Stony Brook University

Heat shock protein, Hsp90α Plays a Role in MT1-MMP's Ability to Activate Substrate Proteins

A Thesis Presented

By

Theresa Buck

to

The Graduate School

In Partial Fulfillment of the

Requirements

For the Degree of

Master of Science

In

Biochemistry and Cell Biology

Stony Brook University

December 2011

Stony Brook University

The Graduate School

Theresa Buck

We, the thesis committee for the above candidate for the

Master of Science degree, hereby recommend

acceptance of this thesis.

Jian Cao – Thesis Advisor
Department of Medicine

Neta Dean
Professor, Department of Biochemistry and Cell Biology

This thesis is accepted by the Graduate School

Lawrence Martin
Dean of the Graduate School

Abstract of the thesis

Heat shock protein, Hsp90α Plays a Role in MT1-MMP's Ability to Activate Substrate Proteins

By

Theresa Buck

Master of Science

In

Biochemistry and Cell Biology

Stony Brook University

2011

Membrane type 1 matrix metalloproteinase (MT1-MMP) plays a major role in cancer invasion and metastasis. The mechanism by which MT1-MMP is trafficked to the plasma membrane where it functions to proteolytically activate its substrate proteins is not fully understood. The gene of heat shock protein (Hsp90α) was identified as being upregulated in HT1080 cells treated with concanavalin A (conA), a lectin that promotes proMMP-2 activation and cell migration in HT1080 cells compared to untreated HT1080 cells. To determine the mechanism underlying MT1-MMP substrate degradation and cell migration, biological and biochemical approaches were carried out. Zymography was preformed to examine the affect of the presence of both Hsp90α and MT1-MMP, on the gelatinase activity of matrix metalloproteinase (MMP-2). The zympograph showed that

when both Hsp90α and MT1-MMP were both present, there was an increase in the active form of MMP-2, a protein that is proteolytically activated by MT1-MMP at the plasma membrane. shRNA knockdown studies of Hsp90α were also done to further establish a role for Hsp90α in effecting the ability of MT1-MMP to activate its target proteins at the plasma membrane. Together these studies indicate that Hsp90α plays a role in the ability of MT1-MMP to activate substrate proteins at the plasma membrane.

Table of Contents

List of Figures

Acknowledgments

Thank you Jian Cao for sponsoring my research and to everyone in the Cao lab for teaching me so much. None of this would be possible without you.

Introduction

Cancer is the second leading cause of death in the United States. Metastasis of cancer usually evolves over time and generally indicates a poor prognosis. Unlike the process of tumorigenesis, the process of metastasis involves similar steps for all cancer cells. The process of cancer metastasis involves many steps and requires that cancer cells acquire many mutations to become metastatic. Understanding the mechanisms involved in cancer metastasis may be important in designing new therapies targeted against cancer metastasis.

Metastasis begins by a piece of a primary tumor breaking off, degrading the basement membrane, a physically tough, highly cross-linked structure that does not contain pores large enough to allow for passive invasion, and invading surrounding normal tissue. Once the tumor cell invades the surrounding tissue it will make its way to nearby lymphatic and/or blood vessels where it will penetrate across the vessel wall and into the circulatory system. The tumor cell must be able to survive in the circulatory system and travel throughout the body where it will eventually arrest in a capillary bed. Once again the tumor cell must penetrate the vessel wall and invade into the underlying tissue where a secondary tumor site will be established.

Matrix metalloproteinases (MMPs) are a family of proteinases that include both secreted and membrane bound proteins that play an important role in both normal physiological processes as well as in numerous diseases including cancer and cancer metastasis. MMPs all share a conserved structure that consists of a signal peptide, a propeptide and a catalytic domain. Several members of the MMP family of proteinases including MT1-MMP, a membrane bound MMP, and MMP2, a secreted MMP, have a

hemopexin (HPX) domain that is involved in protein-protein interactions and substrate recognition (Cao et al. 2004). A positive correlation between expression of multiple MMPs and tumor progression has been demonstrated in numerous studies (Foda and Zucker 2001). MMPs process a variety of substrates, many of which are essential for tumor invasion and metastasis including cell adhesion proteins, extracellular matrix (ECM) proteins, and proMMPs. MMPs facilitate tumor cell invasion and metastasis by several different mechanisms including proteolysis of many ECM molecules such as collagen, laminin, and proteoglycans. Cleavage of these molecules facilitates tumor cell invasion by removal of the physical barrier of the ECM. MMPs regulate cell adhesion through regulating the formation of new cell-matrix and cell-cell attachments as well as regulating the breakdown of existing cell-matrix and cell-cell attachments. Both of which are necessary for tumor cells to move through the ECM and therefore necessary for tumor cell invasion (Kleiner and Stetler-Stevenson 1999).

Both membrane bound and secreted MMPs are synthesized as inactive pro-enzymes that require activation for proteolytic activity. Both the intracellular location and the activation of MT1-MMP are highly regulated. It has been suggested that pro-MT1-MMP is retained in some type of storage pool prior to activation. Upon activation, pro-MT1-MMP is activated by proteolytic cleavage by furin or furin-like proprotein convertases in the trans-Golgi network before trafficking to the plasma membrane (Wu et al. 2007; Yana and Weiss 2000). In contrast, activation of secreted MMP2, a 72kDa protein and the most widely distributed MMP, is mediated by a tri-molecular complex on the cell surface that consists of a homodimer of MT1-MMP and a single molecule of tissue inhibitor of metalloproteinases (TIMP-2). ProMMP2 is secreted as the inactive pro-form and proteolytically cleaved by this

tri-molecular complex into the active 62kDa form at the cell surface (Birkedal-Hansen et al. 1993).

Both MT1-MMP and MMP2 have been shown to accumulate in invadopodia, specialized plasma membrane protrusions enriched in actin, cell-matrix adhesion molecules, actin assembly regulators, tyrosine kinases, and proteases capable of ECM degradation that form at the leading edge of invasive tumor cells (Hofmann et al. 2003; Stylli et al. 2008). Recent findings point to a more direct role for MT1-MMP in proteolytic cleavage of many ECM components and cell surface proteins, a role that does not depend on MT1-MMP activation of MMP2 for its proinvasive qualities (Hotary et al. 2006).

