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HMGA2 overexpression induced ovarian surface epithelial transformation is mediated through regulation of EMT genes

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Abstract

HMGA2, a high-mobility-group AT-hook protein, is an oncogene involved in tumorigenesis of many malignant neoplasms. *HMGA2* overexpression is common in both early and later stage of high grade ovarian serous papillary carcinoma. To test whether HMGA2 participates in the initiation of ovarian cancer and promotion of aggressive tumor growth, we examined the oncogenic properties of *HMGA2* in ovarian surface epithelial (OSE) cell lines. We found that introduction of *HMGA2* overexpression was sufficient to induce OSE transformation *in vitro*. HMGA2-mediated OSE transformation resulted in tumor formation in xenografts of nude mice. By silencing *HMGA2* in HMGA2 overexpressing OSE and ovarian cancer cell lines, the aggressiveness of tumor cell growth behaviors was partially suppressed. Global gene profiling analyses revealed that HMGA2-mediated tumorigenesis was associated with expression changes of target genes and microRNAs that are involved in epithelial-to-mesenchymal transition (EMT). Lumican (LUM), a tumor suppressor that inhibits EMT, was found to be transcriptionally repressed by HMGA2 and was frequently lost in human high-grade serous papillary carcinoma.

Keywords

HMGA2; ovarian cancer; miRNA; oncogene; EMT

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INTRODUCTION

High grade ovarian 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 are frequently detected in high grade serous carcinoma (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 loss of these tumor suppressors, the early tumorigenesis of HG-PSC may require the activation of oncogenes. *HMGA2*, 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 *HMGA2* overexpression was found to be significantly higher in type II ovarian cancer than in type I ovarian cancer (7). Most importantly, about 75% of serous carcinomas in situ in fallopian tube have *HMGA2* overexpression along with *P53* mutations (8).

HMGA2 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 HMGA2 in tumorigenesis and tumor progression remain to be fully understood. The oncogenic properties of HMGA2 are demonstrated to be involved in aggressive tumor growth (10–11), stem cell self-renewal (12–13), the DNA damage response (14), and tumor cell differentiation (6, 15). HMGA2 has also been found to participate in the epithelial-to-mesenchymal transition (EMT) (16–17). Silencing of HMGA2 expression in ovarian cancer cells has also been found to have a therapeutic effect on ovarian cancer (18).

To test the role of HMGA2 in tumorigenesis of OSE cells, we used the immortalized OSE cells as a model system to investigate the oncogenic properties of HMGA2. We found that *HMGA2* 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 high-grade serous carcinoma (HG-PSC) and matched fallopian tube tissues. In addition, 30 ovaries from non-cancer 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 detail information of these two cell lines have been described elsewhere (19). Cell lines were maintained in cell culture medium consisting of 1:1 Medium199 (Sigma-Aldrich, St. Louis, MO) and MCDB105 medium (Sigma-Aldrich) with 10% heat-inactivated fetal bovine serum (USA Scientific, Ocala, FL) 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 Skov3, Hey, and Ovar-3 (from American Type Culture Collection) and T29H (T29 with stable mutant *H-RAS*^{H12} expression) (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 (S Figure 1) (10). The constructs were transfected into the Phoenix amphotropic packaging cell line (from American Type Culture Collection) per manufacturer's protocol with the help of 25 µM of chloroquine (Sigma-Aldrich). The infected cells were subjected to 1.5 µg/ml puromycin for 10–30 days (Detailed information is provided in S Figure 1). Single colonies with *HMGA2* overexpression were isolated and confirmed by reverse transcription–polymerase chain reaction (RT-PCR) and Western blot analysis (Figure 1A).

Soft Agar Assay

The test cells (3×10^4) were suspended in 5 ml of culture medium containing 0.4% agar (USB Corporation, OH) and seeded onto a base layer of 5 ml of 0.7% agar bed in 10-cm tissue-culture dishes. Colonies >0.1 mm in diameter were counted after 2 weeks.

Matrigel Invasion Assay

2×10^5 test cells were placed in Matrigel-coated inserts (8-µm pores) (BD Biosciences, MA) in accordance with the manufacturer's protocol. After 24 hrs, 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 1×10^4 cells per well. Cell proliferation was monitored at 24, 48, 72, and 96 hrs using the colorimetric MTS assay (CellTiter 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 hrs. 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, Germany).

siRNA and microRNA Transient Transfection

Cells were placed in standard medium without antibiotics for 24 hrs. Individual microRNA, siRNA, and control small RNA (Block-iT fluorescent double-stranded random 22mer RNA

from Invitrogen), were 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 (Figure 2A).

