SKI and MEL1 Cooperate to Inhibit Transforming Growth Factor-β Signal in Gastric Cancer Cells

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Chromosomal amplification occurs frequently in solid tumors and is associated with poor prognosis. Several reports demonstrated the cooperative effects of oncogenic factors in the same ampiclon during cancer development. However, the functional correlation between the factors remains unclear. Transforming growth factor (TGF-β) signaling plays important roles in cytostasis and normal epithelium differentiation, and alterations in TGF-β signaling have been identified in many malignancies. Here, we demonstrated that transcriptional co-repressors of TGF-β signaling, SKI and MDS1/EVI1-like gene 1 (MEL1), were aberrantly expressed in MKN28 gastric cancer cells by chromosomal co-amplification of 1p36.32. SKI and MEL1 knockdown synergistically restored TGF-β responsiveness in MKN28 cells and reduced tumor growth in vivo. MEL1 interacted with SKI and inhibited TGF-β signaling by stabilizing the inactive Smad3-SKI complex on the promoter of TGF-β target genes. These findings reveal a novel mechanism where distinct transcriptional co-repressors are co-amplified and functionally interact, and provide molecular targets for gastric cancer treatment.

Transforming growth factor-β (TGF-β) signals through two distinct serine-threonine kinase receptors, termed type I (TβR-I) and type II (TβR-II) receptors. Upon ligand binding, TβR-I activates TβR-I and transduces signals through various proteins, particularly Smad proteins that are the major and specific signal transducers for TGF-β (1, 2). Activated TβR-I phosphorylates receptor-regulated Smads (R-Smads), i.e. Smad2 and Smad3, which interact with the common-mediator Smad (Smad4) and translocate to the nucleus. Nuclear Smad complexes regulate transcription of target genes by binding to various transcription factors and transcriptional co-activators. Moreover, Smad complexes bind transcriptional co-repressors, e.g. SKI (also termed c-Ski), SKIL (also termed SnoN), ectopic virus integration site (EVIII), and TGFIV, which negatively regulate TGF-β signaling.

SKI and SKIL are members of the SKI family and as aproto-oncogenes (3–5). SKI was originally identified as the transforming protein of avian Sloan-Kettering retrovirus (v-Ski) (6, 7). The human cellular SKI homolog and closely related SKIL were later cloned based on sequence similarity to v-Ski (3). Both SKI and SKIL physically interact with Smad3 and Smad4, resulting in the displacement of p300/CREB-binding protein from the Smad proteins (8, 9) and in the recruitment of nuclear hormone receptor co-repressor N-CoR (10, 11) and histone deacetylases (HDACs) (8). SKI and SKIL thus induce histone deacetylation and repress target gene transcription. In addition, SKI has been shown to stabilize inactive Smad complexes on DNA (12).

Murine Evi-1 zinc finger protein was isolated from common sites of viral integration in murine myeloid leukemia (13). The human homolog, EVII, located at chromosomal position 3q26, is also transcriptionally activated by several recurrent chromosomal aberrations in acute myeloid leukemia (AML) (14). EVII was reported to antagonize the growth-inhibitory effects of TGF-β by interacting with Smad3 (15, 16).

MEL1 (MDS1/EVII-like gene I) was originally identified as a member of the EVII gene family and as a PRDI-BF1-RIZ1 homologous (PR) domain member (PRDM16) (17). MEL1 was found in t(1;3)(p36q21)-positive MDS/AML, which has similar clinicopathological features to the 3q21q26 syndrome, as a transcriptionally activated gene near the chromosomal breakpoint. The structure of the MEL1 gene product and MDS1/EVII are very similar, and MEL1S, an alternatively spliced form...
of MEL1 lacking the PR domain, is aberrantly expressed in AML (18). It was reported that MEL1S, but not MEL1, prevented TGF-β-induced growth inhibition in mouse T-cell line CTLL-2 (19). MEL1 has recently been shown to interact with Smad3 (20); however, little is known about the roles of MEL1 in tumorigenesis and TGF-β signaling.

Here, we present the first evidence that SKI is aberrantly expressed in gastric cancer. SKI was overexpressed in clinical cases with well differentiated adenocarcinoma but not in cases with undifferentiated adenocarcinoma, suggesting a role in the pathogenesis of certain gastric cancer subsets. Moreover, high SKI expression in gastric cancer cell lines correlated with MEL1 overexpression. These aberrant expressions resulted from chromosomal amplification of the 1p36.32 locus where SKI and MEL1 co-localize in the MKN28 gastric cancer cell line. Furthermore, MEL1 interacted with SKI and cooperated to inhibit TGF-β-induced cytostasis by enhancing the SKI-Smad3 interaction and promoting binding of Smad3 to the Smad-binding DNA element. These findings reveal a previously unknown mechanism where two distinct transcriptional co-repressors interact, and provide novel molecular targets for the treatment of gastric cancer.

**Experimental Procedures**

**Cell Cultures**—HEK293, COS7, and HaCaT cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 4500 mg/liter glucose, 10% fetal bovine serum, 100 units/ml of penicillin G, and 100 µg/ml of streptomycin. 293FT cells (Invitrogen) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml of penicillin G, and 100 µg/ml of streptomycin. RPMI1640 medium (Invitrogen) supplemented with 5% fetal bovine serum, 100 units/ml of penicillin G, and 100 µg/ml of streptomycin. Cells were grown in a 5% CO₂ atmosphere at 37 °C.