As an important protease, MT1-MMP is regulated at several levels including gene transcription, intracellular trafficking, proteolytic activation, and inhibition by tissue inhibitors of metalloproteinases (TIMPs). Deregulation at any of these levels can lead to diseases involving metalloproteinases including cancer. It has been proposed that MT1-MMP is synthesized and stored in a temporary storage compartment, likely the trans Golgi network or endosomes where it is accessible for rapid trafficking to the cell surface upon stimulation of the cell (Zucker et al. 2002). The mechanism by which MT1-MMP is trafficked from the intracellular compartments to the cell surface has not been fully characterized. The proinvasive role of MT1-MMP requires that the enzyme be activated and inserted into the plasma membrane. The amount of active MT1-MMP at the cell surface is balanced by the rate of trafficking of MT1-MMP to the plasma membrane versus the rate of MT1-MMP removed from the cell surface through endocytosis. MT1-MMP is internalized by dynamin-dependent endocytosis in clathrin-coated pits (Zucker et al. 2002). The cytoplasmic domain of MT1-MMP is necessary for the endocytic process. The

internalization of MT1-MMP may be linked to its activation status; inactive proMT1-MMP is internalized more rapidly than active MT1-MMP. Internalization of MT1-MMP at the cell surface may function as a way to regulate MT1-MMP activity at the plasma membrane (Remacle et al. 2006). Once MT1-MMP is internalized, it can either move to a lysosome for degradation or it can be recycled back to the cell surface as an active enzyme (Remacle et al. 2003). In most cell types, surface expression of MT1-MMP remains low, because MT1-MMP is efficiently removed from the cell surface by clathrin-mediated endocytosis. However, in invasive tumor cells there is increased surface expression of MT1-MMP due to mechanisms that exist in these cells to counteract the removal of MT1-MMP from the cell surface (Artym et al. 2006). In addition to the decrease in removal of MT1-MMP from the cell surface, there is increased trafficking of MT1-MMP to invadopodia in invasive tumor cells. The MT1-MMP seen at invadopodia comes from both the recycling of MT1-MMP that was internalized by the endocytic pathway and the mobilization of MT1-MMP from intracellular compartments. It is likely that both sources of MT1-MMP intersect in a post-trans Golgi network before MT1-MMP is trafficked to the invadopodia (Bravo-Cordero et al. 2007).

Having an intracellular pool of synthesized MT1-MMP available to be trafficked to the plasma membrane upon stimulation allows for a rapid response to stimuli signaling for cell migration and invasion. A recent study has shown that MT1-MMP is moved to the plasma membrane upon stimulation of the cell via an exocytotic pathway regulated by Rab8 GTPase. Recruitment of vesicles containing both MT1-MMP and Rab8 to the plasma membrane is induced by collagen interaction as shown with time-lapsed confocal imaging.

Collagen engagement causes β1 integrin clustering which is the cue that leads to MT1-MMP recruitment to the plasma membrane (Bravo-Cordero et al. 2007).

A previous study showed that CD44H acts as a linker between MT1-MMP and the actin cytoskeleton and that this interaction leads to MT1-MMP trafficking to the plasma membrane from an intracellular storage pool. CD44H interacts with MT1-MMP via the hemopexin-like (PEX) domain of MT1-MMP (Mori et al. 2002). The cytoplasmic tail of CD44H interacts with the actin cytoskeleton and assists in the trafficking of MT1-MMP to the lamellipodia. Rho family GTPases are responsible for the formation of lamellipodia and the localization of CD44H to the lamellipodia, possibly through the reorganization of the actin cytoskeleton. Therefore, CD44H forms a complex with MT1-MMP, linking it to the actin cytoskeleton, and placing MT1-MMP downstream of regulation by the Rho family GTPases (Mori et al. 2002).

Another possible regulator of MT1-MMP trafficking to the plasma membrane is the heat shock protein, Hsp90α. Previous studies suggest that the secreted, extracellular form of Hsp90α interacts with the hemopexin-like domain of MMP2 (Eustace et al. 2004). It has been hypothesized that Hsp90α affects the activation status of MMP2 and not the secretion of MMP2 because pro-MMP2 protein levels show only small changes when Hsp90α is inhibited (Eustace et al. 2004; Song et al. 2010). Here it is hypothesized that Hsp90α assists in the trafficking of MT1-MMP to the plasma membrane where it is then able to activate MMP2. Increased MT1-MMP at the plasma membrane would allow additional MT1-MMP to activate MMP2 as well as other substrate proteins. Since many of the substrate proteins of MT1-MMP are pro-invasive, this increase in MT1-MMP activation would result in an increase in invasion and metastasis.

In this study, we examined the role of Hsp90α in the trafficking of MT1-MMP to the plasma membrane. To determine if there were any significant differences in the genetic profiles of cells stimulated for migration and unstimulated cells we examined the differences in gene expression profiles of HT1080 cells treated with concanavalin A (conA) and HT1080 cells that were untreated. PCR-select cDNA subtraction was used to examine the differences in gene expression profiles of the treated and untreated HT1080 cells. Hsp90α was one of the gene products upregulated in the conA treated cells. It was hypothesized that the role Hsp90α played in cell migration involved an interaction with MT1-MMP and more specifically that Hsp90α played a role in the trafficking of MT1-MMP to the plasma membrane. In order for MT1-MMP to activate the gelatinase activity of MMP2, MT1-MMP has to be present in the active form at the plasma membrane. Zymography was used to determine if Hsp90α affected the ability of MT1-MMP to activate the gelatinase activity of MMP2. To further demonstrate that Hsp90α had an affected on MT1-MMP, small-hairpin RNA (shRNA) knockdown of Hsp90α was also used to determine the effect of a decrease in Hsp90α on MT1-MMP and MMP2 activation.

Materials and Methods

RNA preparation for PCR-select cDNA subtraction – The protocol was adapted from Clontech PCR-select cDNA subtraction protocol. RNA was isolated using the procedure detailed under shRNA isolation. Isolated RNA was run on a 1% agarose to determine if it was denatured.

cDNA synthesis for PCR-select cDNA subtraction – The protocol was adapted from Clontech PCR-select cDNA subtraction protocol. Briefly, three different cDNA synthesis reactions were set up; RNA from ConA treated HT1080 cells and RNA from HT1080 cells untreated with ConA, the latter was the control RNA. cDNA was synthesized using 2μg RNA for each reaction and 10μM cDNA synthesis primer. 2μl 5x first-strand buffer, 10mM dNTP mix, 1μl water, and 20units/μl AMV reverse transcriptase was added to each reaction mixture and the reaction mixtures were centrifuged. For the second-strand cDNA synthesis 48.4μl water, 16μl 5x second-strand buffer, 10mM dNTP mix, and 4μl 20x second-strand enzyme cocktail was added to each first-strand tester and driver. The reaction mixtures were incubated at 16°C for 2 hours. 2μl T4 DNA polymerase was added to each reaction mixture.

