HMG A2 Overexpression-Induced Ovarian Surface Epithelial Transformation Is Mediated Through Regulation of EMT Genes

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Abstract

The AT-hook transcription factor HMG A2 is an oncogene involved in the tumorigenesis of many malignant neoplasms. HMG A2 overexpression is common in both early and late-stage high-grade ovarian serous papillary carcinoma. To test whether HMG A2 participates in the initiation of ovarian cancer and promotion of aggressive tumor growth, we examined the oncogenic properties of HMG A2 in ovarian surface epithelial (OSE) cell lines. We found that introduction of HMG A2 overexpression was sufficient to induce OSE transformation in vitro. HMG A2-mediated OSE transformation resulted in tumor formation in the xenografts of nude mice. By silencing HMG A2 in HMG A2-overexpressing OSE and ovarian cancer cell lines, the aggressiveness of tumor cell growth behaviors was partially suppressed. Global gene profiling analyses revealed that HMG A2-mediated tumorigenesis was associated with expression changes of target genes and microRNAs that are involved in epithelial-to-mesenchymal transition (EMT). Luminic, a tumor suppressor that inhibits EMT, was found to be transcriptionally repressed by HMG A2 and was frequently lost in human high-grade serous papillary carcinoma. Our findings show that HMG A2 overexpression confers a powerful oncogenic signal in ovarian cancers through the modulation of EMT genes. Cancer Res; 71(2); 349–59. ©2011 AACR.

Introduction

High-grade papillary serous carcinoma (HG-PSC) is the most lethal type of gynecologic malignancy. The cause of this disease remains poorly understood. P53 is the most commonly detected mutation in this tumor (1, 2). In addition, mutation or loss of expression of BRCA1 and/or BRCA2 is frequently detected in HG-PSC (3). In mice, conditional inactivation of P53 and BRCA1, the most common genetic changes in HG-PSC, in ovaries, results in the development of leiomyosarcomas but not of ovarian surface epithelial (OSE) carcinomas (4, 5). These findings indicate that in addition to the loss of these tumor suppressors, the early tumorigenesis of HG-PSC may require the activation of oncogenes. HMG A2, the high-mobility group AT-hook 2 gene, is one of a few oncogenes found to be overexpressed in human HG-PSC (6, 7). The rate of HMG A2 overexpression was found to be significantly higher in type II ovarian cancer than in type I ovarian cancer (7). Most importantly, approximately 75% of serous carcinomas in situ in the fallopian tube have HMG A2 overexpression along with P53 mutations (8).

HMG A2 is a nonhistone nuclear-binding protein and an important regulator of cell growth and differentiation (8, 9). It is an oncofetal protein that is overexpressed in embryonic tissue and many malignant neoplasms, including ovarian cancer (6, 7), but rarely in normal adult tissues. However, the roles of HMG A2 in tumorigenesis and tumor progression are still not fully understood. The oncogenic properties of HMG A2 are shown to be involved in aggressive tumor growth (10, 11), stem cell self-renewal (12, 13), DNA damage response (14), and tumor cell differentiation (6, 15). HMG A2 has also been found to participate in the epithelial-to-mesenchymal transition (EMT; refs. 16, 17). Silencing of HMG A2 expression in ovarian cancer cells has also been found to have a therapeutic effect on ovarian cancer (18).

To test the role of HMG A2 in tumorigenesis of OSE cells, we used the immortalized OSE cells as a model system to investigate the oncogenic properties of HMG A2. We found that HMG A2 overexpression was sufficient to induce OSE transformation in vitro and in vivo and resulted in aggressive
tumor cell growth, HMGA2-induced neoplastic transformation is at least partially attributable to a group of target genes that are involved in EMT. Our findings suggest that HMGA2 overexpression can be a critical molecular event responsible for the early tumorigenesis of human HG-PSC.

Materials and Methods

Patients and tissue samples
This study included 30 cases of HG-PSC and matched fallopian tube tissues. In addition, 30 ovaries from noncancer patients were included as normal controls. All cases were collected from Northwestern Memorial Hospital between 2007 and 2010. The study was approved by the Northwestern University institutional review boards. The tissue sections were collected and prepared for tissue microarray (TMA) as described previously (8). Fresh-frozen tissues from 12 HG-PSC with HMGA2 overexpression and matched normal ovarian tissue were also collected for the study.