**cDNA Constructs and Chemicals**—Human MEL1 cDNA (DDBJ/EMBL/GenBank accession number AB078876) deletion mutants were constructed using a PCR-based approach as previously described (21). The original constructs of the constitutively active form of human type I TGF-β receptor (c.a.TBR-1), human Smad2, human Smad3, human Smad4, human SKI, and human HDAC1 cDNAs were described previously (8, 21).

**Tissue Microarray Construction and Immunohistochemistry**—For a total of 120 gastric cancers, we selected one to four representative hematoxylin-eosin-stained sections after reviewing routine histopathological slides and obtaining the corresponding tissue blocks stored in the hospital. To construct tissue microarrays, blocks of tissue microarray blocks were transferred to a recipient paraffin block using a Tissue Microarrayer (Beecher Instruments, Silver Spring, MD) (22). From each tissue-microarray block, 4-µm thick sections were made and subjected to immunohistochemistry.

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue sections as described previously (22). Paraffin-embedded specimens of primary gastric cancer used for immunohistochemistry were obtained from patients treated at the National Defense Medical College Hospital (Saitama, Japan) and Feilong Cytokine Research Institute. Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue sections as described previously (22).

**Quantitative Real-time PCR Analysis**—Total RNAs were purified from gastric cancer and normal stomach tissues using RNaseasy spin columns (Qiagen, Valencia, CA). Samples were obtained from patients treated at the National Defense Medical College Hospital with formal written informed consent from each patient and approval by the local ethics committee. First-strand cDNA was synthesized using the SuperScript VILO cDNA Synthesis Kit (Invitrogen). Total RNAs from culture cells were extracted using TRizol (Invitrogen). First-strand cDNAs were synthesized using the Superscript III First-strand Synthesis System for reverse transcription-PCR (Invitrogen) with oligo(dT)₂₀ primers. Quantitative real-time PCR analysis was performed using Platinum SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen) and ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA).

Specificity of the detected signals was confirmed by the dissociation protocol. The following primer sequences were used: human mannose-phosphate isomerase: forward, 5'-CTTAGCTTCAGCAAGAGAAGG-3', reverse, 5'-CAGGGGCACCTGCCTTCTCTCTACT-3'; human hypoxanthine phosphoribosyltransferase 1 (HPRT1): forward, 5'-TTGGTCTTTCTTGGTCAGGC-3', reverse, 5'-GCTTGGCACCTGTGACCA-TCT-3'; human total MEL1: forward, 5'-AACCTCACCAGAACCCTGAG-3', reverse, 5'-CTTCTCTTGTGTGGATGTTGATGTTG-3'; human MEL1L: forward, 5'-CAAGGAGGAGGAGAGAATG-3', reverse, 5'-CGTTGGGCTCTACATACTACA-3'; human MEL1S: forward, 5'-GTGACACCAGAACC-ACC-3', reverse, 5'-GCTGCGCTAGAAAGACCTG-3'; human PAI-1: forward, 5'-GGCTGACTTACGAGGACGAG-3', reverse, 5'-CCCATGACTTTGAGGATCTCCA-3'; human SKI: forward, 5'-TGCTGCTTCCGCGATTCTCTCA-3'; human SKIL: forward, 5'-CTGAATATGCAGGACAGT-3', reverse, 5'-CCCATGACTTTGAGGATCTCCA-3'; human SKIL: forward, 5'-CTGAATATGCAGGACAGT-3', reverse, 5'-TTCCCGTCTCTGCTAGGTTCTC-3'; human EVII: forward, 5'-AACCTTGGCCACTACTGAG-3', reverse, 5'-TGGCAGACCCTCAGATAAGG-3'; and
FIGURE 1. High expression of SKI and MEL1 in gastric cancer cell lines. A, expression of SKI, EVI1, SKIL, and MEL1 mRNAs in human cancer cell lines determined by quantitative real-time PCR analyses. HaCaT, immortalized normal keratinocytes, and HEK293 (293), transformed embryonal kidney cells, were analyzed for reference. MEL1L and MEL1S transcripts encode long and short forms of MEL1, respectively, and total MEL1 amplifies both long and short forms of MEL1 transcripts. mRNAs expression levels were normalized to HPRT1 expression. Similar results were obtained by normalizing to β-actin expression (data not shown). Error bars represent the S.D.

B, schematic diagrams of MEL1L and MEL1S are shown. PRD indicates PRDI-BF1-RIZ1 homologous (PR) domain; DBD1, DNA-binding domain 1; DBD2, DNA-binding domain 2; AD, acidic domain.

C, schematic diagrams of the SKI and MEL1 genes in human chromosome 1 are shown. The SKI gene is located 800 kbp upstream of the MEL1 gene. D, representative FISH results using BAC clones specific for MEL1 (1p36.32, RP11–22L13), SKI (1p36.32, RP11–181G12), or control MYCL1 (1p34.2, RP1–118J21) on metaphase and interphase chromosomes prepared from the MKN28 cell line. As indicated by the arrowhead, both MEL1 (left and right) and SKI (middle and right) were co-amplified (arrowhead) in this cell line.
human p21: forward, 5′-CGGCAGACCAGCATGACAGA-3′, reverse, 5′-GAAGATCAGCCGGCTTGGTGTG-3′.

