

Upregulation of *MMP-2* by *HMGA1* Promotes Transformation in Undifferentiated, Large-Cell Lung Cancer

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Abstract

Although lung cancer is the leading cause of cancer death worldwide, the precise molecular mechanisms that give rise to lung cancer are incompletely understood. Here, we show that *HMGA1* is an important oncogene that drives transformation in undifferentiated, large-cell carcinoma. First, we show that the *HMGA1* gene is overexpressed in lung cancer cell lines and primary human lung tumors. Forced overexpression of *HMGA1* induces a transformed phenotype with anchorage-independent cell growth in cultured lung cells derived from normal tissue. Conversely, inhibiting *HMGA1* expression blocks anchorage-independent cell growth in the H1299 metastatic, undifferentiated, large-cell human lung carcinoma cells. We also show that the *matrix metalloproteinase-2 (MMP-2)* gene is a downstream target upregulated by *HMGA1* in large-cell carcinoma cells. In chromatin immunoprecipitation experiments, *HMGA1* binds directly to the *MMP-2* promoter *in vivo* in large-cell lung cancer cells, but not in squamous cell carcinoma cells. In large-cell carcinoma cell lines, there is a significant, positive correlation between *HMGA1* and *MMP-2* mRNA. Moreover, interfering with *MMP-2* expression blocks anchorage-independent cell growth in H1299 large-cell carcinoma cells, indicating that the *HMGA1*–*MMP-2* pathway is required for this transformation phenotype in these cells. Blocking *MMP-2* expression also inhibits migration and invasion in the H1299 large-cell carcinoma cells. Our findings suggest an important role for *MMP-2* in transformation mediated by *HMGA1* in

large-cell, undifferentiated lung carcinoma and support the development of strategies to target this pathway in selected tumors. (Mol Cancer Res 2009;7(11):1803–12)

Introduction

Lung cancer is a leading cause of cancer death worldwide and the incidence is increasing, particularly in developing nations where the smoking rate is increasing (1). Therapy is highly toxic and largely ineffective, leading to an overall 5-year survival of only 15% for all types of lung cancer (1). Based on histopathologic findings, lung cancer is classified as small-cell lung cancer and non-small cell lung cancer. Small-cell lung cancer constitutes 10% to 15% of all lung cancers and typically occurs in the setting of cigarette smoke exposure (1). Non-small cell lung cancer makes up the remaining 85% to 90% of lung cancers and is further subdivided into (a) squamous cell carcinoma (~20-30% of all lung cancer cases), (b) adenocarcinoma (~40% of all cases), and (c) large-cell, undifferentiated carcinoma (~10-15% of all cases; ref. 1). The molecular mechanisms that lead to small-cell and non-small cell lung cancer have not been clearly elucidated.

To better understand how lung cancer develops, we are studying molecular pathways involved in transformation. Our focus is the *HMGA1* gene, which encodes the *HMGA1a* and *HMGA1b* chromatin binding proteins. These protein isoforms result from alternatively spliced mRNA and differ by 11 internal amino acids present only in *HMGA1a* (2-8). The low-molecular-weight (high mobility group) proteins contain AT hook DNA-binding domains that enable *HMGA1* proteins to bind to AT-rich regions of DNA (reviewed in ref. 6). Because they function in regulating gene expression and alter chromatin structure, *HMGA1* proteins have been described as architectural transcription factors. *HMGA1* expression is upregulated in diverse human cancers (reviewed in refs. 7, 8), and high levels of expression portend a poor prognosis in some tumors. More recent studies also show that *HMGA1* has oncogenic properties in cultured cells (9-12) and transgenic mice (13-15). How this gene leads to neoplastic transformation is only beginning to be elucidated (8).

Matrix metalloproteinases (MMP) are a family of over 20 extracellular, zinc-dependent proteolytic enzymes capable of degrading multiple components of the extracellular matrix (16-19). These enzymes play important roles not only in normal physiologic conditions, such as matrix homeostasis, but also in pathologic processes, including tumor progression where their

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expression is associated with invasive and metastatic behavior (16-19). A recent study also identified an MMP with tumor suppressor function (20). Here, we show that HMGA1 activates *MMP-2* expression in undifferentiated, large-cell carcinoma cells. Moreover, the HMGA1–MMP-2 pathway is required for anchorage-independent cell growth, cellular invasion, and migration in metastatic, undifferentiated, large-cell carcinoma cells. Taken together, these results support a critical role for *MMP-2* in malignant transformation by HMGA1 and implicate this pathway as a rational therapeutic target in selected lung cancer patients with large-cell carcinomas.

Results

HMGA1a Is Overexpressed in Human Lung Cancer Cell Lines and Primary Lung Tumors

To determine if *HMGA1* is overexpressed in human lung cancer, we initially surveyed human lung cancer cell lines for HMGA1 protein by Western blot analysis compared with cultured cells from normal lung tissue (Fig. 1A). We found that the HMGA1 protein is increased in five of five lung cancer cell lines, including H1299 metastatic, large-cell (undifferentiated), lung carcinoma cells (21); SK-MES-1 metastatic, squamous cell carcinoma cells (22); H358 nonmetastatic, bronchioalveolar carcinoma cells (23, 24); H125 metastatic adenosquamous carcinoma cell line (22); and H82 metastatic small-cell lung cancer cell line (25). These lung cancer cells were compared with the control BEAS-2B cells (26), which are cultured, immortalized cells isolated from normal human lung bronchial epithelium. We also assessed *HMGA1a* mRNA levels by quantitative reverse transcriptase-PCR (RT-PCR) compared with normal lung tissue RNA (Clontech) from pooled, control individuals (Fig. 1B). We included three metastatic, undifferentiated, large-cell carcinoma cell lines (H1299, H460, and H661; refs. 21, 25, 27); two metastatic squamous cell carcinoma cell lines (SK-MES and U-1752; refs. 22, 28); one metastatic adenosquamous carcinoma cell line (H125; ref. 22); and one metastatic, small-cell carcinoma cell line (H82; ref. 25). Notably, *HMGA1a* mRNA was also increased in all the lung cancer cells tested compared with the controls (Fig. 1B). To determine if gene amplification could account for the *HMGA1* overexpression, we performed Southern blot analysis in the H1299, H460, and H661 large-cell carcinoma cells compared with DNA from normal human lymphocytes. There was no evidence for amplification (Supplementary Fig. S1). In addition, sequence analysis of the *HMGA1a* and *HMGA1b* coding regions in the large-cell carcinoma cell lines showed no mutations (not shown).

