Regulation of Matrix Metallopeptidase 1 in Breast Cancer Metastasis

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ABSTRACT

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Matrix Metallopeptidase 1 (MMP-1) expression has repeatedly been correlated to tumorigenesis and metastasis. Yet, MMP-1 regulation in a metastatic context remains largely unknown. Here we confirm differential MMP-1 expression in mammary carcinoma cells with varied metastatic potentials and identify a mechanism differentially regulating MMP-1.

We show that MMP-1 expression is regulated by an AP-1 element in its promoter in highly metastatic MDA-MB-231 mammary carcinoma cell derivatives. Fra-1, an AP-1 family transcription factor, differentially binds this element in highly metastatic derivatives compared to low-metastatic cells and is required for MMP1 expression. Fra-1 mRNA levels are unchanged in the cell variants, however its protein levels are higher in the metastatic cells. There was no change in protein degradation rates, while protein synthesis rates of Fra-1 increased. These results suggest that protein translation of Fra-1 is differentially regulated in these cells.

Consistent with the importance of Fra-1 for tumor growth, we found that Fra-1 overexpression is sufficient to increase cell motility and anchorage independent growth. These results suggest that Fra-1 regulation is critical for regulation of MMP-1 and metastasis.

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Dedication

I would like to dedicate this work to my mother. My family, like millions of others, was devastated by breast cancer. I hope my effort has made some incremental impact in the field, and that others continue the work until fewer lives are lost.

I would also like to dedicate this work to my family. Their time and support saw me through.

Lastly, I would like to dedicate any, and all, success to my wife, Kathryn. Her support, patience, motivation, guidance, intelligence, and love have allowed me to persevere.

Preface

This thesis is divided into five chapters. The first chapter serves as the introduction for the thesis as a whole. The second chapter details methods for the subsequent chapters. Chapter three is focused on data identifying and confirming a gene of interest, which is the basis of this thesis. Evidence from chapter three is confirmed and expanded upon in chapter four. Lastly, chapter five considers potential conclusions and discusses future directions for research.

Chapter 1

Introduction

Nearly all breast cancer mortality results from metastasis (Gupta et al., 2006). Metastasis is a multistep process, with a rate-limiting step being growth of individual tumor cells in secondary organs (Hanahan et al., 2011). These cells may disseminate early in the primary tumor progression and remain dormant for years (Karrison et al., 1999; Klein et al., 2002). Current treatments are effective for a large percentage of primary tumors, but unfortunately a significant number of metastatic tumors evade therapy (Naumov et al., 2003). Understanding the regulation of metastasis may yield opportunities for improved treatments.

Microarrays are a powerful tool for correlating gene expression to metastatic regulation. Microarrays of mammary carcinoma cell line MDA-MB-231 variants with different metastatic potential correlated the expression of several genes to metastasis. Highest among them is matrix metallopeptidase-1 (MMP-1) (Bos et al., 2009; Kang et al., 2003; Minn et al., 2005). Inhibition and overexpression of MMP-1 in cancer cell lines linked expression to tumorigenesis and metastasis (Balduyck et al., 2000; Giambernardi et al., 1998; Kousidou et al., 2004). Furthermore, MMP-1 expression corresponds to tumor progression in patients (Cheng et al., 2008; McGowan et al., 2008; Nakopoulou et al., 1999; Poola et al., 2005).

MMP-1 cleaves collagen, the main structural protein in the extracellular matrix (ECM) (Woessner, 1991). Disruption of the ECM may be critical in tumor invasion, but ECM degradation also frees ECM-sequestered growth factors (Bashkin et al., 1989). In addition, MMP-1 has secondary substrates, including angiogenic factors, which may cause tumor progression (Mazor et al., 2013; Nguyen et al., 2006). MMP-1 regulation in

a metastatic context remains unclear. Activator protein-1 (AP-1) is a known regulator of MMP-1 and may be involved in metastatic regulation of MMP-1, as AP-1 is also correlated to cancer (Young et al., 2006). In this study, we confirm that MMP-1 expression is enhanced in metastatic cell lines and explore the regulatory mechanism responsible.

Breast Cancer

Morbidity/Mortality

Breast cancer is the most common cancer among women and the leading cause of women's cancer mortality (Ferlay et al., 2010). In the developed world, one in 14 women will be diagnosed before the age of 75—nearly one in four of those will succumb to the disease (Jemal et al., 2011).

These statistics are even more daunting when changes in life expectancy and future demographics are considered. Annual breast cancer incidence is expected to increase by 75% to over two million cases in 2030 (Jemal et al., 2011). At that incidence level, even if the mortality rate were to drop by 50%, there would only be a marginal improvement in the annual deaths. The hope, and expectation, is that future breast cancer treatments will continue to improve. Unfortunately, breast cancer is a problem that continues to grow.

Classifications

Breast cancer is a broad term for a number of diseases. The World Health Organization has 17 distinct classifications, and 89 sub-classifications, for breast cancer (Tavassoli et al., 2003). Each cancer is distinct in its origin, differentiation, gene and receptor expression, and progression.

Histological classifications of breast tumors are dominated by invasive ductal carcinoma-not otherwise specified (IDC-NOS) morphologies, comprising 50% – 80% of tumors (Tavassoli et al., 2003). Mammary ductal adenocarcinoma will be the focus of this thesis.

In addition to histopathology, advances in immunohistochemistry correlated surface expression of three receptors with tumor progression: estrogen-α (ER), progesterone (PR), and Erbb2 (Her2). There has been significant evidence for the prognostic value of the expression of these three receptors (Clark et al., 1984; Fisher et al., 1981; McGuire, 1975; Slamon et al., 1987). In addition, hierarchal clustering of microarray data from patients has correlated total gene expression with receptor status (Sorlie et al., 2001). These categories are: A. Luminal A (Her2-, ER+ and/or PR+), B. Luminal B (Her2+, ER+ and/or PR+), C. Her2+ (Her2+, ER- or PR-), and D. Basal Like (Triple Negative).

Progression

Common among all breast cancer types is the relationship between progression and mortality risk. Typically clinicians divide patients into five stages (0 – IV) by primary tumor size, lymph node progression, and metastatic progression (Table 1-1) (Edge et al., 2010).

Table 1-1: Clinical Progression of Breast Cancer

Stage 0:	Abnormal cells are contained to local parental tissue	
	Ductal Carcinoma In Situ (DCIS) Lobular Carcinoma In Situ (LCIS) Paget's Disease	
Stage I	A	Tumor is 2 cm or smaller and has not spread to outside of the breast
	B	Small (0.2 – 2 mm) clusters of cells are found in lymph nodes, with or without a breast tumor
Stage II	A	Cancer (>2 mm) is found in 1 – 3 lymph nodes, with tumor less than 2 cm
		Or, tumor 2 – 5 cm is found but has not spread to lymph nodes
	B	Tumor is 2 – 5 cm with small (0.2 – 2 mm) clusters of cells are found in lymph nodes
		Or, cancer (>2mm) is found in 1 – 3 lymph nodes
		Or, tumor is larger than 5 cm
Stage III	A	Cancer is found in 4 – 9 lymph nodes
		Or, tumor is larger than 5 cm with cancer is less than 4 lymph nodes
	B	Cancer has spread to chest wall or skin
	C	Cancer is found in 10 lymph nodes
		Or, cancer is found in collarbone lymph nodes
		Or, cancer is found in both axillary and breast bone lymph nodes
Stage IV	Cancer has spread to other parts of the body	

Categorizing patients and tumors by histology, receptors, and progression helps standardize assessment and treatment options. However, in a less practical but more absolute sense, it is useful to think of actual progression, as opposed to observable progression. There are three categories for actual progression:

A. Cancer is limited to the primary tumor, or localized invasion.

B. Cancer has spread beyond the primary tumor to distant organs, but secondary tumors have not grown to the point of detection—micrometastases.

C. Cancer is present in the primary and secondary tumors—metastatic tumors.

Clinical and 'true' categories significantly overlap. Stage 0 is almost always limited to the primary tumor, making it the same as category A. Stage 4 progression is the same as category C, where metastatic tumors are diagnosed. There is difficulty in determining whether stages 1 – 3 have metastasized to secondary organs. Recent advances in analyzing bone marrow aspirates can find disseminated tumor cells, which affect prognosis, but beyond this, detection of micrometastases is not currently possible. In

general, higher clinical stages are more likely to have progressed past the primary tumor, to have a higher incidence of mortality, and to be less responsive to treatments.

Treatment

Due to the challenges in determining a patient's 'true' progression, treatment regimens are similar among clinical stages I - IV. Primary tumors are subject to surgical resection and local areas to ablation through radiation, ultrasound or laser. Secondary tumors, if present, are treated specifically for their organ location. Lastly, most patients are subject to adjuvant therapies (Harris, 2010).

Three forms of adjuvant therapy are commonly used in treatment: chemotherapy, hormone therapy, and antibody treatment. Technically, chemotherapy is any treatment of a disease with a chemical substance. Since its widespread use in cancer treatment (DeVita et al., 2008), chemotherapy refers to cancer treatment which targets proliferating cells. Modern treatment regimes are combinations of drugs with varying mechanisms. Cytoskeletal drugs, such as Docataxel and Paclitaxel stabilize microtubules, decreasing free tubulin and inducing apoptosis. Antimetabolites, such as Methotrexate and Fluorouracil inhibit enzymes required for folic acid and thymidine metabolism, respectively—each required for DNA synthesis. Anthracyclines, such as Doxorubicin and Epirubicin, intercalate in DNA and inhibit DNA topoisomerase and DNA replication. Lastly, Cyclophosphamide is an alkylating agent which crosslinks DNA restricting replication. Administering these drugs in combination reduces resistance. In women under 50 years old, combination therapy reduces the annual breast cancer death rate by 38%; in women aged 50 – 69 rates are reduced by 20% (Demicheli et al., 1999; Group, 2005).

Hormonal therapies inhibit estrogen receptors, which are expressed in 65% of breast cancer (Kohail et al., 1985). Tamoxifen inhibits estrogen receptors through direct binding, reducing reoccurrence after five years by 42% (EBTCB, 1998). In postmenopausal women, Letrozole, an aromatase inhibitor, prevents estrogen synthesis in peripheral tissue and has been found to be marginally more effective than Tamoxifen as a monotherapy (Group et al., 2009).

Trastuzamab (Herceptin) is currently the only monoclonal antibody approved for breast cancer treatment after Bevacizumab (Avastin) was found to offer little improvement in life extension (Woodcock, 2010). Herceptin targets and interferes with the Her2 receptor, and its downstream effects. When used in conjunction with chemotherapy, 57% of patients were shown to be disease-free after twelve months, which is a stark improvement considering the poor prognosis of the Her2 positive patients (Vogel et al., 2002).

Unfortunately, as seen in some of the drug efficacy statistics, some patients do not benefit from chemotherapy treatment. The first group is comprised of patients who do not require treatment beyond the primary tumor sites. These patients—in category A—are cured after the primary tumor and localized spread are removed or ablated. Since current diagnostics cannot differentiate between cured patients and patients requiring secondary treatment, all patients are treated similarly. In essence, all patients are treated as though their cancer has spread. Therefore some patients needlessly suffer the side effects of adjuvant therapies to help the population of patients that will respond to treatment (Dowsett et al., 2007; Kalager et al., 2012).

The second group of patients that do no benefit from adjuvant therapy are those who are unresponsive or suffer an unresponsive relapse. Nearly all of these patients have metastatic tumors, the exception being the small fraction of patients with severe primary tumors that have invaded through the chest wall. Metastatic breast cancer that is unresponsive to treatment is the cause of nearly all breast cancer mortality (Gupta and Massague 2006). Therefore the key to improving breast cancer mortality is to better understand and treat metastatic breast cancer.

Metastasis

History

In 1829, the French physician Joseph Claude Anthelme Récamier was the first to use the Latin-Greek word 'metastasis'—to transition or migrate—in reference to cancer (Morton et al., 1997). Forty-five years later, British physician Cambell Greig De Morgan hypothesized that cancer cells would spread from local invasion to the lymph nodes and beyond (Jenner, 1874).

Metastasis was also the foundation for the 'seed and soil' hypothesis set forth by Fuchs and Paget. Ernst Fuchs discovered that uveal melanoma cancer had a predilection to metastasize to the liver (Fuchs, 1882). While performing autopsies on hundreds of breast cancer patients, Steven Paget discovered that metastases to the bone, liver and ovary were more common than other organs (Paget, 1889). Both scientists suggested that when primary tumor cells ('seeds') are challenged to survive outside of the host organ, some metastatic sites ('soil') are less demanding than others. James Ewing later added

caveats to the hypothesis describing mechanical forces in anatomical structures (Ewing, 1922).

In sum, these early works contributed to the modern paradigm (Langley et al., 2011): metastatic tumors are derived from primary tumor cells challenged to progress through a sequence of distinct steps, as follows.

Progression

Progression is generally divided into seven discrete steps (Bross et al., 1975; Gupta et al., 2006; Hanahan et al., 2000): 1) Growth beyond the initial tumor site/ local invasion, 2) intravasation into the blood or lymphatic systems, 3) survival though the vascular system, 4) arrest in a distant organ, 5) extravasation into secondary parenchyma, 6) micrometastatic survival and 7) proliferation and metastatic tumor growth.

The overall process and current research for each segment have been well reviewed (Hanahan et al., 2011; Talmadge et al., 2010; Weiss, 2000). Therefore, I will focus on specific aspects of this process.

Metastatic Inefficiency

Since metastasis is responsible for nearly all breast cancer mortality, it may be counterintuitive to consider metastatic tumors as a highly unlikely and inefficient end. However, evidence indicates that only a small fraction of breast cancer cells become metastatic tumors (Weiss, 1990) and that this process takes several years (Karrison et al., 1999).

As previously described, metastasis progresses through a series of steps. Stepwise process efficiency is the product of the rate of each step. Experiments examining

intravasation demonstrated that rapidly growing xenograft tumors released a large number of cells into the bloodstream. In one study, 36 mg, or roughly $3.6x10^4$ cells, of murine fibrosarcoma cells injected into mouse thighs grew eightfold in volume over several days (Liotta et al., 1974). These tumors released approximately $3.8x10^4$ cells per day into the bloodstream. The number of cells released also increased exponentially to $1.5x10^5$ as the tumor doubled in size. Similar results were observed with intramuscular injection of melanoma or lung cells (Mayhew et al., 1984), murine sarcomas injected intraperitoneally or subcutaneously (Nakadate et al., 1979), and murine mammary carcinomas isolated in a subcutaneous pouch (Butler et al., 1975). These experiments demonstrate that tumors have a high rate of intravasation.

After intravasation, tumor cells must survive in the vasculature, arrest in a secondary organ and extravasate to form metastases. Early research into circulation of cancer cells found that tumor cells could pass the first capillary bed encountered and retain the ability to form tumors. Interestingly, cells which passed through the lung are more likely to pass the lung capillary bed a second time if reinjected intravenously (Zeidman et al., 1952).

Several labs have tracked the fate of individual cells in vasculature. Melanoma cells injected into the tail vein of a mouse were found to arrest in the first capillary bed encountered—the lungs—with a small fraction passing through to other organs. Only 1.5% of cells injected survived more than 24 hours and after 14 days, less than a tenth of those cells became metastases (Fidler, 1970; Mayhew et al., 1984).

Direct lung injection of melanoma cells found high rates of survival (74%) after one day. After 14 days, 3.5% of cells were survived in a solitary latent state, and 12.7%

had formed metastases, mostly on the tumor surface (Cameron et al., 2000). Similar results were found in direct liver injections, where 87.4% extravasated, 36.1% were solitary latent cells, and 0.9% became metastases (Luzzi et al., 1998). More sophisticated cell tracking with high-resolution videomicroscopy of GFP-labeled cells injected intraperitoneally gave analogous results (Naumov et al., 1999). In mammary adenocarcinoma experiments the same pattern was observed (Naumov et al., 2002). Although a large number of cells are capable of initially surviving in the stroma, few are capable of surviving for an extended period of time.

In cell lines which are highly metastatic to the brain, intracardiac injection led to 93.9% of cells extravasating, 4.5% of cells becoming latent, and 1.6% of cells growing into metastatic tumors (Heyn et al., 2006). In comparisons between cell lines with varying metastatic potentials, extravasation rates were similar, but survival and proliferation was greater in more metastatic cells (Morris et al., 1994).

Several conclusions can be made about the metastatic process from these experiments. First, dissemination of tumor cells appears to be fairly efficient, with most cells leaving the primary tumor surviving in circulation, arresting in secondary organs, and extravasating. The rate-limiting step is survival in the secondary organ. Only a small percentage of cells can survive and among the survivors even fewer can proliferate (Wong et al., 2001). A second conclusion is that a population of cells exists which can survive but not proliferate in foreign stroma. These latent cells are considered ‘dormant’ and may be the first step in metastatic progression in patients. Lastly, the metastatic process is overall inefficient.

Inefficiency led to the hypothesis that a large number of cells would have to disseminate from a primary tumor to overcome the odds and metastasize. Early consensus favored this hypothesis, which was supported by findings that the number of tumor cells injected intravenously in mice was proportional to the number of metastases formed (Zeidman et al., 1950). It was hypothesized that 10^5 cells would have to disseminate from primary tumors to form metastatic tumors (Mayhew et al., 1984; Peters, 1975). However, these experiments and the hypothesis they support considered metastasis on a short time scale.

Breast cancer progression occurs over a time period on the order of years. In two studies of breast ductal carcinoma in situ (DCIS) developing into invasive carcinoma, the average interval for progression was 9.7 years (Rosen et al., 1980) and 6.7 years (Page et al., 1982). Furthermore, progression from invasive carcinoma to metastatic tumors typically takes years. In a study of 877 patients, metastasis was observed bimodally one year and five years after primary tumor diagnosis (Demicheli et al., 1999). The final progression step from metastatic tumor discovery to metastatic tumor growth is consistent in timing. Disconcertingly, each two-year interval after metastatic tumor discovery there is a 50% decrease in survival (Engel et al., 2003).

On this longer time scale, an alternative hypothesis has been proposed. If primary tumors disseminate cells early in progression and if disseminated cells can survive in secondary organs for a long period of time, then disseminated cells might adapt and progress in parallel with the primary tumor (reviewed by Klein, 2009). Parallel progression would overcome the inefficiency of metastasis observed in the xenograft assays with fewer cells that adapt over long periods of time.

Dormancy of disseminated cells has been observed in the xenograft studies previously described (Cameron et al., 2000; Heyn et al., 2006; Luzzi et al., 1998; Naumov et al., 2002; Naumov et al., 1999). Additionally, in patients with estrogen receptor positive and Her2 positive breast tumors, disseminated cells discovered in the bone marrow were latent (Pantel et al., 1993). A high proportion of disseminated cells also show 'stem-cell' markers CD24 and CD44 (Balic et al., 2006). Interestingly, latent disseminated breast tumor cells derived from murine livers were capable of expansion if reinjected into a mammary fat pad (Suzuki et al., 2006). This demonstrates that disseminated cells are capable of growth, but are maintained in a dormant state by the secondary organ environment.

Further supporting the parallel progression hypothesis, tumor cell dissemination occurs earlier than previously thought. Genomic comparisons of disseminated cells and primary tumors in patients indicate that tumor cells disseminate before distinct aberrant genomic changes in the primary tumor occur (Klein et al., 2002; Kuukasjarvi et al., 1997; Schardt et al., 2005; Schmidt-Kittler et al., 2003). Early dissemination can also be assumed in patients with breast cancer metastases in the lymph nodes, but with no discernable primary tumor (Holland et al., 1983; Nielsen et al., 1987; Patel et al., 1981). In Her2 transgenic mice, premalignant DCIS were capable of disseminating to the lungs and bone marrow (Husemann et al., 2008).

Taken together, these studies describe a process where benign localized cancer is slow to progress into an invasive tumor. Eventually genetic instability and tumor heterogeneity allow the tumor to overcome the basement membrane, invade the local tissue and disseminate cells into the vasculature and secondary organs. Few extravasated

cells survive; those that do are slow to grow and exist in a dormant state.

This process challenges modern therapy. Disseminated cells have been found to be heterogeneous (Klein et al., 2002), increasing the likelihood of cells resisting treatment. Also, chemotherapeutics target proliferating cells. Therefore, dormant cells may evade therapy. In murine mammary cell lines with different metastatic latencies (Naumov et al., 2002), doxorubicin treatment was less effective on dormant cells (Naumov et al., 2003).

Patient data corroborates these observations. Patients treated with chemotherapy and surgery had less frequent cancer recurrence in the first 24 months compared with patients treated with surgery alone. After three years, however, chemotherapy has much less effect on recurrence (Demicheli et al., 1999). One explanation for the change in efficacy is that dormant disseminated cells were unaffected by chemotherapies targeting rapidly dividing cells. In line with this explanation, the presence of disseminated tumor cells in the bone marrow of breast cancer patients after chemotherapy treatment is a strongly negative prognostic indicator (Braun et al., 2005).

Parallel progression and cell dormancy also present treatment opportunities (Townson et al., 2006). For example, the progression process appears to be selective. Studies of disseminated cells show a high degree of heterogeneity (Klein et al., 2002), while metastatic lesions appear to be clonal (Ding et al., 2010; Talmadge et al., 1982; Teixeira et al., 1996; Woelfle et al., 2003). If the cells that overcome dormancy are more homogeneous, they are also less likely to develop resistance to treatment. Also, disseminated cells are limited in size, making them more available to drugs in the vasculature (Baluk et al., 2005; Naumov et al., 2002). In addition, treatment of dormant

disseminated cells does not require that the cells undergo apoptosis or cell death. Rather, treatment only needs to prevent cells from overcoming dormancy (Rinker-Schaeffer et al., 2006).

Understanding metastasis is critical to improving therapeutics and patient survival. As mentioned, selection and dormancy challenge anti-proliferative adjuvant therapies, but also present an opportunity to attack an exposed clonal tumor population. Understanding metastatic regulation is the first step towards identifying potential therapies.

Regulation

Metastatic regulation occurs at each step in the progression process. The final step, growth from disseminated cells to metastatic tumors, is a rate-limiting step. Therefore, inhibiting this growth is highly relevant for treatment.

Research on disseminated cells is inherently difficult due to the challenges in discerning, observing and manipulating single cells in a secondary organ. As a result, less is know about the regulation of this stage of metastasis. Yet, several mechanisms have been elucidated, including extracellular signals, epigenetic and transcriptional regulators.

Stromal regulation is important in primary tumorigenesis and progression (Tlsty et al., 2006). General observations, dating back to the 19th century, show that primary tumors spread differentially among organs (Fuchs, 1882; Paget, 1889). This phenomenon was observed again in cell culture, where cell populations from a common culture variably hone in on secondary organs (Bos et al., 2009; Kang et al., 2003; Minn et al.,

2005). Specific organ dissemination implies that tumor cells are sensitive to different extracellular interactions in different organs.

Extracellular regulation of disseminated cells has also been observed in a landmark study analyzing the effect of primary tumor excision on metastatic progression. It was found that primary tumors maintain metastatic tumor angiogenic dormancy (Holmgren et al., 1995). Extracellular inhibition of malignant cells has also been shown in injections of tumor cells into blastocysts (Dolberg et al., 1984; Gerschenson et al., 1986; Mintz et al., 1975). *In vitro*, coculture with stem cells dulls aggressive melanoma phenotypes in an unknown epigenetic manner (Postovit et al., 2006).

The advent of systemic approaches to microRNA(miRNA):mRNA research has yielded several putative regulatory miRNAs in patient tumors (Luo et al., 2013) and cell lines with different metastatic potentials (Tavazoie et al., 2008). Overexpression of several miRNAs regulates malignant cell invasiveness and metastatic potential, including: miR-200c, miR-205, miR-375 (Luo et al., 2013), miR-20b (Ma et al., 2007), miR-373, miR-520c (Huang et al., 2008), miR-335, miR-206 (Tavazoie et al., 2008), and miR-34a (Peurala et al., 2011).

Prior to epigenetic study, a large body of work had uncovered transcriptional regulation of metastatic progression (Welch et al., 2000; Yoshida et al., 2000). Systems biology approaches have progressed and complemented this work. The first microarrays performed in breast cancer tumors showed distinct clusters and subtypes, which matched immunohistochemical categorizations (Perou et al., 2000; Sorlie et al., 2001; Sorlie et al., 2003). Since that time, thousands of samples have been deposited in the GEO Database (Barrett et al., 2013). The development of metastatic gene signatures has improved

oncologist's prognostic abilities and yielded a wealth of data for study (Cardoso et al., 2008; Sparano et al., 2008; van 't Veer et al., 2002). However, microarray technology is challenged by limitations. Macroscopic samples contain a diversity of tumor cell populations, which are considered in aggregate. Even among disseminated cancer cells, individual cells are heterogeneous (Klein et al., 2002)

Overcoming this limitation requires more sophisticated analysis or segmenting the input before analysis. Flow sorting for nuclei combined with single cell whole genome amplification and next generation sequencing is one method for considering single cells when analyzing gene copy number (Navin et al., 2011). Alternatively, the development of laser capture microdissection isolates cells of interest (Bonner et al., 1997). Lastly, microarray input can be altered to be less heterogeneous. As will be discussed in the next section, homogenization can also increase expression of the genes of interest, improving the resolution of microarrays.

MDA-MB-231 Cells and Variants

Isaiah Fidler's early work with melanoma cells determined that culturing cells does not abolish metastatic potential (Fidler, 1973). Cell cultures were derived from metastatic lung tumors resulting from intravenous injection of a syngenic melanoma cell line. When these were reinjected into mice they continued to form lung metastases with greater efficiency. In a series of these experiments, 'generations' of cell lines were developed with greater and greater metastatic potential.