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, 8×10^4 cells were plated in each well of 6-well plates along with 2 ml of medium without antibiotics. After overnight incubation, medium was removed and replaced with 1 ml per well of Opti-MEM® I Reduced-Serum Medium containing 12 µg/mL polybrene. Next, 50 µL of concentrated lentiviral particles were added to each well. 48 h later, fresh medium containing 2 µg/ml puromycin was added to each well. Fresh medium containing puromycin was replaced every 3–4 days. Single colonies were obtained after 2 weeks of puromycin selection.

Luciferase Reporter Assay

The constructs were described in S Materials and Methods. Transfection was done in 293T and 293A cells with the aid of Lipofectamine 2000: 7×10^4 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-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 h after transfection using a dual-luciferase reporter assay system (Promega, WI), and results were normalized by Renilla luciferase activity. All experiments were repeated at least in triplicate for each sample.

Western Blotting

Cells were lysed at 4°C in NP40 cell lysis buffer (Invitrogen) supplemented with 1 mM 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 primary antibody at 4°C overnight. The dilution rates of the primary antibodies were 1:100 to 1:1000 for LUM (goat anti-human IgG for LUM, R & D Systems, MN), *HMGA2* (rabbit anti-human IgG (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, MO). The secondary antibody was detected by Western ECL-enhanced luminol reagent (Perkinelmer Inc. MA).

Xenografts in Nude Mice

For xenograft experiments, 6-week old female BALB/c nude mice (NCI-Frederick, MD) were injected with 5×10^6 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, CA). 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, CA) and detected using a chemiluminescence imaging system (Signosis). MiRNA expression data sets have been deposited into the NCBI with accession number of GSE23634.

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) using an miRNA isolation kit (Ambion, TX). RNAs were labeled with Cy5/Cy3 and hybridized to the Human Whole Genome OneArray (Phalanx Biotech Group, Taiwan). The hybridized chips were scanned by an Axon 4000 scanner (Molecular Devices, CA). 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, MI). Gene expression data sets have been deposited into the NCBI with accession number of GSE23599.

Histological and Immunohistochemical Analyses

Formalin-fixed and paraffin-embedded tissues were sectioned at 4 μ m. 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 (Tucson, Arizona, USA).

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 specific primary antibody overnight at 4 °C. After cells were incubated with appropriate FITC-conjugated secondary antibodies (Abcam, MA) and stained by 4,6-diamidino-2-phenylindole (DAPI), the fluorescence images were examined with 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 (1×10^4 cells/well) overnight. Then, cells were treated with indicated dosages of cisplatin or paclitaxel for 48 hrs. Cell proliferation rates were evaluated by MTS assay (Promega, CA).

Statistical Analysis

All data are presented as means and standard errors of at least three independent experiments. Student's *t* test was used for comparisons between two groups of experiments, and one-way ANOVA analysis was used for comparisons among three 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 in immunohistochemistry (19). T29 and T80 cells had very low levels of *HMGA2* expression (Figure 1A). After *pBabe* viral constructs of *HMGA2* were transfected into T29 and T80 cell lines (S Figure 1A), cell lines with stable *HMGA2* overexpression were established and designated as T29A2 and T80A2. Five individual colonies were prepared from each of three cell lines with stable *HMGA2* overexpression with (T29A2+) and without (T29A2- and T80A2-) 3' untranslated region (3'UTR) (S Figure 1B). Levels of *HMGA2* expression were evaluated by RT-PCR and Western blot analysis (Figure 1A, Supplementary Figure 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 (19) (S Figure 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) (Figure 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 regulation of *HMGA2* expression. There was significant increase of colonies in T29H (49±4.21) in comparison to T29, T80 and T29P (Figure 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 of cell proliferation rate between T29/T80 and T29A2/T80A2, due to 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 (Figure 1D), indicating an increase of 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 had (*p*<0.01) (Figure 1C). The number of cells migrating through Matrigel in T29A2- and T80A2- was also significantly 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 three *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. 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 72–96 hours (Figure 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 *HMGA2* expression by shRNA expression revealed a more than 2-fold reduction of tumor cell proliferation (Figure 2D).