Transfection of cDNAs, Immunoprecipitation, and Immunoblotting—Transfection of cDNA was performed using FuGENE6 (Roche Diagnostics, Indianapolis, IN) according to the manufacturer’s recommendations. Cell lysates were prepared in Igepal CA630 lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Igepal CA630, 1 mM phenylmethylsulfonyl fluoride, and 100 units/ml aprotinin). For immunoprecipitation, cell lysates and antibodies were incubated for 2 h to overnight. Immune complexes were then precipitated with Protein G-Sepharose beads (Amersham Biosciences AB, Uppsala, Sweden) or Dynabeads M-280 sheep anti-mouse IgG (Dynal; Invitrogen). SDS-PAGE and immunoblotting were performed as described (23). Horseradish peroxidase-conjugated anti-rabbit IgG (Cell Signaling Technology, Beverly, MA) or horseradish peroxidase-conjugated anti-mouse IgG (Amer sham Biosciences AB) were used as secondary antibodies for ECL detection (Amersham Biosciences AB).

Antibodies—An anti-MEL1 antibody was generated by immunizing a rabbit with human MEL1 (amino acid residues 12–59) fused to glutathione S-transferase. Anti-Smad2 (24) and anti-SKI (25) antibodies were previously described. The following commercially available antibodies used were: mouse anti-FLAG (M2) (Sigma), biotinylated anti-FLAG (M2) (Sigma), anti-α-tubulin (DM1A) (Sigma), rabbit anti-Smad3 (CHEMICON international, Temecula, CA), mouse anti-SKI (G8) (Cascade Bioscience, Winchester, MA), anti-Smad2/3 (BD Bioscience), anti-HDAC1 (Abcam, Cambridge, UK), and anti-myc (9E10) (BD Bioscience), and anti-HA (Invitrogen). Normal mouse IgG1 (MB002) (R&D Systems, Minneapolis, MN) was used as a control.

Luciferase Assay—Cells were transiently transfected with various combinations of promoter-reporter constructs and expression plasmids. Total amounts of transfected DNA were adjusted to the same levels with empty vector. Cell lysates were prepared 24 h later, and luciferase activities were measured by the Dual-Luciferase Reporter System (Promega, Madison, WI) and normalized to pGL4.75-SV40-hRluc. (CAGA)₉-MLP-Luc₂ was derived from (CAGA)₉-MLP-Luc (26) by replacing the plasmid backbone from pGL3 to pGL4.

Fluorescence in Situ Hybridization (FISH)—Metaphase chromosomes were prepared from normal male lymphocytes and the MKN28 cell line. FISH analyses were performed as described previously (27) using BACs located around the region of interest as probes. The copy number and molecular organization of the region of interest were assessed according to the hybridization patterns of both metaphase and interphase chromosomes.

RNA Panel of the Cancer Cell Lines—An RNA panel of cancer cell lines was described previously (28).

Thymidine Incorporation Assay—Cells were seeded at a density of 1.5 × 10⁵ cells/well in 24-well plates and cultured overnight. The serum concentration was then decreased to 1% and
cells were stimulated with TGF-β. Forty-eight hours later, cells were labeled with [3H]thymidine for 4 h. Thymidine incorporation into the trichloroacetic acid-insoluble fraction was analyzed as described previously (23).

Production and Injection of Lentiviral Expression Vectors — The miRNA expression vector was constructed using the BLOCK-iT Pol II miRNAi Expression Vector Kit (Invitrogen) by annealing each oligonucleotide (SKI miR: forward, 5′-TGCTGAGCTTTCTTCTCACGCTGACGTCAGTCCAGGAGAAAGAGCT-3′, reverse, 5′-CTTGAGCTTTCTTCCTGCTGAGCTGTCAGTCAGTGGCCAAAACGTCAGCGAAGAAGAGCT-3′ and MEL1 miR: forward, 5′-TGCTGTATGAAAGGCTTACCGTGCTGTTTTGGCCACTGACACTGACAGCACGGTAGCCTTTCACCGTGCTGTTTTGGCCACTGACACTGACAGCACGGTAGCCAAAACGTCAGCGAAGAAGAGCT-3′; reverse, 5′-CTTGACTGAAAGGCTTACCGTGCTGTTTTGGCCACTGACACTGACAGCACGGTAGCCTTTCATAC-3′) followed by ligation into the pcDNA6.2-GW/EmGFP-miR vector that was pre-linearized and then sequencing. Control miRNA expression vectors were constructed from pcDNA6.2-GW/EmGFP-miR vector by annealing different overhangs (for in vivo experiment) 5′-TATTCAATCCATCTGTTTGAGTTGAGCTGACGTCAGTCCAGGAGAAAGAGCT-3′ and 5′-AAATGCTGAGCTGAGCTGAGCTGACGTCAGTCCAGGAGAAAGAGCT-3′. miRNA coding regions were then transferred to the lentiviral vector CSII-CMV-RfA (29) by the Gateway system. For proliferation-defective lentiviral vectors, 293FT cells were transfected using Lipofectamine 2000 (Invitrogen) with three plasmids: miRNA vector, VSV-G, Rev expressing construct (pCMV-VSV-G-Rev). Culture supernatants were collected 48 h after transfection. For proliferation-defective lentiviral vectors, 293FT cells were transfected using Lipofectamine 2000 (Invitrogen) with three plasmids: miRNA vector, VSV-G, Rev expressing construct (pCMV-VSV-G-Rev) and packaging construct (pCAG-HIVgp) (29). Culture supernatants were collected 48 h after transfection. For infection with the lentiviral vectors, 1 × 10⁵ MKN28 cells/well in 6-well culture plates were infected with lentiviral vectors at 100 plaque-forming units/cell.

