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# Knockdown of *HMGA1* inhibits human breast cancer cell growth and metastasis in immunodeficient mice

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ABSTRACT

The high mobility group A1 gene (*HMGA1*) has been previously implicated in breast carcinogenesis, and is considered an attractive target for therapeutic intervention because its expression is virtually absent in normal adult tissue. Other studies have shown that knockdown of *HMGA1* reduces the tumorigenic potential of breast cancer cells *in vitro*. Therefore, we sought to determine if silencing *HMGA1* can affect breast cancer development and metastatic progression *in vivo*. We silenced *HMGA1* expression in the human breast cancer cell line MDA-MB-231 using an RNA interference vector, and observed a significant reduction in anchorage-independent growth and tumorsphere formation, which respectively indicate loss of tumorigenesis and self-renewal ability. Moreover, silencing *HMGA1* significantly impaired xenograft growth in immunodeficient mice, and while control cells metastasized extensively to the lungs and lymph nodes, *HMGA1*-silenced cells generated only a few small metastases. Thus, our results show that interfering with *HMGA1* expression reduces the tumorigenic and metastatic potential of breast cancer cells *in vivo*, and lend further support to investigations into targeting *HMGA1* as a potential treatment for breast cancer.



## 1. Introduction

Breast cancer is the most frequent type of cancer and a leading cause of cancer death among women worldwide [1–3]. The proteins of the high mobility group A (HMGA) family have been previously implicated in breast carcinogenesis [4]. These are non-histone, DNA-binding proteins often referred to as architectural transcription factors. They contain basic A-T hook domains that mediate binding to the minor groove of AT-rich regions of chromosomal DNA. Upon binding to DNA, HMGA proteins regulate gene expression by organizing the transcriptional complex through protein–protein and protein–DNA interactions (reviewed in [5–7]). The HMGA family includes the products of the *HMGA1* and *HMGA2* genes. Of these, *HMGA1* can generate three different protein isoforms through alternative splicing (HMGA1a, b and c). HMGA1a and HMGA1b are the most abundant isoforms, and differ by only 11 amino acids that are present in HMGA1a but not in HMGA1b [4]. *HMGA1* is expressed almost exclusively during embryonic development [8], but has been found to be abnormally expressed in several types of cancer, including leukemia [9], pancreatic [10,11], thyroid [12], colon [13], breast [14–16], lung [17], ovarian [18], endometrial [19], prostate [20], and head and neck cancer [21]. Several studies have also shown that overexpression of *HMGA1* induces transformation both *in vitro* and in animal models (reviewed in [6,7]).

The causal role of *HMGA1* in breast cancer development and metastasis is supported by studies in cell lines [14,22,23] as well as by the analysis of clinical specimens [15,16]. For example, elevated HMGA1 protein expression has been reported in breast carcinomas and hyperplastic lesions with cellular atypia, in contrast with normal breast tissue where HMGA1 was not detected [15,16]. Similarly, HMGA1 overexpression has been observed in human breast cancer cell lines, with the highest levels in known metastatic lines, such as Hs578T and MDA-MB-231 [14,22,23]. Moreover, exogenous overexpression of HMGA1a was shown to induce transformation of the human non-tumorigenic mammary myoepithelial cell line Hs578Bst *in vitro* [14] and to increase the metastatic ability of MCF7 breast cancer cells *in vivo* [22]. Conversely, decreasing *HMGA1* expression in Hs578T breast cancer cells was shown to cause a reduction in anchorage-independent growth, which is a typical feature of cancer cells [14].

*HMGA1* is considered an attractive target for therapeutic intervention because its expression is virtually absent in normal adult tissue and knockdown of *HMGA1* has been shown to interfere with the tumorigenic growth of multiple cancer cell lines [6,7]. We therefore sought to determine if silencing *HMGA1* can affect breast cancer development and metastatic progression *in vivo* using a human xenograft mouse model.