Cell lines and cell cultures
OSE cell lines T29 and T80 were immortalized using SV40 and hTERT. The cell lines maintained cell biomarkers of normal OSE, and the detail information of these 2 cell lines have been described elsewhere (19). Cell lines were maintained in a cell culture medium consisting of 1:1 Medium199 (Sigma-Aldrich) and MCDB105 medium (Sigma-Aldrich) with 10% heat-inactivated fetal bovine serum (USA Scientific) and 10 ng/mL epidermal growth factor (Sigma-Aldrich) in a humidified atmosphere of 95% air and 5% CO2. Additional ovarian cancer cell lines used in this study included ovarian cancer cell lines SKOV-3, HEY, and OVCAR-3 (from American Type Culture Collection) and T29H (T29 with stable mutant hTERT expression; ref. 19).

T29 and T80 with stable overexpression of HMGA2
pBabe retroviral constructs containing HMGA2 with and without the 3' untranslated region (UTR) were prepared as described previously (Fig. 1A; ref. 10). The constructs were transfected into the Phoenix amphotropic packaging cell line (from American Type Culture Collection) per the manufacturer's protocol. After 24 hours, cells in the upper chamber were removed by cotton swab. Cells on the lower surface of the membrane were fixed by 10% formalin, stained by Giemsa and eosin, and counted under a light microscope of 100× magnification.

Cellular proliferation assay
Cells were seeded in 24-well plates in triplicate at densities of 1 × 104 cells per well. Cell proliferation was monitored at 24, 48, 72, and 96 hours using the colorimetric MTS assay (Cell-Titer 96 Aqueous Assay; Promega). In brief, the cells were rinsed with PBS and then incubated with 20% MTS reagent in serum-free medium for 3 hours. Aliquots were then pipetted into 96-well plates and the samples were read in a spectrophotometric plate reader at 490 nm (FLUOstar OPTIMA; BMG Lab Technologies).

siRNA and microRNA transient transfection
Cells were plated in standard medium without antibiotics for 24 hours. Individual microRNA, siRNA, or control small RNA (Block-iT fluorescent double-stranded random 22mer RNA from Invitrogen) was used at a concentration of 60 pmol/well in a 6-well plate with Lipofectamine 2000 (Invitrogen). Cells receiving only the tagged random sequence double-strand 22mer were used as nonspecific references at all data points. After transfection, cells were harvested and analyzed at the indicated times. HMGA2 siRNAs were purchased from Invitrogen, and the efficacy of HMGA2 inhibition was tested by RT-PCR and Western blot analysis.

shRNA HMGA2 transfection
Human HMGA2 shRNA in pGIPZ was purchased from Open Biosystems (RHS4430-99160621). Lentivirus express HMGA2 shRNA were produced in HEK293T cells packaged by pMD2G and psPAX2. For stable infection, 8 × 104 cells were plated in each well of 6-well plates along with 2 mL of medium without antibiotics. After overnight incubation, the medium was replaced every 3 to 4 days. Single colonies with HMGA2 overexpression were isolated and confirmed by reverse transcription PCR (RT-PCR) and Western blot analysis (Fig. 1A).