A variation of this experiment was performed to determine if a cell line is heterogeneous in its ability to form metastases (Nowell, 1976). Single cells from a 'parental' cell line were expanded clonally into populations and injected intravenously

into mice. These single cell clones exhibited varying metastatic efficiency, suggesting that the initial population was heterogeneous (Fidler et al., 1977).

At the same time, the MDA-MB-231 mammary adenocarcinoma cell line was developed after a series of failures developing solid mammary tumor cell lines. MDA-MB-231 cells were derived from the pleural effusion of a patient with breast carcinoma. The pleural effusion contained a large number of disseminated tumor cells without the "stromal contamination" that had plagued previous studies (Cailleau et al., 1974).

Also near that same time, nude mice were successfully xenografted with human melanoma and cerivical tumor cells intradermally injected into the midback (Giovanella et al., 1973). Tumor cells survived, grew and progressed into metastatic lung lesions. Similarly, mouse mammary adenocarcinoma cell line cells were transplanted into syngenic mice, which led to metastatic tumors. Cells cultured from these metastases had greater metastatic potential than the parental cell line along with altered gene expression. In particular, Fra-1 expression was found to be correlated to metastatic potential, which will be discussed in greater detail below (Kustikova et al., 1998; Tkach et al., 2003).

This process of developing metastatic cell cultures from mouse xenografts was repeated with human MDA-MB-231 cells. The resulting cell lines were organ specific metastatic variants. Among these, brain- or bone- 'seeking' MDA-MB-231 cells had phenotypic changes to transforming growth factor-β (TGFβ) and Insulin-like Growth Factor-1 responses (Yoneda et al., 2001). Analogous experiments derived MDA-MB-231 cell line variants from the bone, brain and lung (Bos et al., 2009; Kang et al., 2003; Minn et al., 2005). In parallel, clonal cell cultures from single MDA-MB-231 cells displayed different metastatic potentials (Bos et al., 2009; Kang et al., 2003; Minn et al.,

2005).

Microarray data using a series of the cell lines described above, resolved input heterogeneity issues. The cell lines were derived from clonal expansion of single parental cells, MDA-MB-231, and are less heterogeneous by definition. Cell lines that were cycled though mouse xenografts endured selection, also reducing heterogeneity. Simultaneously, the amplification of metastatic potential in these cells translated into amplified expression of specific genes, increasing microarray resolution.

Using this research as a foundation gene expression profiles that correlate to metastasis can be found. Once metastatic genes are identified, their regulatory mechanisms can be elucidated, creating opportunities for novel breast cancer treatments.

MMP-1

Collagenase is formally called interstitial collagenase-1. More commonly, it is known as matrix metallopeptidase-1 (MMP-1), as well as matrix metalloprotease-1 and matrix metalloproteinase-1. MMP-1 expression is correlated to the metastatic potential of MDA-MB-231 variants. The MMP family of proteins has been described in great detail in several reviews (Pardo et al., 2005) and in Jacob Woessner's book (Woessner, 1991). Below, MMP-1 will be discussed broadly, with focus on the data describing its role in cancer.

History

Prior to 1962, researchers had hypothesized that tissue development, growth and repair would require enzymes capable of degrading extracellular structural components.

Gross and Lapiere used collagen, the most abundant structural protein, as a substrate to search for an enzyme capable of breaking down matrix proteins. Using metamorphic tadpole tails, they were the first to find evidence of diffusible vertebrate collagenase (Gross et al., 1962). Collagenase was later purified (Nagai et al., 1966) and eventually cloned (Bauer et al., 1970; Goldberg et al., 1986).

Family

MMP-1 is the founding member of the matrix metallopeptidase family (Pardo et al., 2005). Among the 23 human MMP family members (Radisky et al., 2010), several have been shown to be relevant in cancer and metastasis: MMP-2 (Jezierska et al., 2009), MMP-3 (Sternlicht et al., 1999), MMP-7 (Rudolph-Owen et al., 1998), MMP-9 (Sung et al., 2012), MMP-11 (Masson et al., 1998) and MMP-13 (D'Armiento et al., 1995). However, less is known about the function of MMP-1 in breast cancer progression than other MMP family members (McCawley et al., 2000). MMP-1 and its role in metastasis will be the focus of this thesis.

Structure

MMP-1 is an endopeptidase and the eponymous member of the matrix metallopeptidase family. Metallopeptidases are metzincins, which all share similar zinc binding motifs (Bode et al., 1993; Stocker et al., 1995).

MMP-1 is comprised of five domains (Figure 1-1) (Nagase et al., 1999): 1) the predomain, which is hydrophobic and directs the protein to the endoplasmic reticulum. The predomain is removed prior to MMP-1 expression outside the cell; 2) the propeptide domain, which inactivates the proenzyme through the sulfhydryl group of cysteine-73 to

inhibit the catalytic zinc group through a canonical 'cysteine-switch' (Springman et al., 1990); 3) the catalytic domain, which contains the catalytic zinc bound through the triple histidine-sequence HELGHXXGXXH (Spurlino et al., 1994); 4) the linker; and 5) the hemopexin region, which is a four-beta propeller around a calcium ion. This region binds collagen specifically, directing MMP-1 targeting of its substrate (Murphy et al., 1992).

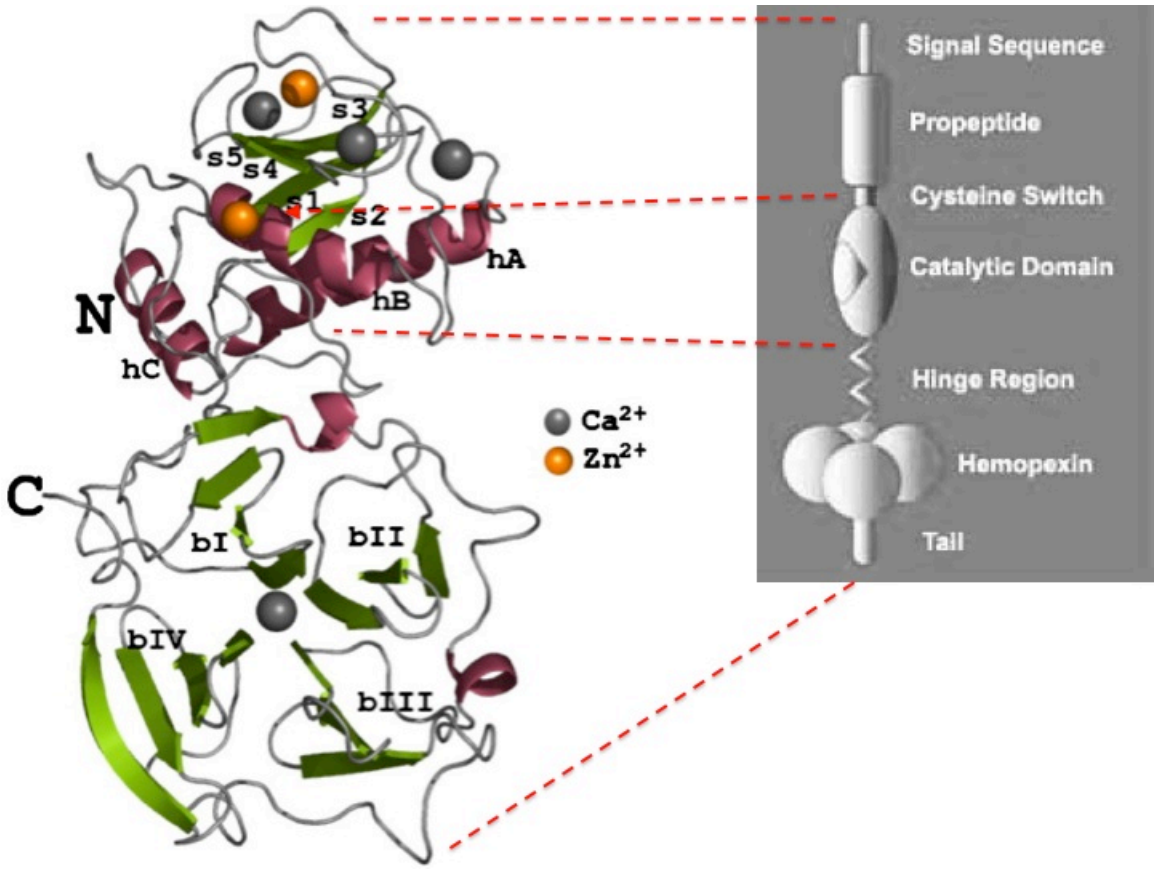

Figure 1–1 Structure of MMP-1 Domains (adapted from Iyer et al., 2006; Whittaker, 2001)

These domains were confirmed by several crystal structures (Figure 1-1) (Iyer et al., 2006; Jozic et al., 2005; Li et al., 1995; Maskos, 2005). From these structures it can be noted that the triple helix of collagen is too large to enter the active cleavage site of

MMP-1. Therefore, MMP-1 must unwind collagen before cleavage takes place (Overall, 2001).

Expression

Since the discovery of collagenase in tadpoles, MMP expression has been found in all species investigated. In humans MMPs exist as a cluster of genes on the 11th chromosome, which suggests evolutionary gene duplication (Fanjul-Fernandez et al., 2010). As a result, sequence homology between species ranges from 55% – 87% among human, murine, leporine, bovine and anuaran MMP-1 (Balbin et al., 2001; Fini et al., 1987; Goldberg et al., 1986; Oofusa et al., 1994; Tamura et al., 1994).

MMP-1 is expressed during tissue remodeling (Nagase et al., 1999) and stress (Shi et al., 2009), but is not at detectable levels in normal tissue (Pardo et al., 2005). In fibroblasts, tuberculosis can induce MMP-1 expression (O'Kane et al., 2010), and in stimulated macrophages MMP-1 is induced (Speidl et al., 2011). MMP-1 expression is also required in uterine resorption (Jeffrey et al., 1970) and reproductive function (Hulboy et al., 1997).

Pathology

MMP-1 is involved in several pathologies, including emphysema (Mercer et al., 2004), wrinkles (Fisher et al., 1996), hip arthroplasty (Godoy-Santos et al. 2009), implant failure (Leite et al. 2008), Alzheimer's disease (Leake et al., 2000), arthritis (Burrage et al., 2006), occlusive peripheral arterial disease (Flex et al. 2007) and coronary artery

disease (Horne et al. 2008). The following pathologies were also described by Parks & Mecham:

> Nephritis, neurological disease, breakdown of blood brain barrier, periodontal disease, skin ulceration, gastric ulcer, corneal ulceration, liver fibrosis, emphysema, fibrotic lung disease (Parks et al., 1998)

Most relevant to this thesis is the role of MMP-1 in breast cancer metastasis, which will be discussed in a later section.

Mechanism

As a zymogen, MMP-1 is not enzymatically active until the inhibitory propeptide domain releases the cysteine switch (Van Wart et al., 1990). Upon activation, MMP-1 functions as an endopeptidase capable of cleaving several substrates. Among them, collagen is the most common, the best studied and the first discovered. MMP-1 is capable of degrading collagen from its native triple-helical form into gelatin (Nagai et al., 1964; Gross and Nagai, 1965; Kang et al., 1966).

Collagen has many subforms, but the major fibril forming collagens are I, II III, and V (and to a lesser extent XI) (Brodsky et al., 1997; Prockop et al., 1995). These fibril collagens are composed of combinations of different fibrils (α peptides) in a triple helix structure (Kolacna et al., 2007). This structure and the hydroxyl group on the pyrollidine ring stabilize the peptide bond. Collagen is a strong and stable protein and is the main component of the extracellular matrix (Holmgren et al., 1998). MMP-1 cleaves collagen fibrils at specific sites—glycine-isoleucine in the α_1 chain and glycine-leucine in the α_2 chain. This cleavage results in an amino terminal ¾ length collagen and a ¼ length carboxy terminal collagen that dentures rapidly into gelatin (Gross et al., 1965).

In addition to acting as a substrate, the collagen interaction can regulate cell migration. In the case of keratinocytes responding to injury, exposure to collagen upregulates MMP-1. MMP-1 binds $\alpha_2\beta_1$ integrin localizing MMP-1 to promote directional migration (Dumin et al., 2001; Pilcher et al., 1997; Stricker et al., 2001). The interaction between $\alpha_2\beta_1$ integrin and MMP-1 was also found to be critical in MMP-1 cytotoxicity in neuronal cultures. Interestingly, MMP-1 does not appear to effect cytotoxicity in these cells by cleavage of a substrate, rather $\alpha_2\beta_1$ integrin interaction influences caspase activity through Akt (Conant et al., 2004).

MMP-1 has several additional interactions and substrates. *In vitro* experiments with extracellular matrix (ECM) coated plates found that MMP-1 released 80% of fibroblast growth factor (FGF) sequestered in the ECM (Bashkin et al., 1989). Basic-FGF (bFGF) bound in human endothelial cells is released by murine collagenase, MMP-1a (Whitelock et al., 1996). Evidence of bFGF in human ECM has been shown in vascular and capillary endothelial cells (Baird et al., 1987). Together, these experiments suggest that MMP-1 releases growth factors from the ECM *in vivo* and possibly in angiogenesis.

Insulin-like growth factor binding protein-2 and -3 (IGFBP-2/ -3) are also MMP-1 targets (Fowlkes et al., 1994a; Fowlkes et al., 1994b; Fowlkes et al., 1995; Thrailkill et al., 1995). Cleavage of IGFBP-2/-3 relinquishes insulin-like growth factor, which correlates to aggressive prostate cancer (Figueroa et al., 1998). MMP-1 has several other substrates, including serpins (Mast et al., 1991), casein, nidogen, versican and tenascin-C (Somerville et al., 2003).

The mechanism of MMP-1 is multifaceted. Varying substrates and indirect effects may explain the breadth and variety of pathologies associated with MMP-1.

Regulation

MMP-1 is regulated at several levels, from transcription to extracellular inhibition (Pardo et al., 2005; Ra et al., 2007). Epigenetic regulation of MMP-1 has been shown in chrondrosarcoma. Histone deacetylase (HDAC) inhibitors blocked MMP-1 induction (Young et al., 2005). Transcriptionally, MMP-1 is regulated by several elements in a context specific manner. Among these elements, activator protein-1 (AP-1) was the first discovered, and will be discussed in more detail in the next section, as it plays a key role in MMP-1 regulation in cancer. A second important promoter site is Ets-1/PEA3. The MMP-1 promoter has a single nucleotide polymorphism (SNP) creating an Ets-1 site in a portion of the population (Gutman et al., 1990). The SNP-created Ets-1 site alters MMP-1 expression (Rutter et al., 1998) and has an impact on cancer susceptibility (McCready et al., 2005).

Several other promoter elements regulate MMP-1 transcription, including retinoid X response element (RXRE) (Pan et al., 1995), Sp-1 (Nelson et al., 2003), TGFβ (White et al., 2000), NFκB (Vincenti et al., 2002) and C/EBP-β (Armstrong et al., 2009). Bioinformatic study of the MMP-1 promoter also suggests sites for Runx2 and Tcf/Let-1 (Clark et al., 2008). Complementing these promoter elements are a series of transcription factors that bind them; for example c-Jun and Fra-1 binding to the AP-1 region (Kimura et al., 2011). Several factors bind the promoter and activate transcription through alternative site binding, such as Bach-1 (Liang et al., 2012) and p53 (Sun et al., 1999).

MMP-1 expression is also induced by receptor pathways. Receptor tyrosine kinases DDR1 and DDR2 upregulate MMP-1 when activated by collagen (Vogel et al., 1997). MMP-1 cleaves collagen closing the feedback loop. In tumors, extracellular matrix metallopeptidase inducer (EMMPRIN) has been found to be enriched on the surface of tumors where it induces stromal expression of MMP-1 (Biswas et al., 1995; Guo et al., 2000; Lim et al., 1998).

At the protein level, MMP-1 is regulated by the previously described the cysteine switch, which inhibits the catalytic zinc in the pro-domain of MMP-1 (Sternlicht et al., 2001). MMP-1 is also a target for inhibition. The first inhibitor was discovered in human skin fibroblasts (Bauer et al., 1975). Eventually, this inhibitor, called tissue inhibitor of metallopeptidases (TIMP) was found to inhibit MMP-1 in a similar manner as the proprotein domain of the inactive proMMP-1. The crystal structure of MMP-1 and TIMP-1 illustrates TIMP-1 binding to the catalytic zinc in MMP-1 (Maskos, 2005).

MMP-1 is regulated at several levels by diverse stimuli and interactions. Many of them are relevant to cancer, such as the overexpression of TIMPs abrogating melanoma tumorigenic and metastatic ability (Khokha, 1994). A specific transcriptional regulator of MMP-1 is AP-1, further discussed below.

AP-1

History

Before AP-1 was discovered, tumor viruses, specifically Avian Sarcoma Virus-17 (ASV-17) (Cavalieri et al., 1985) and Finkel-Biskis-Jinkins—Murine Sarcoma Virus

(FBJ-MSV) (Finkel et al., 1966), were found to have oncogenes. The Vogt group cloned the oncogene v-jun ('jun' being short for 'ju-nana,' which is Japanese for seventeen) (Maki et al., 1987). Similar work found the FBJ-MSV virus contained the oncogene v-Fos ('Fos' being an acronym for FBJ-MSV oncogenic specification) (Curran et al., 1982a; Curran et al., 1982b).

AP-1 was first described as a transcription factor for human metallothionein (Lee et al., 1987a). Later it was found that AP-1 is the factor bound to the TPA (12-O-tetradecanoylphorbol-13-acetate) response element (TRE) (Auble et al., 1991; Wigler et al., 1976) of the MMP-1 promoter (Angel et al., 1987; Lee et al., 1987b).

These paths crossed when endogenous c-Jun and c-Fos were discovered (Bohmann et al., 1987; van Straaten et al., 1983). c-Jun was found to bind the TRE and identified as an AP-1 protein (Angel et al., 1988; Bohmann et al., 1987). Later experiments found that the c-Fos dimerizing protein, p39 (Curran et al., 1982c), is c-Jun (Chiu et al., 1988; Rauscher et al., 1988a; Sassone-Corsi et al., 1988) and that c-Fos also binds AP-1 as an AP-1 protein (Franza et al., 1988; Rauscher et al., 1988b). Later, the remaining AP-1 family members were discovered: JunB (Ryder et al., 1988), JunD (Hirai et al., 1989; Nakabeppu et al., 1988), Fra-1 (Cohen et al., 1988), Fra-2 (Foletta et al., 1994), and FosB (Zerial et al., 1989). Together, these discoveries elucidated the protein dimers of AP-1, which bind the TRE sequence and induce transcription (Chinenov et al., 2001).

Mechanism

AP-1 acts as a dimer (Curran et al., 1988) and its consensus sequence, TGA(C/G)TCA, is found in a plethora of promoter regions (Zhou et al., 2005). Regulation is therefore a product of the expression, regulation, and function of each AP-1 family member. Variations among binding partners also creates redundancy, a phenomenon seen in mouse knockout experiments of AP-1 family members (Fleischmann et al., 2000; Grigoriadis et al., 1994; Hilberg et al., 1993; Johnson et al., 1992; Johnson et al., 1993; Schorpp-Kistner et al., 1999; Schreiber et al., 2000; Thepot et al., 2000; Wang et al., 1992)

Structure

Dimerization occurs through a leucine zipper in each of the AP-1 family members allowing the formation of homo- and hetero-dimers (Landschulz et al., 1988). Non-AP-1 family members with leucine zippers are also capable of forming dimers, but do not bind the AP-1 consensus site (Vinson et al., 2002). Although different AP-1 family dimers have variable DNA binding affinities and transcriptional activations (Halazonetis et al., 1988), sequence comparisons and crystal structures show that key amino acids required for dimerization and DNA binding are conserved (Glover et al., 1995). The crystal structure for a c-Jun—c-Fos dimer binding domain shows coiled coil structures that bind DNA "like a forcep" through the basic domain. In the crystal structure, dimers are linked through the leucine zipper (Glover et al., 1995). The leucine zipper is a long α-helix with a leucine every seventh amino acid facing the same direction, resulting in a hydrophobic surface which aids in binding.

Regulation

AP-1, like MMP-1, is regulated at several levels. Growth factor stimulation results in modification of histone H3, upregulating c-Fos (Cheung et al., 2000). Transcriptionally, a variety of promoter elements and transcription factors regulate AP-1 expression (Angel et al., 1991). Several AP-1 members are autoregulated, including c-Jun (Greenberg et al., 1984) and Fra-1. The Fra-1 promoter has an AP-1 consensus site and two AP-1 like elements (Bergers et al., 1995). c-Fos is the paradigm for immediate early genes and the basis for our understanding of the serum response element (SRE), serum response factor (SRF), and ternary complex factor (TCF) (reviewed by (Cen et al., 2004). Subsequent work demonstrated that most AP-1 members respond to serum (Adiseshaiah et al., 2005; Lazo et al., 1992; Perez-Albuerne et al., 1993; Sonobe et al., 1995).

Post-trascriptionally, several pathways modify AP-1 stability (reviewed by O'Donnell et al., 2012). AP-1 has a high turn-over rate, making it inherently transient unless stabilized by phosphorylation (Basbous et al., 2007; Fuchs et al., 1996; Greenberg et al., 1984; Murphy et al., 2006). Jun family members are phosphorylated by Jun-N-terminal kinase (JNK) (Derijard et al., 1994; Pulverer et al., 1993), while Fos family members are modified by mitogen activated protein kinase and extracellular signal regulated kinases (MAPK/ERK) (Chen et al., 1996; Gruda et al., 1994; Okazaki et al., 1995). Inhibition of phosphorylation results in greater Fra-1 and c-Jun degradation (Casalino et al., 2003; Talotta et al., 2010). In addition, dimerization of Fra-1 with c-Jun increases stability and inhibits c-Jun degradation (Talotta et al., 2010).

The MAPK cascade is one of several canonical pathways regulating AP-1 (Meng et al., 2011). While there are tomes of research defining AP-1 regulation, novel mechanisms are regularly added, particularly in cancer (Eferl et al., 2003). For example, phosphorylation of c-Jun and Fra-1 has been shown to induce MMP-1 expression and increase osteosarcoma cell line invasiveness in collagen (Kimura et al., 2011). AP-1 regulation is a complex mediator of breast cancer tumorigenesis and metastasis.

Fra-1 in Cancer

AP-1 promoter elements have been correlated to cancer since it was discovered that AP-1 is the TPA-regulating element (Matthews et al., 2007). Since then, AP-1 expression has been correlated to many types of tumors (Reviewed by Young et al., 2006). In patients with breast pathologies, Fra-1 is expressed at a higher level in caricinomas compared with benign breast growths (Chiappetta et al., 2007).

Fra-1 expression in patient tumor samples is greater in more differentiated cells (Bamberger et al., 1999). Similarly, in a series of breast cancer cell lines, Fra-1 is upregulated in more invasive cells (Zajchowski et al., 2001). This pattern can also be observed in the metastatic potential of cells, where Fra-1 expression is greater in cells more capable of forming metastases (Belguise et al., 2005; Philips et al., 1998). Similar to cell lines derived from metastatic tumors, metastatic potential is correlated to Fra-1 expression (Kustikova et al., 1998; Tkach et al., 2003).

Development of a DNA vaccine targeting Fra-1 inhibited the expression of Fra-1

in tumors. The vaccine expresses ubiquitinated Fra-1 with immune responsive IL-18 in mice to elicit a Fra-1 specific immune response. After treatment, these mice demonstrated significant inhibition of lung metastases in breast carcinoma cells, as well as regression of established lung metastases (Luo et al., 2005; Luo et al., 2003).

These experiments collectively support Fra-1 impacting cancer progression, however the specific function and regulation of Fra-1 in these systems is yet to be fully elucidated. For example, phospho-ERK 1/2 was found in patient tumors (Milde-Langosch, 2005). Phospho-ERK 1/2 stabilizes Fra-1, so this expression would be expected to increase Fra-1 levels. However, there was no observed difference in Fra-1 levels in tumors compared with normal tissue. Nevertheless, MMP-1, a downstream target of Fra-1, was highly expressed (Milde-Langosch, 2005). These results show the complexity of AP-1 and MMP-1 regulation and the need to determine their mechanisms of regulation in cancer.