In Vivo Tumor Growth Assay — Cell tumorigenicity was examined in 6-week-old female BALB/c nude mice purchased from Charles River Japan (Yokohama, Japan). 6 × 10⁵ MKN28 cells in 100 µl of culture medium and 30 µl of Matrigel (BD Bioscience) were injected subcutaneously into the sides of the stomachs of mice. The mice were sacrificed 3 weeks after injection and tumors were weighed. All animal procedures were performed under specific pathogen-free conditions. Mice were main-

FLAG biotinylated M2, and Alexa Fluor 594-conjugated streptavidin (Invitrogen) were used as secondary antibodies. Cell nuclei were stained with 4',6'-diamidino-2-phenylindole. Cell images were captured with a FV-1000 confocal scanning laser microscope (Olympus, Tokyo, Japan).

DNA Affinity Purification — Proteins were precipitated from transfected cell lysates using a 3 × CAGA probe as described previously (30).

Chromatin Immunoprecipitation (ChIP) Assay — ChIP assay was performed as described previously (31). Briefly, cells were fixed with 1% formaldehyde with gentle shaking for 10 min at room temperature, and cross-linking was stopped by addition of 2.5 mM glycine to a final concentration of 0.125 M glycine. After two washes with cold phosphate-buffered saline, cells were harvested by scraping, pelleted, and suspended in SDS lysis buffer (50 mM Tris-HCl, pH 8.0, 1% SDS, 10 mM EDTA, and protease inhibitors). Samples were sonicated 3 times 15 s each with an interval of 30 s with a UH-50 sonicator (SMT, Japan), and centrifuged at 14,000 rpm at 8 °C for 10 min. After removal of a control aliquot (whole cell extract), supernatants were diluted 10-fold in ChIP dilution buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, and protease inhibitors). Samples were sonicated three times 15 s each with an interval of 30 s with a UH-50 sonicator (SMT, Japan), and centrifuged at 14,000 rpm at 8 °C for 10 min. After removal of a control aliquot (whole cell extract), supernatants were diluted 10-fold in ChIP dilution buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, and protease inhibitors). Samples were immunoprecipitated with Dynabeads M-280 sheep anti–mouse IgG that had been preincubated with 4–8 µg of antibodies in phosphate-buffered saline, 0.5% bovine serum albumin. After five washes by ChIP wash buffer (50 mM Hapes-KOH, pH 7.0, 0.5 mM LiCl, 1 mM EDTA, 0.7% deoxycholate, 1% Igepal CA630) and one wash by TE buffer (pH 8.0), the proteins were eluted from the beads by 0.2 M elution buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS). The DNA samples were extracted with a PCR purification kit (Qiagen) after reversal of cross-linking. The purified DNA was analyzed by quantitative real-time PCR. For

### TABLE 1

Comparison of SKI expression and the histological subtype of gastric cancer tissues

| Histological type | SKI expression level | No. of tumors | p value |
|-------------------|---------------------|---------------|---------|
| Total             |                     |               |         |
| SKI expression level | 2+  | 1+ | 0 |         |
| Differentiated type (Intestinal type) | | | | |
| Papillary adenocarcinoma | 1 | 1 | 0 | 0 |
| W/D tubular adenocarcinoma | 38 | 34 | 4 | 0 |
| M/D tubular adenocarcinoma | 38 | 29 | 9 | 0 |
| Solid-type P/D adenocarcinoma | 5 | 2 | 3 | 0 |
| Undifferentiated type (Gastric type) | | | | |
| Non-solid type P/D adenocarcinoma | 28 | 11 | 17 | 0 |
| Signet-ring cell carcinoma | 10 | 1 | 3 | 6 |
| Total | 38 | 12 | 20 | 6 |

| p value | <0.0001 |

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**RESULTS**

**SKI and MEL1 Are Aberrantly Expressed by Chromosomal Co-amplification of 1p36.32 in MKN28 Gastric Cancer Cells**—To clarify the roles of co-repressors for Smads under pathological conditions, we first examined mRNA expression levels of SKI and other transcriptional co-repressors that inhibit Smad signaling, including SKIL, EVII, and MEL1, in various human cancer cell lines. Interestingly, SKI was highly expressed in gastric cancer cell lines, MKN7, MKN28, and MKN45, and the SKI expression pattern was similar to that of MEL1, but not to that of SKIL or EVII (Fig. 1A).