Luciferase reporter assay
The constructs were described in Supplementary Materials and Methods. Transfection was done in 293T and 293A cells with the aid of Lipofectamine 2000: 7 × 105 cells were seeded in each well of 24-well plates 1 day prior to transfection. Next, 0.1 µg of pRL-TK, 0.5 µg of pGL3-Lumican (LUM), and indicated amounts of pcDNA3.1-HMGA2, together with varied amounts of blank pcDNA3.1 plasmid to keep constant the amounts of total DNA were transfected into cells. Luciferase activity was measured 48 hours after transfection with a dual-luciferase reporter assay system (Promega), and results were normalized by Renilla luciferase activity. All experiments were repeated at least in triplicate for each sample.
Figure 1. HMGA2-induced OSE cell transformation in vitro. A, stable HMGA2 overexpression in OSE T29 and T80 (designated as T29A2− and T80A2−), established by pBabe viral infection, was confirmed by RT-PCR and Western blot (WB) analysis. T29P indicates pBabe viral vector only. B, photomicrographs illustrating examples of soft agar colonies (left) and histobars indicating the statistical significance of the numbers of colonies (right) in OSE cell lines with HMGA2 overexpression (T29A2+, T29A2−, and T80A2−), H-Ras overexpression (T29H), and controls (T29, T80, and T29P). Stable HMGA2 OSE cell lines with (T29A2+) and without (T29A2−, T80A2−) the HMGA2 3’UTR were also analyzed. C, photomicrographs illustrating examples of Matrigel invasion (left) and histobars indicating the statistical significance for the numbers of invasive cells (right) in OSE cell lines with and without HMGA2 overexpression. D, different growth rates of OSE cell lines with (T29A2−) and without (T29) HMGA2 overexpression in cisplatin (left) and paclitaxel (right) treatments. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Western blotting

Cells were lysed at 4°C in an NP40 cell lysis buffer (Invitrogen) supplemented with 1 mmol/L phenylmethylsulfonyl fluoride and protease inhibitor cocktail. Equal amounts of total proteins from each sample were loaded onto a 12.5% SDS-PAGE gel and transferred to PVDF membranes. Membranes were incubated with a primary antibody at 4°C overnight. The dilution rates of the primary antibodies were 1:100 to 1:1,000 for LUM (goat anti-human IgG for LUM; R&D Systems), HMGA2 (rabbit anti-human IgG; ref. 20), VIMENTIN (mouse anti-human IgG; Santa Cruz), N-CADHERIN (rabbit anti-human IgG; Santa Cruz), E-CADHERIN (mouse anti-human IgG; Abcam), and β-ACTIN (mouse anti-human IgG for β-actin; Sigma-Aldrich). The secondary antibody was detected by Western ECL-enhanced luminol reagent (PerkinElmer Inc).

Xenografts in nude mice

For xenograft experiments, 6-week-old female BALB/c nude mice (NCI) were injected with 5 × 10⁶ cells in bilateral flanks. Cells were resuspended in 0.1 mL of PBS for subcutaneous injection. Five types of cell lines were prepared for xenografts: T29 (10 implants), T80 (10 implants), T29H (7 implants), T29A2⁻ (16 implants), T80A2⁻ (18 implants). Tumor sizes were measured weekly, and mice were euthanized when tumors reached 1.5 cm in diameter.

MicroRNA array

Five micrograms of total RNA were annealed with oligo mix from the miRNA profiling assay kit (Signosis). The miRNA/oligo hybrids were purified by beads, and a ligation of miRNA-directed oligos was created to form a single molecule. The ligated molecule was incorporated with biotin-UTP. The labeled RNAs were hybridized with cancer miRNA array membrane (Signosis) and detected using a chemiluminescence imaging system (Signosis). MiRNA expression data sets have been deposited into the NCBI with the accession number GSE23634.

Figure 2. Repression of HMGA2 expression reduced aggressive OSE tumor cell growth in vitro. A, efficacy of HMGA2 repression by HMGA2 siRNAs in OSE cells illustrated by immunofluorescence staining (left) and RT-PCR and Western blot (right) analyses. B and C, transient transfection of HMGA2 siRNA or let-7c substantially reduced T29A2 cell growth rate (B), soft agar colonies (C, left), and the numbers of invasive cells in Matrigel (C, right). ***, P < 0.001. D, stable HMGA2 repression by lentiviral shRNA infection in SKOV-3 cell lines, demonstrated by RT-PCR analysis (left), substantially reduced SKOV-3 cell proliferation (middle), and restored the epithelioid-like cell morphology (top right) and reduced Matrigel invasion (bottom right).
mRNA expression array

Total RNA was isolated from 3 control cell lines (T29-1, T29-2, and T80) and testing cell lines (T29A2-8, T29A2-15, and T80A2-11) with an mRNA isolation kit (Ambion). RNAs were labeled with Cy5/Cy3 and hybridized to the Human Whole Genome OneArray (Phalanx Biotech Group). The hybridized chips were scanned by an Axon 4000 scanner (Molecular Devices). Spot quantification was done with the use of genepix 4.1 software. Hierarchical clustering of the expression profiles was analyzed by Cluster 3.0 (Molecular Devices). Differentially expressed genes with fold changes >2.0 were further analyzed by Pathway-Express (Onto-Tools; Wayne State University, Detroit, MI). Gene expression data sets have been deposited into the NCBI with the accession number GSE23599.

