Macrophage migration inhibitory factor (MIF) is a pluripotent cytokine that is involved in host immune and inflammatory responses, as well as tumorigenesis. However, the regulatory mechanism of MIF function is unclear. Here we report that the NM23-H1 interacts with MIF in cells, as demonstrated by cotransfection and coimmunoprecipitation experiments. Analysis of cysteine (Cys) to serine (Ser) substitution mutants of NM23-H1 (C4S, C109S, and C145S) and MIF (C57S, C60S, and C81S) revealed that Cys145 of NM23-H1 and Cys60 of MIF are responsible for complex formation. NM23-H1-MIF complexes were dependent on reducing conditions, such as the presence of dithiothreitol or β-mercaptoethanol, but not H2O2. NM23-H1 alleviated the MIF-mediated suppression of p53-induced apoptosis and cell cycle arrest by promoting the dissociation of MIF from MIF-p53 complexes. In addition, NM23-H1 significantly inhibited the MIF-induced proliferation of quiescent NIH 3T3 cells through a direct interaction with MIF, and decreased the MIF-induced activation of phosphatidylinositol 3-kinase/PDK1 and p44/p42 extracellular signal-regulated (ERK) mitogen-activated protein kinase. The results of the current study suggest that the NM23-H1 functions as a negative regulator of MIF.

Macrophage migration inhibition factor (MIF) has been shown to be a key mediator in the regulation of host inflammatory and immune responses (1). There have been several studies showing that MIF is involved in a diverse set of biological responses, including cell proliferation, migration, differentiation, angiogenesis, and glucose, and fat metabolism (2, 3). In addition, recent studies strongly suggest that MIF is linked to tumorigenesis through its ability to suppress p53 activity (4, 5). The overexpression of MIF has been documented in human melanoma, breast carcinoma, metastatic prostate cancer, and adenocarcinoma of the lung (6, 7), and MIF has been shown to enhance tumor cell migration and the production of angiogenic factors (8). Recent studies suggest that MIF is a potent regulator of p53- and AP-1 activity, however, the regulatory mechanism of MIF function in cells is largely unknown (5, 9). One way to elucidate the mechanism of MIF function is to analyze the role of MIF-interacting proteins in the regulation of MIF activity.

NM23-H1 was first classified as a metastasis suppressor gene on the basis of its reduced expression in several metastatic melanoma cell lines as compared with their non-metastatic counterparts (10). Overexpression of NM23-H1 in various cancer cell lines has been linked to the suppression of tumor metastasis, and differentiation (10, 11). Extensive work has demonstrated that the NM23 proteins are involved in a broad spectrum of cellular functions, including proliferation (12), differentiation and development (13), signal transduction (14–17), G protein-coupled receptor endocytosis (18), and gene expression (19). Despite considerable effort, however, the molecular mechanisms underlying the functions of NM23 proteins in these cellular processes are not well understood, even though a diverse set of enzymatic activities have been ascribed to NM23 proteins, including nucleoside-diphosphate kinase activity (20), serine/threonine-specific phosphotransferase activity (21, 22), geranyl and farnesyl pyrophosphate kinase activity (23), and histidine protein kinase activity (24, 25).

In the current study, we demonstrated that NM23-H1 and MIF physically interact, and that the interaction is mediated by cysteine residues in both proteins. We also present evidence that the suppression of p53-mediated apoptosis and cell cycle arrest by MIF is alleviated by the direct interaction of NM23-H1 with MIF, and that NM23-H1 inhibits the MIF-induced cell proliferation of quiescent NIH 3T3 cells by binding to MIF.

**MATERIALS AND METHODS**

**Cell Culture, Plasmids, and Reagents—**293T, HEK293, MCF7, NIH 3T3, HCT116, and p53-null HCT116 cells were routinely maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), as described previously (26). The expression plasmid pEBG-MIF, encoding wild-type glutathione S-transferase...
(GST)-tagged MIF (GST-MIF), as well as the expression plasmids for FLAG-tagged MIF (FLAG-MIF) and MIF substitution mutants (MIF(C57S), (C60S), and (C81S)), wild-type NM23-H1 and NM23-H1 substitution mutants (NM23-H1(C4S), (C109S), and (C145S)), and the p53-Luc reporter plasmids were previously defined (15, 16, 27). Anti-GST, anti-hemagglutinin, anti-β-actin, and anti-FLAG (M2) antibodies were previously described (15, 28). For immunoprecipitation and immunoblot analysis, anti-MIF, anti-p53, anti-MDM2, and anti-NM23-H1 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). [γ-32P]ATP was purchased from PerkinElmer Life Sciences (Boston, MA).

