Gastrointestinal, Hepatobiliary and Pancreatic Pathology

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

Sugiko Watanabe, Yasuaki Ueda,
Shin-ichi Akaboshi, Yuko Hino, Yoko Sekita,
and Mitsuyoshi Nakao

*From the Department of Regeneration Medicine, Institute of
Molecular Embryology and Genetics, Kumamoto University,
Kumamoto, Japan*

Pancreatic cancer is a highly aggressive malignancy due to elevated mitotic activities and epithelial-mesenchymal transition (EMT). Oncogenic RAS and transforming growth factor- β signaling are implicated in these malignant features. The mechanisms that underlie EMT need to be addressed since it promotes tissue invasion and metastasis. The high-mobility group A protein 2 (HMGA2) is a non-histone chromatin factor that is primarily expressed in undifferentiated tissues and tumors of mesenchymal origin. However, its role in EMT in pancreatic cancer is largely unknown. Here we report that HMGA2 is involved in EMT maintenance in human pancreatic cancer cells. Specific knockdown of HMGA2 inhibited cell proliferation, leading to an epithelial-state transition that restores cell-cell contact due to E-cadherin up-regulation. Consistently, an inverse correlation between HMGA2-positive cells and E-cadherin-positive cells was found in cancer tissues. Inhibition of the RAS/MEK pathway also induced an epithelial transition, together with HMGA2 down-regulation. Transcriptional repressors of the *E-cadherin* gene, such as SNAIL, decreased after HMGA2 knockdown since HMGA2 directly activated the *SNAIL* gene promoter. The decrease of SNAIL after RAS/MEK inhibition was suppressed by HMGA2 overexpression. Further, *let-7* microRNA-mediated HMGA2 down-regulation had no effect on the prevention of the transformed phenotype in these cells. These data shed light on the importance of HMGA2 in reversibly maintaining EMT, suggesting that HMGA2 is a potential therapeutic target for the treatment of pancreatic cancer. (*Am J Pathol* 2009, 174:854–868; DOI: 10.2353/ajpath.2009.080523)

Pancreatic cancer has the poorest prognosis among human neoplasms due to its highly aggressive and metastasizing features.^{1–3} 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.⁴ 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.^{5,6} In pancreatic cancer cells, EMT is also reported to be a crucial step for tumor cell migration and invasion.⁷ Previous studies suggest that aberrations in pathways emerging from oncogenic RAS and transforming growth factor- β (TGF- β) stimulate the malignant features, including EMT.^{6,8} Several transcriptional factors have been identified that control EMT by repressing *E-cadherin* and other epithelial genes in embryonic morphogenesis and cancer metastasis.^{6,9} These include the Snail-related zinc-finger factors (SNAIL and SLUG), the other zinc-finger factors (SIP1/ZEB2 and ZEB1/ δ 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.⁴ Despite its biological

Supported by a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology (M. N. and S. W.), and in part by a Grant-in-Aid for Global COE (Center of Excellence) "Cell Fate Regulation Research and Education Unit," Kumamoto University.

Accepted for publication November 18, 2008.

Supplemental material for this article can be found on <http://ajp.amjpathol.org>.

Address reprint requests to Mitsuyoshi Nakao, M.D., Ph.D., Department of Regeneration Medicine, Institute of Molecular Embryology and Genetics, Kumamoto University, 2-2-1 Honjo, Kumamoto 860-0811, Japan. E-mail: mnakao@gpo.kumamoto-u.ac.jp.

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 complexes, called enhanceosomes, on the promoter/enhancer regions of several genes.¹¹ 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. 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 overexpressing 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.

Table 1. Antibodies Used in this Study

Name	Antigen	Source	Manufacturer
HMGA1a/1b	HMGA1	rabbit	abcam
HMGA1	HMGA1	goat	Santa Cruz
HMGA1/C (S-15)	HMGA2	goat	Santa Cruz
β -tubulin	β -tubulin	mouse	Amersham
E-cadherin	E-cadherin	mouse	BD trasnduction
N-cadherin	N-cadherin	mouse	BD trasnduction
Vimentin Ab-2	Vimentin	mouse	Neo Markers
K-Ras-2A (C-17)	KRAS	rabbit	Santa Cruz
Anti-FLAG M2	FLAG-tag	mouse	Sigma

Materials and Methods

Cell Culture and Treatment

AsPC1, Capan1, and Panc1 cells (American Type Culture Collection, Manassas, VA), HeLa S3 and MIA PaCa2 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 μ mol/L U0126 (LC Laboratories, Woburn, MA) or dimethyl sulfoxide every 2 days. Human recombinant TGF- β 1 (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 MgCl₂, 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 2. Oligonucleotides Used in this Study