To determine if *HMGA1a* expression is also increased in primary human lung tumors, we assessed *HMGA1a* mRNA levels by quantitative RT-PCR (qRT-PCR). Pooled RNA from normal lungs was included as the control. We found that *HMGA1a* was overexpressed by >1.5-fold in 17 of 24 or 71% of all tumor samples compared with the normal lung tissue (Fig. 1C). There was *HMGA1a* overexpression in 11 of 15 (73.4%) of adenocarcinoma cases and 8 of 9 (88.9%) of squamous cell carcinoma cases. There was a history of exposure to cigarette smoke in 15 of 24 (63%) of these patients, and it is possible that the smoke carcinogens induced aberrant gene expression in the lung tissue that preceded the development of the tumors. To further assess

HMGA1 expression in human lung cancer, we performed *in situ* hybridization on a section from an adenocarcinoma with an antisense *HMGA1* probe and observed significant *HMGA1a* mRNA staining in the malignant tumor, but not the adjacent, normal tissue (Fig. 1D). The control sense probe showed no staining (not shown). In four primary lung tumors with overexpression of *HMGA1*, there was sufficient sample for Western blot analysis, and HMGA1 protein was also elevated in these cases compared with normal lung tissue (not shown).

Inhibiting HMGA1 Expression Blocks Transformation in Large-Cell Lung Carcinoma Cells

To determine if *HMGA1* is required for transformation in the human lung cancer cells, *HMGA1* gene expression was inhibited in the H1299 metastatic, undifferentiated, large-cell lung carcinoma cell line using an RNA interference approach (9, 11). We constructed an HMGA1 ribozyme antisense vector with antisense sequence directed at the amino terminus (HMGA1-AS-1; ref. 9) and the carboxyl terminus (HMGA1-AS-2; ref. 11). The H1299 lung cancer cells were transfected with each construct in separate transfection experiments, and polyclonal cell lines were isolated. Both antisense cell lines had significantly decreased HMGA1 protein compared with the control cell line transfected with the vector alone (Fig. 2A). The loading control protein, β -actin, was unaffected by the antisense construct. In the antisense and control cell lines, the related protein, HMGA2, was also unaffected (Fig. 2A), indicating that the antisense vectors cause a specific, significant decrease in HMGA1 proteins.

The antisense cell lines with decreased HMGA1 proteins were subsequently analyzed in the soft agar assay to determine if transformation was inhibited in these cells. We observed that transformation was significantly blocked in both antisense cell lines by ~65% to 75% ($P = 0.0000995$ for HMGA1-AS-1; $P = 0.0000531$ for HMGA1-AS-2; Student's *t* test) compared with the polyclonal cell line transfected with control ribozyme vector (Fig. 2B and C). These results suggest that HMGA1 is critical for anchorage-independent cell growth in these cells. Cellular growth rates in the antisense and control cell lines were similar (Fig. 2D), indicating that transformation was blocked through a transformation-specific mechanism independent of decreased growth rate.

HMGA1a Induces a Transformed Phenotype with Anchorage-Independent Cell Growth in Lung Cells Derived from Normal Tissue

Because *HMGA1a* is overexpressed in lung cancer cell lines and inhibiting *HMGA1a* expression blocks transformation in H1299 large-cell lung carcinoma cells, we hypothesized that *HMGA1a* induces neoplastic transformation in lung cells. To explore this hypothesis, we constructed a polyclonal cell line overexpressing *HMGA1a* in BEAS-2B (26) immortalized, normal lung bronchial epithelial cells. Western blot analysis of the lung cells transfected with the *HMGA1a* plasmid express high levels of the HMGA1a protein compared with the control cells transfected with pSG5 control vector alone (Fig. 3A). We observed that BEAS-2B cells overexpressing *HMGA1a* formed transformed colonies capable of anchorage-independent growth (or foci formation) in soft agar in a manner similar to H1299

large-cell lung carcinoma cells (Fig. 3B). To determine if the transfected lung cell lines grow similarly in tissue culture, we developed growth curves for all stable cell lines. All cell lines grew at a similar rate, indicating that the transformed phenotype was not a result of an increased growth rate (Fig. 3C). Thus, our results show that *HMGA1a* has similar transforming activity in the cultured lung cells derived from normal lung tissue, indicating that it is oncogenic in these cells. Moreover, the knockdown experiments in H1299 large-cell carcinoma cells indicate that *HMGA1* is required for the transformed phenotype observed in these lung cancer cells.

HMGA1 Binds Directly to the MMP-2 Promoter in Undifferentiated, Large-Cell Carcinoma Lung Cancer Cells In vivo

Because HMGA1 functions in regulating gene expression, it has been postulated that it induces neoplastic transformation by altering expression of critical target genes involved in cell growth and transformation (7, 8). The gene targets that mediate transformation by *HMGA1*, however, are only beginning to be elucidated (8). We previously identified the gene encoding MMP-2 (*MMP-2* or *gelatinase A*) as a candidate HMGA1a target gene upregulated in prostate cancer cells (29). Because MMP-2 has also been implicated in both tumor invasiveness and neoplastic disease of the lung (16-19), we investigated its potential role in lung cancers overexpressing *HMGA1*. To determine if HMGA1 could bind directly to the *MMP-2* promoter, we performed chromatin immunoprecipitation experiments in the H1299 metastatic, undifferentiated large-cell lung carcinoma cells. We found that HMGA1 binds to the *MMP-2* promoter in a highly conserved region near the transcription start sites (Fig. 4A; ref. 30). This region includes two conserved, predicted HMGA1 consensus DNA-binding sites within 200 nucleotides upstream of the two transcription start sites (30). In primary human BJ fibroblasts transduced to overexpress *HMGA1a*, we also found that *MMP-2* expression is significantly increased ($P = 0.00001$; not shown). These findings suggest that HMGA1 binds to the *MMP-2* promoter to upregulate its expression.

