumor progression with the presence of multiple drugs. Demonstrating that inhibition of extracellular Hsp90α reduces cancer metastasis in an *in vivo* model would be an exciting finding. It would jumpstart the extracellular Hsp90α field and likely lead to the development and testing of a number of novel extracellular Hsp90α inhibitors.

Concluding Remarks

Cancer metastasis continues to be the greatest challenge for reducing cancer-related mortality. Successful treatments will most likely involve the combination of various drugs and chemotherapies in a multi-pronged, anti-tumor approach (2,3,14). The role of extracellular proteins in cancer progression, and more specifically in cancer migration and invasion, has become very apparent. Targeting important players in this process will enable the development of inhibitors that could provide a significant reduction in cancer metastasis. Hsp90α, a ubiquitous and essential intracellular protein, has been identified as an important chaperone protein in the extracellular environment (64). Co-chaperones that are known to commonly assist Hsp90α inside the cell have been identified outside of various cell types and I have shown that these co-chaperones interact with extracellular Hsp90α. I hypothesize that extracellular Hsp90α, in a complex with several co-chaperones, assists in the activation of MMP-2 and that this interaction has an important role in cancer migration and invasion.

The export of extracellular proteins is turning out to be much more complex then simply targeting cells with a signal sequence and sending them out via the canonical export pathway. Exosomes, a non-classical form of export first identified in immune cells, has become a crucial way many proteins are exported from cells (78,203). The targeting of these proteins to this pathway is still somewhat of a mystery, but the role of ubiquitination in targeting proteins to exosomes is a likely candidate (139). As described above, Hsp90α has a large number of potential ubiquitination sites and it interacts with the E3 ligase, CHIP (139). In addition, various forms of phosphorylation could also

prove important in the targeting of extracellular proteins. For example, Hsp90α also has several phosphorylation sites that have been shown to be important in its export from the cell (65,187). The other extracellular co-chaperones likely have ubiquitination and phosphorylation sites as well that target them for export through a non-canonical export pathway. Further investigation of these pathways in cancer cells could lead to additional drug targets for the reduction cancer progression and invasion.

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Appendix 1

In Vitro and *In Vivo* Testing of an Hsp90α function-inhibiting Antibody

Abstract

A large number of proteins have been shown to be present in the extracellular space and to be important in a wide variety of cellular functions. A number of these proteins have been found to be upregulated in cancer cells, likely contributing to cancer progression. One such protein is Hsp90α, a protein that has been well-characterized intracellularly, has been found to have an important role outside of the cell in increasing cancer migration and invasion. A number of function-inhibiting drugs targeting Hsp90α have been developed; some of which are cell-impermeable and only target the extracellular form of the protein. One class of these inhibitors is function-inhibiting antibodies. In this thesis, I test one such function-inhibiting antibody, SPS-771, for its ability to reduce breast cancer cell invasion *in vitro* and *in vivo* using cell migration and a breast-to-bone metastasis model. I demonstrated that this antibody can reduce cancer cell invasion by 40-50% in an *in vitro* invasion assay. I also showed that the antibody does not cause any overt toxicity in mice at the concentrations tested and remains at constant levels in mouse serum for at least seven days. I was unable to demonstrate if the antibody reduced cancer cell metastasis *in vivo* due to a small final sample number. The data that was obtained suggests that further *in vivo* testing of the antibody is warranted.

Introduction

Extracellular Hsp90α has recently become a protein of great interest due to the many cellular processes it has been implicated in. In addition, Hsp90α has been shown to be present outside of numerous cell types, including neurons, dermal fibroblasts, macrophages, epithelial cells, vascular smooth muscle cells, and numerous cancer cells

(1,2). Hsp90α was first found to be present outside of fibrosarcoma cells in 1986 by Ulrich *et al* (3). It has since been shown to have an important role in wound healing, immune cell activation, neuronal migration, and cancer invasion (4-6). The importance of Hsp90α in cancer migration and invasion is of great interest to cancer researchers due to its potential to function as an anti-metastatic drug target.

A number of Hsp90α inhibiting drugs have been identified and several of them are currently undergoing clinical testing. The first compound that was found to inhibit Hsp90α function was the anti-bacterial compound geldanamycin (7). This compound inhibited Hsp90α function by binding to the ATP-binding site and preventing the protein from carrying out its chaperoning functions (8). Unfortunately, when geldanamycin was tested *in vivo* it was found to have a high level of toxicity. To circumvent this problem, a number of geldanamycin derivatives have been developed. Two of the most successful were 17-AAG and 17-DMAG (9). 17-AAG will be used as a positive control in some of the experiments in this appendix. There are also a number of other small molecule Hsp90α inhibitors, such as radicocol which is another natural compound that binds to the ATP binding pocket of Hsp90α (10).

As with any drug, a number of side effects have been observed upon treatment with Hsp90α inhibitors. One of the most significant side effects is the upregulation of HSF1; a transcription factor that upregulates Hsp90α, Hsp70, and a number of other heat shock related genes (2,11,12). This side effect causes the inhibitor to be less effective, requiring greater amounts of treatment in order to obtain the same effect. One benefit to targeting only extracellular Hsp90α, is the level of side effects will likely be reduced, and due to intracellular Hs90α remaining functional, HSF1 should not be upregulated. The

important role of extracellular Hsp90α in cancer migration and invasion make it an ideal target for the development of drugs to reduce cancer progression (4-6). There have been a number of cell-impermeable Hsp90α-inhibiting compounds reported ((10) and personal communication). Some of these inhibitors were found to be toxic *in vivo* and cause massive cardiac dysfunction (personal communication), so I did not purse cell-impermeable small molecule inhibitors in this study. Alternatively, a number of function-inhibiting antibodies have been identified (10,13). The majority of these antibodies also bind to the ATP-binding region and likely function in a similar way to geldanamycin- based inhibitors. Function-inhibiting antibodies are also cell-impermeable and are usually well tolerated by animal models, making them an ideal way to specifically inhibit extracellular Hsp90α.

Recently, function-inhibiting antibodies have enjoyed an increase in popularity for anti-cancer treatments, due to their high specificity and lower toxicity profile. Antibodies such as Trastuzumab and Bevacizumab which inhibit HER-2 and VEGF, have been tested in the clinic and found to significantly reduce cancer progression (14,15). Overall, function-inhibiting antibodies have less toxicity than many small molecule inhibitors and are better tolerated by patients. But they are not without side effects. Antibodies have been shown to cause flu-like symptoms and cardiac toxicity in cancer patients, and these symptoms often increase upon combination with other chemotherapies (16). However, one side effect, the activation of the immune system has been found to help reduce cancer progression (17). The success of function-inhibiting antibodies in reducing cancer progression, make them an interesting possibility for the inhibition of extracellular Hsp90α.

In this appendix, I tested the function-inhibiting antibody SPS-771 in an *in vitro* invasion assay and found that it is able to reduce cancer invasion by 40% (13). I then used a cell viability assay to demonstrate that the reduction in invasion is not due to cell death. Next, I performed preliminary toxicology studies of the antibody in mice and did not observe any overt toxicity and found that the antibody remained at high concentrations in the mouse circulation for at least seven days. I next verified that I was able to replicate both tumor formation and tumor metastasis in the mouse breast-to-bone metastasis model developed by Charlotte Kuperwasser (18). Finally, I tested the ability of the function-inhibiting antibody, SPS-771, to reduce cancer metastasis in this model. However, due to a small sample size I was unable to draw any conclusions from the data. The data obtained from this appendix suggests that the function-inhibiting antibody, SPS-771, will be well tolerated in mice and, due to its ability to inhibit cancer cell invasion *in vitro*, warrants further study in *in vivo* models, particularly as part of a combinatorial, anti-metastatic model.

Materials and Methods

Antibodies and Reagents. All antibodies were purchased from Assay Designs (MI) except for Rabbit IgG secondary which was purchased from Jackson Labs (PA). 17-AAG was purchased from R&D (MN). Unless noted, all other reagents were purchased from Sigma (MO).

Cell Culture. MDA-MB-231 cells were obtained from ATCC. Cells were maintained in DMEM supplemented with 10% FBS, 1% non-essential amino acids, and 1%

penicillin-streptomycin. SUM1315 cells were a kind gift from Dr. Charlotte Kuperwasser and were maintained in Hams F12 media supplemented with 5% FBS, 5 μg/mL insulin, 10 ng/ml epidermal growth factor (EGF) and 1% penicillin-streptomycin. All cells were grown at 37ºC under 7.5% CO_2.

Invasion Assay. 2.5 million MDA-MB-231 or SUM1315 cells were plated on five T-75 flasks and incubated for 48 hours. Then $2x10^4$ MDA-MB-231 or SUM1315 cells were labeled with cell tracker orange (CMTMR, Invitrogen), treated with either 10 μg/ml 17AAG, 40 μg/ml Control IgG (rabbit), or the indicated amount of SPS-771, and added to each well of a porous membrane (pore size 8μm (Neuroprobe, Gaithersburg, MD)) with a basement membrane barrier (0.3 μg/μl Matrigel (BD Biosciences, Bedford, MA)). The cells were incubated for 24 hours at 37 °C under 7.5% CO_2. The number of cells that had invaded into the matrigel and migrated through the pores of the membrane was quantified for each well with a fluorescence plate reader (excitation 544 nm, emission 590 nm).

Cell Viability Assay. 20,000 MDA-MB-231 cells were plated per well in a 96-well plate for 24 hours before addition of increasing volumes of 17-AAG, doxorubicin, SPS-771, or Rabbit IgG. The cells were then incubated for an additional 24 hours. Cells were subjected to a Celltiter 96 Aqueous Non-radioactive Cell Proliferation Assay (Promega, WI). The number of viable cells was calculated as described in (19).

Animals and Surgery. Eight-week old, female, NOD-SCID mice were obtained from Jackson Labs (MA). Mice were maintained in a sterile environment and given

autoclaved food and water ad libitum. All surgeries were performed in sterile conditions and mice were anesthetized with isoflurane and given Buprenorphine (Buprenex) post surgery. Mice were given antibiotics (Trimethoprim Sulfa) in their water for two weeks post-surgery.

Function-Inhibiting Antibody Toxicity and Rate of Clearance Assays.

Assay 1. Eight-week old NOD-SCID mice were obtained from Jackson Labs (MA). The mice were divided into six groups of three which were treated as follows: no injection; tail-vein injection of 100 µl PBS; tail vein injection of 25 µg SPS-771 antibody; 50 µg SPS-771 antibody; 75 µg SPS-771 antibody; or 100 µg SPS-771 antibody. Blood samples were collected from each mouse from their tail vein at 1 hour, 24 hours, 48 hours, 96 hours and 7 days post injection. Serum was isolated from each of these samples by incubating on ice for 30 minutes followed by centrifugation on a table top centrifuge for 10 minutes at 10,000 RPM. The blood serum samples were tested for the presence of SPS-771 via dot blot using Rabbit secondary antibody to detect the levels of the antibody. At 7 days post injection blood samples were obtained from each mouse via cardiac puncture and sent for toxicity analysis.