Name	Sequence
Primer	
HMGA1 S	5'-TGAGTCCCGGACAGCACTGGTAG-3'
HMGA1 AS	5'-GCGGCTAAGTGGGATGTTAGCCTTG-3'
HMGA2 S	5'-CAGGATGAGCGCACGCGGTGAGGC-3'
HMGA2 AS	5'-CCATTTCTTAGGTCTGCCTCTTGGC-3'
GAPDH S	5'-GATGCCCCCATGTTTCGT-3'
GAPDH AS	5'-CAGGGGTCTTACTCCTTGA-3'
Primer	
HMGA1 S2	5'-TCCAGGAAGGAAACCAAGG-3'
HMGA1 AS2	5'-AGGACTCCTGCGAGATGC-3'
HMGA2 S2	5'-GCGCCTCAGAAGAGAGGAC-3'
HMGA2 AS2	5'-GGTCTCTTAGGAGAGGGCTCA-3'
E-cadherin S	5'-CCCGGGACAACGTTTATTAC-3'
E-cadherin AS	5'-GCTGGCTCAAGTCAAAGTCC-3'
Vimentin S	5'-TACAGGAAGCTGCTGGAAGG-3'
Vimentin AS	5'-ACCAGAGGGAGTGAATCCAG-3'
SNAIL S	5'-GGAAGCCTAATACAGCGAGCT-3'
SNAIL AS	5'-TCCCAGATGAGCATTTGGCA-3'
SLUG S	5'-TGGTTGCTTCAAGGACACAT-3'
SLUG AS	5'-GTTGCAGTGAGGGCAAGAA-3'
SIP1 S	5'-AAGCCAGGGACAGATCAGC-3'
SIP1 AS	5'-CCACACTCTGTGCATTTGAAC-3'
E12E47 S	5'-CCCAGACCAACTGCTCATC-3'
E12E47 AS	5'-CTGCTTTGGGATTACAGGTTTC-3'
ZEB1 S	5'-CTGCTGGGAGGATGACAGA-3'
ZEB1 AS	5'-ATCCTGCTTTCATCTGCCTGA-3'
Snail Promoter	
SNAIL promoter -1556S	5'-CCTGATTGGAGCTAAATTGACAC-3'
SNAIL promoter -1220AS	5'-CCCAAGTGAGGGGACAGCTGCAC-3'
SNAIL promoter -1220S	5'-GCCCACCCACATGCATTTCAGTC-3'
SNAIL promoter -880AS	5'-AAAAGGCCCGAGGGAAGAAGTGGC-3'
SNAIL promoter -880S	5'-CCCTTGATAATTCTTCACTTCCTC-3'
SNAIL promoter -517AS	5'-AAGCGAGGAAAGGGACACCGCAC-3'
SNAIL promoter -517S	5'-CGCTTCTTCCCAGTGATGTC-3'
SNAIL promoter -223AS	5'-ACTCCTCCGAGGCGGGTTTCGC-3'
SNAIL promoter -223S	5'-CGAACCCCGCTCGGAGGAGTC-3'
SNAIL promoter -94S	5'-CAAAGGGGCGTGGCAGATAAGGC-3'
SNAIL promoter + 59AS	5'-CGCCGATTTCGCGCAGCAGTAG-3'
Name	
HMGA1 si-S	5'-GUGCCAACACCUAAGAGACCUTT-3'
HMGA1 si-AS	5'-AGGUCUCUUAGGUGUUGGCATT-3'
HMGA2 si-S	5'-CGGCCAAGAGGCAGACCUATT-3'
HMGA2 si-AS	5'-UAGGUCUGCCUCUUGGCCGTT-3'
HMGA2 si-S2	5'-CCGGUGAGCCUCUCCUAATT-3'
HMGA2 si-AS2	5'-UUAGGAGAGGGCUCACCGGTT-3'
MISMATCH si-S	5'-AAGCCGCGACGAGAUACCTT-3'
MISMATCH si-AS	5'-GGUAUCUCGUCGCGCUUTT-3'
GL3 si-S	5'-CUUACGUGAGUACUUCGATT-3'
GL3 si-AS	5'-UCGAAGUACUCAGCGUAAGTT-3'

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 micro-

scope 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 μ g 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).²⁶ 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 membrane-associated E-cadherin was used for judging as positive and the absent or weak staining of E-cadherin was counted as negative. HMGA2-positive cells or E-cadherin-positive cells were quantitatively assessed by counting carcinoma cells (mean 286; range 113 to 485) in the same tissue samples.

Chromatin Immunoprecipitation Analysis

HeLa cells (1.5×10^6) were cross-linked with 1% formaldehyde at 37°C for 10 minutes. Crude cell lysates were sonicated to generate 200 to 500 bp DNA fragments. Chromatin immunoprecipitation analysis was performed with anti-FLAG or control IgG, according to the manufacturer's protocol (Upstate Biotechnology, Lake Placid, NY). PCR amplification of human *SNAIL* gene promoters was performed for 35 cycles of 95°C for 30s, 57°C for 30s, and 72°C for 60s, using specific sets of primers (Table 2C).

