

Research Article

Inhibition of Histone Deacetylase Attenuates Hypoxia-Induced Migration and Invasion of Cancer Cells via the Restoration of RECK Expression

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Abstract

Hypoxia is a strong signal for cell migration and invasion in cancer. The reversion-inducing cysteine-rich protein with Kazal motif (RECK), a tumor suppressor, inhibits cancer cell migration and invasion and is frequently silenced in aggressive tumor cells by histone deacetylases (HDAC). However, the effect of RECK silencing in several cancer cells in a hypoxic microenvironment has not been fully delineated. In this report, we investigated whether hypoxia suppressed *RECK* expression and used HDAC inhibitor (HDACI) inhibition to restore *RECK* expression to inhibit cancer cell migration and invasion. HDACIs, including trichostatin A (TSA), completely rescued *RECK* expression, which was suppressed by hypoxia, in the H-Ras-transformed human breast MCF10A and the HT1080 cell lines (human fibrosarcoma). TSA suppressed the activity of matrix metalloproteinase-2 (MMP-2) and MMP-9, induced by hypoxia, and significantly inhibited hypoxia-stimulated migration and invasion of both cancer cells. *RECK* overexpression significantly inhibited the migration and invasion of cancer cells induced by hypoxia. The hypoxic effect on the migration and invasion of cells was equivalent to the effect seen using the small interfering RNA (siRNA) of *RECK* under normoxia, suggesting an inhibitory role for *RECK* in hypoxic conditions. We also showed that siRNA silencing of HDAC1 suppressed hypoxia-induced *RECK* downregulation and inhibited the migration and invasion of cancer cells. In conclusion, the inhibition of HDAC successfully restored the expression of *RECK* under hypoxic conditions. This resulted in the inhibition of cancer cell migration and invasion through the repression of MMP-2 and MMP-9 activity. *Mol Cancer Ther*; 9(5): 1361–70. ©2010 AACR.

Introduction

The reversion-inducing cysteine-rich protein with Kazal motifs (RECK) protein is the only known membrane-bound metalloproteinase (MMP) inhibitor (1). RECK is a 110-kDa glycoprotein that is expressed in many normal tissues, but it is absent in transformed and tumor-derived cells. RECK-transfected HT1080 fibrosarcoma cells produced only low levels of pro-MMP-9 in the culture medium. Previous work has found that purified RECK specifically binds to and inhibits MMP-9 (1). Additionally, RECK is a negative regulator of MMP-2 and MT1-MMP and decreases angiogenesis and tumor growth *in vivo*.

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The discovery of RECK and its ability to inhibit MMPs has implications for the treatment of cancer. Whereas the mechanism underlying the downregulation of RECK in many tumors seems to be multifactorial and tumor specific, a common target of inhibition is the Sp1 site at the *RECK* promoter. Sasahara et al. (2, 3) have speculated that by activating the extracellular signal-regulated kinase pathway, oncogenic Ras facilitates the phosphorylation or other modification of the Sp1/Sp3 factors. This modification increases their affinity for the Sp1 site at the *RECK* promoter, thereby reducing RECK expression. They also hypothesized that the interaction between the histone deacetylases (HDAC) and Sp1 may contribute to the transcriptional repression of *RECK* (2). However, although they used an inhibitor of HDACs, trichostatin A (TSA), in NIH3T3 cells, they did not examine the effect of the HDAC inhibitor (HDACI) on RECK expression. HER-2/neu also recruits HDACs to Sp1 proteins to repress the expression of *RECK* by binding to the promoter region (4).

HDACIs are currently in clinical trials, partly due to their potent antiangiogenesis effects. HDACIs shift the balance of proapoptotic and antiapoptotic genes toward apoptosis, induce ROS generations, and inhibit angiogenesis, all of which contribute to their anticancer

activity (5, 6). These inhibitors have been shown to stimulate the expression of growth inhibitory genes, such as *p21Waf1* and *p27Kip1*, that inhibit the proliferation of cancer cells (7). Additionally, these inhibitors also reduced the expression of antiapoptotic genes, such as *bcl-2* and *bcl-xL* (8, 9). Recent studies have shown that HDACs have antimetastatic and antiangiogenic effects *in vitro* and *in vivo* (10, 11).

Hypoxia is a hallmark of several diverse human malignancies, including breast cancer, head and neck cancer, prostate cancer, pancreatic cancer, brain tumors, and malignant melanoma (12). Hypoxia silences tumor suppressors, such as *MLH1* (13), *RUNX3* (14), and *pVHL* (10), by epigenetic mechanisms, such as histone modification. Hypoxia activates genetic programs that facilitate cellular adaptations (e.g., changes in cell signaling and gene expression) and promotes cell survival, cell migration, invasion, metastasis, and resistance to chemotherapy (15). The activation of hypoxia-inducible factor (HIF)-upregulated proteins, such as lysyl oxidase (16, 17) and MMPs, has been implicated in matrix remodeling, as these proteins disrupt cell-cell and cell-extracellular matrix interactions (18). However, it is noteworthy that HDACs suppress HIF-1 α activity under hypoxic conditions, suggesting that HIF activity may be under the control of HDAC activity (19, 20).

In this report, we showed for the first time that the silencing of RECK expression is induced by hypoxia in cancer cells. In addition, we showed restoration of hypoxia-reduced RECK expression by HDACs, TSA, sodium butyrate (NaB), and suberoylanilide bishydroxamine (SAHA), as well as by small interfering RNA (siRNA) knockdown of HDACs. This restoration of RECK expression potentially inhibited hypoxia-induced cancer cell migration and invasion through the inhibition of MMP-2 and MMP-9 activity.

Materials and Methods

Cell culture and transfection. The human breast epithelial cell line (H-Ras MCF10A) was kindly provided by Dr. A.R. Moon (Duk-sung University), and the human fibrosarcoma cell line (HT1080) was obtained from the American Type Culture Collection. H-Ras MCF10A cells were cultured in DMEM/F12 supplemented with 5% fetal bovine serum (FBS), 0.5 μ L/mL hydrocortisone, 10 μ L/mL insulin, 20 ng/mL epidermal growth factor (R&D Systems), 0.1 μ L/mL cholera toxin (Sigma), 100 units/mL penicillin-streptomycin, 2 mmol/L L-glutamine, and 0.5 μ L/mL fungizone (Invitrogen). HT1080 cells were maintained in DMEM supplemented with 10% FBS and 1 \times antibiotics. The transfection of plasmid DNA was done using the HiPerFect Reagent (Qiagen), Lipofectamine 2000 (Invitrogen), or WefFect-EX reagent (WefGENE) according to the manufacturer's instructions.