Assay 2. Eight-week old NOD-SCID mice were obtained from Jackson Labs (MA). $2x10^6$ SUM1315 breast cancer cells suspended in 200 µL PBS were injected with a 31-gauge needle into the fourth mammary fat pads of each mouse and allowed to establish a primary tumor for 6 weeks. Tumor size was monitored manually three times weekly with a caliper. Mice were separated into four groups and treated as follows over a two-week

period. One group was treated weekly with 100 µl of PBS, one was treated weekly with 100 µl of purified Rabbit secondary (antibody control), one was treated weekly with 100 µg of SPS-771, one was treated twice weekly with 100 µg SPS-771, and one was treated biweekly with 100 µg of SPS-771. Blood was collected from each mouse at 1 hour, 1 day, 7 days and 14 days post first injection to verify the rate of clearance of the antibody from the circulation system. Serum was isolated from each sample as detailed above. The amount of antibody present in the mouse serum was probed using a dot blot and immunoblotted with anti-rabbit secondary antibody. 14 days post first injection, mice were sacrificed and primary tumors, liver, spleen, and lung were excised, paraffin embedded, sliced, and mounted. Immunohistochemistry was performed on the samples to determine where in the mouse the antibody could be detected.

Breast-to-Bone Metastasis Model. Human bone tissue was obtained from discarded femoral heads from patients undergoing total hip replacement surgery. Bone was cut into bone cores (1 cm x 5 mm) using a bone harvester two to four hours after removal of the bone from the patient. The bone cores were then implanted into the right and left dorsal flanks of 8-week-old NOD/SCID mice which were under anesthesia (isoflurane). All human bone tissue for these experiments was procured in compliance with NIH regulations and institutional guidelines, as approved by the Institutional Review Board committee of the New England Baptist Hospital, Beth Israel Deaconess Medical Center, and Tufts University School of Medicine. The bone was allowed to connect into the mouse circulation for four weeks, then fluorescently labeled SUM1315 human breast cancer cells were injected into the fourth mammary fat pads of the bone-engrafted mice

as described above. The mice were then split into two cohorts. One began drug treatment immediately; the other began treatment six weeks after the breast cancer cells were injected. As indicated, animals were injected via tail vein weekly while under anesthesia (isoflurane) using a 27-gauge needle with 100 µl PBS, 100 µl Control IgG or 100 µg SPS-771 (maximum volume of 100 µL). The size of the primary tumor continued to be monitored 3 times weekly. 12 weeks post-cancer cell injection was chosen as the end-point, as breast cancer cells have been shown to metastasize to human bone implants 12 weeks post injection (Kuperwasser *et al*., 2005). Alternatively, if tumor size reached 1 cm in diameter prior to this endpoint, animals were sacrificed. At the end of 12 weeks (or when the primary tumor reached 1 cm) mice were imaged using the Xenogen Imager. At the time of imaging, animals were anesthetized with isoflurane prior to, and during imaging with the gas anesthesia unit included with the Xenogen imager. Mice were then sacrificed and the bone cores, liver, lung, and spleen removed and imaged individually for the presence of metastases. The tissues were sent to be paraffin embedded, sliced, and mounted after which immunohistochemistry was be performed.

Results

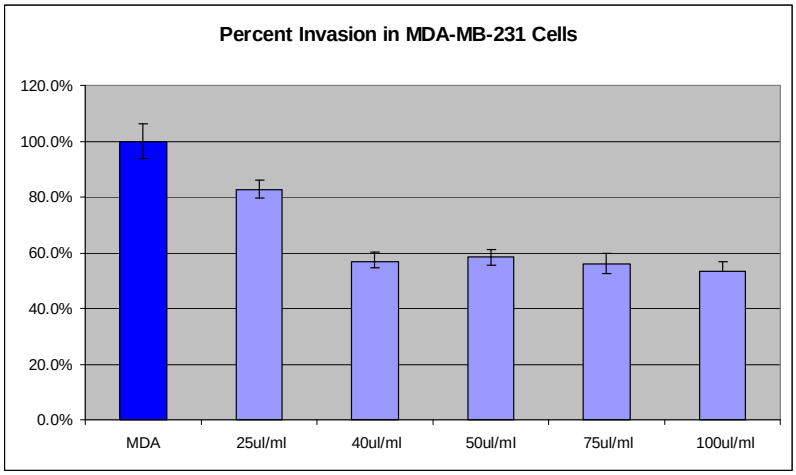
Extracellular Hsp90α has been shown to have an important role in cancer migration and invasion. Specifically inhibiting extracellular Hsp90α could reduce cancer metastasis, while lessening the side effects caused by universally inhibiting Hsp90α. There are two primary drug types for cell-impermeable Hsp90α inhibitors. First are small molecule inhibitors, and second are function-inhibiting antibodies. The small-molecule inhibitor that hand, GAI-1, was found by another researcher to cause sever cardiac toxicity in mice (personal communication). Due to this result, I decided to focus

on the second inhibitor option: function-inhibiting antibodies. Several candidates had already been identified by other researchers, so I opted to test the known inhibitor SPS-771, an antibody that bound to the ATP-binding site, the same area as the small molecule geldanamycin derivatives (10,13). I was able to obtain large amounts of this antibody commercially for the studies detailed in this chapter.

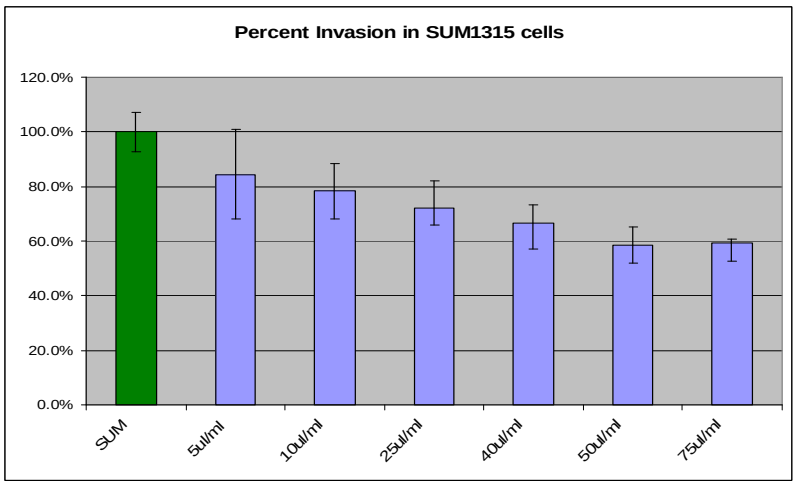
I first tested the ability of this antibody to reduce breast cancer cell invasion in an *in vitro* invasion model. I tested the antibody in two different breast cancer cell lines, MDA-MB-231 and SUM1315, and found that it reduced cancer cell invasion by 40-50% (Figure 1). I next demonstrated that this reduction in invasion was not due to cell death caused by toxicity from the antibody treatment. This was done by performing an MTS Assay to determine cell viability. As can be seen in Figure 1B, I did not observe a reduction in cell viability upon treatment with the function-inhibiting antibody. Interestingly, I found that the viability of the cell slightly increased upon treatment with the antibody, indicating that it may have side effects that promote cell growth.

Figure 1

a.


Percent Invasion in MDA-MB-231 Cells
120.0%
100.0%
80.0%
60.0%
40.0%
20.0%
0.0%
MDA
25ul/ml
40ul/ml
50ul/ml
75ul/ml
100ul/ml

b.


Percent Invasion in SUM1315 cells
120.0%
100.0%
80.0%
60.0%
40.0%
20.0%
0.0%
SUM
5ul/ml
10ul/ml
25ul/ml
40ul/ml
50ul/ml
75ul/ml

c.

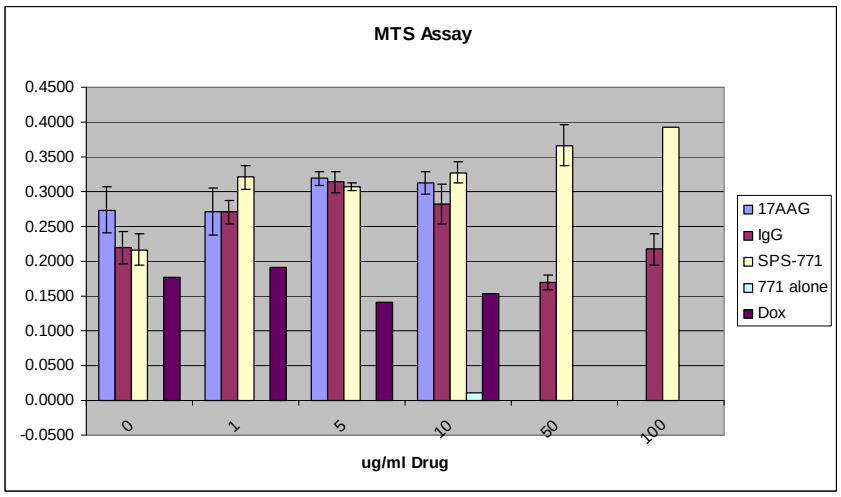

MTS Assay
0.4500
0.4000
0.3500
0.3000
0.2500
0.2000
0.1500
0.1000
0.0500
0.0000
-0.0500
0
1
5
10
50
100
ug/ml Drug
17AAG
IgG
SPS-771
771 alone
Dox

Figure 1-Function-Inhibiting Antibody Reduces Cancer Invasion. Invasion assays with both (a) MDA-MB-231 and (b) SUM1315 cells. In each experiment the cells were treated with an increasing amount of SPS-771. Maximum reduction in invasion (~40%) was obtained at a dosage of 40 ug/ml. (c) MDA-MB-231 cells were tested for viability after being treated with increasing amounts of 17AAG, Rabbit IgG, SPS-771, and doxorubicin using an MTS assay.

The *in vitro* invasion experiments testing SPS-771 demonstrated that the function-inhibiting antibody significantly reduced breast cancer cell invasion without causing cellular toxicity. Due to these encouraging results, I proceeded with testing the compound in an *in vivo* model. I first demonstrated that the antibody was not toxic to mice and determined how long the antibody remained at significant levels in the animal's circulation. To do this, I treated six groups of three mice with either nothing, 100 μl PBS, 25 μg, 50 μg, 75 μg, or 100 μg of SPS-771 antibody. Blood samples were taken from each mouse at 1 hour, 24 hours, 48 hours, 96 hours, and 7 days post injection. Serum was collected from each of these samples and run on a dot blot and probed for SPS-771 with anti-rabbit secondary antibody. I found that at the highest dose of 100 μg per mouse, the level of antibody remained constant in the mouse circulation for at least one week (Figure 2). At the seven day time point, I sacrificed the mice and obtained a large blood sample through cardiac puncture. The blood sample was sent for toxicity analysis of the liver, spleen, heart and kidneys. There were no overt toxicities detected in any of these tests (data not shown). In addition, the mice did not show any outward signs of illness due to the antibody. All of the mice appeared well-groomed and had normal body weight.