Histological and immunohistochemical analyses

Formalin-fixed and paraffin-embedded tissues were sectioned at 4 μm. Antigen retrieval was performed by either heat-induced epitope retrieval or by proteolytic enzyme digestion as previously described (7). All immunohistochemical staining procedures were performed on a Ventana Nexus automated system.

Immunofluorescence analysis

After being fixed with 4% paraformaldehyde, cells were permeabilized with 0.2% Triton X-100 and blocked with 10% goat serum. Then, cells were incubated with a specific primary antibody overnight at 4 °C. After the cells were incubated with appropriate FITC-conjugated secondary antibodies (Abcam) and stained by 4,6-diamidino-2-phenylindole (DAPI), the fluorescence images were examined with a microscope (model IX70; Olympus).

Chemoresistance assay

cis-Diamineplatinum(II) dichloride (cisplatin) and paclitaxel were purchased from Sigma (Sigma-Aldrich). Cultured cells were plated in 96-well plates (1 × 10³ cells/well) overnight. Then, cells were treated with indicated dosages of cisplatin or paclitaxel for 48 hours. Cell proliferation rates were evaluated by MTS assay (Promega).

Statistical analysis

All data are presented as means and standard errors of at least 3 independent experiments. Student’s t test was used for comparisons between 2 groups of experiments, and one-way ANOVA analysis was used for comparisons among 3 or more groups. P < 0.05 was considered significant.

Results

HMGA2 overexpression results in OSE cell transformation in vitro

Immortalized OSE cell lines T29 and T80 showed an epithelioid morphology in culture, and they maintained OSE cell markers, including cytokeratin 7 (CK-7), WT-1, and CA-125, as well as low levels of estrogen and progesterone receptor (ER and PR) expression by immunohistochemistry (19). T29 and T80 cells had very low levels of HMGA2 expression (Fig. 1A). After pBabe viral constructs of HMGA2 were transfected into T29 and T80 cell lines (Supplementary Fig. 1A), cell lines with stable HMGA2 overexpression were established and designated as T29A2 and T80A2. Five individual colonies were prepared from each of 3 cell lines with stable HMGA2 overexpression with (T29A2⁺) and without (T29A2⁻ and T80A2⁻) 3′ untranslated region (3′UTR; Supplementary Fig. 1B). Levels of HMGA2 expression were evaluated by RT-PCR and Western blot analysis (Fig. 1A; Supplementary Fig. 1C). T29 cell line with pBabe virus vector only (designated as T29P) and stable overexpression of mutant H-RAS gene (defined as T29H) was used as controls (ref. 19; Supplementary Fig. 1B).

To test whether HMGA2 overexpression leads to OSE transformation, we examined anchorage-independent cell growth. Numbers of colonies formed by T29A2⁺ (150 ± 8.33) and T80A2⁻ (132 ± 5.69) were significantly higher than those by T29 (7 ± 1.53), T80 (11 ± 2.52), and T29P (12 ± 8.33; Fig. 1B; P < 0.001). The average number of colonies in T29A2⁺ was half of that in T29A2⁻ and T80A2⁻, indicating the functional role of endogenous microRNAs in the regulation of HMGA2 expression. There was a substantial increase in colonies in T29H (49 ± 4.21) in comparison to T29, T80, and T29P (Fig. 1B).

To evaluate the mitogenic role of HMGA2, we compared cell proliferation rates in OSE cell lines with and without HMGA2 overexpression. We did not see a significant difference in cell proliferation rate between T29/T80 and T29A2/T80A2 because of the fast growth rate of T29/T80. However, the growth rates of T29A2⁺ were significantly higher (P < 0.05) than those of T29 when cells were treated with chemotherapy reagents cisplatin and paclitaxel (Fig. 1D), indicating an increase in chemoresistance in the presence of HMGA2 overexpression. This effect was dosage-dependent.