Hypoxia treatment. Cells were placed in an incubator (Astec) calibrated to maintain a hypoxic atmosphere of 1% O₂, 5% CO₂, and 94% N₂ by continuous flow of nitrogen (Thermo Scientific). A standard tissue culture incubator

was used for a normoxic environment (Thermo Scientific; 21% O₂, 5% CO₂).

In vitro cell migration and invasion assays. The migration and invasion assays were done in transwell chambers (Costar). The lower surface of an 8- μ m pore membrane was coated with collagen type I for the migration assay, whereas, for the cell invasion assay, the upper surface of the membrane was coated with Matrigel (BD Biosciences). The transwell was placed in a well with DMEM/F12 containing 5% FBS for the H-Ras MCF10A cells or DMEM containing 10% FBS for the HT1080 cells. The cells were seeded into the upper chamber in serum-free media in the presence or absence of TSA or MMP inhibitor (MMPI; Merck Biosciences). After incubation under normoxia or hypoxia for 24 hours, the cells were fixed with formaldehyde and removed from the top surface of the chamber. The cells on the lower surface of the filter were stained with H&E (Sigma). The cells that migrated and invaded to the lower side of the filter were observed using a light microscope and counted.

Gelatin zymography. After the cells were cultured in serum-free medium for 24 hours, the conditioned medium was collected and centrifuged at 3,000 rpm for 10 minutes to remove the cell debris. The conditioned medium was electrophoresed in 10% SDS-PAGE gels containing 1 mg/mL gelatin under nonreducing condition. After electrophoresis, the gels were incubated overnight in the zymography reaction buffer [50 mmol/L Tris (pH 7.8), 10 mmol/L CaCl₂, 150 mmol/L NaCl, 2 μ mol/L ZnCl₂] at 37°C. The gels were stained with a 0.5% Coomassie Brilliant Blue F-250 solution containing 10% acetic acid and 20% methanol and destained with a 10% acetic acid solution. The areas of gelatinase activity were detected as a clear zone against the blue-stained gelatin background.

RNA extraction and reverse transcription-PCR. Total RNA was isolated from cells after exposure of normoxic and hypoxic conditions using a single-step procedure with TRIzol Reagent (Invitrogen). RECK expression was examined by reverse transcription-PCR (RT-PCR). β -Actin was used as an internal control. The cDNA synthesis was done at 72°C for 5 minutes and then at 42°C for 1 hour using MMLV-RT (Promega). The PCR conditions were 25 to 32 cycles of denaturation (94°C for 1 minute), annealing (60°C for 1 minute), and extension (72°C for 1 minute), followed by a final extension (72°C for 10 minutes). The primers used were as follows: RECK, forward 5'-CCTCAGTGAGCACAGTTCAGA-3' and reverse 5'-GCAGCACACACTGCTG-3'; HDAC1, forward 5'-CTGCTTAGTAGCTTTGGA-3' and reverse 5'-TATCTCAAAAAGGAAACT-3'; and β -actin, forward 5'-GACTACCTCATGAAGATC-3' and reverse 5'-CTGCTGGAATACTGTAAGT-3'. The PCR products were separated in a 1.5% agarose gel in 0.5 \times Tris-borate EDTA buffer, stained with ethidium bromide, and visualized under UV light.

Real-time RT-PCR. Real-time RT-PCR was done in a 7500 Real-Time PCR System (Applied Biosystems) with

SYBR premix Ex Taq (Takara). The PCR program consisted of an initial denaturation step at 95°C for 10 seconds, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The expression level of each specific gene was normalized against the expression of glyceraldehyde-3-phosphate dehydrogenase in the same master reaction. The primers used were as follows: for *RECK*, CCATCTGGAGATCCCTGTCT and GCACCTGGATTA-GTGCCCT; for *glyceraldehyde-3-phosphate dehydrogenase*, GCACCGTCAAGGCTGAGAAC and ACTGGCGTCTT-CACCACCAT. Each experiment was done in triplicate, and the average was calculated.

Whole-cell extract preparation and Western blot. The cells that were treated with or without chemicals under hypoxic or normoxic conditions were harvested in a lysis buffer (iNtRon Biotech). An equal amount of proteins was subjected to SDS-PAGE and transferred to nitrocellulose membrane. After the membrane was blocked with 5% nonfat skim milk in TBS containing 0.1% Tween 20,

it was incubated with the mouse anti-human RECK (R&D Systems, 1:500), mouse anti-human β -actin (Santa Cruz Biotechnology), and rabbit anti-human HDAC1 (Cell Signaling, 1: 2,000) antibodies. The membrane was then incubated with horseradish peroxidase-conjugated antimouse or antirabbit antibodies (1: 10,000) and was then developed using the SuperSignal West Pico Chemiluminescent Substrate (Pierce).

Immunofluorescence assay. After the cells were fixed with 4% paraformaldehyde, immunofluorescence was done as previously described (14). The slides were examined under fluorescence microscopy at 400 \times magnification (Carl Zeiss).

siRNA experiment. The H-Ras MCF 10A cells and HT1080 cells were transfected with siRNAs against HDAC1 or RECK using the HiPerFect transfection reagent (Qiagen). Total RNA was isolated 48 hours after transfection, and RT-PCR was done as described above. When we checked the expression levels of the genes we

