Gastrointestinal, Hepatobiliary and Pancreatic Pathology

HMGA2 Maintains Oncogenic RAS-Induced Epithelial-Mesenchymal Transition in Human Pancreatic Cancer Cells

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Pancreatic cancer has the poorest prognosis among human neoplasms due to its highly aggressive and metastasizing features. Current diagnostic tools have difficulty in detecting the early stage of this disease, and therapeutic applications often remain ineffective at advanced stages. Thus, the mechanisms leading to progression of this cancer especially need to be understood at molecular levels. The epithelial-mesenchymal transition (EMT) is a physiological process, originally found in embryonic development, in which the cells lose epithelial characteristics and gain mesenchymal properties. This process is accompanied by loss of cell-to-cell contact and subsequent increased cell movement. Recent reports have implicated EMT in a malignant conversion of transformed cells, which represents invasive or metastasizing properties in a variety of cancers. In pancreatic cancer cells, EMT is also reported to be a crucial step for tumor cell migration and invasion. Previous studies suggest that aberrations in pathways emerging from oncogenic RAS and transforming growth factor-β (TGF-β) stimulate the malignant features, including EMT. Several transcriptional factors have been identified that control EMT by repressing E-cadherin and other epithelial genes in embryonic morphogenesis and cancer metastasis. These include the Snail-related zinc-finger factors (SNAIL and SLUG), the other zinc-finger factors (SIP1/ZEB2 and ZEB1/EF-1), and the basic helix-loop-helix factors (E12/E47 and TWIST). Further, the mesenchymal-epithelial transition is also known to occur in an opposite direction to that of EMT. Despite its biological

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significance, the precise mechanism of EMT remains to be elucidated in pancreatic cancer.

The high-mobility group A proteins (HMGA1 and HMGA2, formerly HMGI/Y and HMGI/C, respectively) are abundant, non-histone chromatin architectural factors that participate in many biological processes, including cell growth and differentiation.\(^{10}\) HMGA1 and HMGA2 are encoded by two distinct genes and have three DNA binding motifs called AT-hooks that preferentially bind the minor groove of AT-rich DNA sequences.\(^{11}\) HMGA proteins induce conformational changes in bound DNA substrates and promote subsequent recruitment of additional components for transcriptional regulation. In addition, HMGA proteins function in protein-protein interactions and are capable of forming multiple protein complex(es), called enhanceromes, on the promoter/enhancer regions of several genes.\(^{12,13}\) HMGA genes are highly expressed in the embryo and are down-regulated during differentiation,\(^{12,13}\) and both are induced by mitogenic stimuli.\(^{11}\) Interestingly, transgenic mice that overexpress HMGA proteins in all tissues developed lymphomas and some other tumors.\(^{14−17}\) The overexpression of HMGA proteins was also correlated with occurrence of metastasis and poor prognoses in several human cancers.\(^{18−21}\) Thus, HMGA proteins are associated with malignant changes in cancer cells, although the pathological significance of these proteins is unknown. The HMGA genes have a long 3′UTR that can be targeted by some microRNAs including let-7.\(^{22,23}\) Recently, it was reported that disrupting the pairing between let-7 and HMGA2 by chromosomal translocations enhanced oncogenic transformation in soft tissue tumors,\(^{24}\) and that ectopic expression of let-7 reduced HMGA2 and cell proliferation in lung cancer.\(^{25}\) However, the involvement of the let-7 family in pancreatic cancer is largely unknown.

In the present study, we report that HMGA2, in conjunction with the oncogenic RAS signaling pathway, is responsible for cell growth and EMT in human pancreatic cancer cells. HMGA2 depletion inhibited cell proliferation, leading to a transition to epithelial state that restores cell-to-cell contact through up-regulated E-cadherin. The inhibition of the RAS/MEK pathway also induced an epithelial transition, together with down-regulation of HMGA2. HMGA2 enhanced the expression of SNAIL, and the decrease of SNAIL by the RAS/MEK inhibition was suppressed by overexpression of HMGA2, suggesting that HMGA2 maintains RAS-induced EMT in pancreatic cancer cells. These findings are supported by our observations of an inverse correlation between HMGA2-positive cells and E-cadherin-positive cells in pancreatic cancer tissues. Based on the role of HMGA2 in reversibly maintaining EMT of pancreatic cancer cells, we further investigated that HMGA2 is one of the therapeutic targets in pancreatic cancer, from the viewpoint of the post-transcriptional control of HMGA2 and the small molecules acting on HMGA2. Collectively, our data indicate the significance of HMGA2 for considering pathology and emerging therapy of pancreatic cancer.

