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Novel roles for HMGB1 in cancer and its mechanisms of release during Adenovirus infection

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Novel roles for HMGB1 in cancer and its mechanism of release during Adenovirus infection

By

Mike Nehil

DISSERTATION

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DOCTOR OF PHILOSOPHY

in

Cell Biology

in the

GRADUATE DIVISION

of the

Acknowledgements

The text of this dissertation has not been previously published elsewhere.

The coauthor Frank McCormick directed and supervised the research that forms the basis for this dissertation. Mike Nehil provided experimentation and manuscript preparation for the bulk of this dissertation. Collaborators and coauthors are specified later on the relevant chapter title pages.

- Frank McCormick, PhD, FRS

I am grateful primarily to my creative and supportive mentor, Frank McCormick. Frank is always up for a challenge and follows the science to whatever is interesting. I was also lucky to be surrounded by so many great lab mates over the years: Jesse Lyons, Cynthia Lyle, Amy Young, Abby Miller, Tanja Meyer, Jamie Smyth, Irma Stowe, Madhu Macrae, Megan Salt, Sang Lee and many others. I am also grateful to have known so many good UCSF-affiliated people over the years such as the ever-adventurous Temkin family (Paul, Manisha and Elliot) the indefatigable Andrew Houk and all my fellow Tetraders. I have also greatly appreciated all the support from my family living in God's country, or Michigan.

Abstract

Novel roles for HMGB1 in cancer and the mechanism of its release during Adenovirus infection

By

Mike Nehil

High mobility group box 1 (HMGB1) is a nuclear protein first discovered nearly 30 years ago. It was described to aid in transcription by forming complexes with transcription factors. Further studies demonstrated that HMGB1 has the unique ability to act as an extracellular ligand for toll-like receptor (TLR) family proteins and the Receptor for Advanced Glycation End products (RAGE) when released from necrotic cells. Extracellular HMGB1 activates the NFkappaB and MAPK signaling pathways through these receptors. It is thought that a primary function of HMGB1 is to act as an “alarmin” or danger signal to activate immune cells in the presence of tissue damage. The prevailing model has suggested that HMGB1 overexpression in cancer is due to its ability to activate MAPK signaling, an important pathway in several cancers.

We have investigated MAPK-independent functions of HMGB1 in cancer by utilizing cell lines with activating mutations in this pathway in combination with an shRNA-mediated knockdown approach. Using this technique, we have discovered a previously unknown role for HMGB1 in promoting tumor cell growth and migration. We show that HMGB1 remodels the

SEMA3A genomic locus to a more silenced state involving histone deacetylation and decreased CCCTC-binding factor (CTCF) occupancy, and thus promotes tumor cell migration.

In another line of investigation, we have studied the mechanism of release of HMGB1 during Adenovirus infection. Oncolytic viruses are viruses that have been engineered to infect and replicate specifically in tumor but not normal cells. One of the greatest challenges in the field has been the immunogenicity of the viruses. A healthy immune system can eliminate the virus before it is able to spread throughout the tumor. Because HMGB1 is a critical component of immune activation, we investigated how Adenovirus induces its release. We found that poly-ADP-ribose polymerase (PARP) was required for the release of HMGB1 during Adenovirus infection.

We have discovered novel roles for HMGB1 in tumor biology and helped uncover modes of its regulation during Adenovirus infection. It is hoped that these findings will aid in the design of new HMGB1-targeted cancer therapies as well as increase the efficacy of existing oncolytic virus therapies.

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Chapter 1:

Introduction

1.1 Overview

This work encompasses two different projects investigating two separate aspects of the biology of HMGB1. Although different areas, both projects are primarily concerned with increasing our understanding of HMGB1 in order to advance the efficacy of cancer treatment. The first project is focused on the pro-tumor role of HMGB1 in cancer, and the second project investigates the release of HMGB1 as a deleterious aspect of oncolytic viral therapies. Both projects will be discussed, beginning with the role of HMGB1 expression in cancer. It is hoped that these studies will aid in the design of more effective cancer treatments in the future.

1.2 HMGB1 from discovery

HMGB1 was discovered 38 years ago as a non-histone nuclear protein with high acidic and basic amino acid content that ran with “high mobility” on SDS page gels (Goodwin, Sanders et al. 1973). It was later shown that HMGB1 could bind and stabilize DNA and formed physical interactions with histones, suggesting a role in maintaining chromatin structure (Yu, Li et al. 1977). HMGB1 is highly expressed and present in the nucleus of nearly all eukaryotic cells. After the discovery that HMGB1

modulated transcription (Boffa, Walker et al. 1990) it was found that HMGB1 specifically interacts with several transcription factors, in addition to binding DNA directly (Stros, Ozaki et al. 2002; Agresti, Lupo et al. 2003). In 1979 Bustin *et al.* made the interesting observation that in some cases HMGB1 could be found in the cytoplasm of cells (Bustin and Neihart 1979), in addition to the nucleus. Although not initially appreciated, this led to the next fundamental shift in thinking in the HMGB1 field.

1.3 HMGB1 as a cytokine

In 1999, the field was transformed by a study that showed HMGB1 could signal as a cytokine in addition to its nuclear role (Wang, Bloom et al. 1999; Yang, Wang et al. 2001) and played a major role as a pro-inflammatory factor in models of sepsis in mice. In this study, HMGB1 was shown to be both necessary and sufficient for mortality associated with sepsis. Later studies determined that release of extracellular HMGB1 is a highly regulated process. In immune cells, HMGB1 is translocated from the nucleus to the cytoplasm by acetylation (Bonaldi, Talamo et al. 2003) and/or phosphorylation (Youn and Shin 2006) and released by a non-classical secretory pathway (Gardella, Andrei et al. 2002). In immune cells the release of HMGB1 is termed an “active” process, because the cells that release HMGB1 can do so without affecting their own viability. In contrast, HMGB1 can also be released from dying cells in a “passive” process. Although this route of release has been called “passive”, it is still a tightly regulated pathway. Cells that die by apoptosis do not release HMGB1 owing to deacetylation of histones and

sequestration of HMGB1 on the chromatin (Rovere-Querini, Capobianco et al. 2004). However, upon necrotic death HMGB1 is dissociated from the chromatin via a mechanism that requires poly-ADP-ribose polymerase (PARP) activity (Ditsworth, Zong et al. 2007). It is thought that this distinction evolved to promote "danger" signaling from injured tissue that typically dies by necrosis, while reducing inflammation associated with developmentally regulated apoptotic death (El Mezayen, El Gazzar et al. 2007). Extracellular HMGB1 binds and signals through the Receptor for Advanced Glycation End-products (RAGE) as well as Toll-Like family Receptors (TLR) to activate the Ras-MAP kinase and NFkappaB pathways (van Beijnum, Buurman et al. 2008). Downstream effects of HMGB1 signaling include inflammatory gene expression as well as increased release of HMGB1, resulting in an amplification loop.

1.4 HMGB1 in disease

Because of the significant pro-inflammatory functions of HMGB1 previously mentioned, it is not surprising that HMGB1 plays a role in various inflammatory disorders. In the autoimmune disease lupus, in which auto-antibodies cause tissue destruction, anti-HMGB1 antibodies have been detected. Complexes between HMGB1 and nuclear histones have been found in the circulation of these patients. One of the best characterized diseases associated with aberrant serum levels of HMGB1 is septic shock. Septic shock occurs when tissue injury causes an inflammatory chain reaction mediated by cytokine release, immune cell activation, and further cytokine release. This sequence of events results in a severe drop in blood pressure and frequently eventual

death. HMGB1 is associated with septic shock in several models and a critical role is indicated by experiments showing HMGB1 blocking antibodies can rescue mortality associated with septic shock in mouse models in up to 70% of the animals. In cancer, HMGB1 is often over-expressed, and will be discussed in detail later. Elevated levels of HMGB1 have also been detected in the serum of patients suffering from acute viral infection and HMGB1 is thought to contribute to excessive inflammation in these cases. Nonetheless, the mechanism of release during infection has not been well-studied and will be discussed below.

Chapter 2:

HMGB1 promotes tumor cell invasiveness through epigenetic silencing of SEMA3A

Mike Nehil contributed data to all Figures except: Figures 16-19 which were contributed by Taku Tokuyasu, Tables 1 and 2 which were generated with help from Jesse Paquette, and data for Figures 5 and 6 were generated with significant help from Byron Hann and Paul Phojanakong.

2.1 Introduction

High Mobility Group Box 1 (HMGB1) is a 25kD DNA binding protein that is over-expressed in various cancers. Its expression is often associated with poor prognosis, higher tumor grade and metastasis (Ellerman, Brown et al. 2007). In a colon cancer study, HMGB1 was shown to be over-expressed in tumor tissue compared to adjacent normal tissue in 90% of patients (Volp, Brezniceanu et al. 2006). In addition, a causative role for HMGB1 in promoting colon carcinogenesis was shown experimentally (Maeda, Hikiba et al. 2007). Similar results have been reported for hepatocellular carcinoma (Cheng, Jia et al. 2008), nasopharyngeal carcinoma(Wu,

Ding et al. 2008), prostate cancer (Ishiguro, Nakaigawa et al. 2005)and melanoma (Poser, Golob et al. 2003). In a breast cancer study, 2 to 4-fold increased expression of HMGB1 was observed in 50 tumor samples, as compared to 10 normal breast tissue samples (Brezniceanu, Volp et al. 2003). The high prevalence of HMGB1 up-regulation in diverse tumor types suggests a critical role in carcinogenesis; however, despite a strong correlation between HMGB1 expression and negative clinical outcome, the mechanism by which HMGB1 promotes tumor growth and metastasis remains unclear.

HMGB1 binds DNA through its HMG-box motifs without sequence specificity and aids in distorting the DNA structure to allow access for repair and transcription proteins. HMGB1-deficient mice die within 24hrs after birth from hypoglycemia as a result of defective glucocorticoid mediated transcription (Calogero, Grassi et al. 1999). HMGB1 interacts with various transcription factors, including NFkappaB members (Agresti, Lupo et al. 2003), p53 (Jayaraman, Moorthy et al. 1998) and the TATA-binding protein (Ge and Roeder 1994). Interactions with these factors can promote or repress transcription depending on the cellular context. Although promotion of transcription has been attributed to the ability of HMGB1 to bend DNA structure and allow access for transcription factors, the mechanism behind transcriptional repression is much less understood.

In addition to its roles in the nucleus, HMGB1 can also act as a ligand when released from dying or stressed cells. HMGB1 binds to the Receptor for Advanced Glycation End Products (RAGE) or TLR2/4, which are expressed on many types of immune and tumor cells. Although the precise mechanism is unclear, HMGB1 binding to RAGE activates the Ras MAP kinase pathway and increases the level of phosphorylated p42/44 (ERK1/2). Through its interaction with

TLR2/4, HMGB1 can promote transcription of NFkappaB target genes in various types of immune cells (van Beijnum, Buurman et al. 2008). HMGB1 is one of the best studied “alarmin” molecules; alarmins are proteins released from infected or damaged cells to provoke an immune response. HMGB1 has a complex role in cancer due to its ability to act on tumor cells to stimulate the pro-tumor MAP kinase pathway as well as on immune cells to stimulate potential anti-tumor immunity. Nevertheless, the prevalent overexpression of HMGB1 in cancer indicates a strong selection for its pro-tumor functions.

The pro-tumor effects of HMGB1 are often attributed to its extracellular role, through stimulation of the MAPK pathway via RAGE binding (Taguchi, Blood et al. 2000). Functionally, preventing extracellular release of HMGB1 by drug treatment or blocking HMGB1 signaling with antibodies can reduce tumor growth and metastasis in some experimental models of cancer (Maeda, Hikiba et al. 2007). In addition, RAGE-deficient mice are resistant to tumor formation in chemical carcinogen-induced skin cancer models (Gebhardt, Riehl et al. 2008), and RAGE expression is also often associated with poor clinical outcome and metastasis. The HMGB1-RAGE-MAPK signaling axis thus appears to be important in many cancers. However; tumor types with frequent activating mutations in the MAPK pathway such as colon cancer and melanoma still are often found to over-express HMGB1, indicating a possible MAPK-independent function. In this work, we have investigated novel functions for HMGB1 in tumor growth and metastasis that are independent of its role as a ligand for the MAPK pathway.

2.2 Significance

HMGB1 has previously been shown to drive pro-tumor MAPK signaling as a RAGE ligand and therefore several proposed cancer therapies aim to neutralize extracellular HMGB1. We show that HMGB1 has an additional nuclear function to promote an invasive cell phenotype by epigenetically silencing semaphorin 3A (SEMA3A) expression. Although class 3 semaphorins and particularly semaphorin 3A have been increasingly recognized as suppressors of tumor cell invasion, the underlying regulation of their expression has not been investigated. Our findings suggest that targeting extracellular HMGB1 may not be sufficient to ameliorate the full pro-tumor effects of HMGB1 and that epigenetic therapies may be useful for future treatment of tumors with high HMGB1 expression.

2.3 Materials and Methods

Cell lines- MDA-MB-231-luciferase cells were a gift from Byron Hann. SUM159PT and HS578T cells were a gift from Madhu Macrae. A549 cells were a gift from Tony Karnezis. MDA-MB-435 cells were a gift from Amy Young.

Antibodies- Polyclonal rabbit anti-HMGB1 was obtained from AbCam. Anti-SEMA3A was from ECM Biosciences. Biotinylated rat anti-mouse CD31 was from BD Biosciences. Phospho-p42/44 and p42/44 were purchased from Cell Signaling.

Lentiviral shRNA transduction- Five HMGB1-targeted shRNA sequences from the Mission shRNA consortium in the pLKO.1 vector were propagated overnight in bacteria. DNA was then isolated using the Qiagen Maxiprep kit per manufacturer's instructions. DNA was transfected with Fugene into 293T cells in the following amounts: 4.2 ug shRNA, 0.4 ug VSVG, 3.7 ug GAG/POL,

3.7 ug REV. After three days incubation at 37 degrees, the virus-containing supernatant was removed and filtered through a 0.45uM filter before being added to target cells. Four and a half ug per mL polybrene was added to aid in the infection. After 24 hrs, media was replaced with 6ug/mL puromycin-containing media. Transduced cells were selected for by incubating in this media for a minimum of one week. Three sequences that resulted in efficient knockdown of HMGB1 were used in subsequent experiments:

Sh3: CCGGCCGTTATGAAAGAGAAATGAACTCGAGTTCATTTCTCTTTCATAACGGTTTTT

Sh4: CCGGGCAGATGACAAGCAGCCTTATCTCGAGATAAGGCTGCTTGTCATCTGCTTTTT

Sh5: CCGGCCCAGATGCTTCAGTCAACTTCTCGAGAAGTTGACTGAAGCATCTGGGTTTTT

One shRNA that did not have any effect on HMGB1 expression was used as a control vector:

Sh1: CCGGCGAGACTTTCATTACAAGTATCTCGAGATACTTGTAATGAAAGTCTCGTTTTT

Scattering assay- Cells were serially diluted into 6-well plates to a final concentration of ~50-100 cells per well. Cells were grown in 10% Fetal Bovine Serum (FBS) and Dulbecco's Modified Eagle Medium (DMEM) for three to four weeks, with media being changed every three days. After three to four weeks, colonies were imaged by light microscopy.

Scratch assay- Equal numbers of cells were seeded into 6-well plates overnight with full media. Scratches were conducted with a 20ul pipette tip and media was changed. Scratches were imaged every 3 hours using a marked position to return to the same scratch on the plate over time.

F-actin staining- Cells were seeded overnight onto chamber slides and fixed the next day with 4% paraformaldehyde in PBS for 20 minutes. Cells were then washed and permeabilized with 1% Triton-X in PBS for 10 minutes. After washing, 5% goat serum was used to block for one

hour. Rhodamine conjugated to phalloidin was added at 1:500 in 5% goat serum in PBS. After 1 hour incubation in the dark, slides were washed and stained with Hoechst dye.

Metastatic colonization assay- Fifty thousand luciferase-expressing MDA-MB-231 cells were intracardially injected into nude mice, as previously described (Bos, Zhang et al. 2009). Mice were imaged weekly using Xenogen software.

Tumor growth in vivo- Two million luciferase-expressing MDA-MB-231 cells were injected into the mammary fat pads of SCID/Beige (C57BL/6 background) mice obtained from Charles River. Tumors were imaged weekly.

Immunohistochemistry- Tumors were removed from mice and fixed overnight at 4 degrees in Z-fix solution (Anatech). After subsequent dehydration and sectioning, slides were incubated with biotinylated rat anti-mouse CD31 (BD Biosciences) overnight. Slides were developed using HRP-conjugated to streptavidin (R&D Systems).

Microarray- Transcriptional microarray analysis was conducted with help from the David Gladstone Research Center Genome Core. Affymetrix Human Gene 1.0 chips were used in triplicate for each sample. Data was analyzed using the Exploratory Gene Association Network (EGAN) program (http://akt.ucsf.edu/EGAN/).

Real-Time Polymerase Chain Reaction (RT-PCR)- RT-PCR for SEMA3A expression was performed with help from the UCSF Genome Core using the Taqman probes to HMGB1 and SEMA3A RT-PCR for ChIP analysis was performed using Sybr green. The primers used for amplification of the regions in SEMA3A and SEMA4F are as follows:

3A-1:

5’ CCGGATAATGAGGCACAACT 3’

5’ TAGAGACTGCCACCGGCTAT 3’

3A-2:

5’ GTAGTTGGCTGTGGCCTCTC 3’
5’ GGGGTAGGGCAGAATCATTT 3’

4A-1:

5’ TAGGCAGCCGTCCTTAAATG 3’
5’ GGCCACTCCAAAAACTCAAA 3’

Chromatin Immunoprecipitation (ChIP)- ChIP was performed as previously described (Szak, Mays et al. 2001). Briefly, 500ug of protein/chromatin mixture was incubated with 4ug antibody overnight at 4 degrees. After several washes, complexes were eluted and DNA cross-linking reversed. DNA was purified either by phenol/chloroform extraction or using the Biorad ChIP DNA purification kit, per manufacturer’s instructions.

DNAse sensitivity assay- Analysis was performed using the Biorad EpiQ kit as per manufacturer’s instructions. Briefly, cells were seeded into 24-well plates overnight. Cells were permeabilized with a weak detergent and incubated with DNAse for 1 hour at 37 degrees. Genomic DNA was isolated and analyzed by RT-PCR.

Bisulfite sequencing- Bisulfite conversion of genomic DNA was performed using the Qiagen Epitect kit as per manufacturer’s instructions. The SEMA3A promoter region was then amplified using the following primers:

5’ GATTGGTTGATAATGGGAGAATAGG 3’
5’ CACATACAATACACAAATTCAACAAAATTA 3’

The PCR product was subsequently gel purified and ligated using the pGEM-T vector system from Invitrogen. Ligated plasmids were transformed into bacteria and plated overnight. A minimum of 10 colonies were picked and grown further for sequencing.

2.4 Results

HMGB1 knockdown alters cell morphology and decreases *in vitro* migration independently of MAPK signaling

In order to identify MAPK-independent functions of HMGB1, we used a lentiviral shRNA system to stably knock down HMGB1 in cancer cells already harboring mutations in the MAPK pathway. The MDA-MB-231 breast cancer cell line has activating mutations in K-Ras and B-Raf, resulting in high basal MAPK activity. MDA-MB-231 cells have also been well characterized in mouse models of tumor growth and metastasis (Li, Glinskii et al. 2011; Woo, Choi et al. 2011). MDA-MB-435 cells were derived from a melanoma and have an activating B-Raf mutation. HMGB1 expression was reduced to 5-30% control cell levels after lentiviral transduction and selection (Figure 1). All cells were studied after a minimum of 1 week selection with puromycin to ensure an effective and stable knockdown. As expected, HMGB1 knockdown did not reduce basal MAPK signaling in these cell lines as judged by levels of phosphorylated ERK1/2 (Figure 1). However, depletion of HMGB1 caused the cells to adopt a more epithelial morphology with less extended processes in 2D tissue culture (Figure 2A, Figure 2B). Cells with diminished HMGB1 expression also showed reduced F-actin foci at the cell periphery (Figure 2A, Figure 2B) and

significantly reduced cell scattering in a colony formation assay (Figure 3), but did not affect colony forming ability (data not shown). HMGB1-depleted cells were also deficient in migration as measured in a scratch assay (Figure 4). HMGB1-RAGE signaling has been previously reported to affect *in vitro* migration by up-regulating signaling through ERK1/2 (Ranzato, Patrone et al. 2010). These data show that HMGB1 can affect cell migratory behavior without modulating MAPK signaling, suggesting that HMGB1 can drive migration independently from its ability to drive this pathway.

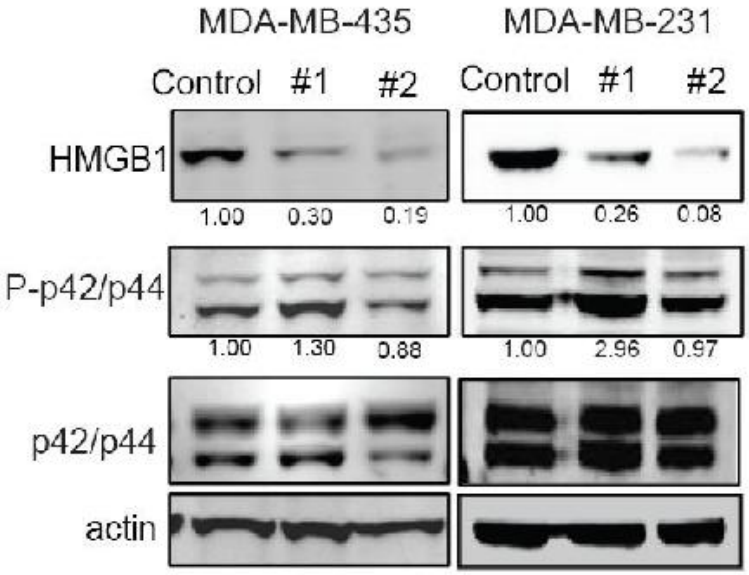

Figure 1. HMGB1 can be stable depleted without decreasing P-p42/44 levels.
MDA-MB-435 or MDA-MB-231 cells were infected with shRNA-expressing lentivirus and selected with puromycin for one week. Cell lysates were collected and analyzed by Western blot.