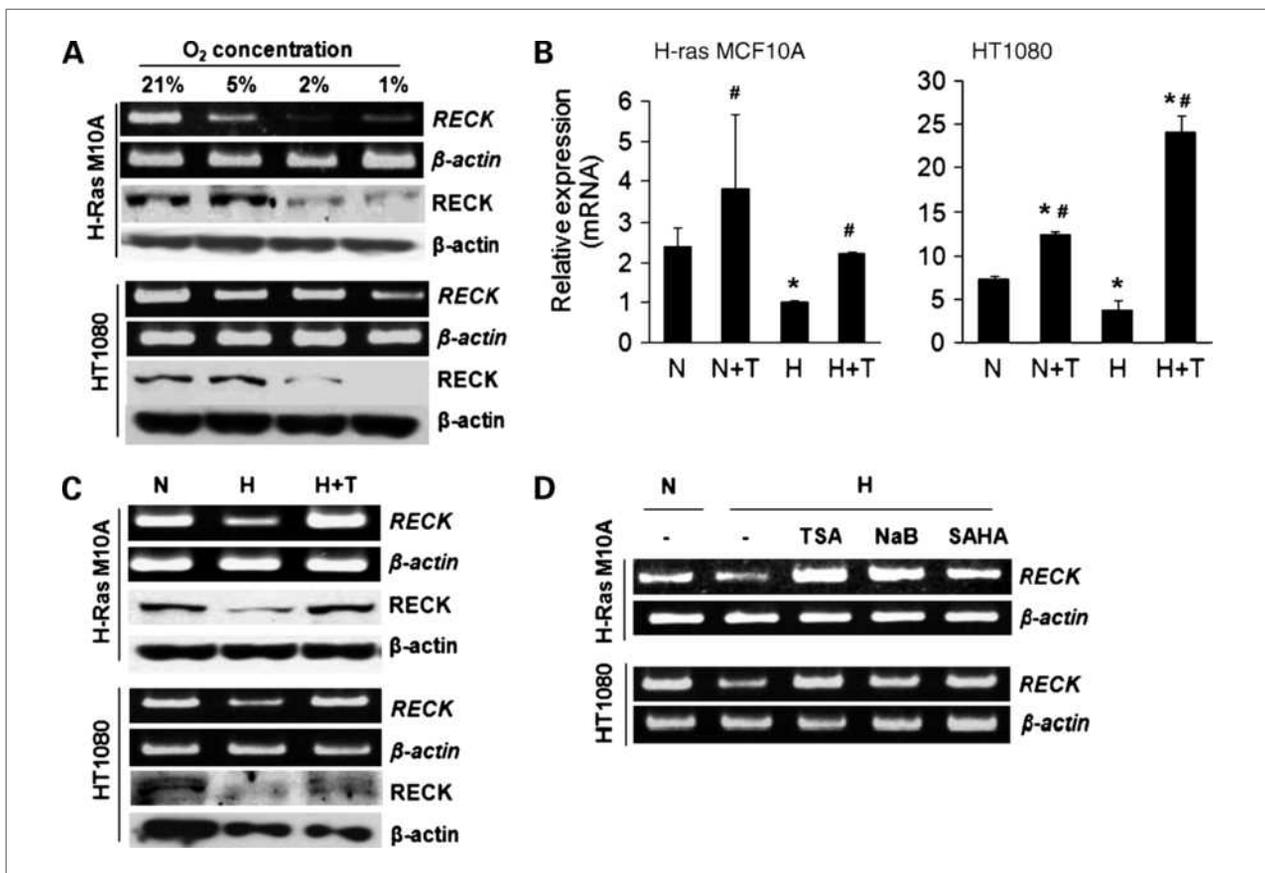


Figure 1. RECK downregulation by hypoxia and its restoration by HDACs. A, H-Ras MCF10A and HT1080 cells were incubated in different oxygen concentration conditions for 24 h. Total RNA and proteins were isolated, and the RECK expression was investigated using RT-PCR and Western blot, respectively. β -Actin was used as an internal control. B, real-time RT-PCR was done after H-Ras MCF10A and HT1080 cells were treated with TSA (330 nmol/L) for 24 h under normoxic or hypoxic conditions. The relative expression of RECK was determined in triplicates, normalized to β -actin expression, and presented. *, $P < 0.001$, significantly different from normoxia; #, $P < 0.001$, significantly different from hypoxia. C, after treatment with an HDACi, TSA (330 nmol/L), for 24 h under hypoxic conditions, RECK expression was examined in H-Ras MCF10A and HT1080 cells using semiquantitative RT-PCR and Western blot analysis. D, after treatment with TSA (330 nmol/L), NaB (1 μ mol/L), and SAHA (1 μ mol/L) under hypoxic condition, RECK expression in both cell lines was examined using semiquantitative RT-PCR.

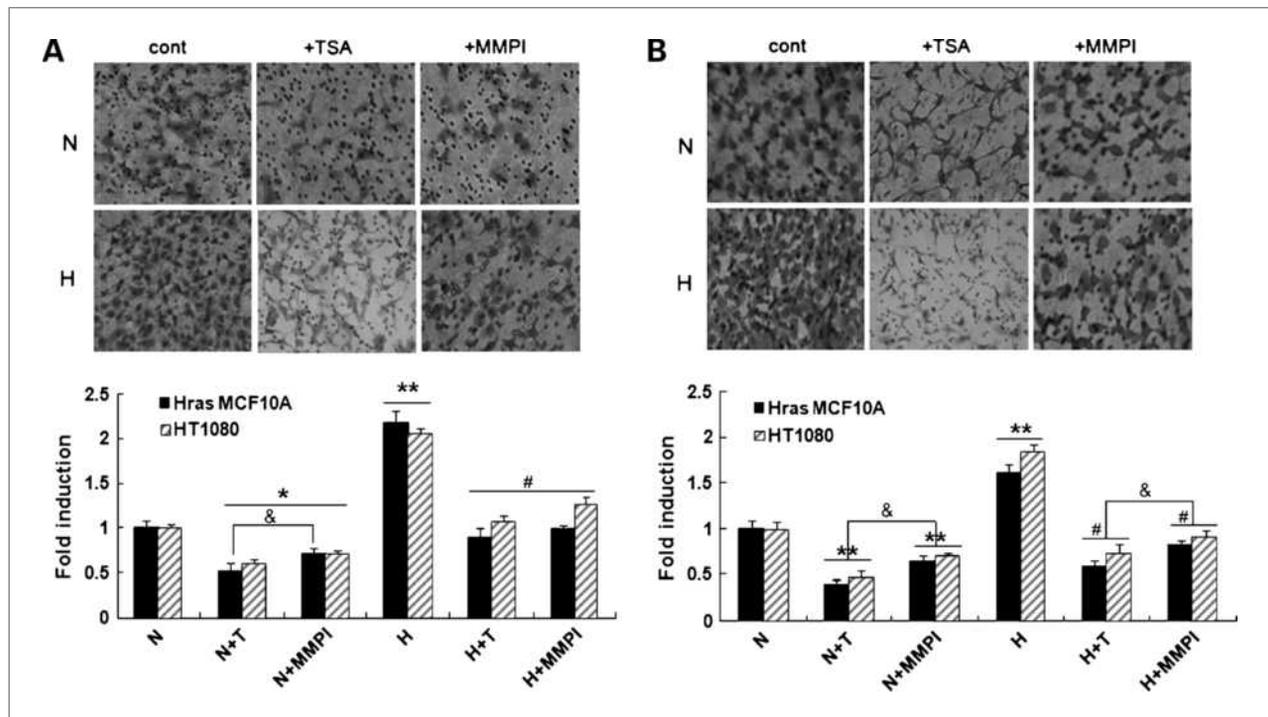


Figure 2. TSA and a MMPI inhibit hypoxia-induced migration and invasion. A, cells were seeded into the upper chamber of a transwell and incubated for 24 h under normoxia (N) or hypoxia (H) conditions. The bottom surface of the filters was stained with H&E, and the cells that migrated were counted. B, similarly, the invasion of H-Ras MCF10A and HT1080 cells through a Matrigel using a transwell was examined. TSA (330 nmol/L) or MMPI (20 μ mol/L) was added to both sides of the transwell membrane for 24 h. Images of HT1080 cells are shown, and those for H-Ras MCF10A cells are in Supplementary Fig. S1. The migrated/invaded cells were counted and are graphed below. *, $P < 0.01$; **, $P < 0.0001$, versus normoxia; #, $P < 0.0001$, versus hypoxia; &, $P < 0.01$, between two groups.

silenced, genes were decreased from 12 hours and peaked at 24 to 48 hours after transfection (data not shown).