MEL1 has two variants, a long form (MEL1L) and a short form (MEL1S) (Fig. 1B), and MELIS was originally identified as a transcriptional co-repressor expressed in AML and adult T-cell leukemia (ATL) patients (18, 19). As shown in Fig. 1A, an ATL cell line, ED, displayed high MELIS expression but very low MELIL expression. In contrast, MELIL expression was higher than that of MELIS in various carcinoma cell lines. Interestingly, total MELI expression levels were low in ED cells, and the expression profile of total MELI was similar to that of MELIL. These findings suggest that MELI, particularly MELIL, is highly expressed in some gastric carcinoma cell lines.

Based on information from public databases (ncbi.nlm.nih.gov and genome.ucsc.edu, Fig. 1C), MELI is located in the vicinity of SKI on the short arm of chromosome 1 (1p36.32). Therefore, we performed FISH to determine the mechanism by which SKI and MEL1 were co-amplified in MKN28 cells. As shown in Fig. 1D, MEL1 and SKI were co-amplified in MKN28 cells.

**SKI and MEL1 Are Highly Expressed in Gastric Cancer Tissues**—To further clarify the roles of SKI in gastric cancer, we next examined SKI protein expression in gastric cancer tissues by immunohistochemistry. Representative immunohistochemical staining of SKI in cases of well differentiated tubular

PCR amplification, the following specific primers were used, 5’-ACTTGTCCTAGGAAAATCC-3’ and 5’-GAAGACCGAGAGTGAGTTT-3’ for p21 promoter; 5’-CTGCTTACGGCATTCAATT-3’ and 5’-CCAGCTGTTGATATGTTTC-3’ for Smad7 promoter; 5’-TTGTGGGCTTTTACTAGTTG-3’ and 5’-ATAAAATGACTTAAGCCAGAG-3’ for HPRT1 first intron were used.

**Statistical Analysis**—For luciferase reporter analysis and thymidine incorporation assay, results are expressed as means. Error bars represent the S.D. For the in vivo tumor growth assay, a Student’s t test was used to calculate significant differences in tumor weights between the two groups. For multiple comparisons of the quantitative real-time reverse transcriptase-PCR data, Tukey-Kramer method was used to calculate the significance of the differences.

**FIGURE 3. SKI and MEL1 cooperate to repress TGF-β signaling.** A, knockdown of SKI and/or MEL1 in MKN28 cells. MKN28 cells constitutively expressing miRNA (miR) control/control, miR MEL1/control, miR control/SKI, and miR MEL1/SKI were generated by lentiviral infection. mRNA expression levels for MEL1 (left upper panel) and SKI (left lower panel) were determined by quantitative real-time PCR and normalized to HPRT1 mRNA levels. Error bars represent the S.D. (left panel), MEL1 and SKI proteins levels were determined by immunoblot analysis (right panel). B, the effects of SKI and/or MEL1 knockdown on TGF-β-responsive gene expression. Cells were treated with 1 ng/ml of TGF-β for 4 h. The induction of target genes, PAI-1 (left panel) and p21 (right panel), was examined by quantitative real-time PCR analyses. Error bars represent the S.D, C, the effects of SKI and/or MEL1 knockdown on TGF-β-induced anti-proliferation in MKN28 cells. Cells were treated with 0, 0.1, or 0.3 ng/ml of TGF-β and labeled with [3H]thymidine for 4 h. Each value represents the mean of duplicate determinations. Error bars represent the S.D. D, SKI and MEL1 knockdown reduced tumor growth in vivo. MKN28 cells stably expressing SKI and MEL1 miRNA or control miRNA by lentiviral infection were inoculated into mice (n = 12 for each group), and tumors were weighed 3 weeks later. Data are represented as box plots.

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Figure 4. MEL1 interacts with Smads to repress TGF-β signaling. A, MEL1 and Smad2/3 co-localized in the nucleus. HaCaT cells were transfected with MEL1-FLAG or empty vector (vector), treated with 1 ng/ml of TGF-β for 30 min, and immunostained with anti-FLAG or anti-Smad2/3 antibodies. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). B, interaction between MEL1 and Smads. COS7 cells transfected with or without MEL1-FLAG and 6Myc-Smads were subjected to anti-FLAG immunoprecipitation (IP) followed by anti-Myc immunoblotting (IB). C, interaction between endogenous MEL1 and Smad2/3 was examined in MKN28 cells treated with or without 1 ng/ml of TGF-β for 30 min. Cells were subjected to immunoprecipitation using anti-Smad2/3 or control IgG, followed by immunoblotting with anti-MEL1. D, MEL1 inhibited TGF-β-induced transcriptional activity. A luciferase reporter assay was conducted in R-2 cells using (CAGA)$_n$-MLP-Luc2. Cells were stimulated by co-expression of constitutively active (c.a.) TpR-I. Error bars represent the S.D.