Figure 2

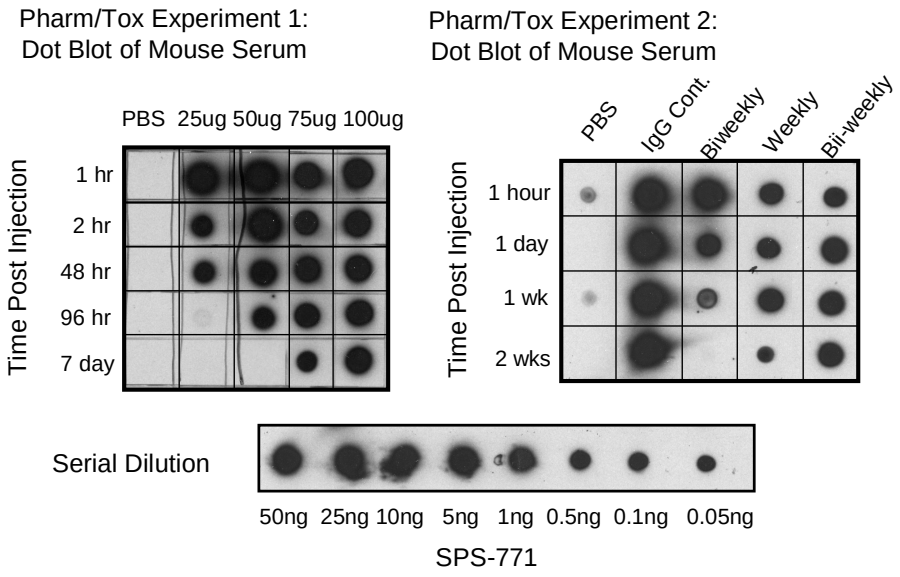


Figure 2-Antibody Remains in Mouse Circulation for one week. NOD-SCID mice were treated with varying concentrations of SPS-771 over a one or two week period. Blood samples were collected from the mice at the indicated times and serum was obtained from the samples. The serum was then run on a dot blot and probed with Rabbit secondary to detect the presence of the SPS-771 antibody.

I next tested if the antibody would specifically home to the primary tumor. Hsp90α-inhibiting drugs have been found to preferentially inhibit Hsp90α in cancer cells (19) and I wanted to verify that this would also occur with the function-inhibiting antibody. I tested this by injecting nine mice with SUM1315 breast cancer cells in their fourth mammary fat pads and allowed the establishment of tumors for six weeks. I then treated the mice weekly with 100 μl PBS, 100 μl of an antibody control (purified Rabbit secondary antibody) or 100 μg of SPS-771. I also had two additional groups that were treated either twice a week or biweekly with 100 μg of SPS-771. These injections took place over a two week time span. I collected blood from each mouse at 1 hour, 1 day, 7

days and 14 days post first injection to verify the rate of clearance of the antibody from the circulation system that I had observed in the previous experiment (Figure 2). Similar to the above results, I found that the antibody remained at a high concentration in the circulation for at least one week. At the end of the experiment the mice were sacrificed and the primary tumors, liver, spleen, and lungs excised from the mice. The tissue samples were sent for paraffin embedding and sectioning and stained for the SPS-771 antibody. I did not observe any significant increase in staining for the SPS-771 antibody over the control tissues (Date not shown). This result may indicate that the antibody did not preferentially home to the tumor tissue; it could also indicate that the mice need longer treatment with the antibody in order for a significant amount of it to be detected. Another possibility is that due to the antibody remaining outside of the cell, it may be present in quantities too small to detect via immunohistochemistry.

After demonstrating that the antibody remained at high levels in the mouse circulation for an extended period of time and did not cause any overt toxicity in the mice, I next tested the ability of the antibody to reduce cancer metastasis in an *in vivo* metastasis model. I chose to use the model developed by Dr. Charlotte Kuperwasser due to it closely recapitulating the process of metastasis that occurs in humans by having human breast cancer cells metastasize to human bone (18).

Prior to testing the function-inhibiting antibody, I verified that I could replicate the breast-to-bone metastasis model and demonstrated that I was able to obtain both primary tumors and metastases of those tumors to the implanted human bone. I did this by implanting five mice with human bone cores and allowing the bone cores to engraft into the mouse circulation system. Four weeks later, I injected 1 million luciferase-

labeled SUM1315 cells into the fourth mammary fat pad on each side of the mouse. The mice were monitored for tumor formation over a three month period. At the end of this period all the mice were sacrificed and the primary tumors and the bone cores were imaged using the Xenogen imager. All of the mice had at least one small primary tumor, several of them developed large tumors that required them to be sacrificed early. I detected metastases in the bone cores of one mouse using the imager (Figure 3). There may have been more metastases present in the bone cores, but I was unable to detect them using the imager. Overall, these results demonstrated that I could replicate the results of the breast-to-bone metastasis model, albeit not with 100% success.

Figure 3

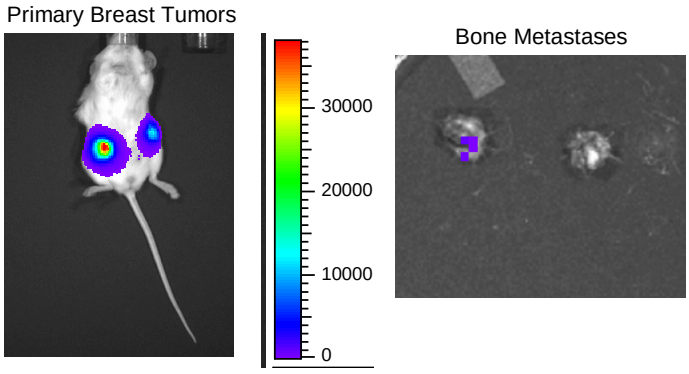


Figure 3-Primary and Secondary Tumors Obtained in *in vivo* Metastasis Model. Mice were implanted with human bone cores and four weeks later injected with SUM1315 breast cancer cells into their fourth mammary fat pad. Twelve weeks later mice were sacrificed and imaged to determine size and location of tumors and metastases.

I next tested the Hsp90α function-inhibiting antibody, SPS-771, in the *in vivo* metastasis model. As detailed above, each mouse had two human bone cores implanted, which were allowed to engraft for four weeks. At this point, each mouse had 1 million

SUM1315 cells implanted into their fourth mammary fat pads. The mice were separated into four groups with five mice in each group. The first two groups served as control groups; one received no treatment and the other received 100 μg of Rabbit IgG weekly for 12 weeks, the other two served as the experimental groups. The first experimental group was treated with 100 μg of the function-inhibiting antibody weekly for 12 weeks. This group was used to test the ability of the antibody to reduce primary tumor growth, as well as the appearance of metastases. The next group only received antibody treatment for the last 6 weeks of the experiment, which allowed the primary tumor to establish itself before treatment began. The second group tested the efficacy of the antibody in reducing or preventing metastases once the primary tumor has been established.

Originally, each of the groups contained five mice, but due to health issues, there were only three mice in each group except for the third group which contained four mice. The sizes of the primary tumors were manually monitored with a caliper three times weekly. At the end of twelve weeks, the mice were imaged to measure primary tumor size and to detect any metastases that were present in the bone cores, liver, or lungs. In order to detect the smaller metastases, mice were sacrificed and the above tissues were removed and imaged individually. The majority of mice had primary tumors, and a number of them had varying sizes of metastases in the liver, lungs, and bone cores. Interesting, the size of the primary tumors varied between the values obtained from the Xenogen imager and those obtained manually (Figure 4). Upon further investigation I found that often the SUM1315 cells lose their ability to produce luciferase and are therefore undetectable by the imager. Therefore even though tumors could be detected manually, they did not show up on the imager.

Figure 4

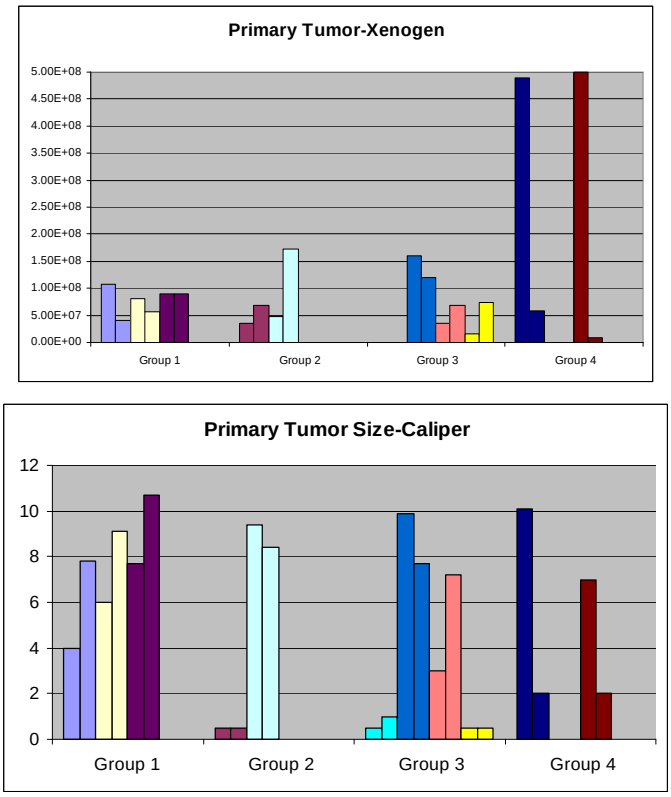


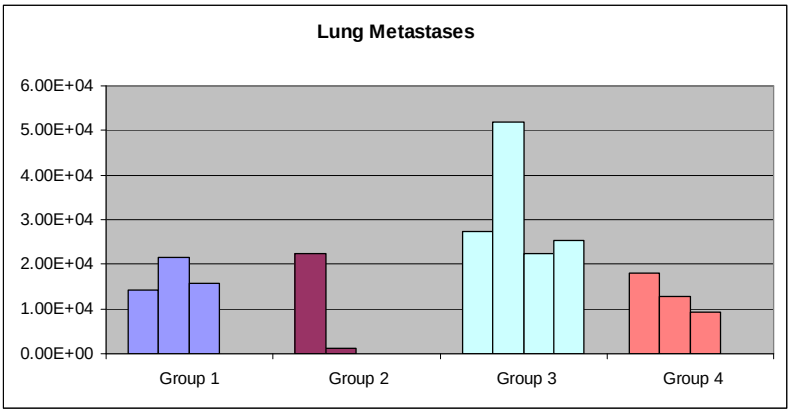
Figure 4-Primary Tumor Sizes Differ Significantly Depending on Form of Measurement. Primary tumors were measured both manually using a caliper and by fluorescence using the Xenogen imager. There was often a large disparity in the sizes of the tumors between the two measurement types. Group 1-PBS Treated; Group 2-IgG Treated; Group 3-12 week SPS-771 treatment; Group 4-6 week SPS-771 treatment.