The scrambled control siRNA sequence was 5'-CU-GAUGACCUGAGUGAAUGdTdT-3', the HDAC1 siRNA sequence was designed to target 5'-AACCCGGAG-GAAAGTCTGTTA-3', and the RECK siRNA sequence was designed to target 5'-CAGATTGAAGCCTGCAATAAA-3' (Bioneer).

Statistical analysis. The ANOVA test was used to assess the significance of the differences among the experimental groups. The results are represented as means \pm SD.

Results

RECK expression is suppressed by low oxygen tension in cancer cells and restored by HDACIs. To examine whether hypoxic conditions could influence the expression of RECK, we cultured two types of cancer cells under different oxygen concentrations (20%, 5%, 2%, and 1%) for 24 hours. Because, H-Ras MCF10A and HT1080 cells are RECK-expressing cells and have similar migratory characteristics but represent different types of tumorigenic cells, we selected these two cell lines for further experiments. In H-Ras transformed MCF10A

human breast epithelial cells and HT1080 human fibrosarcoma cells, RECK expression was downregulated in an oxygen concentration-dependent manner (Fig. 1A). To evaluate the potential contribution of HDACs in the hypoxia-induced inhibition of RECK expression, we treated the cells with an HDACI (TSA) for 24 hours. Treatment with TSA under hypoxic conditions restored RECK expression at the mRNA level, as determined by real-time quantitative RT-PCR in both cell lines (Fig. 1B). In normoxic conditions, the RECK expression was restored by the treatment with TSA in HT1080 cells, but not in the H-Ras MCF10A cells (Fig. 1B). Normoxic restoration of RECK by TSA in HT1080 cells might explain the relative low expression of RECK and that HDAC activity plays a role in regulating RECK expression under normoxic conditions. We therefore continued the experiment without TSA treatment under normoxic conditions. To determine whether the hypoxia-induced inhibition of RECK expression was at the transcriptional level, we transfected a construct encoding luciferase under the control of the RECK promoter. RECK promoter activity was decreased in hypoxic conditions and restored by TSA treatment in a similar pattern to the real-time PCR analysis (data not shown). Furthermore, we confirmed that the RECK protein level was recovered by the treatment of TSA under 1% O₂ conditions (Fig. 1C). To investigate

whether other HDACIs also restored *RECK* expression under hypoxic conditions, we treated both cell lines with SAHA and NaB. We found that *RECK* expression was restored by SAHA and NaB to a level similar to that induced by TSA (Fig. 1D). These data indicate that *RECK* suppression is strongly induced by hypoxia and that inhibition of HDAC activity can reverse this suppression.

TSA inhibits hypoxia-induced cancer cell migration and invasion. To examine whether the inhibition of HDAC activity and MMP activity affects hypoxia-induced cancer cell migration and invasion, H-Ras MCF10A and HT1080 cells were treated with TSA, and their migration and invasiveness were measured with a transwell assay. Cells were allowed to migrate under normoxic or hypoxic conditions as well as hypoxic conditions in the presence of TSA or an MMPi for 24 hours. The hypoxic culture conditions significantly increased the cell migrations to around 2-fold compared with the normoxic controls. However, TSA treatment reduced the hypoxia-induced migration to the level of the normoxic control in both cell lines (Fig. 2A). The treatment of MMPi also significantly reduced the hypoxia-induced migration, and the degree of inhibition did not differ greatly from that of the TSA

treatment. Furthermore, the hypoxia-induced cancer cell invasion (1.6-fold to 1.9-fold compared with normoxic control) was suppressed by TSA to levels lower than that in the normoxic control (Fig. 2B). However, the effect of MMPi was less than that of TSA, indicating that the effect of HDAC inhibition on cancer cell invasion was more global than MMP inhibition. These findings suggest that the inhibition of HDAC activity by TSA under hypoxic conditions could block cancer cell migration and invasion. In addition, cell migration and invasion by MMP inhibition were comparable with that of HDAC inhibition via the restoration of *RECK* function.

TSA inhibits MMP expression under hypoxic conditions. The increased expression of MMPs, particularly MMP-2 (gelatinase A; 72-kDa type IV collagenase) and MMP-9 (gelatinase B; 95-kDa type IV collagenase), has been associated with tumor malignancy and poor survival in patients (21). The main functions of *RECK* in tumor cells are to inhibit the secretion of MMP-9 and MMP-2 from the cell membrane and to inhibit the conversion of MMP-2 into active MMP-2. Therefore, to determine if the restoration of *RECK* expression by an HDACi influences the activity of MMPs, we did gelatin zymography.