In Vivo and In Vitro Binding Assays—Cells were transiently transfected with the indicated expression vectors using WelFect-Ex™ Plus (WelGENE, Daegu, Korea), according to the manufacturer’s instructions. In vivo and in vitro binding assays were performed as previously described (26).

Preparation of Recombinant NM23-H1 and MIF Proteins—Expression vectors for recombinant hexahistidine (His)6-tagged human wild-type NM23-H1 or GST-tagged wild-type and mutant MIF were constructed by subcloning the appropriate cDNA fragments of NM23-H1 and MIF into pQE30 (Qiagen) or pGEX4T-1 (Amersham Biosciences). Recombinant proteins were purified by affinity chromatography on a His-Bind Resin column (Novagen, EMD Biosciences, Inc., San Diego, CA) or a glutathione-Sepharose 4B column (Amersham Biosciences), as described previously (16, 27).

Reporter Assays—MCF7 cells were transiently transfected with the indicated expression vectors together with a reporter plasmid encoding p53-Luc using WelFect-Ex Plus, according to the manufacturer’s instructions. An expression vector for β-galactosidase was also transfected into cells as an internal control. Empty vector was added to each transfection to ensure that the knockdown efficiency of the shRNAs was not due to transfection efficiency. After 24 h of transfection, the cells were treated with 4,000 U/ml of ULTRAVIOLET (UVP), according to the manufacturer’s protocol, as described previously (29). The values were adjusted with respect to expression levels of a cotransfected β-galactosidase reporter control. Data are representative of at least three independent experiments.

RNA Interference Experiments—Small interfering RNA (siRNA) experiments were performed using NM23-H1-specific siRNAs (number 1, 5’-GAGAGUCCGCUUGUGUUUTT-3’; number 2, 5’-GCUUCCAGAGAUUCUCATT-3’) and MIF-specific siRNAs (number 1, 5’-CAACCAAAGGUGC-CAGCGGCAUdTdT-3’; number 2, 5’-CCUUCUGGUGGGAG-AAA UdTdT-3’) (15, 26).

Ubiquitination Assay—The ubiquitination assay was performed as described previously (16). In brief, p53-null HCT116 cells were transiently transfected with expression plasmids for p53, wild-type, and mutant forms of NM23-H1, wild-type MIF, and hemaggulitinin-tagged ubiquitin, as indicated, using WelFect-Ex Plus. After 44 h of transfection, the cells were treated with 10 μg/ml MG132 (Calbiochem) for 4 h, and then washed twice with phosphate-buffered saline, pH 7.4. Cells were lysed in 200 μl of Tris-buffered saline, pH 7.4, containing 2% SDS, and then the lysates were incubated at 95 °C for 10 min, followed by incubation with anti-p53 antibody.

Apoptosis Assay—The apoptosis assay was performed as described previously (29). Briefly, MCF7 cells grown on sterile coverslips were transfected with an expression vector encoding green fluorescent protein, together with the indicated expression vectors. After 24 h of transfection, the cells were treated with 5-fluorouracil (0.38 mM) for 30 h, fixed with ice-cold 100% methanol, and then washed three times with phosphate-buffered saline. The cells were incubated with 1 μg/ml of 4,6-diamidino-2-phenylindole (Sigma), and nuclei were visualized by fluorescence microscopy. The percentage of apoptotic cells was calculated as the number of GFP-positive cells with apoptotic nuclei divided by the total number of GFP-positive cells.

Immunofluorescence Staining and FACS Analysis—Immunofluorescence staining was performed as described previously (15). For FACS analysis for cell cycle distribution (26), transfectants of MCF7 cells expressing the indicated proteins, together with transfectants expressing p53 alone or empty vector as a control, were washed with ice-cold phosphate-buffered saline and then treated with doxorubicin (100 ng/ml) for 24 h. The trypsinized cells were washed twice with ice-cold phosphate-buffered saline and then incubated at 37 °C for 30 min with a solution (1 mM Tris-HCl, pH 7.5) of 50 μg/ml propidium iodide and 1 mg/ml RNase A. Flow cytometry was performed on a FACSCalibur-S system (BD Biosciences).