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).

Cell Proliferation Analyses

Cell proliferation was assessed by seeding AsPC1, BxPC3, Panc1, and MIAPaCa2 cells (1×10^5 /well) into 6-well plates, and 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'-AGGATGAGCGCACGCGGT-GAGGGC-3' and 5'-CTAGTCCTCTTCGGCAGACTCTTG-3'; for *SNAIL* promoter, 5'-CCTGATTGGAGCTAAATTGACAC-3' and 5'-CGCCGATTCGCGCAGCAGTAG-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 *Xba*I site of the pGL3-control vector (Promega). The *let-7a1f1*, *let-7a3b*, and *miR-370* were amplified from genomic DNAs and cloned into pcDNA3.1 (Invitrogen). Sensor or control plasmid (1 μ g) 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.

Antisense 2'-O-Methyloligoribonucleotide

2'-O-methyloligoribonucleotides (*let-7a* antisense and *miR-370* antisense) were synthesized by Japan Bio Services Co., LTD. (Table 3). The 2'-O-methyloligoribonucleotides (80 pmol) were introduced into AsPC1 or Panc1 cells using Lipofectamine 2000 for Western blot and cell proliferation analysis.

Establishment of Stably Expressed Cells

Panc1 cells were introduced with pCAG IRES-Puro, which is kindly gifted by Dr. H. Niwa (RIKEN), that ex-

Table 3. Oligonucleotides Used in MicroRNA Analysis

Name	Sequence
2'-O-methyloligoribonucleotide	
let-7a antisense	5'-AACUAUACAACCUACUACCUCA-3'
miR-370 antisense	5'-CCAGGUUCCACCCAGCAGGC-3'
Oligonucleotide	
complementary to let-7a S	5'-AACTATACAACCTACTACCTCAAACCTATACAACCTACTACCTCA-3'
complementary to let-7a AS	5'-TGAGGTAGTAGGTTGTATAGTTTGAGGTAGTAGGTTGTATAGTT-3'
complementary to miR-370 S	5'-CCAGGTTAAACCCAGCAGGCCAGGTTAAACCCAGCAGGC-3'
complementary to miR-370 AS	5'-GCCTGCTGGGGTGGAACTGGGCCCTGCTGGGGTGGAACTGG-3'
Primer	
RAS 3' UTR S	5'-GACTCTTCTTCCATATTAGTGTC-3'
RAS 3' UTR AS	5'-GTCCTTAAAGGCTGTAATAATTAGG-3'
HMGA1 3' UTR S	5'-GAGGAGCAGTGACCCATGCGTGC-3'
HMGA1 3' UTR AS	5'-AACTGCAAAATAGGAAACCAGAGAGG-3'
HMGA2 3' UTR S	5'-AGAGACATCCTCACAAGAGTCTG-3'
HMGA2 3' UTR AS	5'-ATGAGCTGGCCAATGAGGTTTCT-3'
let-7a1f1 S	5'-CCTGGATGTTCTCTTCACTG-3'
let-7a1f1 AS	5'-TAATGCAGCAAGTCTACTCC-3'
let-7a3b S	5'-ACCAAGACCGACTGCCCTTT-3'
let-7a3b AS	5'-GCTCCTCAGGAAGGCAGTA-3'
miR-370 S	5'-GCTGCATTCTCATTCTAC-3'
miR-370 AS	5'-CTCTGGTGTAGACAGA-3'

presses GFP fused HMGA1 and HMGA2 using FuGENE6 (Roche), and selected in the presence of puromycin (2.0 μ g/ml) (Sigma). HepG2 cells were introduced with pcDNA3.1 that expressed *let-7a1f1* and *let-7a3b* using FuGENE6 (Roche), and selected in the presence of G418 (1.5 mg/ml) (Sigma).

Statistical Analysis

Statistical analyses were performed using JMP 7.0.1 for Windows software (SAS Institute Inc., Cary, NC). The significant difference in real-time PCR quantification was evaluated by two-tailed paired *t*-test. The association between HMGA2-positive cells and E-cadherin-positive cells was analyzed using the Pearson correlation coefficient, which varies from a perfect negative correlation (-1) to a perfect positive correlation ($+1$). Statistical significance was considered at a probability level of 0.05 or less.