To determine if HMGA1 occupies the *MMP-2* promoter in other large-cell carcinoma cells, we investigated two additional cell lines: H661 and H460 (Supplementary Fig. S2). As with the H1299 cells, we found significant HMGA1 binding to the *MMP-2* promoter *in vivo* in these undifferentiated, large-cell carcinoma cells. In contrast, we did not observe HMGA1 binding to the *MMP-2* promoter in neither U-1752 nor SK-MES squamous cell carcinoma cell lines (Supplementary Fig. S2). These findings suggest that the HMGA1–MMP-2 pathway is upregulated in the undifferentiated, large-cell carcinoma cells.

MMP-2 Is Necessary for Anchorage-Independent Cell Growth, Cellular Migration, and Invasion in H1299 Large-Cell Carcinoma Lung Cancer Cells

To determine if *MMP-2* is necessary for transformation in large-cell carcinoma cells overexpressing *HMGA1*, we performed RNA interference experiments to knock down expression of *MMP-2* in H1299 lung cancer cells. Using small interfering RNA (siRNA; Dharmacon), we generated H1299

cells with effective knockdown of *MMP-2* mRNA and protein (Fig. 4B; Supplementary Fig. S3). The cells with decreased *MMP-2* expression had decreased foci formation by 50% ($P = 0.019$; Student's *t* test; Fig. 4C). Because MMP-2 is involved in degrading the extracellular matrix and is thought to contribute to metastatic potential in some tumors, we also assessed migration and invasion in the H1299 lung cancer cells with and without MMP-2 knockdown. We found that migration was decreased by 51% ($P = 0.001$; Student's *t* test; Fig. 4D) and invasion was decreased by 63% ($P = 0.0001$; Student's *t* test; Fig. 4D). The cellular proliferation was similar for lung cancer cells treated with the *MMP-2* or control siRNA (Fig. 4E). Taken together, our studies indicate that *MMP-2* is a critical HMGA1 downstream target required for multiple transformation phenotypes, including anchorage-independent cell growth, migration, and invasion in H1299 human lung cancer cells.

HMGA1 and MMP-2 mRNAs Are Positively Correlated in Cultured, Large-Cell Carcinoma Cells

Because we found that HMGA1 binds to the *MMP-2* promoter in the undifferentiated, large-cell carcinoma cells, we sought to determine if *HMGA1a* expression and *MMP-2* expression correlate in this subset of lung cancer cells. To this end, we assessed the expression of *HMGA1a* and *MMP-2* mRNAs in the large-cell carcinoma cell lines (H1299, H460, H661) by quantitative RT-PCR. Using Pearson's correlation, we found a highly significant positive correlation between *HMGA1a* and *MMP-2* mRNAs in these undifferentiated lung carcinoma cells ($r = 0.96$, $P < 0.00001$; Supplementary Fig. S4). In contrast, there was only a weak correlation between *HMGA1a* and *MMP-2* expression in the squamous cell carcinoma cell lines (U-1752, SK-MES; $r = 0.59$, $P = 0.045$; not shown). To assess *HMGA1a* and *MMP-2* expression in primary tumors, we used data from the GSE2109 public microarray database, which included only five cases of primary large-cell carcinomas. This was the largest number of large-cell carcinoma primary tumors available from a published microarray study, which reflects the relatively low frequency of this tumor type. Using the Affymetrix probes directed at the 3' untranslated region (206074_s_at for HMGA1 and 201069_at for MMP-2), there was no significant correlation between *HMGA1a* and *MMP-2* expression by Pearson's correlation using the bootstrap method (31) in this very small sample size.

Although larger studies are needed to determine if the HMGA1–MMP-2 pathway is upregulated in primary large-cell lung tumors, our results are consistent with the hypothesis that HMGA1 drives tumorigenesis in large-cell lung carcinoma, at least in part by upregulating *MMP-2*. We found a significant, positive correlation between *HMGA1a* and *MMP-2* expression in undifferentiated, large-cell carcinoma cells. In addition, HMGA1 occupies the *MMP-2* promoter *in vivo* in these cells. Moreover, our functional studies show that HMGA1 and MMP-2 are required for anchorage-independent cell growth in large-cell carcinoma cells. Taken together, these studies also suggest that the HMGA1–MMP-2 pathway could be targeted in “personalized therapy” for selected patients with large-cell lung cancers characterized by dysregulation of this pathway.

Discussion

Lung cancer is a leading cause of death that continues to elude successful treatment in most patients. Each year, almost 200,000 new cases of lung cancer are diagnosed and over 150,000 people will die from this disease in the United States alone (1). Thus, research is urgently needed to discover molecular targets that could be exploited in therapy (1). Although progress has been made in understanding some of the molecular aberrations that lead to lung cancer, this has not translated into better therapy or improved survival (32, 33).

Because the *HMGA1* gene is upregulated in malignant transformation (7, 8), we investigated its role in lung cancer. Here, we show that *HMGA1a* is overexpressed in all metastatic human lung cancer cell lines and most primary tumors. Another recent study showed high levels of *HMGA1* expression in human lung cancer primarily by immunohistochemical analysis, including moderate to strong staining for HMGA1 in eight of nine large-cell carcinomas (34). Further, they found a correlation between HMGA1 staining and poor survival, although they did not determine if there was a relationship between HMGA1 staining and differentiation status or tumor grade in this study. Here, we show a functional role for HMGA1 in lung carcinogenesis. Specifically, we found that *HMGA1a* induces a transformed phenotype in lung cells derived from normal lung tissue and inhibiting its expression blocks anchorage-independent cell growth in metastatic, large-cell carcinoma lung cancer cells. Although additional studies with animal models are warranted, these results suggest that blocking HMGA1 function could have therapeutic implications for lung cancer. Of note, another group used an *HMGA1* antisense approach in other cancer cell lines and observed both apoptosis and decreased proliferation (35). We did not observe a significant decrease in growth rates in the H1299 cells with knockdown of *HMGA1*, suggesting that inhibition of transformation was independent of proliferation or apoptosis. Regardless of the mechanisms involved, decreasing HMGA1 proteins interferes with transformation in several malignant cell lines, suggesting that it could serve as a therapeutic target.