Proliferation Assay—Proliferation was assessed by trypan blue exclusion. NIH 3T3 cells were plated in 60-mm dishes at a concentration of 4 × 10⁵ cells per dish and cultured for 24 h. The cells were transfected with the indicated expression vectors using WelFect-Ex Plus, and then cultured for an additional 12–15 h in 10% FBS-containing DMEM. The cells then were re-seeded in 6-well plates at 4–5 × 10⁴ cells per well and allowed to attach for 8 h in 10% FBS-containing DMEM, after which the medium was replaced with 0.5% FBS-containing DMEM. Cell viability was determined by trypan blue exclusion and viable cells were counted with a hemocytometer at the indicated times.

RESULTS

NM23-H1 Interacts with MIF in Vivo—NM23-H1 and MIF have been shown to interact with p53 (16, 26). To determine whether NM23-H1 interacted with MIF in the absence of p53, HEK293 cells were cotransfected with expression vectors for GST-NM23-H1 and FLAG-MIF, and the interaction between NM23-H1 and MIF in GST precipitates was analyzed by immunoblot using an anti-FLAG antibody. As shown in Fig. 1A, MIF was detected in GST precipitates, which suggested that MIF physically interacts with NM23-H1 in cells. To determine whether endogenous NM23-H1 and MIF interacted with each other, the in vivo association of the two proteins was analyzed by coimmunoprecipitation using an anti-NM23-H1 antibody, or preimmune IgG as a control. Endogenous MIF was present in anti-NM23-H1 immune complexes from all cell lines examined, including 293T, HEK293, and MCF7 cells (Fig. 1B). The interaction was also detected in reciprocal experiments in which the immunoprecipitation was carried out using an anti-MIF antibody (data not shown). To determine the subcellular localization of NM23-H1 and MIF, immunofluorescence confocal microscopy was performed using HCT116 cells trans-
Requirement of Cysteine Residues for NM23-H1-MIF Complex Formation—Cysteine residues of NM23-H1 have been shown to participate in STRAP binding (15), and the interaction of both NM23-H1 and MIF with p53 is mediated by cysteine residues (16, 26). To determine whether the NM23-H1-MIF interaction involved cysteine residues in both proteins, and to investigate the effect of cysteine residues of MIF on NM23-H1-MIF complex formation, HEK293 cells were transiently cotransfected with expression vectors for wild-type MIF and NM23-H1. We next examined whether cysteine residues (Cys<sup>4</sup>, Cys<sup>109</sup>, and Cys<sup>145</sup>) of NM23-H1 were involved in complex formation between NM23-H1 and MIF. Coexpression of NM23-H1(C145S) resulted in a significant decrease in complex formation between NM23-H1 and MIF, as compared with control cells expressing wild-type NM23-H1, whereas coexpression of NM23-H1(C4S) and NM23-H1(C109S) had no effect (Fig. 2A, middle). These results indicated that Cys<sup>145</sup> of NM23-H1 is responsible for MIF binding. To verify that the dramatic decrease in the association between NM23-H1 and MIF was due to substitutions of NM23-H1-Cys<sup>145</sup> and MIF-Cys<sup>60</sup>, we performed an in vivo binding assay of NM23-H1(C4S) and MIF(C60S) in HEK293 cells. The coexpression of NM23-H1(C145S) and MIF(C60S) completely abrogated complex formation between NM23-H1 and MIF (Fig. 2A, right). We also carried out non-denaturing polyacrylamide gel electrophoresis to confirm the association between NM23-H1 and MIF in vitro. Purified NM23-H1 was subjected to autophosphorylation using radiolabeled ATP, then incubated with unlabeled, recombinant wild-type and mutant forms of MIF. As shown in Fig. 2B, a shift in the mobility of autophosphorylated NM23-H1 was clearly evident upon incubation in the presence of wild-type MIF, MIF(C57S), and MIF(C81S), but was undetectable in the presence of MIF(C60S). These results provided additional evidence of the physical association of NM23-H1 and MIF through cysteine residues. To determine whether the interaction between NM23-H1 and MIF was redox-dependent, we performed in vivo binding assays under a variety of reducing and oxidizing conditions in HEK293 and MCF7 cells. The pres-
ence of the reductants dithiothreitol and \( \beta \)-mercaptoethanol markedly decreased the amount of endogenous MIF that coimmunoprecipitated with endogenous NM23-H1, whereas the oxidant \( \text{H}_2\text{O}_2 \) had no effect (Fig. 2C). These results indicated that the in vivo association of NM23-H1 with MIF is redox-dependent. Collectively, our results strongly suggest that cysteine residues, particularly Cys\(^{145} \) of NM23-H1 and Cys\(^{60} \) of MIF, play an essential role in the association of NM23-H1 and MIF in vivo.