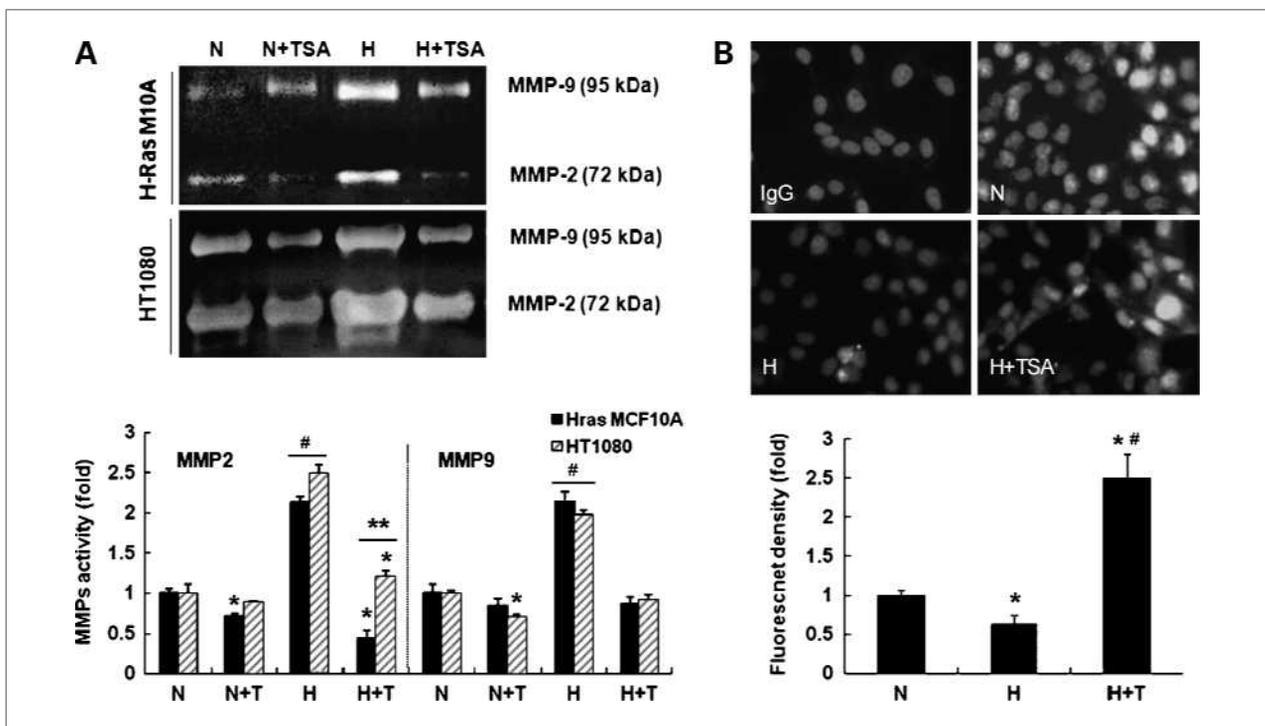


Figure 3. Hypoxia-induced MMP activity was suppressed by an HDACi. A, a gelatin zymography assay was done to analyze the gelatinolytic activity of secreted MMP-2 (72 kDa) and MMP-9 (95 kDa). H-Ras MCF10A cells and HT1080 cells were cultured under normoxic and hypoxic condition with or without TSA (330 nmol/L) for 24 h. A gelatin zymograph assay was done with the supernatant obtained from the cultured cells. The relative expression of MMP-2 and MMP-9 was determined in triplicates and is presented. *, $P < 0.01$; #, $P < 0.001$, versus normoxia; **, $P < 0.001$, versus hypoxia. B, after removal of the supernatant from the cultured cells described in A, cells were fixed with 4% paraformaldehyde and treated with anti-human mouse *RECK* antibody. After washing with PBS, cells were incubated with an antimouse FITC secondary antibody. 4',6-Diamidino-2-phenylindole was used for nuclear staining. Magnification, $\times 400$. The relative expression of *RECK* was determined in triplicates and is presented. *, $P < 0.001$, versus normoxia; #, $P < 0.001$, versus hypoxia.