MMP-1 in Cancer

Expression

MMP-1, like Fra-1, is expressed in patient tumors in several forms of cancer (1-2). Population studies of single nucleotide polymorphisms (SNPs) also show that increased MMP-1 expression raises the likelihood of contracting several forms of cancer. As previously discussed in the MMP-1 regulation subsection, a SNP alters expression of MMP-1 in a portion of the population (Rutter et al., 1998). Consequently, people with greater MMP-1 expression are significantly more likely to be diagnosed with ovarian

Cancer	Reference
Sarcoma	
Osteosarcoma	(Luu et al., 2005)
	(Husmann et al., 2013)
Giant Cell Tumor	(Cowan et al., 2009)
Chondrosarcoma	(Jawad et al., 2010)
	(Berend et al., 1998)
	(Kawashima et al., 1997)
	(Jiang et al., 2003)
	(Yuan et al., 2005)
	(Scully et al., 2000)
Carcinoma	
Oral Squamous Cell	(Gray et al., 1992)
	(Shimizu et al., 2008)
	(Nishizawa et al., 2007)
Nasopharyngeal	(Nasr et al. 2007)
Squamous-Cell (Head & Neck)	(Muller et al., 1991)
	(Polette et al., 1991)
Esophageal	(Murray et al., 1998)
Gastric	(Fujimoto et al., 2008)
	(Kim et al., 2011)
	(Inoue et al., 1999)
	(Kim et al., 2011)
	(Hiraki et al., 2012)
	(Cai et al., 2012)
	(Mizutani et al., 2000)
Colorectal	(Murray et al., 1996)
	(Woo et al. 2007)
Pancreatic	(Ito et al., 1999)
	(Rudroff et al., 2002)
	(Botta et al., 2012)
Bladder	(Gray et al., 1992)
Melanoma	(Weiss et al., 2012)
	(Nikkola et al., 2002)
	(Blackburn et al., 2009)
	(Nikkola et al., 2005)
	(Nierodzik et al., 1998)
	(Even-Ram et al., 2001)
	(Nierodzik et al., 1992)
	(Tellez et al., 2003a)
	(Tellez et al., 2003b)
	(Hazarika et al., 2004)
Ovarian	(Agarwal et al., 2008)
	(Six et al., 2006)
Breast	(McGowan et al., 2008)
	(Kamath et al., 2001)
	(Even-Ram et al., 1998)
	(Korkola et al., 2003)
	(Nakopoulou et al., 1999)
	(Poola et al., 2005)
	(Balduyck et al., 2000)
	(Cheng et al., 2008)
	(Kohrmann et al., 2009)

Table 1-2: MMP-1 Expression in Cancer

cancer (Kanamori et al., 1999), tongue cancer (Shimizu et al., 2008), bladder cancer (Tasci et al., 2008), lung cancer (Hu et al., 2013), colorectal cancer (Ghilardi et al., 2001) or melanoma (Noll et al., 2001).

Metastasis correlates to MMP-1 expression, as well. Gene expression profiling of primary tumors with poor metastatic prognosis correlate MMP-1 expression (van 't Veer et al., 2002). Genomic profiles of cell lines with different metastatic potentials had high MMP-1 expression in metastatic cell lines compared with non-metastatic cell lines (Bos et al., 2009; Kang et al., 2003; Minn et al., 2005).

Stromal MMP-1

Stromal expression of MMP-1 is increased in the presence of tumor cells, which may act to promote cancer progression. For example, MMP-1 is expressed in human dermal fibroblasts when cocultured with MCF-7 breast carcinoma cells (Ito et al., 1995). Also, c-ets-1 expression in lung cancer cells increases MMP-1 expression in neighboring stromal cells (Westermarck et al., 1999).

MMP-1 expression in tumor cells activates neighboring human endothelial cells. Endothelial cell activation (ECA) increases endothelial cell adhesion with circulating cancer cells, marked by increased calcium flux, secretion of IL-8 and secretion of von Willenbrand factor (VHF); responses elicited by MMP-1 expression (Goerge et al., 2006). In circulating tumor cells, inducing ECA might promote tumor cell arrest and extravasation (Enzerink et al., 2011), suggesting that MMP-1 expression is involved in successful tumor dissemination.

Cancer cell driven MMP-1 expression in stromal cells also affects drug sensitivity. MMP-1 expression in tumor associated fibroblast decreases head and neck squamous cell carcinoma (HNSCC) susceptibility to cetuximab, an epidermal growth factor receptor (EGFR) antagonist (Johansson et al., 2012).

MMP-1 in Tumorigenesis and Metastasis

In vitro systems have demonstrated a strong relationship between MMP-1 expression and tumorigenesis, invasiveness and migration (Brinckerhoff et al., 2000). In breast cancer cell lines, metastatic cells express higher levels of MMP-1 than non-metastatic cell lines (Balduyck et al., 2000; Bos et al., 2009; Giambernardi et al., 1998; Kang et al., 2003; Kousidou et al., 2004; Minn et al., 2005). In osteosarcoma cells, MMP-1 expression is greater in metastatic cell lines than non-metastatic cell lines (Kimura et al., 2011). Similarly, overexpression and inhibition of MMP-1 correlates to adhesion and anchorage independent growth—markers for metastatic potential (Husmann et al., 2013). In metastatic breast cancer cell lines, inhibition of MMP-1 represses invasiveness (Benbow et al., 1999)

In vivo models of tumorigenesis and metastasis have also demonstrated MMP-1 involvement in cancer. Transgenic mice overexpressing MMP-1 in the skin, lungs and liver (D'Armiento et al., 1997) through a haptoglobin promoter driver have a carcinogen-sensitive phenotype. When treated with TPA on the skin, MMP-1 expressing mice have enhanced tumor progression than their wild type counterparts (Colandrea et al., 2000; D'Armiento et al., 1995).

In bone surface xenografts of human breast cancer cells, MDA-MB-231, inhibition of MMP-1 attenuated invasion, vascularization, and osteolysis, while overexpression of MMP-1 promoted bone metastasis (Lu et al., 2009). Osteoclasts, which degrade bone, cultured in collagen are activated by MMP-1 from MDA-MB-231 media (Eck et al., 2009). MMP-1 activation of osteolysis *in vivo* may function through similar osteoclast activation (Holliday et al., 1997). MDA-MB-231 cells, in coculture with bone marrow fibroblasts, are more adherent and migratory than less metastatic cell lines (Saad et al., 2000). Counterintuitively, osteoblasts, which generate bone, are induced to produce MMP-1 by media from a human breast cancer line, H31, increasing bone resorption. Interestingly, however, these breast cancer cells showed enhanced migration towards collagen degraded by MMP-1, suggesting a mechanism for breast cancer cell movement (Ohishi et al., 1995)

Inhibition of MMP-1 in MDA-MB-231 cells inhibited growth of mammary fat pad xenografts (Wyatt et al., 2005). In melanoma intradermal xenografts, inhibition of MMP-1 had no impact on primary tumor growth, but significantly decreased metastases (Blackburn et al., 2007).

In mice, inhibition of the endogenous MMP-1 homologue MMP-1a, which has 74% homology, repressed invasion and metastatic growth of murine lung cancer and melanoma cells (Foley et al., 2012). The recent development of a knockout MMP-1a mouse showed that MMP-1a abolition did not affect growth or fertility. However, when these mice and their wild-type littermates were subjected to intraperotineal injection of the carcinogen urethane, MMP-1a-/- mice had significantly smaller tumors (Fanjul-Fernandez et al., 2013)

Function

The complex pathologies and regulation of MMP-1 suggest that its involvement in cancer may be multifaceted. MMP-1 is critical in degrading interstitial collagen, and tumor cells may require that function to invade (Page-McCaw et al., 2007). However, MMP-1 has many substrates. Among them, protease-activated receptor-1 (PAR-1) has been shown to be activated by MMP-1 cleavage in MDA-MB-231 breast cancer cells, promoting tumorigenesis and metastasis (Boire et al., 2005). Similar results were seen in mice with homologous MMP-1a and murine PAR-1 (Foley et al., 2012). Expression of PAR-1 is seen in breast cancer patients (Even-Ram et al., 1998) and is correlated to invasion in breast cancer cell lines (Kamath et al., 2001).

PAR-1 is a G-protein-coupled-receptor which is autoactivated after cleavage, typically through thrombin during coagulation (Coughlin, 2000). PAR-1 activation, in turn, can activate endothelial cells (Goerge et al., 2006) and angiogenesis (Caunt et al., 2003).

Several experiments support PAR-1 increasing angiogenesis in breast cancer. PAR-1 activation functions in a feedback loop with tumor stroma where PAR-1 expression upregulates angiogenic factor Cyr61 (CCN1) in breast cancer cells. CCN1 expression, in turn, activates stromal secretion of MMP-1 (Nguyen et al., 2006). In line with this, MMP-1 expression is required *in vitro* angiogenesis of human endothelial cells (Fisher et al., 1994). Stimulation of endothelial cells with MMP-1 also increases levels of VEGFR2 through PAR-1 activation (Mazor et al., 2013).

Feedback loops, such as the PAR-1/MMP-1/VEGFR2/CCN1 interaction described above, are likely to be major component of MMP-1 regulation and

tumorigenesis. Research focused on these interactions is crucial to understanding breast cancer and metastasis.

Concluding Remarks

MMP-1 has repeatedly been correlated to tumorigenesis and metastasis. Mechanisms for MMP-1 regulation of cancer are beginning to be elucidated. However, less is known about the regulation of MMP-1 in a metastatic context. The AP-1 family is a known regulator of MMP-1. Specific AP-1 members are also known to be regulated in metastasis, and therefore may regulate MMP-1 in that context. Nevertheless, as described above, there are many other transcriptions factors reported to regulate MMP-1, so its complex mechanism of regulation during metastasis is far from clear.

It is critical that, in addition to correlating expression to cancer progression, we follow up on leads gathered from genomic profiling. Our aim here is to confirm the function of MMP-1 in cancer and metastasis, detail MMP-1 regulation in a metastatic context, and shed light on the regulatory process. The hope is that better understanding of MMP-1 regulation may be an opportunity to find novel treatments to better manage a devastating disease.

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Chapter 2

Methods

Analysis of Microarray Data

Microarray gene expression data was available as supplemental data in several publications (Bos et al., 2009; Kang et al., 2003; Minn et al., 2005). To parse the data, Affymetrix comparison sheets were used with Microsoft Excel Vlookup functions to match primer coding with gene name, symbol and reference sequence ID. Expression values from cell lines with high metastatic potential to the bone (1833, Scp-2, Scp-25 and Scp-46), to the lung (1834, 3481, 4142, 4173, 4175, 4180, Scp-3 and Scp-28), or with low metastatic potential (MDA-MB-231, Scp-6, Scp-21 and Scp-26) were averaged for each gene. The ratio of high to low metastatic potential expression levels was calculated and ordered by highest ratio. A T-test was used to calculate the p value for the significance of the differences between each group.

Cell Culture

Scp-2, Scp-3, Scp-21, Scp-26, Scp-28, and MDA-MB-231 cell lines were a generous gift from Joan Massague (Memorial Sloan Kettering Research Institute)(Minn et al., 2005). Cells were grown in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal bovine serum. Phoenix amphotropic helper cells (Pear et al., 1993) were grown in DMEM supplemented with 10% Fetal Bovine Serum.

Cell lines stably expressing Fra-1 or a control vector were made in Scp-21 cells. The Fra-1 expression vector, p6599 MSCV-IP N-HAonly FosL1 (White et al., 2012), and pBabe-Puro vector (Morgenstern et al., 1990) plasmids were independently transfected

into Phoenix amphotropic helper cells (Pear et al., 1993) using Lipfectamine LTX (Life Technologies) per manufacturer's instructions. The transfection media was changed after 16 hours to DMEM/10% fetal bovine serum. After 24 hours the media containing virus was removed and polybrene was added to 4 μg/mL. This viral media was filtered with 0.45 μm polyethersulfone filters (Thermo Scientific) and added to Scp-21 cells. This infection media was removed after 24 hours and selection in puromycin (10 μg/mL; Sigma Aldrich) started 24 hours later. These Scp-21 cells expressing Fra-1 or control vector were maintained in DMEM supplemented with 10% fetal bovine serum and 5 μg/mL puromycin.

Scp-2 and Scp-21 cells used to measure protein degradation with cycloheximide were plated at $2x10^6$ cells in a 6 cm plate overnight. Plates were then treated with cycloheximide (10 μg/mL) for the indicated times.

RNA Purification, cDNA Reverse Transcription, DNase Treatment

RNA was purified from adherent cells with Trizol Reagent (Life Technologies) per the manufacturer's instructions. RNA was reverse transcribed with the ImProm Reverse Transcriptase (Promega) per the manufacturer's instructions with random hexamer primers (Integrated DNA Technologies).

In some cases, where signal from contaminating genomic DNA would be problematic, samples to be measured by quantitative RT-PCR were treated with DNase I (Sigma) per the manufacturer's instructions.

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

qRT-PCR was performed with standard protocols with the StepOne Plus System (Life Technologies) with Power SYBR master mix (Life Technologies) per the manufacturer's instructions. Briefly, cDNA samples were combined with master mix and primers (final concentration 0.5 μM; shown in table below). Expression was normalized to 18S rRNA expression. Standard deviations were calculated from three independent experiments. p-values were determined by Student's two-tailed t-tests with significance thresholds as labeled.

Primers (Integrated DNA Technologies) were designed using Primer Express (Life Technologies) with standard parameters. Primer sequences for the human genes were as follows:

RNA	Sequence (5' – 3')	
18S	F	TCGAGGCCCTGTAATTGGAAT
	R	CCCTCCAATGGATCCTCGTTA
MMP-1	F	CCTAGTCTATTCATAGCTAATCAAGAGGATGT
	R	AGTGGAGGAAAGCTGTGCATAC
MMP-1 Pre-mRNA	F	GCTGTGCTGTTACCCTAGTCCCT
	R	GGCAGCCAATCCCTTTGTT
c-Fos	F	CTGGCGTTGTGAAGACCATGA
	R	CCCTTCGGATTCTCCTTTTCTC
FosB	F	AGCAGCAGCTAAATGCAGGA
	R	TTTTGGAGCTCGGCGATCT

Fra-1	F	CCGGGCATGTTCCGAGACTT
	R	ACTCATGGTGTTGATGCTTGGCAC
Fra-2	F	AACTTTGACACCTCGTCCCG
	R	CCAGGCATATCTACCCGGAAT
c-Jun	F	AGATGAACTCTTTCTGGCCTGCCT
	R	ACACTGGGCAGGATACCCAAACAA
JunB	F	AGTCCTTCCACCTCGACGTTTA
	R	TGAATCGAGTCTGTTTCCAGCA
JunD	F	GACAAGCTTATGGAAACACCCTTCTACGG
	R	CCGGGATCCTCAGTACGCGGGCACCTGG

Conservation Mapping

The matrix metallopeptidase-1 (MMP-1) promoter was analyzed with the UCSC Genome Browser (Kent et al., 2002). Analysis was performed on the following tracks: 1) Base Position, 2) Human mRNAs, 3) Placental Mammal Conservation by PhastCon with all 23 species, and 4) Vertebrate Conservation by PhastCon with all 46 species.

Luciferase Assays

All constructs were made with the pGL3-Basic promoter backbone with inserts at the BglII and HindIII sites of the multiple cloning sequence. The MMP-1 promoter regions were amplified from human genomic DNA (Bioline). Amplified inserts spanned -819/+71, -514/+71, -174/+71, -810/-174, -172/-27, -115/-27, and -94/-27 bases from the

transcription start site. Shorter promoter inserts were annealed from oligonucleotide sequences, as follows:

-107/-57	
F	AGCTGTCTATTCATAGCTAATCAAGAGGATGTTATAAAGCATGAGTCAGA CAGCCT
R	GATCAGGCTGTCTGACTCATGCTTTATAACATCCTCTTGATTAGCTATGAA TAGAC
-74/-27	
F	AGCTAGCATGAGTCAGACAGCCTCTGGCTTTCTGGAAGGGCAAGGACTCT CGTACTCAGTCTGTCGGAGACCGAAAGACCT TCCCGTTCCTGAG
R	GATCCTCAGGAACGGGAAGGTCTTTCGGTCTCCGACAGACTGAGTACGAG AGTCCTTGCCCTTCCAGAAAGCCAGAGGCTGTCTGACTCATGCT
-59/-27	
F	AGCTCCTCTGGCTTTCTGGAAGGGCAAGGACTCTCGTACTCAGTCTGTCGG AGACCGAAAGACCT TCCCGTTCCTGAG
R	GATCCTCAGGAACGGGAAGGTCTTTCGGTCTCCGACAGACTGAGTACGAG AGTCCTTGCCCTTCCAGAAAGCCAGAGG
AP-1 Point Mutant	
F	AGCTGTCTATTCATAGCTAATCAAGAGGATGTTATAAAGCATGCCACAG ACAGCCT
R	GATCAGGCTGTCTGTGGCATGCTTTATAACATCCTCTTGATTAGCTATGA ATAGAC

PEA3 Point Mutant	
F	AGCTGTCTATTCATAGCTAATCAAGATCTTGTTATAAAGCATGAGTCAGAC AGCCT
R	GATCAGGCTGTCTGACTCATGCTTTATAACAAGATCTTGATTAGCTATGAA TAGAC
HoxA5 Point Mutant	
F	AGCTGTCTATTCATAGCATGCCAAGAGGATGTTATAAAGCATGAGTCAGA CAGCCT
R	GATCAGGCTGTCTGACTCATGCTTTATAACATCCTCTTGGCATGCTATGAA TAGAC
3xAP-1	
F	AGCTCATGAGTCAGACATGAGTCAGACATGAGTCAGA
R	GATCTCTGACTCATGTCTGACTCATGTCTGACTCATG
3xPEA3	
F	AGCTAATCAAGAGGATGTTAAGCTAATCAAGAGGATGTTAAGCTAATCAA GAGGATGTTA
R	GATCTAACATCCTCTTGATTAGCTTAACATCCTCTTGATTAGCTTAACATCC TCTTGATT

The -819/-174, -172/-27, -115/-27, -94/-27, -74/-27, and -59/-27 inserts and synthetic promoters were added upstream of a c-Fos minimal promoter insert (Wang et al., 2000) in the pGL3-Basic backbone. -819/+71 AP-1 point mutations were made by PCR driven overlap extension (Heckman et al., 2007). pRL-SV40P with the SV40

promoter driving Renilla luciferase (Chen et al., 1999) served as an internal control. pCMV-Luciferase (Selvaraj et al., 2003) served as a positive control.

The luciferase plasmids were transfected into cells using Lipofectamine 2000 (Life Technologies) per the manufacturer's instructions. Cell were lysed in passive lysis buffer (Promega) 16 hours post transfection and analyzed using the Dual-Luciferase Reporter Assay System (Promega) per the manufacturer's instructions with a 20/20 luminometer (Turner Biosystems) for a 10-second interval measurement. Mean and standard deviation for the ratio of firefly-luciferase to renilla-luciferase signals were calculated from three independent experiments. p-values were determine by Student's two-tailed t-tests, with significance thresholds as indicated.

Immunoblot Analysis

Whole cell lysates were prepared with RIPA buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton-100, 1 mM DTT, 1 mM PMSF, Protease Inhibitor Cocktail III [1:200; Calbiochem], pH 7.6). After 10 minutes at 4°C, the lysates were centrifuged at 20,000 g for 15 minutes at 4°C, and lysate supernatants were normalized for protein levels with BCA Assays (Pierce) per the manufacturer's instructions. Normalized lysates were separated by SDS-polyacrylamide gel electrophoresis (PAGE), transferred onto Trans-Blot transfer medium (Bio-Rad), and immunoblotted with primary antibody at 4°C for 16 hours. Antibodies used were against Fra-1 (sc-605X), MMP-1 (sc-12348), JunD (sc-74X), c-Jun (sc-1694X), HSP-90 (sc-101494) and Actin (sc-1616) from Santa Cruz Biotechnology. Generally 1:1000 dilutions were used. Membranes were then washed three times with Tris-buffered saline (TBS) and

incubated with secondary antibody at a 1:10,000 dilution for one hour. Secondary antibodies used were: Goat anti-Rabbit IRDye 800CW, Goat anti-Rabbit IRDye 680LT, and Donkey anti-Goat IRDye 800CW from LiCor. Membranes were then washed three times with TBS. Membranes were measured for fluorescence with an Odyssey infrared imager (LiCor). Means and standard deviations were calculated from Odyssey quantitation of specific band intensities in three independent experiments. p-values were determined by Student's two-tailed t-tests with significance thresholds as indicated.

siRNA treatment

Double stranded siRNA duplexes (Integrated DNA Technologies, Sigma-Aldrich), as indicated below, were transfected with RNAiMax Lipofectamine transfection reagent (Life Technologies) per the manufacturers instructions. Duplexes were designed as follows:

Name	**Catalog Number**	**Manufacturer**
Control	DS NC1	Integrated DNA Technologies
CGUUAAUCGCGUAUAAUACGCGU AUACGCGUAUUAUACGCGAUUAACGAC		
dsiRNA-Fra-1 A	HSC.RNAI.N005438.12.1	Integrated DNA Technologies
GGCGGAGACUGACAAACUGGAAGAT GUCCGCCUCUGACUGUUUGACCUUCUA		
dsiRNA-Fra-1 B	HSC.RNAI.N005438.12.2	Integrated DNA Technologies
CCACUUUACCCACCUAGAACACUAA		

ACGGUGAAAUGGGUGGAUCUUGUGAUU		
dsiRNA-JunD A	HSC.RNAI.N005354.12.1	Integrated DNA Technologies
CGAGUCCACAUUCCUGUUUGUAATC AUGCUCAGGUGUAAGGACAAACAUUAG		
dsiRNA-JunD B	HSC.RNAI.N005354.12.3	Integrated DNA Technologies
GCCGACGAGGCUCACAGUUCCUCUAC UGCGGCUGCUCGAGUGUCAAGGAGAUG		
dsiRNA-c-Jun A	SAS_Hs02_00333461	Sigma-Aldrich
dsiRNA-c-Jun B	SAS_Hs01_00150279	Sigma-Aldrich

Electrophoretic Mobility Shift Assays (EMSA)

Nuclear extracts were made from $4x10^7$ cells grown on four 15 cm plates. Cells were washed with cold PBS, and scraped into 3 mL of phosphate buffered saline (PBS). Samples were centrifuged at 400 g for 1 minute at 4°C in a J6B centrifuge (Beckman). The cell pellets were resuspended in 4 mL of Buffer A (10 mM Tris, 1.5 mM $MgCl_2$, 10mM KCl, 0.4 mM DTT, .04 mM PMSF, pH 7.9) and incubated for 10 minutes at 4°C. Samples were dounced 50 times with a type B 15 mL glass douncer (Kontes Glassware Co.). Dounced samples were centrifuged at 400 g for 10 minutes at 4°C in the J6B centrifuge. The nuclear pellets were resuspended in 300 µl Buffer C (20 mM Tris, 0.3 M KCl, 1.5 mM $MgCl_2$, 25% Glycerol, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, pH 7.9) and rotated at 4°C for 30 minutes. Samples were centrifuged at 20,000 g for 15 minutes at 4°C. Nuclear extract supernatants were then removed, normalized for total protein levels by BCA Assays (Pierce) and used for DNA binding reactions.

Probes and competitors for DNA binding assays were made with annealed complementary oligonucleotides (Integrated DNA Technologies), as listed below:

MMP-1 Probe (with AP-1 consensus site)	
F	AGCTGTCTATTCATAGCTAATCAAGAGGATGTTATAAAGCATGAGTCAGA CAGCCT
R	GATCAGGCTGTCTGACTCATGCTTTATAACATCCTCTTGATTAGCTATGAA TAGAC
Non-specific Competitor:	
F	TGTCGAATGCAAGCCACTAGAA
R	TTCTAGTGGCTTGCATTCGACA
Probe with Mutant AP-1 Site:	
F	AGCTGTCTATTCATAGCTAATCAAGAGGATGTTATAAAGCATGCCACAG ACAGCCT
R	GATCAGGCTGTCTGTGGCATGCTTTATAACATCCTCTTGATTAGCTATGA ATAGAC

The annealed probes were end-labeled with **γ-**^{32}P-ATP (Perkin Elmer) and poly nucleotide kinase (New England Biolabs), per the manufacturer's instructions, to a final concentration of 1 ng/ul.

DNA binding reactions contained 5 µl nuclear extract (approximately 10 µg),1 ng of ^{32}P-labeled probe, 50 ng poly dI-dC, 250 ng of competitor (as indicated), and 2 µg antibody (in supershift experiments) for 30 minutes at room temperature with binding buffer (final concentration: 10 mM Tris HCl (pH 8.0), 50 mM KCl, 0.5 mM EDTA, 0.1%

Triton-X 100, 12.5% Glycerol, 0.2 mM DTT). Samples were then loaded on a 5% polyacrylamide gel in 1/4x TBE, and run for 2.5 hours at 100 V with 1x TBE running buffer. The gel was then dried and exposed to x-ray film (Kodak) for 16 hours.

Chromatin Immunoprecipitation (ChIP)

ChIP was performed as described (Beckerman et al., 2009), with minor modifications. Briefly, $4x10^7$ cells were crosslinked with 1% formaldehyde for 15 minutes at 25°C and quenched with 125 mM glycine. Crosslinked plates were lysed in RIPA buffer (as described in immunoblot methods above), sonicate with a Sonicator 3000 (Misonix) for 1 minute total, in 5 seconds on – 15 seconds off intervals, and centrifuged at 20,000 g for 15 minutes at 4°C. Lysates were normalized by BCA Assay (Pierce), per the manufacturer's instructions, and immunoprecipitated with 2 μg of anti-Fra-1 antibody (Santa Cruz Biotechnology; Catalog #: sc-605) overnight rotating at 4°C. Protein-A agarose beads (7.5 μL)(Santa Cruz Biotechnology) diluted with 22.5 μL RIPA were added to purify immunoprecipitated protein for 90 minutes rotating at 4°C. The beads were washed three times in RIPA buffer and reconstituted in 200 μL elution buffer (70 mM Tris HCl pH 8.0, 1 mM EDTA, 1.5% SDS) for 10 minutes at 65°C. Beads were centrifuged at 1700 g for 1 minute at room temperature. The salt of the transferred supernatant was adjusted to a concentration of 200 mM NaCl and incubated for 5 hours at 65°C to reverse the crosslinks. DNA from ChIP samples was then purified with Qiaquik PCR Purification Kits (Qiagen) per the manufacturer's instructions.