HMGA1 is widely overexpressed in aggressive human cancers, which underscores the importance of elucidating the molecular mechanisms that drive *HMGA1a*-mediated tumorigenesis (7, 8). Because HMGA1 functions in transcriptional regulation, it has been postulated that it induces transformation by dysregulating specific gene targets (7, 8, 12, 15, 29, 36). The repertoire of target genes regulated by HMGA1, however, is only beginning to emerge and is likely to depend on the cellular milieu. We previously showed that *HMGA1a* upregulates *MMP-2* in prostate cancer cells (29), although the role of *MMP-2* in transformation mediated by HMGA1 was not explored. A previous study showed that *MMP-2* protein expression predicts an unfavorable outcome with decreased survival in early-stage non-small cell lung cancer (37). In addition, *MMP-2* has been implicated in tumor invasiveness in lung cancer (16, 17). Thus, we investigated the functional significance of the HMGA1–*MMP-2* pathway in lung cancer. Interestingly, we found a highly significant, positive correlation between *HMGA1a* and *MMP-2* mRNA in the undifferentiated, large-cell carcinoma cells. Moreover, our chromatin immunoprecipitation experiments show that HMGA1 binds directly to the *MMP-2*

promoter in all three undifferentiated, large-cell carcinoma cell lines studied. In addition, inhibiting *MMP-2* expression blocks anchorage-independent cell growth, migration, and invasion in undifferentiated, large-cell carcinoma cells. We recently reported a similar correlation between HMGA1 protein levels and poor differentiation status in pancreatic cancer (38). Another group also identified HMGA1 as a key transcription factor in embryonic stem cells and undifferentiated or high-grade breast, bladder, and brain cancer, further implicating HMGA1 in driving tumor progression and a primitive, undifferentiated state in normal stem cells and cancer (39).

The MMP family of zinc-dependent proteinases was originally characterized based on the ability of these proteinases to degrade extracellular matrix and basement membrane proteins (16–19). By degrading the basement membrane, MMPs are thought to enhance cell mobility in a stationary tumor cell and promote metastases. In fact, MMP activity correlates with cellular invasiveness and metastatic potential in some solid tumors (16–19). More recently, MMPs were shown to exert other important biological effects relevant to cancer by cleaving critical proteins involved in angiogenesis, apoptosis, chemotaxis, cell migration, and cell proliferation (16). Thus, it has become clear that MMPs not only function in cancer progression and metastasis but may also contribute to steps of cancer development (16–19). These diverse activities may account for the role of *MMP-2* in mediating anchorage-independent cell growth, migration, and invasion observed in our studies. Another group recently showed that HMGA1 may modulate *MMP-9* activity in pancreatic cancer (40) and putative HMGA1 DNA-binding sites are present in the promoter regions of several *MMP* family members (41). This suggests that HMGA1 could promote metastatic progression by orchestrating the activity of varied *MMP* genes depending on the cellular context. Along these lines, a recent study also found that *MMP-2* immunostaining is a more sensitive predictor than *MMP-9* in lung cancer progression, metastasis, and survival (42).

In conclusion, we discovered that *HMGA1* is an important oncogene that seems to drive transformation in human lung cancer. Moreover, we show that *MMP-2* is a critical downstream target activated by HMGA1 in undifferentiated, large-cell carcinoma lung cancers. Our findings identify a molecular mechanism for increased *MMP-2* expression and implicate this pathway as a rational therapeutic target in selected cases of lung cancer.

Materials and Methods

Cell Culture and Transfection

BEAS-2B (26), H1299 (21), SK-MES-1 (22), H358 (23, 24), H125 (22), H82 (25), H460 (27), and H661 (25) were obtained from the American Type Culture Collection and grown according to American Type Culture Collection guidelines. U-1752 cells (28) were grown as previously described (43). Cells were transfected using Lipofectin as described by the manufacturer (Life Technologies). Polyclonal, pooled, resistant cell lines overexpressing *HMGA1a* or control vector were selected in a medium containing puromycin (0.75 µg/mL). H1299 cells transfected with the *HMGA1* (HMG-I) ribozyme antisense (9, 11) and control constructs were selected in a

