HMGA1: A Master Regulator of Tumor Progression in Triple-Negative Breast Cancer Cells

Sandeep N. Shah¹, Leslie Cope²,³, Weijie Poh⁴, Amy Belton¹, Sujayita Roy¹, C. Conover Talbot, Jr.⁵, Saraswati Sukumar⁶,⁷, David L. Huso⁷, Linda M. S. Resar¹,²,⁴,⁸,⁹,⁎

1 Hematology Division, Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland, United States of America, 2 Department of Oncology, Johns Hopkins University School of Medicine, Baltimore, Maryland, United States of America, 3 Department of Biostatistics, Johns Hopkins University School of Medicine, Baltimore, Maryland, United States of America, 4 Pathobiology Graduate Program, Johns Hopkins University School of Medicine, Baltimore, Maryland, United States of America, 5 Institute for Basic Biomedical Sciences, Johns Hopkins University School of Medicine, Baltimore, Maryland, United States of America, 6 Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, Maryland, United States of America, 7 Department of Comparative Molecular & Pathobiology, Johns Hopkins University School of Medicine, Baltimore, Maryland, United States of America, 8 Department of Pediatrics, Johns Hopkins University School of Medicine, Baltimore, Maryland, United States of America, 9 Institute for Cellular Engineering, Johns Hopkins University School of Medicine, Baltimore, Maryland, United States of America

Abstract

Emerging evidence suggests that tumor cells metastasize by co-opting stem cell transcriptional networks, although the molecular underpinnings of this process are poorly understood. Here, we show for the first time that the high mobility group A1 (HMGA1) gene drives metastatic progression in triple negative breast cancer cells (MDA-MB-231, Hs578T) by reprogramming cancer cells to a stem-like state. Silencing HMGA1 expression in invasive, aggressive breast cancer cells dramatically halts cell growth and results in striking morphologic changes from mesenchymal-like, spindle-shaped cells to cuboidal, epithelial-like cells. Mesenchymal genes (Vimentin, Snail) are repressed, while E-cadherin is induced in the knock-down cells. Silencing HMGA1 also blocks oncogenic properties, including proliferation, migration, invasion, and orthotopic tumorigenesis. Metastatic progression following mammary implantation is almost completely abrogated in the HMGA1 knock-down cells. Moreover, silencing HMGA1 inhibits the stem cell property of three-dimensional mammosphere formation, including primary, secondary, and tertiary spheres. In addition, knock-down of HMGA1 depletes cancer initiator/cancer stem cells and prevents tumorigenesis at limiting dilutions. We also discovered an HMGA1 signature in triple negative breast cancer cells that is highly enriched in embryonic stem cells. Together, these findings indicate that HMGA1 is a master regulator of tumor progression in breast cancer by reprogramming cancer cells through stem cell transcriptional networks. Future studies are needed to determine how to target HMGA1 in therapy.

Introduction

Despite advances in our ability to detect and treat breast cancer, it remains a leading cause of death in women with cancer, and the incidence is rising [1]. Approximately 15–20% of all cases are classified as triple negative breast cancer, a subtype that is frequently associated with rapid progression and poor outcomes [1–2]. Triple negative breast cancer refers to the lack of detectable markers for the estrogen receptor (ER), progesterone receptor (PR), and Her2/neu amplification. These tumors do not respond to our most effective and least toxic therapies, including hormonal therapy (tamoxifen) or herceptin. Thus, further studies are needed to elucidate the molecular pathways that lead to tumor progression in triple negative breast cancer and could be targeted with novel therapies.

Recent studies identified the high mobility group A1 (HMGA1) oncogene as a key factor enriched in embryonic stem cells, adult stem cells, and refractory or high-grade/poorly differentiated tumors [3–32]. The HMGA1 gene encodes the HMGA1a and HMGA1b chromatin remodeling proteins, which result from alternatively spliced messenger RNA [4,14,22,27]. These low molecular weight (thus high mobility group) protein isoforms bind to the minor groove of chromatin at AT-rich regions. HMGA1 proteins modulate gene expression by altering chromatin structure and orchestrating the assembly of transcription factor complexes to enhanceosomes within enhancer or promoter regions throughout the genome. These proteins are highly expressed during embryogenesis with low or absent levels in adult tissues. HMGA1 is overexpressed in all aggressive cancers studied to date, and high levels portend a poor prognosis in diverse tumors [3–31]. In fact, HMGA1 proteins are the most abundant nonhistone chromatin binding proteins found in cancer cells. A recent landmark paper demonstrated that HMGA1 is essential for the cellular reprogramming of somatic cells to induced pluripotent stem cells by the four Yamanaka factors (Oct4, Sox2, Klf4, c-Myc) [32]. HMGA1 induces expression of key stem cell transcriptional networks in
normal embryonic stem cells and during cellular reprogramming. Together, these findings suggest that HMGA1 could function in tumor progression by reprogramming differentiated cells into poorly differentiated, stem-like cancer cells.