Rsa I digestion for PCR-select cDNA subtraction – The protocol was adapted from Clontach PCr-select cDNA subtraction protocol. Briefly, following procedure was preformed with cDNA from the HT1080 cells treated with conA and the untreated HT1080 cells as well as with the driver cDNA. 43.5μl ds cDNA, 5μl 10x Rsa I restriction buffer, and 1.5μl Rsa I (10units/μl) were combined and incubated at 37°C for 1.5hours. 50μl phenol:choloroform:isoamyl alcohol (25:21:1) was added to the reaction mixture. 50μl chloroform:isoamyl alcohol (24:1) was added. The aqueous layer was collected and saved. 25μl 4M NH_4OAc and 187.5μl 95% ethanol was added to the remaining phases. 200μl 80% ethanol was added to the pellets and centrifuged at 14,000rpm for 5minutes. The supernatant was removed and the pellets were air-dried.

Adaptor ligation for PCR-select cDNA subtraction - 1μl of each Rsa I-digested experimental tester cDNA was diluted with 5μl water. A ligation master mix (3μl water, 2μl 5x ligation buffer, and 1μl T4 DNA ligase for each reaction) was prepared for each tester cDNA. For each experimental tester cDNA, two ligation reaction mixtures were set up with the following; 2μl diluted tester cDNA, 2μl adaptor 1, 6μl master mix and 2μl diluted tester cDNA, 2μl adaptor 2R, 6μl master mix. The ligation reactions were centrifuged and incubated at 16°C overnight. 1μl EDTA/glycogen mix was added to stop the ligation reaction. The samples were heated at 72°C for 5minutes to inactivate the ligase and briefly centrifuged.

First hybridization for PCR-select cDNA subtraction – Two different reaction mixtures were set up for each experimental tester cDNA and control cDNA. One reaction mixture contained 1.5μl Rsa I-digested driver cDNA, 1.5μl adaptor 1-ligated tester, and 1μl 4x hybridization buffer. The second reaction mixture contained 1.5μl Rsa I-digested driver cDNA, 1.5vl adaptor 2R-ligated tester, and 1μl 4x hybridization buffer. A drop of mineral oil was laid over each sample and then they were centrifuged briefly. The samples were incubated at 98°C for 1.5minutes in a thermal cycler and then incubated at 68°C for 8hours.

Second hybridization for PCR-select cDNA subtraction - 1μl driver cDNA, 1μl 4x hybridization buffer, and 2μl water was added for each experimental tester cDNA and the control cDNA. 1 drop of mineral oil was placed on 1μl of each reaction mixture. The reaction mixtures were incubated at 98°C for 1.5minutes in the thermal cycler. Denatured driver was added to hybridization samples 1 and 2 simultaneously for each experimental tester cDNA and the control cDNA. The reactions were incubated at 68°C overnight. Then

200μl dilution buffer was mixed with each reaction. The reactions were heated at 68°C for 7minutes in the thermal cycler.

PCR amplification for PCR-select cDNA subtraction – A PCR master mix was created with 19.5μl water, 2.5μl 10x PCR reaction buffer, 0.5μl 10mM dNTP mix, 1μl 10μM PCR primer, and 0.5μl 50x advantage cDNA polymerase mix per reaction. 24μl master mix was added to each reaction and overlaid with 50μl mineral oil. The reaction mixtures were incubated at 75°C for 5minutes in the thermal cycler. The reaction mixtures were incubated at 94°C for 25 seconds followed by 27 cycles of 94°C for 10seconds, 66°C for 30seconds, and 72°C for 1.5minutes. 8μl from each reaction mixture was analyzed on a 2% agarose/EtBr gel. 3μl of each primary PCR mixture was diluted in 27μl water. A PCR master mix for the secondary PCR reactions containing 18.5μl water, 2.5μl 10x PCR reaction buffer, 1μl 10μM nested PCR primer 1, 1μl 10μM nested PCR primer 2R, 0.5μl 10mM dNTP mix, and 0.5μl 50x advantage cDNA polymerase mix per reaction was created. 24μl master mix was added to each reaction and overlaid with 1 drop of mineral oil. The reaction mixtures were incubated at 94°C for 10seconds, 68°C for 30seconds, and 72°C for 1.5minutes for 12 cycles. 8μl from each reaction was analyzed on a 2% agarose/EtBR gel.

Zymography – Six 3cm plates of Cos-1 cells each with 105 cells per plate were transiently transfected using 75μl NaCl and 2μl polyethylenimie (PEI) per plate and 75μl NaCl with varying combinations of GFP cloned into pcDNA3.1, MT1-MMP in pcDNA3.1, Hsp90α in pcDNA3.1, and the empty pcDNA3.1 vector as a control. After 24hr the media was changed with 600μl FBS free media and 20μl gelatinase (MMP2) was added. After 24hr the conditioned media was collected, samples were examined by gelatin zymography as

follows; after electrophoresis, the SDS in the gel was removed by washing in renaturing buffer (2.5% Triton 100X in dH_2O). The gel was developed in developing buffer (50mM Tris-Bff, pH8.0, 200mM NaCl, 5mM $CaCl_2$, 0.02% Brij-35) at 37°C overnight. The gel was stained in staining solution (2.5g coomassie blue dissolved in 1.25l methanol, 0.5l acetic acid, .75l water) for 15-30minutes, destained for 2 hours in destaining solution (20% methanol and 10% acetic acid) with multiple changes of destaining solution. Zones of gelatinase activity were detected as clear bands against a blue background. The relative amount of gelatinase activity from each sample can be determined by the size of the clear band, the larger the band, the more gelatinase activity is present.