Purified DNA was measured by qPCR (as previously described in qRT-PCR method) with primers, as follows:

MMP-1		
AP-1	F	TCTGCTAGGAGTCACCATTTCT
	R	ATAGAGTCCTTGCCCTTCCAG
Control	F	AGTGACTACCGCTCTGCTGTGT
	R	GTTCCGTCAGTCCTCATGGTT
IL-6		
AP-1	F	CTTCGTGCATGACTTCAGCTTT
	R	AGCGCTAAGAAGCAGAACCACT
Control	F	ATAGACGGATCACAGTGCACG
	R	GCAACGTAGACACTCCTGAACC

Samples were normalized to input DNA purified from reversed cross-linked input samples and measured through qPCR. Mean and standard deviations were calculated from three independent experiments. p-values were determine by Student's two-tailed t-tests, with significance thresholds as indicated.

Metabolic Labeling

Cells ($1x10^5$) were plated in 6 cm plates for labeling. After 16 hours, cells were washed twice with warm PBS and starved for 30 minutes at 37°C with 4 mL methionine and cysteine free DMEM (Life Technologies). Media was changed to 2 mL ^{35}S-Translabel metabolic labeling reagent (100 μCi/mL; MP Biomedicals) in methionine and cysteine free DMEM and incubated at 37°C for the indicated times. Plates were washed twice with cold PBS, lysed in ice cold RIPA buffer, centrifuged at 20,000 g for 15 minutes at 4°C, and immunopreciptated with 2 μg of anti-Fra-1 antibody (Santa Cruz

Biotechnology; #sc-605) overnight rotating at 4°C. Protein-A agarose beads (Santa Cruz Biotechnology) were used to purify immunoprecipitated protein by incubation for 90 minutes at 4°C. Washed beads were reconstituted in SDS-PAGE sample buffer (described above) and boiled for 5 minutes. Boiled samples were centrifuged at 1700 g for 1 minute at 4°C, and resolved on 12% SDS-PAGE for 2.5 hours at 150 V. The gel was placed in fixative (50% Methanol/10% Acetic Acid) for 30 minutes rocking at 25°C. The gel was then enhanced with Amplify Fluorographic Reagent (GE) for 30 minutes rocking at 25°C. After enhancement, the gel was dried and exposed to film (Kodak) for 5 days. Autoradiographs were quantitated by ImageJ software analysis. Means and standard deviations were calculated from three independent experiments. p-values were determine by Student's two-tailed t-tests with significance thresholds as indicated.

Soft Agar Assay

Soft agar plating of the cell lines was performed as described (Freed-Pastor et al., 2012), with minor modifications. Briefly, 35-mm plates were coated with 1.5 mL 0.6% agar in DMEM. Cells ($5x10^3$) were reconstituted in 1.5 mL 0.3% agar in DMEM, and plated on top of the 0.6% agar layer. Agar layers were then covered with 1.5 mL DMEM/10% Fetal Bovine Serum. Cells were grown for 21 days with the media being changed every 5 days. Colonies were stained with 0.005% Crystal Violet in water for one hour and counted. Mean colony number and standard deviation were calculated from three independent experiments. p-values were determined by Student's two tailed t-tests with significance thresholds as indicated.

Scratch-Wound Assays

Cells were grown to confluency and the monolayer was scratched and monitored by phase contrast microscopy. Triplicate images at each time point were used to count the number of cells that passed the scratch threshold. Mean and standard deviation were calculated from three independent experiments. p-values were determined by Student's two-tailed t-tests, with significance thresholds as indicated.

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Chapter 3

Differential Expression and Transcriptional Regulation of Interstitial Collagenase (MMP-1) in MDA-MB-231 Cell Variants

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Introduction

Breast cancer will affect one in eight women in the United States (Siegel et al., 2012). Unfortunately for many patients, after the primary tumor is excised or ablated, cancer can remain in secondary organs as micrometastases. Compounding this issue, micrometastases often exist in a nonproliferative state (Braun et al., 2005; Pantel et al., 1993), making them less sensitive to adjuvant therapies targeting proliferating cells. Nearly all breast cancer mortality is the result of tumor cells metastasizing to vital organs (Gupta et al., 2006). Therefore, understanding breast cancer metastasis is critical to improving treatment and prognoses.

Genomic expression technology has yielded a tremendous amount of data for breast cancer. Several metastatic gene profiles have been discovered (van 't Veer et al., 2002). Some profiles have also been commercialized and are used for diagnostic purposes in the clinic (Ross et al., 2008). However, genomic expression technology also presents limitations and challenges. For example, microarray data may uncover a correlation between gene expression and a metastatic phenotype, yet how these genes are regulated and what role their expression has in cancer must still be determined using other approaches.

To further complicate diagnosis and treatment, cancer, while clonal in origin, often exists as heterogeneous tumors (Ding et al., 2010; Farabegoli et al., 2001; Navin et al., 2011; Shipitsin et al., 2007; Teixeira et al., 1996). Statistically, this heterogeneity can obscure microarray signals and increase background noise. Physiologically, relevant cell populations or gene expression patterns may be lost in the aggregate.

Two methods were used to increase the homogeneity and robustness of the tumor phenotype. In the first method, tumor heterogeneity was reduced by the development of clonal cultures from single B16 melanoma cells, each with a distinct metastatic potential in syngenic mouse intravenous injections (Fidler et al., 1977). In a second set of experiments, metastatic B16 cell populations were selected using syngenic mouse injections. After injection, cells were cultured from the resulting metastatic lung tumors. These derivative B16 cell lines exhibited greater metastatic potential when reinjected into mice. Repeating this process led to a series of cell lines with increasing metastatic potential and greater homogeneity (Fidler, 1973).

The Massague group developed a similar system for breast cancer xenografts. The parental cell line, MDA-MB-231, is a breast adenocarcinoma derived from a pleural effusion. These cells cause a low level of metastasis when injected into the mammary pads of immunocompromised mice. Metastatic cells from these xenografts had higher metastatic potential and subsequent reinjection of these secondary xenografts resulted in cells with even higher metastatic potential. Alternatively, MDA-MB-231 cells were cloned into single cell populations (Scp cell lines). The Scp cell lines were found to have varied metastatic potential (Minn et al., 2005). The metastatic potential to specific tissues also varied. Cells derived from metastatic tumors in the lung or the bone were more likely to metastasize to same organ if reinjected in mouse xenografts (Kang et al., 2003; Minn et al., 2005). Microarrays on these cell lines of varying metastatic potential[1] provide an

[1] As a brief aside, we note that experiments were performed in Scp-2, Scp-21 and MDA-MB-231 cell lines. For the sake of brevity, we will refer to the number of metastatic tumors in mouse xenograft models (Minn *et al*, 2005) as 'metastatic potential.' Scp-2 cells, which were in the top quinitile of metastatic potential, will be called 'metastatic.' MDA-MB-231 cells, which were in the fourth lowest quintile of

opportunity to discover genes correlated to, and potentially causative of, breast cancer metastasis (Bos et al., 2009; Kang et al., 2003; Minn et al., 2005). In addition, comparison of these cells provides a well-controlled system to understand the mechanisms that alter gene expression and lead to higher metastatic potential.

We have analyzed microarray gene expression data from the MDA-MB-231 derived cell lines with varied metastatic potential and focused on the expression of matrix metallopeptidase-1 (MMP-1), which correlated strongly with high metastasis to both the bone and the lung. We show that MMP-1 expression is differentially regulated by an AP-1 element in its promoter and the transcription factor Fra-1.

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metastatic potential, will be called 'low-metastatic.' Scp-21 cells, which were in the bottom quintile of metastatic potential, will be called 'non-metastatic.'

Low-metastatic MDA-MB-231 cells were derived from breast cancer carcinoma cells collected from the pleura of the lung, and are therefore metastatic by definition. The focus of this research is to determine the regulators of metastasis and therefore a higher threshold for metastatic potential will be used.

Results

Correlation of MMP-1 Expression with Cell Line Metastatic Potential

We analyzed publicly available microarray gene expression data from a set of breast carcinoma cell lines with well-characterized metastatic potential (Minn et al., 2005) for a correlation between gene expression and metastasis. To identify genes that were specifically upregulated in cells with high bone or lung metastatic potential, we grouped cell lines as either highly metastatic to the bone, to the lung, or neither (i.e. with low metastatic potential) (Minn et al., 2005). We determined the ratio of average expression in highly metastatic cell lines (bone or lung) to the low metastatic cells (Tables 3-1 and 3-2; Appendix 1). Microarray data from four highly metastatic bone lines were used, eight lung metastatic lines and four low or non-metastatic lines. The highest differential expression was found for the MMP-1 gene. MMP-1 was expressed nearly an average of 100 fold more in bone metastatic cells than non-metastatic cells. Expression was also strongly higher in lung metastatic cells (27 fold), albeit with a weaker p value (0.056).

We confirmed the microarray data for MMP-1 by measuring expression by quantitative RT-PCR in three cell lines with varying metastatic potential: Scp-2 (highly metastatic), Scp-21 (non-metastatic) and MDA-231 (low-metastatic). As was shown by the microarray data, MMP-1 mRNA expression was 90 fold higher in Scp-2 cells than in Scp-21 cells and 17 fold higher than in MDA-MB-231 cells (Figure 3-1A). Immunoblot analysis confirmed that MMP-1 protein levels were commensurate with mRNA expression (Figures 3-1C and D). These data indicate that MMP-1 is differentially regulated in cells with different metastatic potentials.

In order to test whether differential mRNA expression of MMP-1 is transcriptionally regulated, we used qRT-PCR to measure the relative amounts of MMP-1 pre-mRNA. Pre-mRNA levels preceding splicing is a more direct indicator of transcription. Pre-mRNA levels of MMP-1 were also greatly elevated in Scp-2 metastatic cells compared to the non-metastatic Scp-21 cells, suggesting that this difference is due to changes in transcription (Figure 3-1B).

Mapping of Gene Regulatory Elements

We examined the human MMP-1 promoter for sequence conservation and found blocks of conserved elements in the proximal promoter region (Figure 3-2). These conserved regions overlap some consensus transcription factor binding sites that have previously been identified for the MMP-1 promoter (Overall, 2001; Pardo et al., 2005; Ra et al., 2007).

To determine if the MMP-1 promoter is sufficient to reproduce differential transcription in reporter assays, we inserted sections of the MMP-1 promoter in luciferase reporter constructs (Figure 3-3A) and measured luciferase expression in Scp-2 and MDA-MB-231 cells (high and low metastatic cells, respectively). The MMP-1 promoter region from -819 to +71 was sufficient for five fold greater expression in the highly metastatic Scp-2 cells (Figure 3-3B).

In order to determine which region of the MMP-1 promoter was required for differential transcription of MMP-1 in Scp-2, Scp-21 and MDA-MB-231 cells, 5' and 3' deletions were made (Figure 3-3A). Both -514/+71 and -174/+71 constructs were sufficient to drive significant differential expression, similar to -819/+71 (Figure 3-

3B). As a control, we used a CMV promoter-luciferase construct that gave similar expression in the two cell lines. These results suggest that key regulatory elements for differential expression are in the -174/+71 promoter region.

To demonstrate that the -174/+71 region is required for differential expression, we designed a 3' deletion -819/-174 construct. The -819/-174 MMP-1 region was inserted into a luciferase plasmid upstream of the c-Fos minimal promoter. The c-Fos minimal promoter includes the TATA box and transcription start site to give baseline expression. The -819/-174 construct was not able to drive significant expression (Figure 3-3B). Together, the 5' and 3' deletion constructs identified the -174/+70 MMP-1 promoter region as necessary and sufficient for MMP-1 transcriptional regulation.

We also used the c-Fos minimal promoter with a series of 5' MMP-1 promoter deletions to -27, to further isolate the region required for expression in -174/+70 (Figure 3-4A). We found that the -94/-27 region was the minimal region required for differential expression between Scp-2 and MDA-MB-231 cells, with little differential expression seen with the -74 construct (Figure 3-4B). However, while the ratio of expression between Scp-2 and MDA-MB-231 was consistent among -172/-27, -115/-27 and -94/-27, overall expression was significantly lower in -94/-27 and -115/-27 compared to -172/-27, suggesting that there are positively acting regulatory elements between -74 and -172. These constructs showed that the -94 to -27 region was sufficient for differential expression by the MMP1 promoter.

Having isolated a small regulatory region of the MMP-1 promoter, we sought to determine the specific transcription factor binding sites involved. Previous findings and conservation mapping pointed to several potential regulators in the -94/-27 region of the

MMP-1 promoter: HoxA5, PEA3, and AP-1 (Auble et al., 1991; Gutman et al., 1990)(Figure 3-2). To determine which, if any, of these sites are required for regulation, synthetic promoters were made with the region that contains the three consensus sites, -107 to -59, upstream of the c-Fos minimal promoter (Figure 3-5A). The -107/-59 region drove significantly higher expression in Scp-2 than in MDA-MB-231 cells (Figure 3-5B). Point mutations were made to each of the three conserved consensus regions (Figure 3-5A). Among them, only the AP-1 site mutation significantly decreased expression and decreased differential expression. While Scp-2 cells did have greater luciferase expression than MDA-MB-231 cells for the AP1 mutant, this low level was variable and the difference was not statistically significant (Figure 3-5B).

To confirm that the AP-1 site is required for expression in the context of the fuller promoter, we created a -819/+71 MMP-1 promoter construct with point mutations in the AP-1 consensus site (Figure 3-6A). These point mutations were sufficient to completely abrogate luciferase expression in both Scp-2 and MDA-MB-231 cells (Figure 3-6B). The AP1 site alone was not sufficient to drive expression in Scp-2 cells as the site is present in the -74/-27 construct that was not expressed (Figure 3-4). To determine whether multiple copies of the AP1 site were sufficient, we made a synthetic promoter construct with a triple MMP-1 AP-1 consensus site and found that it gave a robust signal with significant differences between Scp-2 and MDA-MB-231 (Figure 3-6A and B). The ratios of luciferase expression in Scp-2 versus MDA-MB-231 cells were similar with the triple AP-1 synthetic promoter and the -819/+71 region of MMP-1 (Figure 3-6B). In contrast, a triple PEA3 site did not drive luciferase expression, suggesting that it is not sufficient for differential expression (Figure 3-6B). Together these experiments

demonstrated that the AP-1 region of the promoter is both necessary and sufficient for differential transcriptional regulation of MMP-1 in Scp-2 and MDA-231 cell lines.

Characterization of AP-1 family members in MDA-MB-231 derived cell lines

The AP-1 consensus site is bound by a dimer of AP-1 family members (reviewed in (Ozanne et al., 2007). There are seven AP-1 family genes: three Jun genes (c-Jun, JunB and JunD) and four Fos related genes (c-Fos, Fra-1, Fra-2, and FosB). Dimers are comprised of at least one Jun family member, but can be homo- or hetero-dimers (Halazonetis et al., 1988; Verde et al., 2007). To determine which AP-1 family members were expressed in Scp-2, Scp-21 and MDA-MB-231 cells, and would therefore be candidates for MMP-1 regulation, we performed qRT-PCR in each of the cell lines. Fra-1, Fra-2 and JunD had the highest expression levels, with lower levels of c-Jun and nearly undetectable JunB, FosB and c-Fos (Figure 3-7A). However, unlike the differential mRNA expression seen for MMP-1 (Figure 3-1A), all the detectable AP-1 family members had comparable mRNA expression among the different cell lines (Figure 3-7A).

To explore whether AP-1 family member protein expression is consistent with their mRNA expression, we performed immunoblots. Specifically, we looked at Fra-1, Fra-2, c-Jun and JunD in Scp-2, Scp-21 and MDA-MB-231 cell lines. The remaining AP-1 family members, c-Fos, FosB and JunB, that were not expressed at the mRNA level were not considered further. Interestingly, contrary to Fra-1 mRNA expression levels, Fra-1 protein levels were significantly higher in Scp-2 cells than Scp-21 and MDA-MB-231 cells (Figure 3-7B). However, there was no significant change in protein expression levels of the Fra-2, JunD or c-Jun. These results suggest the possibility that differences

in Fra-1 protein expression in Scp-2, Scp-21 and MDA-MB-231 cell lines are responsible for regulation of MMP-1 transcription.

To test the hypothesis that Fra-1 regulates MMP-1, we inhibited expression of Fra-1 with short interfering RNAs (siRNA). Two siRNA duplexes decreased Fra-1 mRNA expression by over 80% (Figure 3-8A) and Fra-1 protein levels by about 70% (Figures 3-8B and C). This inhibition greatly reduced MMP-1 mRNA expression (Figure 3-8D), supporting Fra-1's role in MMP-1 regulation.

Though other AP-1 family members were not differentially expressed in the Scp-2, Scp-21 and MDA-MB-231 cell lines, we sought to determine which other AP-1 family members were required for MMP-1 expression. As JunD is the most strongly expressed Jun family member in these cells, we first inhibited its mRNA expression with siRNA duplexes (Fig. 9A and B). However, despite efficient reduction in JunD levels, this inhibition did not have an effect on MMP-1 mRNA expression (Figure 3-9C).

We had difficulty efficiently depleting Fra-2 and c-Jun with siRNAs, perhaps because mRNA expression of these genes was relatively low. As such, partial inhibition c-Jun and Fra-2 had no statistically significant impact on MMP-1 (data not shown). Therefore, it was not possible for us to assess whether c-Jun or c-Jun acting redundantly with JunD were required for MMP-1 expression. Nevertheless, the requirement of the AP-1 site in the MMP1 promoter and depletion of Fra-1 clearly show that this factor is required for expression of MMP1 in the metastatic MDA-MB-231 derived cells. The differential expression of Fra-1 protein levels suggests that this mechanism may at least partially account for differences in MMP1 expression.

Discussion

Genomic expression technology has yielded a tremendous amount of data regarding altered gene expression in cancer cell lines and patient tumors (Atlas, 2012). Expression patterns have identified expression "signatures" and advanced diagnostic tools (Ross et al., 2008; van 't Veer et al., 2002), but how these varying expression patterns are achieved is often unknown. With the abundance of correlation data available, we sought to explore the regulatory mechanism of expression in metastasis.

As described in the introduction, analysis of microarray studies poses major challenges partially because the samples are often heterogeneous, which can dilute the expression signal from cells of interest and increase the background noise from other irrelevant cells. In metastatic studies of tumors, only a fraction of the cell population may be capable of metastasizing. Among this metastatic cell population there may be expression variation due to varied mechanisms, such as degree of metastatic potential or preference for metastasis to specific organs. In addition, in cases where samples come from different individuals, there can be variability due to uncontrolled differences in genetic background.

The solution to this challenge is to use a well defined system to limit the heterogeneity of the samples and to correlate expression with defined and selected changes in metastatic potential. The MDA-MB-231 metastasis system defined by Massague and colleagues provides a large number of closely related cell lines with variable metastatic potential (Minn et al., 2005). The cell lines are all derived from MDA-MB-231 breast carcinoma cells, either by selection of metastatic clones in mouse xenograft or by analysis of single cell clones (the Scp lines). The result that the Scp cell

clones have vastly different, but reproducible, metastatic potentials suggests that the cells with these properties were pre-existing in the MDA-MB-231 cultures (Kang et al., 2003; Minn et al., 2005).

MDA-MB-231 cell variants provided us with an opportunity to understand how the changes translated to altered gene expression. The generations of xenografted metastatic tumor cell lines, together with the Scp cell lines, yielded a list of genes correlated to metastatic potential. We have found that among these genes, MMP-1 expression is strongly correlated to metastatic potential in cells and that this expression is transcriptionally regulated by Fra-1 interaction with the AP-1 site of the MMP-1 promoter.

Minimal Promoter Region Sufficient for Differential Expression

Luciferase data confirmed that AP-1 was required for differential expression between highly metastatic and non-metastatic cell lines (Figure 3-6B). However, while the synthetic triplicate AP-1 site promoter construct was sufficient for differential expression, the -74/-27 MMP-1 promoter fragment with the c-Fos minimal promoter was not. Therefore, although required, the single AP-1 consensus site in the -74/-27 region was insufficient for differential expression without the neighboring upstream sequence (-96/-74). Initial studies of phorbol ester induction of the MMP-1 promoter in fibroblasts had a similar result (Auble et al., 1991). Therefore, it is likely that additional factor binding to the -96/-74 region is also required. However, transcription factors with known binding sites in that region, HoxA5 and PEA3, were not required (Figure 3-5B).

In addition, differential expression was significantly greater with the -172/-27 region compared to the -115/-27 region. While this region is not required for differential expression, it is likely that an additional factor binding in this region increases expression (Figure 3-4B).

Role of AP-1 in MMP-1 Expression and Metastasis

In line with our findings, AP-1 regulation of MMP-1 has been well studied in several systems (Angel et al., 1987; Lee et al., 1987; Vincenti et al., 1996) and AP-1 expression has been implicated in tumorigenesis (Bamberger et al., 1999; Milde-Langosch, 2005; Song et al., 2006). In particular, expression of Fra-1 has been shown to be correlated to plastic proliferative breast disorders (Chiappetta et al., 2007) and aggressive breast cancer cells (Zajchowski et al., 2001).

We have shown that Fra-1 is required for MMP-1 expression in the MDA-MB-231 derivatives. However, Fra-1 binds to the AP-1 consensus sequence as a heterodimer with a Jun protein (Cohen et al., 1989). Therefore, a Jun protein should also be required for MMP-1 expression. However, depletion of the most highly expressed Jun protein, JunD, had no effect on MMP-1 expression. JunB could not be detected by immunoblotting and showed very low expression by qRT-PCR. The final Jun protein, c-Jun, was detected by immunoblot and qRT-PCR. However, five siRNA duplexes were unable to significantly reduce c-Jun expression (data not shown). Challenges inhibiting c-Jun expression may be due to its low levels or, alternatively, to a cell requirement for c-Jun expression—making c-Jun inhibition toxic to the cell. Due to the inability to strongly deplete c-Jun levels, we cannot determine whether it is required for MMP-1 expression or

whether it fulfills a redundant requirement with JunD. It is also possible that there is a novel partner for Fra-1 in these cells.

Conclusion

In summary, we correlated MMP-1 expression to the metastatic expression of MDA-MB-231 cell line variants. We found that MMP-1 expression is regulated in these cells by the AP-1 consensus site on the promoter and that the AP-1 family member, Fra-1, is required for MMP-1 expression. AP-1 binding and regulation will be described further in the chapter four.

Figure Legends

Figure 3-1

Differential expression of MMP-1 in MDA-MB-231 derivative cell lines. A. qRT-PCR of MMP-1 in Scp-2 (high metastatic potential), Scp-21 (non-metastatic) and MDA-MB-231 (low metastatic) cells. B. qRT-PCR of MMP-1 mRNA and pre-mRNA in Scp-2 and Scp-21 cells. Mean relative values +/- standard deviation from three independent experiments. C. Immunoblot with anti-MMP-1 antibody of whole cell lysates from Scp-2, Scp-21, and MDA-MB-231 cells. Anti-actin antibody served as a loading control. D. Mean MMP-1 protein band intensity +/- standard deviation from three independent experiments. **, $p < 0.005$ for two-tailed t-tests.

Figure 3-2

Schematic of conservation and consensus regions of the MMP-1 promoter. Genome Browser (Kent et al., 2002) analysis of placental mammalian and vertebrate conservation by PhastCon. Regions of conservation were compared to known transcription factor consensus sequences (shown in gray, with unmatched bases in red); numbers represent base position in reference to MMP-1's transcription start site.

Figure 3-3

The MMP-1 promoter region from -174 to +71 bases from the site of transcription start is necessary and sufficient for differential expression in Scp-2 and Scp-21 cells. A. Schematic of MMP-1 promoter region sequence inserts in a pGL3-luciferase backbone. B. Luciferase signal from Scp-2 and MDA-MB-231 cells transfected with the indicated

reporter constructs. The signal was normalized to the Renilla luciferase levels from the co-transfected pRL-SV40P. The pCMV-luciferase construct was included as a control and its values were normalized to 1.0 for the Scp-2 cells. Its expression is approximately 10 times stronger than the MMP-1 luciferase reporters. The data shown represent mean +/- standard deviation for three independent experiments. *, $p < 0.05$ for two-tailed t-tests.

Figure 3-4

The minimum MMP-1 promoter region for differential expression in Scp-2 and Scp-21 cells is -94 to -27 bases from transcription start. A. Schematic of MMP-1 promoter region sequence inserts in a pGL3-luciferase backbone. B. Luciferase signal from Scp-2 and MDA-MB-231 cells transfected with the indicated reporter constructs. The signal was normalized to the Renilla luciferase levels from the co-transfected pRL-SV40P. The data shown represent mean +/- standard deviation for three independent experiments. **, $p < 0.005$ for two-tailed t-tests.

Figure 3-5

The AP-1 site of the MMP-1 promoter is necessary for differential expression in Scp-2 and Scp-21 cells. A. Schematic of MMP-1 promoter region sequence inserts in a pGL3-luciferase backbone. B. Luciferase signal from Scp-2 and MDA-MB-231 cells transfected with the indicated reporter constructs. The signal was normalized to the Renilla luciferase levels from the co-transfected pRL-SV40P. The data shown represent

mean +/- standard deviation for three independent experiments. *, $p < 0.05$; **, $p < 0.005$ for two-tailed t-tests.

Figure 3-6

The AP-1 site of the MMP-1 promoter is necessary and sufficient for differential expression in Scp-2 and Scp-21 cells. A. Schematic of MMP-1 promoter region sequence inserts in a pGL3-luciferase backbone. B. Luciferase signal from Scp-2 and MDA-MB-231 cells transfected with the indicated reporter constructs. The signal was normalized to the Renilla luciferase levels from the co-transfected pRL-SV40P. The 3x AP-1 construct values were normalized to 1.0 for the Scp-2 cells. Its expression is approximately three times stronger than the MMP-1 luciferase reporter. The data shown represent mean +/- standard deviation for three independent experiments. **, $p < 0.005$ for two-tailed t-tests.