Here, we discovered that HMGA1 is a central factor in reprogramming poorly differentiated, triple-negative breast cancer cells. Our findings further implicate HMGA1 as a master regulator in tumor progression and suggest that targeting HMGA1 pathways could be effective in poorly differentiated, metastatic tumors.

Results

Silencing HMGA1 halts cell proliferation and reprograms invasive, mesenchymal-like cells

To define the role of HMGA1 in oncogenic properties and tumor progression, we silenced HMGA1 expression using lentiviral-mediated delivery of short hairpin RNA (shRNA) [32] in cell lines derived from aggressive, triple negative breast cancers (MDA-MB-231, Hs578T; Fig. 1A). Control cells were transduced with a control lentiviral vector [10,32]. We discovered that cell proliferation was rapidly halted in both cell lines (Fig. 1B) within the first 4 days. Surprisingly, there was a dramatic change in cell morphology whereby the spindle-shaped, fibroblast-like cells became cuboidal and epithelial-like in appearance (Fig. 1C). Because these morphologic changes are consistent with a mesenchymal-epithelial transition, we investigated the expression of genes involved in a mesenchymal-epithelial transition [10,33]. In MDA-MB-231 cells, we found that silencing HMGA1 led to a significant repression in the mesenchymal genes, Snail and Vimentin, while there was an increase in the gene expressing the epithelial marker, E-Cadherin (Fig. 1D). Similarly, in Hs578T cells, E-Cadherin was induced when HMGA1 was silenced. We also assessed tumor progression properties, including invasion and migration. In both cell lines, there was a marked reduction in migration and invasion in cells with silencing of HMGA1 (Fig. 1E). Together, these findings indicate that silencing HMGA1 results in a profound decrease in proliferation, migration, and invasion, as well as morphologic and gene expression changes consistent with a mesenchymal-epithelial transition.

Silencing HMGA1 interferes with orthotopic tumorigenicity and metastatic progression

Next, we assessed the role of HMGA1 on tumorigenesis using in vivo models of triple negative breast cancer. First, we assessed tumor growth following mammary fat pad implantation. We found that silencing HMGA1 in the aggressive MDA-MB-231 cells leads to a dramatic decrease in tumor growth following mammary fat pad implantation (Fig. 2Aii). Specifically, cells (10^5) transduced with control virus reached a volume of 0.53 cm^3 ±0.34 at 8 weeks following mammary fat pad implantation. In contrast, the tumors from cells transduced with HMGA1 shRNA (shHMGA1) were significantly smaller at 8 weeks following implantation (0.037 cm^3 ±0.058; p = 0.016). Because there was a dramatic effect on primary tumorigenesis, we also sought to determine if silencing HMGA1 interferes with metastatic progression. We therefore evaluated the lungs histopathologically for tumor foci after necropsy. Strikingly, we discovered almost no metastatic lesions to the lungs in the mice implanted with the shHMGA1 cells as compared to the mice implanted with control cells in which there were extensive, coalescing sheets of metastatic tumor cells throughout the lungs following mammary implantation with 10^5 cells (Fig. 2Aii). We also assessed metastatic progression following mammary fat pad implantation with a greater number of cells (10^6) from a repeat transduction experiment, and sacrificed the mice after 5 weeks. With the higher number of cells, tumors formed from all injections (3/3 in controls and 3/3 in shHMGA1 cells) (Fig. 2Bi). Although tumors were slightly smaller from the shHMGA1 cells, the difference was not significant (0.64±0.27 in controls versus 0.17±0.072 in shHMGA1 cells, p = 0.08). Despite the similar tumor volumes, we observed a dramatic decrease (>100-fold) in metastatic foci in the shHMGA1 cells as compared to controls (0.67±1.15 versus >100 in all controls; p = 0.00004; Fig. 2Bi & 2Bii). We also assessed lung foci following tail vein injection of control or shHMGA1 cells (10^6) after 3 weeks. Only one lung focus was observed after injection of the shHMGA1 cells, while there were numerous foci in the control cells (0.25±0.5 versus 99.3±15.0; p = 0.007; Fig. S1).