Malignant tumors were classified according to the Lauren classification into intestinal, diffuse, and signet-ring types. Adenocarcinoma (Fig. 2A), moderately differentiated tubular adenocarcinoma (Fig. 2B), poorly differentiated adenocarcinoma (Fig. 2C), and signet-ring cell carcinoma (Fig. 2D) are shown. The SKI immunoreactivity score was 2+ in 78 (65%), 1+ in 36 (30%), and 0 in 6 (5%) gastric cancer cases (Table 1). SKI expression correlated with the histological type: the incidence of score 2+ cancers was 80% (66 of 82) in the differentiated type (or intestinal type) but only 32% (12 of 38) in the undifferentiated type (or gastric type). The incidence of high SKI expression was significantly higher in differentiated than in undifferentiated cases ($p < 0.0001$). In particular, well differentiated tubular adenocarcinoma showed a high incidence (89%, 34 of 38) of score 2+. In contrast, score 2+ was detected in only one (10%) of 10 signet-ring cell carcinoma cases.

We next examined mRNA expression of SKI and MEL1 in tumor tissues. As shown in Figs. 2, E and F, in three of six gastric cancer patient samples, both MEL1 and SKI were highly expressed in tumor tissues compared with normal tissues.

**SKI and MEL1 Cooperatively Repress TGF-β Signaling in Gastric Cancer Cells**—To determine the contribution of SKI and/or MEL1 in gastric cancer progression, we investigated the effect of SKI and/or MEL1 loss-of-function on TGF-β responsiveness. We generated MKN28 cells in which endogenous SKI and/or MEL1 were knocked down by an miRNA system. These cells exhibited at least a 60% reduction in SKI and MEL1 mRNA levels (Fig. 3A, left panel), and SKI and MEL1 protein levels were greatly decreased in these cells (Fig. 3A, right panel). Knockdown of SKI and MEL1 expression by miRNA did not affect phosphorylation and nuclear accumulation of Smad2/3 (data not shown). Interestingly, although SKI or MEL1 knockdown only weakly enhanced PAI-1 (Fig. 3B, left panel) and p21 (Fig. 3B, right panel) expression induced by TGF-β, knockdown of both SKI and MEL1 synergistically enhanced their expression. Similar results were obtained using another gastric cancer cell, MKN7 cells (supplementary Fig. S1). We further examined the effect of SKI and MEL1 on the anti-proliferative responses to TGF-β. As shown in Fig. 3C, knock-
**MEL1 Interacts with Smad2/3 and Represses TGF-β Signaling**—Next, to elucidate the mechanisms by which MEL1 represses TGF-β-Smad signaling, we examined the intracellular localization of MEL1 using immunofluorescence labeling. As shown in Fig. 4A, transfected MEL1 localized to the nucleus with or without TGF-β stimulation. Moreover, the nuclear colocalization of MEL1 and Smad2/3 depended on TGF-β stimulation, because Smad2/3 accumulated in the nucleus upon TGF-β stimulation.

We then studied whether MEL1 physically interacts with Smads. Fig. 4B shows that MEL1 interacted with Smad2 and Smad3 but not with Smad4. The endogenous interaction of MEL1 with Smad2/3 was also confirmed in a TGF-β-dependent fashion in MKN28 cells (Fig. 4C).

Because other transcriptional corepressors that inhibit Smad signaling, such as SKI and EVI1, recruit the HDAC complex to Smads to inhibit TGF-β signaling, we next examined the interaction between MEL1 and HDAC1. As shown in supplementary Fig. S2A, MEL1 interacted with HDAC1 in transfected COS7 cells. Moreover, MEL1 enhanced the interaction between Smad3 and HDAC1 (supplementary Fig. S2B).

We then investigated the interaction between MEL1 and Smad3 in more detail using a glutathione S-transferase pull-down assay. Smad3 bound MEL1 through its C-terminal Mad homology 2 domain (20) (supplementary Fig. S3). Binding of Smad3 to MEL1-(1339–3774) and MEL1-(2224–3774) was weaker than to MEL1 (Full), and Smad3 did not bind to MEL1-(2845–3774) (supplementary Fig. S4A). C-terminal MEL1 deletion mutants, MEL1-(1–2214) and MEL1-(1–1680), bound more weakly to Smad3 than MEL1 (Full) and MEL1-(1–2850) (supplementary Fig. S4B). These results suggest that MEL1 interacts with Smad3 through DNA-binding domain 1 and the repressor domain (RD). Of note, EVI1 has been reported to bind Smad3 through DNA-binding domain 1 (15).

We next examined the effects of MEL1 on TGF-β-Smad-induced transcriptional activity. In a TGF-β-responsive (CAGA)₉-MLP-Luc reporter assay, MEL1 (MEL1L) repressed the transcriptional activity induced by a constitutively active TGF-β type I receptor (c.a.TBR-I) in a dose-dependent manner (Fig. 4D). Similar results were obtained using MEL1S or two other TGF-β-responsive reporters, p3TP-Luc and ARE-Luc (data not shown).

**MEL1 Represses TGF-β Signaling Mainly by Enhancing the SKI-Smad Interaction and the Binding of Inactive Smad3 Complexes to Target Gene Promoters**—To elucidate the mechanism by which MEL1 reduces TGF-β signaling with SKI, we examined whether MEL1 physically interacts with SKI in a co-immunoprecipitation assay. In transfected COS7 cells, MEL1-FLAG interacted with 6Myc-SKI (Fig. 5A, left panel). Endogenous MEL1 also interacted with SKI in a ligand-independent fashion.
in MKN28 cells (Fig. 5A, right panel). To further address how MEL1 represses TGF-β signaling by binding to Smad3 and SKI, we examined the effect of MEL1 on SKI and Smad3 binding. As shown in Fig. 5, B and C, MEL1 enhanced SKI and Smad3 binding, and SKI enhanced MEL1 and Smad3 binding. In addition, knockdown of SKI reduced the interaction of MEL1 with Smad2/3 in MKN28 cells (supplementary Fig. S5).