shRNA synthesis – Primers were designed and synthesized. The annealed double stranded oligos for the target gene were inserted into pSIREN plasmid containing U6 promoter to enhance shRNA synthesis. The constructs were selected by a PCR approach using 10μl PCR master mix (3.30μl si 5' primer, 3.30μl si 3' primer, 11μl dNTP, 13μl 10X PCR buffer, 1.30μl *Taq* polymerase, 98μl water) and half of a colony was used for the colony PCR. The 10μl PCR samples were run on a 1.2% agarose gel. Two colonies were chosen and sequenced to confirm the presence of the shRNA in the pSIREN plasmid. Maxiprep and RNA isolation was preformed on one of the selected colonies

shRNA transfection – One 10cm plate of GP2-293 cells was transfected using 400μl NaCl + polyethylenimie (PEI), 400μl NaCl + 4μg vesicular stomatitis virus protein G (VSV-G), and 400μl NaCl + shRNA-Hsp90α. A second 10cm plate of GP2-293 cells was transfected using 400μl NaCl + PEI, 400μl NaCl + 4μg VSV-G, and 400μl NaCl + shRNA-Luciferase. Conditioned media was collected over two days from both plates and filtered with a

0.65μM filter. Media containing the virus was added to HT1080 cells. The HT1080 were grown for 48hrs. Transduced cells were selected using 3μg/ml puromycin.

shRNA isolation - RNA was isolated using the Qiagen RNeasy kit from HT1080 cells containing shLuciferase and HT1080 cells containing shHsp90α. 1μg of RNA from the shRNA-Hsp90α transfected cells and the shRNA-Luciferase transfected cells was run on a 1% agarose gel for 25minutes.

cDNA synthesis – Two reaction mixtures were set up, one for the RNA from the HT1080 shLuciferase cells and one for the RNA from the HT1080 shHsp90α cells. Each reaction mixture contained 1μg of the respective RNA isolated from the cells, 2μl 5X buffer, 0.5μl reverse transcriptase, and water for a total a reaction mixture of 10μl. The reaction mixtures were incubated at 42°C for 30minutes followed by incubation at 85°C for 5minutes.

Real-Time PCR – Two reaction mixtures were set up, one for the Luciferase cDNA and one for the Hsp90α cDNA. Each reaction mixture contained 2μl of the respective cDNA, 0.2μl forward primer (5'GCAGAAATTGCCCAGTTGAT), 0.2μl reverse primer (5'TGCAGCTCTTTCCCAGAGTC), 10μl 2X Biorad iCycler SYBR-Green super mix, and water to have a total reaction mixture of 20μl. Both reaction mixtures were incubated at 95°C for 5minutes, followed by incubation at 94°C for 30seconds 30X, followed by incubation at 60°C for 30seconds 30X, followed by incubation at 72°C for 30seconds 30X, followed by one 7minute incubation at 72°C.

Results

Upregulation of the Hsp90α gene in HT1080 cells treated with concanavalin A PCR-select cDNA subtraction, a powerful technique used to examine differences in genetic profiles of two different populations of cells. To examine differences in the genetic profiles of cells stimulated to migrate and non-migrating cells, the genetic profiles of HT1080 cells treated with concanavalin A (conA) and HT1080 cells untreated were compared. ConA is a lectin that promotes cell migration in HT1080 cells by stimulating MT1-MMP mediated activation of proMMP2 in vitro. ConA treatment stabilizes the tissue inhibitor of metalloproteinases-2 (TIMP2) and MT1-MMP complex at the plasma membrane thereby promoting MMP2 activation (Shofuda et al. 1998). The PCR-select cDNA subtraction identified six genes that differed between the HT1080 treated and untreated cells. We identified Hsp90α as a gene upregulated in HT1080 cells treated with conA (figure 1).

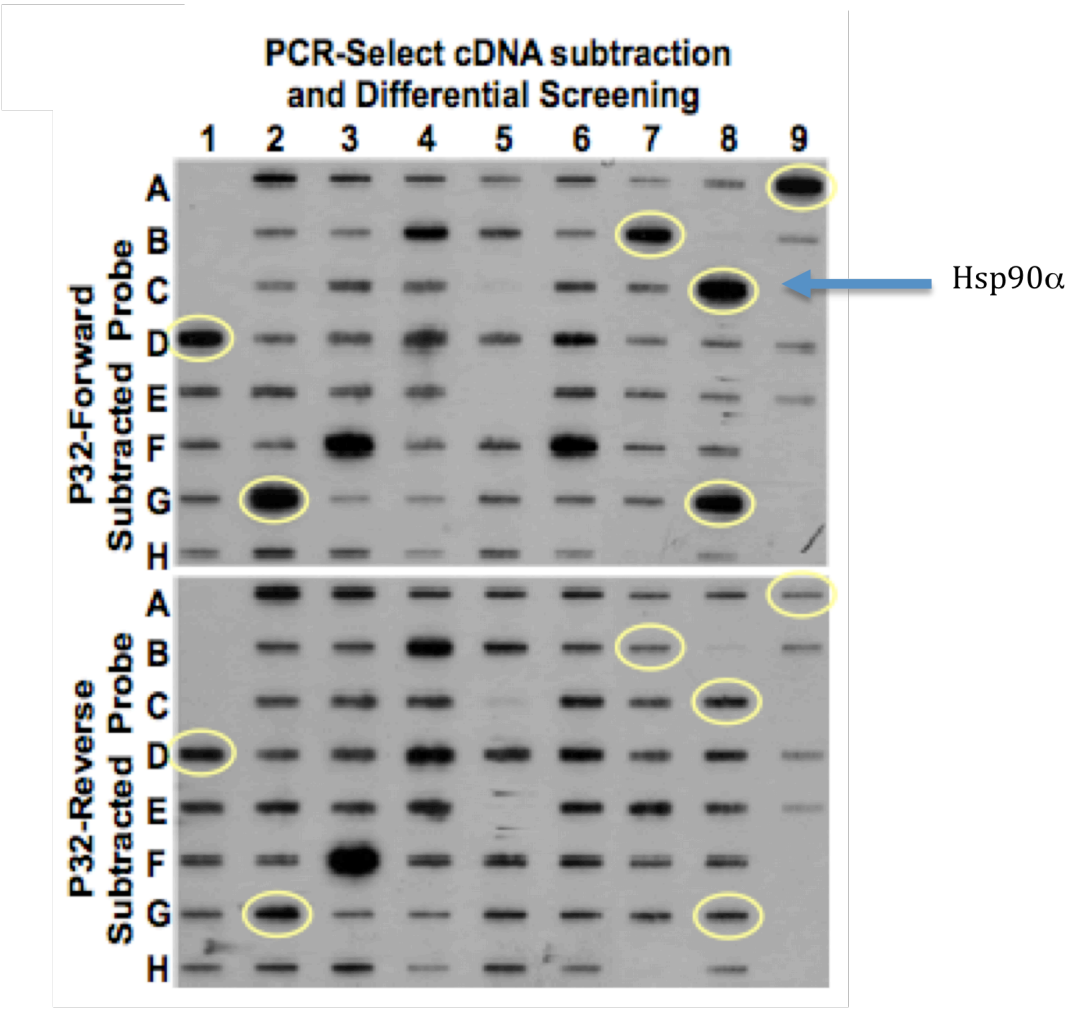

Figure 1 PCR-select cDNA subtraction of HT1080 cells vs. HT1080 cells treated with conA. Circled bands represent differences in gene expression in HT1080 untreated cells vs. HT1080 cells treated with conA. Band C8 represents Hsp90α which appeared to be upregulated in the HT1080 cells treated with conA.