Figure 3-7

AP-1 family mRNA and protein expression. A. qRT-PCR of AP-1 family members in Scp-2, Scp-21 and MDA-MB-231 cells. Mean relative values +/- standard deviation from three independent experiments. **, $p < 0.005$ for two-tailed t-tests. B. Immunoblots with anti-AP-1 antibodies of whole cell lysates from Scp-2, Scp-21, and MDA-MB-231 cells. Anti-MMP-1 is included for comparison and anti-actin antibody served as a loading control.

Figure 3-8

Fra-1 depletion with siRNAs decreases MMP-1 mRNA expression. A. qRT-PCR of Fra-1 in Scp-2 cells transfected with control or siRNA duplexes for Fra-1. Mean relative values +/- standard deviation from three independent experiments are shown. B. Immunoblot with anti-Fra-1 antibodies of Scp-2 cells transfected with control or siRNA duplexes for Fra-1. Anti-actin antibody serves as a loading control. C. Mean Fra-1 band intensity +/- standard deviation for immunoblots from three independent experiments. D. qRT-PCR of MMP-1 and GAPDH control expression of Scp-2 cells transfected with control or double-stranded siRNA duplexes for Fra-1. Mean relative values are +/- standard deviation from three independent experiments. *, $p < 0.05$; **, $p < 0.005$ for two-tailed t-tests.

Figure 3-9

JunD depletion with siRNAs has no effect on MMP-1 mRNA expression. A. Immunoblot with anti-JunD antibody of Scp-2 cells transfected with control or siRNA duplexes for JunD. B. Mean JunD band intensity +/- standard deviation for immunoblots from three independent experiments. C. qRT-PCR of JunD, MMP-1 and Fra-1 in Scp-2 cells transfected with control or siRNA duplexes for JunD. Mean relative values are +/- standard deviation from three independent experiments. **, $p < 0.005$ for two-tailed t-tests.

Figures & Tables

Gene	Bone (High/Low)	p-Value	Lung (High/Low)	p-Value
MMP1	98.28	0.000241	26.63	0.056189
SPANX(A1/A2/B1/B2/C)	18.23	0.006807	22.04	0.005703
SPANXC	14.59	0.003343	14.92	0.007564
SPANX(B1/B2)	12.82	0.017419	15.82	0.008754
CXCR4	9.33	0.002383	0.14	0.052374
IL11	8.44	0.001599	3.38	0.155692
SRGN	5.75	0.000088	0.99	0.970525

Table 3-1 Top Genes Correlated to Metastatic Potential in Bone.

Gene	Lung (High/Low)	p-Value	Bone (High/Low)	p-Value
SPARC	104.03	0.058864	2.01	0.222907
MMP1	26.63	0.056189	98.28	0.000241
SPANX(A1/A2/B1/B2/C)	22.04	0.005703	18.23	0.006807
SPANXC	14.92	0.007564	14.59	0.003343
SPANX(B1/B2)	15.82	0.008754	12.82	0.017419
KRT81	13.49	0.001711	3.32	0.101475
SOX4	11.39	0.003905	4.67	0.038471

Table 3-2 Top Genes Correlated to Metastatic Potential in Lung.

Tables 3-1 & 3-2

Top five genes with greatest expression differential in genes of high metastatic potential and low metastatic potential in bone and lung. High bone metastatic cell lines are: 1833, Scp-2, Scp-25 and Scp-46. High lung metastatic cell lines are: 1834, 3481, 4142, 4173, 4175, 4180, Scp-3 and Scp-28. Low metastatic cells lines are: MDA-MB-231, Scp-6, Scp-21 and Scp-26. Standard deviations for each were calculated and used in a two-tailed t-test to calculate relative p-value. Genes with p-values over 0.06 were not included.

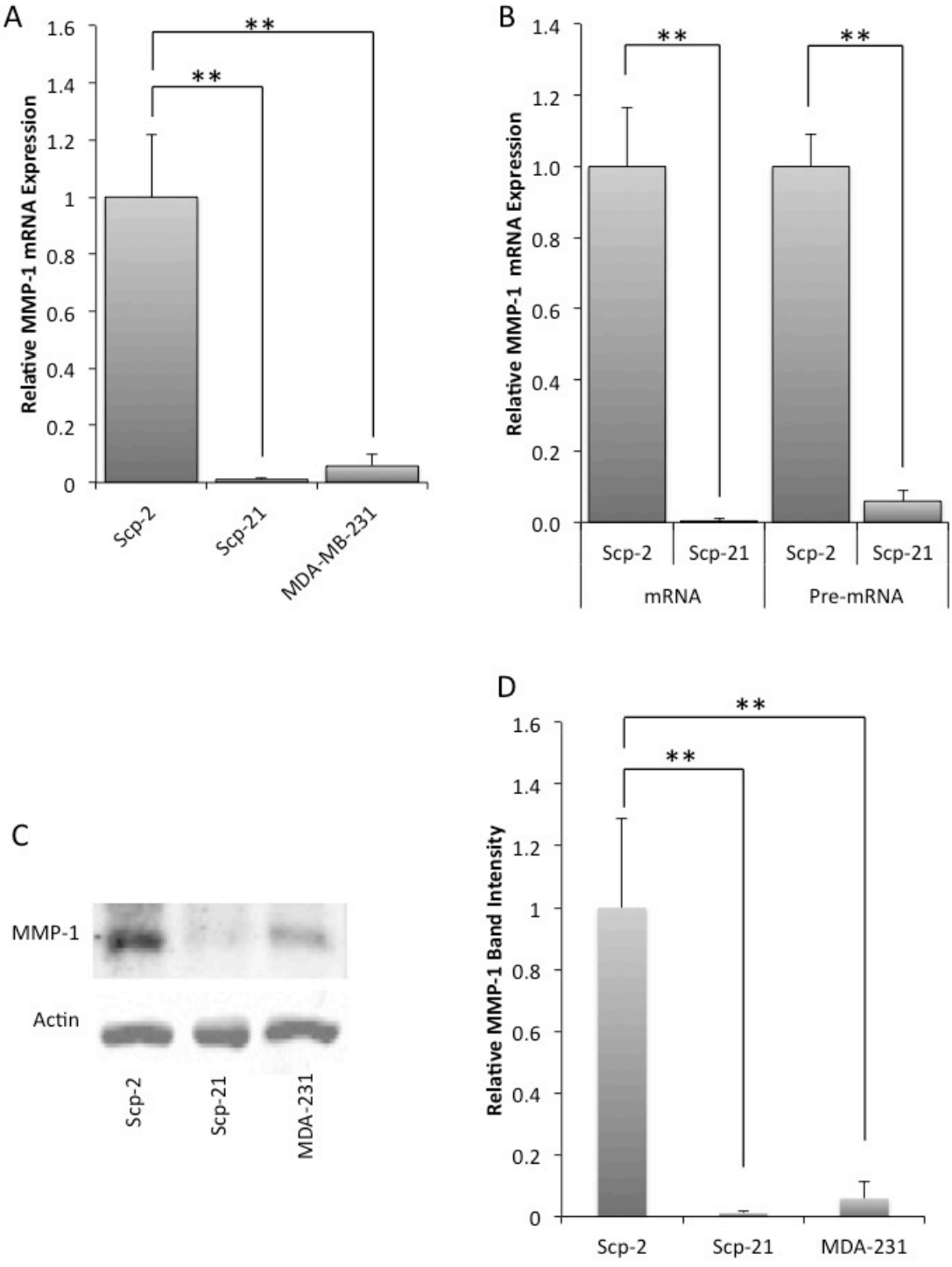

Figure 3–1 Differential expression of MMP-1 in Scp-2, Scp-21 and MDA-MB-231 cells.

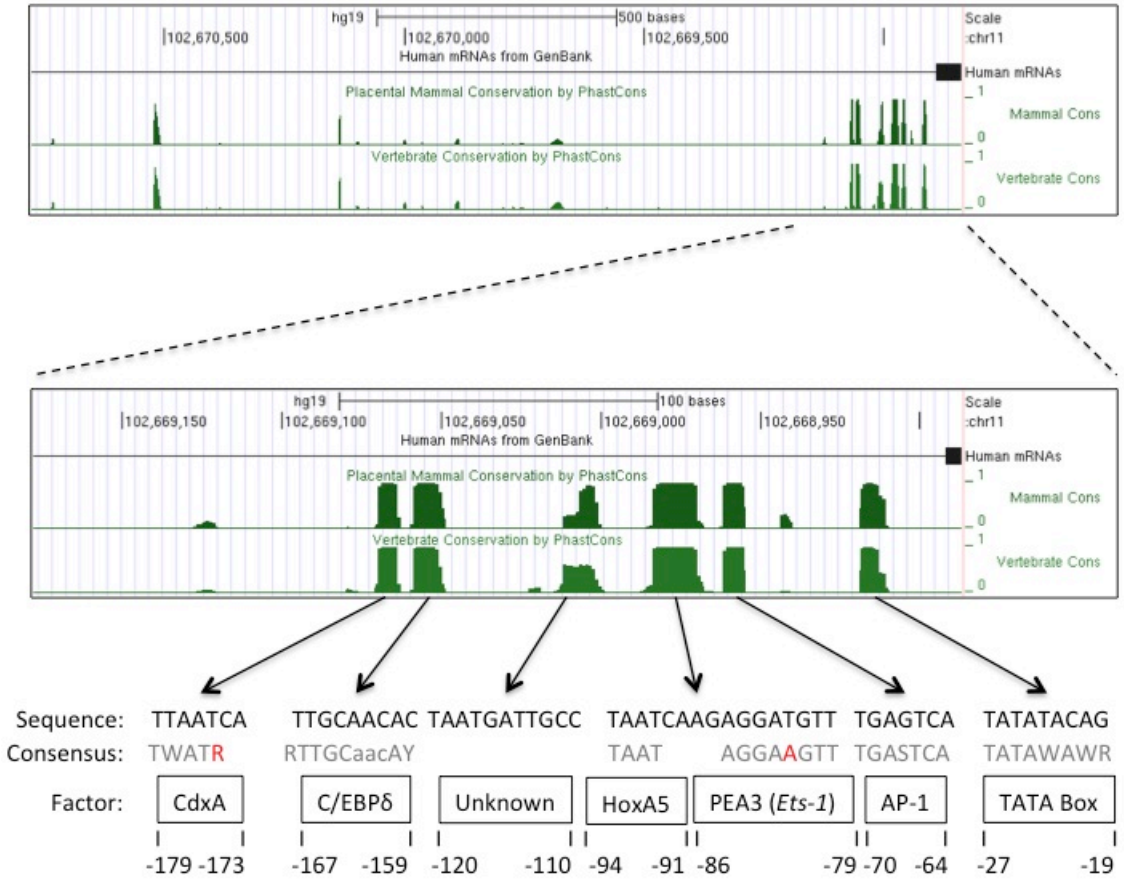

Figure 3–2 Conservation.

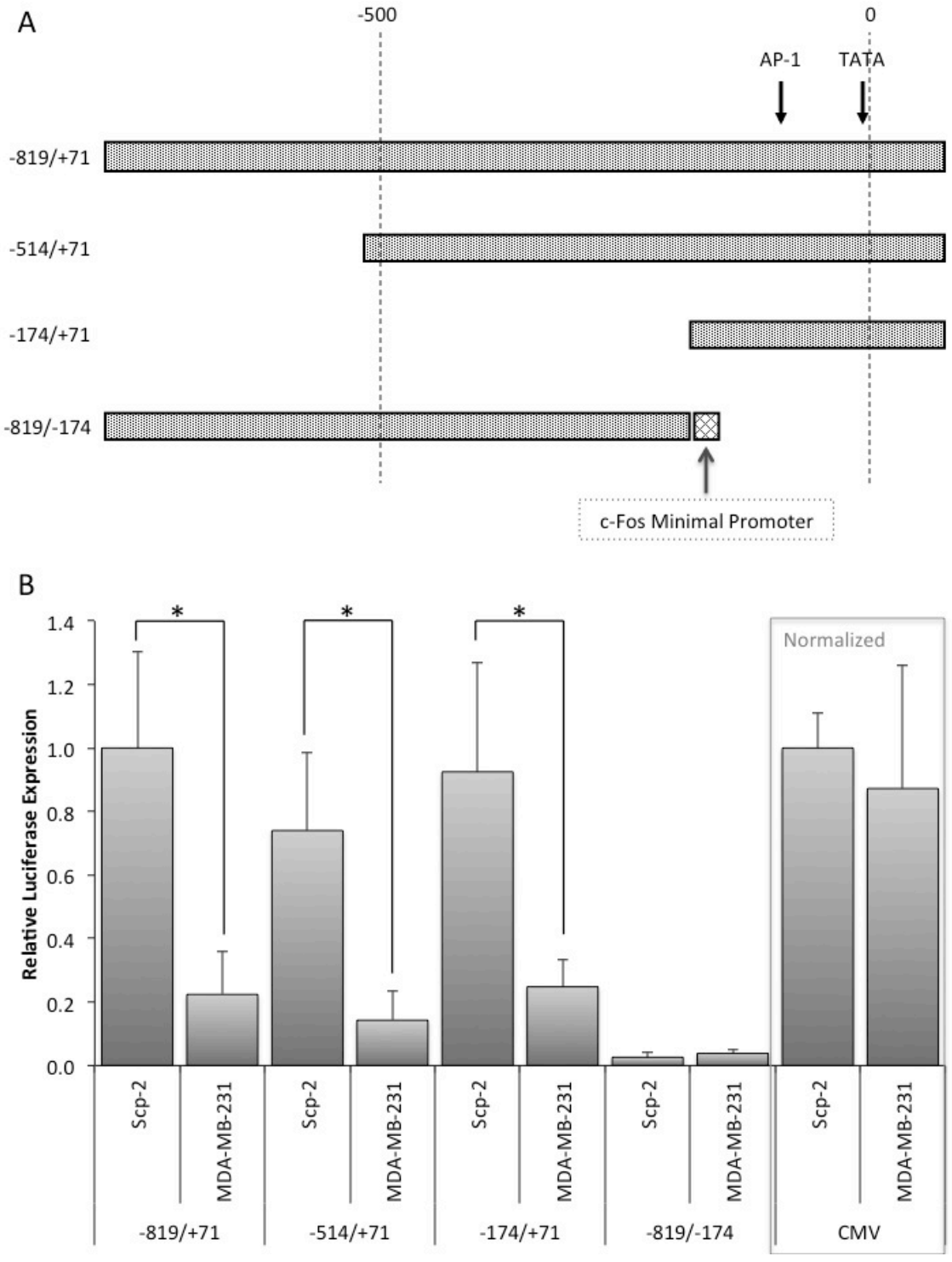

Figure 3–3 The -174/+71 region of the MMP-1 promoter is necessary and sufficient for differential expression in Scp-2 and MDA-MB-231 cells.

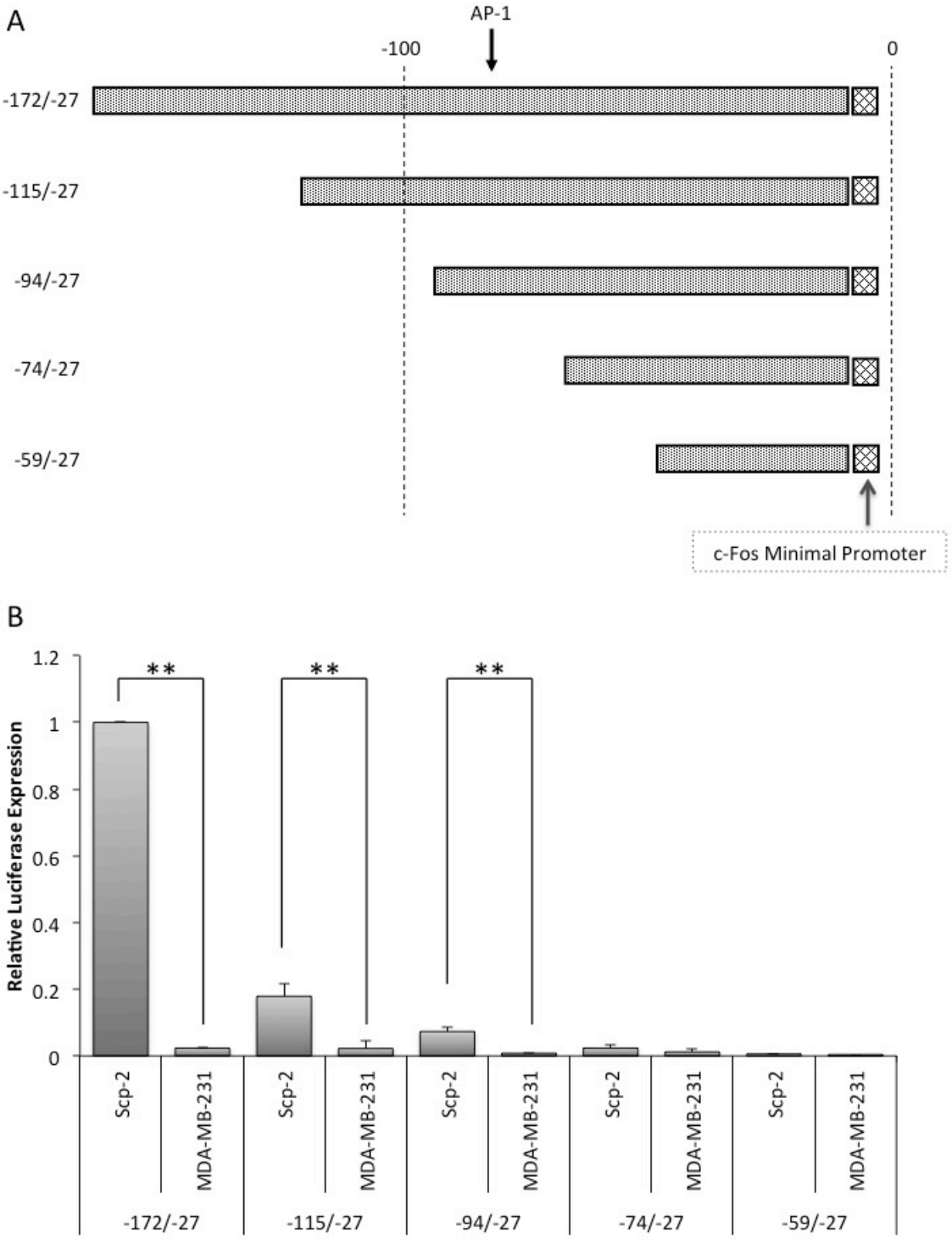

Figure 3–4 The -94/-27 region of the MMP-1 promoter is necessary and sufficient for differential expression in Scp-2 and MDA-MB-231 cells.

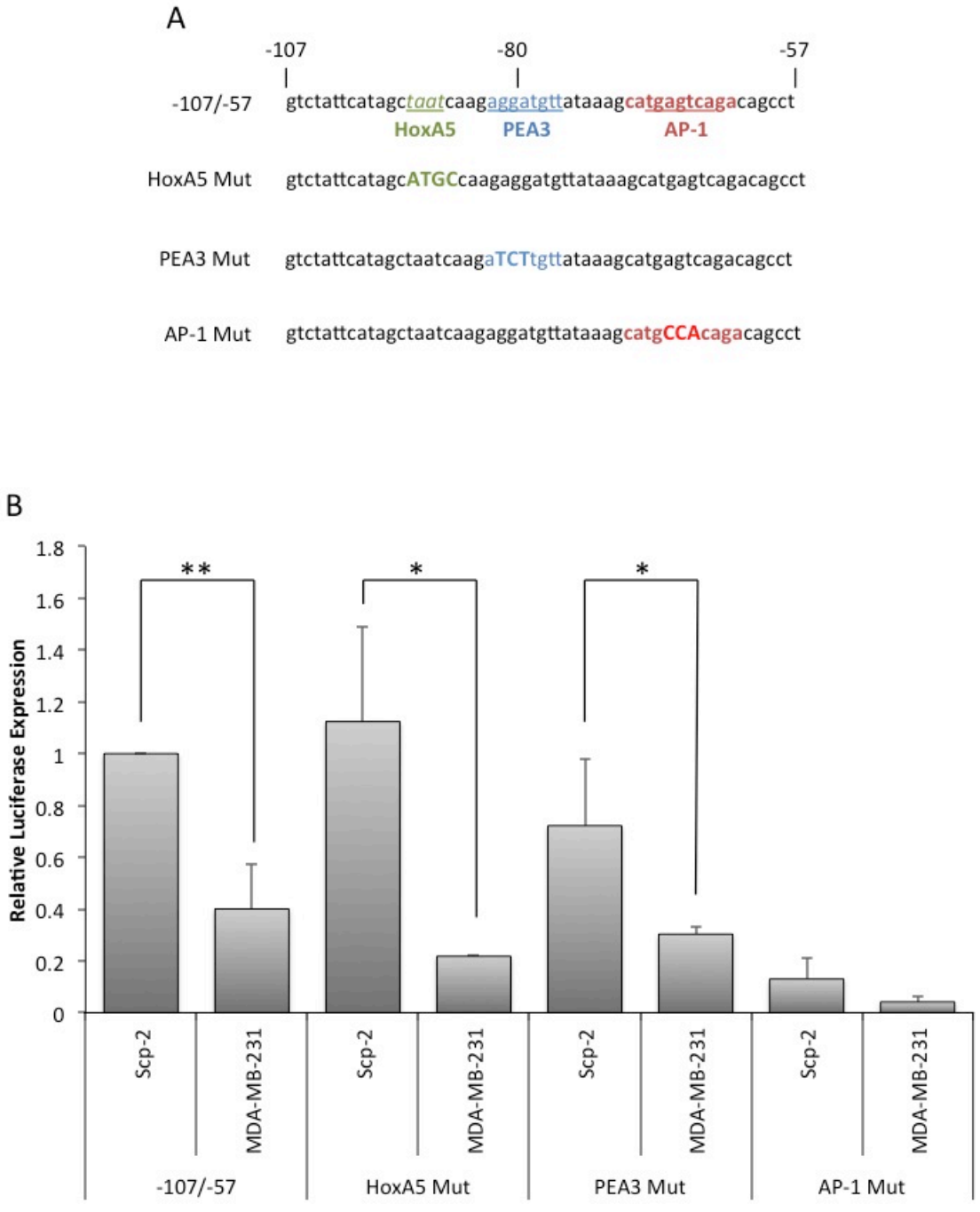

Figure 3–5 The AP-1 site of the MMP-1 promoter is necessary for differential expression in Scp-2 and MDA-MB-231 cells.

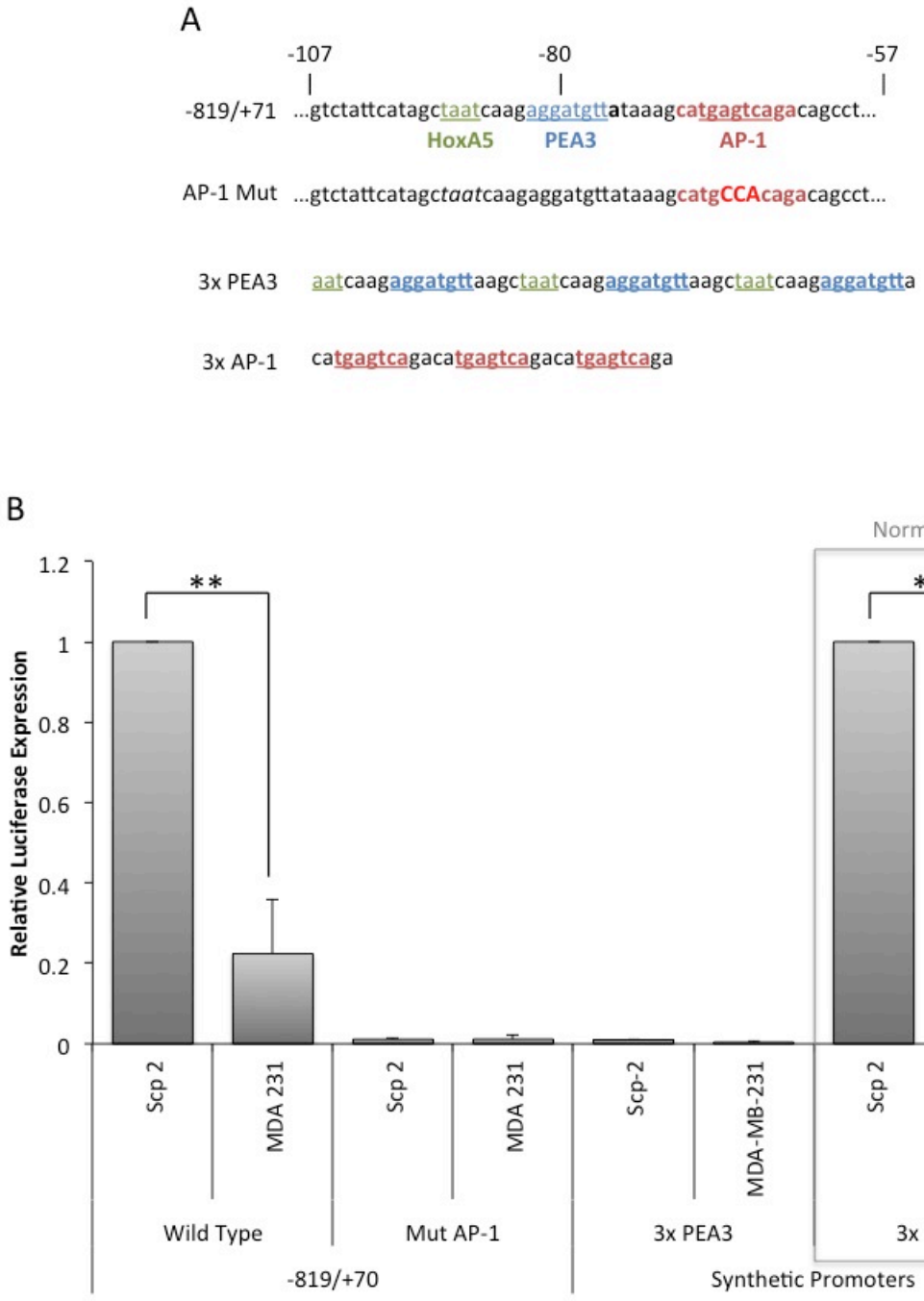

Figure 3–6 The AP-1 site of the MMP-1 promoter is necessary and sufficient for differential expression in Scp-2 and MDA-MB-231 cells.