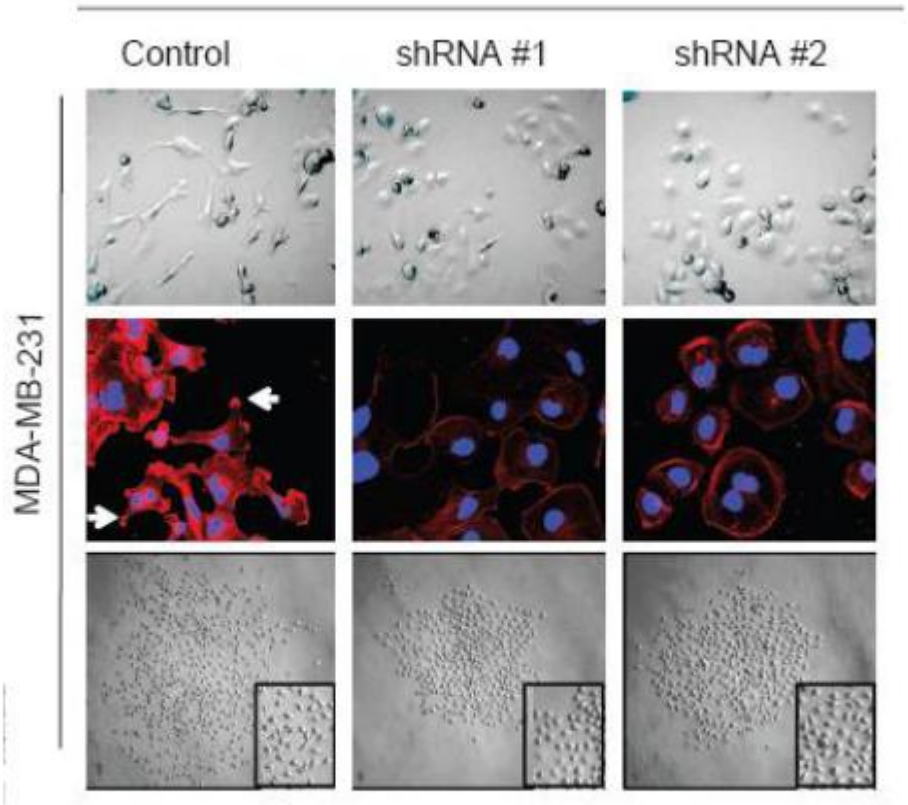

Figure 2 (A). HMGB1 depletion causes cells to adopt an epithelial morphology, reduces F-actin foci and decreases scattering ability. (top) MDA-MB-231 cells expressing HMGB1 or control shRNA were visualized with phase contrast microscopy or (middle) phaolloidin-rhodamine to visualize F-actin. (bottom) Scattering ability was measured by Imaging cell expansion from single colonies over 3-5 weeks.

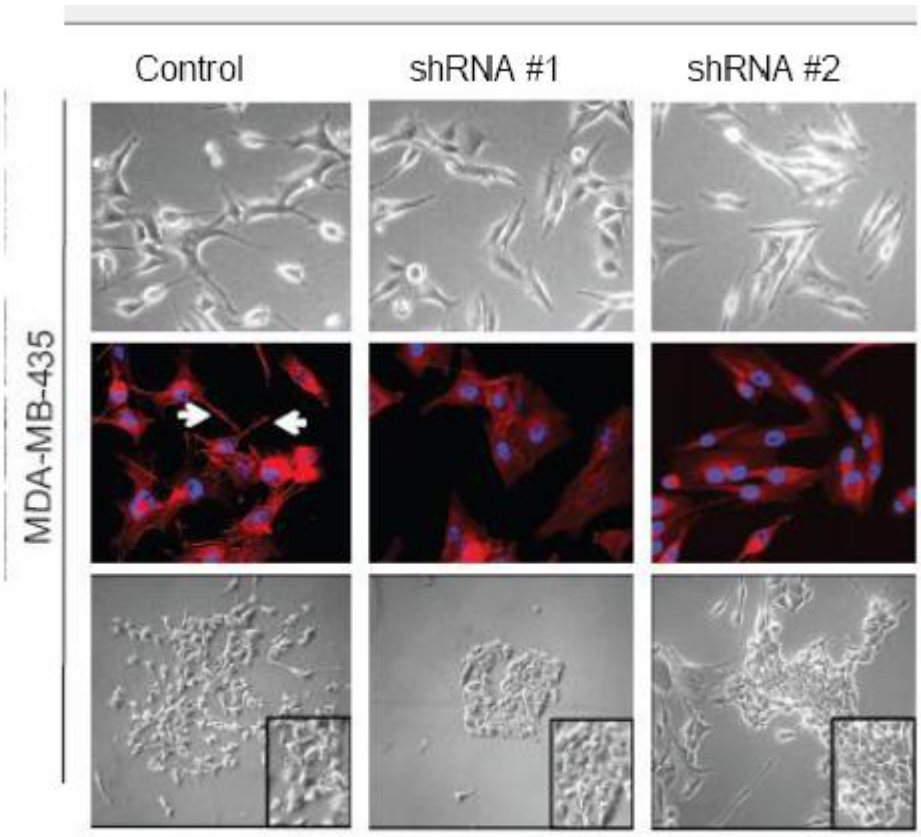

Figure 2 (B). HMGB1 depletion causes cells to adopt an epithelial morphology, reduces F-actin foci and decreases scattering ability. As described in Figure 2(A) but with MDA-MB-435 cells.

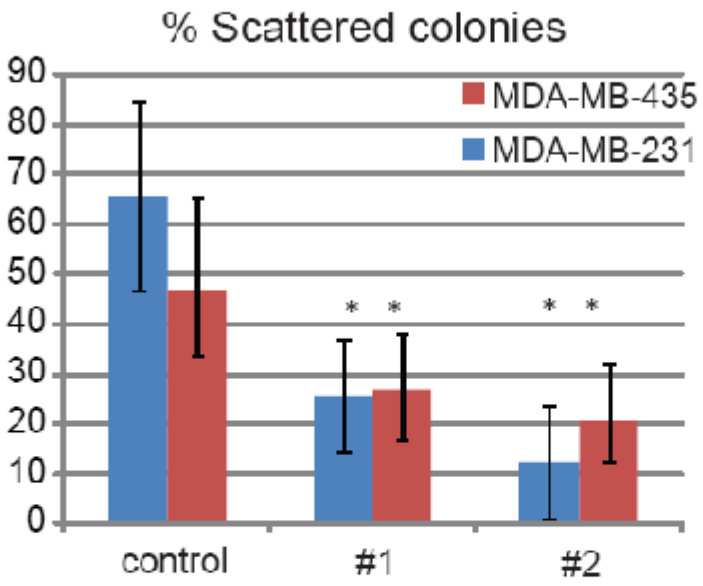

Figure 3. In vitro scattering ability is decreased by HMGB1 knockdown. Colonies were seeded as described in Figure 2. Scattering was quantified by counting scattered or non-scattered colonies for 10 fields of view per condition. Significance analysis was performed using a two-tailed Students T-test, * = < 0.05, ** = < 0.01, *** = < 0.001.

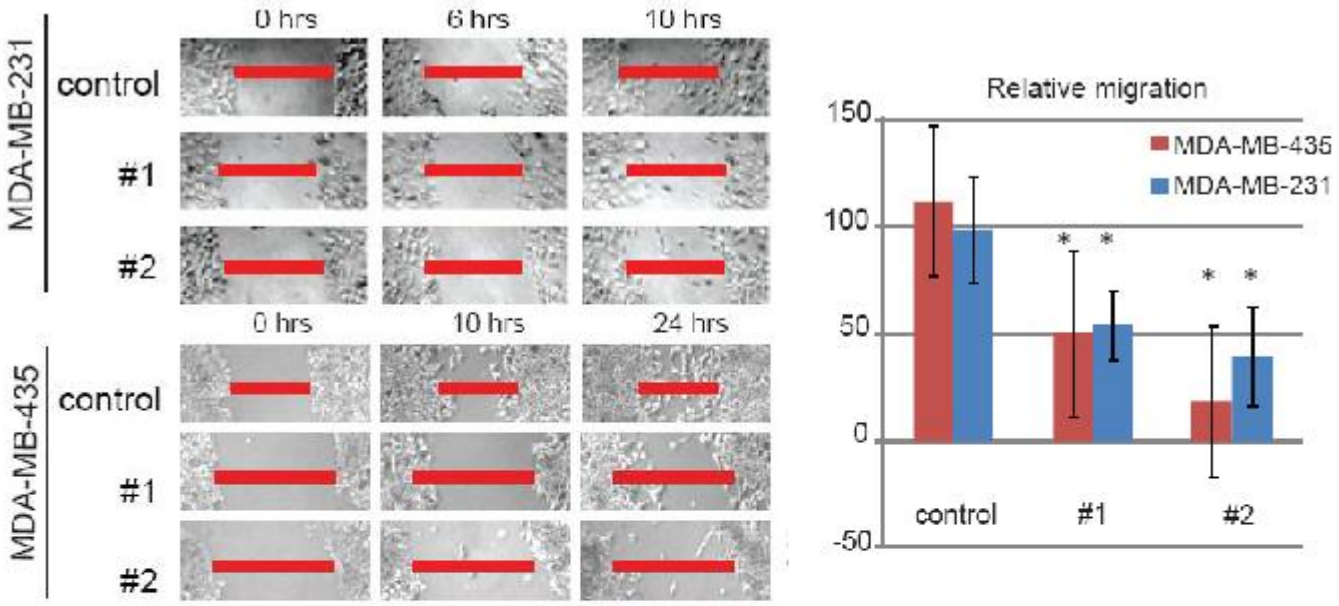

Figure 4. HMGB1 depletion decreases migratory ability in vitro in a scratch assay. MDA-MB-231 or MDA-MB-435 cells were depleted for HMGB1 as previously described and plated at equal densities overnight. Scratches were made with pipette tips and imaged at specific times. Migration was quantified by measuring 10 different scratch widths per scratch at each time point.

HMGB1 is required for tumor cell colonization from the circulatory system in vivo

Tumor metastasis is thought to occur in several steps. Initially, the cells must detach from the primary tumor and degrade the stroma to gain access to the circulatory system. Once in circulation the cells must adhere to the endothelial cell wall and migrate through the surrounding tissue to arrive at and populate new organ sites. HMGB1 is correlated with metastasis in human tumors (Yao, Zhao et al. 2010) and has been shown to be required for aspects of the metastatic process in some experimental models (Taguchi, Blood et al. 2000).

Because our *in vitro* results suggested a deficiency in migration, we tested whether HMGB1 knockdown also affected *in vivo* metastatic capacity. To investigate metastatic potential, we used a system that has been well described previously for MDA-MB-231 cells (Bos, Zhang et al. 2009). In this colonization/metastasis model the cells are injected into the circulatory system of an immune compromised mouse and allowed to invade and populate new locations, therefore recapitulating the later steps of metastasis.

HMGB1 knockdown cells with a luciferase reporter were injected intracardially into nude mice. The resulting tumors were allowed to grow for 10-12 weeks and monitored by bioluminescence. In order to account for differences in rates of tumor growth and thus detection between HMGB1 knockdown and control tumors, metastases were counted at a time point normalized to equal bioluminescence of the largest tumor. HMGB1 knockdown cells formed 40% fewer tumors in this assay ($p= 0.01$) (Figure 5). There was no significant preference for sites of metastases between cell groups, with bone, kidney and lung constituting the majority in both groups. These data are consistent with the *in vitro* defects in migration and less invasive morphology of the knockdown cells. Collectively, these results demonstrate that HMGB1 has a role in modulating cell migration and metastatic potential that is independent from its function as a ligand for the MAPK signaling pathway.

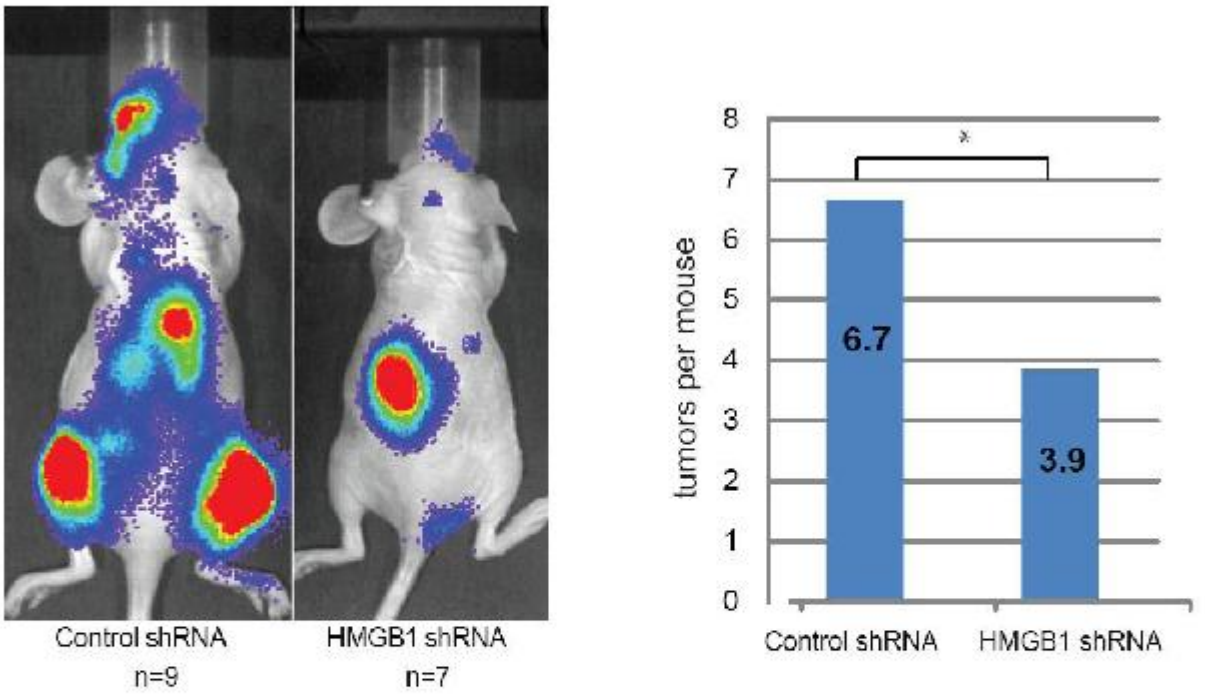

Figure 5. HMGB1 is required for metastatic colonization *in vivo*. 50,000 HMGB1-depleted or control cells with a luciferase reporter were intracardially injected into nude mice. Mice were imaged weekly for 10 weeks. At a point normalized for tumor size, the number of metastases were counted in each of the groups.

HMGB1 is required for tumor growth in vivo

Previous models have shown that HMGB1 is required for *in vivo* tumor growth owing to its ability to drive MAPK activation. Using our system, we tested whether HMGB1 was still required for tumor growth in the presence of activating MAPK mutations. Stable populations of MDA-MB-231 cells carrying a luciferase reporter gene and HMGB1 knockdown or control shRNA were orthotopically injected into the mammary fat pads of 6 week-old SCID mice and monitored for growth up to 8 weeks. Cells expressing HMGB1 shRNA showed a significantly reduced growth rate as measured by bioluminescent signal (Figure 6). In contrast to previous

studies (Taguchi, Blood et al. 2000), we did not detect a difference in doubling time *in vitro* (Figure 7), indicating that the reduced growth rate we observe is specific to *in vivo* conditions (Figure 8). Interestingly, both cell types grew at comparable rates during the first week (Figure 6). This suggests that HMGB1 is dispensable for tumor initiation, but is required for tumor maintenance after a certain size is reached.

Because angiogenesis is a well established event required for tumors in late, but not early development, and because HMGB1 has been reported to contribute to angiogenesis in other systems (Lin, Yang et al. 2011), we investigated the vasculature of these tumors. There was a large reduction in the tumor associated blood in HMGB1 knockdown vs. control tumors by macroscopic observation. In addition, knockdown tumors showed a disorganized and less dense structure (Figure 9). These observations are consistent with decreased angiogenesis and increased necrosis in the absence of HMGB1. To confirm these observations on the molecular level, endothelial cell infiltration and necrosis were examined by immunohistochemistry and hematoxylin and eosin staining, respectively. To assess the effects of HMGB1 deficiency on angiogenesis, we measured endothelial cell recruitment to the tumors using immunohistochemical staining for the endothelial cell marker, CD31. In the absence of HMGB1, tumors showed decreased CD31+ cell infiltration and increased evidence of necrosis (Figure 10 and Figure 9). Consistent with the *in vitro* data, HMGB1-deficient tumors did not have significantly reduced levels of phosphorylated ERK1/2 as judged by Western blot (Figure 15). This data shows that HMGB1 is required for cancer cell growth *in vivo* but not *in vitro* via a mechanism that is independent of MAPK signaling but associated with angiogenesis.

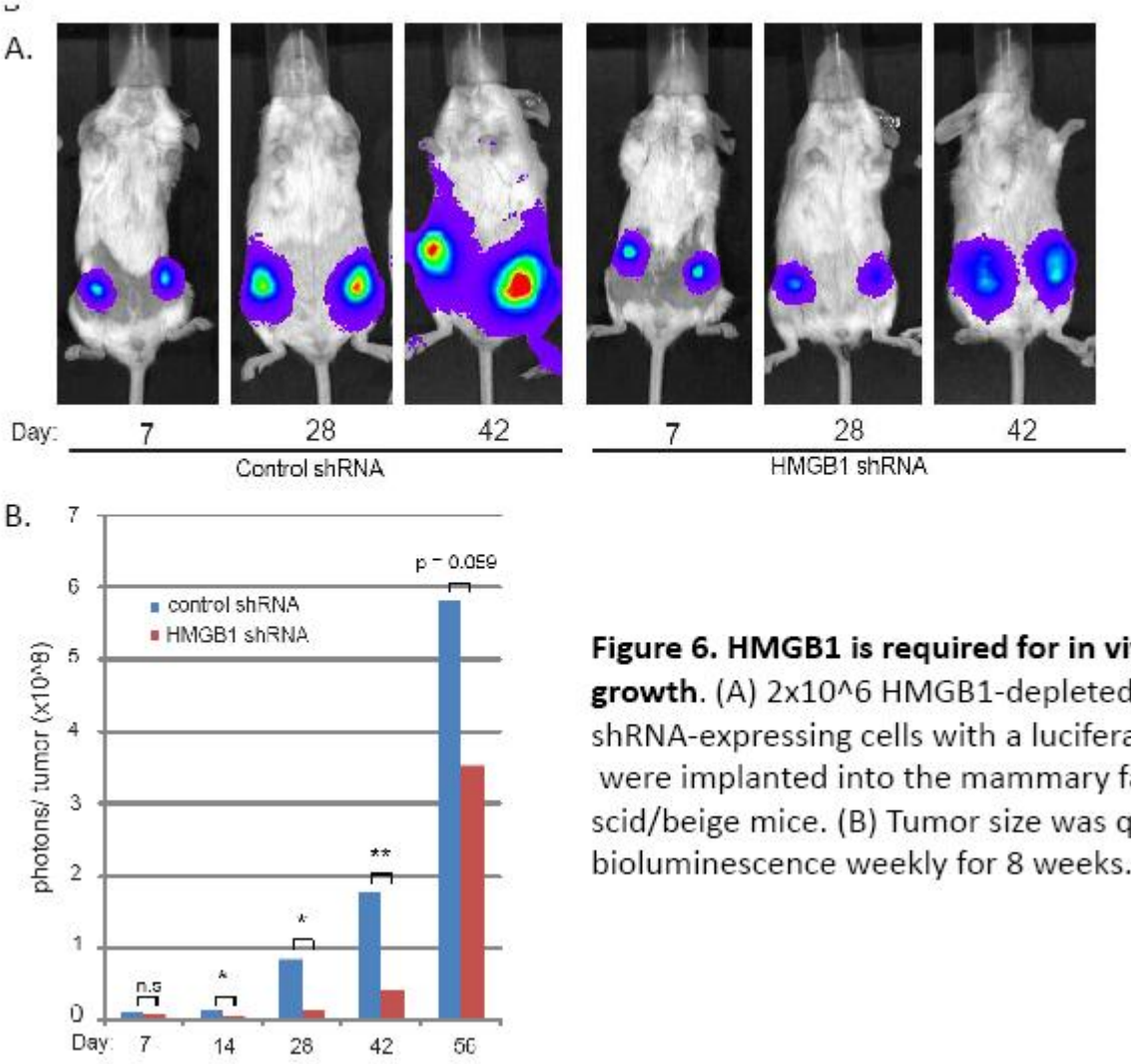

Figure 6. HMGB1 is required for in vivo tumor growth. (A) 2x10^6 HMGB1-depleted or control shRNA-expressing cells with a luciferase reporter were implanted into the mammary fat pads of scid/beige mice. (B) Tumor size was quantified by bioluminescence weekly for 8 weeks.

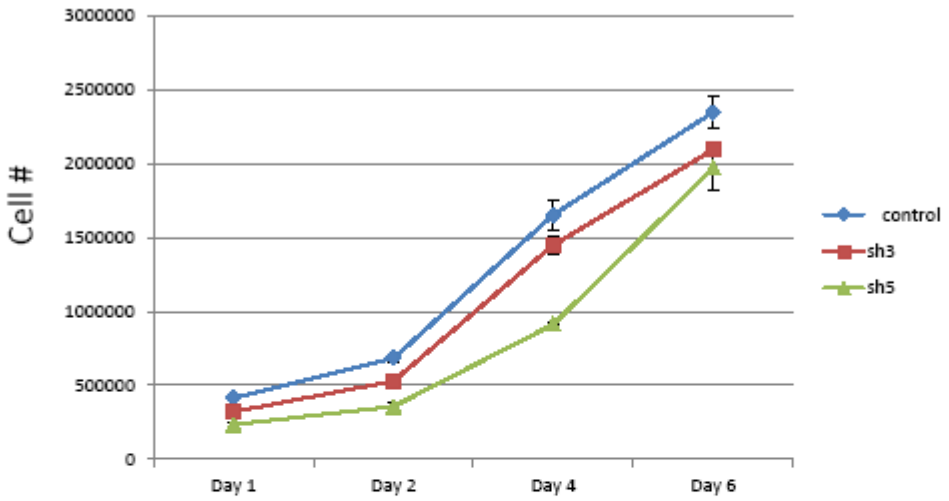

Figure 7. HMGB1 knockdown does not affect *in vitro* proliferation rate. MDA-MB-231 cells expressing control shRNA or two independent HMGB1-targeted shRNAs were seeded at equal densities overnight. 3 wells per condition were counted on indicated days, media was changed on Day 3.

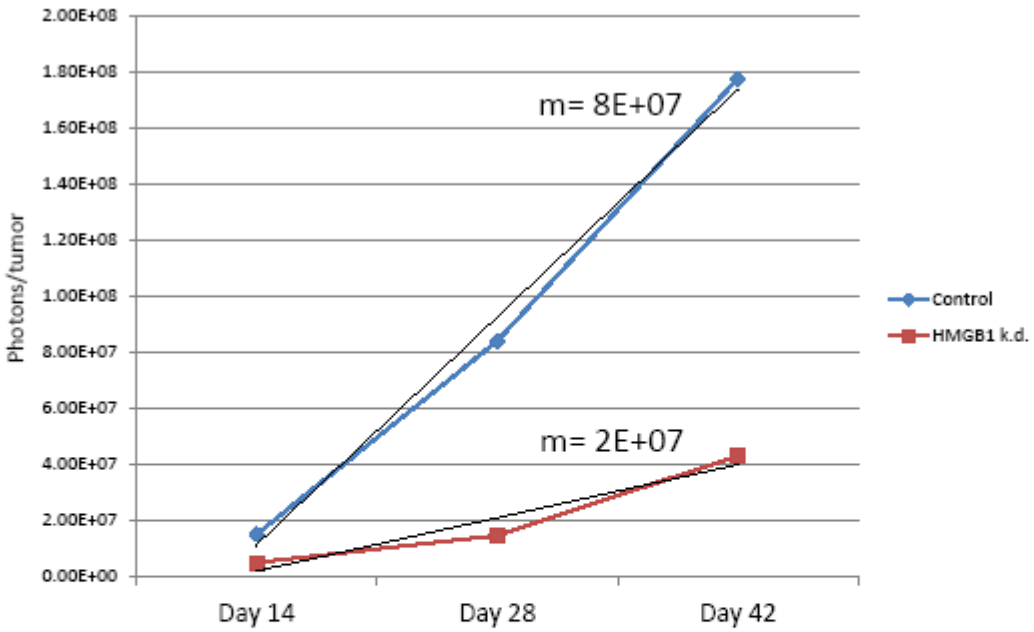

Figure 8. The growth rate of HMGB1 knockdown cells is reduced *in vivo*. Tumor size was quantified by bioluminescence as described in Figure 6.