To test whether HMGA2 overexpression induced a more aggressive growth of OSE cells, we examined the rate of OSE cell migration and invasion through Matrigel. T29A2⁻ and T80A2⁻ cells had significantly higher numbers of cells migrating through Matrigel than T29 and T80 (P < 0.01; Fig. 1C). The number of cells migrating through Matrigel in T29A2⁻ and T80A2⁻ was also substantially higher than that in T29H (data not shown), indicating more aggressive tumor invasion in HMGA2-mediated tumor transformation.

Repression of HMGA2 overexpression can reduce the oncogenic activities of OSE and ovarian cancer cell lines in vitro

To determine whether inhibition of HMGA2 expression can reduce the oncogenic activities in OSE and ovarian cancer cell lines, we prepared and tested 3 HMGA2-interfering RNA targeting molecules, including let-7s, HMGA2 siRNA, and shRNA (see Materials and Methods). As shown in Figure 2A, transient transfection of HMGA2 siRNAs significantly inhibited HMGA2 expression at both transcription and translational levels. The repression of HMGA2 expression by let-7s was also reproducible in T29A2⁺ (data not shown).

Repression of HMGA2 expression by siRNA in T29A2⁺ significantly (P < 0.01) reduced the rate of cell proliferation.
at 72 to 96 hours (Fig. 2B). The rate of cell proliferation can also be reduced in T29A2 cells by let-7c (data not shown). Similar results were found in SKOV-3 cells in which complete repression of HMG A2 expression by shRNA expression revealed a reduction of more than 2-fold in tumor cell proliferation (Fig. 2D).

To test whether inhibition of HMG A2 overexpression interrupts anchorage-independent cell growth of OSE cell lines, we examined the cell growth in soft agar. The repression of HMG A2 expression by HMG A2 siRNA in T29A2 cells (Fig. 2C) significantly decreased the numbers of colonies compared with controls (Block-it; \( P < 0.001; \) Fig. 2C). The same results were obtained in T29A2 treated with let-7 (Fig. 2C). Matrigel invasion was partially blocked by the repression of HMG A2 expression with either siRNA or let-7 mimics (Fig. 2C). Notably, efficient repression of HMG A2 expression by shRNA in SKOV-3 cells substantially reduced cell proliferation (Fig. 2D) and changed cell morphology in culture from a spindle shape to a polygonal type differentiation (Fig. 2D). These findings suggested a role for HMG A2 in the EMT and in promoting cell migration through Matrigel.

HMGA2 overexpression in OSE cell lines enhances tumor formation in xenografts in nude mice

To explore whether HMGA2-mediated tumor transformation in OSE cell lines can promote tumor formation in vivo, we inoculated OSE cell lines with and without HMGA2 overexpression as xenografts in nude mice. We found that T29H, the positive control, required approximately 3 months to reach a tumor size of 1.5 cm in diameter and that 71.4% (5/7) of mice developed tumors (Fig. 3A and B). T29H tumors showed mostly a solid growth pattern with extensive tumor necrosis. T29A2 and T80A2 grew visible tumors in approximately 8 weeks and reached 1.5 cm in 20 weeks (Fig. 3C). Of the injection sites, 50% (8/16) of T29A2 and 72.2% (13/18) of T80A2 had tumor formation (Fig. 3B). The tumor growth rates are summarized in Figure 3C. By the end of 20 weeks, no tumor was identified in mice receiving T29 (0/10), and 3 tumors of <0.5 cm were found in mice receiving T80 (3/10).

To characterize the pathologic features of HMGA2-induced OSE tumors, we collected all xenograft tumors (29 tumors) and prepared a TMA from formalin-fixed and paraffin-embedded tumor tissues. T29A2 and T80A2 tumors had solid growth (A). Tumor formations in xenografts of OSE cell lines with HMGA2 overexpression in nude mice. A, photomicrographs illustrated examples of tumor formation in flanks of nude mice. Dissected tumors are listed below. B, the numbers of mice and the rate of tumor formation in each OSE cell type are summarized. C, growth curves of xenograft tumors in OSE with H-Ras (T29H) and HMGA2 overexpression (T29A2 and T80A2) were measured biweekly (x-axis) in cm (y-axis). D, immunophenotypes of xenograft tumors in OSE cell lines with (T29A2 and T80A2) and without (T80) HMGA2 overexpression. Left, examples of immunostains in selected markers; right, statistical results. n represents the number of xenograft tumors collected for analysis. VIM, vimentin; E-CAD, E-cadherin; CK7, cytokeratin 7; WT1, Wilm's tumor 1.