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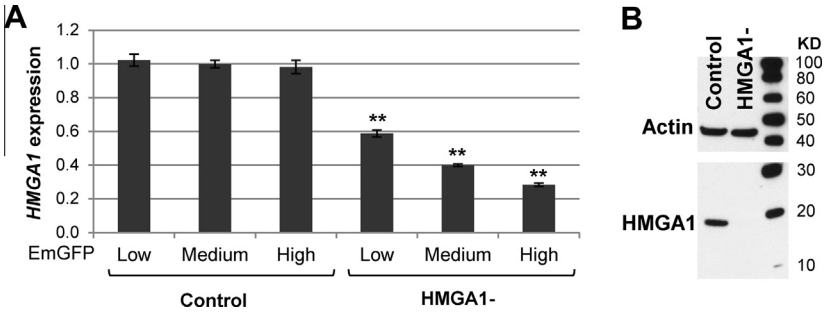


**Fig. 1.** *HMGA1* knockdown in MDA-MB-231 breast cancer cells. *HMGA1* expression analysis by qRT-PCR (A), and western blot (B) in MDA-MB-231 cells transfected with the *HMGA1* silencing vector (HMGA1−). Bars represent means ± standard deviation (SD) of three replicates; $^{**}P < 0.001$ by Student's *t*-test. Expression values are relative to the average of the control samples.

## 2. Materials and methods

### 2.1. Cell lines, transfections and proliferation assays

The MDA-MB-231 breast cancer cell line was obtained from American Type Culture Collection, and cultured in DMEM (Cellgro 10-013), supplemented with 10% FBS and 5 μg/ml gentamicin. Cells were propagated for two weeks, aliquoted in media supplemented with 5% DMSO and stored in liquid nitrogen. Each aliquot was used for less than six months. MDA-MB-231 cells were transfected using Lipofectamine 2000 (Invitrogen). Stable transfectants were selected by adding 50 μg/ml of blasticidin to the media, and propagated in media without blasticidin. Anchorage-independent growth was assessed on soft agar as previously described [17]. Tumorsphere formation from single cell suspensions was assessed using MammoCult media (STEMCELL Technologies) in ultra-low adherence plates (Corning). Spheres were counted using an inverted microscope after seven days of growth. Secondary tumorspheres were generated from single cells suspensions obtained by enzymatic digestion of the primary tumorspheres using 0.05% trypsin (Invitrogen). All kits and reagents were used according to the manufacturer's instructions.

### 2.2. HMGA1 silencing construct

The *HMGA1* silencing construct pHMGA1-394-EmGFP-miR was generated using the BLOCK-iT Pol II miR RNAi with EmGFP system (Invitrogen), following the manufacturer's instructions. Briefly, a silencing microRNA (miRNA) RNA interference (RNAi) oligonucleotide was designed based on the reference sequence NM_145899.1, using Invitrogen's RNAi designer. The *HMGA1* target sequence with the best rank score (5′-AGCGAAGTGCCAACACCTAAG) was incorporated into a pre-miRNA sequence and cloned into the pcDNA6.2-GW/EmGFP-miR vector using the reagents and competent *E. coli* supplied with the kit. Five *E. coli* clones were isolated and the constructs sequenced. One construct with verified miRNA sequence was selected for transfection. A vector harboring a non-targeting miRNA sequence provided with the kit was used as control.

### 2.3. Gene expression analysis

RNA expression was assessed by quantitative Real-Time PCR (qRT-PCR) after reverse transcription. RNA was extracted using the RNeasy Mini Kit (Qiagen) applying the on-column DNase treatment. The amount and quality of the RNA were verified by measuring the absorbance at 260 and 280 nm. Reverse transcription was performed with the High Capacity cDNA Reverse Transcription Kit, and duplex qPCR of the resulting cDNA was performed on a 7500 Real Time PCR System, using the TaqMan Gene Expression Master Mix, and the Human RPLP0 Endogenous Control (Applied Biosystems). The TaqMan primers and probes for *HMGA1* were previously described [19]. Reverse Transcriptase negative (RT−) samples were analyzed in parallel to verify the absence of contaminating genomic DNA. All qPCR reactions were performed in triplicate. Protein expression was assessed by western blot. Cell lysates were prepared using RIPA buffer (Sigma–Aldrich) with added cOmplete, EDTA-free Protease Inhibitor Cocktail (Roche Applied Science), and run on 4–12% NuPAGE Bis-Tris Gels using NuPAGE MES SDS Running Buffer (Invitrogen). Gels were blotted on PVDF membranes and HMGA1 was detected using 0.3 μg/ml of a goat primary antibody (EB06959, Everest Biotech); Beta Actin (loading control) was detected using a 1:1000 dilution of rabbit primary antibody (4967, Cell Signaling Technology). HRP-conjugated secondary antibodies were detected using the SuperSignal West Pico Chemiluminescent Substrate (Pierce). All kits were used according to the manufacturer's instructions.