### Materials and Methods

#### Cell Culture and Treatment

AsPC1, Capan1, and Panc1 cells (American Type Culture Collection, Manassas, VA), HeLa S3 and MiaPaCa2 cells (Health Science Research Resources Bank, Osaka, Japan), and BxPC3 cells (European Collection of Cell Culture, Salisbury, UK) were obtained. The culture conditions were AsPC1 and BxPC3 cells (RPMI 1640 medium, from Sigma-Aldrich, St. Louis, MO, supplemented with 10% (v/v) heat-inactivated fetal bovine serum), Capan1 cells (1:1 mixture of Dulbecco's modified Eagle's minimum essential medium and Ham's F-12 nutrient [DMEM/F12] medium supplied with 20% fetal bovine serum), Panc1, and HeLa S3 cells (DMEM/F12 medium supplied with 10% fetal bovine serum), and Mia PaCa2 and HepG2 cells (DMEM medium supplied with 10% fetal bovine serum). AsPC1 and Panc1 cells (2 × 10\(^5\)) were treated with 10 μmol/L U0126 (LC Laboratories, Woburn, MA) or dimethyl sulfoxide every 2 days. Human recombinant TGF-β1 (R&D systems, Inc., Minneapolis, MN) was dissolved in 4 mmol/L HCl with 1 mg/ml bovine serum albumin and was used at a concentration of 5 ng/ml.

#### Western Blot Analysis

The denatured cell lysates were separated by SDS-polyacrylamide gel electrophoresis on a 5% to 20% polyacrylamide gradient gel and transferred to a nitrocellulose membrane (Amersham Bioscience, Freiburg, Germany). The membranes were then blocked with 10% nonfat dry milk in PBS for 1 hour and probed with the primary antibodies (Table 1). After the washings, the membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences) for 1 hour. The results were visualized by an enhanced chemiluminescence kit (Amersham Biosciences). For analyzing HMGA2, nuclear extracts were prepared with high salt RIPA buffer (1% Triton X-100, 0.1% SDS, 500 mmol/L NaCl, 50 mmol/L Tris-HCl [pH 7.4], 10 mmol/L MgCl\(_2\), 0.5% sodium deoxycholate) and protease inhibitors. Protein content was determined by the Bradford assay (Bio-Rad Laboratories, Hercules, CA). Each band was quantified using ImageJ software (version 1.38; National Institutes of Health, Bethesda, MD) and the normalization with β-tubulin and histone H3.

<table>
<thead>
<tr>
<th>Table 1. Antibodies Used in this Study</th>
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<tr>
<td>Name</td>
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<tr>
<td>------</td>
</tr>
<tr>
<td>HMGA1a/1b</td>
</tr>
<tr>
<td>HMGA1</td>
</tr>
<tr>
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</tr>
<tr>
<td>β-tubulin</td>
</tr>
<tr>
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</tr>
<tr>
<td>N-cadherin</td>
</tr>
<tr>
<td>Vimentin Ab-2</td>
</tr>
<tr>
<td>K-Ras-2A (C-17)</td>
</tr>
<tr>
<td>Anti-FLAG M2</td>
</tr>
</tbody>
</table>
Immunoﬂuorescent Analysis

After being ﬁxed with 4% paraformaldehyde, cells were permeabilized in 0.2% Triton X-100 and blocked with 0.5% bovine serum albumin in PBS. The cells were then incubated with speciﬁc primary antibodies, listed in Table 1, for 1 hour at room temperature. After being washed, the cells were incubated with appropriate ﬂuorescein isothiocyanate-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) and then with 4',6-diamidino-2-phenylindole (DAPI) (Roche Diagnostics, Mannheim, Germany) for DNA staining. The images were visualized with an Olympus IX71 microscope using Lumina Vision software (version 2.2; Mitani Corporation, Tokyo, Japan).

Reverse Transcription PCR and Quantitative Real-Time Reverse Transcription PCR

After incubation with DNase I (Invitrogen, Carlsbad, CA), 5 µg of the total RNAs was reverse-transcribed using Superscript III (Invitrogen) and random hexamers (Operon Biotechnologies, Tokyo, Japan). The cDNAs from normal pancreatic tissue were obtained from Toyobo Co., Ltd. (Osaka, Japan). PCR ampliﬁcation was
then performed with specific primers (Tables 2A and B). Real-time PCR analysis was performed using Power SYBR Green PCR Master Mix on an ABI Prism 7500 Sequence Detector (Applied Biosystems, Foster City, CA). PCR amplification was repeated by more than three independent experiments. The relative fold induction was quantified by the comparative threshold cycle method, and β-actin was used as a control.

**Immunohistochemistry**

Immunohistochemistry was performed with human pancreas tumor tissue array I (BioChain Institute, Inc., Hayward, CA). The array slides were deparaffinized, and antigens were retrieved by autoclaved heating at 120°C for 15 minutes for vimentin and HMGA proteins, or by heating in the microwave oven at less than 95°C for 15 minutes for E-cadherin, in a buffer (1 mmol/L EDTA/PBS [pH 9.0] for HMGA proteins and E-cadherin; 0.01M/L sodium citrate [pH 6.0] for vimentin). The slides then were incubated in methanol with 0.3% hydrogen peroxide for 30 minutes to block the endogenous peroxidase activity. Thereafter, tissue sections were immersed in 0.5% Block-Ace (Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan) in PBS for 30 minutes, covered with primary antibodies, and incubated overnight at 4°C. Visualization of the immunoreactions was performed using Histofine Simple Stain MAX-PO (Nichirei Bioscience Inc., Tokyo, Japan) and 3,3-diaminobenzidine tetrahydrochloride (Dako, Glostrup, Denmark). The slides were counterstained with hematoxylin and mounted with Malinol (Muto Pure Chemicals Co., Ltd., Tokyo, Japan).