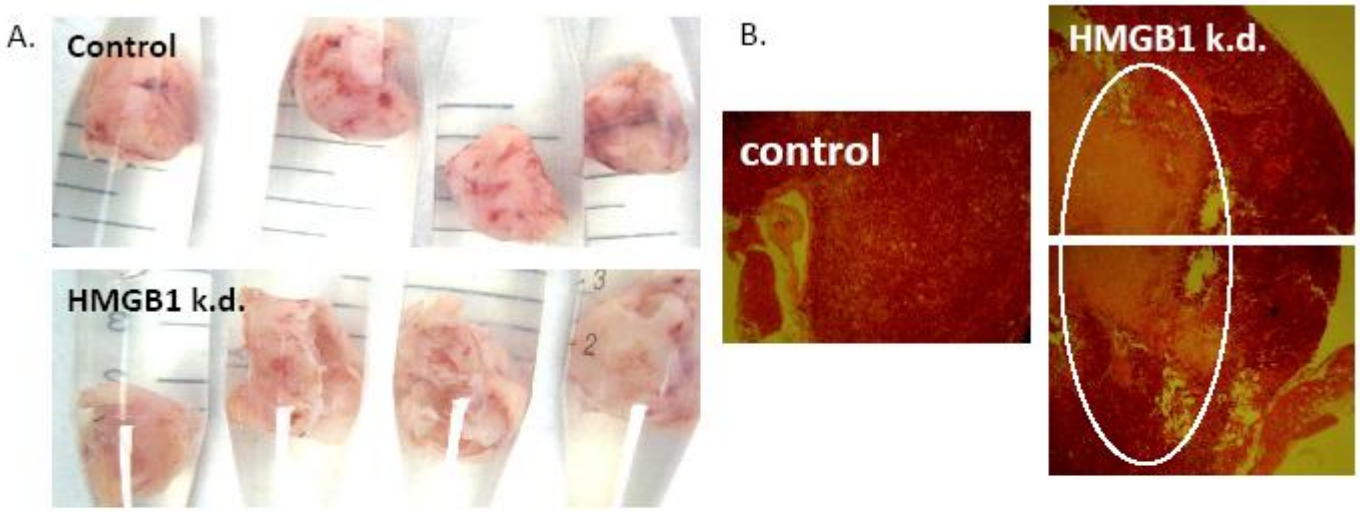

Figure 9. HMGB1 knockdown tumors form disorganized structures with increased necrosis. Tumors described in Figure 6 were removed at 8 weeks post-implantation and (A) macroscopically visualized or (B) stained with hematoxylin and eosin. Necrotic areas are circled.

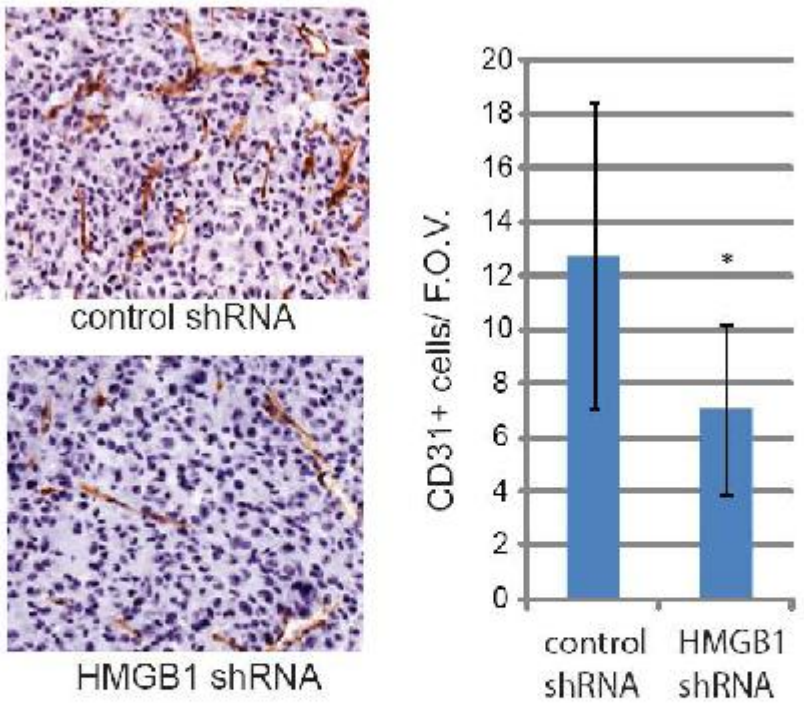

Figure 10. HMGB1 is required for CD31+ endothelial cell infiltration into tumors. HMGB1-depleted or control tumors, as described in Figure 6, were removed at 8 weeks post-implantation and fixed in Z-fix solution. CD31+ cells were visualized by immunohistochemistry and quantifed by counting 10 fields of view per tumor for 5 tumors of each condition.

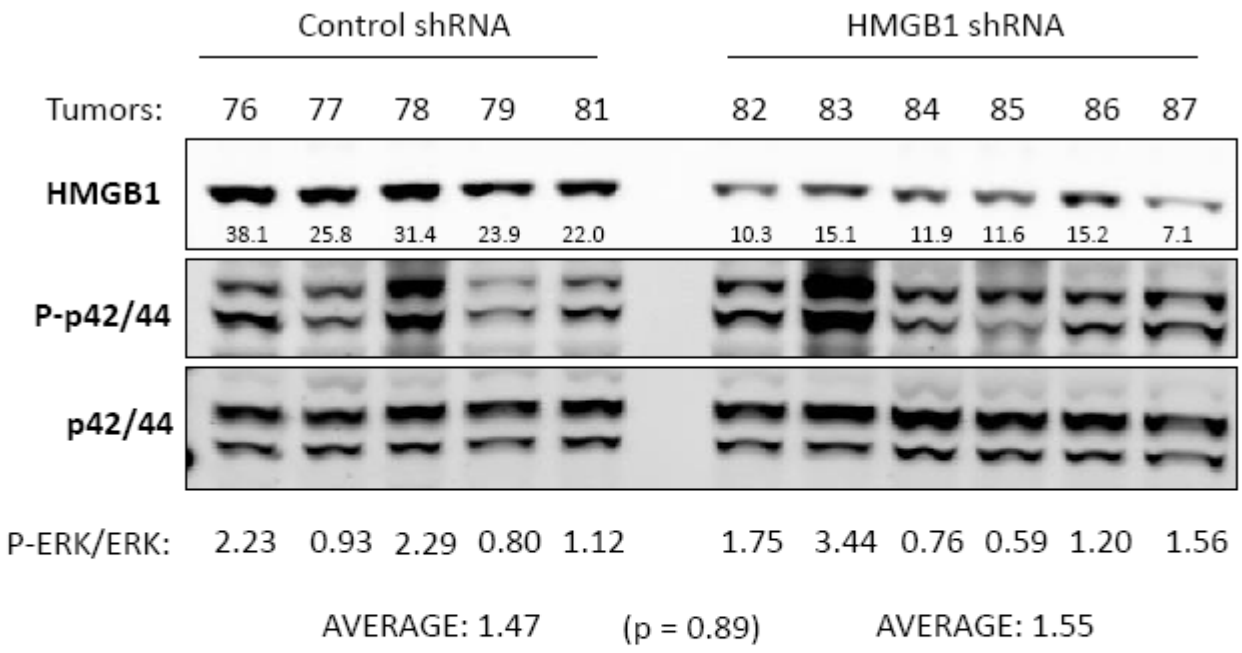

Figure 15. P-ERK is not reduced in HMGB1-knockdown implanted tumors. Tumors were removed as described in Figure 13 and analyzed by Western blot for phosphorylated ERK.

HMGB1 regulates 200 genes including class 3 semaphorins

Given that the phenotypes we observed could not be explained by MAPK signaling differences, we sought to investigate the transcriptional functions of HMGB1. HMGB1 is known to cooperate with various transcription factors to promote or repress gene expression depending on the cellular context. We used an array-based approach to identify genes whose expression was altered by HMGB1 depletion. We performed a transcriptional microarray in triplicate using HMGB1 knockdown or control cell populations in log phase growth. Using a cutoff of 1.25-fold difference in expression and a q value <0.05 ($p<0.001$), we identified 44

genes that were down-regulated by HMGB1 knockdown and 167 genes that were up-regulated (Figure 11). These results were confirmed with a second HMGB1 targeting shRNA and microarray (data not shown). Interestingly, although HMGB1 binds DNA without sequence specificity, its depletion affects the expression of only a specific set of genes (Table 1, Table 2). The majority of previous studies have shown HMGB1 to be a positive acting factor for transcription by bending DNA to give greater access to transcription factors. Loss of HMGB1 would thus be expected to result in more down-regulated genes than up-regulated genes. However, our identification of more up-regulated than down-regulated genes by knockdown (Figure 11) shows that HMGB1 acts largely as a repressor of transcription in this system. Among the top up-regulated genes were 2 members of the class 3 semaphorin family, semaphorin 3A (SEMA3A) and semaphorin 3E (SEMA3E). Class 3 semaphorins are secreted proteins that act as inhibitors of angiogenesis and cellular migration (Neufeld and Kessler 2008). They are often down-regulated in more aggressive tumors (Staton, Shaw et al. 2011) and suppress tumor growth and metastasis in experimental models (Casazza, Fu et al. 2011). As such, the loss of their expression is increasingly being recognized as a critical step in the evolution of more aggressive tumors. SEMA3A is the best characterized member of this family. SEMA3A binds to neuropilin 1 and causes dimerization with plexinA1. This leads to activation of plexinA1 GAP activity and inhibition of R-Ras, resulting ultimately in inactivation of Beta1 integrin (Schmidt and Strittmatter 2007). Additionally, SEMA3A can initiate F-actin depolymerization through Plexin-A1/MICAL interactions (Schmidt, Shim et al. 2008). Because of this link between the functions of semaphorins and the phenotype of the HMGB1 knockdown, we chose to further investigate this potential connection.

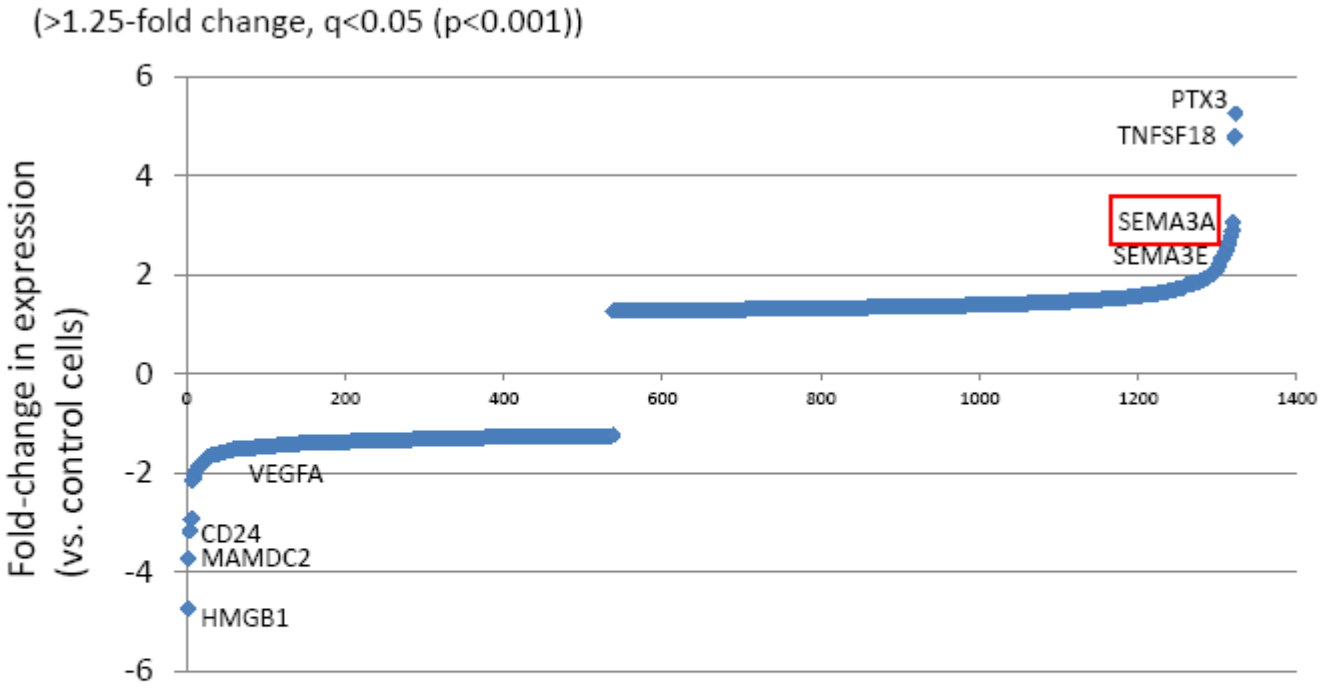

Figure 11. 43 genes are down-regulated and 167 are up-regulated by HMGB1 knockdown. Total RNA was isolated from MDA-MB-231 cells expressing HMGB1 shRNA or control shRNA. A microarray was performed in triplicate using the Affymetrix Human Gene 1.0 platform.

Table 1. Upregulated genes after HMGB1 knockdown.

Symbol	Fold-Change	Associated Processes
PTX3	5.2	inflammation, extracellular, tumor suppressor, anti-angiogenic, FGF antagonist
TNFSF18	2.7	inflammation, extracellular, stimulates anti-tumor immunity
PTPN22	2.7	phosphatase, signaling
LIPH	2.5	lipase, makes LPA
F2RL2	2.5	phosphoinositide phospholipase activity
C7orf58	2.4	unknown
STC1	2.3	calcium homeostasis
SEMA3A	2.3	inhibition of angiogenesis, axon guidance, migration
DSC2	2.3	calcium binding, adhesion
RGS4	2.2	GAP, inhibition of G-protein signaling

Table 2. Downregulated genes after HMGB1 knockdown.

Symbol	Fold-Change	Associated Processes
HMGB1	-4.7	DNA repair, transcription, signaling, angiogenesis
MAMDC2	-3.7	glycosoaminoglycan binding
CD24	-3.1	cell adhesion, migration, inflammation
GKN2	-3.1	extracellular binding, adhesion
CCDC86	-2.1	nuclear, inflammation
ID2	-1.9	transcription, angiogenesis
KDELR3	-1.8	ER protein retention
CDH11	-1.8	adhesion
TSPAN31	-1.8	cell growth, signaling
KRT81	-1.8	structural, extracellular

HMGB1 silences SEMA3A expression

In order to validate our transcriptional array data, semaphorin gene expression during HMGB1 knockdown was examined with real-time PCR in different tumor cell lines, including a melanoma, lung and two breast cancer cell lines in addition to MDA-MB-231. These cell lines showed significant up-regulation of SEMA3A mRNA when HMGB1 was depleted, by as much as 5-fold, which correlated with the level of HMGB1 knockdown (Figure 12). Interestingly, two of these cell lines showed a reduction in basal P-ERK levels upon knockdown (Figure 13), consistent with the known role for HMGB1 in this pathway. In these cells SEMA3A was still up-

regulated by HMGB1 knockdown, demonstrating that MAPK pathway activation and SEMA3A repression by HMGB1 are independent functions.

We further validated these findings on the protein level with Western blot analysis and immunohistochemistry on the implanted tumors shown in Figure 6. As shown in Figure 14, SEMA3A protein was up-regulated in MDA-MB-231 and MDA-MB-435 cell lines after HMGB1 knockdown. In the implanted tumors, loss of HMGB1 caused up-regulation of SEMA3A as seen by Western blotting and immunohistochemistry (Figure 14). This demonstrates that HMGB1 negatively regulates SEMA3A expression at both the mRNA and protein levels in diverse cancer cell lines in culture, including breast, lung, and melanoma, as well as in xenograft tumors.

Although we identified HMGB1 as a repressor for SEMA3A in cell lines, its role in primary human tumors was still not clear. To investigate whether HMGB1 silenced SEMA3A in primary tumors, we analyzed publicly available transcriptional microarray data from breast and lung tumors. Using transcriptional microarray data from 140 primary breast tumors we observed a significant direct correlation between HMGB1 and tumor grade, as has been previously reported (Kang, Koh et al. 2006). There was also a significant inverse correlation (-0.2, p=0.02) between HMGB1 and SEMA3A (Figure 16). A similar relationship between HMGB1 and SEMA3A was detected in a cohort of bladder, prostate and cervical tumors (Figures 17-19). Collectively, our data show that HMGB1 is required for SEMA3A silencing in various cell lines, and is significantly inversely correlated with SEMA3A in primary human tumors. This strongly suggests that HMGB1-mediated repression of SEMA3A occurs in human cancers and may contribute to their aggressiveness and grade.

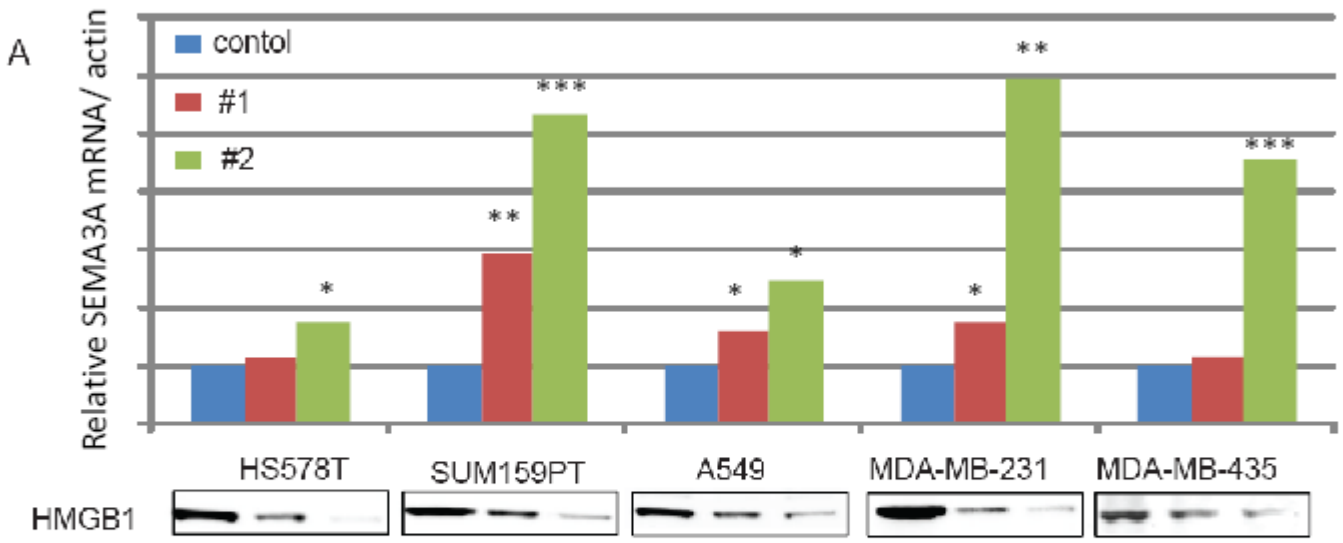

Figure 12. HMGB1 is required for SEMA3A silencing. Control shRNA or two HMGB1-targeted shRNAs were applied to indicated cell lines as described previously. Total RNA was isolated and SEMA3A expression was analyzed using RT-PCR. All experiments were conducted in triplicate, and RT-PCR was performed in triplicate. HMGB1 protein was analyzed by Western blotting.

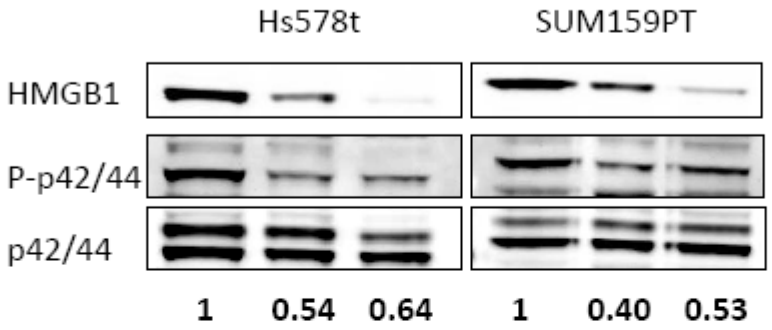

Figure 13. HMGB1 depletion in Hs578t and SUM159PT cell lines causes a decrease in basal P-ERK levels. HMGB1 knockdown was performed as previously described and P-ERK was analyzed by Western blot.

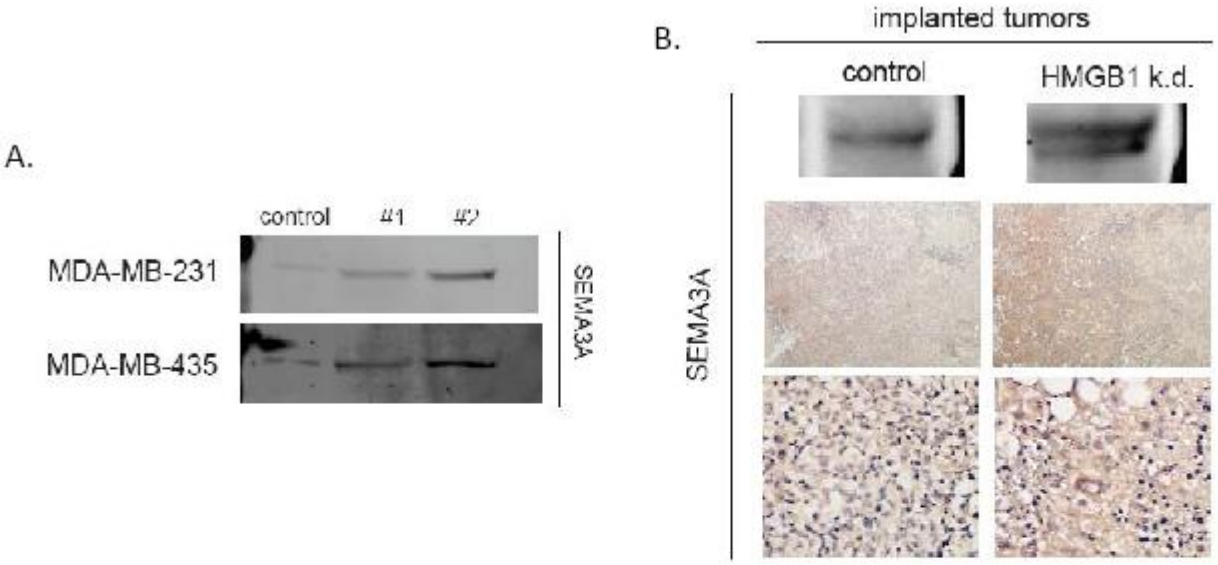

Figure 14. SEMA3A protein is upregulated in cell lines and implanted tumors after HMGB1 depletion. (A) HMGB1 was depleted from indicated cell lines and SEMA3A protein was measured by Western blot. (B) Tumors from Figure 6 were removed and analyzed by (top) Western blot or (bottom) immunohistochemistry for SEMA3A expression.