Figure 3. Tumor formations in xenografts of OSE cell lines with HMGA2 overexpression in nude mice. A, photomicrographs illustrated examples of tumor formation in flanks of nude mice. Dissected tumors are listed below. B, the numbers of mice and the rate of tumor formation in each OSE cell type are summarized. C, growth curves of xenograft tumors in OSE with H-Ras (T29H) and HMGA2 overexpression (T29A2 and T80A2) were measured biweekly (x-axis) in cm (y-axis). D, immunophenotypes of xenograft tumors in OSE cell lines with (T29A2 and T80A2) and without (T80) HMGA2 overexpression. Left, examples of immunostains in selected markers; right, statistical results. n represents the number of xenograft tumors collected for analysis. VIM, vimentin; E-CAD, E-cadherin; CK7, cytokeratin 7; WT1, Wilm’s tumor 1.
patterns with areas of nesting and papillary features (Supplementary Fig. 2). Tumor cells had moderate to high-grade nuclei, brisk mitosis, and areas of tumor necrosis. Immunoprofiling showed that T29A2- and T80A2- tumor cells were strongly immunoreactive for HMGA2 and P53. Tumor cells maintained CK-7 and WT-1, characteristics of Mullerian epithelial origin. T29A2- and T80A2- showed increased immunoreactivity for VIMENTIN and decreased immunoreactivity for E-CADHERIN compared with RAS-induced tumor (T29H) and T80 (Fig. 3). These findings supported a functional role for HMGA2 in the EMT in OSE tumors.

**HMGA2-induced tumor transformation is associated with dysregulation of a group of target genes and microRNAs in OSE cell lines**

HMGA2 can induce OSE transformation in vitro (Fig. 1) and in vivo (Fig. 3). Identification of HMGA2-regulated target genes may increase the understanding of the oncogenic properties of HMGA2 in ovarian tumorigenesis. To test whether HMGA2-mediated tumor transformation acts through the regulation of its target genes and microRNAs, we examined and compared global gene expression of OSE cell lines with and without HMGA2 overexpression (T29/T80 vs. T29A2- /T80A2-).

Of 124 cancer-related microRNAs, we found that 18% (22/124) were expressed differently between T29 and T29A2- by at least a 2-fold change (Fig. 4A and B). Among these, 4 microRNAs were downregulated (miR-15a, miR-18a, miR-22, and miR-29b). Differential expression of the selected microRNAs was validated by end-product (Fig. 4C) and real-time (data not shown) RT-PCR. MiR-29b showed an almost 9-fold reduction in HMGA2-transformed OSE cell lines. MiR-29b is an important tumor suppressor in regulating the P53 pathway (21), apoptosis, and tumor growth and was downregulated in HG-PSC (22). In the selected 12 HG-PSC tissues with HMGA2 overexpression, significant downregulation of miR-29b expression was evident compared with expression in matched normal ovarian tissues (Fig. 4D).

Seven of 11 let-7 family members were upregulated in T29A2- and T80A2- (Fig. 4B). HMGA2-mediated upregulation of let-7 family members in OSE cell lines could be related to a cellular response to HMGA2 overexpression. MiR-196 and miR29, the direct targets of HMGA1 (23), were dysregulated in HMGA2-transformed OSE cell lines. Whether HMGA2 can directly regulate miR-196 and miR-29 deserves further investigation. Among 22 miRNAs dysregulated by HMGA2 transduction, 73% (16/22) were found to be substantially dysregulated in ovarian carcinomas (24). The findings suggest that HMGA2-induced tumor transformation in vitro might share many of the molecular changes seen in human ovarian cancer.
HMGA2, a nuclear binding protein, directly regulates or interferes with the expression of many genes (25). We further examined the global gene expression of OSE cell lines with and without HMGA2 overexpression. Three OSE cell lines without HMGA2 overexpression (T29-1, T29-2, and T80) were used as controls, and 3 OSE cell lines with HMGA2 overexpression (T29A2-8, T29A2-15, and T80A2-11) were used as the testing group. Among a total of 32,000 genes, 621 were significantly dysregulated in OSE cell lines with HMGA2 overexpression ($P < 0.05$). Among these, 36 genes showed at least 2-fold changes (Fig. 6A; Supplementary Table 2), of which 25 genes were upregulated and 11 genes were downregulated. The genes most dysregulated in OSE cell lines with HMGA2 overexpression were confirmed by RT-PCR (data not shown).