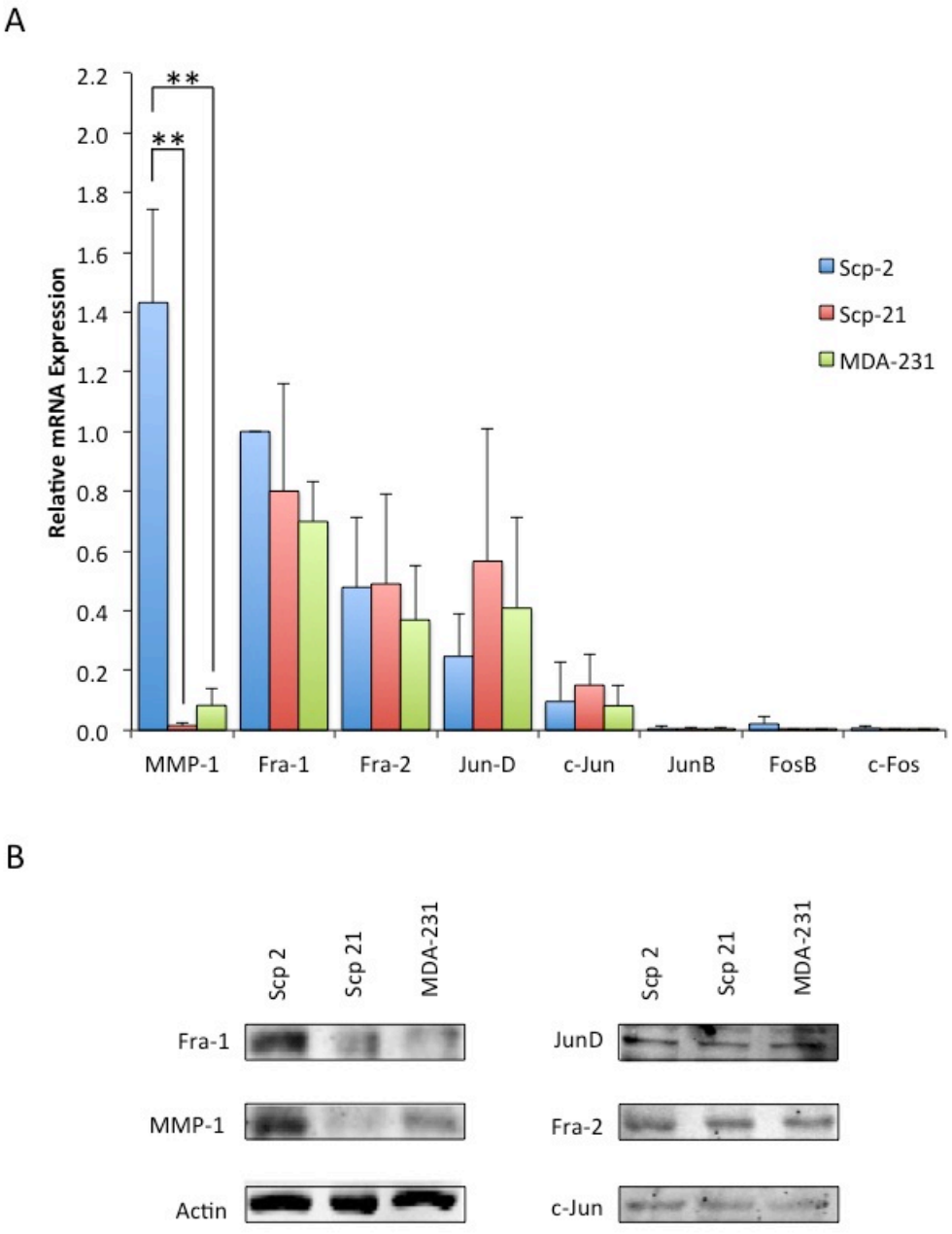

Figure 3–7 AP-1 mRNA and Protein Expression.

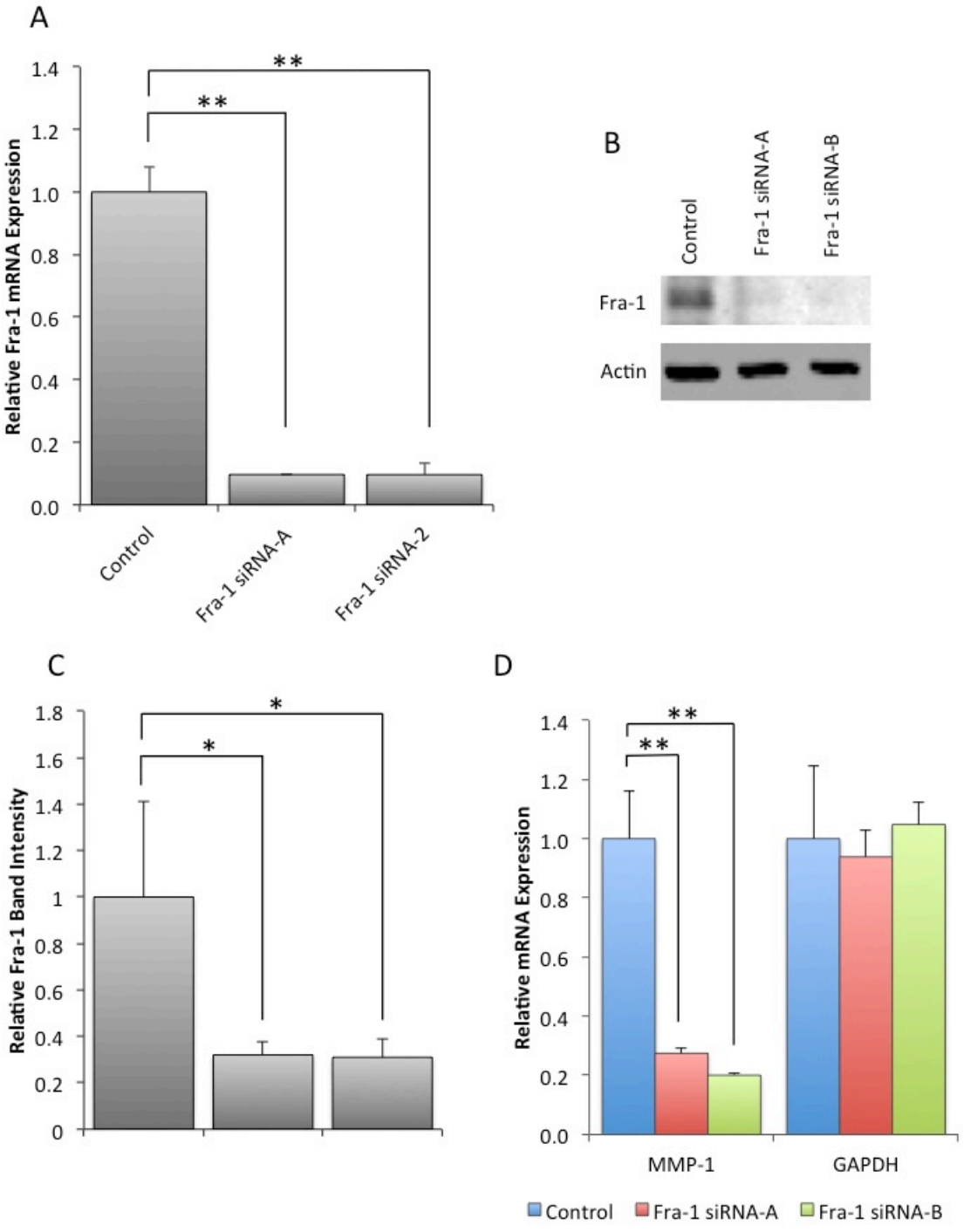

Figure 3–8 Inhibition of Fra-1 decreases MMP-1 Expression.

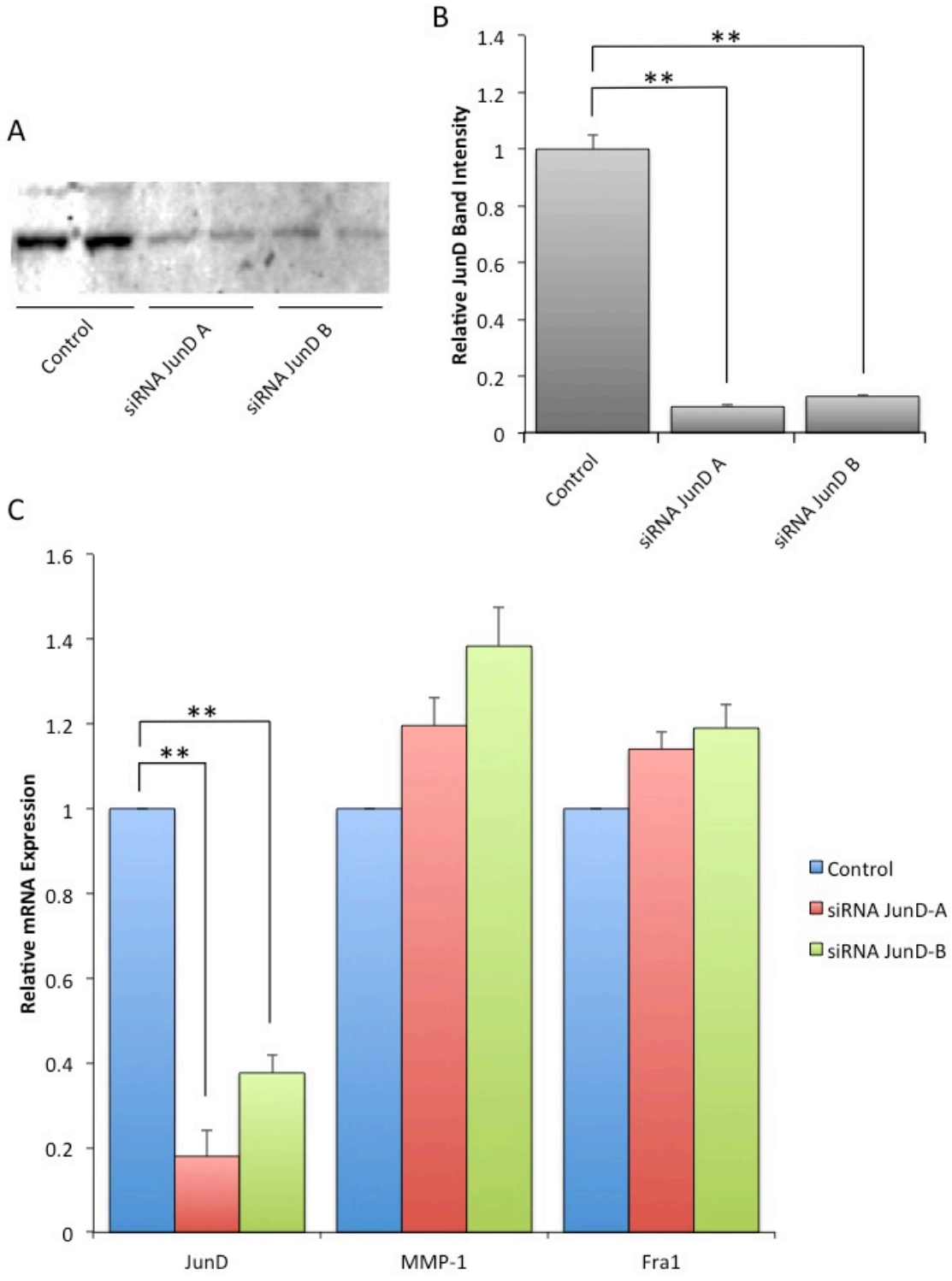

Figure 3–9 Other AP-1 family siRNAs have no effect on MMP-1 expression.

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Chapter 4

Translational Regulation of Fos-Like Protein-1 (Fra-1) Regulates MMP-1 in MDA-MB-231 Cell Line Variants

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Introduction

Matrix metallopeptidase-1 (MMP-1) expression is highly correlated to several forms of cancer (Table 1-2). In breast cancer patients, MMP-1 expression has been correlated to primary tumor progression, metastatic potential, and survival (Cheng et al., 2008; McGowan et al., 2008; Nakopoulou et al., 1999; Poola et al., 2005). In addition, expression in tissue paired from the same patient showed significantly higher MMP-1 expression in tumors than in normal breast tissue (Kohrmann et al., 2009). Further, in glioblastoma, melanoma and breast cancer, higher incidence has been associated with a single nucleotide polymorphism in an Ets-binding site which increases MMP-1 expression (McCready et al., 2005; Rutter et al., 1998).

Outside the clinic, MMP-1 expression has been measured in a variety of breast cancer cell lines. In general, expression is greater in cells with higher metastatic potential (e.g. MDA-MB-231) when compared to cells of low metastatic potential (e.g. MCF-7) (Balduyck et al., 2000; Giambernardi et al., 1998; Kousidou et al., 2004). Data comparing MCF-7 and MDA-MB-231 is similar to results with MDA-MB-231 cell variants discussed in Chapter 3 (Bos et al., 2009; Kang et al., 2003; Minn et al., 2005).

MMP-1 regulation has been well studied in HeLa and other cell culture systems (Brinckerhoff et al., 2002). However, less is known about how MMP-1 is regulated in metastasis. Recent studies have identified several promoter regions and transcription factors that may play a role in MMP-1 regulation. For example, in melanoma cells, Twist binding to the MMP-1 promoter was found to increase expression of MMP-1 (Weiss et al., 2012). In MCF-7 cells, Her2, which is upregulated in 15% – 20% of breast tumors

and associated with poor prognosis, was found to upregulate MMP-1 through the ERK1/2 pathway (Kim et al., 2012).

The AP-1 consensus site is the archetype for tumor associated gene expression. It was discovered in the MMP-1 promoter as being activated by tumor promoting phorbol esters (Angel et al., 1987a; Angel et al., 1987b). Since its discovery, the role of AP-1 in tumorigenesis has been further substantiated (Young et al., 2006). Tissue immunohistochemistry reveals that Fra-1, an AP-1 family member, expression correlates to breast cancer malignancy (Chiappetta et al., 2007; Song et al., 2006). As discussed in the previous chapter, we have shown that Fra-1 is required for MMP-1 expression in metastatic MDA-MB-231 cell line variants. In line with this work, Fra-1 expression has been shown to be higher in MDA-MB-231 cells when compared to less metastatic cells (Zajchowski et al., 2001).

In this study, we show that greater protein expression of Fra-1 in metastatic versus non-metastatic MDA-MB-231 variants correlates with its greater binding to the AP-1 site in the MMP-1 promoter. We also found that Fra-1 regulation is post-transcriptional and independent of the rate of protein degradation, suggesting that Fra-1 is regulated by increased translation in highly metastatic cells. Consistent with Fra-1's importance for tumor growth, we found that Fra-1 overexpression is sufficient to increase cell motility and anchorage independent growth.

Results

Fra-1 in Scp-2 and Scp-21 Nuclear Lysates Binds the MMP-1 AP-1 site In Vitro

Previous experiments supported Fra-1 being required for MMP-1 expression. We confirmed protein binding of the MMP-1 AP-1 site in the highly metastatic Scp-2 and non-metastatic Scp-21 cells using electrophoretic mobility shift assays (EMSA). The -107 to -57 region of the MMP-1 promoter, containing the AP-1 consensus sequence, was used as a probe for binding with nuclear extracts from Scp-2 and Scp-21 cells. Specific binding was observed which was competed by excess non-labeled competitor (Figure 4-1). Mutations in the AP-1 binding site abolished this competition, suggesting that the band is indeed AP-1.

Interestingly, a stronger AP-1 complex was detected in the highly metastatic Scp-2 cells compared with the low metastatic Scp-21 cells (Figure 4-1, compare lanes 1 and 2, and 5 and 6). This is consistent with higher Fra-1 protein expression in Scp-2 cells and higher expression of MMP1 (Chapter 3).

To determine which proteins in the nuclear extracts were present in the bound band, we used antibodies specific for AP-1 family members. Anti-Fra-1 antibody supershifted the band (Figure 4-1, lane 7), indicating that Fra-1 is a major component of the bound complex. In contrast, Fra-2 antibodies had little effect (lane 8). Antibodies to c-Jun strongly shifted the complex into multiple bands, suggesting that it too is in the complex. We did not observe a shift with antibodies to JunD, however the antibodies may be ineffective for supershifts (data not shown). These EMSA experiments support the conclusion that Fra-1 and c-Jun are the predominant members of the AP-1 complex bound to the MMP-1 site.

Fra-1 Binds the AP-1 Site of the MMP-1 Promoter in Scp-2 and Scp-21 Cells

To confirm Fra-1 binding and regulation of the MMP-1 promoter *in vivo*, we performed chromatin immunoprecipitation (ChIP) in Scp-2 and Scp-21 cells. Antibodies to Fra-1 demonstrated higher binding to the MMP-1 promoter in Scp-2 cells than Scp-21, consistent with the relative MMP-1 expression in these cells (Figure 4-2). A similar, though slightly weaker, difference was seen at a known AP-1 binding site in the IL-6 gene. Background signal was seen at distal control sites in the MMP-1 and IL-6 genes or with a non-specific control antibody.

Fra-1 Regulation is Translational

Since Fra-1 is required for MMP-1 expression and binds preferentially to the MMP-1 promoter in Scp-2 cells, we analyzed Fra-1 regulation. As shown Chapter 3, and again in Figure 4-3, Fra-1 mRNA levels did not vary significantly among the metastatic variant cell lines, while Fra-1 protein levels were higher in Scp-2 cells. To better understand the post-transcriptional regulation of Fra-1, we analyzed Fra-1 protein degradation and translation.

We first measured the degradation rate using the protein synthesis inhibitor cycloheximide. By measuring protein levels over time, without de-novo translation, we could compare degradation rates of Fra-1 in Scp-2 and Scp-21 cells. We found that Fra-1 protein was more abundant in Scp-2 than Scp-21 cells, as previously seen (Figure 4-4A). However, over 6 to 24 hours, when normalized for initial protein levels, the degradation rates of Fra-1 showed no significant difference in Scp-2 and Scp-21 cells (Figure 4-4B).

Comparisons at shorter time points also showed no significant difference (Figure 4-4 A & B).

With no difference in Fra-1 degradation, we focused on measuring Fra-1 translation rates. Scp-2 and Scp-21 cells were labeled with ^{35}S-labeled amino acids to measure amino acid incorporation into proteins over a one-hour interval. Fra-1 protein was purified by immunoprecipitation, separated by SDS-PAGE and visualized by autoradiography. Interestingly, more Fra-1 was labeled over a one-hour interval in Scp-2 cells than Scp-21 cells, suggesting that the rate of Fra-1 translation is higher in Scp-2 than Scp-21 cells (Figures 4-5A and B). There was no significant difference in general protein synthesis (Appendix 3).

Stable Expression of Fra-1 in Scp-21 Cells Increases MMP-1 Expression

To determine the effect of Fra-1 expression in non-metastatic cells, we created Scp-21 cells that stably express Fra-1. Control Scp-21 cells that stably express a control vector, have low Fra-1 protein expression, while the cells infected with a Fra-1 retrovirus expressed high levels, several fold higher than that in Scp-2 cells (Figure 4-6). We also found that MMP1 expression was elevated in the Fra-1 expressing cells, suggesting that higher levels of Fra-1 are sufficient for increased MMP1 expression (Figure 4-6).

Stable expression of Fra-1 increases motility and anchorage independent growth.

Since increased Fra-1 protein expression correlated with increased MMP-1 expression and metastasis, we tested whether it is sufficient to drive properties of metastatic cells, in particular cell motility and anchorage independent growth. A scratch

assay, or wound healing assay, can be used to measure cell motility. We found that Scp-2 had greater motility than Scp-21 cells, and Scp-21 cells expressing Fra-1 had significantly greater motility than vector control Scp-21 cells (Figure 4-7). Surprisingly, Scp-21 cells expressing Fra-1 had even greater motility than highly metastatic Scp-2 cells. Therefore, Fra-1 expression increases motility.

Non-metastatic cells are unable to grow in soft agar, while metastatic cells often display anchorage independent growth (Hamburger et al., 1977; Yoneda et al., 2001). Similar to the pattern seen in motility assays, Scp-2 showed significantly greater potential to grow in soft agar than Scp-21 cells (Figure 4-8, Appendix 2). Interestingly, Scp-21 cells expressing Fra-1 greatly increased the growth of the cells in soft agar, similar to the Scp-2 levels. These results indicate that increased Fra-1 expression in Scp-21 cells was sufficient to gain anchorage independent growth.

Discussion

MMP-1 and Fra-1 Expression Correlate to Metastatic Potential

MMP-1 expression is strongly elevated in a series of breast carcinoma cell variants with high metastatic potential compared to variants with low metastatic potential (Bos et al., 2009; Kang et al., 2003; Minn et al., 2005 and Chapter 3). This is consistent with studies showing elevated MMP-1 expression in metastatic breast carcinomas (as reviewed in Table 1-2). In addition, several studies have found higher MMP-1 expression in estrogen receptor (ER) negative cell lines with high metastatic potential, such as MDA-MB-231, compared to cell lines with lower metastatic potential (Balduyck et al., 2000; Giambernardi et al., 1998; Kousidou et al., 2004). The MDA-MB-231 variants we describe here have the advantage that they are more closely controlled, having been derived from a single cell population, compared to disparate cell lines from different people.

Our analysis of the MMP-1 promoter has revealed that its expression is regulated by an AP-1 site in MDA-MB-231 cells and requires the AP-1 family member Fra-1. We did not detect altered Fra-1 mRNA expression in the MDA-MB-231 cell variants, however higher mRNA expression has been observed in more metastatic ER negative cell lines when compared to less metastatic cells (Belguise et al., 2005; Philips et al., 1998). Differences in Fra-1 expression were also observed in breast cancer patients, where expression was higher in carcinomas compared with benign tumors (Chiappetta et al., 2007).

Translational Regulation of Fra-1 Regulates MMP-1

While Fra-1 mRNA levels were not significantly regulated in the MDA-MB-231 cell variants, immunoblots, EMSAs and chromatin immunoprecipitations show that the metastatic variant Scp-2 has higher Fra-1 protein expression and higher DNA binding *in vitro* and *in vivo* to the MMP-1 AP-1 site. As Fra-1 was the only AP-1 family factor that varied in the metastatic variants, we propose that it is responsible for the difference in MMP-1 expression. The EMSAs show clearly that Fra-1 is the predominant factor in the AP-1 complex in these cells, while c-Jun also appears to be in the complex. We could not detect JunD, but this may be due to limitations of the antibodies.

We found that Fra-1 protein levels were regulated by altered translation rates. There were little differences in the rates of protein degradation. However, short metabolic labeling showed increased synthesis of Fra-1 in the metastatic cell variant. Several studies have demonstrated that phosphorylation of Fra-1 by ERK1/2 increases its protein stability (Basbous et al., 2007; Casalino et al., 2003; Gruda et al., 1994; Vial et al., 2003a). However, this mechanism does not appear to be functioning in the MDA-MB-231 cells, since we did not detect a change in degradation rates.

Recently, data from human cancer cell lines pointed to evidence of miRNA-34a regulation Fra-1 and MMP-1 (Wu et al., 2012; Yang et al., 2012). In breast cancers, miRNA-34a was inversely correlated to the metastatic potential of cell lines and tumor samples, but was not found to be different in paired tumor and normal breast tissue samples (Yang et al., 2012). Strikingly, expression of miRNA-34a in MDA-MB-231 cells reduced Fra-1 expression, matrigel invasion, and tumors in mouse xenografts. Further, coexpression of Fra-1 and miRNA-34a in MDA-MB-231 cells rescued migration

and invasion (Yang et al., 2012). While miRNA-34a regulation of Fra-1 is a strong hypothesis for MMP-1 regulation in MDA-MB-231 variants, a major difference is that we did not observe changes in Fra-1 mRNA expression as reported with miRNA-34a (Wu et al., 2012; Yang et al., 2012). Separately, miRNA-143 was also found to target Fra-1 mRNA, suggesting that this and other miRNAs are also candidates for Fra-1 regulation (Horita et al. 2012).

Despite initial work supporting miRNA translational regulation without impact on mRNA levels (Olsen et al., 1999), more recent evidence supports miRNA regulation of both protein and mRNA expression (Eulalio et al., 2008; Filipowicz et al., 2008). As our experiments only show translational regulation, it is possible that Fra-1 is a case where miRNA regulation is entirely translational. There are several examples of other genes being regulated by miRNA without discernable differences in mRNA levels (Bhattacharyya et al., 2006; Cimmino et al., 2005; Fazi et al., 2005; Poy et al., 2004). Alternatively, it is possible that Fra-1 translation is regulated by a mechanism other than miRNA.