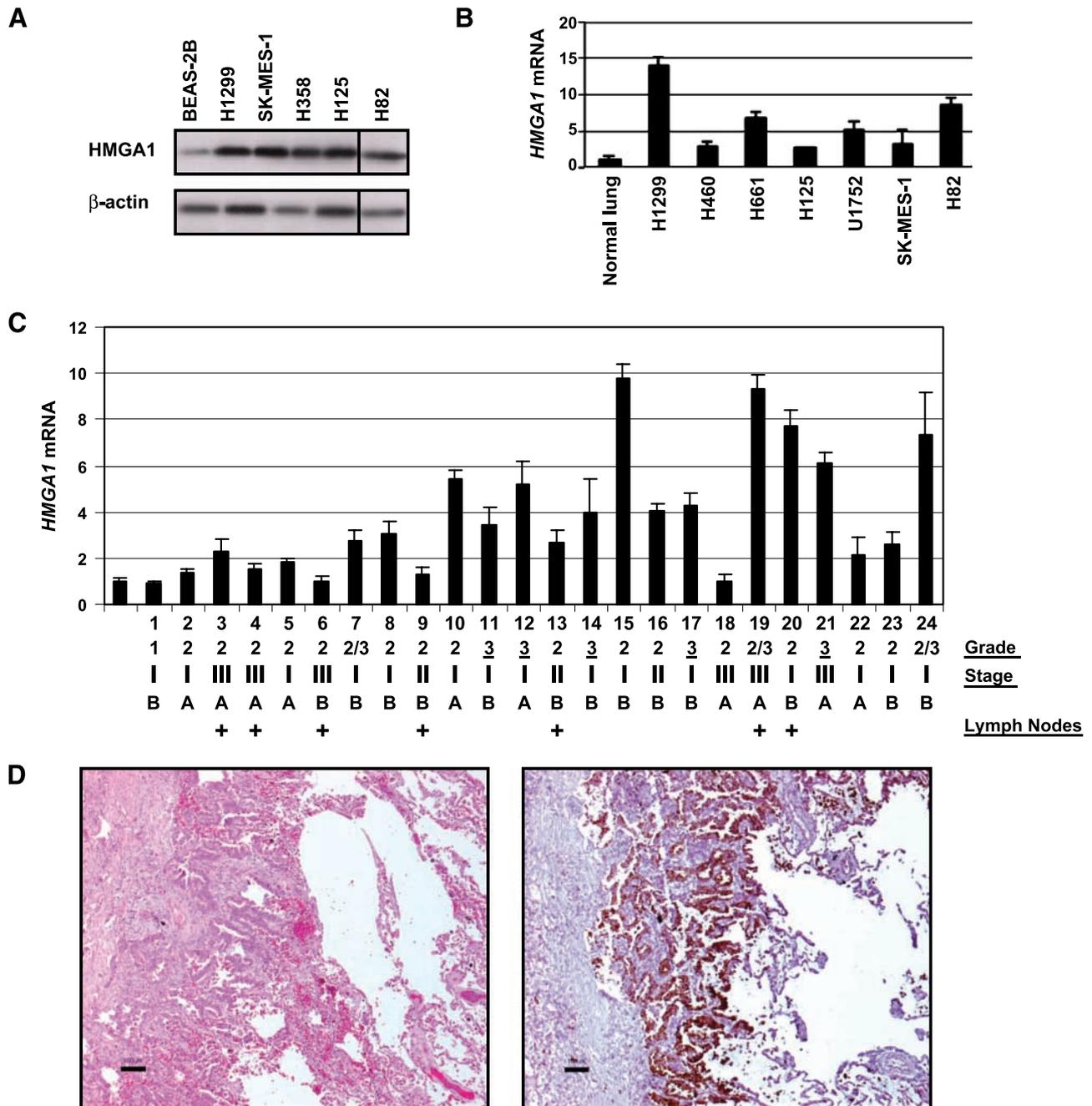


FIGURE 1. *HMGA1* is overexpressed in cultured human lung cancer cells and primary lung tumors. **A.** *HMGA1* protein is increased in the cultured human lung cancer cell lines H1299, SK-MES-1, H358, H125, and H82 compared with the control BEAS-2B cells derived from normal human bronchial lung. **B.** *HMGA1a* mRNA is increased in the cultured human lung cancer cell lines H1299, H460, H661, H125, U1752, SK-MES-1, and H82 compared with RNA from pooled, normal lung tissue from normal individuals. **C.** *HMGA1a* mRNA is increased in 71% of primary lung cancer tumors by qRT-PCR. C, control RNA from normal lung derived from three normal individuals who died from causes other than lung cancer. Samples 1 to 15 are from primary lung tumors diagnosed histopathologically as adenocarcinoma, and samples 16 to 24 are from primary lung tumors diagnosed as squamous cell carcinoma. The bottom panel shows tumor grade, stage, and lymph node metastases (+) if present. Additional clinical data are available in Supplementary Table S1. **D.** *HMGA1* mRNA is elevated in this case of adenocarcinoma by *in situ* hybridization.

medium containing zeocin (75 μ g/mL). Polyclonal, pooled, resistant cell lines with decreased *HMGA1* proteins by Western blot analysis were used for soft agar analysis. H1299 cells were transfected with siRNA (Dharmacon) according to the manufacturer's instructions as we previously described (12, 15).

Plasmids

The *HMGA1* antisense constructs were previously described (9, 11). Briefly, both vectors were made using the vector pU1/RIBOZYME, which incorporates an autocatalytic hammerhead ribozyme structure within the complementary sequence. The NT-antisense-*HMGA1* vector included an antisense sequence