### 2.4. Animal experiments

NOD.CB17-*Prkdc*$^{scid}$/J (NOD SCID) mice were purchased from The Jackson Laboratory, and maintained in Johns Hopkins AAALAC accredited facilities, with all procedures approved by the ACUC. All efforts were made to minimize suffering. Mice were injected subcutaneously at the inguinal mammary fat pad with $2 \times 10^6$ cells in 50 μl of serum-free media (one injection per mouse). Tumor growth was monitored for 10 weeks. Mice were euthanized by intraperitoneal Avertin overdose, followed by cardiac perfusion with heparinized PBS, then 10% neutral buffer formalin for fixation. Tissues were routinely processed for paraffin embedment, sectioned at 5 μm, and stained with hematoxylin and eosin (H&E).

## 3. Results and discussion

We generated an *HMGA1*-silencing construct that uses microRNA (miRNA) for RNA interference (RNAi), and co-expresses the fluorescent molecule EmGFP (co-cistronic) for easy detection of the transfected cells (pHMGA1-394-EmGFP-miR). We selected the breast cancer cell line MDA-MB-231 because it was previously shown to overexpress *HMGA1* [14], and it is tumorigenic in immunodeficient mice. Stable, polyclonal MDA-MB-231 transfectants were generated, and sorted by flow cytometry. We isolated three fractions with different degrees of EmGFP fluorescence, which were analyzed for *HMGA1* expression by qRT-PCR. The analysis showed that knockdown of *HMGA1* correlated with fluorescence intensity in MDA-MB-231 cells transfected with the silencing vector (Fig. 1A), as it was expected since the sequences for the miRNA and EmGFP are co-cistronic. There was no decrease in *HMGA1*

mRNA in cells transfected with the control vector (Fig. 1A). This allowed us to select a cell population with efficient knockdown of *HMGA1* without isolating individual clones. The fractions with the highest fluorescence were expanded and further analyzed by western blot. HMGA1 was detected exclusively in the control cells