I next performed analysis on the data I obtained from the *in vivo* metastasis model experiment. Group 1, the control group that received no treatment, appeared to have the largest number of tumors, but the small number of data points made any statistically significant conclusions impossible. The lung metastases appeared to be centered in group 3, the group treated with antibody for the full twelve weeks (Figure 5a), but the bone and liver metastases were primary in the two control groups (Figure 5b and 5c). This data indicates that there might be a slight anti-metastatic effect of the antibody in the bone and

liver. As mentioned previously, the small number of data points makes any definite conclusions impossible.

Figure 5

a.



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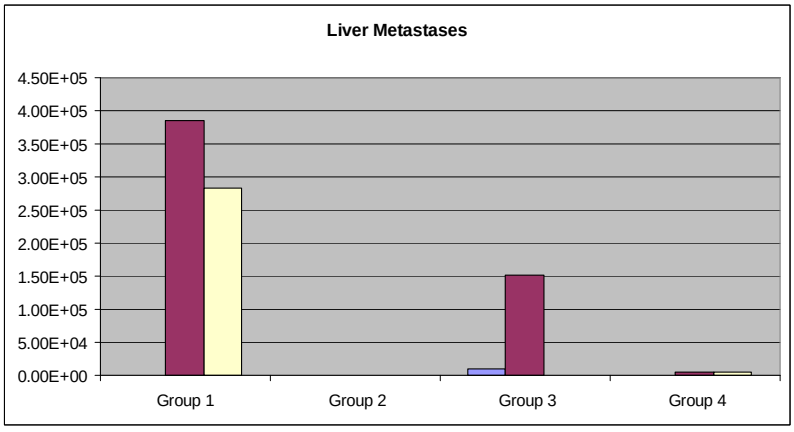

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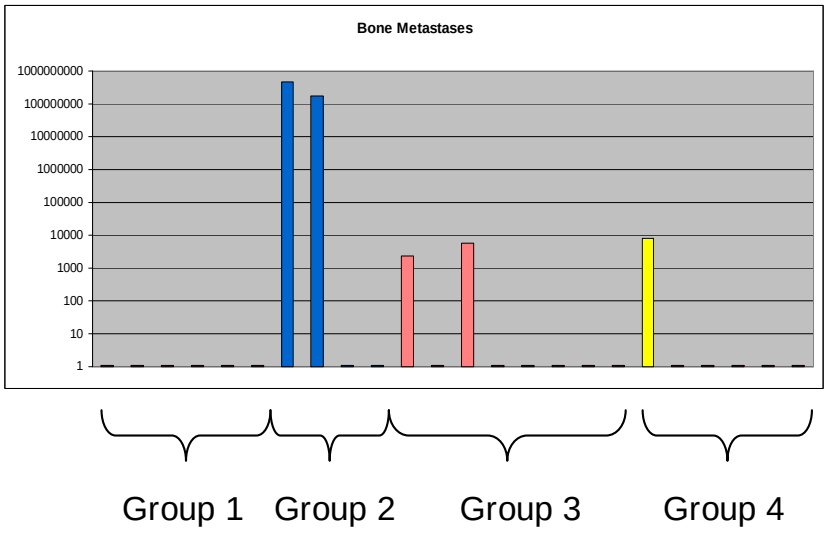
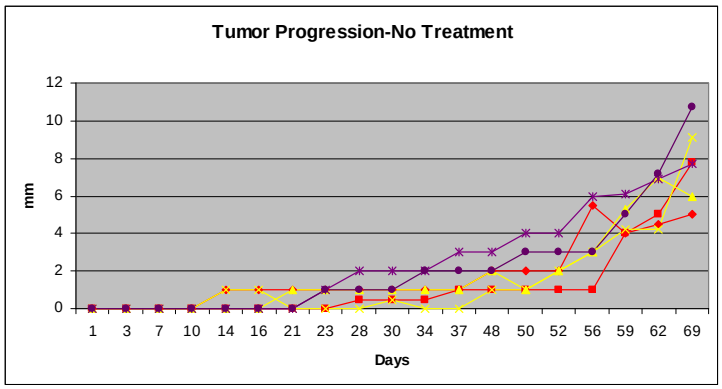
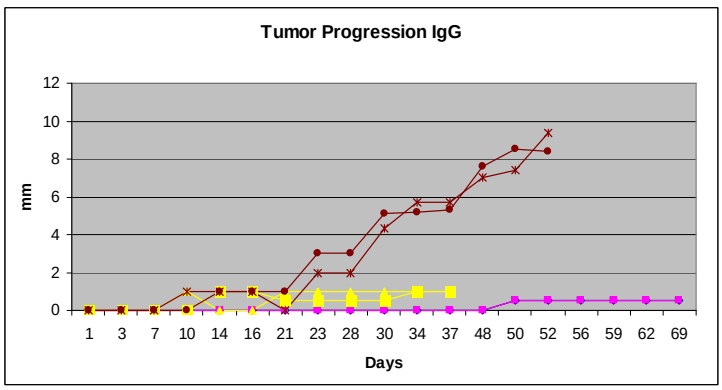


Figure 5-Tumor Metastases in Lung, Liver, and Bone. Relative sizes of lung, liver, and bone metastases for each mouse as measured by Xenogen imager. Tumors are arranged according to group. Group 1-PBS Treated; Group 2-IgG Treated; Group 3-12 week SPS-771 treatment; Group 4-6 week SPS-771 treatment.

Although the data I obtained from the Hsp90α function-inhibiting antibody *in vivo* metastasis experiment was inconclusive, we hypothesized that we may be able to demonstrate that the anti-hsp90α treatment may have affected the progression of the primary tumor. To ascertain this, I analyzed the data to determine if the antibody affected the progression of the primary tumors. I graphed tumor growth over time and no obvious trends presented themselves (Figure 6). Once again, due to the small sample number, I was unable to make any definitive conclusions as to the ability of the Hsp90α-inhibiting antibody, SPS-771, to reduce cancer progression or metastasis.

Figure 6





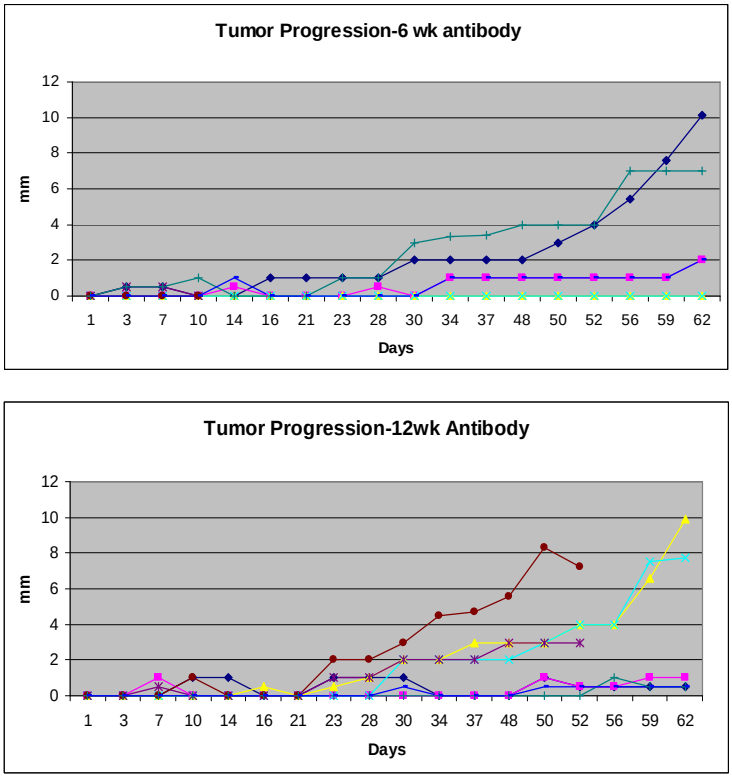


Figure 6-Primary Tumor Progression over 12 weeks. The size of the primary tumors was obtained manually with a caliper for 12 weeks. The data was graphed for each experimental group. Lines of the same color represent left and right tumors of the same mouse. Lines that terminate early represent mice that were lost due to surgical complications or were sacrificed early due to a large tumor burden.

Discussion

Metastasis continues to be the number one cause of cancer related death (14). Developing new treatments that will inhibit novel proteins both inside and outside the cell are crucial to reducing the rate of cancer mortality. Extracellular Hsp90α has been identified as protein important for cancer migration and invasion, two important steps in cancer metastasis (6,13). A number of intracellular Hsp90α inhibitors have been identified and tested both in *in vivo* models and in the clinic (9,20,21). However, extracellular Hsp90α inhibitors are only just beginning to be characterized. In this

appendix, I tested an Hsp90α-function inhibiting antibody for its ability to reduce cancer invasion *in vitro* and cancer progression and metastasis *in vivo*. I demonstrated that the antibody was able to reduce cancer invasion *in vitro* by about 40% using two different breast cancer cell lines. I was unable to demonstrate any significant reduction in cancer progression or metastasis *in vivo* due to a small sample size at the end of the experiment.

In these assays I used SPS-771, a function-inhibiting antibody that had been previously demonstrated to reduce cell migration in other *in vitro* models (13), to test if an extracellular Hsp90α inhibitor could reduce cancer metastasis. I chose to use a function-inhibiting antibody in these assays instead of a small molecule inhibitor due to the greater likelihood of the antibody having a low toxicity profile and remaining in the circulation for an extended period of time. It had been previously shown that IgG antibodies have a half life between 7 and 23 days in the circulation, much longer than the half-life of most small molecule inhibitors. Function-inhibiting antibodies also have some drawbacks, including the high cost of production, and their large size makes them unsuitable for treatment of brain metastases (15,22). Further investigation of small molecule cell-impermeable Hsp90α inhibitors would be worthwhile in order to identify a compound that could be affordably produced, enabling it to be extensively tested for its ability to inhibit cancer progression and metastasis.

In this appendix, I chose to test the Hsp90α function-inhibiting antibody in a breast-to-bone metastasis model due to the model closely simulating the metastatic process that occurs in human patients (18). The ability to observe human breast cancer cells metastasizing to human bone makes this an invaluable metastasis model. Even with its many benefits, this model also has a number of downsides. These downsides include

being unable to determine what part of metastasis a particular drug inhibits, only that it inhibits overall metastasis. Also, troubleshooting the model is difficult due its complexity and the possibility of many confounding factors affecting the results. In addition, there are other complications including the difficulty of obtaining the human bone samples and the long duration of the experiment.