Silencing HMGA1 blocks mammosphere formation and depletes tumor-initiator cells

Because silencing HMGA1 has profound effects on oncogenic properties in vitro, primary tumorigenesis and metastatic progression in vivo, and expression of genes involved in epithelial-mesenchymal transition, we sought to determine its role in cancer stem cell characteristics. To this end, we explored the epithelial stem cell property of mammosphere formation [34] in the control and shHMGA1-treated cells (Fig. 3A–B). We found that growth of primary, secondary, and tertiary mammospheres was significantly impaired in the MDA-MB-231 cells with silencing of HMGA1. Similarly, we observed that there was a significant decrease in primary mammosphere formation in the Hs578T cells treated with shHMGA1. (Secondary or tertiary mammospheres do not form in control Hs578T cells, precluding analysis of these phenotypes). Next, we performed orthotopic implantations and assessed tumorigenicity with limiting dilutions. As presented above, tumors formed in both control and shHMGA1 cells when 10^5 or 10^6 cells were implanted. In contrast, no tumors formed in the MDA-MB-231 cells with silencing of HMGA1 when 10^5 cells were injected (0/3), while tumors formed in all control injections (3/3; Fig. 3C). These results indicate that silencing HMGA1 in MDA-MB-231 cells depletes the tumor-initiator or cancer stem-like cells and further underscores the role of HMGA1 as a key regulator of stem cell properties in aggressive, triple-negative breast cancer cells.

HMGA1 induces a stem cell signature in triple negative breast cancer cells

To globally define the transcriptional networks regulated by HMGA1, we performed gene expression profile analysis in MDA-MB-231 cells with or without HMGA1 knock-down. To this end, we used siRNA [21,25] and observed a rapid and significant reduction in HMGA1 expression (Fig. S2A). HMGA1 mRNA falls dramatically by 48 hours, with persistent decreases at 72 hours (Fig. S2A). There was also a marked decrease in HMGA1 protein at 48 and 72 hours (Fig. S2B). We therefore performed global gene expression profile analysis at 48 hours using an Affymetrix exon array (GeneChip Human exon 1.0 ST Array) with RNA from three independent replicates of each experimental condition. To define an HMGA1 signature in breast cancer, we identified the 100 transcripts that were most differentially expressed. These 100 transcripts correspond to 63 unique genes. Because HMGA1 is enriched in embryonic stem cells and our functional studies showed that it is required for cancer stem cell properties, we compared the HMGA1 signature of 63 genes to gene expression profiles from diverse pluripotent stem cells and differentiated cells, including embryonic stem cells (ESCs), induced pluripotent stem cells, embryoid bodies, and fibroblasts [35]. As shown, un-
supervised cluster analysis of these genes separates the samples by cell type with a clear distinction between pluripotent stem cells and differentiated cells. Moreover, the HMGA1 signature is highly enriched in pluripotent/embryonic stem cells ($p < 0.001$; Fig. 4A).

We validated a subset of the HMGA1 signature genes using quantitative RT-PCR, and found differential expression similar to the microarray gene expression results in all cases (Fig. S2C). These findings suggest that HMGA1 drives tumor progression by inducing stem cell transcriptional networks.