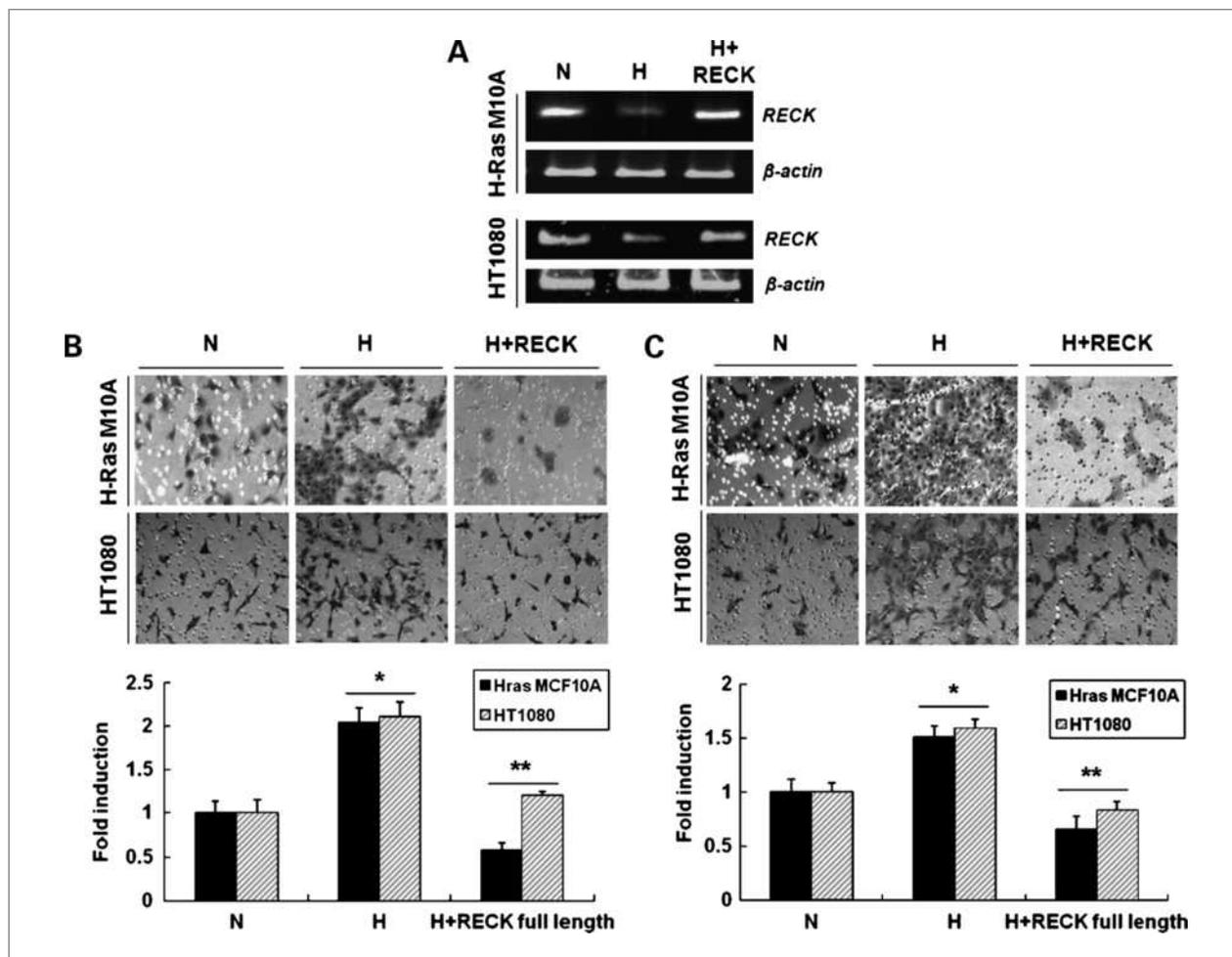


Figure 4. RECK overexpression inhibits hypoxia-induced migration and invasion. A, H-Ras MCF10A and HT1080 cells were transfected with a plasmid encoding the full-length human RECK and cultured under normoxic (N) or hypoxic (H) conditions. RECK expression was examined by semiquantitative RT-PCR. β -Actin was used as an internal control. B, transwell migration assays were done at 24 h under normoxic or hypoxic condition in the absence or presence of TSA. The migrated cells were counted and are graphed below. *, $P < 0.0001$, normoxia (N) versus hypoxia (H); **, $P < 0.001$, hypoxia versus plus RECK full-length (+RECK). C, similarly, transwell invasion assays were done, and the cells that invaded the Matrigel were counted and are graphed below. *, $P < 0.0001$, normoxia (N) versus hypoxia (H); **, $P < 0.001$, hypoxia versus plus RECK full-length.

The results clearly showed that MMP-2/MMP-9 and active MMP-2 were significantly increased under hypoxic conditions but were blocked by TSA. In addition, active MMP-2 did not increase in H-Ras MCF-10A cells under hypoxic conditions (Fig. 3A). These data support the hypothesis that MMP-2/MMP-9 define the minimal essential components for hypoxia-induced cancer cell migration and invasion and may be crucial for TSA-induced activation of RECK function under hypoxic conditions.

To confirm that the inhibition of MMP-2 and MMP-9 by TSA was caused by RECK restoration, we did an immunofluorescence assay with the same cells after supernatant media were collected for zymography. As shown in Fig. 3B, RECK expression under normoxia was high in the cytosol; however, hypoxic conditions dramatically decreased RECK expression. TSA completely rescued RECK expression to levels greater than

those found in normoxic conditions. These results also revealed that the TSA treatment recovered RECK under hypoxic conditions, which in turn inhibited MMP-2/MMP-9 activity.

RECK is involved in hypoxia-induced cell migration and invasion. To obtain direct evidence in support of the negative regulation of RECK in hypoxia-induced migration and invasion, H-Ras MCF10A and HT1080 cells were transfected with a full-length RECK expression vector under hypoxic conditions. Hypoxia-suppressed RECK expression was restored at the mRNA levels by ectopic expression of RECK in both cell lines (Fig. 4A). Furthermore, as seen in Fig. 4B, the transient transfection of full-length RECK under hypoxic conditions significantly decreased the cell migration of the H-Ras MCF10A and HT1080 cells. The hypoxia-induced cell invasion was also blocked by the ectopic expression of RECK (Fig. 4C).

To further confirm the role of RECK and to link RECK with HDAC in hypoxia-induced migration and invasion, we did a siRNA experiment. Although we generated two different RECK-specific siRNAs, the more efficient siRNA is shown in Fig. 5A and was used for the migration and invasion assay. When transfected with the RECK siRNA

under normoxic conditions, the HT1080 cells migrated more and were more invasive than normoxic conditions (Fig. 5B), similar to the hypoxic cells (Fig. 5B, graph). However, cells transfected with siRECK under hypoxic conditions displayed a similar degree of migration and invasion capability compared with hypoxic cells. TSA had a similar