Other more simplistic metastatic models may be more useful for the initial testing of extracellular Hsp90α inhibitors. One model, for example, tests the ability of the Hsp90α-inhibiting antibody to reduce cancer cell extravasation from the mouse circulation system to the lungs. In this experiment mice are pre-treated with the Hsp90α inhibitor and then human breast cancer cells that have been selected to metastasize to the lung (such as the MDA-MB-231 s4175 cell line from Juan Massague's Lab (23)) are injected into the mouse circulation via the tail vein. The mice are treated weekly with the antibody and after six weeks the mice are sacrificed and their lungs are examined for metastases. The other benefit of this model is that other cell lines that specifically metastasize to other areas of the mouse, such as the bone or liver, can be used in place of the line that metastasizes to the lung and various inhibitors can be tested for their efficacy in reducing metastasis to one organ or another.

In addition to using other mouse models of metastasis, another way to test the efficacy of Hsp90α inhibitors would be to use them in combination with other chemotherapies. Inhibiting extracellular Hsp90α alone may only result in a 40-50% reduction in metastasis, as seen in the *in vitro* experiments in figure 1, but if an Hsp90α inhibitor is combined with known chemotherapeutics, such as doxorubicin or paclitaxel (24), it may have a much greater effect in both reducing cancer progression and cancer

metastasis. The above models of metastasis could be used to test these combinatorial therapies and determine if they result in a greater reduction of metastasis than either of the treatments given alone. Combinatorial therapies such as these have been increasingly used in cancer research and clinical treatments, and in general they have been found to have a synergistic effect on the reduction of cancer progression (14,15,24). The only downside to these therapies is the increase in negative side effects (14,24). Ideally, the use of the extracellular Hsp90α inhibitor with other therapies will result in less side effects than if an intracellular inhibitor had been used, making the inhibition of extracellular Hsp90α an exciting potential anti-cancer therapeutic.

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Appendix 2

Secretion of extracellular hsp90alpha via exosomes increases cancer cell motility: a role for plasminogen activation

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RESEARCH ARTICLE Open Access

Secretion of extracellular hsp90α via exosomes increases cancer cell motility: a role for plasminogen activation

Jessica McCready[1], Jessica D Sims[1], Doug Chan[2] and Daniel G Jay*[1]

Abstract

Background: Metastasis is a multi-step process that is responsible for the majority of deaths in cancer patients. Current treatments are not effective in targeting metastasis. The molecular chaperone hsp90α is secreted from invasive cancer cells and activates MMP-2 to enhance invasiveness, required for the first step in metastasis.

Methods: We analyzed the morphology and motility of invasive cancer cells that were treated with exogenous exosomes in the presence or absence of hsp90α. We performed mass spectrometry and immunoprecipitation to identify plasminogen as a potential client protein of extracellular hsp90α. Plasmin activation assays and migration assays were performed to test if plasminogen is activated by extracellular hsp90α and has a role in migration.

Results: We found that hsp90α is secreted in exosomes in invasive cancer cells and it contributes to their invasive nature. We identified a novel interaction between hsp90α and tissue plasminogen activator that together with annexin II, also found in exosomes, activates plasmin. Extracellular hsp90α promotes plasmin activation as well as increases plasmin dependent cell motility.

Conclusions: Our data indicate that hsp90α is released by invasive cancer cells via exosomes and implicates hsp90α in activating plasmin, a second protease that acts in cancer cell invasion.

Background

Approximately 90% of cancer deaths are not from the primary tumor but due to metastasis to distant sites [1]. Current treatments do not target metastatic disease. Towards developing anti-metastasis drugs, a functional proteomic screen was performed to identify surface proteins required for tumor cell invasion, the first step in metastasis [2]. One of the proteins identified was the molecular chaperone heat shock protein 90α (hsp90α) [2]. Intracellular hsp90α aids in the folding, assembly-disassembly and activation of a variety of client proteins including kinases, steroid hormone receptors and transcription factors [3]. We discovered that extracellular hsp90α acts in tumor cell invasion through its activation of the pro-invasive protein matrix metalloproteinase-2 (MMP-2). Since the publication of this study, additional reports in the literature have demonstrated the importance of extracellular hsp90α in both physiological and pathological states. Extracellular hsp90α is required for both dermal fibroblast [4] and neuronal motility [5] as well as for melanoma migration [6], invasion and metastasis [7].

The secretion method of extracellular hsp90α from invasive cancer cells has not been fully elucidated. Hsp90α has been found in exosomes in immune and other physiologically normal cell types [8-11] and suggested to be in exosomes in diabetic cells [12]. Exosomes are small vesicles, approximately 30-100 nM in diameter, that are part of the endocytic pathway. They are secreted as intact vesicles that form within multivesicular bodies (MVB) and are released from cells when the membrane of the MVB fuses with the plasma membrane. Exosomes function in the immune system and in acellular communication [13]. Recent reports indicate that exosomes contribute to the aggressive nature of gliomas by transferring

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the mutated EGFRvIII receptor between cells [14]. The presence of hsp90α in exosomes of other cells types and the observation that exosomes contribute to glioma aggressiveness suggested to us that hsp90α in exosomes might contribute to cancer invasiveness.

In this study, we demonstrate that hsp90α is secreted from invasive cancer cells via exosomes and increases cancer cell migration. We show that extracellular hsp90α is necessary for the activation of a second extracellular protease, plasmin, and that fibrosarcoma cell movement is dependent on this activation.

Methods

Cell culture

A172, HT-1080, and MDA-MB231 cells were obtained from ATCC and maintained in DMEM supplemented with 10% FBS, 1% NEAA, and 1% P/S. SUM159 cells were a kind gift from Charlotte Kuperwasser and were maintained in Hams F12 media supplemented with 5% FBS, 5 μg/mL insulin, 10 ng/mL EGF and 1% P/S. All cells were grown in a 37°C incubator with 7.5% CO_2.

Quantitative Real time PCR

Total RNA was extracted from MDA-MB231 breast cancer cell lines with TRIzol (Invitrogen, California) and 2 μg of RNA was reverse transcribed into cDNA with Superscript III (Invitrogen) following the instructions supplied by the supplier. Real time PCR was performed at the Tufts Univesity Center for Neuroscience Research using the Stratagene real time cycler. Primer sequences were as follows: HSP90AA1-1 forward 5'-GGCAGAGGCTGATAAG-AACG-3' and reverse 5'CCCAGACCAAGT TTGATCATCC-3'; HSP90AA1-2 forward 5'-CATCTGATGGTGTCTGGATCC-3' and reverse 5'-AATGGCTGCAGATCCTTGTAG-3'. Samples were analyzed using the 2-ΔΔCT method (29) with GAPDH as the reference.

Brefeldin A Treatment

MDA-MB231 cells were treated with 10 μg/mL Brefeldin A (BFA), (Sigma, Missouri) or vehicle control for 16 hours. Conditioned media was collected, concentrated and subjected to SDS-PAGE followed by a Western blot probed with MMP-2 antibody (EMD Biosciences, New Jersey), anti-hsp90α or β-actin antibody (Sigma, Missouri). β-actin protein should be absent in conditioned media samples isolated from intact, alive cells.

RNAi Treatment

MDA-MB231 cells were transfected with either control siRNA (non-targeting) or 100 nM siRNA directed against the HSP90AA1-2 (sense 5'-GTTAACTGGTACCAAGAAA-dTdT-3') isoform using Oligofectamine (Invitrogen). RNA was extracted as indicated above and the results are graphed as percentage knockdown setting the control at 100%.

Exosome isolation

Exosomes were isolated from A172, HT-1080, MDA-MB231, and SUM159 cells as previously described [8]. Briefly, 5 × 10^6 cells were plated in 10% DMEM and allowed to settle overnight. Cells were then washed with HBSS and re-fed with serum free media or serum free media containing 15 nM dimethyl amiloride (Sigma). Media was collected 48 hours after the addition of serum free DMEM and spun at 300 × g to collect any cellular debris. This media was then filtered with a 0.2 μM filter and spun for 1 hour at 110,000 × g. The pellet was washed with PBS and spun for 1 hour at 110,000 × g. One μg of protein was subjected to Western Blot probed for hsp90α (Assay Designs, Michigan). Samples were also probed with an anti-Annexin II antibody (BD Biosciences, California) and Flotillin (Cell Signaling Technology, Massachusetts) as positive controls and vATPase subunit B (Molecular Probes, California) as a negative control.

Immunostaining

1 × 10^4 MDA-MB231 cells were plated into an 8-well chamber slide and treated with exosomes isolated from MDA-MB231 cells or 0.5 μg recombinant hsp90α (Assay Designs) for 16 hours. Cells were fixed in PBS/4% paraformaldehyde/4% sucrose, permeabilized in 0.1% TritonX-100/PBS, blocked in 1%BSA/PBS and stained with Alexa546-labeled phalloidin (Invitrogen, CA) for 30 minutes to visualize F-actin.

Cell shape analysis

The cell shape and area of MDA-MB231 cells were measured and calculated with OpenLab software (Improvision). Cell shape was defined using the equation (4 × cell area)/cell perimeter^2, where greater than 1 indicates a perfect circle and values less than 1 indicate a more irregular shape.

Wound healing assay

1 × 10^5 SUM159 breast cancer cells or A172 glioma cells were plated in an 8 well chamber slide. Cells were wounded by scratching a sterile yellow pipette tip lengthwise along the chamber. The cells were washed twice with 1× PBS and serum free media was placed in each well with either the vehicle control PBS, 0.5 μg recombinant hsp90α protein, 1 μg exosomes isolated from SUM159 cells, or 1 μg exosomes isolated from SUM159 cells plus 40 μg/mL anti-hsp90 antibody (SPS-771, Assay Designs). Pictures were taken immediately after cell wounding (0 hours) and 16 hours after cell wounding. Wound width was calculated using OpenLab software and is represented as μm between the cells at 16 hours for each treatment.

Immunoprecipitation/Mass spectrometry

4×10^6 MDA-MB231 breast cancer cells were plated in a 150 mm tissue culture dish and allowed to settle for 24 hours. Cells were then refed with serum free media and incubated for 48 hours at 37°C. Conditioned media was concentrated by centrifugation (Millipore, MA) and a protein assay was performed (BioRad, CA). 1 mg of protein was pre-cleared with protein A beads after which 1 ug of hsp90α antibody (Assay Designs) was added to the samples. Samples were washed with RIPA B buffer (50 mM Tris, 150 mM NaCl, 0.5% NP40, 0.25% DOC) boiled, subjected to SDS PAGE, stained with Coomassie Blue and removed from the gel for mass spectrometry analysis. The excised gel bands were analyzed by mass spectrometry as previously described [15]. MS results were verified using antibodies for hsp90α and tPA (Abcam, MA).