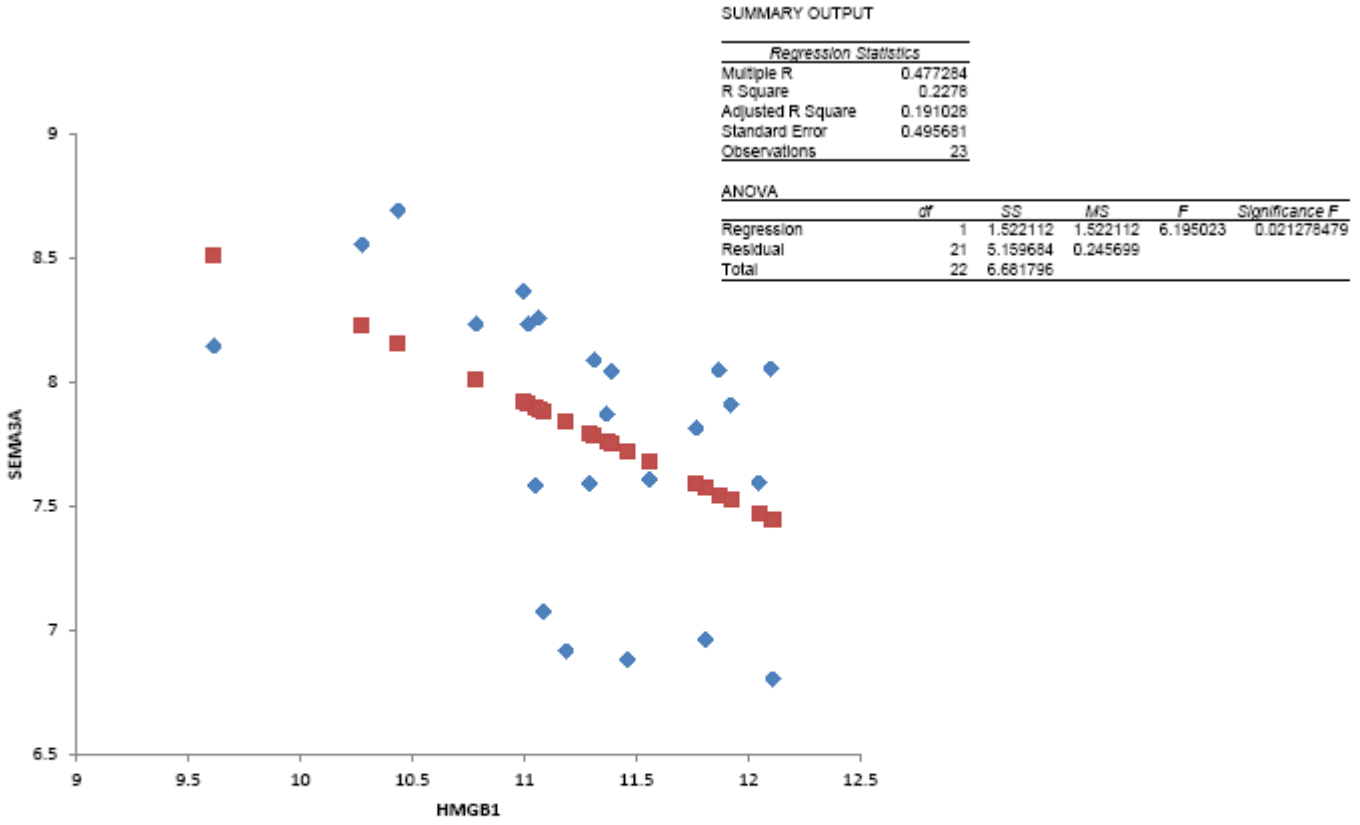

Figure 16. HMGB1 expression inversely correlates with SEMA3A in primary human breast cancers. Data was analyzed using the GEO dataset GSE20194. Actual tumor samples are in blue, optimal correlation is in red.

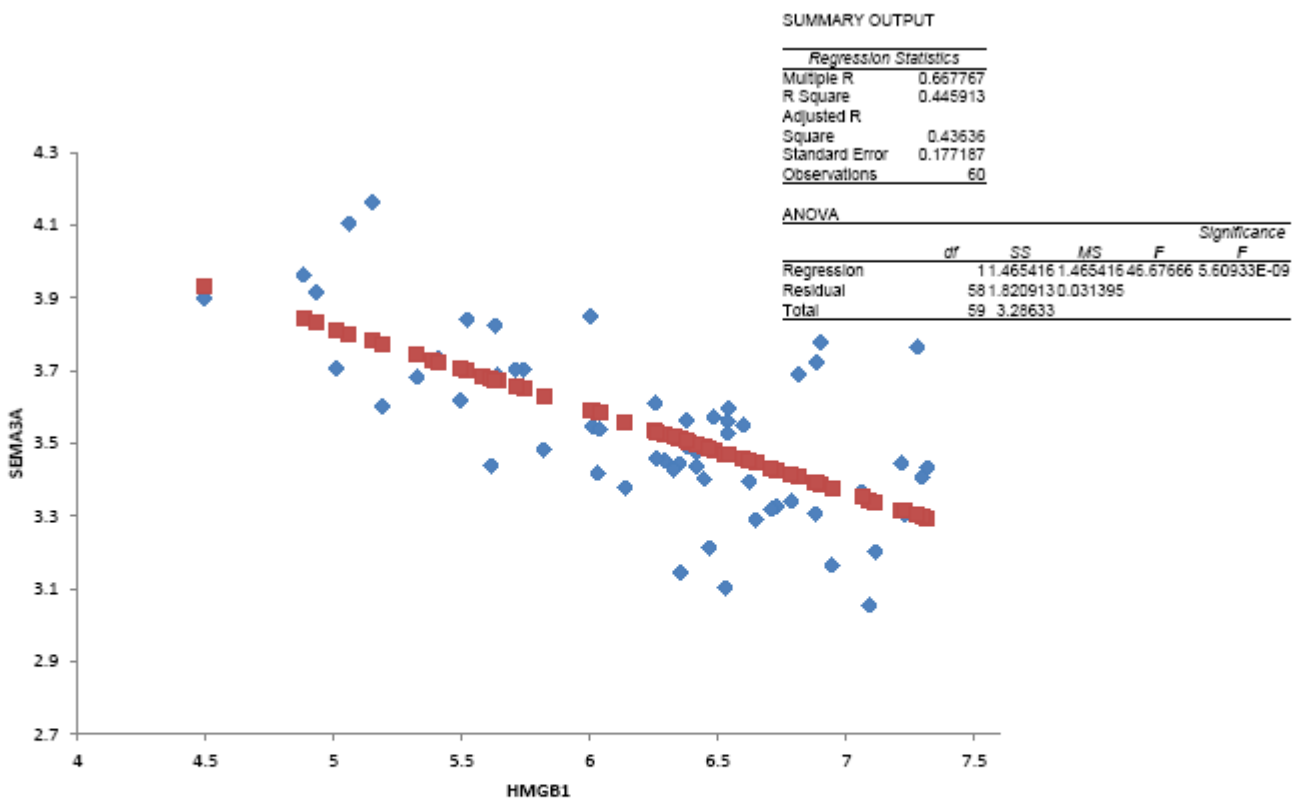

Figure 17. HMGB1 expression inversely correlates with SEMA3A in primary human bladder cancers. Data was analyzed using the GEO dataset GDS1479. Actual tumor samples are in blue, optimal correlation is in red.

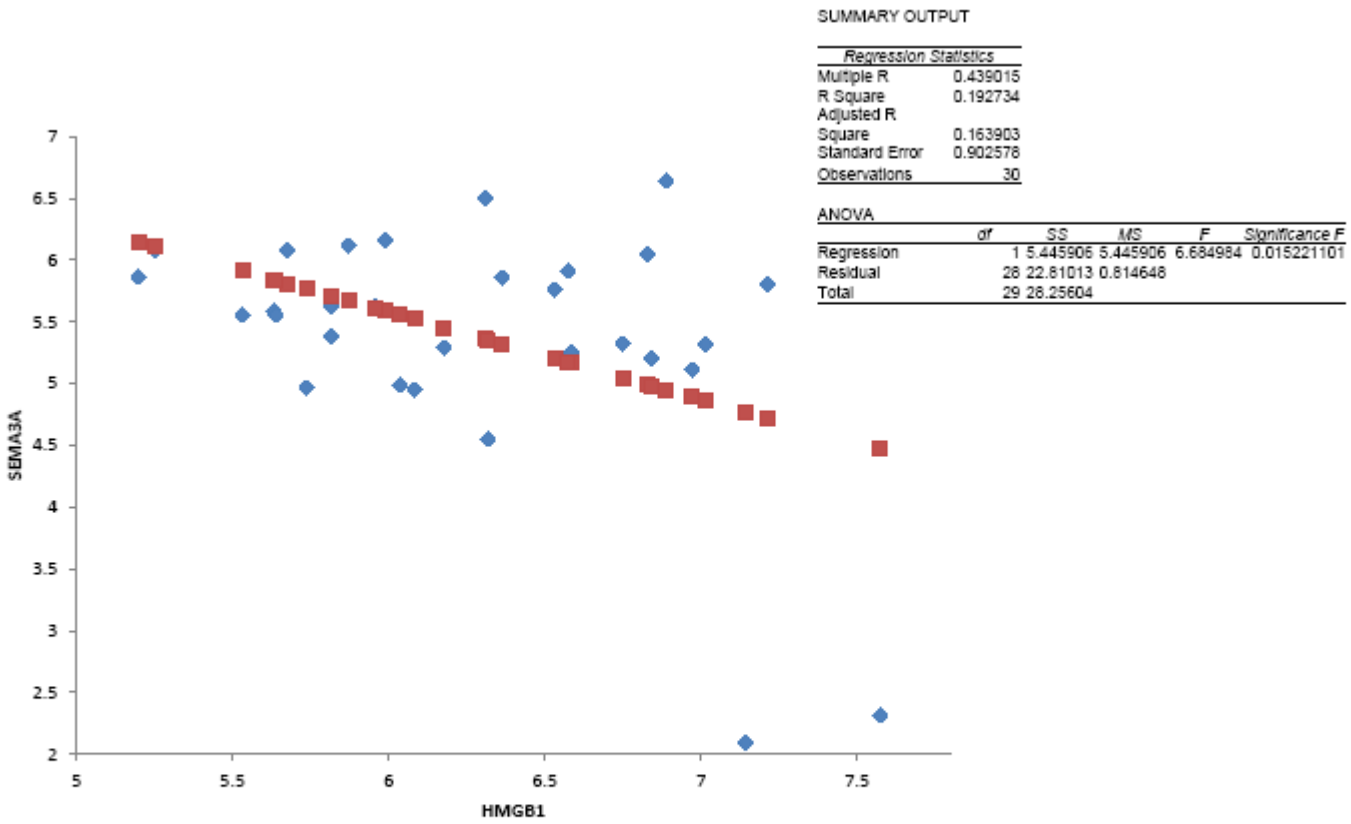

Figure 18. HMGB1 expression inversely correlates with SEMA3A in primary human prostate cancers. Data was analyzed using the GEO dataset GDS1746. Actual tumor samples are in blue, optimal correlation is in red.

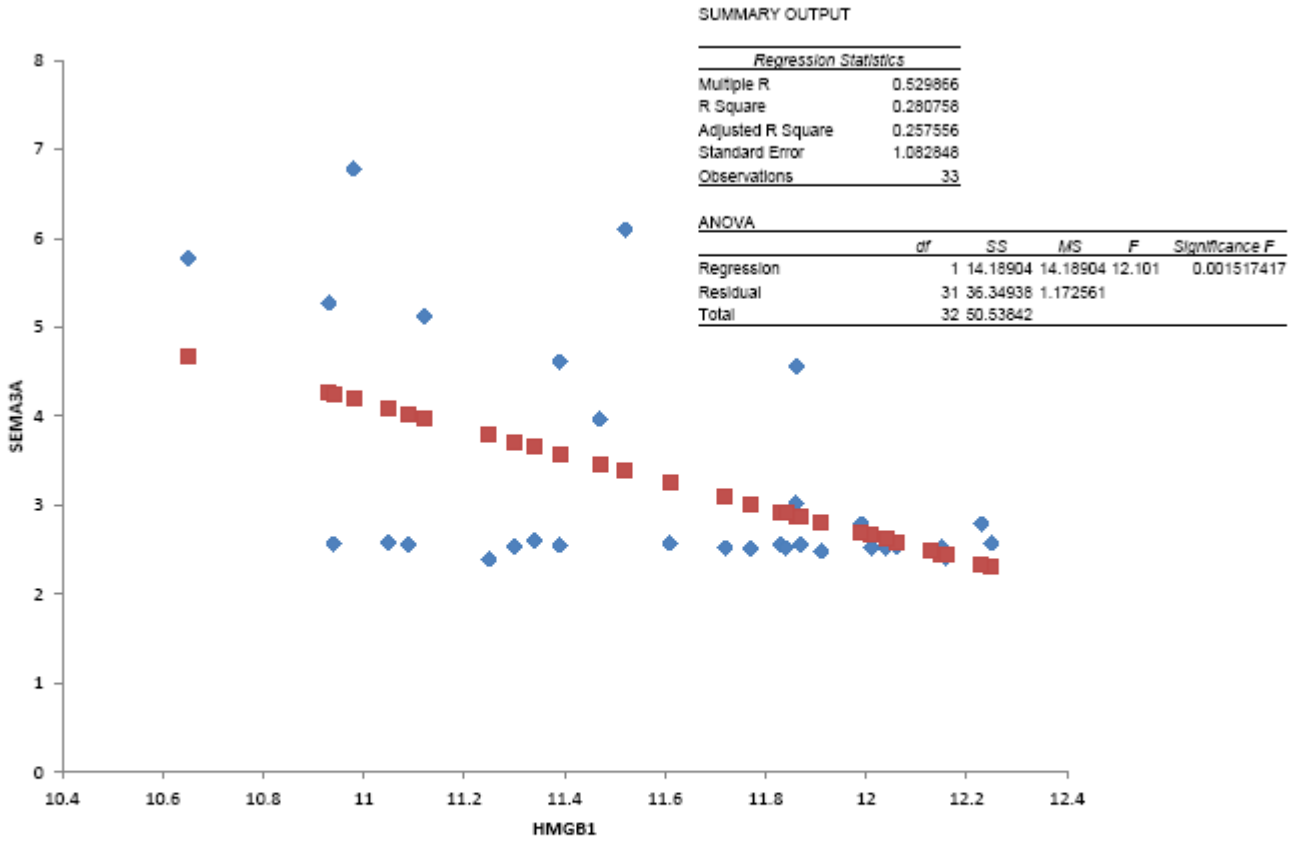

Figure 19. HMGB1 expression inversely correlates with SEMA3A in primary human cervical cancers. Data was analyzed using the GEO dataset GDS2416. Actual tumor samples are in blue, optimal correlation is in red.

SEMA3A knockdown restores F-actin localization, invasive morphology, and in vitro migration of HMGB1-deficient cells

HMGB1 knockdown caused a significant change in cell morphology and migration and also altered the expression of 167 genes, including SEMA3A. To determine the contribution of SEMA3A up-regulation to the more epithelial phenotype of HMGB1 knockdown cells, we performed double knockdowns of HMGB1 and SEMA3A (Figure 20). Double-knockdown cells largely regained the invasive morphology of the parent cells (Figure 21). F-actin was restored to the leading edges of cell processes and scattering ability in vitro was partially restored (Figure

23). As judged by scratch assay, migration was also significantly rescued by knockdown of SEMA3A (Figure 22). Although these *in vitro* characteristics were rescued by double knockdown, we were unable to observe a difference in metastatic potential *in vivo*. This was largely due to the fact that double knockdown cells showed a reduced growth rate and poor adhesion. This is likely because of adaptations that occur during adjustment to loss of HMGB1 and widespread gene expression changes. Nonetheless, our data indicates that HMGB1-mediated silencing of SEMA3A contributes to the invasive phenotype of tumor cells. This observation is consistent with previous studies that show an essential role for SEMA3A in suppressing experimental models of metastasis and invasive cell morphology. These data, in addition to the correlation between HMGB1 and SEMA3A in primary tumors, led us to further investigate the mechanism of HMGB1-mediated SEMA3A silencing.

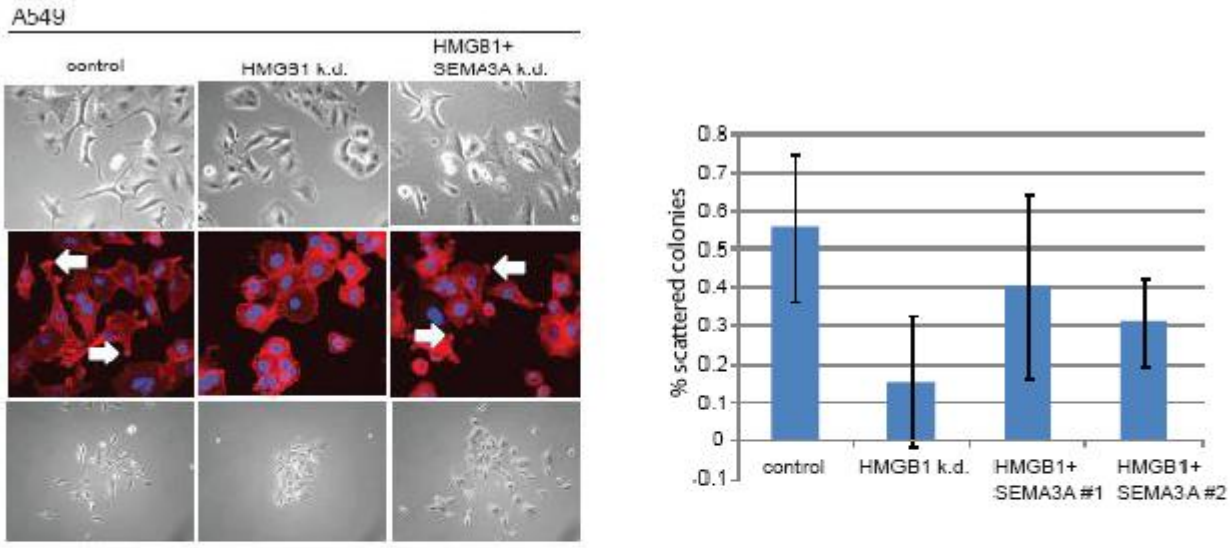

Figure 23. HMGB1/SEMA3A double-knockdown rescues morphology, F-actin and scattering ability *in vitro*. A549 cells were treated as described in Figure 20 and analyzed as described in Figure 2 and 3.

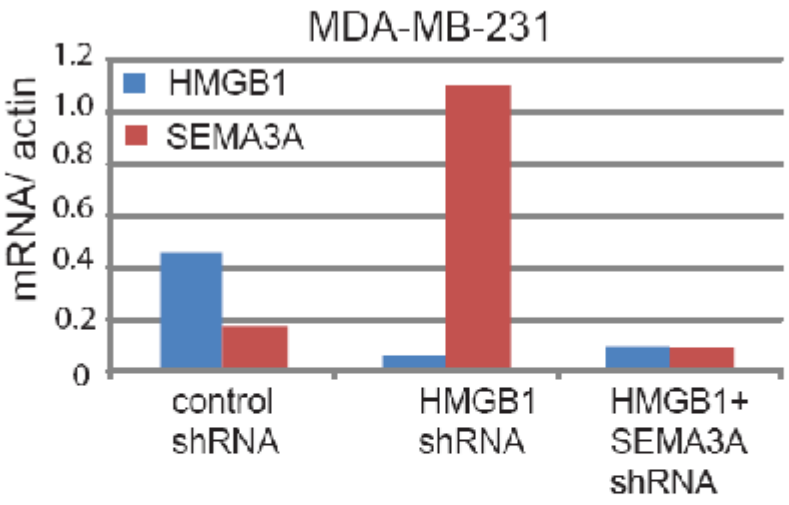

Figure 20. HMGB1 SEMA3A double knockdown cells were constructed. MDA-MB-231 cells were infected as described previously with HMGB1 or control shRNA with puromycin resistance (-puro), or HMGB1-puro plus SEMA3A shRNA with hygromycin resistance. Cells were selected with puromycin and hygromycin for one to two weeks and subsequently total RNA was isolated and analyzed by RT-PCR.

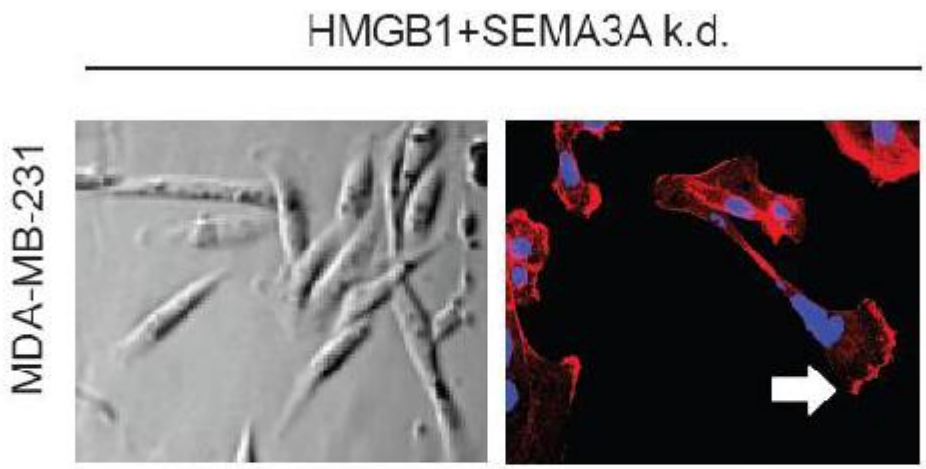

Figure 21. MDA-MB-231 HMGB1/SEMA3A double knockdown cells revert cell morphology and F-actin staining to the parental phenotype. Double-knockdowns were performed as described in Figure 20, and cells were analyzed as described in Figure 2.

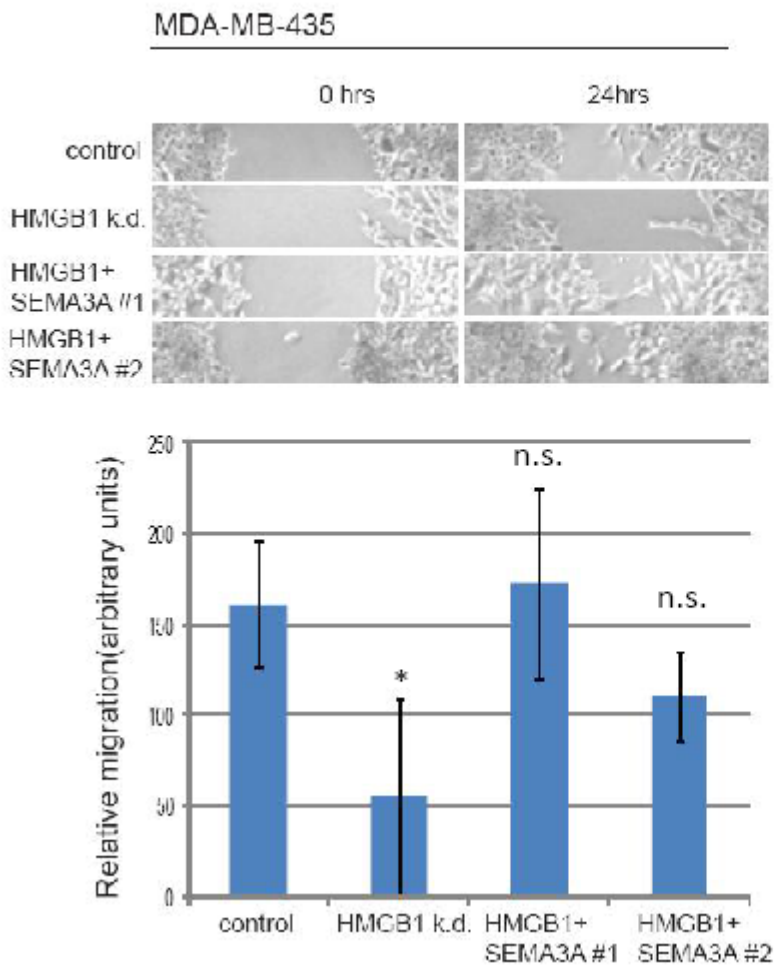

Figure 22. HMGB1/SEMA3A double knockdown rescues migration deficiency of HMGB1 single knockdown in a scratch assay. Double knockdown was performed in MDA-MB-435 cells as described in Figure 20 and migration was quantified as described in Figure 4. Two independent SEMA3A-targeted shRNAs were used. Significance was evaluated relative to control cell migration.