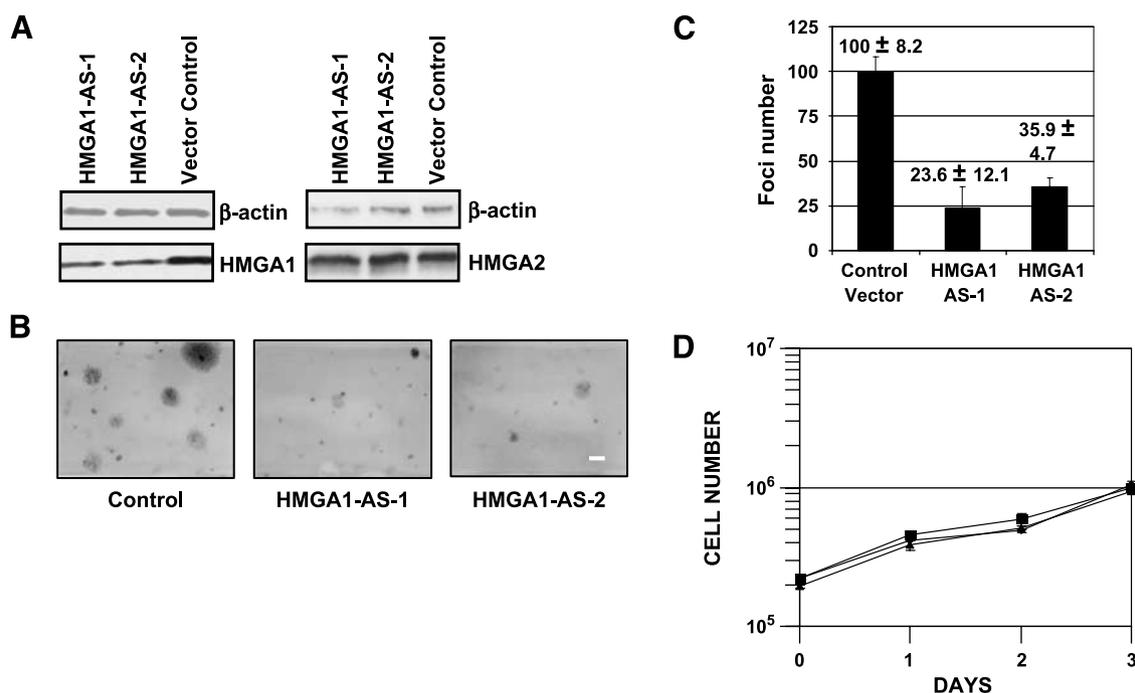


FIGURE 2. Inhibiting *HMGA1* expression blocks transformation in cultured human lung cancer cells derived from metastatic disease. **A.** Western blot analysis shows decreased *HMGA1* protein in the antisense cell lines. The lane marked *HMGA1-AS-1* represents H1299 polyclonal cells transfected with the amino-terminal antisense *HMGA1* construct; the lane marked *HMGA1-AS-2* represents H1299 polyclonal cells transfected with the carboxyl-terminal antisense construct; and the lane marked vector control represents H1299 polyclonal cells transfected with control vector alone. The blot was probed with the *HMGA1* antibody as well as an antibody to β -actin to control for protein loading. Western blot analysis of *HMGA2* protein in the control and antisense cell lines shows no decrease in *HMGA2* protein. This blot was also probed with a β -actin antibody as a loading control. **B.** Decreased anchorage-independent cell growth or foci formation in cells with decreased *HMGA1* proteins ($P = 0.0000995$ for *HMGA1-AS-1* and $P = 0.0000531$ for *HMGA1-AS-2*; Student's *t* test). This experiment was done thrice in duplicate. Columns, average; bars, SD. **C.** Graphical representation of decreased foci formation in the cells with decreased *HMGA1* proteins ($P = 0.0000995$ for *HMGA1-AS-1* and $P = 0.0000531$ for *HMGA1-AS-2*; Student's *t* test). This experiment was done thrice in duplicate. Columns, average; bars, SD. **D.** Cell growth rates in control cells and cells with decreased *HMGA1* proteins were similar. This experiment was done in duplicate and repeated at least once. Points, average; bars, SD.

directed at the amino terminus (AS-1), and the CT-antisense-*HMGA1* vector (AS-2) included an antisense sequence directed at the carboxyl terminus (11). The parent vector pU1/RIBOZYME was used as a control vector for both antisense vectors.

The plasmids pSG5-*HMGA1a* and pSG5-*HMGA1b* have been previously described (9, 10).

Western Blot Analysis

For Western blot analysis of *HMGA1*, total cell lysates collected from growing cells were boiled in 2 \times Laemmli buffer and analyzed by SDS/4% to 20% gradient PAGE and subjected to Western blot analysis using a chicken polyclonal antibody raised against the amino terminus of *HMGA1* diluted 1:500 as described previously (9, 10). For analysis of *HMGA2*, a rabbit polyclonal antibody raised against the amino terminus of *HMGA2* was diluted 1:500 (9, 10). The actin monoclonal antibody AC15 (Sigma Immunochemicals) was diluted 1:5,000 and used to control for sample loading. Reactive proteins were detected by enhanced chemiluminescence (Amersham).

In situ Hybridization

To assess *HMGA1* gene expression in lung cancer specimens, *in situ* hybridization was done as previously described (44).

Riboprobe Preparation for Nonradioactive In situ Hybridization

PCR was used to generate 400- to 500-bp DNA templates for antisense or sense riboprobes by incorporating the T7 promoter into the 5' end of the antisense or sense primer as described (44). The primers for *HMGA1* were forward 5'-GAAGGGAAGATGAGTGAGTCGAGCTCG-3' and reverse 5'-GGTAAATCTGCTCCTCACTCAGCCC-3'. The primers incorporating T7 for *HMGA1* were forward 5'-CTAATACGACTCACTATAGGGAAGCGAAGATGAC-3' and reverse 5'-CTAATACGACTCACTATAGGGG-TAAATCTGCTC-3'.

Chromatin Immunoprecipitation Experiments

Chromatin immunoprecipitation experiments were done as we previously described (15) using H1299 cells. Proteins cross-linked to chromatin were immunoprecipitated with the following antibodies: *HMGA1* (12, 15, 45), Pol II or histone H3 (as positive controls), or IgG (as a negative control). The *MMP-2* promoter region with the consensus *HMGA1* DNA-binding site was amplified from the immunoprecipitated protein-DNA complexes using the forward and reverse primers 5'-GGGAAAAGAGGTGGAGAAA-3' and 5'-CGCCTGAG-GAAGTCTGGAT-3', respectively. The *HPRT1* promoter was amplified as a negative control promoter with no *HMGA1* binding sites as described (12, 15, 45).

MMP-2 Knockdown Experiments

siRNA was used to knock down expression of *MMP-2* according to the instructions of the manufacturer. Specifically, the H1299 cells were incubated with siRNA to *MMP-2* (20 $\mu\text{mol/L}$; Dharmacon) compared with the control siRNA (20 $\mu\text{mol/L}$; siCONTROL nontargeting siRNA pool that contains four negative control siRNAs without matches to human, mouse, or rat gene also by Dharmacon). After 18 to 20 h of incubation, cells were washed and placed in soft agar or maintained in culture dishes to harvest for protein or mRNA at the indicated time points. Protein levels for *MMP-2* were assessed by gelatin zymography with conditioned medium at 24 h, 48 h, 8 d, and 10 d after transfection with each siRNA. *MMP-2* and control β -actin mRNA levels were assessed at the same time points.

Gelatin Zymography of Conditioned Medium

The medium was collected and subjected to gelatin zymography as previously described (46, 47). Further identification of the MMPs was done using low-molecular-weight protease inhibitors added to the Triton X-100 buffer during the 2-d incubation required for protease activation as previously described (46, 47).

Soft Agar Assay

Soft agar assays were done as previously described (9, 10), but with the following exceptions. For the BEAS-2B cells, 2×10^5 were seeded in 8 mL of top agarose composed of 1.2 mL of 2% agarose and 6.8 mL of BEGM medium and layered on top of 10 mL of bottom agarose composed of 2.5 mL of 2% agarose and 7.5 mL of BEGM medium (Clonetics CC-3170). For the H1299 experiments, $2 \times \text{RPMI}$ containing 5% fetal bovine serum was used instead of $2 \times \text{DMEM}$. To determine the effects of *MMP-2* knockdown, H1299 cells were transfected with siRNA to *MMP-2* or control siRNA and 5,000 cells/mL in suspension were mixed with 0.4% agarose-RPMI supplemented with 10% fetal bovine serum. Cells were then seeded on a 0.8% agarose base. Colony growth was assayed 2 wk later by counting colonies under the microscope. Experiments were done in duplicate and repeated at least once.