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, MIAPaCa2, and Panc1), and that HMGA2 was expressed in four cells

but not MIAPaCa2 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 reports^{18–21} 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). On the other hand, growth suppression did not appear to be evident in MIAPaCa2 cells that originally expressed undetectable HMGA2, suggesting that HMGA2 has a role in maintaining growth activities of pancreatic cancer cells. Further, we observed that AsPC1 cells had morphological changes under the HMGA2 knockdown. As shown by phase contrast image, the cells were grown in a scattered pattern (Figure 1D; control),

Figure 1. Involvement of HMGA2 in phenotype of pancreatic cancer cells. **A:** Expression of HMGA1 and HMGA2 transcripts. RT-PCR was performed using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control. Pancreatic cancer cell lines include AsPC1, BxPC3, Capan1, MIA PaCa2, and Panc1. Normal pancreatic tissue, HeLa and HepG2 cells are used as a control. **B:** Specific siRNA-mediated knockdown of HMGA proteins. The data are presented from AsPC1, BxPC3 and Panc1 cells. β -tubulin is shown as a control. **C:** The effect of HMGA knockdown on cell proliferation. The cell numbers were determined on day 0, 2, 4, and 6 after the knockdown. Results were obtained from three independent experiments. Error bars indicate SD. **D:** 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.

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* mutations²⁷ and were used for further study. To test a conversion to the epithe-

Figure 2. Requirement of HMGA2 for maintaining a mesenchymal state in pancreatic cancer cells. **A:** Membranous localization of E-cadherin in HMGA2-knockdown cells. Immunofluorescent analysis was performed using anti-E-cadherin antibodies on day 5 after siRNA transfection. Nuclei were counterstained with 4',6-diamidino-2-phenylindole. **B:** Expression of E-cadherin. Western blot analysis was performed using AsPC1 and Panc1 cells on day 5 after distinct siRNA transfection. The relative amount of E-cadherin and HMGA proteins was normalized with β -tubulin and histone H3. The densitometrical data were quantitatively shown. **C:** Expression of E-cadherin and vimentin. Quantitative RT-PCR analysis of E-cadherin and vimentin was performed using HMGA-knockdown cells. The relative mRNA levels with the use of control siRNAs were normalized to 1. Values are given as means and standard deviations from more than three independent experiments when compared with control cells * $P < 0.05$; ** $P < 0.0001$.

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.²⁸ In addition, HMGA proteins were found to be induced by the RAS signaling pathway, possibly leading to alteration of various expressed genes.²⁹⁻³¹ 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 ERK1/2.³² AsPC1 and Panc1 cells

Figure 3. Expression status of E-cadherin and HMGA2 in pancreatic cancer tissues. **A:** Serial sections of hyperplastic duct of pancreas (**a-c**) and pancreatic adenocarcinoma (**d-f**, **g-i**, and **j-l**). Normal or hyperplastic duct shows E-cadherin expression, while HMGA2 is highly stained in the nuclei of cancer cells. H&E staining (**a**, **d**, **g**, **j**); immunostaining for E-cadherin (**b**, **e**, **h**, **k**) and HMGA2 (**c**, **f**, **i**, **l**). Scale bar = 50 μ m. **B:** Percentage correlations between HMGA2-positive cells and E-cadherin-positive cells in pancreatic cancer tissues. Carcinoma cells (mean, 286; range, 113 to 485) were counted in the same samples, and HMGA2-positive cells or E-cadherin-positive cells were quantitatively assessed. Data were analyzed using the Pearson's correlation coefficient. Table 3 shows the summary of tumor grade and immunohistochemical data.

were treated with 10 μ mol/L of U0126 for 4 days. As previously reported,³³ 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. AsPC1 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

ID	Diagnosis	Grade	% of E-cadherin positive cells	% of HMGA2 positive cells
1	Ductal adenocarcinoma	I	100	13.3
2	Adenocarcinoma	I	100	11.4
3	Ductal adenocarcinoma	II	100	8.7
4	Ductal adenocarcinoma	II	70.6	61.7
5	Ductal adenocarcinoma	II	90.4	64
6	Ductal adenocarcinoma	II	84.2	65.6
7	Ductal adenocarcinoma	II	82.1	79.6
8	Ductal adenocarcinoma	II	6.2	74.3
9	Adenocarcinoma	II	8.1	91.2
10	Ductal adenocarcinoma	II	10.8	83.3
11	Ductal adenocarcinoma	II	14.4	64
12	Ductal adenocarcinoma	III	88.5	67.6
13	Adenocarcinoma	III	44.7	41.5
14	Ductal adenocarcinoma	III	0.4	92.7