Nuclear HMGB1 remodels chromatin and promotes binding of acetylated histone H4 and H3 at the SEMA3A promoter

HMGB1 can influence gene expression directly in the nucleus via interactions with transcription factors, or indirectly by binding receptors and activating signaling pathways that ultimately affect transcription. Although we have ruled out the RAGE-MAPK axis in this system, extracellular HMGB1 can also signal through other receptors, such as TLR family members, to initiate a transcriptional response. In order to discriminate between the extracellular and the

nuclear effects of HMGB1 on SEMA3A regulation, we applied conditioned media from HMGB1 expressing cells to HMGB1 knockdown cells. After 4 days of treatment, cells were analyzed by RT-PCR for SEMA3A expression. Extracellular HMGB1 from control cell supernatant was not able to repress SEMA3A expression (Figure 24). These data suggest that nuclear HMGB1 is responsible for SEMA3A silencing, and led us to further investigate the mechanism of silencing at the *SEMA3A* genomic locus.

The mechanism of nuclear HMGB1-mediated transcription stimulation has been well-characterized and involves the intrinsic ability of HMGB1 to bend DNA, thus allowing greater access to transcription factors (McCauley, Zimmerman et al. 2007). In contrast, the mechanism of HMGB1-mediated gene silencing is much less understood. HMGB1 has previously been reported to associate with silenced chromatin at the TNF-alpha promoter in models of endotoxin tolerance (El Gazzar, Yoza et al. 2009). In this model, HMGB1 is recruited with histone H1 via RelB to the TNFalpha promoter, where it associates with heterochromatin protein 1 and silences transcription. In this study, HMGB1 was specifically recruited to the TNFalpha promoter and not the IkappaB promoter, explaining the locus specific effect on transcription. However, other studies that have looked globally at HMGB1 binding have not seen any locus-specific enrichment of HMGB1 (Sapojnikova, Maman et al. 2005). As in many cases of gene silencing, the El Gazzar *et al.* (El Gazzar, Yoza et al. 2009) study found increased CpG DNA methylation that correlated with reduced TNFalpha expression. Although HMGB1 was required for TNFalpha silencing in this model, it is not clear whether HMGB1 drives heterochromatin formation or is just one component of the silenced chromatin.

To investigate the mechanism of HMGB1-mediated gene silencing further, we focused on a region of SEMA3A between the transcription start site and 600bp upstream (Figure 25). This region is bound by p300, KAP1, and contains a TATA box, suggesting a key role in regulation of gene expression (http://genome.ucsc.edu/). As a control, we analyzed the homologous region in the *SEMA4F* gene, whose expression was not affected by HMGB1 knockdown in our microarray analysis. In contrast to the TNFalpha model, in our system HMGB1 bound at roughly equal levels to both HMGB1-dependent SEMA3A and HMGB1-independent SEMA4F, as revealed by chromatin immunoprecipitation experiments and RT-PCR analysis (Figure 25). This suggests that promoter binding alone is not sufficient to explain the locus-specific transcriptional repression by HMGB1 in our system, and is consistent with previous studies showing HMGB1 binding to DNA without gene specificity. Therefore, we investigated whether HMGB1 altered chromatin structure in a locus-specific manner. To study this we examined chromatin sensitivity to DNAse digestion in control or HMGB1 shRNA-expressing cells. Open chromatin or euchromatin is characterized as being more sensitive to nuclease digestion and more transcriptionally active, while closed chromatin or heterochromatin is resistant to digestion and associated with silenced genes. As shown in Figure 27, in the absence of HMGB1, the *SEMA3A* promoter became sensitized to DNAse digestion while the *SEMA4F* promoter was not significantly affected. This shows that HMGB1 participates in locus specific chromatin remodeling and is required for heterochromatin formation selectively at the *SEMA3A* promoter region, despite promiscuous binding at other promoters.

Heterochromatin is often associated with several types of epigenetic modification, including histone hypoacetylation and DNA methylation. To analyze the potential epigenetic

regulation of SEM3A expression, we first used chemical inhibitors of histone deacetylases and DNA methyltransferases trichostatin A (TSA) and 5-Azacytidine (5-Aza), respectively. If SEMA3A expression was subject to this level of regulation, we hypothesized these treatments should increase its expression in the parental MDA-MB-231 cells, which normally have low levels of SEMA3A. 5-Aza treatment increased expression of SEMA3A mRNA significantly and showed an even greater increase when used in combination with TSA (Figure 26). These data suggest that SEMA3A expression is regulated at least partially by epigenetic modification.

To test if HMGB1 altered the presence of acetylated histones in a locus specific manner, we used chromatin immunoprecipitation with an antibody to acetylated histone H4 with or without HMGB1 knockdown. As shown in Figure 28, loss of HMGB1 increased acetylated histone H4 (Ac-H4) occupancy at the *SEMA3A* promoter by 9-fold compared IgG control immunoprecipitation. In contrast, binding of Ac-H4 increased to a lesser degree at the *SEMA4F* promoter at about 3-fold.

Although it has been noted that HMGB1 can associate with methylated DNA and the methyl-CpG binding protein, a causative role for HMGB1 in DNA methylation has not been specifically addressed. To determine the effect of HMGB1 on methylation we used bisulfite sequencing to analyze genomic DNA from control and knockdown cells. We focused on an area of the *SEMA3A* promoter that undergoes remodeling to an open state in the absence of HMGB1 (Figure 27). This region contains five CpG residues. CpG dinucleotides are recognized by DNMT family members, which then methylate the cytosine on its 5’ position. For all of the CpG residues examined, we observed no detectable methylation in the presence or absence of HMGB1, indicating that heterochromatin formation and gene expression at this site is

independent of CpG methylation in this region (Figure 29). However, in a few samples, we were able to observe non-CpG methylation in distinct positions within this region. Intriguingly, both sites occurred at an almost identical position respective to a CCCTC-binding factor (CTCF) motif (Figure 29). CTCF is a transcriptional regulator that is capable of exerting either a positive or negative influence on transcription depending on which cofactors it binds (Zlatanova and Caiafa 2009; Weth and Renkawitz 2011). CTCF has also been previously reported to bind near this genomic locus in several studies (http://genome.ucsc.edu/). These connections led us to investigate whether HMGB1 influenced binding of CTCF at the *SEMA3A* promoter region.

To test for this, we performed a chromatin immunoprecipitation for CTCF in control or HMGB1 knockdown cells. As shown in Figure 30, loss of HMGB1 resulted in a significant increase in the occupancy of CTCF at the *SEMA3A* promoter. Although occupancy increased at *SEMA3A*, there was not as large of an increase at the *SEMA4F* control region, despite both regions harboring CTCF-binding sequences. This binding pattern is very similar to what we observed for Ac-H4 in the absence of HMGB1. These data support the conclusion that HMGB1 is required for heterochromatin formation at the SEMA3A promoter that is independent of CpG DNA methylation, but is associated with decreased binding of acetylated histone H4 and CTCF.

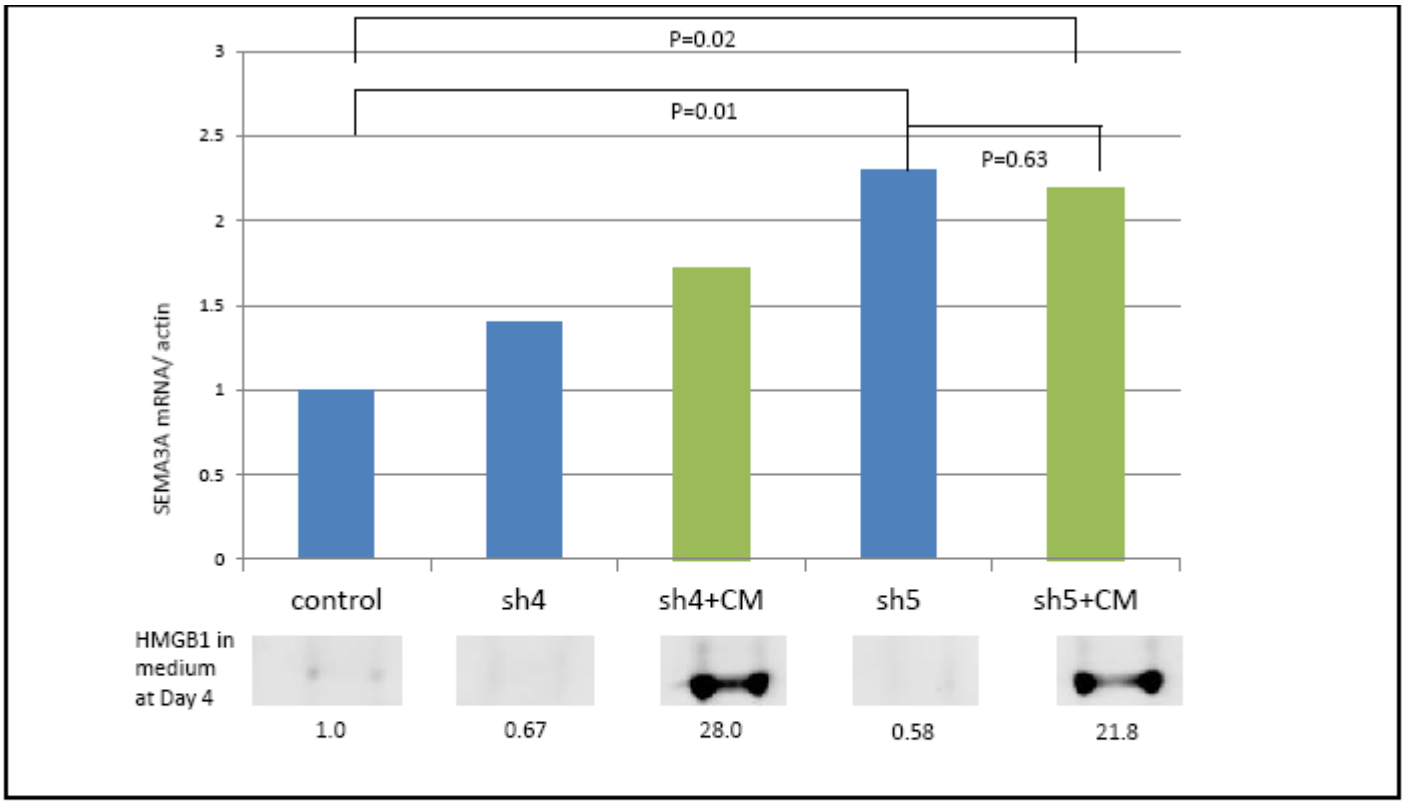

Figure 24. Conditioned media containing high levels of extracellular HMGB1 is not sufficient to silence SEMA3A expression. Conditioned media from confluent control shRNA-expressing MDA-MB-231 cells was collected after 3 days in culture, filtered through 0.45 uM filter and applied to HMGB1-knockdown cells. Two independent shRNA knockdowns (sh4, sh5) were analyzed. SEMA3A expression after 4 days with conditioned or normal media applied daily was detected by RT-PCR analysis.

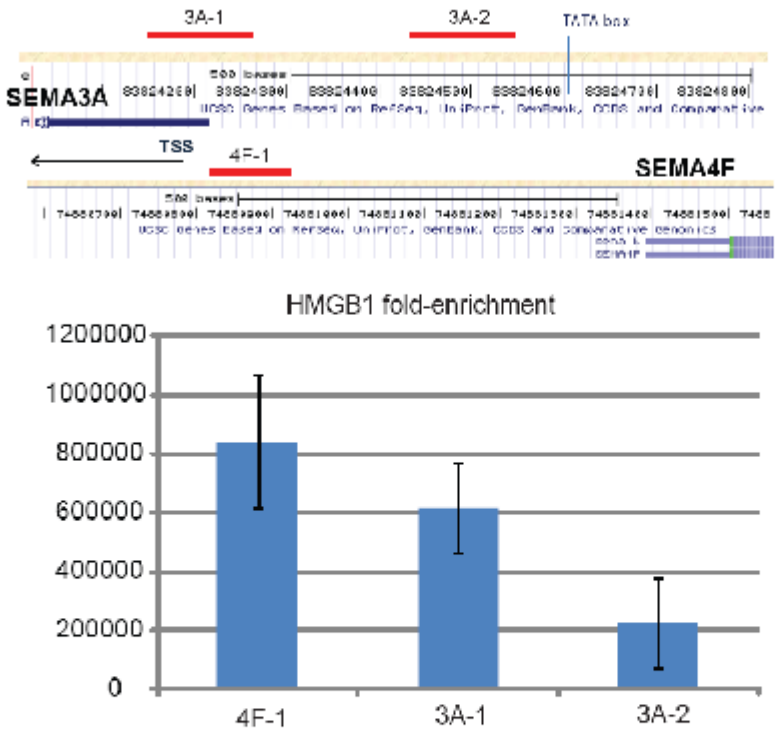

Figure 25. HMGB1 binds at the *SEMA3A* and *SEMA4F* genomic loci. (A) Regions of *SEMA3A* and *SEMA4F* analyzed. Genomic regions and transcriptional start sites (TSS) are as annotated in the Santa Cruz Genome Browser. Primer-amplified regions are highlighted in red. (B) Chromatin immunoprecipitation and subsequent RT-PCR analysis was performed as described in methods. Fold-enrichment was calculated relative to isotype control.

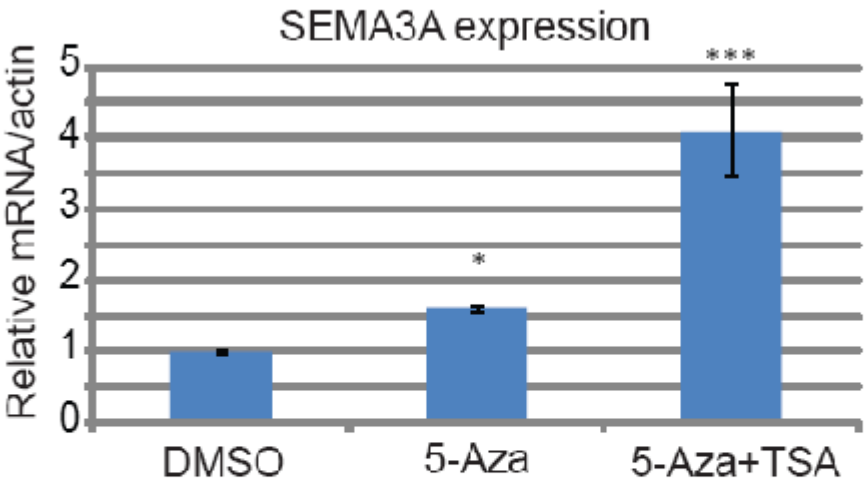

Figure 26. 5-Azacytidine (5-Aza) and Trichostatin A (TSA) increase SEMA3A mRNA expression. 5uM 5-Aza, DMSO or 5-Aza + TSA was applied to MDA-MB-231 cells for three days. 100 nM TSA was applied on Day 2. Total mRNA was collected and analyzed by RT-PCR.

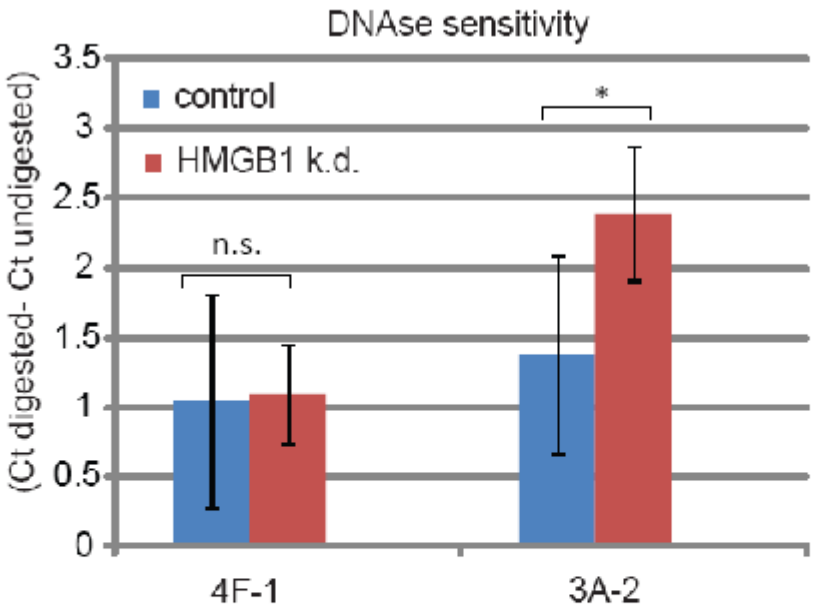

Figure 27. HMGB1 depletion increases DNAse sensitivity at the *SEMA3A* locus. HMGB1 knockdown was performed as previously described in MDA-MB-231 cells. DNAse sensitivity was performed using the BioRad EpiQ kit per manufacturers instructions.

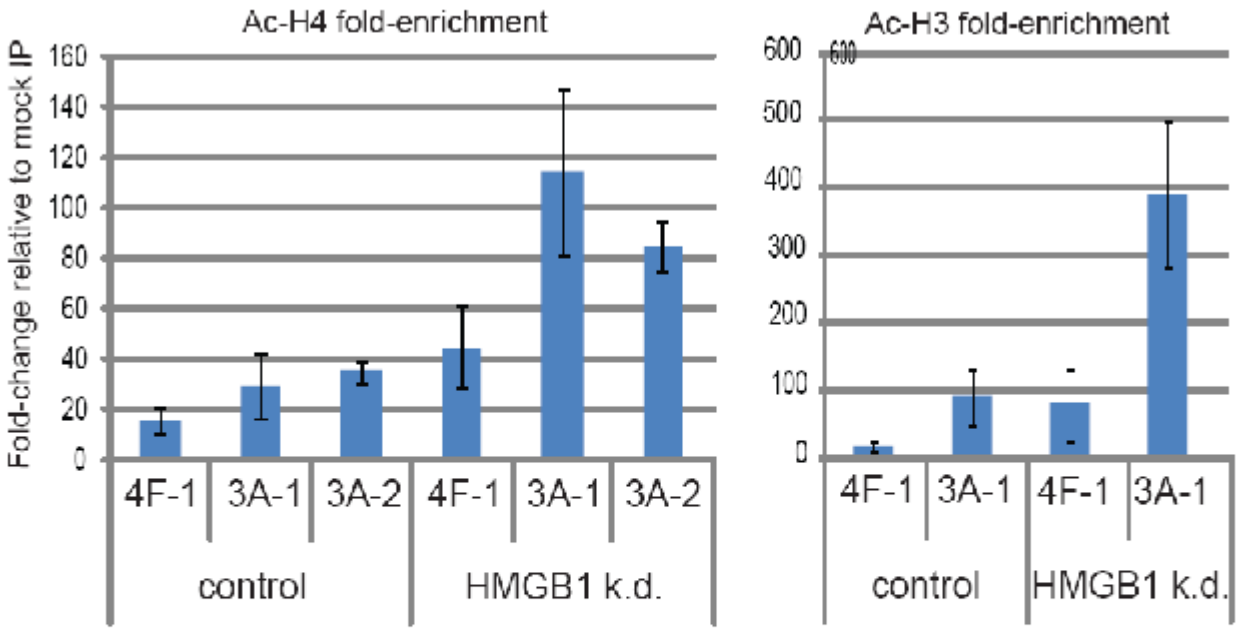

Figure 28. Acetylated histone H4 and H3 increase occupancy at the *SEMA3A* locus after HMGB1 knockdown. HMGB1 knockdown in MDA-MB-231 cells was performed as previously described. Chromatin immunoprecipitation and RT-PCR analysis was performed as described in Figure 24.

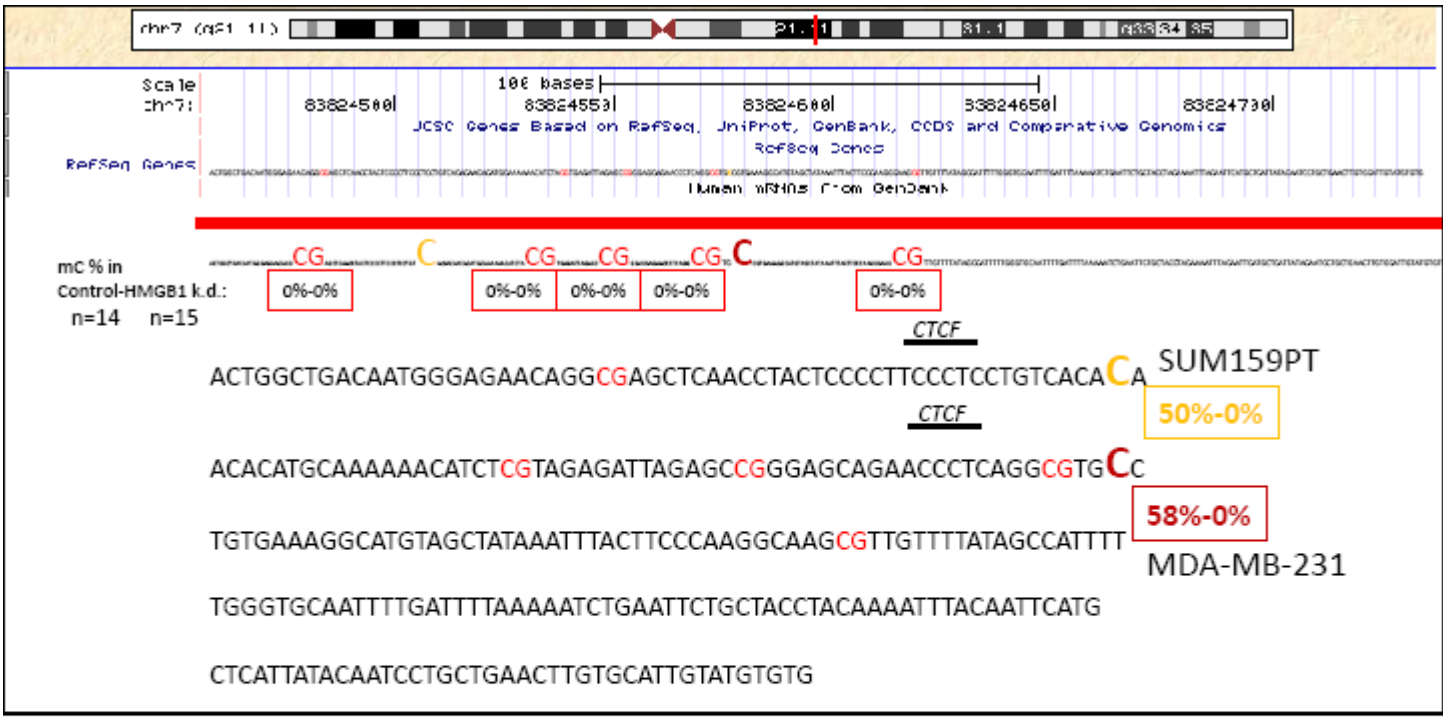

Figure 29. HMGB1 knockdown does not affect CpG methylation in the SEMA3A promoter but decreases non-CpG methylation. Methylation analysis of displayed region was performed using bisulfite conversion, subcloning and sequencing. A minimum of 10 clones were used for each experiment. Red boxes (top) indicate CpG residues in which no difference in methylation was detected after HMGB1 knockdown. Orange and red boxes (right) indicate non-CpG cytosine methylation detected, shown as % control-% HMGB1 knockdown. CTCF binding sequences are italicized.