To test whether inhibition of *HMGA2* overexpression interrupts anchorage-independent cell growth of OSE cell lines, we examined the cell growth in soft agar. Repression of *HMGA2* expression by *HMGA2* siRNA in T29A2– (Figure 2C) significantly decreased the numbers of colonies compared with controls (Block-iT) ($p<0.001$) (Figure 2C). The same results were obtained in T29A2+ treated with *let-7* (Figure 2C). Matrigel invasion was partially blocked by repression of *HMGA2* expression with either siRNA or *let-7* mimics (Figure 2C). Notably, efficient repression of *HMGA2* expression by shRNA in SKOV-3 cells significantly reduced cell proliferation (Figure 2D) and changed cell morphology in culture from a spindle shape to a polygonal-type differentiation (Figure 2D). These findings suggested a role for *HMGA2* 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 about 3 months to reach a tumor size of 1.5 cm in diameter and that 71.4% (5/7) of mice developed tumors (Figure 3A and 3B). T29H tumors showed mostly a solid growth pattern with extensive tumor necrosis. T29A2– and T80A2– grew visible tumors in about 8 weeks and reached 1.5 cm in 20 weeks (Figure 3C). Of the injection sites, 50% (8/16) of T29A2– and 72.2% (13/18) of T80A2– had tumor formation (Figure 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 three tumors of <0.5 cm were found in mice receiving T80 (3/10).

To characterize the pathological 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 patterns with areas of nesting and papillary features (S Figure 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 (Figure 3D). These findings supported a functional role of 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* (Figure 1) and *in vivo* (Figure 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 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 (Figure 4A and 4B). Among these, four microRNAs were down-regulated (*miR-15a*, *miR-18a*, *miR-22*, and *miR-29b*). Differential expression of the selected microRNAs was validated by end-product (Figure 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 down-regulated in HG-PSC (22). In the selected 12 HG-PSC tissues with *HMGA2* overexpression, significant down-regulation of *miR-29b* expression was evident compared with expression in matched normal ovarian tissues (Figure 4D).

Seven of 11 *let-7* family members were up-regulated in T29A2– and T80A2–. *HMGA2*-mediated up-regulation 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 significantly 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 three 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 (Figure 6A, S Table 2), of which 25 genes were up-regulated and 11 genes were down-regulated. The genes most dysregulated in OSE cell lines with *HMGA2* overexpression were confirmed by RT-PCR (data not shown).

Of the 11 down-regulated genes, 4 were functionally related to the EMT, including *IDI*, *LUM*, *POSTN*, and *WNT2*. Of the 25 up-regulated 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 morphological changes in cultured ovarian cancer cell lines from a more spindle type (high HMGA2) to an epithelial type (low HMGA2) (Figure 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.

LUM, an EMT-Associated Gene, Is a Target of HMGA2

A near 3-fold reduction in *LUM* expression was found in all three HMGA2-induced OSE cell lines (S Table 2). Western blot analysis confirmed that *HMGA2* induced a 2–5-fold reduction in *LUM* expression in OSE cell lines (Figure 5A). Interestingly, in the six OSE and ovarian cancer cell lines used in this study, *HMGA2* expression was inversely correlated with *LUM* expression (Figure 5A). To determine 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 (S Table 1), the first 800 nt, immediately adjacent to the *LUM* transcription start site, showed a significant reduction in luciferase expression when cotransfected with *HMGA2* expression (Figure 5B). Reduction in luciferase activity was dose dependent (Figure 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 up-regulated in OSE cells with transient transfection of *HMGA2* siRNA or with shRNA (Figure 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 very low level of endogenous LUM expression, Figure 5A) and established stable *LUM* overexpression (Figure 5D). We found that induction of *LUM* overexpression in ovarian cancer cell line HEY significantly reduced tumor cell migration through Mitragel (Figure 5D).

We examined LUM expression in 30 HG-PSC samples with matched fallopian tubes and 30 normal ovaries by immunohistochemistry. Overall, LUM expression was found to be significantly higher in stroma than in epithelial cells (Figure 6B). Furthermore, HG-PSC tumor cells showed significant down-regulation of LUM compared with normal ovarian surface and fallopian tube epithelia (Figure 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 (Figure 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 a few gene markers that can distinguish most type I (mesenchymal gene signature) from type II (epithelial gene signature) cancer cell lines (6, 15). In addition, Thauvau et al identified HMGA2 to be a transcriptional regulator of *SNAIL1* by directly binding to the promoter (16). *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 epithelioid phenotype (Figure 2D). Second, HMGA2 expressing OSE xenograft tumors had a significant loss of E-cadherin and increase of vimentin (Figure 3D). Third, miRNAs *miR-200s* (31) and *miR-29b* (32), critical microRNAs in the EMT, were significantly dysregulated in OSE cells with *HMGA2* overexpression (Figure 4B). Fourth, we found that induction of HMGA2 overexpression in OSE cell lines (T29 and T80) and inhibition of HMGA2 expression in ovarian cancer cell line SKOV3 had an impact on EMT gene expression, including vimentin, N-cadherin and E-Cadherin (Figure 6C).