Figure 4. Involvement of HMGA2 in the RAS/MEK pathway in pancreatic cancer cells. **A:** Expression of epithelial and mesenchymal markers and HMGA proteins. AsPC1 and Panc1 cells were treated with MEK inhibitor U0126 for 4 days. **B–E.** Quantitative RT-PCR analysis of HMGA and EMT markers (**B, D**), and EMT-stimulating transcription factors (**C, E**). U0126-treated cells (**B, C**) and TGF-β-treated cells (**D, E**) were used. The cells were treated with 5 ng/ml TGF-β1 for 8 or 24 hours. The relative mRNA levels with the use of dimethyl sulfoxide were normalized to 1. Values are given as means and standard deviations from more than three independent experiments when compared with control cells **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

atic cancer, we checked the expression of EMT-stimulating transcription factors (SNAIL, SLUG, SIP1/ZEB2, E12/E47, and ZEB1/δEF-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 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.³⁴ In pancreatic cancer, it was reported that TGF-β itself is overexpressed,²⁸ 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-β1 on Panc1 cells, which have an intact Smad-mediated transcription pathway (Figure 4D). Using a quantitative RT-PCR analysis of the TGF-β1-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-β1 treatment. We then examined the expression of EMT-stimulating transcription factors (Figure 4E). TGF-β1 treatment markedly increased the expression of both SNAIL and SLUG. Further, we treated HMGA-knockdown Panc1 cells with TGF-β1 and checked the expression of these EMT regulators (see supplemental Figure S2, B and C 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-β1 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

Figure 5. HMGA2-enhanced expression of SNAIL in pancreatic cancer cells. **A:** Expression of EMT regulators in HMGA2-knockdown cells. Quantitative RT-PCR analysis of SNAIL, SLUG, SIP1, E12/E47, and ZEB1 was performed. The relative mRNA levels with the use of control siRNAs were normalized to 1. Values are given as means and standard deviations from more than three independent experiments when compared with control cells * $P < 0.05$; ** $P < 0.005$. **B:** Effect of HMGA2 on *SNAIL* gene promoter. HMGA2 expression vector was introduced into HeLa cells, together with a pGL3-SNAIL reporter (1 μ g) and pRL-SV40 (1 ng), for 48 hours. Using a dual luciferase reporter assay, the activities of the mock vectors were normalized to 100. **C:** Localization of HMGA2 in the endogenous *SNAIL* gene promoter. Schematic presentation of *SNAIL* gene promoter (**left panel**). 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). **D:** Reversion of U0126-mediated *SNAIL* inhibition by exogenous HMGA2. Panc1 cells were expressed exogenous HMGA2 and treated with U0126 for 4 days. The relative mRNA levels with the use of mock vector and dimethyl sulfoxide were normalized to 1. Values are given as means and standard deviations from more than three independent experiments when compared with control cells ** $P < 0.005$. **E:** Model for EMT in pancreatic cancer cells. HMGA2 links constitutively active mutant KRAS to SNAIL, leading to maintenance of EMT.

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 a promoter-associated CpG island (~500bp of GC-rich) and an AT-rich upstream region.³⁵ Six genomic regions (I to VI) in the *SNAIL* promoter were tested in this experiment. In agreement with the binding property of the protein, HMGA2 was localized on the AT-rich region (IV to VI) in the *SNAIL* promoter.

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.^{26,36} The *KRAS* gene is mostly mutated in pancreatic cancer, and

Figure 6. The *let-7* microRNA family targets *HMGA1*, *HMGA2*, and *KRAS* for inhibition. **A:** Schematic presentation of transcripts encoding human *HMGA1*, *HMGA2*, and *KRAS*. The siRNA-target sites used in this study, *let-7* family complementary sites, and *miR-370* complementary sites are indicated by gray, black, and open triangles, respectively. **B:** Expression of *let-7a1f1*, *let-7a3b*, and *miR-370* in pancreatic cancer cells. Total RNAs were isolated for RT-PCR of indicated microRNA transcripts. The expression of GAPDH and no reverse transcriptase (RT⁻) are used as controls. **C:** Effect of the 3' untranslated regions (3'UTR) of *HMGA1*, *HMGA2*, and *KRAS* on translated products. The luciferase reporters containing the 3'UTR of *HMGA1*, *HMGA2*, and *KRAS* were introduced into HeLa, BxPC3, and Panc1 cells, which originally expressed the *let-7* microRNA family. Luciferase activities from the pGL3-control were normalized to 100. Data are from three independent experiments; error bars represent SD. **D, E:** Effect of the *let-7* family on the 3'UTR of *HMGA1*, *HMGA2*, and *KRAS*. Exogenous *let-7a1f1*, *let-7a3b*, and *miR-370* were expressed in HepG2 or HeLa cells (**D**), AsPC1 or Panc1 cells (**E**). The luciferase reporters containing a *let-7a* complementary sequence or the 3'UTR of *HMGA1*, *HMGA2*, and *KRAS* were used as a sensor. phRL-SV40 was used as a transfection control.

the implication of the *let-7* microRNAs remains unknown. Similar to the *KRAS*, *HMGA1* and *HMGA2* have a long 3'UTR,³⁷ and are targeted by the *let-7* microRNAs in mammalian cells.^{22,24,25,38} 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 9,³⁹ 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.²⁶ 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 addition, *miR-370* was expressed