Cellular Growth Rate Determinations

The growth rates of the BEAS-2B and H1299 lung cells were determined as previously described (9, 10). Cells were seeded at 2×10^5 into six separate 10-cm tissue culture dishes. Duplicate dishes were harvested every 24 h for 3 d and the cells were counted. For the *MMP-2* knockdown experiments, cell proliferation was evaluated at 24-h intervals by measuring the mitochondrial-dependent conversion of the tetrazolium salt MTS to a colored formazan product using a CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (Promega) according to the manufacturer's instructions. Briefly, cells were seeded in 96-well plates at a density of 1,000 per well and grown for 1 wk. A volume of 20 μL of CellTiter 96 AQueous One Solution Reagent was pipetted directly into each well of the 96-well assay plate containing cells in 100 μL of culture medium and cells were incubated for 2 h at 37°C in a humidified, 5% CO_2 atmosphere. Absorbance was read at 490 nm using a 96-well microplate reader (Mode 680, Bio-Rad).

Migration and Invasion Assays

Migration and invasion assays were done in 24-well plates containing 0.8- μm -pore cell culture inserts with polyethylene terephthalate membranes according to the manufacturer's

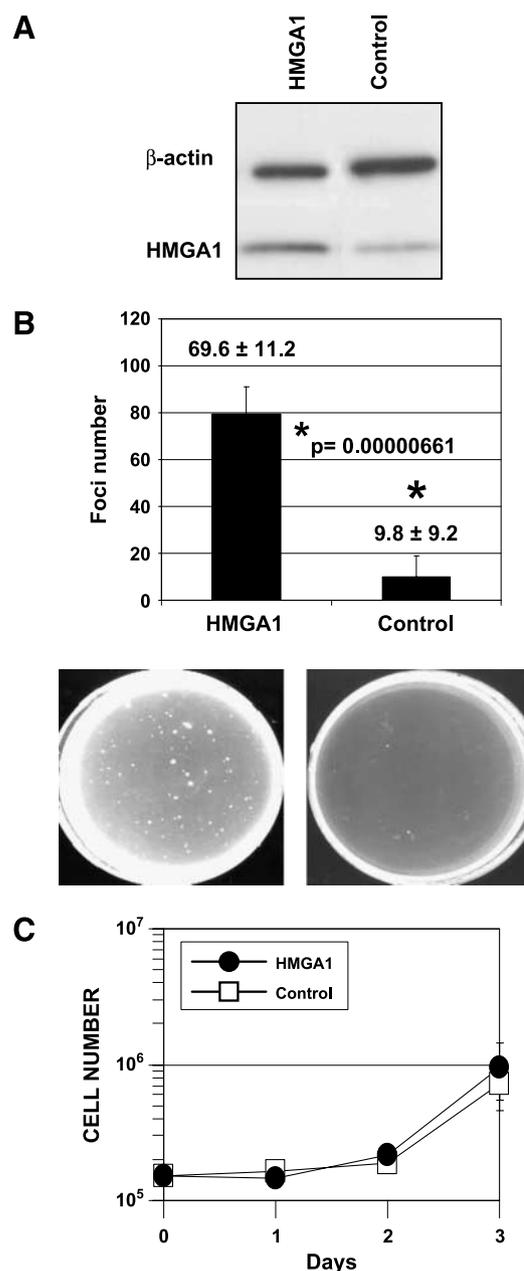


FIGURE 3. *HMGA1a* induces a transformed phenotype with anchorage-independent cell growth in cultured cells derived from normal, human lung tissue. **A.** BEAS2-B cells transfected with the *HMGA1a* vector overexpress the *HMGA1a* protein compared with cells transfected with control, empty vector by Western blot analysis. Membranes were blotted with the *HMGA1* antibody as well as a β -actin antibody to control for sample loading. **B.** Lung cells overexpressing *HMGA1* exhibit anchorage-independent cell growth (foci formation) in soft agar. Columns, average from two different experiments done in duplicate; bars, SD. A representative plate from each condition is shown. **C.** Cell growth rates of the transfected BEAS-2B cell lines. This experiment was done with duplicate plates and repeated twice. The error bars depict the SDs from a representative experiment.