We previously reported that SKI enhances the binding of Smad complexes to DNA, and that the stabilization of inactive Smad complexes on DNA is a critical event in SKI-mediated inhibition of TGF-β signaling (12). We thus investigated how MEL1 and SKI cooperatively affected binding of Smad3 to the Smad binding element. As shown in Fig. 5D, MEL1 and SKI dramatically enhanced the binding of Smad3 to the Smad binding element judged by DNA pull down assay (Fig. 5D).

For further investigation, the association of Smad3-HDAC1 complexes with the promoter regions of TGF-β target genes was examined by ChIP assay. The recruitment of Smad2/3 to the Smad binding element in the p21 and Smad7 promoters was increased in response to TGF-β, whereas double knockdown of MEL1 and SKI resulted in reduced Smad2/3 binding to these promoters (Fig. 6A). On the other hand, HDAC1 recruitment to the p21 and Smad7 promoters was hardly affected upon TGF-β stimulation in control small interfering RNA-transfected MKN28 cells (Fig. 6B), suggesting that the inactive Smad3 complexes including HDAC1 occupied TGF-β target gene promoters in the absence or presence of TGF-β signals. However, double knockdown of MEL1 and SKI resulted in the dissociation of HDAC1 from the p21 and Smad7 promoters in unstimulated cells. Moreover, the release of HDAC1 from these promoters was induced by TGF-β stimulation in double knockdown cells.

These results suggest that MEL1 and SKI cooperatively inhibit TGF-β signaling by stabilizing the inactive Smad3-HDAC1 complexes on the promoter of TGF-β target genes.

We then defined the SKI-interacting regions in MEL1. As shown in Fig. 7A, SKI bound MEL1 (Full) but not MEL1-(1–2214), indicating that MEL1 interacts with SKI through the C-terminal domain containing RD and DBD2. Moreover, although MEL1-(1–2214) could bind to HDAC1 (data not shown), MEL1 (Full) but not MEL1-(1–2214), enhanced the binding of HDAC1 to Smad3 (Fig. 7B). We finally identified the MEL1 domain that represses TGF-β signaling. Interestingly, a C-terminal truncation of MEL1 attenuated the inhibitory effect (Fig. 7C), suggesting that binding to SKI may be important for MEL1 to repress TGF-β signaling (Fig. 7D).

**DISCUSSION**

**TGF-β Signaling and Its Alternations in Gastric Cancer**—It is widely recognized that TGF-β acts as a tumor suppressor through its cytostatic and pro-apoptotic functions on the epithelium, and many reports have demonstrated alterations in the TGF-β signaling pathway in various cancers (32, 33). Although several reports suggest that TGF-β signaling is involved in the pathogenesis of gastric cancer (34–36), the molecular mechanisms are still unclear.

We report that the transcriptional co-repressors SKI and MEL1 are highly expressed in gastric cancer cells and define their roles in repressing TGF-β signaling. Increased levels of SKI or its family SKIL have been detected in many human tumor cell lines derived from neuroblastomas, melanomas, breast cancers, chorions, thyroid cancers, and epidermoid tumors (3, 37, 38). High SKI or SKIL expression levels have also been found in human tumor tissues from melanoma, breast cancer, esophageal cancer, lung cancer, and carcinomas of the vulva (3, 38–41), but to our knowledge, this is the first evidence that SKI is overexpressed and involved in gastric cancer. Moreover, there are limited reports on aberrant MEL1 expression in cancer, including some reports on aberrant MEL1 expression in leukemia and a report of MEL1 genomic amplification in osteosarcoma tissues (42). Therefore, we have identified new key molecules that regulate TGF-β signaling in gastric cancer.

**Mechanisms of MEL1 in the Regulation of TGF-β Signaling**—Because MEL1 and MDS1/EVI1 share structural similarity, it is predicted that these molecules will have similar functions in leukemogenesis and TGF-β signaling. Indeed, MEL1S, which lacks the PR domain, has been reported to be expressed in AML cells. The MEL1S form inhibited myeloid cell differentiation, and thus, like EVI1, exhibited oncogenic functions. Similarly, forced expression of MEL1S in ATL cells reduced TGF-β responsiveness (19). In this paper, we have further investigated the molecular mechanisms of MEL1 in TGF-β signaling. In contrast to ATL cells, MEL1L expression was higher than MEL1S in various carcinoma cell lines, particularly gastric cancer cell lines. Moreover, both MEL1S and MEL1L reduced transcriptional activity induced by TGF-β in several cells (data not

**Figure 6. Double knockdown of SKI and MEL1 decreases the recruitment of Smad2/3 and HDAC1 to TGF-β target gene promoters.** A and B, MKN28 cells were transfected with the indicated small interfering RNAs for 48 h, and incubated with 1 ng/ml TGF-β for 1.5 h. Cells were fixed with 1% formaldehyde, and the cell lysates were immunoprecipitated with the indicated antibodies. DNA fragments eluted from immunoprecipitates were quantified by quantitative real-time PCR. Data were normalized by input DNA.
SKI and MEL1 Cooperate to Inhibit TGF-β Signal