Stable Expression of Fra-1 in Non-Metastatic Cells

Stable expression of Fra-1 in non-metastatic MDA-MB-231 derivative cells led to greater MMP-1 expression, motility and anchorage-independent growth. This supports Fra-1 as an upstream regulator of MMP-1 and potentially of other genes required for increased metastatic properties. These results are in line with previous colon cancer data correlating Fra-1 expression with escape from anoikis (Vial et al., 2003a) and motility (Vial et al., 2003b). In spontaneous murine mammary adenocarcima variants with

different metastatic potential, Fra-1 expression also correlated to invasiveness (Kustikova et al., 1998). Transient transfections of Fra-1 in MDA-MB-231 and MCF-7 similarly increased matrigel cell invasion (Milde-Langosch, 2005). Contrary to our findings, this overexpression had no impact on MMP-1 expression. In other experiments, however, overexpression of Fra-1 in MCF-7 cells increased cell invasion and MMP-1 expression (Belguise et al., 2005)

Potential functions of Fra-1 or MMP-1 in Invasion and Migration

Fra-1 has many direct and indirect targets (Chinenov et al., 2001). Fra-1 depletion in a highly metastatic MDA-MB-231 variant line altered the expression of 1,234 genes (Desmet et al., 2013). Among these, E-cadherin has an inverse correlation with Fra-1, confirming previous results (Zajchowski et al., 2001). Fra-1 expression has also been previously shown to alter morphology and invasiveness (Kustikova et al., 1998) in a manner similar to the epithelial to mesenchymal transition (EMT). As such, Fra-1 regulation may function as a keystone regulator, impacting several aspects of tumorigenesis and metastasis (Fleischmann et al., 2000).

How MMP-1 function is coopted by tumor cells is an open question. MMP-1 is critical in degrading interstitial collagen, and tumor cells may require that function to invade (Brinckerhoff et al., 2002; Page-McCaw et al., 2007). However, MMP-1 function is also more diverse and complex. MMP-1 has been shown to be required for migration and xenograft tumor formation by MDA-MB-231 cells through cleavage and activation of protein activated receptor-1 (PAR-1) (Boire et al., 2005). Previously, PAR-1 expression was found to be greater in MDA-MB-231 than MCF-7 cells (Kamath et al.,

2001). Interestingly, MMP-1 expression was found to be insufficient in MDA-MB-231 conditioned media to promote cell migration (Boire et al., 2005). It is possible that the higher MMP-1 expression in highly metastatic MDA-MB-231 variants is high enough to induce and activate PAR-1 and subsequent signaling pathways.

In summary, we have shown that MMP-1 regulation by Fra-1 is greatly enhanced in highly metastatic variants of MDA-MB-231 cells. This is mediated by greater translation of Fra-1which results in its higher occupancy of the AP-1 site in the MMP-1 promoter. Increased Fra-1 expression in non-metastatic cells increased cell motility and anchorage independent growth, suggesting these cells would be metastatic.

Figure Legends

Figure 4-1

Fra-1 binds the AP-1 promoter site of MMP-1 *in vitro*. Electrophoretic mobility shift assay with the MMP-1 AP-1 site. Scp-2 and Scp-21 nuclear extracts were bound to a ^{32}P-end labeled MMP-1 promoter double-stranded oligonucleotide probe spanning the AP-1 consensus site (-107 to -57 bases relative to the transcription start site). Nonspecific competitor, unlabeled, and point mutant AP-1 site oligonucleotides were added in 250 fold excess of the probe. The final three lanes included anti-AP-1 family member antibodies.

Figure 4-2

Fra-1 binding to the MMP-1 promoter is greater in Scp-2 than Scp-21 cells. Chromatin immunoprecipitation with Scp-2 and Scp-21 cells immunoprecipated with anti-Fra-1 antibody or mock antibody control. The immunoprecipitated DNA from the samples was measured by qRT-PCR for binding of Fra-1 to the MMP-1 AP-1 promoter sites, an upstream non-AP-1 control MMP-1 site, the IL-6 AP-1 site, or an upstream non-AP-1 control IL-6 site. The data shown represent the mean fold over control DNA values +/- standard deviation for three indendent experiments. **, $p < 0.005$ for two-tailed t-tests.

Figure 4-3

Fra-1 protein levels are higher in Scp-2 than in Scp-21 cells while mRNA expression is unchanged. A. Quantitative RT-PCR of Fra-1 in Scp-2, Scp-21 and MDA-231 cells. Data represents the mean +/- the standard deviation of three independent experiments. B. Scp-2, Scp-21 and MDA-MB-231 cell lysates were immunobloted with anti-Fra-1 antibody. Anti-actin immunoblotting is included as a loading control. C. The mean relative Fra-1 band intensity +/- standard deviation for three independent immunoblotting experiments. **, $p < 0.005$ for two-tailed t-tests.

Figure 4-4

The degradation rates of Fra-1 is similar in Scp-2 and Scp-21 cells. Scp-2 and Scp-21 cells were treated with cycloheximide and whole cell lysates collected at the indicated times post-treatment. A. Immunoblot with anti-Fra-1 of a representative experiment. B. The mean relative Fra-1 band intensity +/- standard deviation for three independent experiments.

Figure 4-5

Translation of Fra-1 protein is greater in Scp-2 than Scp-21 cells. A. ^{35}S-metabolic labeling of Fra-1. Cells were depleted of cysteine and methionine for 30 minutes and labeled with ^{35}S-cysteine and -methionine for the indicated times and immunoprecipitated. B. Fra-1 protein levels were quantitated and normalized to total protein labeling. Data represents the mean band intensity +/- standard deviation for three independent experiments. **, $p < 5x10^{-5}$ for two-tailed t-tests.

Figure 4-6

Stable HA-tagged Fra-1 expression in Scp-21 cells. A. qRT-PCR of MMP-1 in Scp-2, Scp-21, Scp-21 control vector (Puro), and Scp-21 stably expressing Fra-1 Mean relative values are +/- standard deviation from three independent experiments. **, $p < 0.005$ for two-tailed t-tests. B. Scp-2, Scp-21, Scp-21 control vector (Puro), and Scp-21 stably expressing Fra-1 cells were immunoblotted with anti-MMP-1, anti-Fra-1 and anti-HA antibodies. Anti-HSP-90 served as a loading control.

Figure 4-7

Fra-1 expression increases cell motility of the non-metastatic Scp-21 cells. A. Scratch-wound assay of Scp-2, Scp-21 cells and Scp-21 cells stably expressing vector (Puro) or Fra-1 was performed with motility measured 0 and 18 hours after the scratch. Cells were grown to 95% confluency and scratched with a pipette tip. B. Quantitation of the number of cells crossing the initial scratch threshold at 18 hours. Data represents the mean of three fields in three independent experiments +/- standard deviation. *, $p < 0.05$. **, $p < 0.005$ for two-tailed t-tests.

Figure 4-8

Fra-1 expression increases anchorage independent growth in non-metastatic Scp-21 cells. Scp-2, Scp-21, and Scp-21 cells stably expressing Fra-1 or vector (puro) were grown in soft agar for 21 days. Data represent the mean +/- standard deviation of the relative number of cells which formed colonies in three independent experiments. *, $p < 0.05$. **, $p < 0.005$ for two-tailed t-tests.

Figures

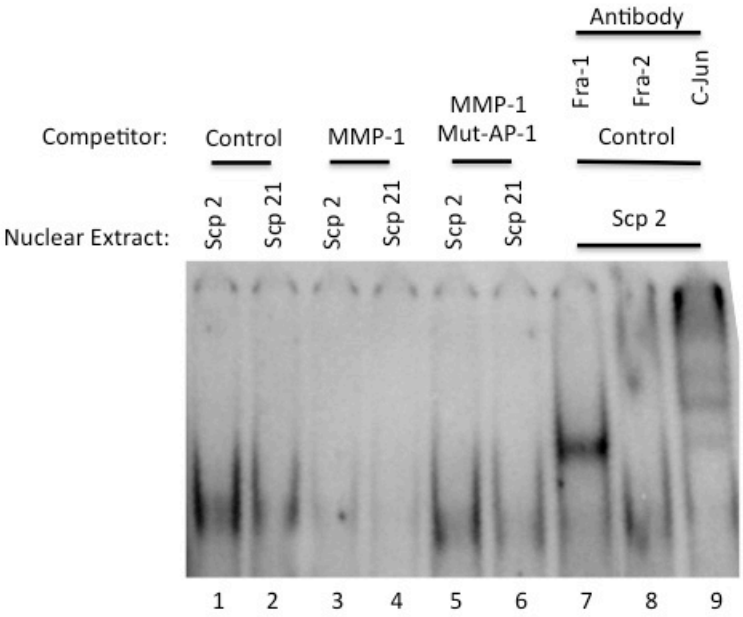

Figure 4–1 Fra-1 binds the AP-1 promoter site of MMP-1.

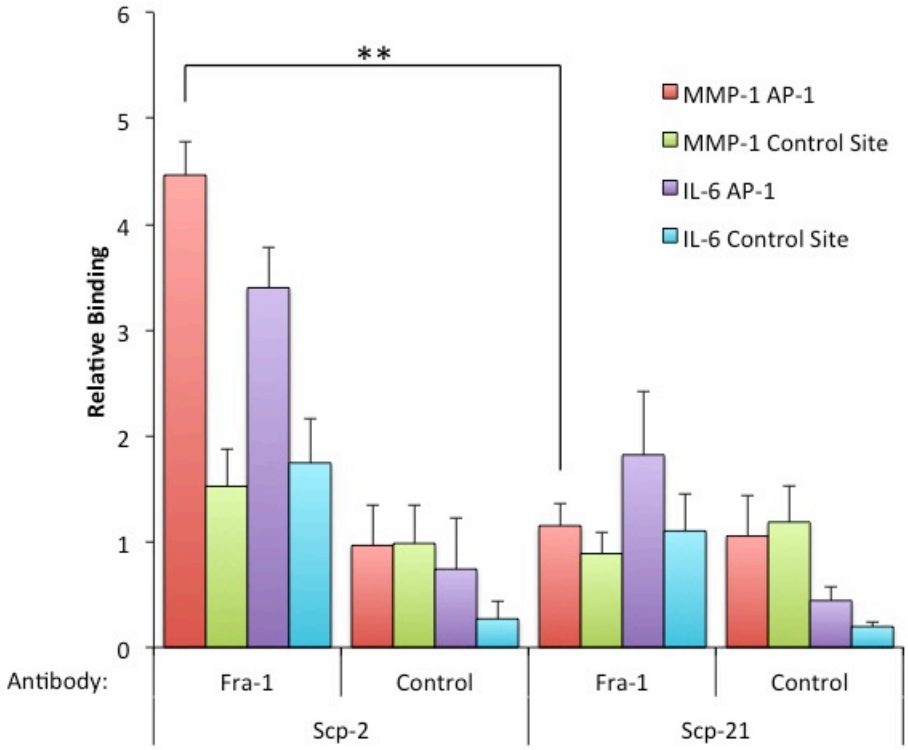

Figure 4–2 Fra-1 binding to the MMP-1 promoter is greater in Scp-2 than Scp-21 cells.

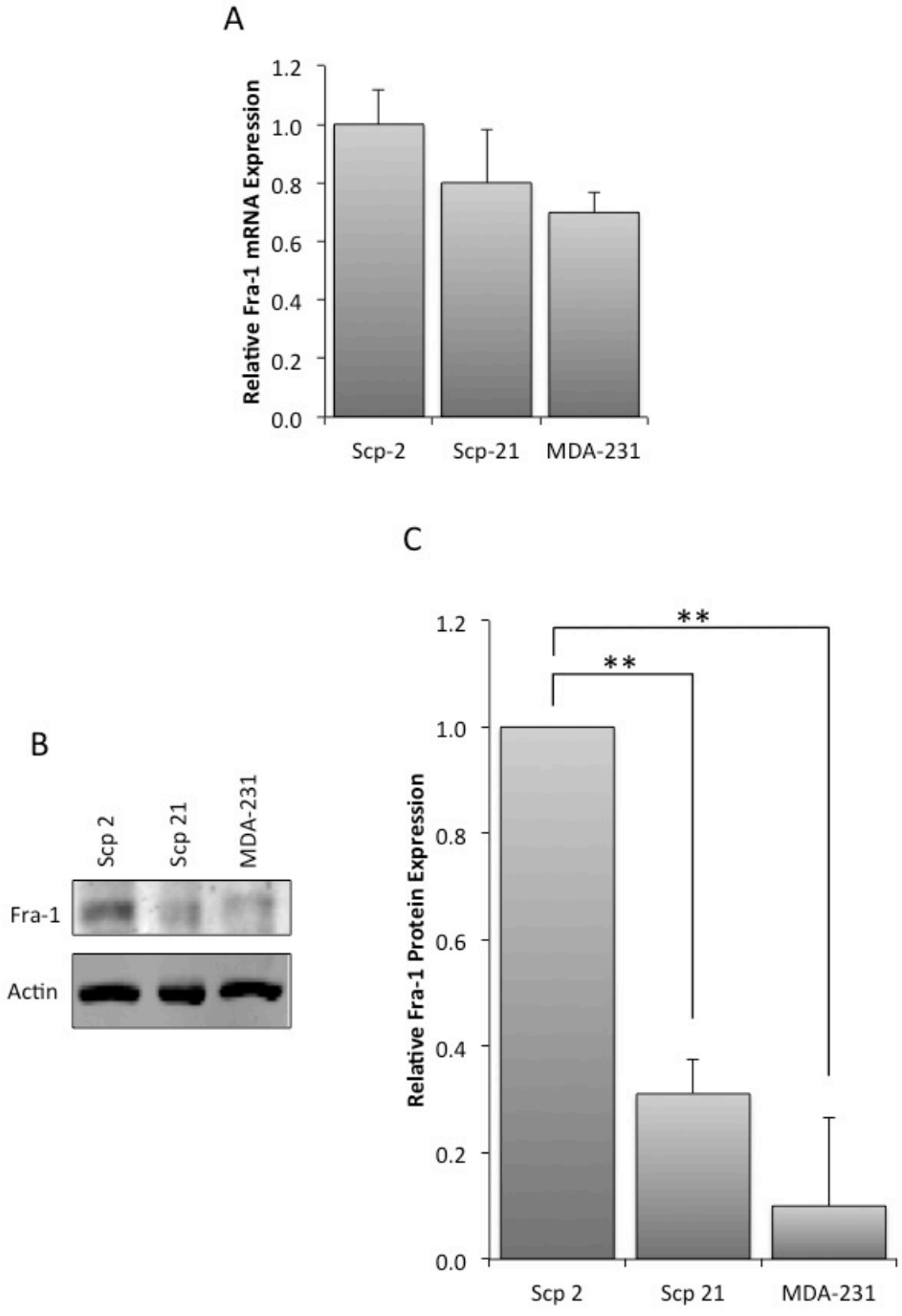

Figure 4–3 Fra-1 protein levels are higher in Scp-2 than in Scp-21 cells while mRNA expression is similar.

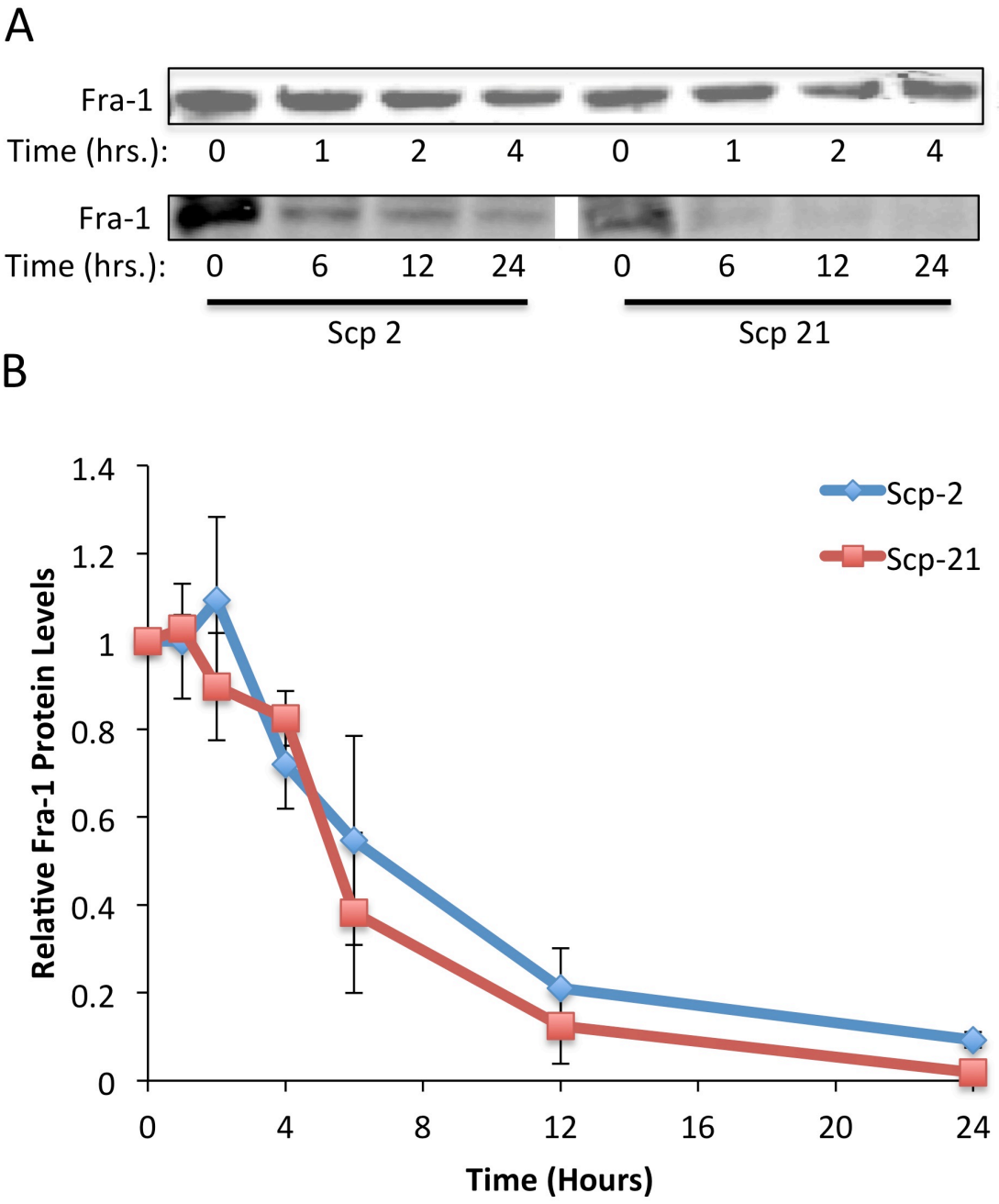

Figure 4–4 Degradation of Fra-1 is similar in Scp-2 and Scp-21.

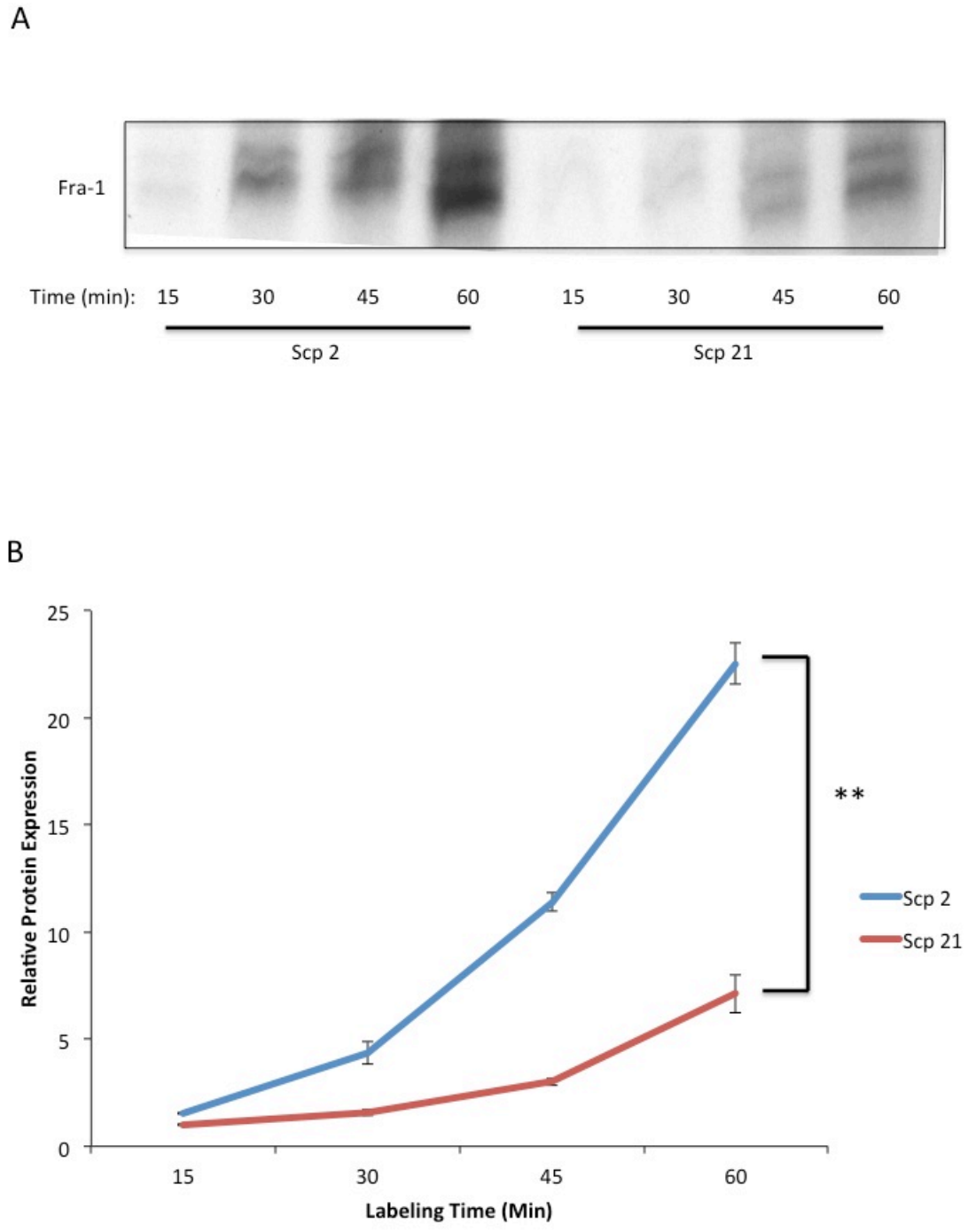

Figure 4–5 Translation of Fra-1 protein is greater in Scp-2 than Scp-21 cells.

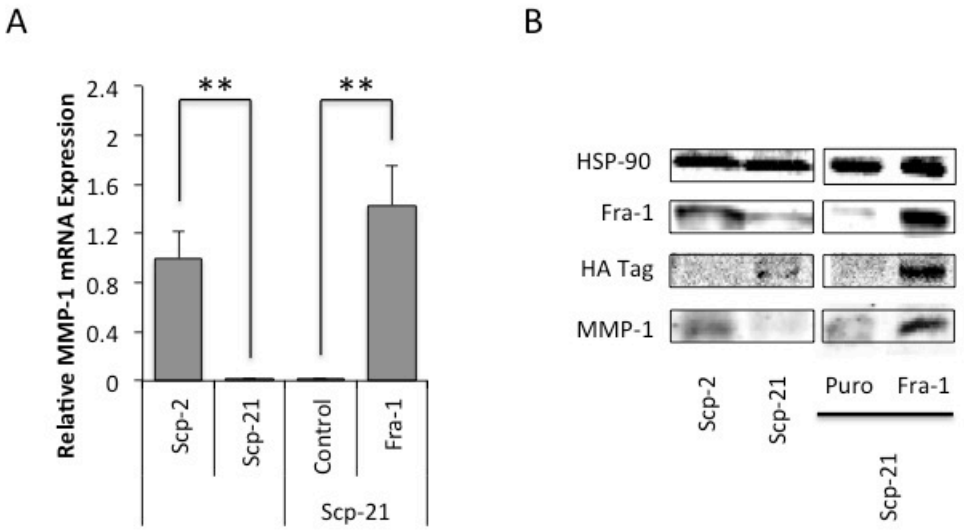

Figure 4–6 Stable HA-tagged Fra-1 expression in Scp-21 cells.