Carcinoma cells with moderate or strong nuclear staining of HMGA2 were counted as positive, while the cells with weak nuclear staining and/or diffuse cytoplasmic staining were counted as negative. The presence of HMGA2 in EMT of Pancreatic Cancer. The cells were transfected with HMGA1, HMGA2, or control siRNAs (200 nmol/L) using Oligofectamine according to the manufacturer's protocols (Invitrogen). Apoptotic or dead cells were stained with 0.2% trypan blue and counted using a hemocytometer. The data were obtained from three independent experiments.

**Plasmids and Luciferase Assay**

The cDNA for human HMGA2 was cloned into pcDNA3.1-FLAG (Invitrogen). The genomic DNAs from human SNAIL promoter were amplified and cloned into pGL3-basic vector (Promega, Madison, WI). Primer sequences are as follows: for HMGA2, 5'-AGGATGACGCACCGGTT-GAGGCC-3' and 5'-CTAGTCCTCCTGCGAAGACTCTTG-3'; for SNAIL promoter, 5'-CTGGATGAGCGCTAAATTGACAC-3' and 5'-CGCCGATTGCGAGACAGTAG-3'. For constructing sensor and control luciferase reporter plasmids, oligonucleotides containing two copies perfectly complementary to let-7a or miR-370, and the 3'UTR of KRAS, HMGA1, or HMGA2 were ligated into the XbaI site of the pGL3-control vector (Promega). The let-7a, let-7a3b, and miR-370 were amplified from genomic DNAs and cloned into pcDNA3.1 (Invitrogen). Sensor or control plasmid (1 μg) and phRL-SV40 (1 ng) (Promega) were introduced into cells per well in a 6-well plate using Lipofectamine (Invitrogen). In combination with 2'-O-methyloligoribonucleotides, sensor or control plasmid (400 ng) and phRL-SV40 (0.5 ng) were transfected to cells per well in a 12-well plate using Lipofectamine 2000 (Invitrogen). Primer sequences are listed in Table 3. Luciferase activities were checked at 48 hours after the transfection using the dual luciferase reporter assay system (Promega). Firefly luciferase activities were normalized by renilla luciferase activities. Luciferase activities were determined by more than three independent assays.

**Small Interfering RNA-Mediated Knockdown**

More than two small interfering (si)RNA duplexes were designed for targeting mRNAs encoding human HMGA1 and HMGA2 (Japan Bio Services Co., LTD., Saitama, Japan), listed in Table 2D. The selected siRNA sequences were submitted to human genome and EST databases to ensure the target specificities. The siRNAs were transfected into the cells by using Oligofectamine (Invitrogen).

**Establishment of Stably Expressed Cells**

Panc1 cells were introduced with pCAG IRES-Puro, which is kindly gifted by Dr. H. Niwa (RIKEN), that ex-
expresses GFP fused HMGA1 and HMGA2 using FuGENE6 (Roche), and selected in the presence of puromycin (2.0 µg/ml) (Sigma). HepG2 cells were introduced with pcDNA3.1 that expressed let-7a1f1 and both are reactivated in various cancer cells.18 –21 We first examined the expression status of HMGA genes in human pancreatic cancer cells (Figure 1A and supplemental Figure S1 available at http://ajp.amjpathol.org). Reverse transcription (RT)-PCR and Western blot analyses showed that HMGA1 was expressed in five pancreatic cancer cells (AsPC1, BxPC3, Capan1, MIA PaCa2, and Panc1), and that HMGA2 was expressed in four cells but not MIA PaCa2 cells. An immunofluorescent analysis indicated the endogenous existence of HMGA proteins in the nuclei of these cells (See supplemental Figure S1 available at http://ajp.amjpathol.org). In normal pancreatic tissues, HMGA1 was expressed at low levels, and HMGA2 transcripts were not detected (Figure 1A). Consistently, the gene expression profiles showed that HMGA2 mRNA is undetectable in normal pancreatic tissues (National Center for Biotechnology Information, Gene Expression Omnibus profiles; accessions GDS1086, GDS426, GDS422 and GDS181). Since previous reports18 –21 and our present study show that HMGA2 is overexpressed in pancreatic cancer, enhanced expression of HMGA2 may contribute to malignant phenotype in these cancer cells.