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,^{26,36} 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 inhibitor^{40,41} 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 (Fig-

ure 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

Figure 7. *let-7*-mediated inhibition of HMGA2 has no effective role in preventing the transformed phenotypes of pancreatic cancer cells. **A:** Effect of the *let-7a* inhibitor on the 3'UTR of *HMGA1*, *HMGA2*, and *KRAS*. The *let-7a* inhibitor, *let-7a* antisense 2'-O-methyloligoribonucleotides, was introduced to AsPC1 and Panc1 cells that expressed the *let-7* family, together with the sensor reporters. As previously described,⁴⁸ the ratio of normalized sensor to control luciferase activities is shown. **B:** Expression of HMGA proteins and KRAS under the use of the *let-7a* inhibitor. *let-7a* or *miR-370* antisense 2'-O-methyloligoribonucleotides (80 pmol) were introduced into AsPC1 and Panc1 cells for 5 days. The relative amount of these proteins was normalized with β -tubulin. The densitometric data were quantitatively shown. **C:** Effect of the *let-7a* inhibitor on growth and expression of EMT regulators in AsPC1 and Panc1 cells. The cell numbers were counted on days 0, 2, 4, and 6 after the transfection (left panel). Quantitative RT-PCR analysis was similarly performed (right panel). **D, E:** Effect of the exogenous *let-7* family on proliferation and expression of EMT regulators in Panc1 (**D**) and HepG2 cells (**E**). The cell numbers were counted on days 0, 2, 4, and 6 (left panels). The expression of regulators of EMT was analyzed by quantitative RT-PCR analysis using β -actin as a normalization control (right panels). The mRNA levels in the control cells were normalized to 1. Values are given as means and SD from more than three independent experiments. The expression levels of the exogenous *let-7a1f1* and *let-7a3b* increased approximately 10-fold compared with the endogenous levels.

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.^{18–20} 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.⁴² On the other hand, HMGA2 is predominantly expressed in proliferating, undifferentiated mesenchymal cells, and is not found in adult tissues.^{10,11} In addition, *Hmga2*^{−/−} mice had a deficiency in fat tissue, termed a *pygmy* phenotype.^{12,43} HMGA2 was also found to transactivate the *cyclin A* gene and stimulate cell growth.⁴⁴ 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 down-regulated 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.^{22,24,25,38} 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.²⁵ 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-pos-

itive cells 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.

Acknowledgments

We thank Drs. Takaaki Ito and Takaya Ichimura (Kumamoto University Graduate School of Medical Sciences) for teaching immunohistochemistry, members of our laboratory for helpful discussions, and Astellas Pharma Inc. (Osaka, Japan) for providing FK317 agent.

References

1. Sener SF, Fremgen A, Menck HR, Winchester DP: Pancreatic cancer: a report of treatment and survival trends for 100,313 patients diagnosed from 1985–1995, using the National Cancer Database. *J Am Coll Surg* 1999, 189:1–7
2. Li D, Xie K, Wolff R, Abbruzzese JL: Pancreatic cancer. *Lancet* 2004, 363:1049–1057
3. Matsuno S, Egawa S, Fukuyama S, Motoi F, Sunamura M, Isaji S, Imaizumi T, Okada S, Kato H, Suda K, Nakao A, Hiraoka T, Hosotani R, Takeda K: Pancreatic Cancer Registry in Japan: 20 years of experience. *Pancreas* 2004, 28:219–230
4. Thiery JP, Sleeman JP: Complex networks orchestrate epithelial-mesenchymal transitions. *Nat Rev Mol Cell Biol* 2006, 7:131–142
5. Thiery JP: Epithelial-mesenchymal transitions in tumour progression. *Nat Rev Cancer* 2002, 2:442–454
6. Huber MA, Kraut N, Beug H: Molecular requirements for epithelial-mesenchymal transition during tumor progression. *Curr Opin Cell Biol* 2005, 17:548–558
7. Jungert K, Buck A, von Wichert G, Adler G, Konig A, Buchholz M, Gress TM, Ellenrieder V: Sp1 is required for transforming growth factor-beta-induced mesenchymal transition and migration in pancreatic cancer cells. *Cancer Res* 2007, 67:1563–1570
8. Grunert S, Jechlinger M, Beug H: Diverse cellular and molecular mechanisms contribute to epithelial plasticity and metastasis. *Nat Rev Mol Cell Biol* 2003, 4:657–665
9. Peinado H, Portillo F, Cano A: Transcriptional regulation of cadherins during development and carcinogenesis. *Int J Dev Biol* 2004, 48:365–375
10. Sgarra R, Rustighi A, Tessari MA, Di Bernardo J, Altamura S, Fusco A, Manfioletti G, Giannotti V: Nuclear phosphoproteins HMGA and their relationship with chromatin structure and cancer. *FEBS Lett* 2004, 574:1–8
11. Reeves R: Molecular biology of HMGA proteins: hubs of nuclear function. *Gene* 2001, 277:63–81
12. Zhou X, Benson KF, Ashar HR, Chada K: Mutation responsible for the mouse pygmy phenotype in the developmentally regulated factor HMGI-C. *Nature* 1995, 376:771–774
13. Chiappetta G, Avantaggiato V, Visconti R, Fedele M, Battista S, Trapasso F, Merciai BM, Fidanza V, Giannotti V, Santoro M, Simeone A, Fusco A: High level expression of the HMGI (Y) gene during embryonic development. *Oncogene* 1996, 13:2439–2446
14. Fedele M, Pentimalli F, Baldassarre G, Battista S, Klein-Szanto AJ, Kenyon L, Visone R, De Martino I, Ciarmiello A, Arra C, Viglietto G, Croce CM, Fusco A: Transgenic mice overexpressing the wild-type form of the HMGA1 gene develop mixed growth hormone/prolactin cell pituitary adenomas and natural killer cell lymphomas. *Oncogene* 2005, 24:3427–3435
15. Xu Y, Sumter TF, Bhattacharya R, Tesfaye A, Fuchs EJ, Wood LJ, Huso DL, Resar LM: The HMGI oncogene causes highly penetrant,