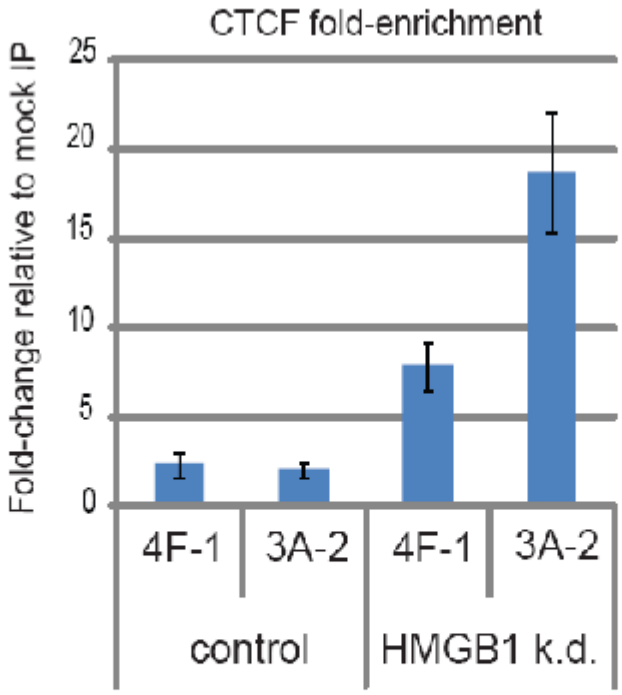

Figure 30. HMGB1 knockdown increases CTCF occupancy at the SEMA3A locus. Knockdown was performed in MDA-MB-231 cells as previously described. Chromatin immunoprecipitation and RT-PCR analysis was performed as described in Figure 24.

2.5 Discussion

We have identified a role for HMGB1 in promoting tumor growth, cell migration and metastasis that is independent of its previously reported function as an extracellular ligand that activates the MAPK pathway. Although HMGB1 can contribute to MAPK signaling, in the presence of constitutive activation of this pathway, HMGB1 is still required for tumor cell migration and metastasis, as well as tumor growth. We have shown that at least part of the HMGB1 migratory phenotype is mediated by transcriptional repression of SEMA3A. SEMA3A, and generally the class 3 semaphorins are increasingly being recognized as key suppressors of

tumor growth and metastasis. Our findings suggest that overexpression of HMGB1 is one mechanism that contributes to semaphorin silencing in cancer.

In vivo, we showed a significantly reduced growth rate of HMGB1 knockdown tumors despite no difference in proliferation *in vitro*. This reduction in growth correlated with increased necrosis and reduced angiogenesis in the HMGB1-deficient tumors. These tumors also showed higher levels of SEMA3A *in vivo*. SEMA3A is a well-established inhibitor of angiogenesis in various experimental systems. Although we did not formally investigate the possibility here, it is plausible that increased SEMA3A expression contributed to the reduced angiogenesis and defective growth of HMGB1 knockdown tumors.

Although SEMA3A was clearly important for reduced F-actin staining and the epithelial morphology of the HMGB1 knockdown phenotype, it was only one of ~200 genes targeted by HMGB1 and it is likely that the other HMGB1 targets also contribute to the pro-tumor phenotype associated with HMGB1 expression. It is of note that although HMGB1 has no intrinsic sequence specificity in binding DNA, we nonetheless observed altered expression at only specific gene targets during knockdown. We and others have shown that HMGB1 is not differentially enriched for binding at specific loci, regardless of its differential effect on specific gene expression. It is possible that HMGB1 achieves gene specificity due to being a required cofactor only at certain loci, making these genes especially sensitive to HMGB1 expression levels. Our work shows that HMGB1 participates in gene silencing through driving chromatin remodeling independently of CpG methylation, but associated with binding of acetylated histone H4. These findings suggest that those gene targets that are particularly sensitive to histone deacetylase inhibitors may also be sensitive to HMGB1 inhibition.

The inverse correlation between HMGB1 and SEMA3A that we observed in primary tumor transcriptional microarray data suggest that HMGB1-mediated repression of SEMA3A might occur in human tumors. Although correlative data are not definitive evidence of a mechanistic relationship; in light of the causative and mechanistic role for HMGB1 in cell lines, this correlation may be more relevant. SEMA3A is normally expressed in the developing brain where it regulates dendrite and axon branching. HMGB1 also is expressed highly in neurons and plays a role in these processes. Although past studies have focused on extracellular HMGB1 signaling in the brain, it is interesting to speculate that nuclear HMGB1 may also have key functions in this context with respect to SEMA3A regulation.

HMGB1 has been recognized as a tumor promoting factor for many years, but the majority of work has focused on its role as an extracellular ligand. Owing to this, several proposed therapies seek to neutralize, block release of or inhibit signaling by extracellular HMGB1. Our studies shed light on the significant role nuclear HMGB1 has in promotion of tumor growth and metastasis, independently of its role as a ligand. Our findings suggest that future cancer therapies should consider targeting nuclear HMGB1 and emphasize the important pro-tumor functions of HMGB1 even in malignancies that harbor MAPK mutations.

Chapter 3:

PARP-dependent release of HMGB1 during Adenovirus infection

The data contributing to this work was all generated by Mike Nehil, with the exception of mass-spectrometry analysis and protein identification, which was performed by Robert Chalkey.

3.1 Introduction

Oncolytic viruses hold great promise as a cancer therapy. By selectively replicating in cancer cells and spreading to and killing other nearby tumor cells, they have the potential to be both highly cancer-targeted and self-amplifying. Cancer specificity can be achieved in many ways, and several diverse approaches are currently underway to achieve this (Toth and Wold 2010). They include tumor-specific promoters (Huang, Kaku et al. 2010) and deletion mutants that can replicate only in permissive tumor cells (McCormick 2003). There are presently several viruses in clinical trials for cancer therapy (Breitbach, Burke et al. 2011). In earlier trials with these viruses, it was shown that one of the main complications in making an effective treatment is the host immune response. Several lines of evidence have demonstrated that the immune response can target and eliminate oncolytic viruses, thus preventing them from fully spreading within and killing the tumor cells (Bortolanza, Bunuales et al. 2009; Dhar, Spencer et al. 2009; Raki, Sarkioja et al. 2011; Weibel, Raab et al. 2011). However, in other contexts an immune response can effectively break tolerance to the tumor and promote rejection of the

tumor (Melcher, Parato et al. 2011). The immune response during oncolytic therapy is complex and requires better understanding for more effective future therapies.

Adenovirus is one of the earliest and best studied viruses used as an oncolytic therapy. It is an encapsidated double-stranded DNA virus that typically causes mild respiratory infections in young people. It is not usually a fatal virus because of a robust immune response and antibody production in reaction to infection that quickly neutralizes the virus (Ginsberg and Prince 1994). An immune response to viral infection is complex and is elicited by both viral proteins directly and host factors released from infected cells. The importance of host proteins in promoting a response is underscored by several examples of an inability to eliminate virus in animals that are deficient for these signal proteins (Chintakuntlawar and Chodosh 2009; Nguyen, Procario et al. 2011).

One such “danger” signal protein is HMGB1. When cells die by necrosis, HMGB1 is released from the chromatin and subsequently into the extracellular space (Scaffidi, Misteli et al. 2002). Outside of the cell, HMGB1 interacts with the Receptor for Advanced Glycation End Products (RAGE) as well as TLR2 and 4 receptors on a number of types of immune cells and triggers a powerful inflammatory response (van Beijnum, Buurman et al. 2008). The essential role for HMGB1 in inflammation is highlighted by the fact that neutralizing antibodies against HMGB1 alone are able to prevent up to 70% of the lethality from a septic shock response due to LPS (Wang, Bloom et al. 1999). Since this discovery, many different studies have shown that HMGB1 is critical in a variety of inflammatory diseases. There are a few examples of viruses

inducing the release of HMGB1 (Alleva, Budd et al. 2008), but the mechanism of release is unknown. HMGB1 release has not yet been examined during Adenovirus infection.

In light of the fact that the immune system is a major barrier for effective oncolytic viral therapies and HMGB1 is a major activator of the immune system, we examined the mechanism of HMGB1 release during Adenovirus infection.

3.2 Materials and Methods

Cells and Adenovirus- Wild-type Adenovirus type 5 was purchased from ATCC. All infections were performed at a multiplicity of infection (MOI) of 25 in 10% FBS containing DMEM. MDA-MB-231 and SUM159PT cells were gifts from Amy Young and Madhu Macrae, respectively. RAW264.7 cells were purchased from ATCC.

Antibodies- Rabbit polyclonal anti-HMGB1, anti-H2B and anti-Ad5 late proteins were purchased from Abcam. Anti-PAR was purchased from EMD Biosciences.

MTS assay- MTS assay was purchased from Promega and performed according to the manufacturer's directions.

2-dimensional (2D) Western blotting- Whole cell lysates were made in buffer containing 8 M urea. Protein content was quantified with Bradford assay. Three hundred micrograms total protein was loaded onto 3-10pH 2D strips (BD Biosciences) and focused for a total of 60,000 volt-hours. Strips were then loaded onto a 4-12% Tris-glycine gel (Invitrogen) and separated by

electrophoresis. Gels were then transferred to nitrocellulose and blotted by standard Western blot protocol for the indicated proteins.

Immunoprecipitation- Lysates were made using 1% Triton-X lysis buffer (1% Triton-X, 150mM NaCl, 1mM EDTA, 1mM EGTA, 10mM Tris-HCl (pH 7.4), 10mM NaF, and protease inhibitors (Complete mini, Roche)). Protein was quantified with Bradford assay and 0.5mg-1mg of total protein was incubated overnight at 4 degrees with 4ug total of indicated antibodies or isotype controls. After several washes, beads were boiled in SDS containing loading buffer and subsequently gel separated and Western blotting performed.

3.3 Results

HMGB1 release during Adenovirus infection is PARP-dependent

Adenovirus very effectively infects and replicates in many cancer cell lines in vitro. This system has been used extensively to study Adenovirus life cycle and host cell interactions with the virus (O'Shea 2005). We focused on 2 breast cancer cell lines that are productively infected by Adenovirus, MDA-MB-231 and SUM159PT. In both of these cell lines, Adenovirus infection caused HMGB1 translocation from the nucleus to cytoplasm and subsequent release into the extracellular medium (Figure 34, Figure 31). HMGB1 was first detected in the cell supernatant by western blot at about 72 hours post-infection, following translocation to the cytoplasm at about 48 hours. Previous work has shown that during DNA damage-induced necrosis, HMGB1 cytoplasmic translocation and release is dependent on the nuclear enzyme poly-ADP-ribose

polymerase (PARP) (Ditsworth, Zong et al. 2007). In this model, high levels of MNNG, a DNA-damaging agent, causes PARP activation and release of HMGB1. Adenovirus is also known to elicit a DNA-damage response through viral replication (Cuconati, Mukherjee et al. 2003), raising the possibility that HMGB1 release during infection was also PARP-dependent. To investigate this possibility, we first analyzed PARP activity during infection. One of the main targets for poly-ADP-ribosylation is PARP itself through automodification (Soldatenkov and Smulson 2000). As shown in Figure 32, PARP shifts to a series of higher molecular weight species during infection as judged by western blot. These species also react with poly-ADP-ribose antibody, as do many other proteins during infection. Importantly, both the higher molecular weight species of PARP and the poly-ADP-ribose reactive bands are abolished by treatment with either of two PARP inhibitors 1,5-Dihydroxyisoquinoline (DIQ) or 3-aminobenzamine (3AB) (Figure 32), indicating these modifications truly represent poly-ADP-ribosylation. These data show that PARP becomes activated during Adenovirus infection.

To test the requirement for PARP activity in Adenovirus-mediated HMGB1 release, we used two different PARP inhibitors during Adenovirus infection. Cells were infected with Adenovirus and treated with PARP inhibitors at the start of infection and translocation and release of HMGB1 were examined over time. Treatment with either DIQ or 3AB caused a significant decrease in release of HMGB1 into the medium (Figure 33) as well as preventing cytoplasmic translocation (Figure 34). Importantly, HMGB1 becomes translocated to the cytoplasm at least 24 hours prior to detectable levels of HMGB1 in the supernatant. As discussed below, cytoplasmic translocation also occurs prior to significant cell death. This

finding closely mimics what was observed in the Ditsworth *et al.* study (Ditsworth, Zong et al. 2007), and suggests an active process in which HMGB1 is shuttled out of the nucleus prior to being released during cell death, as opposed to "passive" release from the nucleus concurrent with cell death. Our findings show that HMGB1 is translocated to the cytoplasm and subsequently released from cells via a mechanism that is dependent on PARP enzymatic activity.

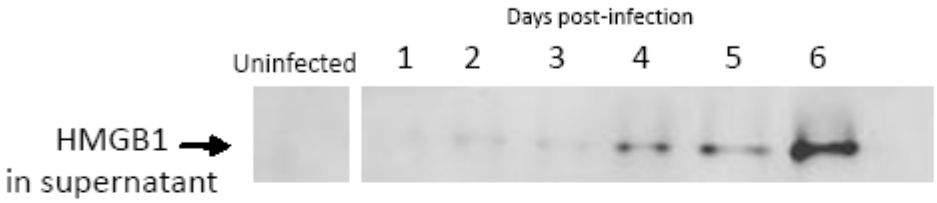

Figure 31. HMGB1 is released from Adenovirus infected cells. MDA-MB-231 cells were infected with wild-type Adenovirus Type 5 at a multiplicity of infection (MOI) of 25. Cells were infected in 6-well plates with 1.5mls of 10% FBS-containing DMEM media. At indicated times, 80uL of supernatant was removed, filtered through a 0.45uM filter, and analyzed by Western blot.

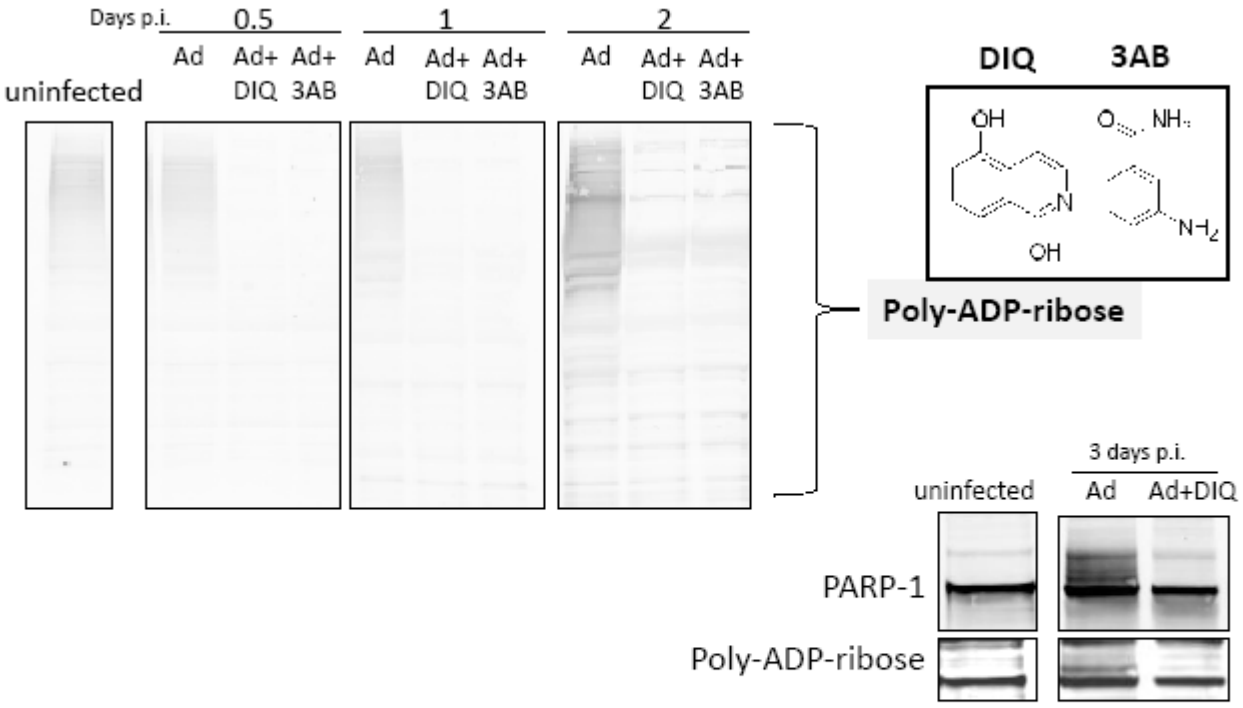

Figure 32. PARP is activated during Adenovirus infection. MDA-MB-231 cells were infected as described in Figure 31 with or without 300 uM DIQ or 500uM 3AB. Total lysates were collected at indicated times and analyzed by Western blot for Poly-ADP-ribose or PARP-1.

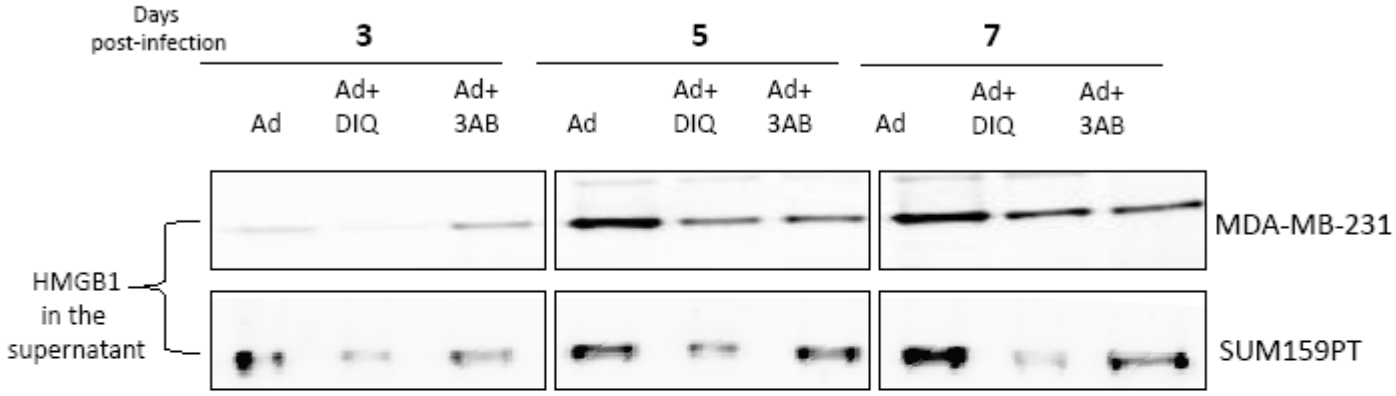

Figure 33. Adenovirus-mediated release of HMGB1 is PARP-dependent. MDA-MB-231 or SUM159PT cells were infected as previously described with or without 300uM DIQ or 500uM 3AB. Supernatant levels of HMGB1 were measured as described in Figure 31.

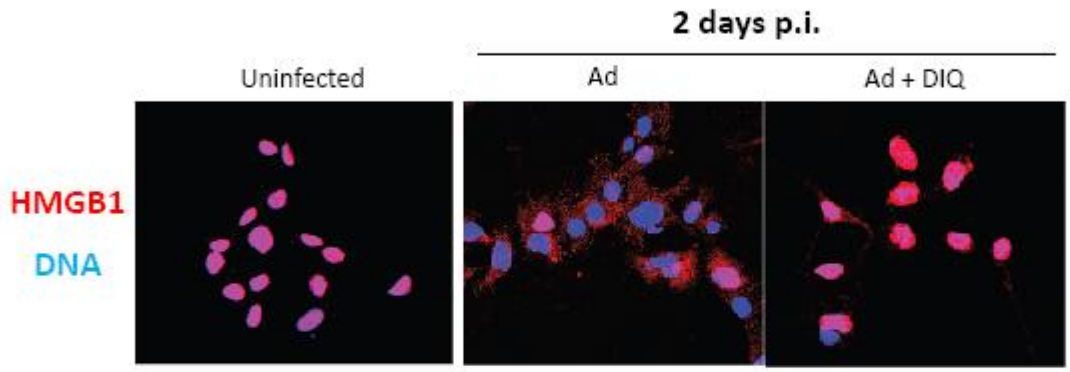

Figure 34. PARP inhibitors block cytoplasmic translocation of HMGB1 during Adenovirus infection prior to release. Adenovirus (Ad) infected cells were analyzed by immunofluorescence at two days post-infection with or without 300uM DIQ.

PARP inhibitors block HMGB1 release independently of affecting cell death or lysis

Although we observed a decrease in HMGB1 release during PARP inhibition, it was not clear whether this was a specific effect on the mechanism of HMGB1 release, or a general inhibition of cell death and lysis, which would also be expected to delay release. Indeed, previous studies have shown that PARP-inhibition can delay cell death induced by DNA damaging agents (Zong, Ditsworth et al. 2004). To examine cell death during Adenovirus infection we used MTS and propidium iodide assays. MTS measures mitochondrial reducing

potential and thus cell viability. Propidium iodide measures membrane permeability to negatively charged molecules and thus the level of intact electronegative potential. With both assays, we observed no significant difference in cell death during Adenovirus infection in the presence or absence of PARP inhibition (Figure 35, Figure 36). These data show that PARP inhibitors block Adenovirus-mediated HMGB1 release without affecting the ability of the virus to kill the cells. Consistent with this result, we also observed no difference in levels of late viral proteins released into the medium during cell lysis (Figure 37). Since late viral protein production is dependent on early protein production and DNA replication, this shows that the phases of the viral life cycle leading up to cell lysis and death are similarly largely not affected by PARP inhibition.

Previous work has shown that cells release HMGB1 specifically after necrotic death and not apoptotic death (Scaffidi, Misteli et al. 2002). During apoptosis, HMGB1 becomes permanently associated with the DNA bodies. The mode of death during Adenovirus is not well understood but is independent of the classical apoptotic proteases, the caspases. Therefore, it was possible that PARP inhibition caused HMGB1 to remain associated with the chromatin as a result of activating apoptotic pathways. To test for this possibility, we examined caspase targets for cleavage during infection with or without PARP inhibition. As shown in Figure 38, infection in the presence of DIQ does not cause any substantial increase in cleaved caspase 3, PARP-1 itself, or spectrin. Etoposide was used as a positive control and clearly induces these cleavage products. These data show that the ability of PARP inhibitors to block release of HMGB1 during infection cannot be explained by an increased propensity toward apoptotic death.