Increase in the activated form of MMP2 when both Hsp90α and MT1-MMP are present To examine the effect of Hsp90α on MT1-MMP, the relative amount of the active form of MMP2 was compared to the amount of inactive form of MMP2 (Pro-MMP) as determined by the amount of gelatinase activity in both the presence and absence of Hsp90α using zymography. Using zymography, the relative amount of gelatinase activity is determined by comparing the size of the bands produced by gelainase activity, the larger

the band; the more gelatinase activity is present. In this experiment, an increase in gelatinase activity was seen when both Hsp90α and MT1-MMP were present compared to when either Hsp90α or MT1-MMP were present in the absence of the other. In lanes 1 and 2, which contained conditioned media from Cos-l cells transiently transfected with GFP in pcDNA3.1 + empty pcDNA3.1 and Hsp90α in pcDNA3.1 + empty pcDNA3.1 respectively, no activated form of MMP2 was present as expected. In lanes 3 and 5 which contained conditioned media from Cos-1 cells transiently transfected with MT1-MMP in pcDNA3.1 + empty pcDNA3.1 and MT1-MMP in pcDNA3.1 + Hsp90α in pcDNA3.1 respectively, there was an increase in the amount of the activated form of MMP2 present in lane 5, which contained conditioned media containing both MT1-MMP and Hsp90α compared to lane 3, which contained conditioned media only containing MT1-MMP. An increase in the amount of the activated form of MMP2 was also seen in lane 6 compared to lane 4, which contained conditioned media from Cos-l cells transiently transfected with different amounts of MT1-MMP in pcDNA3.1 + Hsp90α in pcDNA3.1 and MT1-MMP in pcDNA3.1 + empty pcDNA3.1 respectively compared to lanes 3 and 5 (figure 2).

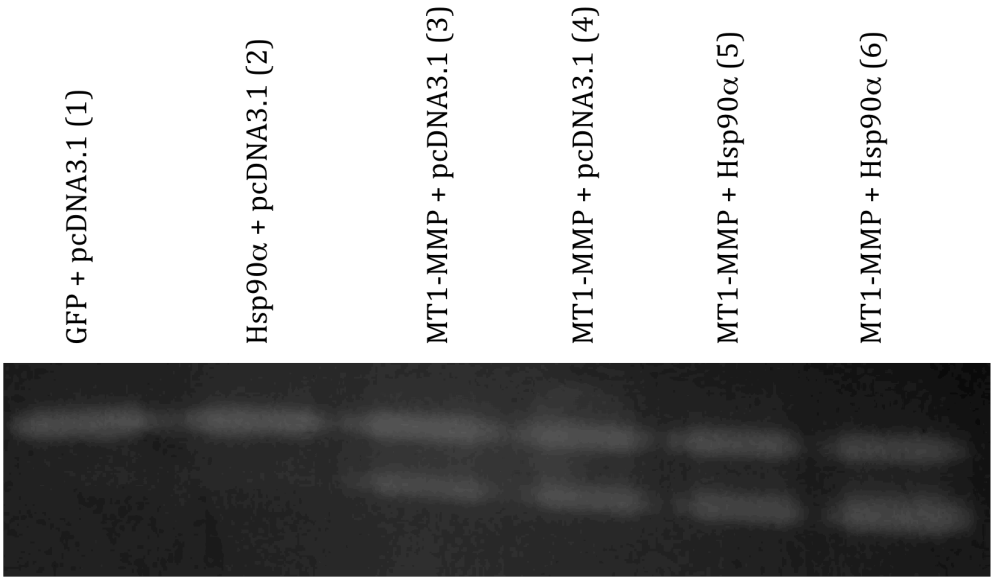

Figure 2 Gelatin zymograph of MMP2 in conditioned media from Cos-1 cells. The only digested areas correspond to MMP2 activity. An increase in activity is seen when both Hsp90α and MT1-MMP are present. Each lane contained 20μl conditioned media from Cos-l cells transiently transfected with the following DNA; lane 1 contained 1μg GFP in pcDNA3.1 + 1μg empty pcDNA3.1, lane 2 contained 1μg Hsp90α in pcDNA3.1 + 1μg empty pcDNA3.1, lane 3 contained 0.25μg MT1-MMP in pcDNA3.1 + 1.75μg empty pcDNA3.1, lane 4 contained 1μg MT1-MMP in pcDNA3.1 + 1μg empty pcDNA3.1, lane 5 contained 0.25μg MT1-MMP in pcDNA3.1 + 1.75μg Hsp90α in pcDNA3.1, lane 6 contained 1μg MT1-MMP in pcDNA3.1 + 1μg Hsp90α in pcDNA3.1.

These results show that in the presence of both MT1-MMP and Hsp90α there is an increase in the active form of MMP2, in contrast when only MT1-MMP or only Hsp90α is present, active MMP2 is reduced indicating a possible role either directly or indirectly for Hsp90α in MT1-MMP activation of MMP2.

shRNA knockdown of Hsp90α To further examine the effect of Hsp90α on MT1-MMP, Hsp90α was knocked down using shRNA against Hsp90α (shRNA-Hsp90α). DH5α cells were transformed with a pSIREN plasmid containing shRNA-Hsp90α. PCR was preformed to determine which colonies contained the shRNA in the pSIREN plasmid. It was determined that colonies from PCR samples run in lanes 2 and 4-11 contained the shRNA in

the pSIREN plasmid. Colonies from samples run in lanes 7 and 8 were selected and sent for sequencing (figure 3).