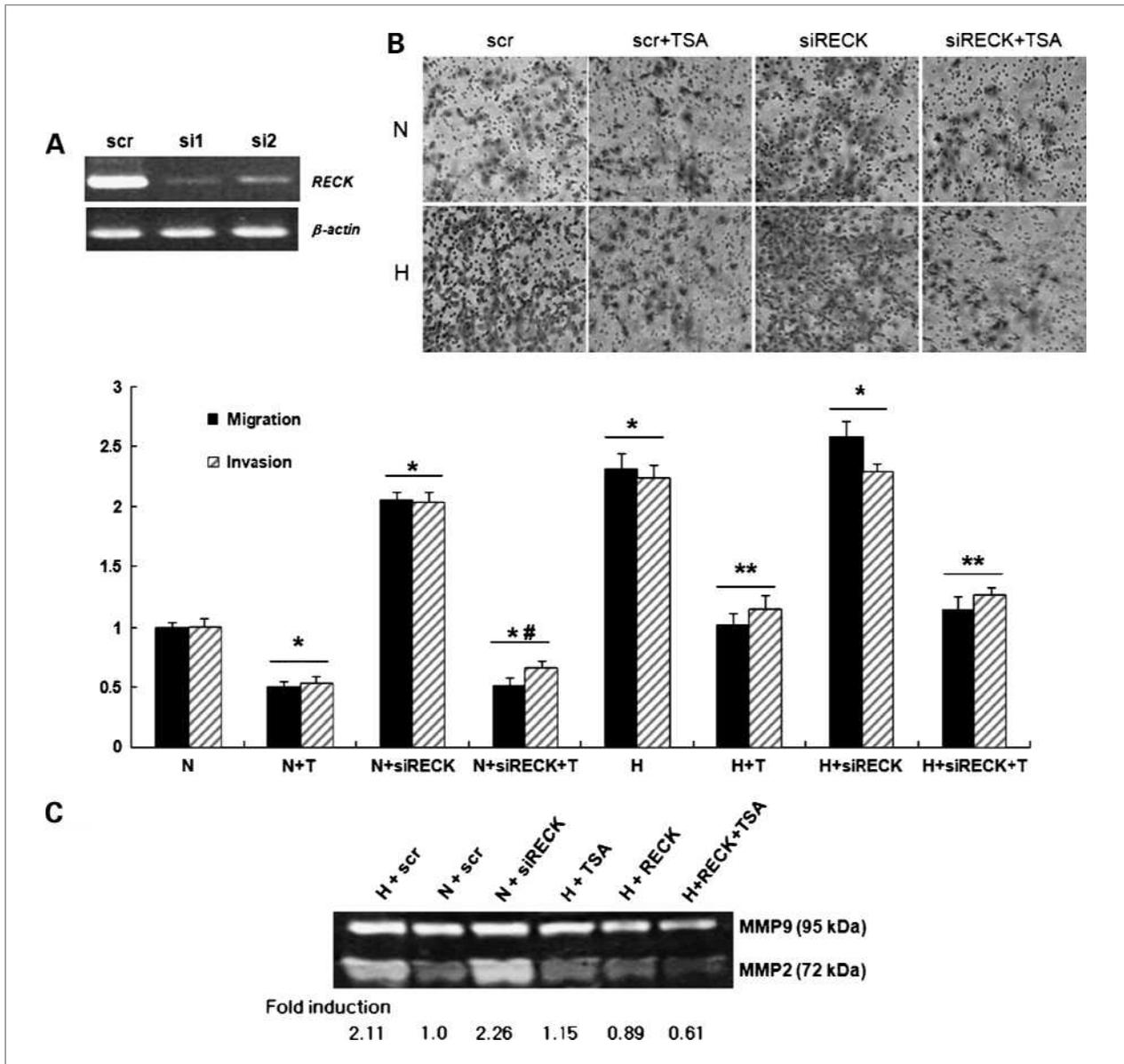


Figure 5. Knockdown of RECK with siRNA stimulates migration, invasion, and MMP secretion in HT1080 cells. **A**, after transfection with siRNA to RECK, HT1080 cells were analyzed for RECK expression by RT-PCR. siRNA1 was more effective than siRNA2 for downregulating RECK. **B**, HT1080 cells were seeded in the transwell chamber for the migration and invasion assay under normoxic (N), hypoxia (H), and control (scr) or RECK siRNA (siRECK) conditions with or without TSA (+TSA, 330 nmol/L). Images of H-Ras MCF10A cells are shown, and those for HT1080 cells are presented in Supplementary Fig. S2. The migrated/invaded cells were counted and are graphed below. The black bars indicate migrated cells, whereas the slashed bars indicate invaded cells. *, $P < 0.0001$, significantly different from normoxia; **, $P < 0.0001$, from hypoxia; #, $P < 0.0001$, from siRECK transfectant. **C**, after transfection with scrambled (scr) siRNA, RECK siRNA (siRECK), and full-length RECK (RECK) plasmid, HT1080 cells were cultured under normoxic (N) and hypoxic (H) condition with or without TSA (330 nmol/L) for 24 h. A gelatin zymograph assay was done with the supernatant obtained from the cultured cells. Fold induction of MMPs activity is shown below the picture.

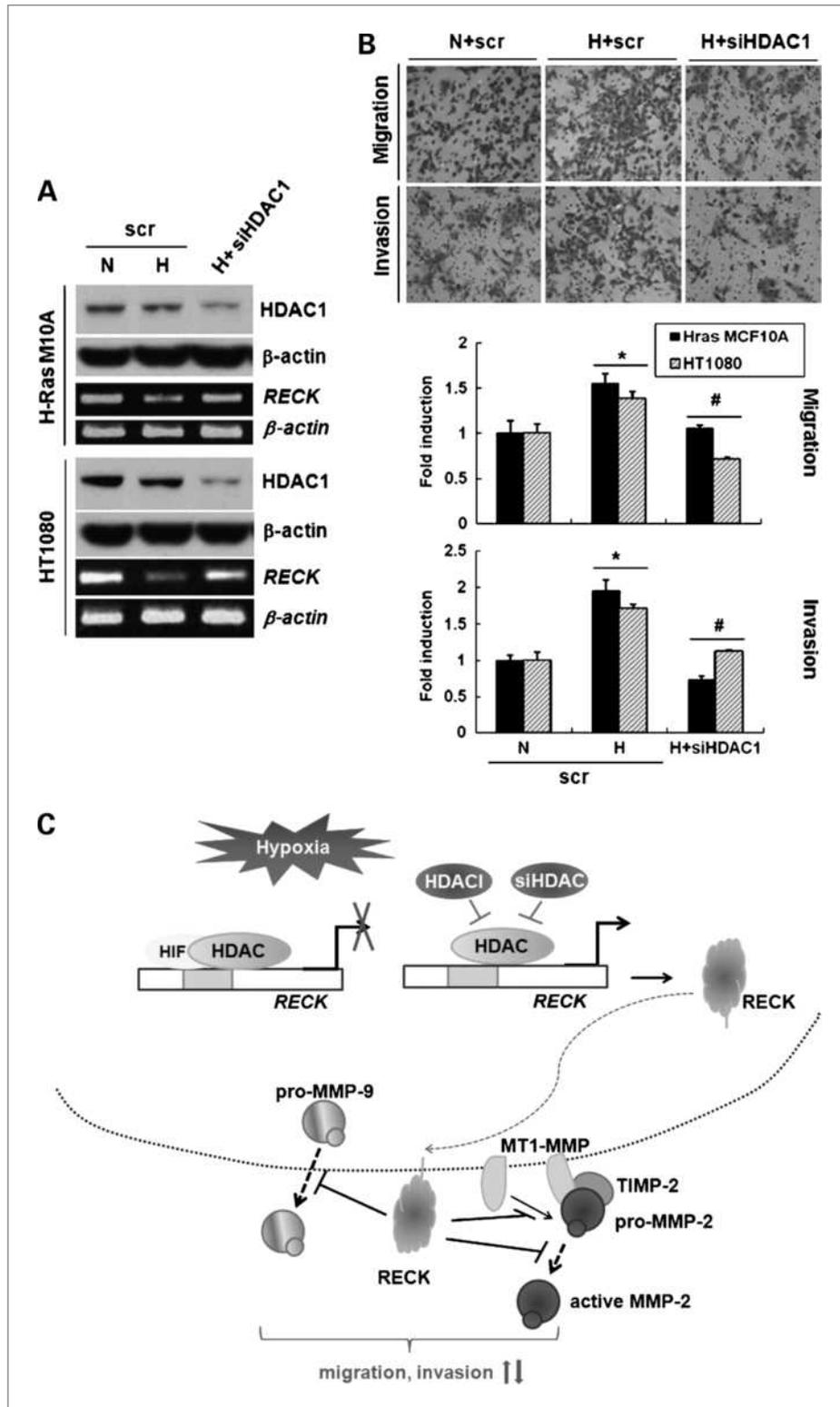


Figure 6. Hypoxia-reduced RECK expression is controlled by HDAC1. **A**, H-Ras MCF10A and HT1080 cells were transfected with HDAC1 siRNAs, and mRNA and protein expression were investigated using semiquantitative RT-PCR and Western blot, respectively. β -Actin was used as an internal control. **B**, H-Ras MCF10A and HT-1080 cells were transfected with siRNAs specific for HDAC1, and migration/invasion assays were done. Top, migrated and invaded H-Ras MCF10A cells; bottom, quantification of the migrated and invaded cells for each cell line. *, $P < 0.001$, normoxia versus hypoxia; #, $P < 0.001$, hypoxia versus plus siRNAs. **C**, schematic summary of our hypothesis and results. Under hypoxic conditions, RECK was downregulated through HDAC in collaboration with HIF-1 at the RECK promoter region (26). However, if the activity of HDAC was blocked by HDACi or by siRNA, RECK expression could be restored. RECK could then translocate to the cell membrane to block MMPs turnover. This might be a mechanism of RECK restoration by HDAC inhibition to attenuate cancer cell migration and invasion under hypoxic conditions.