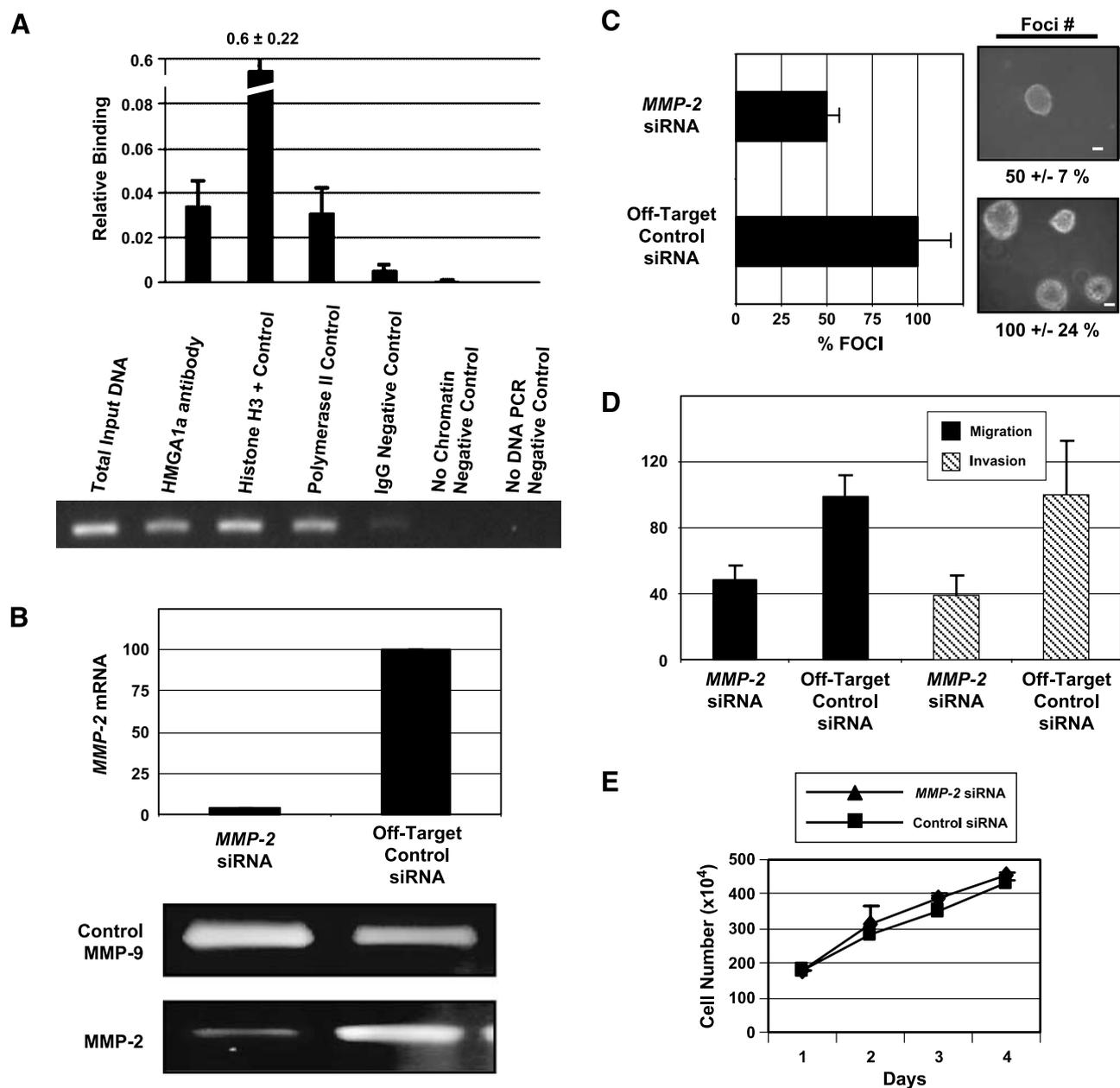


FIGURE 4. The HMGA1–MMP-2 pathway contributes to anchorage-independent cell growth, migration, and invasion in metastatic lung cancer cells. **A.** Chromatin immunoprecipitation experiments with sheared chromatin from H1299 cells after cross-linking proteins bound to DNA with formaldehyde (15). Columns, quantity of immunoprecipitated DNA with the following antibodies (all from Upstate, except for the HMGA1 antibody): HMGA1 (35), polymerase II (Pol II) or histone H3, both as positive controls, or rabbit IgG (as a negative control); bars, SD. Additional negative controls included no chromatin and no DNA. The *HPRT* promoter sequence was also used as a negative control because there are no HMGA1 DNA-binding sites in the region amplified and previous chromatin immunoprecipitation experiments showed no binding by HMGA1 to the amplified region (15, 35). The gel shows total input DNA compared with the DNA immunoprecipitated with the same antibodies. **B.** *MMP-2* mRNA and protein are decreased in the H1299 cells transfected with siRNA to *MMP-2* (20 nmol/L; Dharmacon) compared with cells transfected with the off-target control siRNA (siCONTROL nontargeting siRNA pool at 20 nmol/L; Dharmacon). The mRNA levels for *MMP-2* and control β -actin (β -actin primer/probe set; Applied Biosystems) were assessed at 24 h, 48 h, 8 d, and 10 d after adding the siRNA. *MMP-2* mRNA was normalized to β -actin as the loading control. *MMP-2* mRNA was decreased at all time points; only the day 8 time point is shown here (see Supplementary Fig. S1 for the remaining time points). All SDs were less than 5%. Protein was assessed for secreted *MMP-2* by zymography at the same time points as the mRNA. The *MMP-9* protein was also measured as a loading control. The zymogram shows decreased *MMP-2* protein at 8 d (see Supplementary Fig. S1 for the remaining time points). **C.** Anchorage-independent cell growth or foci formation are decreased in the H1299 cells with knockdown in *MMP-2* expression. The bar graph shows the decreased number of foci in the cells incubated with siRNA to *MMP-2* compared with the control siRNA. Columns, mean from three experiments done in triplicate ($100 \pm 41\%$ versus $49 \pm 19\%$; $P = 0.0019$; Student's *t* test); bars, SD. The photograph shows actual foci from the experiment (*bar*, 100 μ m). **D.** Migration and invasion were also assessed in the H1299 non–small cell lung carcinoma cells with and without *MMP-2* knockdown. Twenty-four hours after transfection with the siRNA, 3×10^4 cells per well were seeded onto the wells for migration/invasion insert. After 48 h, inserts were stained and counted for cells that migrated or invaded. There was a significant decrease in migration ($P < 0.0001$; Student's *t* test) and invasion ($P = 0.0006$; Student's *t* test). All experiments were done in triplicate and repeated at least once. **E.** Growth curves showed that cells incubated with siRNA to *MMP-2* or control siRNA proliferate at similar rates.

instructions (BioCoat Cell Culture Inserts, BD Biosciences) as previously described (48). Briefly, for invasion, filters were coated with 100 μ L growth factor-reduced Matrigel at 0.5 to 0.8 mg/mL protein on ice. For migration, the experiment was identical except that the filters were not coated with Matrigel. The cells were seeded in 500 μ L of 10% fetal bovine serum-RPMI at 30,000 per well into the upper chamber. The lower chamber was filled with 750 μ L of the same medium. After 48 h, migration or invasion was assessed by counting cells on the underside after fixation with 70% ethanol and staining with hematoxylin as described (48).

qRT-PCR Analysis

Cells were stored in RLT buffer (Qiagen) and tissues were stored in TRIzol at -80°C , and total RNA was subsequently extracted from cell lines and tumors as we described (13, 15). RNA (10 ng) and *HMGA1* primers used for PCR were previously described (13, 15). For *MMP-2*, commercially available primers and probe set were used (Applied Biosystems). For control RNA from normal lung, we used commercially available RNA (Clontech) harvested from lung tissue from three normal individuals (ages 19-50 y) who died with no history of lung disease or lung cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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