Plasminogen activation assay

Plasminogen activation assays were performed as previously described [16]. Briefly, HT-1080 fibrosarcoma cells were plated in 10% DMEM and refed with serum free media 24 hours after plating. DMSO, 0.5 μM [Glu] plasminogen (American Diagnostica Inc, CT), or 40 μg/mL DMAG-N-oxide (a gift from Len Neckers) were added for five hours at 37°C. DMAG-N-oxide was used in this experiment because the large amount of antibody required for this experiment precluded its use. It has been previously characterized as an inhibitor of extracellular hsp90α [17]. Conditioned media was concentrated (Millipore) and a protein assay was performed (Bio-Rad). 25 μg of each sample was loaded into a 0.1% gelatin zymogram. The zymogram was washed twice for two hours each in wash buffer (50 mM Tris-HCl, 150 mM NaCl, 2.5% (v/v) Triton X-100, pH 7.4), three times for 5 minutes each in water and then incubated in wash buffer for 12 hours at 37°C. The zymogram was stained with 0.5% coomassie, destained, and densitometry was performed to determine the plasminogen activation levels of each condition.

Migration assay

HT-1080 fibrosarcoma cells were plated in 10% DMEM. 48 hours after plating the cells were labeled with CMTMR (Invitrogen) and 1×10^5 labeled cells were plated into a 24-well Fluoroblok plate (BD Biosciences, CA). Cells were treated with either 40 μg/mL rabbit IgG, 40 μg/mL anti-hsp90α (SPS-771, Assay Designs), or 0.5 μg plasmin (Molecular Innovations, MI). Cells were allowed to migrate for 24 hours after which the number of cells that migrated to the bottom chamber were photographed and counted.

Results

Hsp90α is secreted via exosomes in invasive cancer cells

While the importance of extracellular hsp90α for tumor cell migration, invasion and metastasis has been recognized, the mechanism that invasive tumor cells use to secrete hsp90α is still unclear. In response to our work (2), it was hypothesized that alternative splicing of hsp90α created multiple isoforms and that perhaps there was a mainly intracellular hsp90α isoform and a mainly extracellular isoform of hsp90α (18). This was of interest because one could then target drugs to the extracellular isoform without interfering with the important intracellular functions of hsp90α. We verified that the two hypothesized Hsp90α isoforms existed in MDA-231 cells and determined the relative amounts using Real Time PCR (Figure 1a). We found that the primary isoform present in MDA-231 cells was the classical ten-exon isoform (AA1-1). The other isoform, AA1-2, with two additional exons, was present in only very small quantities. Even though there was only a small amount of the second isoform present, we hypothesized that this isoform could be targeted outside of the cell since the amount of Hsp90α inside of the cell is much greater than the amount outside of the cell. In order to test if Hsp90α was indeed being exported via a signal sequence in the two extra exons, we treated MDA-231 cells with Brefeldin A, a compound that inhibits the export of proteins through the canonical pathway. We collected the conditioned media from the cells, subjected it to SDS-PAGE and blotted for Hsp90α (Figure 1b). Hsp90α was detected outside

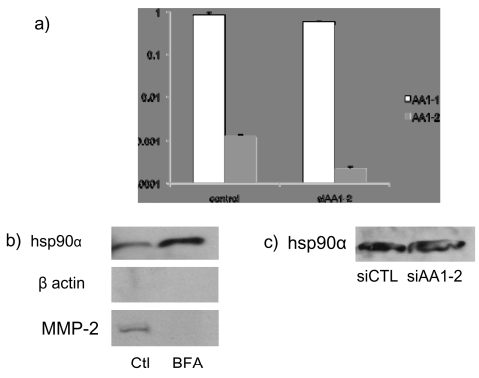

Figure 1 Hsp90α is not secreted either through the classic secretory pathway or in an isoform specific fashion. (a) We identified two isoforms of hsp90α in breast cancer cell lines using quantitative real time PCR. Cells were transfected with either control siRNA (non-targeting) or 100 nM siRNA directed against the HSP90AA1-2 isoform. Data from one of the three cell lines tested are represented (A172 and HT1080 not shown). (b) MDA-MB231 cells were treated with BFA or vehicle control for 16 hours and conditioned media was collected, concentrated and subjected to SDS-PAGE followed by a Western blot probed with anti-hsp90α, MMP-2 or β-actin antibody. (c) Conditioned media from cells treated with siRNA directed against the HSP90AA1-2 isoform was collected, concentrated and subjected to SDS-PAGE followed by a Western blot probed with an anti-hsp90α antibody.

of the cell after BFA treatment in both cell lines indicating that it is not exported through the canonical signal sequence pathway. In fact, the extracellular hsp90α protein levels were markedly increased by Brefeldin A, probably in response to cellular stress caused by this inhibitor. As a positive control we probed for MMP-2, a protein known to be exported via a signal sequence, and we found that its secretion was markedly inhibited by BFA (Figure 1b).

Even though Hsp90α is not exported via a signal sequence, it may still be exported in an isoform specific manner. In order to test this we used siRNA to knock down the AA1-2 isoform. We obtained approximately 80% knock down of this isoform in the cells (Figure 1a) but did not see any reduction in the amount of Hsp90α outside of the cell (Figure 1c). These results indicate that hsp90α is not secreted by the classical secretory pathway or in an isoform specific manner. This led us to explore other non-classical secretory pathways such as exosomes. We isolated exosomes from MDA-MB231 cells and showed by immunoblot that they contain hsp90α (Figure 2a). To test the generality of this, we isolated exosomes from three other invasive tumor cell lines from different lineages, A172 glioblastoma cells, HT-1080 fibrosarcoma cells, and SUM159 breast cancer cells. The exosomes isolated from these cells also contain hsp90α (Figure 2a). Purity of our exosome preparations was verified by the presence of annexin II and flotillin, two markers for exosomes, as well as by the absence of the large B subunit of the v-ATPase (Figure 2b), which is found in lysosomes and plasma membrane but not in exosomes [18]. When cells were treated with dimethyl amiloride (DMA), which blocks the exosome pathway[19], isolated exosome preparations from all cell lines tested showed marked reduction in hsp90α as well as exosome markers (Figure 2a). Both hsp90α and annexin II were degraded in a protease protection assay suggesting that they are easily accessible to extracellular proteins (data not shown). Therefore, hsp90α in exosomes is accessible to activate secreted extracellular proteins such as MMP-2 [2].

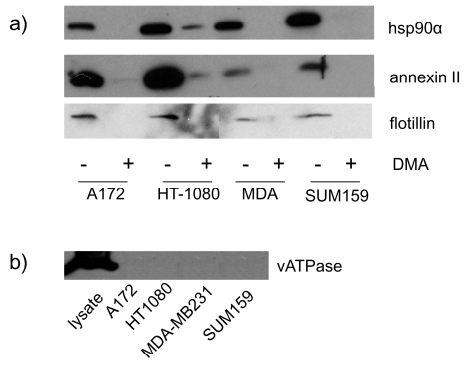

Figure 2 Hsp90α is present in the exosomes of four cancer cell lines. Western blot analysis of exosomes isolated from invasive cancer cell lines treated with vehicle (-) or DMA (+), which blocks the exosome pathway. Proteins were resolved by electrophoresis on 10% SDS-PAGE and transferred to a nitrocellulose membrane. Western blot was performed using hsp90α, annexin II, flotillin, and vATPase antibodies. Representative images taken from three independent experiments are shown.

Exosomes induce a change in morphology of breast cancer cells

Recent reports indicate that cancer cells secrete exosomes containing annexins and major histocompatibility complex proteins, which are normally associated with exosomes but also contain proteins involved in cell adhesion and motility such as integrins and fibronectin [20-22]. These reports, along with our previously published data regarding the role of extracellular hsp90α and tumor cell motility, suggest that exosomes containing hsp90α released by invasive cancer cells could increase tumor cell motility. To test this hypothesis we isolated exosomes from the breast cancer cell line MDA-MB231, added them to the media of MDA-MB231 cells plated the previous day and stained the cells with phalloidin to analyze the morphology of the cells in the presence of exosomes (Figure 3a). Cells exposed to the control vehicle, PBS, display normal morphology, whereas cells exposed to exosomes for 16 hours show a more polarized shape associated with a motile phenotype. We also exposed MDA-MB231 cells to recombinant hsp90α protein to determine if the effect we see with the exogenous exosomes is due in part to the presence of hsp90α in the exosomes. Hsp90α treated cells are more polarized than control-treated cells but less polarized than the cells treated with exosomes. To quantitate these changes, we measured both the cell shape and cell area using the OpenLab software program (Figure 3b). A motile cell can have a more linear cell shape as well as a large cellular area. We measured the perimeter of cells treated with either PBS, recombinant hsp90α protein or exosomes. The data are represented in a Chang plot that depicts the shape of cells in which a perfect circle is greater than 1 and a perfect line is 0. Cells treated with PBS have the most circular shape (average = 0.79 ± 0.02), cells treated with exosomes have the least circular shape (average = 0.49 ± 0.02) and cells treated with recombinant hsp90α show intermediate values between those seen for control and exosome treated cells (average = 0.61 ± 0.03). Cells treated with either the recombinant hsp90α or exosomes are statistically significantly different in shape when compared to the control treated cells ($p < 0.001$). Exosome treatment also significantly increases the cell area when compared to cells treated with PBS or recombinant