inhibitory effect on both hypoxia alone and in combination with siRECK (Fig. 5B), indicating that HDAC inhibition had a global effect on the inhibition of migration and invasion of the cancer cells. Furthermore, the results indi-

cated that HDAC inhibition was enough to counterbalance the effect of RECK silencing. The results suggest that RECK might be a major molecule in regulating migration and invasion under hypoxic conditions. The RECK

siRNA-transfected cells secreted more MMP-2/MMP-9 to a similar level as that of scrambled siRNA-transfected cells under hypoxic condition compared with normoxic cells. In particular, the levels of MMP-2 and active MMP-2 were clearly increased by knockdown of RECK under hypoxic conditions. We confirmed RECK overexpression and validated that TSA treatment inhibited MMP activity in HT1080 cells (Fig. 5C). These results indicate that RECK silencing by hypoxia plays a critical role in hypoxia-induced cancer cell migration and invasion and that the restoration of RECK blocks these hypoxia-induced effects in these cells.

Knockdown of HDAC1 inhibits hypoxia-induced cell migration and invasion. We showed that HDACs dramatically inhibited hypoxia-induced cancer cell migration and invasion. In addition, the downregulation of RECK in hypoxic conditions was critically involved in this biological function. To confirm that HDACs were crucial for the hypoxia-induced downregulation of RECK and cancer cell migration and invasion, we generated two HDAC1-specific siRNAs. The more efficient HDAC1-specific siRNA was transfected into H-Ras MCF10A and HT1080 cells. The knockdown of HDAC1 by siRNA clearly restored RECK expression (Fig. 6A). We subsequently did migration and invasion assays with these siRNA-treated cells under normoxic and hypoxic conditions. The knockdown of HDAC1 resulted in a significant decrease in the migration and invasion of both cell lines compared with scrambled siRNA-transfected cells under hypoxic conditions (Fig. 6B). Overall, these results indicate that the hypoxia-induced migration and invasion of these cancer cell lines is caused by RECK suppression through the activity of HDAC1 under hypoxic conditions.

Discussion

In this report, we show for the first time that loss of RECK expression is induced by hypoxia in cancer cells. This suppression of RECK expression stimulated the migration and invasion of the cancer cells. HDACs (e.g., TSA), as well as HDAC1 siRNA, potently suppressed the hypoxia-induced migration and invasion of the cancer cells through RECK restoration.

RECK is a membrane-bound protein that, at least in the mouse, has been found to be important in suppressing two key components of the metastatic cascade: MMPs and angiogenesis (22). A limited number of studies have also shown that RECK levels are significantly downregulated in common human malignancies when compared with the levels found in the normal surrounding tissue. In contrast, in the minority of tumor samples wherein RECK levels are normal or elevated, there is generally a reduction in the local invasion and metastasis and an improved prognosis (23, 24).

It has been reported that hypoxia downregulates tumor suppressor genes, such as *BNIP3*, *p53*, *pVHL*, *MLH1*, *BRCA1*, *BRCA2*, and *RUNX3* in cancer cells, as well as in normal cells (10, 13, 14, 25). Conversely, HDAC

levels have been shown to be elevated in various cancer tissues and have been reported to increase following hypoxia (11). The increased expression of HDACs may lead to the inhibition of tumor suppressor genes and the stimulation of angiogenesis, because HDACs restore the expression of tumor suppressors, such as *MLH1*, *p53*, and *pVHL* (10, 13). Our recent report suggests that histone deacetylation and methylation are involved in hypoxia-induced *RUNX3* silencing (14). Thus, an epigenetic mechanism seems to be involved in the hypoxia-induced repression of tumor suppressors. When we treated cells with epigenetic regulating agents (5-aza-2'-deoxycytidine and TSA) under hypoxic conditions, 5-aza-2'-deoxycytidine did not restore *RECK* expression (data not shown) but TSA did restore *RECK* expression (Fig. 1), suggesting that histone deacetylation, but not methylation, was involved in the hypoxia-induced suppression of *RECK*. In this report, we used two methods to inhibit the activity of HDACs: treatment with inhibitors (e.g., TSA) and silencing by an HDAC1-specific siRNA. Both the HDACs and the HDAC1 siRNA recovered *RECK* expression (Figs. 1 and 6). This suggests that HDACs play a critical role in the hypoxia-induced suppression of *RECK* and that the inhibition of HDACs is a key part of *RECK* recovery under hypoxic conditions. Furthermore, we found that HDAC1 was recruited to the *RECK* promoter region in the presence of HIF-1 α under hypoxic conditions (26).

Hypoxia increases the metastatic potential of a variety of cancer cells through HIF-1 (18, 27–30). Although HIF-1 α is also involved in cancer cell migration and invasion, we did not investigate HIF-1 α involvement. However, our previous report (19) and other studies have already shown that the inhibition of HDAC1 destabilizes HIF-1 α , but not vice versa (31–34). Therefore, we evaluated only HDAC inhibition to target the suppression of *RECK* under hypoxic conditions. The inhibition of both HIF-1 α and HDAC1 might more potently restore *RECK* expression and suppress cancer cell migration and invasion. Therefore, whereas targeting both HDACs and HIF-1 α simultaneously would be beneficial, if only one component could be targeted by a therapy, the inhibition of HDAC activity is preferable to the inhibition of HIF-1 α (Fig. 6C).

As previously outlined, the new evidence that HDACs and the knockdown of HDAC1 are able to increase *RECK* levels provides a therapeutic approach for the inhibition of metastasis as well as an improvement in the prognosis of cancer treatment through inhibition of MMPs. With the discovery of the role of the HDAC in oncogenic Ras-mediated *RECK* suppression, the use of HDACs, such as TSA, offers a possible means to manipulate *RECK* expression *in vivo* to block various oncogenic processes (3), particularly in the hypoxic microenvironment present within the tumor (Fig. 6C). This treatment may be a promising alternative to the difficulties encountered with gene therapy. Therefore, the control of *RECK* gene expression should be studied further to gain insights into the mechanisms of tumor progression and to facilitate the design of new strategies for cancer prevention and treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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