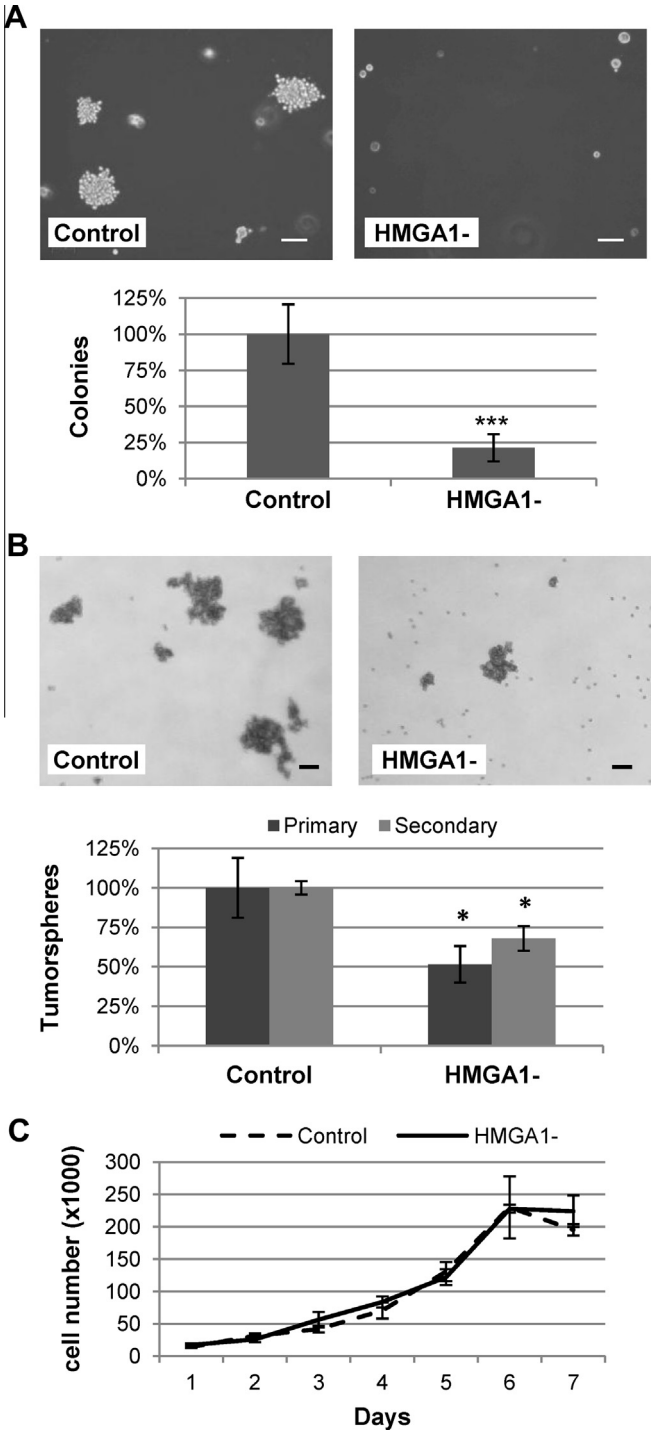


**Fig. 2.** Knock down of *HMGA1* inhibits anchorage-independent growth and tumorspheres formation in MDA-MB-231 breast cancer cells. Colonies on soft agar (A), tumorspheres (B) and cell growth in standard tissue culture conditions (C) of MDA-MB-231 cells transfected with the *HMGA1* silencing vector (HMGA1−) or the non-silencing vector (control). Size bar = 100 μm. Colonies larger than 100 μm and tumorspheres larger than 50 μm were counted. The experiments were performed in triplicate and repeated twice. Value shown are means ± SD; $^{*}p < 0.05$, $^{***}p < 0.001$ by Student's *t*-test.