Regulation of NM23-H1-MIF Complex Formation by 5-FU and Doxorubicin—The p53 activity is induced by 5-fluorouracil (5-FU) and doxorubicin (30, 31), and MIF has been shown to inhibit p53-mediated apoptosis and cell cycle arrest (26). To determine whether 5-FU and doxorubicin influenced NM23-H1-MIF complex formation, HEK293 cells were transfected with expression vectors for GST-NM23-H1 and FLAG-MIF, and then treated with 5-FU or doxorubicin, and the association of NM23-H1 and MIF was analyzed by immunoblot. Complex formation between NM23-H1 and MIF in total cell lysate were determined by immunoblot analysis using anti-Nm23-H1 (middle panels) and anti-MIF antibodies (bottom panels), respectively. WB, Western blot.

FIGURE 2. Role of cysteine residues of NM23-H1 and MIF on NM23-H1-MIF complex formation. A, HEK293 cells were transiently transfected with the indicated expression plasmids (1.5–2 \( \mu \)g each). GST fusion proteins were purified using glutathione-Sepharose beads (GST purification) and resolved by SDS-PAGE. Immune complexes were visualized by ECL. Complex formation between NM23-H1 and MIF was determined by immunoblot analysis using an anti-FLAG antibody (top panels). The blot was re-probed with an anti-GST antibody to demonstrate that equivalent amounts of wild-type and mutant GST-MIF were precipitated (middle panels); the level of FLAG-NM23-H1 in total cell lysate was analyzed by immunoblot using an anti-FLAG antibody (bottom panels, Lysate). B, native polyacrylamide gel electrophoresis of NM23-H1-MIF complexes. Autophosphorylation of purified recombinant NM23-H1 was performed in TMD buffer, as described previously (15). Autophosphorylated NM23-H1 (3 \( \mu \)g) was incubated with unlabeled recombinant MIF (5 \( \mu \)g each), as indicated, at room temperature for 1 h. For native polyacrylamide (8%) gel electrophoresis, the procedure was the same as SDS-PAGE, with the exception that SDS and \( \beta \)-mercaptoethanol were not included in any solutions, and samples were not boiled before loading. C, cell lysates from HEK293 and MCF7 cells were treated with the indicated concentrations of \( \text{H}_2\text{O}_2 \), DTT, and \( \beta \)-mercaptoethanol (\( \beta \)-ME) on ice for 0.5–1 h, and then subjected to immunoprecipitation using an anti-NM23-H1 antibody (IP), followed by immunoblot analysis using an anti-MIF antibody (top panels). The amounts of NM23-H1 in the immune complexes and MIF in total cell lysate were determined by immunoblot analysis using anti-Nm23-H1 (middle panels) and anti-MIF antibodies (bottom panels), respectively. WB, Western blot.
Negative Regulation of MIF by NM23-H1

NM23-H1 Alleviates MIF-mediated Suppression of p53-induced Transcription by Destabilizing MIF-p53 Complexes—MIF forms a complex with p53 and suppresses p53-induced transcription (26). To determine whether NM23-H1 affected the MIF-mediated suppression of p53-induced transcription, MCF7 cells were cotransfected with an expression vector for MIF, and increasing amounts of expression vectors for wild-type NM23-H1 and MIF is presented. The data are representative of at least three independent experiments.