To check the effect of HMGA proteins on cell proliferation, we knocked-down HMGA1 and HMGA2 using synthesized small interfering RNAs (siRNAs). Western blot analysis confirmed that HMGA1 and HMGA2 were depleted by the specific knockdown in AsPC1, BxPC3, and Panc1 cells (Figure 1B and immunofluorescent data not shown). The depletion of HMGA2 significantly reduced the growth rate of these pancreatic cancer cells, in comparison with control and HMGA1 knockdown (Figure 1C). The cell death, which was assessed by trypan blue staining, did not increase in these knockdown cells. In addition, the anti-proliferative effect of a single knockdown of HMGA2 was persistent in these cells for more than 7 days (data not shown). The depletion of HMGA2 significantly reduced the growth rate of these pancreatic cancer cells, in comparison with control and HMGA1 knockdown (Figure 1C).

**Results**

**Involvement of HMGA2 in Transformed Phenotype of Pancreatic Cancer Cells**

HMGA1 and HMGA2 are expressed in most undifferentiated tissues and mesenchymal cells, respectively,10,11 and both are reactivated in various cancer cells.18 –21 We first examined the expression status of HMGA genes in human pancreatic cancer cells (Figure 1A and supplemental Figure S1 available at http://ajp.amjpathol.org). Reverse transcription (RT)-PCR and Western blot analyses showed that HMGA1 was expressed in five pancreatic cancer cells (AsPC1, BxPC3, Capan1, MIA PaCa2, and Panc1), and that HMGA2 was expressed in four cells.
and the depletion of HMGA2, but not HMGA1, restored the cell-to-cell contact leading to the adhesive growth on days 3 and 9 after the knockdown. We frequently observed the appearance of multiple clusters of adherent cells under the HMGA2 knockdown (as indicated by triangles). Other pancreatic Panc1 cells appeared to be originally grown at adhesive state, and they tended to enhance adhesive growth to some extent by the HMGA2 depletion (data not shown).

Requirement of HMGA2 for Maintaining the Mesenchymal State of Pancreatic Cancer Cells

Among the cells studied in Figure 1A, AsPC1 cells were originally established from disseminated cells in ascites of a patient with pancreatic adenocarcinoma, while Panc1 cells were derived from primary pancreatic adenocarcinoma. These cell lines have KRAS mutations and were used for further study. To test a conversion to the epithelial-like changes induced by HMGA2 knockdown. The data are represented with AsPC1 cells. Phase contrast studies were done on day 3 and 9 after the siRNA transfection. Arrowheads highlight the appearance of clusters of adherent cells.
lial state, an immunofluorescent analysis using anti-E-cadherin antibodies was performed in AsPC1 cells (Figure 2A). The HMGA2-knockdown induced a change of E-cadherin from a disorganized to organized (membrane-accumulated) localization. In contrast, E-cadherin was faintly stained in control and the HMGA1-knockdown cells. Western blot analysis further showed that the amount of E-cadherin increased approximately 1.6- and 2.4-fold in HMGA2-knockdown AsPC1 and Panc1 cells, respectively (Figure 2B, upper panels), suggesting a switch to the epithelial state under the HMGA2 knockdown. In addition, similar results were obtained using distinct siRNAs against HMGA2 (Figure 2B, lower panels). These data suggest that HMGA2 is actively involved in maintaining EMT of pancreatic cancer cells. To check expression of epithelial and mesenchymal markers, we analyzed relative mRNA levels of E-cadherin and vimentin, using a quantitative RT-PCR (Figure 2C). Using more than three independent assays, the mRNA levels in controls were normalized to 1. The knockdown of HMGA2, but not HMGA1, increased E-cadherin and decreased vimentin in both AsPC1 and Panc1 cells. These data suggest that HMGA2 is actively involved in maintaining EMT in pancreatic cancer cells.

Expression Status of E-Cadherin and HMGA2 in Pancreatic Cancer Tissues

To investigate the involvement of HMGA2 in EMT in vivo, we then examined the expression status of E-cadherin and HMGA2 in human pancreatic carcinoma tissues and normal control tissues, using immunohistochemical analysis (representative data in Figure 3A, and summarized in Table 3). Expression of E-cadherin was found in normal or hyperplastic ductal cells, while there seemed to be at very low levels of HMGA2 or to lack nuclear concentration of the protein in the control tissues (Figure 3A; a-c). Among 14 human primary pancreatic adenocarcinomas studied, high expression of HMGA2 was found in 11 out of 14 (78.6%), where HMGA2 was densely stained in the nuclei of more than 40% of cancer cells. Low expression of E-cadherin was detected in 5 out of 14 cancer tissues (35.7%), where E-cadherin was stained in less than 15% of cancer cells, and HMGA2 was highly expressed in these five carcinoma tissues (Figure 3A; d-f, g-i, and j-l). To assess the correlation of HMGA2 with EMT, we counted the percentage of HMGA2-positive cells and compared it with the percentage of the cells with E-cadherin staining in the same samples. Using the Pearson correlation coefficient analysis, there was an inverse correlation between HMGA2-positive cells and E-cadherin-positive cells in the pancreatic cancer tissues (P < 0.01) (Figure 3B). In addition, low expression of HMGA2 coexisted with the low grade of pancreatic cancers that retained E-cadherin expression (Table 4). On the other hand, HMGA1 was expressed in most cancer tissues, and vimentin was found dominantly in stromal cells around the carcinoma cells (data not shown). These data suggest that enhanced expression of HMGA2 is at least in part correlated with EMT in pancreatic cancer tissues.