Mechanisms by which SKI and MEL1 cooperatively repress TGF-β signaling. A, the binding of MEL1 to SKI via the C-terminal domain of MEL1. COS7 cells were transfected with or without 6Myc-SKI, FLAG-MEL1, and FLAG-MEL1-(1–2214). The cell lysates were subjected to FLAG immunoprecipitation (IP) followed by Myc immunoblotting (IB). The top panel shows the interaction and the lower two panels show the expression of each protein as indicated. B, MEL1, but not MEL1-(1–2214), enhanced the interaction of Smad3 with HDAC1. COS7 cells were transfected with or without 6Myc-Smad3, 6Myc-MEL1, 6Myc-MEL1-(1–2214), and FLAG-HDAC1. Cells were treated with 1 ng/ml TGF-β for 30 min. The cell lysates were then subjected to FLAG immunoprecipitation followed by Myc immunoblotting. The top panel shows the interaction and the lower three panels show the expression of each protein as indicated. C, MEL1-(1–2214) failed to inhibit TGF-β-induced transcription. A luciferase reporter assay was conducted in R4-2 cells using (CAGA)₉-MLP-Luc₂. Cells were stimulated by co-expression of constitutively active (c.a.) TGFRI. Error bars represent the S.D. D, schematic diagrams of MEL1(Full) and MEL1-(1–2214). The results of the binding assay and luciferase assay are summarized at the right.

The results shown suggest a functional difference between the two spliced forms in ATL cells and gastric cancer cell lines. Transcriptional co-repressors, including SKI, SKIL, and EVI1, inhibit TGF-β signaling through multiple mechanisms. Like other transcriptional co-repressors, MEL1 interacted with and recruited HDAC1 to Smad3. Moreover, MEL1 repressed TGF-β signaling through its C-terminal domain, including the RD and DBD2. Interestingly, a MEL1 mutant lacking the C-terminal domain still bound to Smad3 as well as HDAC1. In addition, MEL1 interacted with SKI through the C-terminal domain to enhance SKI binding to Smad3, and stabilize the inactivated Smad3 complex on target gene promoters. These results suggest that SKI binding may be important for MEL1 to repress TGF-β signaling. Of note, MEL1 did not stabilize SKI protein (data not shown) or Smad2/3 localization.

RUNX3 is a tumor suppressor gene that is frequently silenced in gastric cancer by hypermethylation of CpG islands in the exon 1 region. The silenced gene is reactivated by a combination of 5′-aza-2′-deoxycytidine, a DNA methyltransferase inhibitor, and trichostatin A, a histone deacetylase inhibitor, in gastric cancer cell lines, including MKN28 cells (43). Because SKI and MEL1 act as co-repressors of the TGF-β-Smad pathway together with HDAC1, we determined the contribution of SKI and MEL1 to RUNX3 silencing in MKN28 cells. However, SKI and MEL1 knockdown did not affect RUNX3 expression in MKN28 cells (data not shown), suggesting that they are not involved in regulating RUNX3 expression in gastric carcinoma.

Co-amplification of SKI and MEL1—In the present study, we demonstrated that SKI and MEL1 were aberrantly expressed by chromosomal co-amplification of 1p36.32 in MKN28 gastric cancer cells. Moreover, 21% of gastric cancers had a gain in copy number at 1p36 (44), suggesting that SKI and MEL1 are co-amplified in gastric cancer cell lines as well as in gastric cancer tissues. It is interesting to note that these two oncogenic genes are embedded within the same focal amplification, and cooperatively inhibit TGF-β signaling in gastric cancer cells. Such cooperative factors in the same amplicon are also observed in recent studies of ZNF217 and PFDN4 (prefoldin 4) in the 20q13.2 breast cancer amplicon (45) and BIRC1 and YAP1 (cIAP) in the 11q22 liver cancer amplicon (46), although the functional correlation between these molecules remains unclear. Moreover, amplification of the 3q26 region has been reported to occur frequently among esophageal squamous cell carcinomas (40). Indeed, SKIL and EVI1 are involved in this region and co-amplified, suggesting that these two molecules may function in a cooperative relationship similar to the SKI-MEL1 interaction in gastric cancers. The present study proposed the necessity to re-examine the well characterized amplification events for the search of novel mechanisms of co-amplified oncogenic factors and potential drug targets.

In conclusion, we have demonstrated that SKI and MEL1 were aberrantly expressed in gastric cancer cells by chromosomal co-amplification. SKI and MEL1 knockdown synergistically restored TGF-β responsiveness in two gastric cancer cell lines, MKN7 and MKN28 cells. Moreover, knockdown of SKI and MEL1 in MKN28 cells reduced tumor growth in vivo. Biochemical analysis demonstrated that MEL1 interacts with SKI and inhibits TGF-β signaling by stabilizing the inactive Smad3-SKI complex on the promoter of TGF-β target genes. These
findings reveal a novel mechanism where distinct transcriptional co-repressors are co-amplified and functionally interact, and provide molecular targets for gastric cancer treatment.

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