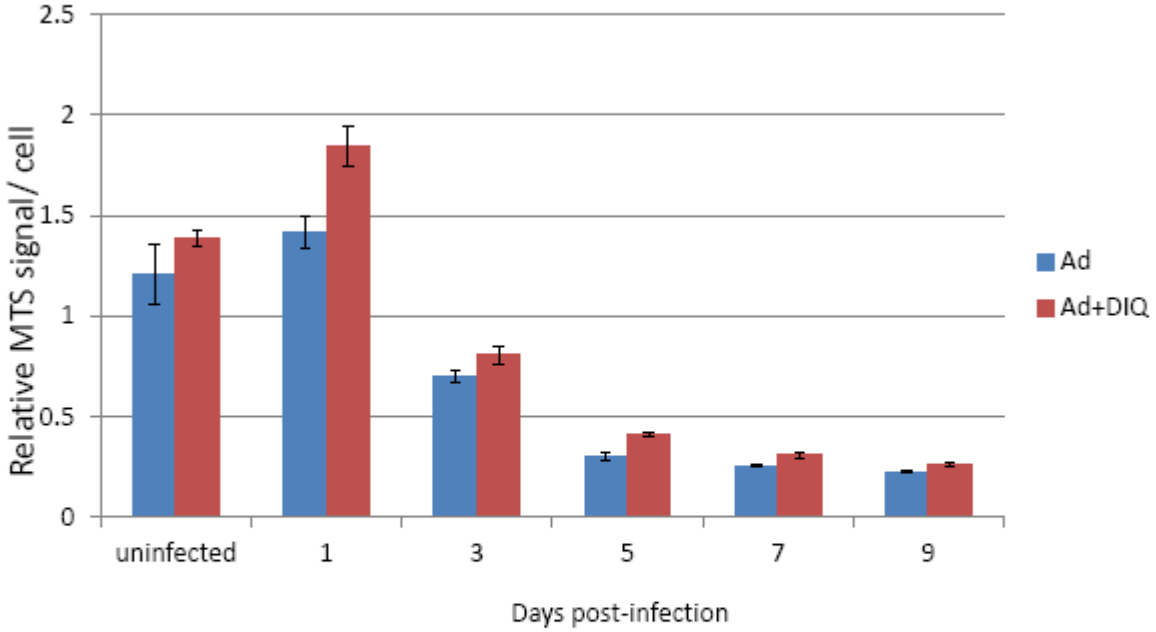

Figure 35. PARP inhibitors do not significantly prevent Adenovirus-mediated cell death as judged by MTT assay. MDA-MB-231 cells were infected as previously described in the presence or absence of 300 uM DIQ. Cell viability was measured using MTS assay per manufacturers instructions.

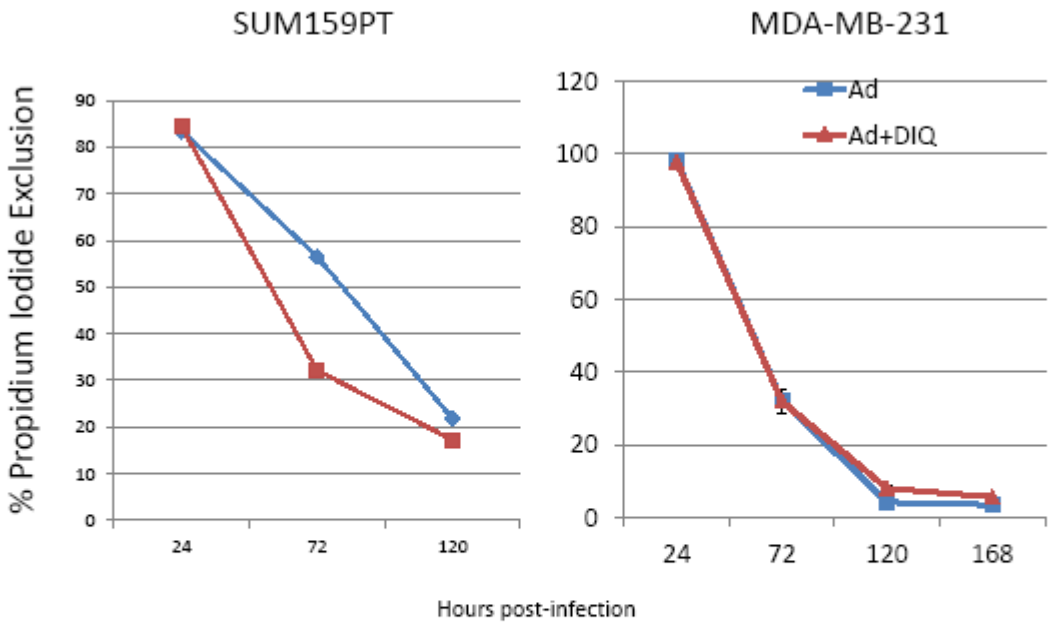

Figure 36. PARP inhibitors do not significantly prevent Adenovirus-mediated cell death as judged by propidium iodide (PI) exclusion assay. SUM159PT or MDA-MB-231 cells were infected with Adenovirus in the presence or absence of 300uM DIQ as previously described. PI positive cells were measured and compared to negative cells using flow-cytometry.

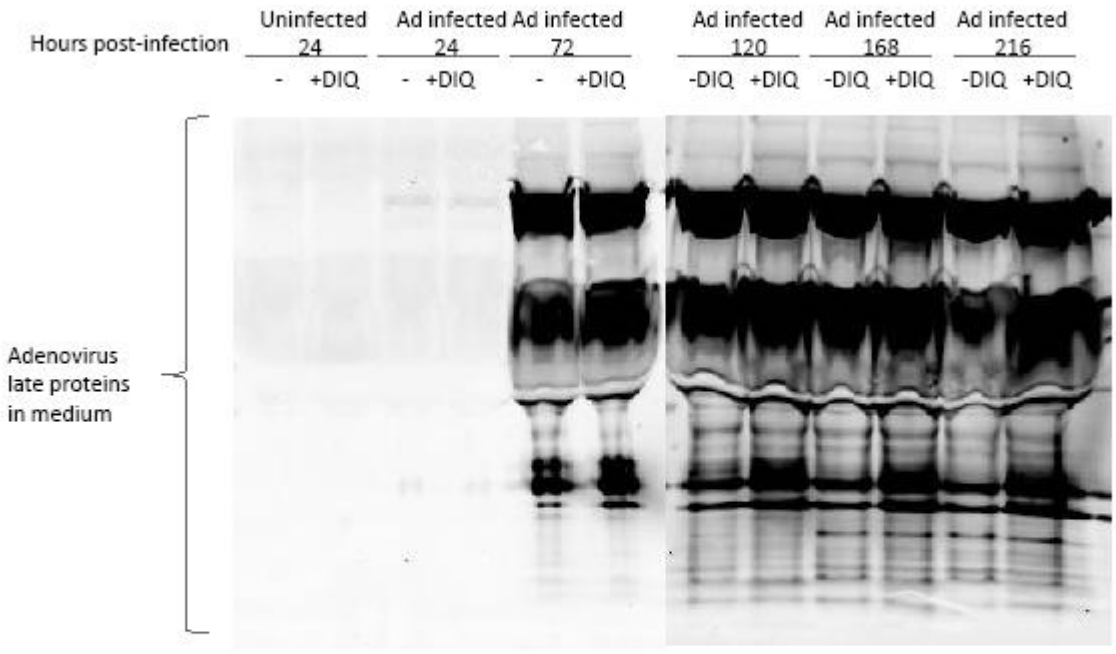

Figure 37. PARP inhibitors do not decrease late viral protein production or cell lysis. MDA-MB-231 cells were infected as previously described. Supernatant was analyzed by Western blot using a rabbit polyclonal antibody to Ad5 late viral proteins.

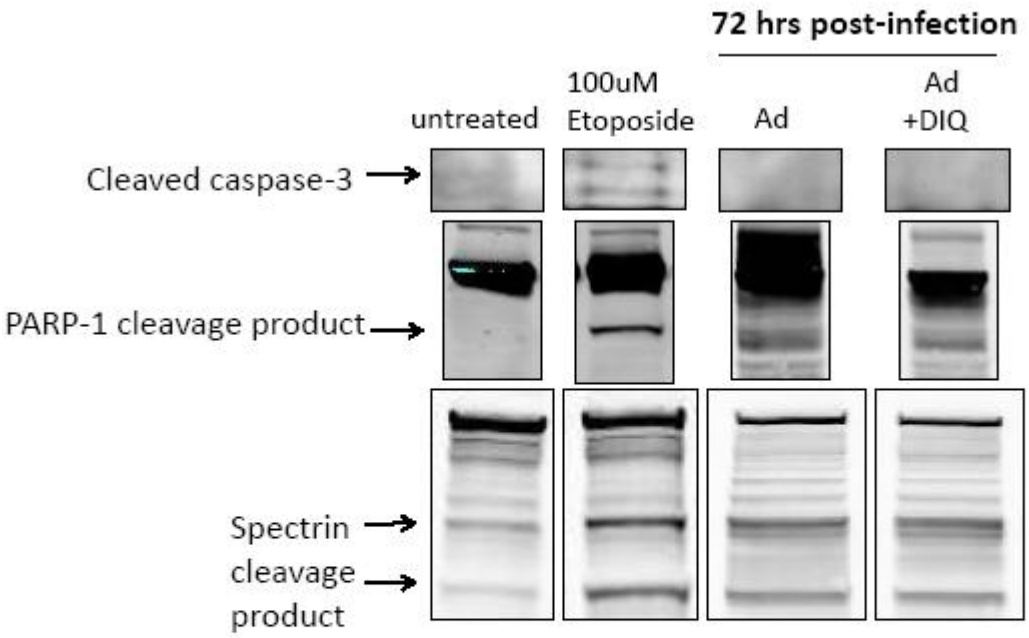

Figure 38. PARP inhibition does not induce apoptosis in Adenovirus-infected cells. To measure apoptosis in infected cells, Western blotting was performed for cleaved Caspase-3, PARP-1, and Spectrin. As a positive control, cells were treated for 24hrs with 100uM Etoposide.

HMGB1 is not a target for poly-ADP-ribosylation during Adenovirus infection

HMGB1 has been reported to undergo various post-translational modifications such as phosphorylation (Youn and Shin 2006), acetylation (Bonaldi, Talamo et al. 2003), methylation (Ito, Fukazawa et al. 2007) and ADP-ribosylation (Ditsworth, Zong et al. 2007) during different experimental conditions. It has been previously suggested that direct modification of HMGB1 by PARP mediates its release during DNA damage-induced necrosis. To examine if HMGB1 was targeted for ADP-ribosylation during Adenovirus infection we utilized 2-dimensional (2D)

Western blotting. As shown in Figure 40, HMGB1 shifts in isoelectric point to at least 3 distinct species during infection. If these modifications represented poly-ADP-riboslyation, we hypothesized that treatment with PARP inhibitors should change the isoelectric point of HMGB1, and therefore alter the position of these shifted species. However, PARP inhibition did not affect the positions of these species (Figure 40), indicating that poly-ADP-ribosylation is likely not a modification that occurs to HMGB1 during Adenovirus infection. To further validate this finding, we purified HMGB1 by immunoprecipitation from cells and examined poly-ADP-ribosylation by Western blot. Consistent with the 2D data, no detectable ADP ribosylation was seen either in uninfected or infected cells (Figure 39). Together, these results suggest that HMGB1 is not a direct target of PARP activity during Adenovirus infection.

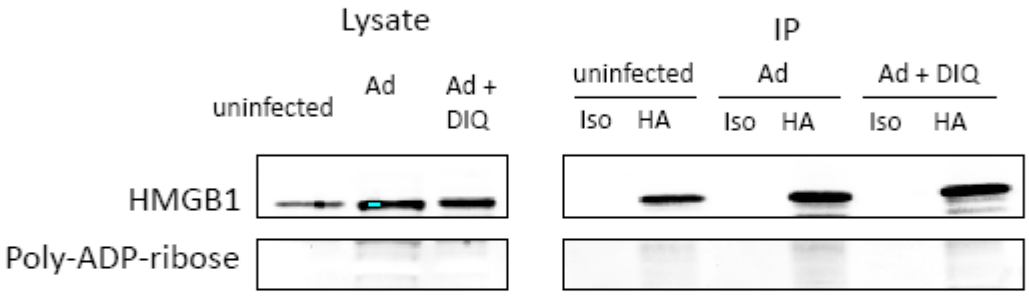

Figure 39. HMGB1 is not poly-ADP-ribosylated during Adenovirus infection. HA-HMGB1 was transfected into cells for 24hrs and immunprecipitated at 48 hours post-infection. Cells were subsequently Western blotted for poly-ADP-ribose.

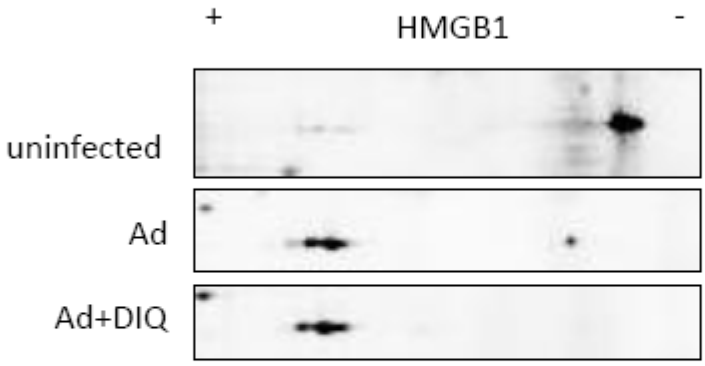

Figure 40. HMGB1 is not poly-ADP-ribosylated during Adenovirus infection. HMGB1-infected cells were lysed in urea buffer at 48 hours post-infection. Whole cell lysates were separated by 2-dimensional gel electrophoresis and Western blotted for HMGB1. The relative charge on each end of the gel is indicated above the blot.

H2B undergoes PARP-dependent post-translational modification

Because we could not detect direct poly-ADP-ribosylation of HMGB1 during infection, and PARP activity is required for release during infection, we sought to identify novel PARP targets during Adenovirus infection. To accomplish this, we used 2D protein separation during infection in the presence or absence of PARP inhibition, as previously described. Cell lysates were then stained by Coomassie blue to identify proteins that significantly change isoelectric

point during Adenovirus infection (Figure 41). We focused on 2 major species that became diffuse during infection but that remained concentrated single spots during PARP inhibition (Figure 41, outlined). We then used mass spectrometry to identify these proteins. Both spots were identified as histone H2B. H2B has been previously well established as a PARP target and is modified to facilitate chromatin remodeling that occurs in response to DNA damage (Malanga and Althaus 2005). ADP-ribosylation adds significant negative charge to H2B and thus weakens its interaction with the negatively charged backbone of DNA.

Because H2B is a well-established target for ADP-ribosylation, we sought to determine if H2B was ADP-ribosylated during infection using the same techniques we employed to analyze HMGB1 modification. In contrast to what was observed with HMGB1, PARP inhibition caused H2B to change its distribution of charged species during infection, as observed by 2D Western blot (Figure 42). H2B shows a wide range of charged species, consistent with the various post-translational modifications reported to occur on H2B. As shown in Figure 42 (outlined), in the presence of PARP inhibition during infection, there is an increase in a positively charged spot (right-shifted) compared to infection alone. This direction of shift is consistent with a decrease in negatively charged poly-ADP-ribose modifications, although it is likely other modifications such as acetylation occur and may be indirectly altered by PARP inhibition. In order to validate that H2B was modified by poly-ADP-ribose, we performed immunoprecipitation of HMGB1 and Western blotting to poly-ADP-ribose. However, we were unable to confirm poly-ADP-ribose modification using this method (data not shown). The antibody used has a higher affinity for long 20-30 residue ADP-ribose polymers, and it is possible that H2B is modified with shorter

polymers of ADP-ribose. Nonetheless, these data show that unlike HMGB1, histone H2B undergoes PARP-dependent post-translational modification during Adenovirus infection.

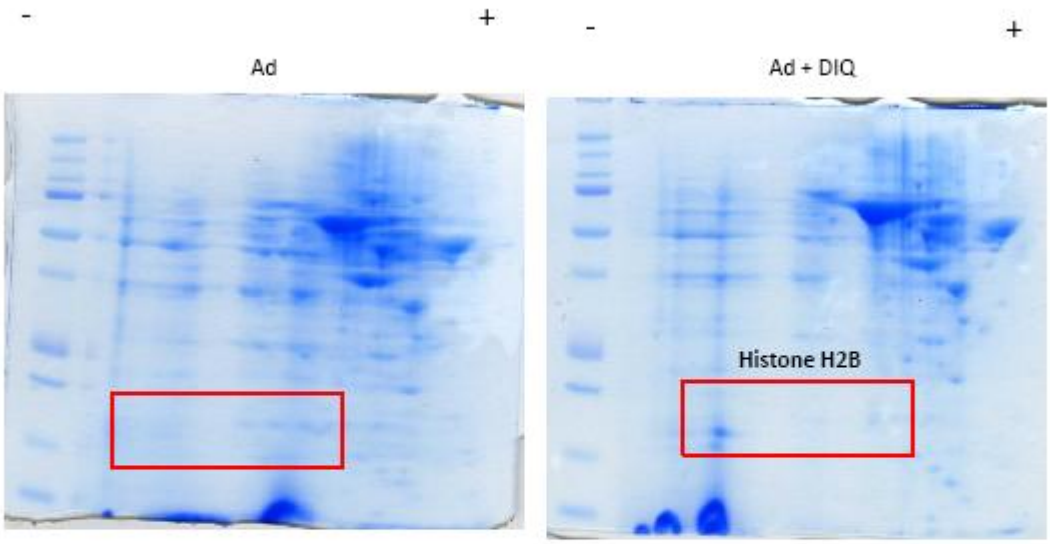

Figure 41. Histone H2B was identified by mass-spectrometry as a protein that underwent PARP-dependent post-translational modification. 2D gel electrophoresis during Adenovirus infection in the presence or absence of 300uM DIQ was performed. Whole cell lysates were stained by coomassie blue and subsequently analyzed by mass spectrometry.

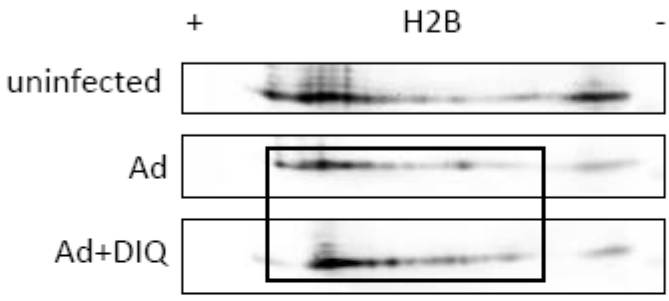

Figure 42. Histone H2B is post-translationally modified in a PARP-dependent manner. Lysates were prepared and analyzed by 2D electrophoresis as in Figure 40. Western blotting was performed for histone H2B. Differentially charged species with PARP-inhibiton are outlined.

HMGB1 interacts with H2B in a PARP-dependent manner during infection

HMGB1 has previously been reported to interact with histones as a component of the chromatin (Cato, Stott et al. 2008). Because we identified H2B as an abundant protein that undergoes PARP-dependent modification during Adenovirus infection, and HMGB1 has been previously reported to interact with histones, we examined whether HMGB1 physically interacted with H2B during infection and the role of PARP activity in this process. To investigate this we used co-immunoprecipitation and Western blotting during Adenovirus infection in the

presence or absence of PARP inhibition. As shown in Figure 5, in uninfected cells HMGB1 interacts physically with H2B. However, at 48 hours post-infection, a time when the majority of HMGB1 has translocated to the cytosol (Figure 34), HMGB1 and H2B show a considerably reduced interaction, despite comparable levels in the lysate. When a PARP inhibitor is used during infection, this interaction is restored to levels similar to uninfected conditions (Figure 43). Collectively, these data suggest that H2B and HMGB1 lose the ability to interact during Adenovirus infection in a mechanism that is dependent on PARP activity.

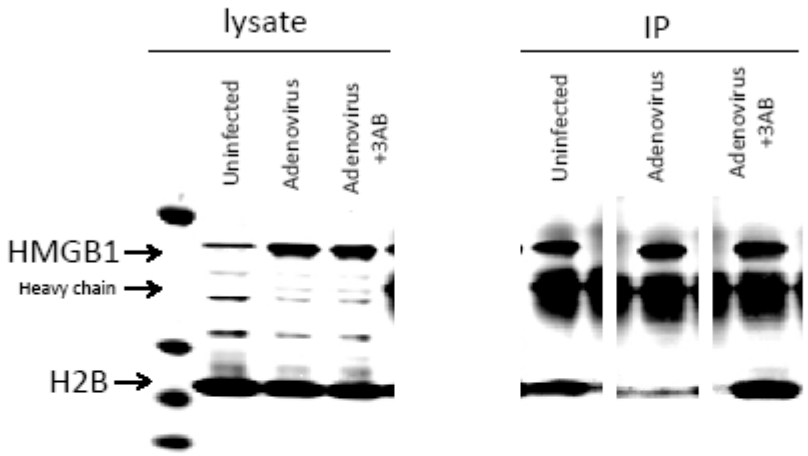

Figure 43. HMGB1 interacts with H2B in a PARP-dependent manner. MDA-MB-231 cells were infected with Adenovirus with or without 3AB as previously described. At 48 hours post-infection, cells were lysed and immunoprecipitated with anti-HMGB1.

PARP inhibition or HMGB1 depletion reduces the *in vitro* ability of Adenovirus conditioned media to induce NFkappaB signaling in macrophages

HMGB1 has been demonstrated to be a powerful activating signal to immune cells in several models of pathogenic inflammation. HMGB1 is required for systemic inflammation that ultimately results in death in models of septic shock and ischemia/repurfusion injury (Wang, Bloom et al. 1999; Lu, Hartono et al. 2007). In addition to systemic effects, HMGB1 can activate many types of immune cells including macrophages *in vitro*, through binding TLR and activating the NFkappB pathway (Robert, Sjodin et al. 2010).

Adenovirus mediated immune suppression is well-documented, with down-regulation of Natural Killer ligand expression and the Major Histocompatibility Class I (MHCI) pathway being two salient features (Horwitz 2004). Adenovirus-mediated immune activation has not been studied as extensively. These studies have largely focused on innate activation by the capsid and DNA of the virus, prior to viral replication and productive infection (Ahtiainen, Mirantes et al. 2010; Ahi, Bangari et al. 2011). Although HMGB1 has an established role in immune activation in other systems, it has not been previously studied in the context of Adenovirus infection. In order to test if HMGB1 contributed to the immune activating potential of Adenovirus conditioned media, we used the mouse macrophage cell line RAW264.7. This cell line expresses TLR receptors and is capable of activating the NFkappaB pathway through these receptors. Filtered media was collected from Adenovirus infected cells at 7 days post-infection, corresponding to a peak in HMGB1 release (Figure 33). This media was then applied to serum starved macrophages for 30 minutes and subsequently macrophages were lysed and NFkappaB activation was analyzed by Western blotting. The canonical NFkappaB complex is composed of two subunits, p65 and p50. The p65 subunit is modified by phosphorylation at several sites. Because it has been shown previously that phosphorylation at serine 536 is specifically required for NFkappaB-mediated transcription (Hu, Nakano et al. 2004), we used this as a readout for NFkappaB activity. As shown in Figure 44, Adenovirus conditioned media induced phosphorylation of p65 compared to untreated cells. In contrast, if media from Adenovirus infected cells in the presence of DIQ was used, this phosphorylation was greatly reduced. To further validate a role for HMGB1 in Adenovirus-conditioned media macrophage activation, we also used stable lentiviral shRNA to deplete HMGB1 expression, as described in Figure 1. HMGB1 was stably depleted from cells to 5-25% of control shRNA-treated cell levels. These cells were then infected with Adenovirus, and media was collected and applied to macrophages as previously described. As shown in Figure 6, depletion of HMGB1 by two different HMGB1-targeting shRNAs but not control shRNA caused a decrease in p65 phosphorylation. These data

show that by decreasing HMGB1 release, either by PARP inhibition or HMGB1 depletion, the ability of Adenovirus-conditioned media to activate NFkappaB signaling in macrophages *in vitro* is decreased.