HMGA2 Is Involved in the RAS/MEK Signaling Pathway in Pancreatic Cancer Cells

KRAS mutations and activated RAS/ERK signaling pathway play an essential role in initiation and progression of pancreatic cancer. In addition, HMGA proteins were found to be induced by the RAS signaling pathway, possibly leading to alteration of various expressed genes. To investigate the effect of a constitutively active RAS/ERK pathway on EMT in pancreatic cancer cells, we used the agent U0126, which selectively inhibits the ability of MEK1/2 to activate ERK1/2.
were treated with 10 μmol/L of U0126 for 4 days. As previously reported, the treatment blocked activation of ERK1/2 and induced inhibition of cell proliferation (data not shown). As was the case of the HMGA2 knockdown (Figure 1D), the U0126-treated cells enhanced the adhesive growth and formed multiple clusters of adherent cells (See supplemental Figure S2A available at http://ajp.amjpathol.org), suggesting that the RAS signaling pathway is linked to EMT in pancreatic cancer cells. To check the effect of RAS/MEK inhibition, Western blot analysis was performed using the U0126-treated cells (Figure 4A). Under the RAS/MEK inhibition, E-cadherin was markedly up-regulated 1.7- to 3.8-fold, whereas expression of mesenchymal markers N-cadherin and vimentin decreased approximately 0.6- to 0.7-fold. A5P1 cells did not originally express N-cadherin. In addition, HMGA1 and HMGA2 were down-regulated approximately 0.2- to 0.5-fold by this treatment. To check expression of mRNA levels of HMGA and EMT markers under the RAS/MEK inhibition, a quantitative RT-PCR analysis was performed using the U0126-treated cells. By more than three independent assays, the mRNA levels in controls were normalized to 1. Compared with the control cells, E-cadherin was up-regulated, while vimentin, as well as HMGA1 and HMGA2, was down-regulated (Figure 4B). To further investigate the mechanisms of maintaining EMT in pancre-

Table 4. Summary of Immunohistochemical Analysis of Human Pancreatic Cancer Specimens

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<tr>
<th>ID</th>
<th>Diagnosis</th>
<th>Grade</th>
<th>% of E-cadherin positive cells</th>
<th>% of HMGA2 positive cells</th>
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<td>Ductal adenocarcinoma</td>
<td>I</td>
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atic cancer, we checked the expression of EMT-stimulating transcription factors (SNAIL, SLUG, SIP1/ZEβ2, E12/E47, and ZEB1/αEF-1) that potentially repress E-cadherin\(^6\),\(^9\) using a quantitative RT-PCR analysis (Figure 4C). The U0126 treatment reduced the expression of EMT-stimulating transcription factors tested, especially SLUG.

Previous reports showed that TGF-β\(^1\) signaling promotes the metastasizing and invasive properties of some cancers, presumably by inducing EMT of the cells.\(^6\),\(^8\) In addition, HMGA2 was reported to control epithelial differentiation of mouse mammary epithelial cells as one of the mediators in the TGF-β signaling.\(^3\)\(^4\) In pancreatic cancer, it was reported that TGF-β\(^1\) itself is overexpressed,\(^2\)\(^8\) and that TGF-β-induced EMT requires an intact Smad-mediated transcription pathway.\(^7\) To clarify the relationship between HMGA and TGF-β signaling in pancreatic cancer cells, we checked the effect of recombinant TGF-β\(^1\) on Panc1 cells, which have an intact Smad-mediated transcription pathway (Figure 4D). Using a quantitative RT-PCR analysis of the TGF-β\(^1\)-treated cells, E-cadherin was down-regulated, while vimentin was up-regulated. In contrast, the expression of HMGA1 and HMGA2 was not affected by TGF-β\(^1\) treatment. We then examined the expression of EMT-stimulating transcription factors (Figure 4E). TGF-β\(^1\) treatment markedly increased the expression of both SNAIL and SLUG. Further, we treated HMGA-knockdown Panc1 cells with TGF-β\(^1\) and checked the expression of these EMT regulators (see supplemental Figure S2B available at http://ajp.amjpathol.org). Using the specific knockdown, the expression level of endogenous HMGA1 and HMGA2 was reduced to less than 10% of the control (see supplemental Figure S2B available at http://ajp.amjpathol.org). The HMGA knockdown did not affect the down-regulation of E-cadherin or the up-regulation of SNAIL and SLUG, which was induced by the TGF-β\(^1\) treatment (see supplemental Figure S2C available at http://ajp.amjpathol.org). These data suggest that HMGA proteins are unlikely to be related with TGF-β signaling pathway in pancreatic cancer cells. Taken together, the lines of evidence suggest that HMGA2 is involved in RAS/MEK-induced mesenchymal state in pancreatic cancer cells.