- aggressive lymphoid malignancy in transgenic mice and is overexpressed in human leukemia. *Cancer Res* 2004, 64:3371–3375
16. Fedele M, Battista S, Kenyon L, Baldassarre G, Fidanza V, Klein-Szanto AJ, Parlow AF, Visone R, Pierantoni GM, Outwater E, Santoro M, Croce CM, Fusco A: Overexpression of the HMGA2 gene in transgenic mice leads to the onset of pituitary adenomas. *Oncogene* 2002, 21:3190–3198
17. Fedele M, Visone R, De Martino I, Troncone G, Palmieri D, Battista S, Ciarmiello A, Pallante P, Arra C, Melillo RM, Helin K, Croce CM, Fusco A: HMGA2 induces pituitary tumorigenesis by enhancing E2F1 activity. *Cancer Cell* 2006, 9:459–471
18. Abe N, Watanabe T, Masaki T, Mori T, Sugiyama M, Uchimura H, Fujioka Y, Chiappetta G, Fusco A, Atomi Y: Pancreatic duct cell carcinomas express high levels of high mobility group I(Y) proteins. *Cancer Res* 2000, 60:3117–3122
19. Abe N, Watanabe T, Suzuki Y, Matsumoto N, Masaki T, Mori T, Sugiyama M, Chiappetta G, Fusco A, Atomi Y: An increased high-mobility group A2 expression level is associated with malignant phenotype in pancreatic exocrine tissue. *Br J Cancer* 2003, 89:2104–2109
20. Reeves R, Edberg DD, Li Y: Architectural transcription factor HMGI(Y) promotes tumor progression and mesenchymal transition of human epithelial cells. *Mol Cell Biol* 2001, 21:575–594
21. Evans A, Lennard TW, Davies BR: High-mobility group protein 1(Y): metastasis-associated or metastasis-inducing? *J Surg Oncol* 2004, 88:86–99
22. Wang T, Zhang X, Obijuru L, Laser J, Aris V, Lee P, Mittal K, Soteropoulos P, Wei JJ: A micro-RNA signature associated with race, tumor size, and target gene activity in human uterine leiomyomas. *Genes Chromosomes Cancer* 2007, 46:336–347
23. Hebert C, Norris K, Scheper MA, Nikitakis N, Sauk JJ: High mobility group A2 is a target for miRNA-98 in head and neck squamous cell carcinoma. *Mol Cancer* 2007, 6:5
24. Mayr C, Hemann MT, Bartel DP: Disrupting the pairing between let-7 and Hmga2 enhances oncogenic transformation. *Science* 2007, 315:1576–1579
25. Lee YS, Dutta A: The tumor suppressor microRNA let-7 represses the HMGA2 oncogene. *Genes Dev* 2007, 21:1025–1030
26. Takamizawa J, Konishi H, Yanagisawa K, Tomida S, Osada H, Endoh H, Harano T, Yatabe Y, Nagino M, Nimura Y, Mitsudomi T, Takahashi T: Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. *Cancer Res* 2004, 64:3753–3756
27. Sipos B, Moser S, Kalthoff H, Torok V, Lohr M, Kloppel G: A comprehensive characterization of pancreatic ductal carcinoma cell lines: towards the establishment of an in vitro research platform. *Virchows Arch* 2003, 442:444–452
28. Rustgi AK: The molecular pathogenesis of pancreatic cancer: clarifying a complex circuitry. *Genes Dev* 2006, 20:3049–3053
29. Zentner MD, Lin HH, Deng HT, Kim KJ, Shih HM, Ann DK: Requirement for high mobility group protein HMGI-C interaction with STAT3 inhibitor PIAS3 in repression of alpha-subunit of epithelial Na⁺ channel (alpha-ENaC) transcription by Ras activation in salivary epithelial cells. *J Biol Chem* 2001, 276:29805–29814
30. Treff NR, Pouchnik D, Dement GA, Britt RL, Reeves R: High-mobility group A1a protein regulates Ras/ERK signaling in MCF-7 human breast cancer cells. *Oncogene* 2004, 23:777–785