One of most important findings in this study was that about 16% of the HMGA2-regulated genes were associated with EMT (S Table 2). Among them is LUM which is down regulated by *HMGA2* overexpression. LUM, a member of a small leucine-rich proteoglycan family, constitutes a fraction of noncollagenous extracellular matrix proteins (33). LUM can bind type I collagen and regulate the assembly of collagen fibers. LUM functions in the wound healing process (34) and affects the bioavailability of SMAD 2 activators (TGF- β) (35). Nikitovic et al (35) reported that a human osteosarcoma cell line with a higher level of LUM expression displays low metastatic capability. In breast cancer, low LUM expression was shown to be correlated with rapid progression and poor survival (36). Although the functional role of LUM in cancer has not been fully characterized, the

accumulating evidence suggests that it may serve as a tumor suppressor by inhibiting EMT. Thus, the identification of *LUM* as target of *HMGA2* in ovarian cancer provides further evidence that *HMGA2* promotes ovarian tumorigenesis through regulation of EMT.

Several other EMT-associated genes identified in this study may also be directly regulated by *HMGA2*. For example, T29A2– and T80A2– cell lines have *STC2* overexpression (Supplementary Table 2). Recent study demonstrates that *STC2* overexpression promotes epithelial and stromal transition in ovarian cancer cell lines in the hypoxic condition (37). Further characterization of how *HMGA2* regulates these gene expressions and their EMT function will provide new insight for our understanding of HG-PSC.

In summary, we have, to our knowledge for the first time, 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 short latency of noninvasive carcinoma to the rapid progression of ovarian serous carcinoma. 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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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 histograms 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 histograms 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 (T29) and without (T29A2–) *HMGA2* overexpression in cisplatin (left) and paclitaxel (right) treatments. *: $p < .05$; **: $p < .01$; ***: $p < .001$.

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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* significantly 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), significantly reduced Skov-3 cell proliferation (middle) and restored the epithelioid-like cell morphology (right upper) and reduced Matrigel invasion (right lower).

Figure 3. Tumor formations in xenografts of OSE cell lines with *HMGA2* overexpression in nude mice. **A.** Photomacrographs illustrated examples of tumor formation in flanks of nude mice. Dissected tumors are listed below. **B.** Numbers of mice and the rate of tumor formation in each OSE cell type are summarized in this table. **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 without (T80) and with (T29A2- and T80A2-) *HMGA2* overexpression. Left panel: examples of immunostains in selected markers; right panel: 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 4.

Differential expression of microRNAs in OSE cells with (T29A2-) and without (T29) *HMGA2* overexpression. **A.** Dot-blot analysis of cancer-related microRNA expression (a total of 128 microRNAs) in T29 and T29A2-. Boxed dots illustrate significantly down-regulated miRNAs in T29A2-. **B.** List of microRNAs significantly up- or down-regulated (>2-fold) in OSE cell lines with *HMGA2* overexpression. **C.** Differential expression of the selected miRNAs was validated by RT-PCR. **D.** Differential expression of *miR-29b* was examined in 12 human HG-PSC samples with *HMGA2* overexpression and in 10 normal ovaries. A significant inverse correlation of *HMGA2* and *miR-29b* expression was noted (right panel).

Figure 5.

HMGA2 regulates target gene *LUM* expression. **A.** Western blot analysis confirmed the down-regulation 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 Ovar-3) revealed an inverse correlation of *HMGA2* and *LUM* expression (right). **B.** Five 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 panel) significantly reduces the cell migration in Matrigel (middle and right panel) in comparison to the controls.

Figure 6.

A. Dendrogram and cluster analysis revealed that 36 genes were differentially expressed (>2-fold) between in OSE cell lines with (right) and without (left) *HMGA2* overexpression. Green indicates down-regulation and red up-regulation. **B.** Photomicrographs (left) illustrate examples of immunoreactivity for LUM in human high grade serous papillary carcinoma (HG-PSC), matched normal fallopian tubes and ovaries. Histobars (right) show the relative expression level of LUM scaled by immunointensity as mean values of LUM (y-axis) in HG-PSC (PSC, blue), fallopian tube (FT, red) and ovary (OV, green). * $p < 0.05$; ** $p < 0.01$;

*** $p < 0.001$. **C.** Western blot analysis of Vimentin (VIM, upper), N-cadherin (N-Cad, mid) and E-cadherin (E-Cad, lower) in cells with (T29A2–, T80A2–, SKOV3) and without (T29, T80, SKOV3+*shHMGA2*) *HMGA2* overexpression. β -actin is protein loading control.

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