**HMGA2 Enhances SNAIL Expression as a Downstream of Oncogenic RAS Signaling Pathway in Pancreatic Cancer Cells**

Based on our present data, it is suggested that HMGA2 is required for maintaining oncogenic RAS-induced EMT, together with the decrease of E-cadherin, in pancreatic cancer cells. To investigate whether HMGA2 has a role in the expression of EMT-stimulating transcription factors, we analyzed relative mRNA levels of them in HMGA2-depleted AsPC1 cells, using a quantitative RT-PCR (Figure 5A). By more than three independent assays, the mRNA levels in controls were normalized to 1. HMGA2 knockdown caused a decrease of these factors except for SLUG, and the depletion of HMGA2 constantly down-regulated the SNAIL gene. In addition, the expression of TWIST1 and TWIST2 were detected only at low levels in the control cells (data not shown). These data suggest that HMGA2 at least controls SNAIL, and that HMGA2
may not be involved in SLUG induction by the RAS signaling pathway.

To confirm a transcriptional role of HMGA2, we performed a luciferase assay using a reporter construct containing human SNAIL gene promoter (position −1556 to +59) upstream of the luciferase gene (Figure 5B). FLAG-tagged HMGA2 was expressed in HeLa cells that originally had low level of HMGA2 expression (Figure 1A). HMGA2 increased the luciferase activities in a dose-dependent manner, suggesting that HMGA2 stimulates the SNAIL promoter. To examine whether HMGA2 localizes to the endogenous SNAIL promoter, we performed a chromatin immunoprecipitation analysis (Figure 5C). The SNAIL gene has AT-rich sequences upstream of the promoter-associated CpG island. The promoter region of this gene is divided into six subregions (I to VI). Chromatin immunoprecipitation analysis of HMGA2 proteins on SNAIL gene promoter (right panel). FLAG-tagged HMGA2 was expressed in HeLa cells and precipitated with anti-FLAG antibodies, followed by PCR amplification with specific primers for these sites. Genomic DNAs were used as a control (input).

To conclude the relationship between HMGA2 and RAS signaling pathway, we finally prepared exogenous HMGA2-overexpressing Panc1 cells and treated them with U0126 for 4 days (Figure 5D). Exogenous HMGA2 was expressed under the control of cytomegaloviral promoter in the vector. Importantly, the overexpression of HMGA2 reversed the decrease of endogenous SNAIL expression by U0126 (see additional observations in the Discussion). Together with the results of the down-regulation of HMGA2 by U0126 treatment (Figure 4) and the suppression of oncogenic RAS-associated SNAIL expression by HMGA2 knockdown (Figure 5A), our results collectively suggest that HMGA2 maintains RAS-induced EMT by stimulating SNAIL expression in pancreatic cancer cells (Figure 5E).

**Effect of the let-7 MicroRNAs on the Phenotypes of Pancreatic Cancer Cells**

RAS is known to be translationally down-regulated by the let-7 microRNA family, and the loss of let-7 expression led to the progression of some human cancers. The KRAS gene is mostly mutated in pancreatic cancer, and
the implication of the let-7 microRNAs remains unknown. Similar to the KRAS, HMGA1 and HMGA2 have a long 3′UTR and are targeted by the let-7 microRNAs in mammalian cells. Using the miRanda (http://www.microrna.org/) and TargetScan (http://genes.mit.edu/targetscan/), as shown in Figure 6A, HMGA1, HMGA2, and KRAS commonly had let-7 family and miR-370 complementary sites in their 3′UTR. In particular, HMGA2 and KRAS showed seven and eight let-7 target sites, respectively. Previous reports indicated that let-7a1 and let-7f1 are clustered within 517 bp in human chromosome 9, and that let-7a3 and let-7b are also clustered within 1047 bp in chromosome 22. Let-7a1f1 and let-7a3b are the most abundant species among the let-7 family, and can be amplified together by RT-PCR. We first analyzed the expression status of let-7 and miR-370 microRNAs in pancreatic cancer cell lines (Figure 6B). The let-7 family tested was expressed in these cells, while HepG2 hepatocellular carcinoma cells did not express let-7b and let-7a3b.
in Panc1 cells alone. Thus, we identified that the let-7 microRNAs are expressed in pancreatic cancer cells. Because the effect of let-7 has been previously demonstrated in endogenous let-7-lacking cancer cells, the biological significance needs to be evaluated in let-7-expressing pancreatic cancer cells.

To test whether the 3′UTRs of HMGA1, HMGA, and KRAS are functional, we generated firefly luciferase construct containing each of the 3′UTRs, and introduced these vectors to HeLa, BxPC3, and Panc1 cells (Figure 6C), where the let-7 family was expressed. When compared with the controls, the addition of 3′UTRs of these genes, especially HMGA2 as well as KRAS, decreased the luciferase activities. We confirmed that 3′UTRs of HMGA1, HMGA2, and KRAS are targets of the let-7 family by expressing let-7a1f1 and let-7a3b driven by a cytomegalovirus promoter in HepG2 cells that had the let-7 family at low levels (Figure 6B). As was the case of the let-7a complementary sequences (Figure 6D; left panel), the 3′UTR of HMGA2 showed inhibition in the presence of either let-7a1f1 or let-7a3b (Figure 6D; middle panel). On the other hand, the expression of miR-370 did not affect the luciferase activities even in the presence of miR-370 complementary sequences, suggesting that miR-370 does not function as a translational block (Figure 6D; right panel). In addition, using pancreatic cancer cells, the expression of the let-7 family moderately decreased the luciferase activities in the presence of let-7a complementary sequences or 3′UTRs of HMGA1, HMGA2, and KRAS, when compared with the mock vector in AsPC1 and Panc1 cells (Figure 6E).