To elucidate cellular pathways regulated by HMGA1 in breast cancer, we analyzed the HMGA1 signature with Ingenuity Pathway Analysis (IPA, Ingenuity Systems, http://www.ingenuity.com). From the top list of differentially regulated genes, 2 pathways had significant network scores (69 and 46, respectively; Fig. 4B and Fig. S3). The highest scoring network was embryonic development, tissue development, and cellular development. The top molecular and cellular functions were cell death and survival and cellular movement, while the top physiologic system development and functions included: 1. nervous system development and function, 2. organ morphology, and 3. embryonic development. In this network, the most down-regulated molecule was ARL2BP or ADP-ribosylation factor (ARF)-like 2 binding protein. This protein is a member of a functionally distinct group of RAS-related GTPases, called the ARF family. ARL2BP protein binds to ARL2.GTP with high affinity and plays a role in the nuclear translocation, retention and transcriptional activity of STAT3 [36]. Notably, we showed that HMGA1 induces STAT3 expression in lymphoid tumorigenesis, and STAT3 inhibitors are cytotoxic to the HMGA1-driven tumor cells [24]. TMCO1 or transmembrane and coiled-coil domains 1 protein was the most up-regulated protein in this network. Although its function is not known, it is associated with breast cancer cells [8882811 GEO Profiles-NCBI]. TGF$\beta$1 is a major node and this protein is up-regulated in diverse cancers and thought to promote invasion, migration, EMT and tumor progression [37]. EGFR and MAPK are other important nodes that are activated in cancer and mediate proliferative signals [9]. Another central node was HIF-1 alpha, a key factor involved in angiogenesis during tumor progression and vascular development during embryogenesis [38]. In addition, Myc was identified as a major node and prior studies found that not only does cMYC induce HMGA1 expression [12], but HMGA1 also directly up-regulates cMYC expression [32]. Myc also has a well-defined role in breast [39–40] and other diverse cancers [41] as well as in embryonic stem cells [41–42]. Thus, our pathway analysis further confirms the important role for

**Figure 1. Silencing HMGA1 expression halts cell growth and induces dramatic changes in cell morphology and gene expression.**

A) Lentiviral-mediated delivery of shRNA to HMGA1 (denoted shHMGA1) results in a marked decrease in HMGA1 mRNA and protein in triple negative breast cancer cell lines (MDA-MB-231, Hs578T). B) Proliferation is disrupted in cancer cell lines following silencing of HMGA1. C) Mesenchymal, fibroblast-like cancer cells undergo dramatic morphologic changes within 4 days after treatment with shHMGA1. Striking changes were observed in MDA-MB-231 (top panels) and Hs578T cells (bottom panels). Bar: 50 μm. D) Alterations in EMT genes with silencing of HMGA1. E) Migration and invasion is decreased with silencing of HMGA1. * $p < 0.05$; ** $p < 0.01$. doi:10.1371/journal.pone.0063419.g001
Discussion

Here, we report for the first time that silencing HMGA1 induces a rapid and dramatic reprogramming of highly proliferative, invasive, mesenchymal-like breast cancer cells to more differentiated, slowly growing, epithelial-like cells. We also found that knock-down of HMGA1 has profound effects on oncogenic properties associated with both tumor initiation (orthotopic tumorigenesis) and tumor progression (migration, invasion, and metastatic progression). In fact, the in vivo effects on metastatic progression were even more pronounced than the effects on in vivo reversion of orthotopic tumorigenicity and metastatic progression. In Figure 2, silencing HMGA1 interferes with orthotopic tumorigenicity and metastatic progression. A) Silencing HMGA1 impairs orthotopic tumorigenicity. Tumor volumes ± standard deviations are shown. No tumors formed from shHMGA1 cells when 10⁴ cells were implanted. (For injections with 10⁴ cells, n = 3 for control or shHMGA1 cells; for injections with 10⁵ cells, n = 5 for control and n = 8 for shHMGA1 cells; and for injections with 10⁷ cells, n = 3 for control and shHMGA1 cells. B) Metastatic progression is almost completely abrogated in cells that do not express HMGA1. This graph shows the number of metastatic foci in the lungs 5 weeks following implantation of MDA-MB-231 cells (10⁷) into mammary fat pads following treatment with control shRNA or shHMGA1. C) The top photographs show the lungs 8 weeks following implantation into mammary fat pads. There are coalescing sheets of metastatic tumor cells in the lungs of mice injected with control cells (left) as compared to mice injected with shHMGA1 cells (right). Due to the widespread tumor cells, individual foci could not be counted. Bar: 50 μm. D) The bottom panels show multiple, discreet foci in the lungs 5 weeks following implantation of control cells into mammary fat pads (left) as compared to mice injected with shHMGA1 cells (right). *P < 0.05; **P < 0.0001.