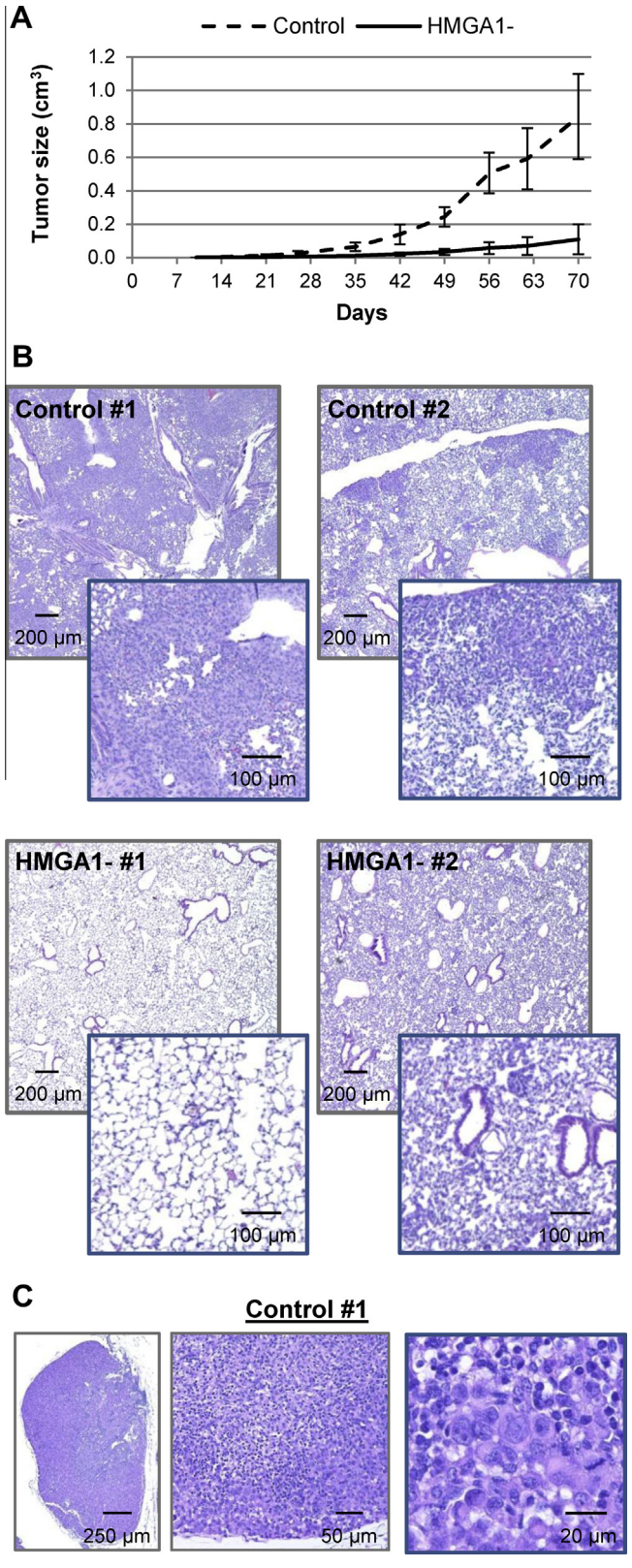


**Fig. 3.** Knockdown of *HMGA1* in MDA-MB-231 breast cancer cells impairs tumorigenesis and metastasis in immunodeficient mice. (A) Primary tumor size in immunodeficient mice injected with control or *HMGA1*-silenced (HMGA1−) MDA-MB-231 breast cancer cells; values shown are means ± SD; $n = 10$. (B) Histological analysis shows extensive metastases in the lungs of mice injected with control cells, and only small metastases or intravascular neoplastic emboli in the lungs of animals injected with *HMGA1*-silenced cells; two representative mice from each cohort, and two magnifications for each slide are shown. (C) Representative lymph node from one mouse injected with control cells; the lymph node is expanded and largely effaced by neoplastic cells.

(Fig. 1B), confirming the efficient knockdown of *HMGA1* by this silencing construct.

Decreasing *HMGA1* expression has been shown to inhibit transformation *in vitro* in various established cancer cells lines [6,7], including Hs578T breast cancer cells [14]. Thus, we tested our *HMGA1*-silenced MDA-MB-231 cells for anchorage-independent growth, which is indicative of the tumorigenic and metastatic potentials of cancer cells [24], and the ability to form tumorspheres, which reflects stem/progenitor cell properties [25,26]. Knockdown of *HMGA1* reduced colony formation on soft agar by 80% ($P < 0.001$; Fig. 2A) and formation of primary tumorspheres by almost 50% ($P = 0.019$; Fig. 2B). The effect of *HMGA1* knockdown on the formation of secondary tumorspheres was less pronounced but still significant (Fig. 2B; $P = 0.047$). Since we used a polyclonal cell population, the reduced response in the secondary tumorspheres may result from the selection of clones with less efficient *HMGA1* knockdown during the formation of the primary tumorspheres. Cell growth in standard tissue culture conditions was not affected (Fig. 2C), indicating that silencing *HMGA1* does not cause a general inhibition of cell proliferation in this cell line but rather affects specific growth features associated with transformation, similarly to what was previously observed in Hs578T cells [14].

To investigate if knockdown of *HMGA1* affects breast cancer development and metastatic progression *in vivo*, we injected *HMGA1*-silenced and control MDA-MB-231 cells subcutaneously at the inguinal mammary fat pads of NOD SCID immunodeficient mice. Each cohort consisted of 10 mice, injected at one single site with $2 \times 10^6$ cells. Control MDA-MB-231 cells generated large tumors as expected. In contrast, growth of *HMGA1*-silenced xenografts was significantly inhibited ($P < 0.0001$; two-way anova) indicating that *HMGA1* is necessary for *in vivo* tumorigenesis in this breast cancer cell line (Fig. 3A). Tumor growth was monitored for 10 weeks. Post-mortem examination showed that the animals injected with control MDA-MB-231 cells had extensive necrosis of the primary tumors, numerous extensive lung metastases exceeding 1 mm in diameter (Fig. 3B), and distant metastases exceeding 5 mm diameter in axillary and contralateral prefemoral (subiliac) lymph nodes (Fig. 3C). Animals injected with *HMGA1*-silenced cells had less necrosis, far less lung metastases (Fig. 3B) and only one lymph node metastasis less 2 mm in diameter was observed. This striking difference in metastasis may be the result of a general inhibition of tumorigenesis since the primary tumors generated by *HMGA1*-silenced cells are significantly smaller than those formed by control cells. However, *HMGA1* overexpression is known to affect the transcription of many genes involved in metastatic progression [22,27] and has recently been associated with repression of *MTSS1*, which encodes the metastasis suppressor protein 1 [28]. Thus, it is possible that knockdown of *HMGA1* in our model is affecting metastatic potential through specific pathways and not only because of the reduced tumor growth.

In summary, we show that interfering with *HMGA1* expression reduces breast tumorigenesis *in vivo*. Taken together with the existing literature, our results indicate that *HMGA1* could be a viable target for the development of new therapeutic strategies for breast cancer treatment.

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