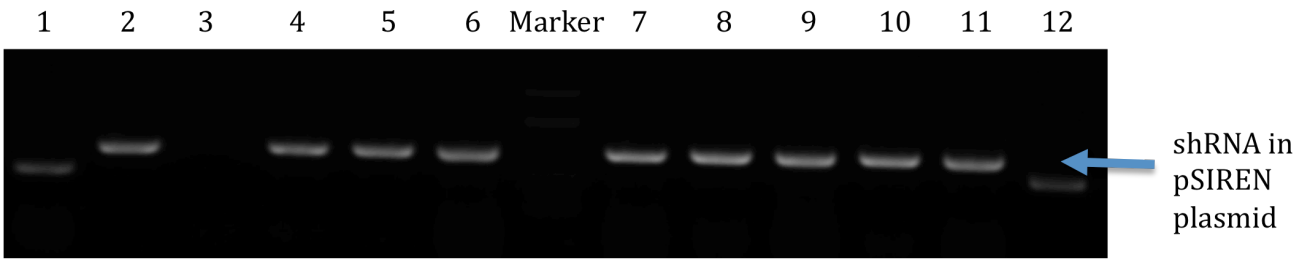

Figure 3 Colony PCR screen for shRNA in pSIREN plasmid. Lanes 1-12 contained 10μl PCR reaction from 12 different colonies. Lanes 2 and 4-11 contained the shRNA in pSIREN plasmid. Colonies run in lanes 7 and 8 were sent for sequencing.

DNA was isolated from one of the selected colonies and used to transfect GP2-293 cells. A viral transfection approach was used deliver shRNA into the cells because viral transfection creates a stably expressed shRNA, which allows for long-term gene knockdown experiments to be conducted. The approach used involved the specialized cell line, GP2-293, which stably expresses the viral *gag* and *pol* proteins. VSV-G is a viral envelope protein that is slightly toxic to cells. A plate of GP2-293 cells was transfected with VSV-G and shRNA-Hsp90α and a second plate of GP2-293 cells, to be used as a control, was transfected with VSV-G and shRNA-Luciferase in order to create a retro virus. Viral transduction was preformed. The media containing the virus was then added to HT1080 cells, a human fibrosarcoma invasive tumor cell line expressing endogenous MT1-MMP, MMP2 and Hsp90α in order to express the shRNA in the HT1080 cells. The cells were grown for 48hrs and selected using puromycin. RNA was isolated from both the HT1080 cells containing shRNA-Hsp90α and the HT1080 cells containing shRNA-Luciferase. The isolated RNA from both the shRNA-Hsp90α containing cells and the shRNA-Luciferase

containing cells were run on an agarose gel to check for RNA degradation. Two sharp bands corresponding to different parts of the ribosome, some of the most abundant RNA in the cell were present, indicating that the RNA was not degraded (figure 4).

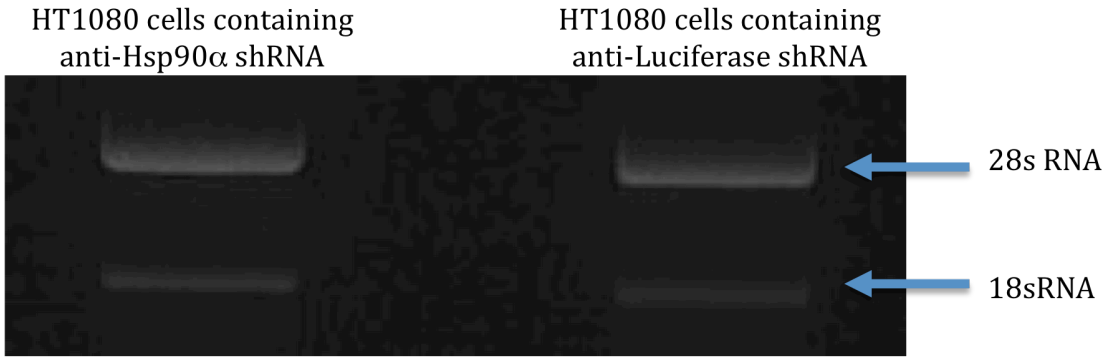

Figure 4 Degradation of isolated RNA check. The first lane contained 1μg of RNA isolated from HT1080 cells that were transfected with shRNA against Hsp90α. The second lane contained 1μg of RNA isolated from HT1080 cells that were transfected with shRNA against Luciferase. Both lanes showed a sharp band for 28s RNA and 18s RNA, indicating that the RNA was not degraded.

The isolated RNA was synthesized to cDNA using reverse transcriptase in order to measure the ability of the shRNA-Hsp90α to knock down Hsp90α. Real-time PCR was used to determine the ability of the shRNA to knockdown Hsp90α. There was a 10 fold decrease in Hsp90α RNA compared to Luciferase RNA seen in the real-time PCR. Luciferase RNA appeared in cycle 16 whereas Hsp90α RNA did not appear until cycle 22 indicating the ability of shRNA-Hsp90α to knockdown Hsp90α mRNA (figure 5).