doi:10.1371/journal.pone.0063419.g002

HMGA1 in regulating embryonic stem cell networks during tumor progression in breast cancer.
primary tumorigenesis, thus highlighting the role of HMGA1 in tumor progression. The changes induced by silencing HMGA1 are among the most striking alterations reported to date with knockdown of HMGA1 or most other oncogenes for that matter, both in degree and rate of onset. The profound effects could be related to our efficient, viral-mediated delivery of shRNA to repress HMGA1. In addition, triple negative breast cancer cells may be highly dependent upon HMGA1 and related pathways for their oncogenic properties. Indeed, a study from the Broad Institute at MIT identified HMGA1 as a key transcription factor enriched in triple negative breast cancer [3]. Moreover, expression of HMGA1 and 8 additional genes predicted poor outcomes in breast cancer, as well as brain and bladder cancer. Prior studies using antisense or dominant-negative approaches in triple-negative breast cancer cells (MDA-MB-231 or Hs578T) also showed that anchorage-independent cell growth or colony formation are inhibited by HMGA1 repression [15–16]. We also have preliminary evidence demonstrating that HMGA1 expression correlates with more advanced nuclear grade in primary tumors (Asch & Resar, unpublished data).

Emerging evidence further indicates that HMGA1 is important in maintaining a de-differentiated, pluripotent stem-like state [32]. A recent landmark paper demonstrated that HMGA1 is required for cellular reprogramming of somatic cells to induced pluripotent stem cells (iPSCs) by the Yamanaka factors [31]. Blocking HMGA1 expression or function prevents the derivation of iPSCs. In normal embryonic stem cells in culture and during the reprogramming process to iPSCs, HMGA1 activates expression of stem cell transcriptional networks. Recent studies also found that tumor progression and an epithelial-mesenchymal transition (EMT) involves transcriptional networks important in stem cells [3,9–10,32–33]. The first evidence linking HMGA1 to EMT came from an important study in 2001 in MCF-7 breast cancer cells, which demonstrated that forced expression of HMGA1 results in metastatic progression and histologic changes consistent with EMT in the epithelial MCF-7 breast cancer cell line [16]. This group also found that HMGA1 induces changes in classes of genes involved in tumor progression. More recently, studies in colon cancer showed that HMGA1 is required for tumor progression and stem cell properties [10]. Here, we discovered that HMGA1 is required for mammosphere formation, including secondary and tertiary mammospheres in MDA-MB-231 cells. We also found that silencing HMGA1 depletes tumor initiator/cancer stem cells, indicating that targeting HMGA1 in breast cancer therapy could have an important impact on the cancer stem cell population, which is believed to be the basis for refractory disease in diverse tumors. These functional studies are corroborated by the HMGA1 signature and pathway analysis demonstrating that HMGA1 orchestrates transcriptional networks important in stem cells and metastatic progression.

There is a dire need to understand the molecular underpinnings of metastatic progression because this is the major cause of death in patients with cancer. Although cancer is a highly complex and heterogeneous disease, with significant heterogeneity even within a single tumor, increasing evidence indicates that common, central pathways exist that could serve as “Achilles heels” or rational therapeutic targets in diverse tumors. Our studies presented here underscore the fundamental role for HMGA1 in tumor progression in preclinical models for aggressive, triple negative breast cancers. This work, together with prior studies in diverse tumors,
provide compelling evidence that HMGA1 is a master regulator in the evolution of primary tumors to metastatic disease. Further studies are now needed to develop approaches to target HMGA1 in cancer.

Materials and Methods

Ethics statement

All animal experiments were conducted in accordance with a protocol approved by the Johns Hopkins University Animal Care and Use Committee (protocol #MO111M270). Mice were housed in a sterile environment where they had free access to food and water as outlined in our institutional guidelines.

Cell culture and proliferation assay

Cells (MDA-MB-231 and Hs578T) were cultured as recommended (ATCC). For proliferation assays, cells were seeded (7,500/well) and counted using an automated cell counter (Nexcelom). Each experiment was done in triplicate and performed at least twice.