31. Cleynen I, Huysmans C, Sasazuki T, Shirasawa S, Van de Ven W, Peeters K: Transcriptional control of the human high mobility group A1 gene: basal and oncogenic ras-regulated expression. *Cancer Res* 2007, 67:4620–4629
32. Ahn NG, Nahreini TS, Tolwinski NS, Resing KA: Pharmacologic inhibitors of MKK1 and MKK2. *Methods Enzymol* 2001, 332:417–431
33. Gysin S, Lee SH, Dean NM, McMahon M: Pharmacologic inhibition of RAF→MEK→ERK signaling elicits pancreatic cancer cell cycle arrest through induced expression of p27Kip1. *Cancer Res* 2005, 65:4870–4880
34. Thuault S, Valcourt U, Petersen M, Manfioletti G, Heldin CH, Moustakas A: Transforming growth factor-beta employs HMGA2 to elicit epithelial-mesenchymal transition. *J Cell Biol* 2006, 174:175–183
35. Barbera MJ, Puig I, Dominguez D, Julien-Grille S, Guaita-Esteruelas S, Peiro S, Baulida J, Franci C, Dedhar S, Larue L, Garcia de Herreros A: Regulation of Snail transcription during epithelial to mesenchymal transition of tumor cells. *Oncogene* 2004, 23:7345–7354
36. Johnson SM, Grosshans H, Shingara J, Byrom M, Jarvis R, Cheng A, Labourier E, Reinert KL, Brown D, Slack FJ: RAS is regulated by the let-7 microRNA family. *Cell* 2005, 120:635–647
37. Borrmann L, Wilkening S, Bullerdiek J: The expression of HMGA genes is regulated by their 3'UTR. *Oncogene* 2001, 20:4537–4541
38. Young AR, Narita M: Oncogenic HMGA2: short or small? *Genes Dev* 2007, 21:1005–1009
39. Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T: Identification of novel genes coding for small expressed RNAs. *Science* 2001, 294:853–858
40. Meister G, Landthaler M, Dorsett Y, Tuschl T: Sequence-specific inhibition of microRNA- and siRNA-induced RNA silencing. *RNA* 2004, 10:544–550
41. Cheng AM, Byrom MW, Shelton J, Ford LP: Antisense inhibition of human miRNAs and indications for an involvement of miRNA in cell growth and apoptosis. *Nucleic Acids Res* 2005, 33:1290–1297
42. Liao SS, Jazag A, Whang EE: HMGA1 is a determinant of cellular invasiveness and in vivo metastatic potential in pancreatic adenocarcinoma. *Cancer Res* 2006, 66:11613–11622
43. Anand A, Chada K: In vivo modulation of Hmga reduces obesity. *Nat Genet* 2000, 24:377–380
44. Tessari MA, Gostissa M, Altamura S, Sgarra R, Rustighi A, Salvagno C, Caretti G, Imbriano C, Mantovani R, Del Sal G, Giancotti V, Manfioletti G: Transcriptional activation of the cyclin A gene by the architectural transcription factor HMGA2. *Mol Cell Biol* 2003, 23:9104–9116
45. Brueckner B, Stresemann C, Kuner R, Mund C, Musch T, Meister M, Sultmann H, Lyko F: The human let-7a-3 locus contains an epigenetically regulated microRNA gene with oncogenic function. *Cancer Res* 2007, 67:1419–1423
46. Beckerbauer L, Tepe JJ, Cullison J, Reeves R, Williams RM: FR900482 class of anti-tumor drugs cross-links oncoprotein HMG I/Y to DNA in vivo. *Chem Biol* 2000, 7:805–812
47. Reeves R, Nissen MS: The A.T-DNA-binding domain of mammalian high mobility group I chromosomal proteins. A novel peptide motif for recognizing DNA structure. *J Biol Chem* 1990, 265:8573–8582
48. O'Donnell KA, Wentzel EA, Zeller KI, Dang CV, Mendell JT: c-Myc-regulated microRNAs modulate E2F1 expression. *Nature* 2005, 435:839–843