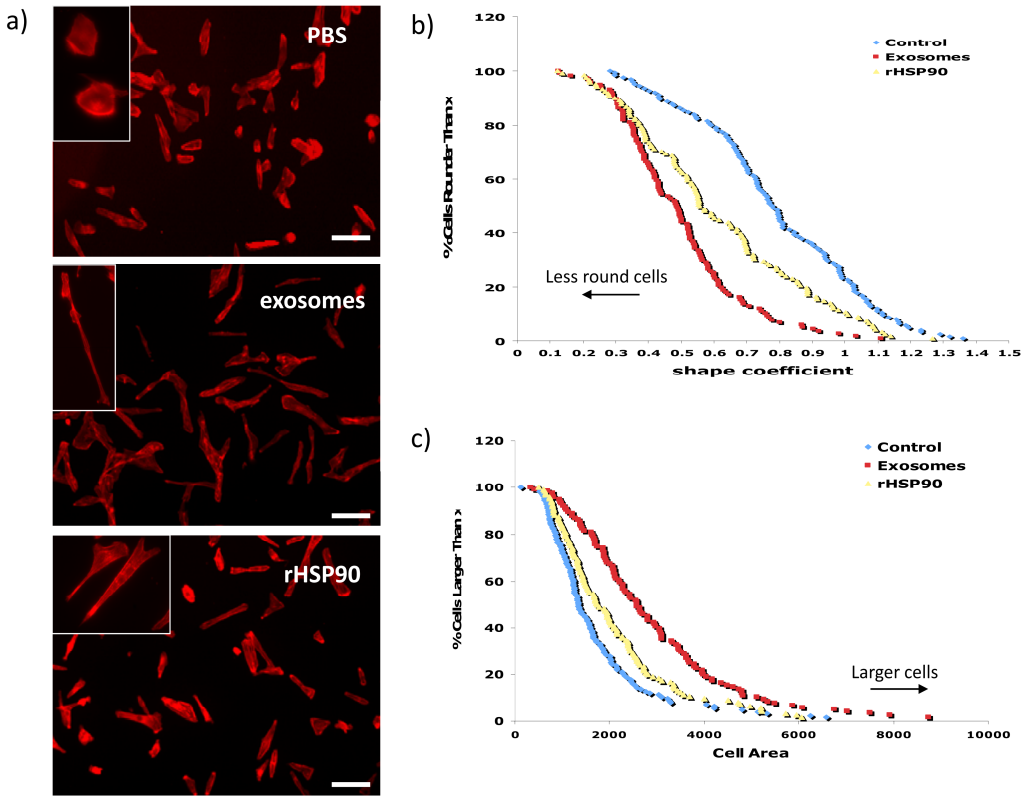

Figure 3 Exosomes alter the morphology of invasive breast cancer cells. Immunofluorescence and cell morphological analysis of MDA-MB231 cells. (a) Cells were treated with PBS (top panel), exosomes isolated from MDA-MB231 cells (middle panel) or recombinant hsp90α protein (bottom panel) and stained with phalloidin to visualize F actin. Images taken at 10× magnification from three independent experiments. Scale bar = 100 μM. Inset: Representative images at 20× magnification. (b) Chang plot showing cell shape measurements. Cell shape is defined using the equation (4 × cell area)/cell perimeter^2, where greater than 1 indicates a perfect circle and smaller values indicate a more irregular shape. Cell shape was calculated using OpenLab software. n = 130. (c) Cell area analysis of MDA-MB231 cells treated with exogenous exosomes or recombinant hsp90α. Cell area was calculated using OpenLab software. n = 130.

hsp90α (Figure 3c). Cell area increased from an average of 1137 ± 121 μm^2 to an average of 1398 ± 138 μm^2 with the addition of recombinant hsp90α ($p < 0.02$) and further increased to an average of 1914 ± 167 μm^2 with the addition of exosomes ($p < 0.001$). Taken together these results indicate that exogenous exosomes cause changes in both cell shape and cell area, consistent with motile behavior. We performed this experiment with A172 glioma cells (data not shown), however since they display a polarized morphology under normal growing conditions, the addition of exosomes did not affect their morphology.

Exosomes increase cancer cell motility

To determine if the changes in cell morphology translate to an increase in cell motility we performed wound healing assays. Since MDA-MB231 cells are not well suited to the wound healing assay we used two highly motile and invasive cell lines originating from different tumors: SUM159 breast cancer cells (Figure 4a) and A172 glioma cells (Figure 4b). We imaged the wound immediately after wounding the cells (0 hours) and added either recombinant hsp90α, exosomes, or exosomes plus a function inhibiting hsp90α antibody [4]. We captured a second set of images 16 hours later. SUM159 control cells moved significantly less than cells treated with either 0.5 μg recombinant hsp90α ($p < 0.05$) or 1 μg exosomes ($p < 0.01$). The effect of exosomes on SUM159 cells was reduced by the hsp90α function inhibiting antibody suggesting that hsp90α is required for an exosome-dependent increase in cell movement. Control cells moved significantly less than cells treated with either recombi-

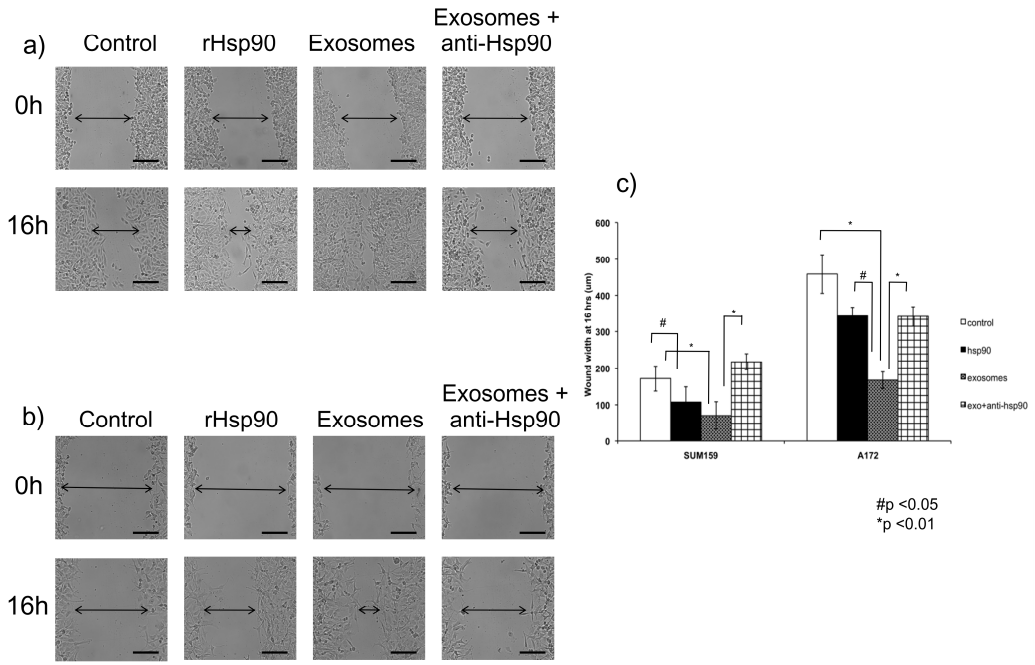

Figure 4 Exosomes increase hsp90α dependent glioma and breast cancer cell movement. (a) SUM159 breast cancer cells were wounded and images captured immediately and 16 hours after wounding. Cells were treated with either PBS, recombinant hsp90α protein, exosomes isolated from SUM159 cells or exosomes isolated from SUM159 cells plus anti-hsp90α antibody. Representative images from three independent experiments are shown for each time point and condition. Scale bar = 150 μM (b) A172 glioma cells were wounded and images captured immediately or 16 hours after wounding. Cells were treated with either PBS, recombinant hsp90α protein, exosomes isolated from A172 cells or exosomes isolated from A172 cells plus anti-hsp90α antibody. Representative images from three independent experiments are shown for each time point and condition. Scale bar = 150 μM (c) Graphical representation of the wound healing assay with SUM159 and A172 cancer cell lines. The addition of both recombinant hsp90α protein and anti-hsp90α antibody results in a significant decrease in wound closure at 16 hours ($p < 0.05$, $p < 0.01$ respectively, two tailed t test).

nant hsp90α ($p < 0.05$) or exosomes ($p < 0.01$). A172 cells acted similarly to SUM159 cells in the wound healing assay. Data from the wound healing assay is represented graphically in Figure 4c.

Extracellular hsp90α immunoprecipitates with tissue plasminogen activator

Our findings established that the addition of exogenous exosomes changed both the morphology and motility of cancer cells. We had previously implicated hsp90α in the activation of MMP-2, but it is possible that hsp90α secreted via exosomes could activate other extracellular proteins. We hypothesized that extracellular hsp90α was interacting with other extracellular proteins to increase cancer cell motility. To identify proteins that associate with hsp90α we performed immunoprecipitation with conditioned media from MDA-MB231 cells followed by mass spectrometry (Figure 5a). We discovered ten proteins bound to extracellular hsp90α. The identified proteins are in their precursor form suggesting that they might be potential clients for this chaperone. We focused on one that could be linked to both exosomes and cell motility: tissue plasminogen activator protein (tPA). Annexin II, a protein secreted via exosomes, binds to both tPA and plasminogen and has been shown to associate with hsp90α [23]. This binding initiates conversion of plasminogen to the protease plasmin [24]. We verified the mass spectrometry result with co-immunoprecipitation of Hsp90α and tPA in conditioned media from HT-1080 fibrosarcoma cells (Figure 5b), the cell line for which we originally discovered the interaction of extracellular hsp90α and MMP-2, and MDA-MB231 cells (data not shown). tPA and hsp90α weakly interact in both MDA-MB231 and HT-1080 cells perhaps because client and chaperone proteins interact transiently [25]. This raises the possibility that tPA is a novel client protein for extracellular hsp90α.

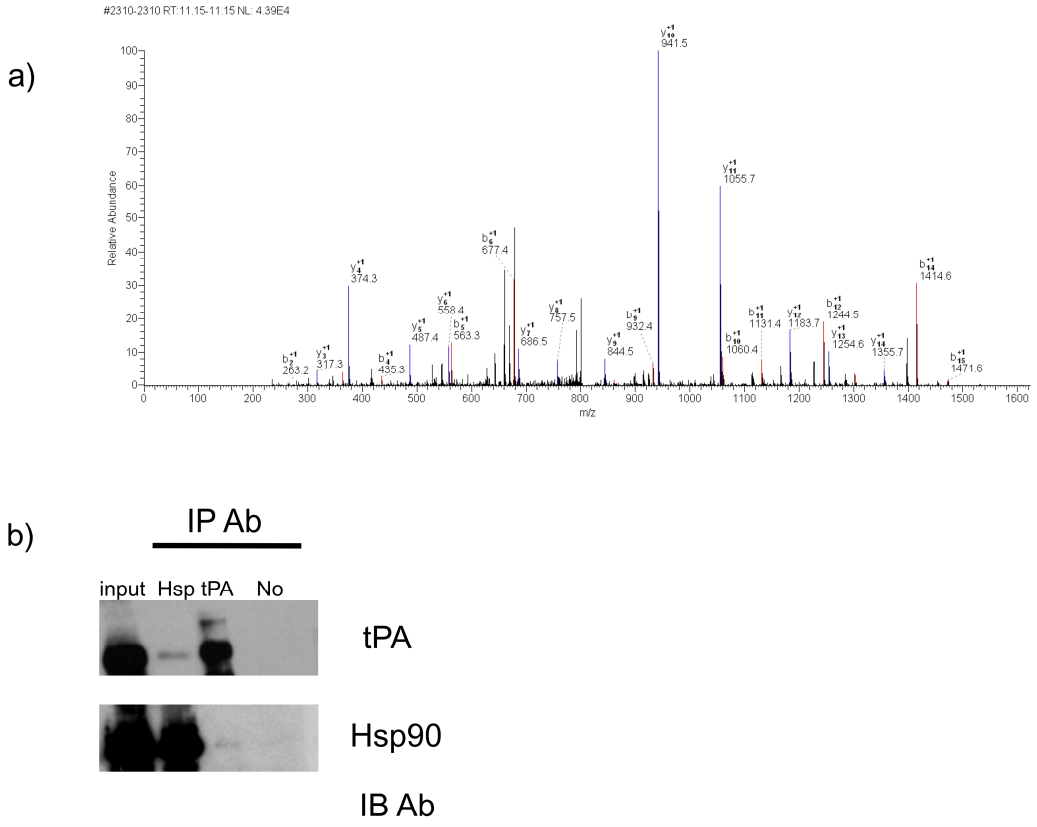

Figure 5 Extracellular hsp90α co-immunoprecipitates with tissue plasminogen activator. (a) MS/MS spectrum of the peptide ([248]VYTAQNPSAQALGLGK[263]) that was used to identify tPA as a HSP90α interacting protein. (b) Immunoprecipitation of hsp90α from the conditioned media of HT-1080 fibrosarcoma cells. Hsp90α was immunoprecipitated from the media of HT-1080 cells and resolved on 10% SDS-PAGE. Western blot analysis was performed using anti-hsp90α or anti-tPA antibodies. The input lane contains approximately 1/100th of the total media collected. "IP Ab" indicates the antibody used during immunoprecipitation (hsp90α, tPA or no antibody as the negative control). "IB Ab" indicates which antibody was used the probe the immunoblot. Representative images from three independent experiments are shown.