RNA Interference

The short-hairpin RNA interference vector for HMGA1 targets 5'-CAACTCCAGAAGGAAACCAA-3' and has been described elsewhere [43]. Virus was prepared as previously described. The empty vector was used as a negative control as we described [10]. Polyclonal, transduced cells were selected and maintained in puromycin (1 ug/ml). To ensure that the effects of silencing HMGA were not a result of a single clone, independent, polyclonal transductions were done at least twice for each experiment. All functional experiments were performed in duplicate or triplicate and replicated after a repeat transduction experiment and polyclonal selection of shRNA or control cells. Repression of HMGA1 was confirmed in each case at the level of gene expression (qRT-PCR) and Western analysis.

Migration and invasion assays

Invasion assays were performed as previously described [25] with the following modifications. Briefly, 15,000 cells were resuspended in serum-free media (500 µl) and placed in the upper chamber of a 24-well BD BioCoat™ Matrigel™ Invasion Chamber coated with Matrigel. Invasion was calculated as the percentage of total cells that invaded into the bottom chamber containing complete media with serum. Migration was performed similarly, except that Matrigel was omitted.

Orthotopic tumorigenicity and metastatic foci experiments

Cells (suspended in 75 µl phosphate-buffered saline (PBS)) were implanted into murine (NOD-scid IL2Rgamma<sup>−/−</sup>) mammary fat pads with an equal volume of Matrigel. Tumor volumes (calculated by 4/3 πx length/2 x width/2 x depth/2) were monitored daily until they reached 1–1.5 cm<sup>3</sup>, at which time mice were euthanized. The presence of tumor foci within the lung was analyzed histopathologically. For tail vein injection experiments, cells (10<sup>7</sup>) were resuspended in PBS (150 µl). Mice were euthanized after 3 weeks, and lungs were examined histopathologically.

Mammosphere Assay

Mammosphere assays were performed as previously described [34] and spheres (>50 µm) were counted.

Western Analysis

Western blots were performed as previously described [10,12], using commercial antibodies to HMGA1 (Abcam) and β-Actin (Cell Signaling), both at a 1:1000 dilution.

Gene Expression Analysis with Quantitative, Reverse Transcription PCR

Total RNA was isolated using the Direct-zol RNA MiniPrep kit (Zymo) and analyzed by qRT-PCR as we previously described. The expression level of each gene was normalized to the human RPLP0 (Applied Biosystems) or β-actin gene. Primers for E-Cadherin, Snail, and Vimentin were previously described [10,33].

HMGA1 knock-down and gene expression profile analysis

HMGA1 was knocked-down in MDA-MB-231 cells using siRNA as we previously described [10,21,25]. RNA was isolated and hybridized to the Affymetrix exon array (GeneChip Human Exon 1.0 ST Array) as previously described. Expression data was pre-processed using the robust microarray (RMA) algorithm [44], as implemented in the oligo software package [45] available from Bioconductor [46] and annotated to the most recent human genome using the getNetAffx function in the oligo package. Microarray data were uploaded to Gene Expression Omnibus (GSE45483). Expression profiles from HMGA1 knock-down cells were compared to control cells treated with the vector RNA using Bayes modified t-tests and the limma package from Bioconductor [47]. We focused our analysis on the 100 most differentially expressed transcripts.

To compare this signature to genes expressed in a panel of embryonic stem cells, induced pluripotent stem cells, embryoid bodies, and fibroblasts, we downloaded expression profiles for a 43 sample study (GSE25970) [35] from the Gene Expression...
Pathway analysis of differentially expressed genes was performed using Ingenuity Pathway Analysis (IPA, Ingenuity Systems, http://www.ingenuity.com) as we described [9]. IPA scores were generated for each network and indicate the likelihood that the focus genes present in the network could occur by chance alone. A score of ≥3 is considered significant because it represents a 1/1,000 chance that the network contains specific focus genes by random chance alone [9].

**Supporting Information**

**Figure S1** Silencing HMGA1 in MDA-MB-231 blocks the formation of foci to the lung following tail vein injections. A) Lung foci were enumerated 3 weeks following tail vein injections of control or shHMGA1 MDA-MB-231 cells (n = 3 for control mice; n = 4 for shHMGA1 mice). B) Graph of the mean number of tumor foci for control mice; n = 4 for shHMGA1 mice).