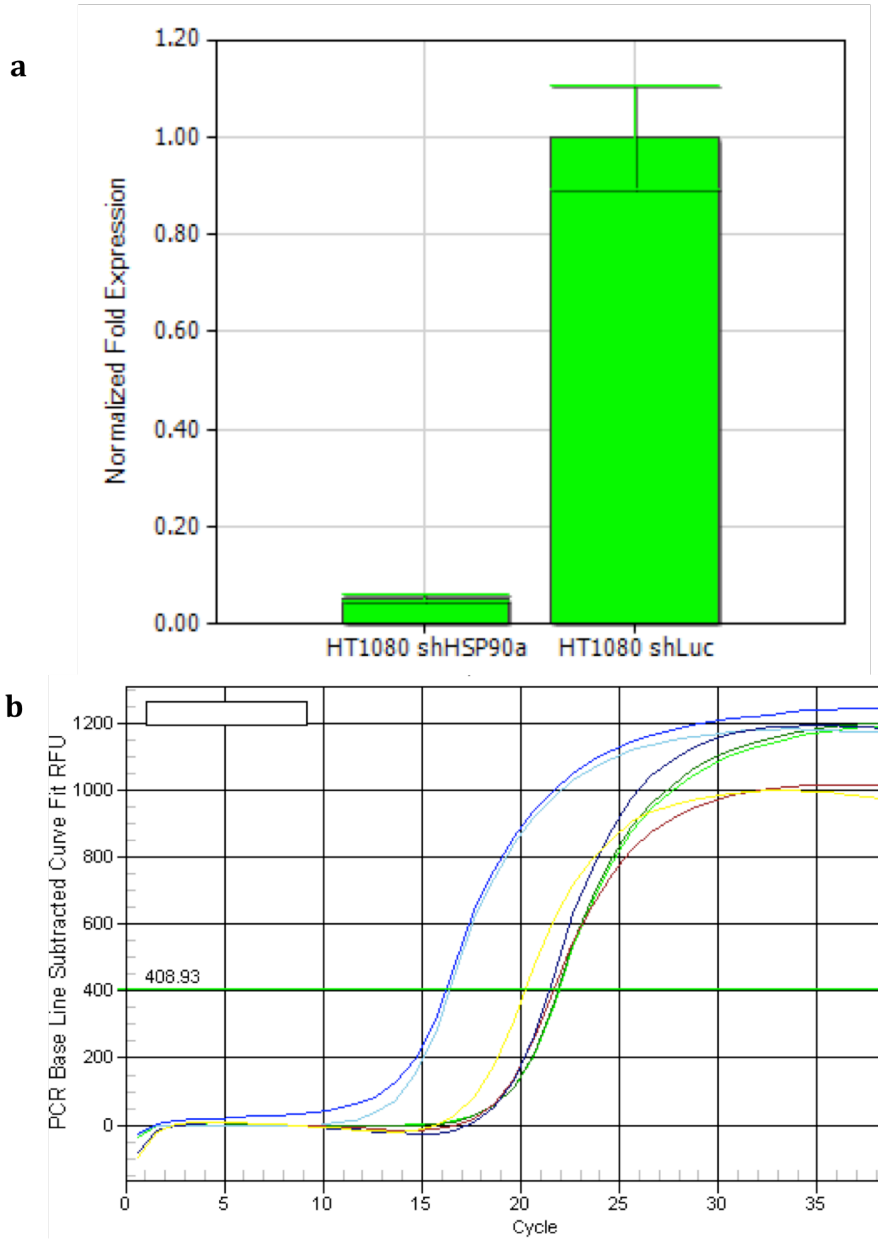

Figure 5 Real-time PCR for shRNA knockdown of Hsp90α. (**a**) Gene expression chart for shRNA knockdown of Hsp90α. HT1080 cells were transfected with a virus containing either shRNA-Hsp90α or shRNA-Luciferase. SYBR-green was used to view the level of RNA present. A 10fold knockdown of the level of Hsp90α RNA was seen compared to the level of Luciferase RNA. (**b**) PCR cycle chart for shRNA knockdown of Hsp90α. Hsp90α did not appear until the cycle 22 whereas Luciferse appeared in cycle 16, indicating a significant decrease in the amount of Hsp90α RNA present compared to Luciferase RNA.

Completion of the RNA knockdown of Hsp90α began in this study is necessary to determine the ability of shRNA-Hsp90α to knockdown the Hsp90α protein. If shRNA-Hsp90α is able to successfully knockdown the Hsp90α protein further experiments will need to be done to determine the effect of this knockdown on MT1-MMP.

Discussion

The small heat shock protein, Hsp90α is known to have many client proteins. A current list of known Hsp90 client proteins is maintained on-line by the Picard lab (http://www.picard.ch/downloads/Hsp90facts.pdf). It is also know that Hsp90α plays a significant role in cancer (Whitesell and Lindquist 2005). Known Hsp90α client proteins that play a role in cancer invasion and metastasis include MMP2 and MMP9, here we propose an additional Hsp90α client protein that plays an essential role in cancer invasion and metastasis, MT1-MMP. We propose that Hsp90α assists in the intracellular trafficking of MT1-MMP to the plasma membrane where it is than able to proteolytically activate pro-MMP2. Currently, the mechanism of MT1-MMP trafficking to the plasma membrane is not fully understood. Previous studies have shown the existence of an intracellular storage pool of pre-synthesized MT1-MMP available for trafficking to the plasma membrane upon stimulation of the cell. Currently there are two alternative hypotheses that explain how MT1-MMP is trafficked to the plasma membrane; use of an exocytic pathway that is regulated by Rab8 GTPase and the linkage of MT1-MMP to the actin cytoskeleton via CD44H (Bravo-Cordero et al. 2007; Mori et al. 2002). Here we propose an additional player in MT1-MMP trafficking, Hsp90α.

The upregulation of the Hsp90α gene seen in the PCR-select cDNA subtraction study comparing gene expression profiles of HT1080 cells both treated and untreated with conA suggests that Hsp90α plays a role in cell migration. The increase in the activated form of MMP2 when both Hsp90α and MT1-MMP were present in the gelatin zymograph further supports a role for Hsp90α in cell migration and more specifically a role for Hsp90α in enhancing the ability of MT1-MMP to activate MMP2. Completion of the shRNA knockdown of Hsp90α began in this study would provide additional evidence that Hsp90α has an effect on the ability of MT1-MMP to activate MMP2. In this study it was only determined that the shRNA synthesized against Hsp90α had the ability to knockdown Hsp90α RNA compared to Luciferase RNA in HT1080 cells. Further experiments need to be performed to determine if the knockdown of Hsp90α RNA using the shRNA-Hsp90α has any effect on the level of MMP2 activation. If our hypothesis that Hsp90α effects the trafficking of MT1-MMP to the plasma membrane is correct, then we predict that the knockdown of Hsp90α RNA using the shRNA-Hsp90α will result in a decrease in MMP2 activation since less Hsp90α would be available to enhance the ability of MT1-MMP to activate MMP2.

The results presented in this study support the hypothesis that Hsp90α affects the ability of MT1-MMP to activate MMP2. Further study is required to determine if the increase in MMP2 activation seen when both MT1-MMP and Hsp90α are present as seen in the gelatin zymograph is due to increased MT1-MMP trafficking to the plasma membrane do to direct assistance of Hsp90α or to a possible indirect effect of Hsp90α on MT1-MMP trafficking to the plasma membrane. Co-immunoprecipitation studies should be done to determine if there is a direct interaction between Hsp90α and MT1-MMP. If no direct interaction is seen between MT1-MMP and Hsp90α then it is possible that Hsp90α affects

MT1-MMP trafficking to the plasma membrane indirectly by interacting with another component of the MT1-MMP trafficking mechanism such as the cytoskeleton. Previous studies have shown that Hsp90α interacts with components of the cytoskeleton, including actin (Fostinis et al. 1992; Mounier and Arrigo 2002). Further study will be required to determine if these interactions with the cytoskeleton have any effect on the trafficking of MT1-MMP to the plasma membrane.