We then examined whether the endogenous let-7 family targets the complementary sequences in the 3′UTRs of KRAS, HMGA1, and HMGA2 by using the let-7a antisense inhibitor40-41 in AsPC1 and Panc1 cells that expressed the let-7 microRNAs. Into these cells we introduced a sensor construct containing let-7a complementary sites and the let-7a antisense inhibitor (or a control oligonucleotide). Cells transfected with the let-7a inhibitor relieved repression exerted on the reporter, relative to the control transfections (Figure 7A; left panel). In addition, the use of the miR-370 inhibitor did not affect the sensor construct for miR-370 in Panc1 cells (data not shown). The use of the let-7a inhibitor markedly reduced the inhibition by the 3′UTRs of HMGA2 (Figure 7A; middle and right panels), suggesting that the let-7 family down-regulates the expression of HMGA2. To determine the inhibitory effect of the let-7 microRNAs on HMGA proteins and KRAS, the let-7a inhibitor was introduced into AsPC1 and Panc1 cells (Figure 7B). Western blot analysis showed that expression of HMGA2 increased approximately 4.5- and 2.9-fold in the let-7a-inhibited AsPC1 and Panc1 cells, respectively. The amount of HMGA1 and KRAS was not significantly affected by the let-7a inhibitor. These data suggest that let-7 family dominantly targets HMGA2 transcripts in pancreatic cancer cells.

To assess the effect of the let-7 family on cell growth and EMT, we introduced the inhibitors against let-7a or miR-370 into AsPC1 and Panc1 cells that expressed these microRNAs (Figure 7C). However, there were no significant differences in growth rate in AsPC1 cells (Figure 7C; left panel). Similar results were found by using MTS assays, and in Panc1 cells (data not shown). A quantitative RT-PCR analysis showed that inhibition of let-7 family did not significantly alter expression levels of repressors of E-cadherin (Figure 7C; right panel). Next, we introduced the let-7 clusters (let-7a1f1 or let-7a3b) into Panc1 cells (Figure 7D), which originally expressed endogenous let-7 microRNAs. The exogenous expression of the let-7 did not significantly affect proliferation (Figure 7D; left panel). Although the expression levels of the repressors of the E-cadherin gene, except for SNAIL, might decrease to some extent (Figure 7D; right panel), additional expression of the let-7 microRNAs did not cause any changes in endogenous HMGA1, HMGA2, and KRAS levels, and in the EMT phenotype (data not shown). These data suggest that let-7-mediated inhibition of HMGA2 does not evidently alter the transformed phenotype in pancreatic cancer cells. As a control, we used HepG2 cells (Figure 7E), since this cell line did not originally have the let-7a3b (Figure 6B). The exogenous expression of the let-7 microRNAs reduced cell proliferation to about 65.7% (let-7a1f1) and 49.4% (let-7a3b) (Figure 7E; left panel), and SNAIL expression, compared with the other EMT regulators, to approximately 80.0% (let-7a1f1) and 62.8% (let-7a3b) (Figure 7E; right panel). Collectively, these data suggest that let-7 microRNAs do not inhibit cell growth and EMT in pancreatic cancer cells that maintain the expression of endogenous let-7 microRNAs. In addition, ectopically expressed let-7 may not evidently have a therapeutic role in pancreatic cancer cells that originally express let-7.

**Discussion**

Our study revealed that HMGA2, in association with the oncogenic RAS/MEK signaling pathway, is required for maintenance of proliferation and the mesenchymal state in pancreatic cancer cells. Similar to the inhibition of the RAS/MEK pathway, the knockdown of HMGA2 caused growth suppression and the epithelial transition of these cells, together with an increase of E-cadherin and down-regulation of vimentin. Thus, EMT itself is reversibly maintained in pancreatic cancer cells. Transcriptional repressors of the E-cadherin gene, such as SNAIL, decreased under the HMGA2 knockdown, and exogenously expressed HMGA2 activated the SNAIL gene promoter by binding to the upstream AT-rich region and overcame the down-regulation of the SNAIL gene by the U0126 treatment. On the other hand, the U0126 treatment constantly caused the decrease of SLUG, rather than SNAIL, together with down-regulation of HMGA proteins. Thus, the mechanisms of maintaining the transformed phenotype of pancreatic cancer cells by activated RAS signaling or by HMGA2 may be partly overlapping but not identical. With regard to the data in Figure 5D, overexpression of HMGA2 reversed SNAIL down-regulation by U0126 and tended to decrease the expression level of E-cadherin, although there were no evident differences in EMT and growth patterns of HMGA2- and mock-transfected Panc1
cells with or without U0126 treatment (data not shown). These observations may be explained by a complementary involvement of other EMT regulators such as SLUG, and by effects of U0126 on multiple signaling molecules including HMGA2. As shown in Figure 5E, we collectively proposed a current model showing that HMGA2 maintains EMT in pancreatic cancer cells, in conjugation with oncogenic RAS signaling.