**Figure S2** Silencing HMGA1 in MDA-MB-231 results in significant repression in HMGA1 mRNA and protein, with alterations in gene expression. A) Independent replicate experiments of MDA-MB-231 cells with or without HMGA1 knock-down result in silencing HMGA1 at the level of mRNA. B) HMGA1 protein is also repressed following treatment with siRNA. C) Validation of genes in the HMGA1 signature shows that gene expression assessed by quantitative RT-PCR (qRT-PCR) parallels that of the microarray results. D) Table comparing differential expression of the HMGA1 signature identified by microarray and qRT-PCR.

**Figure S3** HMGA1 network derived from differentially expressed genes in MDA-MB-231 with or without HMGA1 knock-down. From 63 differentially expressed genes as the focus gene set, the second highest-scoring network was Cardiovascular Disease, Cell Death and Survival, and Nervous System Development and Function (score = 46). Colors, arrows, lines and abbreviations are described under Figure 4B. NF-κB, ERK, and MAPK are major nodes, which have been identified in prior studies of global gene expression profiles mediated by HMGA1 [9].

**Acknowledgments**

This work is dedicated to the memory of Dr. Wenlan Wang, a friend and colleague who courageously battled breast cancer. She will forever inspire all who had the opportunity to know her personally or through her work.

**Author Contributions**

Conceived and designed the experiments: SNS SS DLH LMSR. Performed the experiments: SNS WP AB SR. Analyzed the data: SNS. Wrote the paper: SNS LMSR.

**References**

1. Siegel R, Naishadham D, Jemal A (2013) Cancer statistics. CA Cancer J Clin 63: 11–30.
2. Lee JS, Fackler MJ, Lee JH, Choi C, Park MH, et al. (2010) Basal-like breast cancer displays distinct patterns of promoter methylation. Cancer Biol Ther 9:1017–1024.
3. Ben-Porath I, Thomson MW, Carey VJ, Ge R, Bell GW, et al. (2000) An embryonic stem-cell gene-expression signature in poorly differentiated aggressive human tumors. Nat Genet 40: 499–507.
4. Resar LMS (2010) The High Mobility Group A1 Gene: Transforming Inflammation and Cancer? Cancer Research 70: 436–439.
5. Chou B-K, Mali P, Huang X, Ye Z, Dowsey S, et al. (2011) Efficient human iPSC cell derivation by a non-integrating plasmid from blood cells with unique epigenetic and gene expression signatures. Cell Res 21: 518–529.
6. Zhou G, Chen J, Lee S, Clark T, Rowley JD, et al. (2001) The pattern of gene expression in human CD34(+)/stem/progenitor cells. Proc Natl Acad Sci U S A 98: 13966–13971.
7. Karp JE, Smith BD, Resar LS, Greer JM, Choi C, Park MH, et al. (2010) Basal-like breast cancer displays distinct patterns of promoter methylation. Cancer Biol Ther 9:1017–1024.
8. Nelson DM, Joseph B, Hillion J, Segal J, Karp JE, et al. (2011) Flavopiridol induces BCL-2 expression and represses oncogenic transcription factors in leukemic blasts from adults with refractory acute myeloid leukemia. Leuk Lymphoma 52: 1999–2006.
9. Schuldiner A, Belton A, Kowalski J, Talbot CC, Di Cello F, et al. (2011) HMGA1 Drives Stem Cell, Inflammatory Pathway, and Cell Cycle Progression During Lymphoid Tumorigenesis. BMC Genomics 12: 549.
10. Belton A, Gabrovsky A, Iacobuzio-Donahue C, Huo DL, Resar LMS (2012) HMGA1 induces intestinal polyps in transgenic mice and drives tumor progression and stem cell properties in colon cancer cells. PLoS One 7: e30034.
11. Shah SN, Resar LMS (2012) High Mobility Group AI and Cancer: Potential Biomarker and Therapeutic Target. Histol Histopathol 27: 567–579.
12. Wood LJ, Mukherjee M, Dolle CE, Xu Y, Maher JF, et al. (2000) HMGA1 knock-down abrogates c-Myc transcription and represses oncogenic transcription factors in leukemia blasts from adults with refractory acute myeloid leukemia. Leuk Lymphoma 32: 51–58.
13. Pedulla ML, Trefil NR, Resar LM, Reeves R (2001) Sequence and analysis of the murine Hmg1 (Hmg1a) gene locus. Genom 271: 51–58.
14. Reeves R, Beckerbauer L (2001) HMGA1/2 promoter flexible regulators of transcription and chromatin structure. Biochem Biophys Acta 1519: 1–29.
15. Reeves R, Edberg DJ, Li Y (2001) Architectural transcription factor HMGI(Y) promotes tumor progression and mesenchymal transition of human epithelial cells. Mol Biol Cell 11: 217–224.
30. Pomeroy SL, Tamayo P, Gaasenbeek M, Sturla LM, Angelo M, et al. (2002) Prediction of central nervous system embryonal tumour outcome based on gene expression. Nature 415: 436–442.