Of the 11 downregulated genes, 4 were functionally related to the EMT, including ID1, LUM, POSTN, and WNT2. Of the 25 upregulated genes, 1 was EMT genes (STC2), 2 were mitogenic factors (CDKN1a and IFI27L2), and 4 were ribosomal protein complex genes (SNORD58B, RPS15A, RPS27L, and RPS14). In the list of HMGA2 target genes, 5 (16%) were associated with the EMT, suggesting the strong role of HMGA2 in EMT. This was compatible with our observation of frequent morphologic changes in cultured ovarian cancer cell lines from a more spindle type (high HMGA2) to an epithelial type (low HMGA2; Fig. 2D). Our experiment identified a list of possible HMGA2 target genes in association with ovarian carcinogenesis. We selected LUM for further validation as shown below.

Figure 5. HMGA2 regulates target gene LUM expression. A, Western blot analysis confirmed the downregulation of LUM in OSE cell lines T29 and T80 with HMGA2 overexpression (left). RT-PCR analysis of HMGA2 and LUM expression in several ovarian cancer cell lines (T29H, HEY, SKOV-3, and OVCAR-3) revealed an inverse correlation of HMGA2 and LUM expression (right). B, 5 constructs containing up to 2,200 nt of genomic DNA upstream of the LUM promoter were prepared for the luciferase assay. Significant reduction of luciferase expression was noted when the region from 0 to −800 nt was included in the construct and cotransfected with HMGA2 expression (left). Reduced luciferase expression rendered by the −800 nt DNA sequence was HMGA2 dosage–dependent (right). C, repression of HMGA2 expression by transfection of HMGA2 siRNA (right) or shRNA (left) enhanced LUM expression in T29A2, 293T, and SKOV-3 cell lines. D, ovarian cancer cell line HEY with stable LUM overexpression by Western blot (left) substantially reduces the cell migration in Matrigel (middle and right) in comparison to the controls.
LUM, an EMT-associated gene, is a target of HMGA2

A near 3-fold reduction in LUM expression was found in all 3 HMGA2-induced OSE cell lines (Supplementary Table 2). Western blot analysis confirmed that HMGA2 induced a 2- to 5-fold reduction in LUM expression in OSE cell lines (Fig. 5A). Interestingly, in the 6 OSE and ovarian cancer cell lines used in this study, HMGA2 expression was inversely correlated with LUM expression (Fig. 5A). To find whether this HMGA2-mediated LUM reduction was through transcription regulation, we examined LUM promoter regions.

Computer analysis revealed that there were multiple HMGA2 AT binding domains (ATATT) along a 2,200 nt upstream region from the 5' transcription start site. In several constructs representing different parts of 5' LUM promoter regions for a luciferase assay (Supplementary Table 1), the first 800 nt, immediately adjacent to the LUM transcription start site, showed a substantial reduction in luciferase expression when cotransfected with HMGA2 expression (Fig. 5B). Reduction in luciferase activity was dose-dependent (Fig. 5B). In contrast, the upstream region starting from −1,000 nt showed minimal activity in driving luciferase expression, indicating that HMGA2 regulatory region is mostly confined to the 0 to −1,000 nt promoter region.

To examine whether HMGA2 interferes with LUM expression, we examined LUM expression in different cell types. LUM expression was upregulated in OSE cells with transient transfection of HMGA2 siRNA or with shRNA (Fig. 5C). These findings further support that HMGA2 can directly regulate LUM expression.