The overexpression of HMGA proteins was reported to be associated with progression and metastasis in several cancers, as a diagnostic molecular marker. A recent study reported that HMGA1 is a determinant of cellular invasiveness and in vivo metastatic potential, in part due to PI3K/Akt-dependent modulation of MMP-9 activity in pancreatic adenocarcinoma. On the other hand, HMGA2 is predominantly expressed in proliferating, undifferentiated mesenchymal cells, and is not found in adult tissues. In addition, Hmga2−/− mice had a deficiency in fat tissue, termed a pygmy phenotype. HMGA2 was also found to transactivate the cyclin A gene and stimulate cell growth. Our present study demonstrated that HMGA2 is responsible for EMT in pancreatic cancer cells, and that the EMT-regulator SNAIL is one of the HMGA2-target genes. In addition, knockdown of HMGA2 reduced expression of three other EMT regulators except for SLUG. Thus, the reactivation of HMGA2 is
significantly implicated in EMT and malignant transformation of the pancreatic ductal cells.

Based on the role of HMGA2 in reversibly maintaining EMT of pancreatic cancer cells, we further investigated that HMGA2 is one of the therapeutic targets in this cancer. First, RAS is known to be translationally downregulated by the let-7 microRNA family, and the loss of let-7 expression led to the progression of some human cancers.26,36 Similar to the KRAS, HMGA1 and HMGA2 mRNAs have a long 3'UTR,37 and are targeted by the let-7 microRNAs in mammalian cells.22,24,26,38 Because constitutively active mutations of KRAS mostly occur during early development of pancreatic cancer,28 it is of great interest as to whether let-7 can inhibit mutant KRAS or HMGA in pancreatic cancer cells. In AsPC1 and Panc1 cells, the 3'UTRs of HMGA2, as well as KRAS transcripts, were targeted by let-7 for down-regulation (Figures 6 and 7). However, the use of exogenous let-7 microRNAs or let-7 inhibitor did not affect cell growth and EMT. In contrast, ectopic expression of let-7 was reported to reduce HMGA2 and cell proliferation in lung cancer lacking original let-7 expression.29 Another report showed that epigenetic activation of let-7a-3 changed gene expression profile and contributed to human lung tumorigenesis.45 These reports and our data suggest that the effect of let-7 may be distinct among cancer cell types, and that the inhibitory effect of let-7 on RAS and HMGA2 may be ineffective for tumor suppression in the let-7-expressing cancer cells. Thus, ectopically expressed let-7 may not have a therapeutic effect on pancreatic cancer cells that originally express let-7.

Second, pancreatic cancer cells express both epithelial and mesenchymal markers and may be intermediates between these two states in nature. Our findings highlight the role of HMGA2 in maintaining EMT as a novel therapeutic target in pancreatic cancer. Notably, this study showed that single knockdown of HMGA2 induced long-term growth suppression and epithelial transition in pancreatic cancer cells. Specific siRNA delivery is likely to be useful to develop HMGA2-targeted cancer therapy. Furthermore, competitors against HMGA proteins, which include either functional or physical inhibitors, may provide potential therapeutic applications. In fact, FK317 and its derivatives were able to act on HMGA proteins by cross-linking bound DNAs.46 Minor groove DNA-binding drugs such as netropsin and distamycin A compete with HMGA proteins.47 Our preliminary data showed that AsPC1 cells, and to a lesser extent Panc1 cells, were sensitive to FK317, and this agent had growth suppressive and cytotoxic effects on these cells in dose-dependent manners (data not shown). Further studies are required to discover a new type of specific HMGA2 inhibitors, such as chemicals acting on AT hook of each HMGA protein.

In conclusion, our study indicates that: (i) HMGA2 promotes proliferation in pancreatic cancer cells, (ii) HMGA2 is required for maintaining EMT of pancreatic cancer cells, (iii) HMGA2 directly binds SNAIL gene promoter for transcriptional activation, leading to possible repression of E-cadherin gene, (iv) oncogenic RAS signaling pathway induces EMT via up-regulation of HMGA2, (v) an inverse correlation between HMGA2-positive and E-cadherin-positive cells was found in pancreatic cancer tissues. In addition, (vi) the let-7 microRNAs are expressed in pancreatic cancer cells but do not effectively contribute to preventing EMT in pancreatic cancer cells. Taken together, our study provides mechanistic insight into the role of HMGA2 in RAS-induced EMT of pancreatic cancer cells, together with therapeutic potential.

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