31. Flohr AM, Rogalla P, Bouk U, Pauwtmann B, Buerger H, et al. (2003) High mobility group protein HMGA1 expression in breast cancer reveals a positive correlation with tumour grade. Histol Histopathol 18: 999–1004.

32. Shah SN, Kerr C, Cope L, Zambidis E, Liu C, et al. (2012) HMGA1 reprograms somatic cells into pluripotent stem cells by inducing stem cell transcriptional networks. PLoS One 7: e40533.

33. Mani SA, Guo W, Liao MJ, Ng E, Ayyanan A, et al. (2008) The epithelial–mesenchymal transition generates cells with properties of stem cells. Cell 133: 704–715.

34. Shaw FL, Harrison H, Spence K, Ablett MP, Simões BM, et al. (2012) A detailed mammosphere assay protocol for the quantification of breast stem cell activity. J Mammary Gland Biol Neoplasia 17: 111–117.

35. Bock C, Kisinis E, Verstappen G, Gu H, Boulting G, et al. (2011) Reference maps of human ES and iPS cell variation enable high-throughput characterization of pluripotent cell lines. Cell 144: 439–452.

36. Muramoto R, Sekine Y, Imoto S, Ikeda O, Okayama T, et al. (2008) BART is essential for nuclear retention of STAT3. Int Immunol 20: 395–403.

37. Massague J (2008) TGFβ in cancer. Cell 134: 215–230.

38. Semenza GL (2012) Cancer stromal cell interactions mediated by hypoxia-inducible factors promote angiogenesis, lymphangiogenesis, and metastasis. Oncogene in press.

39. Thibodeaux CA, Liu X, Dishbroe GL, Zhang Y, Rone JD, et al. (2009) Immortalization and transformation of human mammary epithelial cells by a tumor-derived Myc mutant. Breast Cancer Res Treat 116:281–294.

40. Trout JS, Huang Y, Fornace AJ, Hoffman B, Liebermann D (2010) Gadd45a functions as a promoter or suppressor of cancer dependent on the oncogenic stress. Cancer Res 70:9671–9681.

41. Dang CV (2012) MYC on the path to cancer. Cell 2:304–307.

42. Nie Z, Hu G, Wei G, Yamane A, Roch A, et al. (2012) c-Myc as a universal amplifier of expressed genes in lymphocytes and embryonic stem cells. Cell 151:68–79.

43. Liu S-S, Jazag A, Whang EE (2006) HMGA1a is a determinant of cellular invasiveness & in vitro metastasis in pancreatic adenocarcinoma. Cancer Res 66: 11615–11622.

44. Irizarry RA, Bolstad BM, Collin F, Cope LM, Hobbs B, et al. (2003) Summaries of Affymetrix GeneChip Probe Level Data. Nucleic Acids Res 31: e15.

45. Carvalho BS, Irizarry RA (2010) A framework for oligonucleotide microarray preprocessing. Bioinformatics 26: 2363–2367.

46. Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, et al. (2004) Bioconductor: open software development for computational biology and bioinformatics. Genome Biol 5: R80.

47. Smyth GK (2005) Limma: linear models for microarray data, in R. Gentleman; R. V. Carey; S. Dudoit; R. Irizarry, concordance at the top (CAT) plot & W. Huber, eds., ‘Bioinformatics and Computational Biology Solutions using R and Bioconductor’, Springer-Verlag: 397–420.

48. Barrett T, Suzek TO, Troup DB, Wilhite SE, Ngau W-C, et al. (2005) NCBI GEO: mining millions of expression profiles database and tools. Nucleic Acids Res 33: D562–D566.