Hsp90α aids in the conversion of plasminogen to plasmin

Previous data from our lab indicated that extracellular hsp90α increases cancer cell invasion by assisting in the activation of MMP-2 [2]. To test if extracellular hsp90α can promote the activation of other extracellular proteins involved in cancer cell motility we assessed whether the activation of plasmin through its association with tPA requires extracellular hsp90α. We performed plasmin activation assays with HT-1080 fibrosarcoma cells in the presence or absence of an inhibitor of extracellular hsp90α (Figure 6a). We used DMAG-N-oxide, an impermeable form of the hsp90α inhibitor geldanamycin [17] to determine if extracellular hsp90α activates plasmin. The addition of DMAG-N-oxide resulted in a 32% decrease in activated plasmin when compared to cells that were treated with vehicle alone (Figure 6b, $p < 0.02$). These findings indicate that extracellular hsp90α is involved in the conversion of plasminogen to plasmin.

Inhibition of extracellular hsp90α decreases tumor cell migration

While extracellular hsp90α can activate plasmin, it was not known if this activation contributes to increased tumor cell motility. We performed transwell migration assays using a function-inhibiting antibody against hsp90α (Figure 7). This antibody will only inhibit the extracellular hsp90α because antibodies are membrane impermeant. HT-1080 fibrosarcoma cells treated with the hsp90α antibody migrated 37% less than control treated cells ($p < 0.01$). Interestingly, the addition of plasmin alone did not increase cell motility. Perhaps the cells are

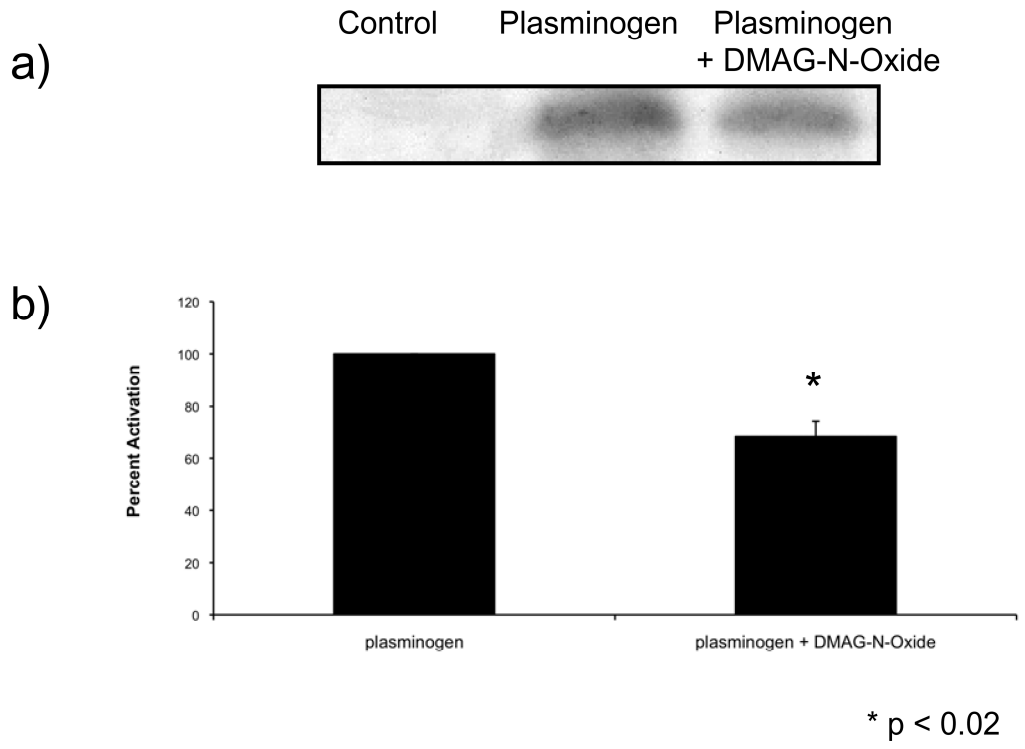

Figure 6 Extracellular hsp90α assists in the conversion of plasminogen to plasmin. (a) HT-1080 fibrosarcoma cells were treated with either DMSO, plasminogen or plasinogen plus DMAG-N-oxide, a membrane impermeant inhibitor of hsp90α. Samples were added to a gelatin zymogram. A representative image taken from three independent experiments is shown. (b) Graphical representation of the plasminogen activation assay. Densitometry results are expressed as the percent decrease in plasmin activation (control set to 100%) and are from three independent experiments. Inhibition of extracellular hsp90α with DMAG-N-oxide results in a 32% decrease in plasmin activation ($p < 0.02$, two tailed t test).

already saturated with active plasmin such that additional plasmin would not affect migration. Normal migration was recovered when we added activated plasmin to the cells treated with hsp90α antibody. Together, these findings indicate that hsp90α can activate plasmin and this activity stimulates cell motility.

Discussion

In this study we present evidence that extracellular hsp90α, secreted via exosomes, activates a novel client protein and increases tumor cell motility. Previously published work from our lab and others indicate that extracellular hsp90α contributes to the activation of both MMP-2 [2] and HER-2 [26], two proteins involved in cancer metastasis. We now present data indicating that extracellular hsp90α is necessary for the activation of a second protease, plasmin, also involved in tumor metastasis [27]. Inhibiting extracellular hsp90α *in vivo* inhibits both wound healing [4] and tumor invasion [17]. Our current findings suggest however that other exosomal proteins may also contribute to these processes. Hsp90α protein alone does not elicit as complete an effect on cell morphology or movement as the addition of exosomes (Figures 3 and 4). Hsp90α binds to tPA and inhibiting hsp90α decreased plasmin activation and cell migration. Therefore, we speculate that hsp90α is part of an extracellular complex including annexin II, tPA and plasminogen that functions to increase cell movement. Annexin II is found in exosomes and has an established role in aggressive tumors and binds both tPA and plasminogen thereby enhancing the conversion of plasminogen to active plasmin [24]. Extracellular hsp90α increases annexin II at the cell surface in rat aortic cells, leading to an increase in plasmin production in these cells [23]. Also, cell surface annexin II expression levels are increased in metastatic tumors and it interacts with multiple extracellular pro-

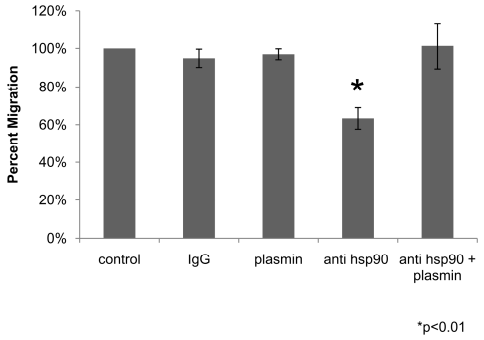

Figure 7 Inhibition of extracellular hsp90α inhibits cancer cell motility by preventing plasmin activation. HT-1080 cells were treated with control, IgG, plasmin, anti-hsp90α antibody, or plasmin plus anti-hsp90α antibody. A transwell migration assay was performed and cells that migrated through the chamber were counted. Results are from four independent experiments and expressed as percentage decrease from control (100%). Anti-hsp90α antibody results in a 37% decrease in migration ($p < 0.01$, two tailed t test).

teases that have been implicated in tumor progression [28]. Although plasmin is known for its role in cellular invasion it has not been well studied in migration, one component of the multi-step process of tumor invasion. Plasmin may be contributing to cell migration by contributing to the local remodelling of the extracellular matrix exposing cryptic cell attachment sites necessary for cellular migration, similar to that seen in smooth muscle cells during wound healing [29]. It is also possible that plasmin contributes to cell migration by interacting with currently unknown targets.

We suggest that exosome contents are released outside the tumor cell in close proximity to each other and other inactive extracellular pro-invasive proteins such as plasminogen. Once released from the exosomes, extracellular hsp90α assists in the activation of pro-MMP2 as well as plasminogen. Beyond MMP-2 and plasmin it is possible that hsp90α could activate other extracellular proteins as most of the proteins identified by mass spectrometry in this study were found in their inactive pro-forms. It is therefore interesting to speculate that extracellular hsp90α could activate a cassette of proteins that function collectively in cancer cell migration. These proteins would act in concert to enhance breakdown and remodeling of the extracellular matrix and permit the tumor cell to invade its microenvironment. Thus, inhibition of extracellular hsp90α could inhibit a growing number of proteins that are responsible for increased tumor cell movement making extracellular hsp90α an attractive target for drug therapy to limit tumor invasion.

Conclusions

In summary, we have identified that exosomes increase cell motility. One mechanism for this increased motility is the activation of plasmin by extracellular hsp90α. The discovery of a second protease activated by extracellular hsp90α suggests the possibility that the one role of extracellular hsp90α in cancer cells is the activation of precursor proteins that contribute to cellular migration and invasion.

Competing interests

JM, JDS, DC, DGJ: none declared.

Authors' contributions

JM contributed to study design, data interpretation, carried out all of the experiments and drafted the manuscript. JDS contributed to experiments in Figure 1, to study design, data interpretation and helped revise the manuscript. DC carried out the mass spectrometry. DGJ contributed to study design, data interpretation and editing of the manuscript. All authors have read and approved the final manuscript.

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