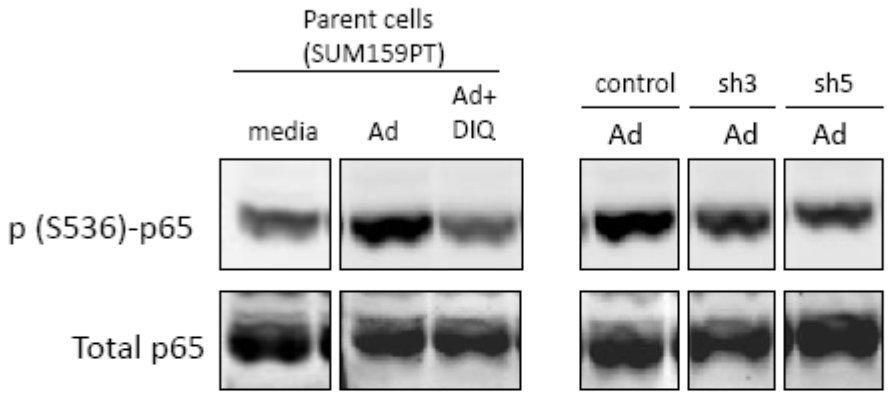

Figure 44. Inhibition of HMGB1 release by DIQ or shRNA during Adenovirus infection reduces activation of macrophages. SUM159PT cells were infected with DIQ as previously described or depleted for HMGB1 by two independent shRNAs as described in Figure 2. Conditioned media was collected after 5 days of infection, filtered through a 0.45uM filter and applied to serum-starved RAW264.7 macrophages. Macrophages were lysed after 30 minutes and phosphorylated p65 was evaluated by Western blot.

3.4 Discussion

We have demonstrated that the pro-inflammatory molecule HMGB1 is released from Adenovirus-infected cells in culture. We have further shown that this release is dependent on the

activity of the PARP enzyme. In contrast to previous studies, PARP inhibition during infection does not inhibit the ability of Adenovirus to kill and lyse infected cells. This finding is especially important with respect to the potential therapeutic application of PARP inhibitors in combination with oncolytic viruses. There are currently several clinical trials investigating the use of PARP inhibitors as a cancer therapy. Our work suggests that the combination of these two treatments is not likely to affect the efficacy of virus-mediated cell killing.

Although previous studies have shown HMGB1 to be a direct target for poly-ADP-ribosylation during DNA damage-induced necrosis, we were not able to find evidence of this modification during Adenovirus infection. Although the antibody used is not expected to recognize low-molecular weight polymers of ADP-ribose, the 2D Western blotting method is likely to have detected this modification, as it has been used to detect acetylation events on HMGB1 in previous studies (Bonaldi, Talamo et al. 2003). Indeed, although HMGB1 is not poly-ADP-ribosylated during infection, it is post-translationally modified, as observed by 2D analysis. HMGB1 post-translational modifications were analyzed by mass-spectrometry (data not shown) and acetylation was identified. It is likely that HMGB1 acetylation occurs during infection, however this modification is not alone sufficient to promote release of HMGB1 because PARP inhibition is able to prevent release while not affecting HMGB1 modification. It is however still possible that acetylation of HMGB1 is required upstream of the requirement for PARP in the release process.

Our work shows that HMGB1 interacts with the histone H2B in a PARP-dependent manner during infection and that H2B undergoes PARP-dependent modification during Adenovirus infection. Although we have not proven that H2B is the relevant target for PARP mediated modification in HMGB1 release, the data are suggestive this is the case. Further studies will have to determine whether H2B is directly modified by ADP-ribosylation during infection and clarify its role in the release mechanism. Histones and HMGB1 have previously been shown to form complexes in studies on the autoimmune

disease lupus (Urbonaviciute, Furnrohr et al. 2008). Our work suggests that inhibition of PARP activity causes HMGB1 to increase association with H2B. One possible model is that PARP modification of H2B causes dissociation of HMGB1 and subsequent release of HMGB1 from the chromatin. Once dissociated from chromatin, cytoplasmic HMGB1 is expected to be passively released from cells after membrane integrity is lost during cell lysis.

Importantly, we have shown that HMGB1 released from Adenovirus infected cells is a significant component of Adenovirus conditioned media's ability to activate of macrophages *in vitro.* Adenovirus infection causes the release of various pro-inflammatory factors that would be expected to activate macrophages, such as DNA and double-stranded RNA; however, HMGB1 deficiency alone is sufficient to significantly decrease activating potential. This is not wholly surprising in light of recent reports demonstrating that HMGB1 is a critical component of innate responses to infection owing to its ability to complex with nucleic acids (Yanai, Ban et al. 2009). HMGB1-nucleic acid complexes were shown to be capable of greater activation of TLR pathways that nucleic acids alone, and is consistent with our preliminary observations. Further work will be required to identify the relative contribution of HMGB1 and other innate activators such as viral DNA during infection.

Collectively, our work shows that HMGB1 is released from cells *in vitro* during Adenovirus infection in a PARP-dependent manner that likely involves differential interaction between HMGB1 and histone H2B. We also demonstrate that HMGB1 contributes to the inflammatory potential of in vitro Adenovirus infection. Future work is needed to extend these findings into *in vivo* settings and investigate the efficacy of PARP inhibition in combination with oncolytic virus therapy in a clinical setting.

Chapter 4:

Overall Conclusions

4.1 Discussion

In this work, we have determined some previously unknown functions of HMGB1 in cancer as well as defined a mechanism of its release during Adenovirus infection. Although these were different areas of investigation, they both focused on increasing our understanding of HMGB1 biology with the ultimate goal of creating more effective cancer treatments. Our work on the role of nuclear HMGB1 suggests that targeting extracellular HMGB1, as has been proposed in the literature, may not fully counter the pro-tumor effects of over-expressed HMGB1. Our findings suggest that interfering with nuclear HMGB1-driven gene silencing may be one way to limit its ability to promote cancer development. Further, based on our work with inhibitors of DNA methyltransferase and histone deacetylase, we propose that these types of epigenetic therapies may be useful in high HMGB1 expressing tumors. It is predicted that these inhibitors will prevent HMGB1 from silencing the tumor suppressor SEMA3A, and re-expression of SEMA3A will inhibit tumor cell migration and angiogenesis. Importantly, although we have discovered a subset of tumors that appear to have a mechanistic relationship between HMGB1 and SEMA3A, this is likely not universally true. Future work will be needed to specifically identify those cancers that support HMGB1-mediated silencing of SEMA3A. By identifying

primary human tumors that fall in this subset and employing epigenetic treatments, it is hoped that SEMA3A will regain expression and limit tumor progression.

Our work on Adenovirus-mediated release of HMGB1 has several important implications for cancer therapy. Oncolytic viruses continue to go forward in clinical trials; however, the largest barrier to date in effectiveness of these treatments has been the immune system. The relationship between oncolytic viruses and immunity is complex and multifaceted. As discussed above, in some cases immune-mediated viral clearance prevents the virus from spreading throughout and eliminating the tumor. In other cases though, viral replication in the tumor recruits the immune system to the tumor site and promotes recognition of the cancer cells by the immune cells, in a process known as breaking tolerance. Therefore, immune recognition of tumor-targeted viruses can have both pro and anti-tumor results. Because of this complexity, it is important to understand the various ways in which the virus can stimulate immunity. We have described one such mechanism as a PARP-dependent release of HMGB1 during Adenovirus infection. Therefore, PARP inhibitors used in tandem with oncolytic viral therapy would be expected to decrease the immunogenicity of the infection. Whether this results in inhibition of breaking tolerance or better virus-mediated tumor death likely depends on the individual setting and the tumor type. Although not formally presented in this work, we have found evidence that Adenovirus replication causes a DNA damage checkpoint upstream of PARP activation. Therefore, blocking this checkpoint may be another viable strategy for reducing PARP activity and preventing HMGB1 release. This new understanding of the role of HMGB1 during Adenovirus infection and its mechanism of release will help aid in the design of more effective clinical trials with oncolytic virus-based therapies.

This work has been conducted with the ultimate goal of furthering the effectiveness of cancer treatments. By elucidating novel roles for nuclear HMGB1 in tumor growth and metastasis and identifying the mechanism by which HMGB1 is released in Adenovirus infection, we hope to aid in the development of more productive cancer therapies.

4.2 References

Agresti, A., R. Lupo, et al. (2003). "HMGB1 interacts differentially with members of the Rel family of transcription factors." Biochem Biophys Res Commun **302**(2): 421-426.

Ahi, Y. S., D. S. Bangari, et al. (2011). "Adenoviral vector immunity: its implications and circumvention strategies." Curr Gene Ther **11**(4): 307-320.

Ahtiainen, L., C. Mirantes, et al. (2010). "Defects in innate immunity render breast cancer initiating cells permissive to oncolytic adenovirus." PLoS One **5**(11): e13859.

Alleva, L. M., A. C. Budd, et al. (2008). "Systemic release of high mobility group box 1 protein during severe murine influenza." J Immunol **181**(2): 1454-1459.

Boffa, L. C., J. Walker, et al. (1990). "Factors affecting nucleosome structure in transcriptionally active chromatin. Histone acetylation, nascent RNA and inhibitors of RNA synthesis." Eur J Biochem **194**(3): 811-823.

Bonaldi, T., F. Talamo, et al. (2003). "Monocytic cells hyperacetylate chromatin protein HMGB1 to redirect it towards secretion." EMBO J **22**(20): 5551-5560.

Bortolanza, S., M. Bunuales, et al. (2009). "Deletion of the E3-6.7K/gp19K region reduces the persistence of wild-type adenovirus in a permissive tumor model in Syrian hamsters." Cancer Gene Ther **16**(9): 703-712.

Bos, P. D., X. H. Zhang, et al. (2009). "Genes that mediate breast cancer metastasis to the brain." Nature **459**(7249): 1005-1009.

Breitbach, C. J., J. Burke, et al. (2011). "Intravenous delivery of a multi-mechanistic cancer-targeted oncolytic poxvirus in humans." Nature **477**(7362): 99-102.

Brezniceanu, M. L., K. Volp, et al. (2003). "HMGB1 inhibits cell death in yeast and mammalian cells and is abundantly expressed in human breast carcinoma." FASEB J **17**(10): 1295-1297.

Bustin, M. and N. K. Neihart (1979). "Antibodies against chromosomal HMG proteins stain the cytoplasm of mammalian cells." Cell **16**(1): 181-189.

Calogero, S., F. Grassi, et al. (1999). "The lack of chromosomal protein Hmg1 does not disrupt cell growth but causes lethal hypoglycaemia in newborn mice." Nat Genet **22**(3): 276-280.

Casazza, A., X. Fu, et al. (2011). "Systemic and targeted delivery of semaphorin 3A inhibits tumor angiogenesis and progression in mouse tumor models." Arterioscler Thromb Vasc Biol **31**(4): 741-749.

Cato, L., K. Stott, et al. (2008). "The interaction of HMGB1 and linker histones occurs through their acidic and basic tails." J Mol Biol **384**(5): 1262-1272.
Cheng, B. Q., C. Q. Jia, et al. (2008). "Serum high mobility group box chromosomal protein 1 is associated with clinicopathologic features in patients with hepatocellular carcinoma." Dig Liver Dis **40**(6): 446-452.
Chintakuntlawar, A. V. and J. Chodosh (2009). "Chemokine CXCL1/KC and its receptor CXCR2 are responsible for neutrophil chemotaxis in adenoviral keratitis." J Interferon Cytokine Res **29**(10): 657-666.
Cuconati, A., C. Mukherjee, et al. (2003). "DNA damage response and MCL-1 destruction initiate apoptosis in adenovirus-infected cells." Genes Dev **17**(23): 2922-2932.
Dhar, D., J. F. Spencer, et al. (2009). "Pre-existing immunity and passive immunity to adenovirus 5 prevents toxicity caused by an oncolytic adenovirus vector in the Syrian hamster model." Mol Ther **17**(10): 1724-1732.
Ditsworth, D., W. X. Zong, et al. (2007). "Activation of poly(ADP)-ribose polymerase (PARP-1) induces release of the pro-inflammatory mediator HMGB1 from the nucleus." J Biol Chem **282**(24): 17845-17854.
El Gazzar, M., B. K. Yoza, et al. (2009). "Chromatin-specific remodeling by HMGB1 and linker histone H1 silences proinflammatory genes during endotoxin tolerance." Mol Cell Biol **29**(7): 1959-1971.
El Mezayen, R., M. El Gazzar, et al. (2007). "Endogenous signals released from necrotic cells augment inflammatory responses to bacterial endotoxin." Immunol Lett **111**(1): 36-44.
Ellerman, J. E., C. K. Brown, et al. (2007). "Masquerader: high mobility group box-1 and cancer." Clin Cancer Res **13**(10): 2836-2848.
Gardella, S., C. Andrei, et al. (2002). "The nuclear protein HMGB1 is secreted by monocytes via a non-classical, vesicle-mediated secretory pathway." EMBO Rep **3**(10): 995-1001.
Ge, H. and R. G. Roeder (1994). "The high mobility group protein HMG1 can reversibly inhibit class II gene transcription by interaction with the TATA-binding protein." J Biol Chem **269**(25): 17136-17140.
Gebhardt, C., A. Riehl, et al. (2008). "RAGE signaling sustains inflammation and promotes tumor development." J Exp Med **205**(2): 275-285.
Ginsberg, H. S. and G. A. Prince (1994). "The molecular basis of adenovirus pathogenesis." Infect Agents Dis **3**(1): 1-8.
Goodwin, G. H., C. Sanders, et al. (1973). "A new group of chromatin-associated proteins with a high content of acidic and basic amino acids." Eur J Biochem **38**(1): 14-19.
Horwitz, M. S. (2004). "Function of adenovirus E3 proteins and their interactions with immunoregulatory cell proteins." J Gene Med **6 Suppl 1**: S172-183.
Hu, J., H. Nakano, et al. (2004). "Insufficient p65 phosphorylation at S536 specifically contributes to the lack of NF-kappaB activation and transformation in resistant JB6 cells." Carcinogenesis **25**(10): 1991-2003.
Huang, P., H. Kaku, et al. (2010). "Potent antitumor effects of combined therapy with a telomerase-specific, replication-competent adenovirus (OBP-301) and IL-2 in a mouse model of renal cell carcinoma." Cancer Gene Ther **17**(7): 484-491.
Ishiguro, H., N. Nakaigawa, et al. (2005). "Receptor for advanced glycation end products (RAGE) and its ligand, amphoterin are overexpressed and associated with prostate cancer development." Prostate **64**(1): 92-100.
Ito, I., J. Fukazawa, et al. (2007). "Post-translational methylation of high mobility group box 1 (HMGB1) causes its cytoplasmic localization in neutrophils." J Biol Chem **282**(22): 16336-16344.

Jayaraman, L., N. C. Moorthy, et al. (1998). "High mobility group protein-1 (HMG-1) is a unique activator of p53." Genes Dev **12**(4): 462-472.
Kang, H. J., K. H. Koh, et al. (2006). "Differentially expressed proteins in gastrointestinal stromal tumors with KIT and PDGFRA mutations." Proteomics **6**(4): 1151-1157.
Li, F., O. V. Glinskii, et al. (2011). "Identification and analysis of signaling networks potentially involved in breast carcinoma metastasis to the brain." PLoS One **6**(7): e21977.
Lin, Q., X. P. Yang, et al. (2011). "High-mobility group box-1 mediates toll-like receptor 4-dependent angiogenesis." Arterioscler Thromb Vasc Biol **31**(5): 1024-1032.
Lu, C. Y., J. Hartono, et al. (2007). "The inflammatory response to ischemic acute kidney injury: a result of the 'right stuff' in the 'wrong place'?" Curr Opin Nephrol Hypertens **16**(2): 83-89.
Maeda, S., Y. Hikiba, et al. (2007). "Essential roles of high-mobility group box 1 in the development of murine colitis and colitis-associated cancer." Biochem Biophys Res Commun **360**(2): 394-400.
Malanga, M. and F. R. Althaus (2005). "The role of poly(ADP-ribose) in the DNA damage signaling network." Biochem Cell Biol **83**(3): 354-364.
McCauley, M. J., J. Zimmerman, et al. (2007). "HMGB binding to DNA: single and double box motifs." J Mol Biol **374**(4): 993-1004.
McCormick, F. (2003). "Cancer-specific viruses and the development of ONYX-015." Cancer Biol Ther **2**(4 Suppl 1): S157-160.
Melcher, A., K. Parato, et al. (2011). "Thunder and lightning: immunotherapy and oncolytic viruses collide." Mol Ther **19**(6): 1008-1016.
Neufeld, G. and O. Kessler (2008). "The semaphorins: versatile regulators of tumour progression and tumour angiogenesis." Nat Rev Cancer **8**(8): 632-645.
Nguyen, Y., M. C. Procario, et al. (2011). "Limited effects of Muc1 deficiency on mouse adenovirus type 1 respiratory infection." Virus Res **160**(1-2): 351-359.
O'Shea, C. C. (2005). "Viruses - seeking and destroying the tumor program." Oncogene **24**(52): 7640-7655.
Poser, I., M. Golob, et al. (2003). "Upregulation of HMG1 leads to melanoma inhibitory activity expression in malignant melanoma cells and contributes to their malignancy phenotype." Mol Cell Biol **23**(8): 2991-2998.
Raki, M., M. Sarkioja, et al. (2011). "Switching the fiber knob of oncolytic adenoviruses to avoid neutralizing antibodies in human cancer patients." J Gene Med **13**(5): 253-261.
Ranzato, E., M. Patrone, et al. (2010). "Hmgb1 promotes wound healing of 3T3 mouse fibroblasts via RAGE-dependent ERK1/2 activation." Cell Biochem Biophys **57**(1): 9-17.
Robert, S. M., H. Sjodin, et al. (2010). "Preconditioning with high mobility group box 1 (HMGB1) induces lipoteichoic acid (LTA) tolerance." J Immunother **33**(7): 663-671.
Rovere-Querini, P., A. Capobianco, et al. (2004). "HMGB1 is an endogenous immune adjuvant released by necrotic cells." EMBO Rep **5**(8): 825-830.
Sapojnikova, N., J. Maman, et al. (2005). "Biochemical observation of the rapid mobility of nuclear HMGB1." Biochim Biophys Acta **1729**(1): 57-63.
Scaffidi, P., T. Misteli, et al. (2002). "Release of chromatin protein HMGB1 by necrotic cells triggers inflammation." Nature **418**(6894): 191-195.
Schmidt, E. F., S. O. Shim, et al. (2008). "Release of MICAL autoinhibition by semaphorin-plexin signaling promotes interaction with collapsin response mediator protein." J Neurosci **28**(9): 2287-2297.
Schmidt, E. F. and S. M. Strittmatter (2007). "The CRMP family of proteins and their role in Sema3A signaling." Adv Exp Med Biol **600**: 1-11.
Soldatenkov, V. A. and M. Smulson (2000). "Poly(ADP-ribose) polymerase in DNA damage-response pathway: implications for radiation oncology." Int J Cancer **90**(2): 59-67.

Staton, C. A., L. A. Shaw, et al. (2011). "Expression of class 3 semaphorins and their receptors in human breast neoplasia." Histopathology **59**(2): 274-282.
Stros, M., T. Ozaki, et al. (2002). "HMGB1 and HMGB2 cell-specifically down-regulate the p53- and p73-dependent sequence-specific transactivation from the human Bax gene promoter." J Biol Chem **277**(9): 7157-7164.
Szak, S. T., D. Mays, et al. (2001). "Kinetics of p53 binding to promoter sites in vivo." Mol Cell Biol **21**(10): 3375-3386.
Taguchi, A., D. C. Blood, et al. (2000). "Blockade of RAGE-amphoterin signalling suppresses tumour growth and metastases." Nature **405**(6784): 354-360.
Toth, K. and W. S. Wold (2010). "Increasing the efficacy of oncolytic adenovirus vectors." Viruses **2**(9): 1844-1866.
Urbonaviciute, V., B. G. Furnrohr, et al. (2008). "Induction of inflammatory and immune responses by HMGB1-nucleosome complexes: implications for the pathogenesis of SLE." J Exp Med **205**(13): 3007-3018.
van Beijnum, J. R., W. A. Buurman, et al. (2008). "Convergence and amplification of toll-like receptor (TLR) and receptor for advanced glycation end products (RAGE) signaling pathways via high mobility group B1 (HMGB1)." Angiogenesis **11**(1): 91-99.
Volp, K., M. L. Brezniceanu, et al. (2006). "Increased expression of high mobility group box 1 (HMGB1) is associated with an elevated level of the antiapoptotic c-IAP2 protein in human colon carcinomas." Gut **55**(2): 234-242.
Wang, H., O. Bloom, et al. (1999). "HMG-1 as a late mediator of endotoxin lethality in mice." Science **285**(5425): 248-251.
Weibel, S., V. Raab, et al. (2011). "Viral-mediated oncolysis is the most critical factor in the late-phase of the tumor regression process upon vaccinia virus infection." BMC Cancer **11**: 68.
Weth, O. and R. Renkawitz (2011). "CTCF function is modulated by neighboring DNA binding factors." Biochem Cell Biol **89**(5): 459-468.
Woo, J. K., Y. Choi, et al. (2011). "Mucin 1 enhances the tumor angiogenic response by activation of the AKT signaling pathway." Oncogene.
Wu, D., Y. Ding, et al. (2008). "Increased expression of high mobility group box 1 (HMGB1) is associated with progression and poor prognosis in human nasopharyngeal carcinoma." J Pathol **216**(2): 167-175.
Yanai, H., T. Ban, et al. (2009). "HMGB proteins function as universal sentinels for nucleic-acid-mediated innate immune responses." Nature **462**(7269): 99-103.
Yang, H., H. Wang, et al. (2001). "HMG-1 rediscovered as a cytokine." Shock **15**(4): 247-253.
Yao, X., G. Zhao, et al. (2010). "Overexpression of high-mobility group box 1 correlates with tumor progression and poor prognosis in human colorectal carcinoma." J Cancer Res Clin Oncol **136**(5): 677-684.
Youn, J. H. and J. S. Shin (2006). "Nucleocytoplasmic shuttling of HMGB1 is regulated by phosphorylation that redirects it toward secretion." J Immunol **177**(11): 7889-7897.
Yu, S. S., H. J. Li, et al. (1977). "Interaction of non-histone chromosomal proteins HMG1 and HMG2 with DNA." Eur J Biochem **78**(2): 497-502.
Zlatanova, J. and P. Caiafa (2009). "CTCF and its protein partners: divide and rule?" J Cell Sci **122**(Pt 9): 1275-1284.
Zong, W. X., D. Ditsworth, et al. (2004). "Alkylating DNA damage stimulates a regulated form of necrotic cell death." Genes Dev **18**(11): 1272-1282.

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