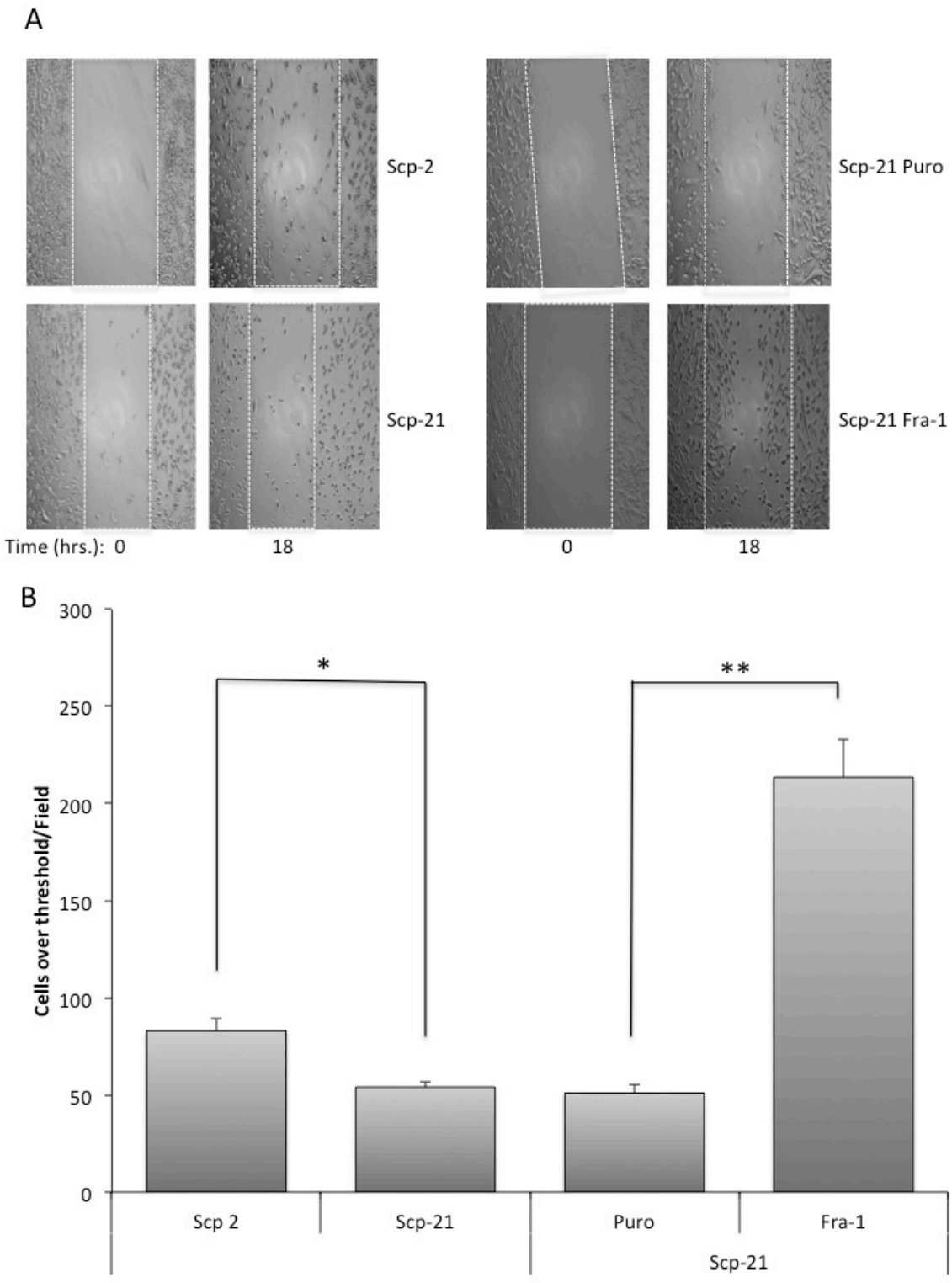

Figure 4–7 Fra-1 expression increases non-metastatic cell line motility.

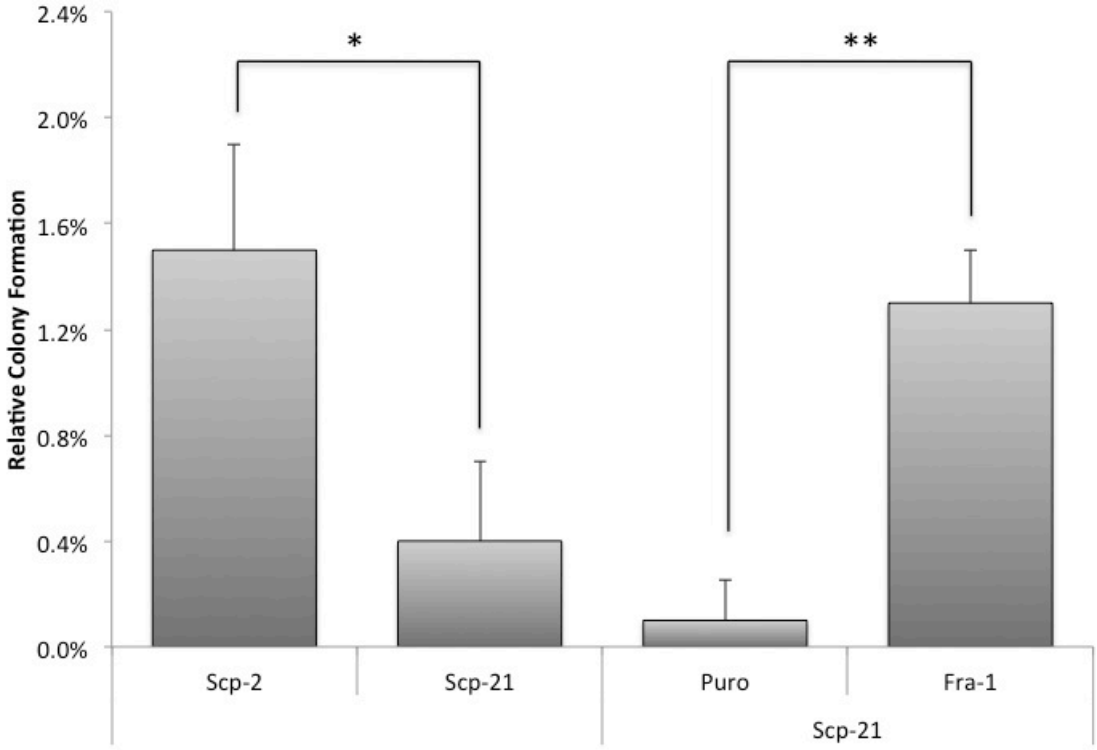

Figure 4–8 Fra-1 expression increases anchorage independent growth in non-metastatic cells

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Chapter 5

Conclusions

MMP-1 Upregulation is Common among Organ Specific Metastases

Evidence from our experiments have shown that Fra-1 regulation of MMP-1 is highly correlated to metastatic potential. Since MMP-1 expression has been linked to bone formation and osteosclerosis (Jochum et al., 2000), it might have been expected that Fra-1 regulation of MMP-1 is specific to bone metastases. However, in mouse xenograft models MMP-1 is highly correlated to the metastatic potential of cell lines regardless of the metastatic organ site (Bos et al., 2009; Kang et al., 2003; Minn et al., 2005). Since very few genes are implicated across organ metastases, MMP-1 expression is likely to be important to metastasis independent of organ site.

Two hypotheses can be drawn from the expression of MMP-1 in different organ metastases. First, MMP-1 is critical for the early stages of metastasis that are common to all metatastic tumors, independent of organ site. For example, primary tumor invasion, intravasation, or survival in the circulation.

An alternative hypothesis is that MMP-1 is required for a common mechanism in late stage metastatic progression in different organs. For example, in various foreign tissue, MMP-1 expression may allow cells to arrest in the vasculature, extravasate, survive in new parenchyma, or overcome dormancy and proliferate.

MMP-1 functions in primary tumor growth and angiogenesis in xenografts of highly metastatic variants of MDA-MB-231 (Gupta et al., 2007). Simultaneous inhibition of four genes—EREG, Cox2, MMP-1 and MMP-2—was sufficient to reduce tumor size and vascularization. Inhibition of MMP-1 alone was unable to reduce the

metastatic potential of these highly metastatic cells. However, the 30% residual MMP-1 expression in the highly metastatic cell lines was twenty-fold higher than normal MDA-MB-231 cell expression. Therefore, the residual MMP-1 may have been sufficient for elevated metastasis (Gupta et al., 2007). Later work demonstrated that more complete inhibition of MMP-1 expression was sufficient to reduce the number of bone metastases after intracardiac injection of highly bone metastatic MDA-MB-231 variants (Lu et al., 2009).

MMP-1 might function after tumor cells have arrested in various organs, but serve some purpose common to brain, lung and bone metastatic mammary tumors. For example, we could speculate that MMP-1 expression might foster interactions with foreign stroma. Several studies have shown that upon reaching secondary organs, disseminated tumor cells become dormant (Aguirre-Ghiso, 2007). Preliminary evidence points to the possibility of MMP-1 degradation of the extracellular matrix (ECM) releasing growth factors (Mazor et al., 2013). Fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) have been shown to have a strong angiogenic response (Baeriswyl et al., 2009). One hypothesis is that MMP-1 allows disseminated tumor cells to overcome dormancy in an unconditioned stromal environment with limited growth signaling.

Future Directions

Several conclusions were made from the sum of the work described, such as the role of MMP-1 and Fra-1 in causing elevated metastasis in MDA-MB-231 mammary carcinoma cells. Potential directions for this research are discussed below.

miRNA Regulation of Fra-1

Fra-1 is translationally regulated and this may be due to the presence of a regulatory miRNA. To determine if this hypothesis is correct, several experiments could be performed.

First, luciferase expression constructs with the Fra-1 3' UTR together with a constitutively active promoter might show evidence of repression through the UTR sequence. Truncations and mutation in the UTR might abolish repression and yield a specific miRNA binding site sequence, which could be matched to a miRNA. Also, repression might be differential in highly metastatic and non-metastatic cell lines. Second, sequencing of UTR regions of Fra-1 in metastatic and non-metastatic cells would eliminate the possibility of a novel mutation causing differential repression. Third, Fra-1 and MMP-1 expression can be compared in cells with individual miRNAs inhibited or overexpressed. Overexpression can be performed by transfection of miR-mimics and inhibition by transfection of LNA-antimirs. LNA-antimirs are RNA analogs modified to bind with higher affinity than endogenous RNA, resulting in improved miRNA inhibition (Elmen et al., 2008).

Mimics and antimirs are limited by the total number of known miRNAs. There are too many to audit the impact of each one, therefore a limited set must be selected. The first strategy for this selection is to use evidence from previous studies. As described in the previous chapter, miR-34a regulates breast cancer through Fra-1 (Yang et al., 2012) making it an ideal candidate. Other candidates include miRNA-146a, which regulates MMP-1 in breast cancer metastasis, and several miRNAs broadly correlated to cancer (Lu et al., 2005). A second strategy is to use bioinformatics to analyze the Fra-1

sequence for miRNA target sites and any corresponding miRNAs (Lewis et al., 2003; Sethupathy et al., 2006). Lastly, miRNA sites implicated in previously described luciferase experiments would be high priority candidates.

MMP-1 Activation of PAR-1

MMP-1 cleaves protease-activated receptor-1 (PAR-1), promoting tumorigenesis and metastasis (Boire et al., 2005; Foley et al., 2012). PAR-1 is also aberrantly expressed in patient tumors (Even-Ram et al., 1998; Kamath et al., 2001).

As previously discussed, PAR-1 may also play a role in angiogenesis through angiogenic factors like cysteine-rich angiogenic inducer-61 (CCN1), which upregulates MMP-1 in tumor associated stromal cells (Nguyen et al., 2006). Simlarly, endothelaial cells stimulated with MMP-1-activated PAR-1 express vascular endothelial growth factor receptor (VEGFR), another angiogenic factor (Mazor et al., 2013). PAR-1 and MMP-1 have both been previously associated with angiogenesis (Caunt et al., 2003; Fisher et al., 1994). An interesting line of study would test the hypothesis that MMP-1 activation of PAR-1 promotes breast cancer metastasis through increased angiogenesis.

Improved Gene Signatures

Recent work has demonstrated the value of novel gene signatures in metastasis based on confirmed regulatory factors. Fra-1 was poorly correlated with metastatic potential in microarrays, yet targets of Fra-1 (the Fra-1 transcriptome) are highly correlated. The Fra-1 transcriptome is a better prognostic indicator of patient hazard than

several published and commercialized gene signatures (Desmet et al., 2013). This work can be expanded upon with MMP-1. If confirmed, the role of miRNAs and PAR-1 might yield novel transcriptomes with new targets for study and treatment.

Improved Treatments

Based upon our work and previous findings (Balduyck et al., 2000; Giambernardi et al., 1998; Kousidou et al., 2004), MMP-1 and Fra-1 increase tumorigenesis and metastasis. Therefore, both are also targets for breast cancer treatment. While developing novel therapies is a difficult task, from our work there are opportunities to evaluate the efficacy of novel treatment combinations.

To date, inhibition of the Fra-1/MMP-1 pathway has been unsuccessful in clinical trials. Small molecule inhibition of Fra-1 has not been possible (Desmet et al., 2013). However, exciting work in DNA vaccines showed promise in mouse studies (Reisfeld et al., 2004). Tumor cells overexpressing Fra-1 present ubiquitinated Fra-1 (Ub-Fra-1) antigens. In mice treated with the vaccine, cytotoxic T-cells are activated to respond to the Ub-Fra-1, which prevented and reduced metastatic tumors. Unfortunately, the vaccine has not progressed in clinical trials (Luo et al., 2005; Luo et al., 2003). This lack of clinical progress is particularly frustrating, as inhibition of Fra-1 has been shown to inhibit breast cancer (Liu et al., 2002).

Small molecule inhibition of MMP-1 has also been unsuccessful in clinical trials (Chaudhary et al., 2013). Several inhibitors showed promise, including Batimastat (Lee et al., 2001). In mice, mesenateric intravenous injection of murine melanoma cells resulted in liver metastases, which when treated with Batimastat tumors were smaller and

less vacularized than tumors in untreated mice (Wylie et al., 1999). Unfortunately, because of Batimastat's poor water solubility it did not progress past phase II clinical trials (Chaudhary et al., 2013). Other MMP-1 inhibitor treatments had prohibitive side effects, including Marimastat with severe inflammatory polyarthritis (Tierney et al., 1999), Salimastat with musculoskeletal pain (Wall et al., 2004), and MMI-270-B with cutaneous rash and arthralgia (Levitt et al., 2001). It is possible that more effective and specific MMP-1 inhibitors might avoid these side effects.

Naturally occurring MMP-1 inhibitors are also potential treatments, but none have reached clinical trials. Neovastat, from shark cartilage, inhibited xenografts of MDA-MB-231 cell metastases (Weber et al., 2002). Berberine, a component of the herbal medicine Goldenseal, has been shown to reduce MMP-1 expression in carcinogen-induced mammary carcinoma cells, MCF-7 (Kim et al., 2012). Similar examples include the chinese herbal medicine běi qí (Hong et al., 2013), decursin from herbal dang gui (Hwang et al., 2013), and nobiliten from citrus peel (Sato et al., 2002).

PAR-1, a downstream MMP-1 effector, also presents a target for therapy. PAR-1 inhibitor, P1pal-7 pepducin, together with Docetaxel, a chemotherapeutic, reduced metastasis in murine MDA-MB-231 xenografts (Yang et al., 2009). Similarly, downstream targets of Fra-1 have been identified as potential treatment targets. In synthetic lethal small molecule screens, Fra-1 inhibition in MDA-MB-231 cells increases toxicity when treated with adenosine receptor A2B (ADORA2B) antagonists. Treatment with theophylline, an adenosine receptor inhibitor, together with Docetaxel reduced metastatic tumor burden significantly (Desmet et al., 2013).

P1pal-7 and theophylline demonstrate that treatments can be ineffective alone but synergistically efficacious. An interesting direction for future research would be to study if combinations of Fra-1/MMP-1 pathway inhibitors and current breast cancer treatments are effective in inhibiting breast cancer.

In-Vitro Model of Disseminated Tumor Growth

We have developed a pilot model of disseminated cell growth in secondary organs that might be suitable for high-throughput screens. As previously discussed, inhibiting disseminated tumor growth in the secondary tumor environment is an important point of regulation and treatment. Unfortunately, this stage is also difficult to imitate in *in vitro* assays, particularly in models capable of being used in high-throughput screening. One solution to better emulate tumor cells in secondary organs is co-culture with secondary organ fibroblasts.

Preliminary experiments show that a low volume of metastatic breast cancer cells will proliferate on lung fibroblast monolayers, while non-metastatic cells do not survive. Fluorescent labeling of tumor and fibroblast cells would allow for this model system to be used in high-throughput siRNA and small molecule screens, where tumor and fibroblast cells can be measured by fluorescent signal. If successful, this model could potentially be expanded to other tumor cell lines, fibroblast cells, and culture systems, including 3D cultures. Similar work in high volume coculture experiments with luciferase expression have yielded interesting results (McMillin et al., 2010). Future research might confirm these preliminary experiments and further develop this system.

Concluding Remarks

Our work, and the work of others, have clearly demonstrated Fra-1 and MMP-1 involvement in cancer. In breast cancer, Fra-1 is translationally upregulated in metastatic cells, perhaps through the loss or inhibition of a miRNA. Increased Fra-1 protein binds the AP-1 site of the MMP-1 promoter, increasing MMP-1 transcription and translation. Ectopic expression of Fra-1 in non-metastatic cells drives a metastatic phenotype, perhaps through MMP-1 and PAR-1.

From these data we can begin to hypothesize a pathway in metastasis. Fra-1 upregulates MMP-1, which is known to further cleave PAR-1, rendering the tumor cells more aggressively metastatic through stromal invasion and increased angiogenesis. In metastasis, Fra-1 could potentially be upregulated by the loss of a specific miRNA inhibition. More research will be necessary to further support the links in this pathway, which will be an interesting research direction for the future.

On a personal note, this thesis has been written in the context of improving breast cancer treatment, which has also been the purpose for the work itself. My hope is that the work during my tenure has made some incremental impact on reducing the impact of a devastating disease.

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Appendices

Appendix 1: Top genes with greatest expression differential in genes of high metastatic potential and low metastatic potential in bone and lung. High bone metastatic cell lines are: 1833, Scp-2, Scp-25 and Scp-46. High lung metastatic cell lines are: 1834, 3481, 4142, 4173, 4175, 4180, Scp-3 and Scp-28. Low metastatic cells lines are: MDA-MB-231, Scp-6, Scp-21 and Scp-26. Standard deviations for each were calculated and used in a two-tailed t-test to calculate relative p-value. Genes are over two fold greater in bone and lung with p-values less than 0.06. Data sourced from (Minn et al., 2005)

Gene	Bone (High/Low)	p-Value	Lung (High/Low)	p-Value
MMP1	98.28	0.00024	26.63	0.05619
SPANXA1/A2/B1/B2/C	18.23	0.00681	22.04	0.00570
SPANXC	14.59	0.00334	14.92	0.00756
ROBO1	13.99	0.00012	10.00	0.00013
SPANXB1B2	12.82	0.01742	15.82	0.00875
FOXA2	11.27	0.00233	9.69	0.00025
RGS2	8.32	0.00176	7.68	0.00014
NLRP3	8.24	0.00075	3.28	0.00775
CFH /// CFHR1	6.66	0.01548	5.75	0.00003
PTGS2	6.26	0.00840	5.54	0.00272
KCNK1	5.27	0.04210	3.73	0.00265
ANK3	5.05	0.02726	3.23	0.00995
KYNU	4.98	0.02145	10.12	0.01040
NR2F1	4.87	0.00088	5.36	0.00001
HAS2	4.83	0.00083	3.11	0.05186
LGR5	4.82	0.04688	3.82	0.00197
SOX4	4.67	0.03847	11.39	0.00391
RBM5	4.52	0.01639	2.13	0.05365
MOCS1	4.41	0.00053	2.36	0.00509
PRSS3	4.41	0.00751	3.21	0.00091
KHDRBS3	4.08	0.00183	2.59	0.00181
MEF2C	4.05	0.00620	2.49	0.00892
GPR37	3.99	0.02415	2.44	0.00030
ARHGDIB	3.95	0.00194	4.14	0.00001
MUSK	3.88	0.01237	3.03	0.02432
ZDHHC17	3.86	0.04522	2.49	0.05806
ODZ3	3.82	0.00299	3.61	0.00101

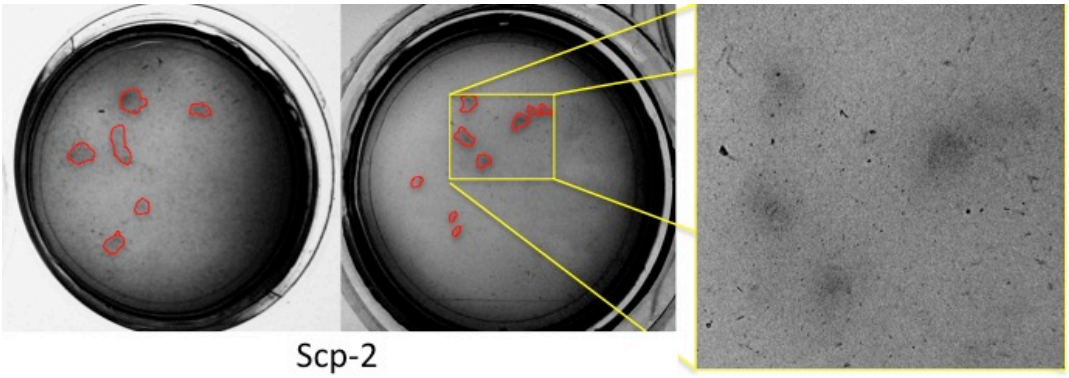
Gene	Bone (High/Low)	p-Value	Lung (High/Low)	p-Value
ENPP4	3.75	0.00026	2.88	0.00073
SULT1C2	3.74	0.00065	3.59	0.00021
MMP16	3.73	0.02076	2.15	0.04840
KCNK1	3.69	0.02844	3.01	0.00000
SLCO1B3	3.64	0.00058	2.63	0.00636
PLCE1	3.64	0.00065	2.41	0.01816
FOXA2	3.60	0.00241	3.11	0.00022
PELO	3.54	0.04780	3.28	0.02047
LPXN	3.45	0.00108	3.05	0.00005
NSBP1	3.43	0.05792	2.95	0.01074
CASP1	3.34	0.01960	2.32	0.04582
PRSS3	3.34	0.02957	2.43	0.00078
TSPAN13	3.28	0.01774	2.55	0.00022
MAGEH1	3.25	0.04480	2.03	0.03014
ANGPTL4	3.19	0.04369	3.68	0.00043
NR3C2	3.12	0.00236	2.18	0.01802
FLJ20489	3.07	0.02672	2.76	0.05765
SERPINI1	3.07	0.02671	2.87	0.00013

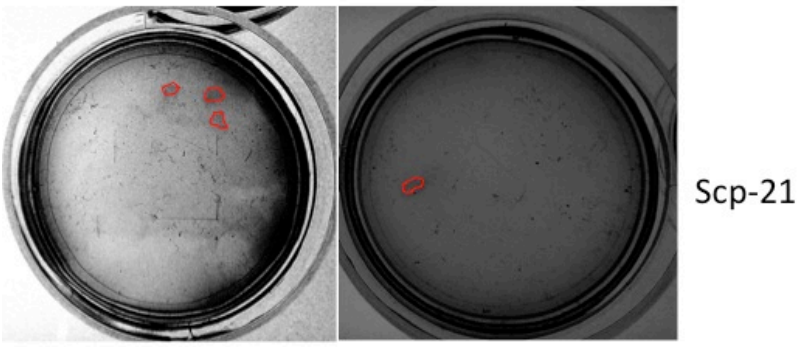
Reference:

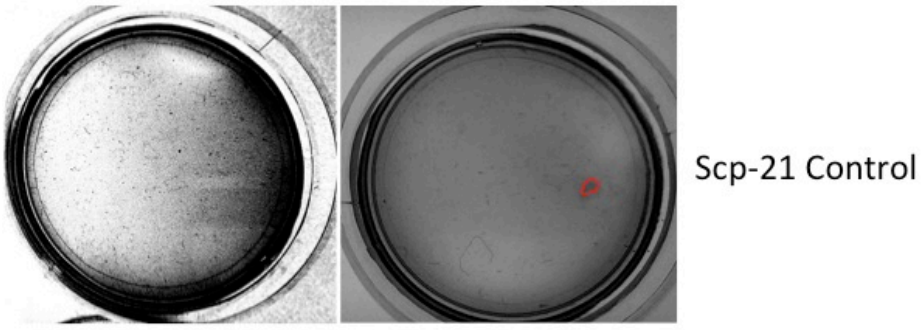
Minn, A. J., Gupta, G. P., Siegel, P. M., Bos, P. D., Shu, W., Giri, D. D., . . . Massague, J. (2005). Genes that mediate breast cancer metastasis to lung. *Nature, 436*(7050), 518-524. doi: 10.1038/nature03799

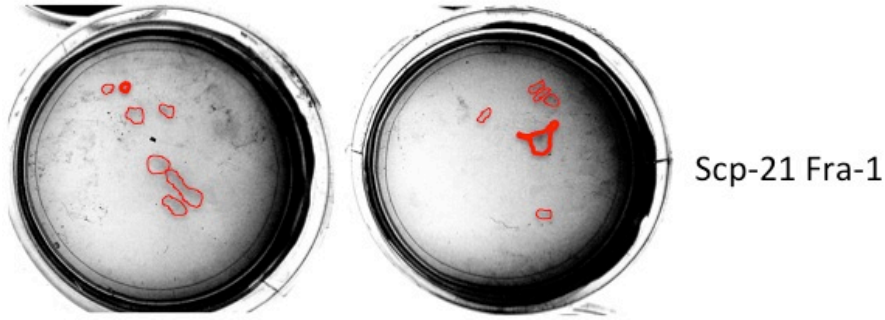
Appendix 2: Images for Figure 4-8, Soft Agar Colony Staining

Photographs of representative soft agar plates. Cell colonies are outlined in red, yellow inset is a magnification of colonies without outline.









Appendix 3: Supplemental Image for Figure 4-4

Autoradiograph of total protein from cells depleted of cysteine and methionine for 30 minutes and labeled with ^{35}S-cysteine and -methionine for the indicated times.