If it is determined that MT1-MMP and Hsp90α directly interact, additional experiments will address where the interaction takes place. Studies using Fluorescence Resonance Energy Transfer (FRET) would be beneficial in determining where Hsp90α and MT1-MMP interact. Determining the location of the interaction would help elucidate the role Hsp90α plays in the increase in MMP2 seen in the presence of both Hsp90α and MT1-MMP seen in the zymograph study.

From the results presented in this study it is not possible to conclude that Hsp90α assists in the trafficking of MT1-MMP to the plasma membrane. The results presented indicate that Hsp90α has an effect on the ability of MT1-MMP to activate MMP2. The use of co-immunoprecipitation and FRET could prove invaluable in further determining the role Hsp90α plays in affecting the ability of MT1-MMP to activate MMP2.

References

Artym V. Zhang Y, Seillier-Moiseiwitsch F, Yamada K, and Mueller S. 2006. Dynamic interactions of cortactin and membrane type 1 matrix metalloproteinase at inavdopodia: defining the stages of invadopodia formation and function. Cancer Research. 66: 3034-3043.

Birkedal-Hansen H, Moore W, Bodden M, Windsor L, Birkedal-Hansen B, DeCarlo A, and Engler J. 1993. Matrix metalloproteinases: A review. Critical Reviews in Oral Biology and Medicine. 4(2): 197-250.

Bravo-Cordero J, Marrero-Diaz R, Megias D, Genis L, Garcia-Grande A, Garcia M, Arroyo A, and Montoya M. 2007. MT1-MMP proinvasive activity is regulated by a novel Rab8-dependent exocytic pathway. The EMBO Journal. 26: 1499-1510.

Cao J, Kozarekar P, Pavlaki M, Chiarelli C, Bahou W, and Zucker S. 2004. Distinct roles for the catalytic and hemopexin domains of membrane type 1-matrix metalloproteinase in substrate degradation and cell migration. Journal of Biochemistry. 279(14): 14129-14139.

Eustace B, Sakurai T, Stewart J, Yimlamai D, Unger C, Zehetmeier C, Lain B, Torella C, Henning S, Beste G, Scroggins B, Neckers L, Ilag L, and Jay D. 2004. Functional proteomic screens reveal an essential extracellular role for hsp90α in cancer cell invasiveness. Nature. 6(6): 507-514.

Foda H and Zucker S. 2001 Matrix metalloproteinases in cancer invasion, metastasis and angiogenesis. Drug Discovery Today. 6(9): 478-482.

Fostinis Y, Theordoropoulos P, Gravanis A, and Stournaras C. 1992 Heat shock protein HSP90 and its association with the cytoskeleton: a morphological study. Biochemistry and Cell Biology. 70(9): 779-786.

Hofmann U, Eggert A, Blass K, Brocker E, and BeckerJ. 2003. Expression of matrix metalloproteinases in the microenvironment of spontaneous and experimental melanoma metastases reflects the requirements for tumor formation. Cancer Research. 63(23): 8221-8225.

Hotary K, Li X, Allen E, Stevens S, and Weiss S. 2006. A cancer cell metalloprotease triad regulates the basement membrane transmigration program. Genes & Development. 20: 2673-2686.

Kleiner D and Stetler-Stevenson W. 1999. Matrix metalloproteinases and metastasis. Cancer Chemotherapy Pharmacology. 43: S42-S51.

Mori H, Tomari T, Koshikawa N, Kajita M, Itoh Y, Sato H, Tojo H, Yana I, and Seiki M. 2002. CD44 directs membrane-type 1 matrix metalloproteinase to lamellipodia by associating with its hemopexin-like domain. The EMBO Journal. 21(15): 3949-3959.

Mounier N, and Arrigo A. 2002. Actin cytoskeleton and small heat shock proteins: how do they interact?. Cell Stress Chaperones. 7(2): 167-176.

Poincloux R, Lizarraga F, and Chavrier P. 2009. Matrix invasion by tumour cells: a focus on MT1-MM trafficking to invadopodia. Journal of Cell Science. 122(17): 3015-3024.

Remacle A, Murphy G, and Roghi C. 2003. Membrane type 1-matrix metalloproteinase (MT1-MMP) is internalised by two different pathways and is recycled to the cell surface. Journal of Cell Science. 116(19): 3905-3916.

Remacle A, Rozanov D, Fugere M, Day R, and Strongin A. 2006. Furin regulates the intracellular activation and the uptake rate of cell surface-associated MT1-MMP. Oncogene. 25: 5648-5655.

Shofuda K, Moriyama K, Nishihashi A, Higashi S, Mizushima H, Yasumitsu H, Miki K, Sato H, Seiki M, and Miyazaki K. 1998. Role of tissue inhibitor of metalloproteinases-2 (TIMP-2) in regulation of pro-gelatinase A activation catalyzed by membrane-type matrix metalloproteinase-1 (MT1-MMP) in human cancer cells. Journal of Biochemistry. 124: 462-470.

Song X, Wang X, Zhou W, Shi H, Feng D, Sun Y, Liang Y, Fu Y, Zhou D, and Luo Y. 2010. The regulatory mechanism of extracellular Hsp90α on matrix metalloproteinase-2 processing and tumor angiogenesis. Journal of Biological Chemistry. 285(51): 40039-40049.

Stylli S, Kaye A, and Locke P. 2008. Invadopodia: at the cutting edge of tumour invasion. Journal of Clinical Neuroscience. 15(7): 725-737.

Whitesell L, and Lindquist S. 2005. Hsp90 and the chaperoning of cancer. Nature. 5: 761-772.

Wu Y, Munshi H, Snipas S, Salvesen G, Fridman R, and Stack S. 2007. Activation –coupled membrane-type 1 matrix metalloproteinase membrane trafficking. Biochemistry Journal. 407: 171-177.

Yana I and Weiss S. 2000. Regulation of membrane type-1 matrix metalloproteinase activation by propeptide convertase. Molecular Biology of the Cell. 11(7): 2387-2401.

Zucker S, Hymowitz M, Conner C, DiYanni E, and Cao J. 2002. Rapid trafficking of membrane type 1-matrix metalloproteinase to the cell surface regulates progelatinase A activation. Laboratory Investigation. 82(12): 1673-1684.