To test whether LUM is associated with invasion and migration in ovarian cancer, we selected HEY cell line (with a very low level of endogenous LUM expression; Fig. 5A) and established stable LUM overexpression (Fig. 5D). We found that induction of LUM overexpression in ovarian cancer cell line HEY substantially reduced tumor cell migration through Matrigel (Fig. 5D).
immunohistochemistry. Overall, LUM expression was found to be substantially higher in stroma than in epithelial cells (Fig. 6B). Furthermore, HG-PSC tumor cells showed significant downregulation of LUM compared with normal ovarian surface and fallopian tube epithelia (Fig. 6B). When we compared whether HMGA2 overexpression was associated with LUM expression in the 30 cases of HG-PSC, a weak correlation \( r = 0.29 \) was noted. The weak correlation was largely owing to the very low level of LUM immunoreactivity in almost all HG-PSC samples.

**Discussion**

HMGA2 was shown to enhance tumor transformation in different cell types (26, 27). It has been found that HMGA2 overexpression is associated with aggressive tumor growth, early metastasis, and a poor prognosis, typically seen in papillary thyroid carcinoma (28), pancreatic cancer (11), breast cancer (29), and HG-PSC (18). In this study, we found that HMGA2 enhances OSE cell transformation (Figs. 1 and 3) and that the oncogenic properties of HMGA2 in ovarian cancer may be mediated by its regulation of genes involved in EMT.

EMT, a complex process involving multiple extracellular signal pathways (30), confers tumor plasticity by converting adherent epithelial cells to motile mesenchymal cells through the functional loss of E-cadherin, which is required to maintain epithelial cell–cell adhesion (30). EMT plays a key role in embryonic development and is important in the pathogenesis of cancer. Several EMT-associated genes have been identified and their functions have been characterized in breast and ovarian cancers (30). Until recently, HMGA2 has not been linked to EMT regulation. Shell et al. found that HMGA2 is one of few gene markers that can distinguish most type I (mesenchymal-like phenotype) from type II (epithelial-like phenotype) cancer cell lines (6, 15). In addition, Thauault et al. identified HMGA2 to be a transcriptional regulator of \( \text{SNAIL1} \) by directly binding to the promoter (16). \( \text{SNAIL1} \), a key EMT molecule, is associated with aggressive tumor growth in pancreatic cancer (17).

In this study, we found that HMGA2-induced OSE transformation displays many cellular and molecular features common to the EMT process. First, we observed that OSE cells with high HMGA2 expression had a spindle-like cell morphology in culture and that repression of HMGA2 restored their epithelial phenotype (Fig. 2D). Second, HMGA2 expressing OSE xenograft tumors had a significant loss of E-cadherin and increase of vimentin (Fig. 3D). Inactivation of \( \text{HMGA2} \) expression in early ovarian serous carcinoma may be responsible for the functional loss of E-cadherin, which is required to maintain epithelial cell–cell adhesion (30). EMT plays a key role in embryonic development and is important in the pathogenesis of cancer. Several EMT-associated genes have been identified and their functions have been characterized in breast and ovarian cancers (30). Until recently, HMGA2 has not been linked to EMT regulation. Shell et al. found that HMGA2 is one of few gene markers that can distinguish most type I (mesenchymal-like phenotype) from type II (epithelial-like phenotype) cancer cell lines (6, 15). In addition, Thauault et al. identified HMGA2 to be a transcriptional regulator of \( \text{SNAIL1} \) by directly binding to the promoter (16). \( \text{SNAIL1} \), a key EMT molecule, is associated with aggressive tumor growth in pancreatic cancer (17).

In this study, we have for the first time, to our knowledge, achieved HMGA2 overexpression in human OSE cell lines, and we found that HMGA2 overexpression was sufficient to induce OSE cell transformation and tumor formation. HMGA2-induced ovarian carcinogenesis occurs, at least in part, through controlling tumor-associated EMT gene expression. We postulate that induction of HMGA2 overexpression in early ovarian serous carcinoma may be responsible for the latent latency of invasive carcinoma to the rapid progression of ovarian cancer. Further characterization of the functional relationship between HMGA2 and HMGA2-mediated EMT target gene regulation will help us understand the tumorigenesis of ovarian serous carcinoma.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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