FIGURE 3. Modulation of NM23-H1-MIF complex formation by p53 stimulation. HEK293 cells were transfected with the indicated expression vectors (1.5–2 μg each), then incubated with or without 5-FU (0.38 mM, 30 h) or doxorubicin (Dox) (100 ng/ml, 24 h). Complex formation was determined by immunoblot analysis using an anti-FLAG antibody (top panels) as described in the legend to Fig. 1A. The relative levels of NM23-H1-MIF complexes were quantitated by densitometry, and -fold increase relative to untreated cells expressing wild-type NM23-H1 and MIF is presented. The data are representative of at least three independent experiments.

the association between MIF and p53, because MIF inhibits p53 activity through a direct interaction with p53 (26). HEK293 cells were cotransfected with expression vectors for MIF and p53 in the presence or absence of the wild-type and mutant form of NM23-H1. Compared with control cells expressing MIF and p53 in the absence of NM23-H1, the expression of wild-type NM23-H1, NM23-H1(C4S), or NM23-H1(C109S) significantly decreased the association between MIF and p53 (Fig. 4C, left). In contrast, NM23-H1(C145S), which is unable to bind to p53 or MIF, had no effect on the association of MIF and p53, which indicated that NM23-H1 alleviates the MIF-mediated suppression of p53-induced transcription by dissociating MIF from MIF-p53 complexes. To elucidate the mechanism underlying the diminished effect of NM23-H1 on p53-induced transcription in the presence of MIF(C60S), we analyzed the effect of NM23-H1 on the association of MIF(C60S) and p53 using an in vivo binding assay. The coexpression of wild-type NM23-H1, NM23-H1(C4S), or NM23-H1(C109S) slightly diminished the association between MIF(C60S) and p53, most likely due to the failure of NM23-H1 to associate with MIF(C60S), even though the physical interaction between NM23-H1 and p53 was still present, whereas NM23-H1(C145S) had no effect on the association between MIF(C60S) and p53 (Fig. 4C, right).

NM23-H1 Alleviates the MIF-mediated Suppression of p53-induced Apoptosis and Cell Cycle Arrest through Direct Binding to MIF—To determine whether the expression of NM23-H1 affected p53-induced apoptosis and cell cycle arrest in the presence of MIF, we first examined the effect of wild-type and mutant forms of NM23-H1 on the MIF-mediated suppression of p53-induced apoptosis using a GFP-based assay system in MCF7 cells. In cells that expressed p53 alone, ~42% of the cells were apoptotic (Fig. 5A, upper, second lane). The coexpression of wild-type NM23-H1, NM23-H1(C4S), or NM23-H1(C109S) with wild-type MIF considerably increased the percentage of apoptotic cells by ~1.8-fold relative to control cells that expressed wild-type MIF alone (Fig. 5A, upper, lane 3 versus lanes 4–6). The coexpression of NM23-H1(C145S) had no effect on the MIF-mediated suppression of p53-induced apoptosis. The stimulatory effect of NM23-H1 on p53-induced apoptosis in the presence of MIF(C60S) was less pronounced than in the presence of wild-type MIF (Fig. 5A, upper, lanes 3 and 8 versus lanes 4–6 and 9–11), which was consistent with earlier results (Fig. 4B). We also examined whether NM23-H1 affected the MIF-mediated suppression of p53-induced cell cycle arrest. G0/G1 arrest induced by p53 was lower in cells that expressed wild-type NM23-H1, NM23-H1(C4S), or NM23-H1(C109S) in the presence of MIF(C60S) than in the presence of wild-type MIF (Fig. 5B, upper, lanes 4–6 versus lanes 9–11), whereas the coexpression of NM23-H1(C145S) had no effect on
Negative Regulation of MIF by NM23-H1

FIGURE 4. Alleviation of the MIF-mediated suppression of p53-induced transcription by NM23-H1. A, effect of NM23-H1 on p53-induced transcription in the presence of wild-type MIF. MCF7 cells were transfected with increasing amounts of expression vector for wild-type or mutant forms of NM23-H1, as indicated, together with 0.2 μg of p53-Luc reporter plasmid and 0.2 μg of β-galactosidase expression vector as an internal control, in the presence of wild-type (WT) MIF and p53. Luciferase activity was normalized to β-galactosidase activity, and data from duplicate samples are presented. Fold increase relative to control, untransfected cells is presented. B, effect of NM23-H1 on the MIF-mediated suppression of p53-induced transcription in the presence of MIF(C60S). MCF7 cells were cotransfected with increasing amounts of expression vector (0.2, 0.4, and 0.6 μg) for wild-type or mutant forms of NM23-H1 and either wild-type MIF (0.6 μg) (white bars) or MIF(C60S) (black bars), as indicated. Fold increase relative to control cells untreated with p53 is presented. Data represent the mean ± S.E. of three independent experiments. p < 0.05 relative to control untransfected cells; significance was calculated by Student’s t test (A and B). C, effect of NM23-H1 on the formation of MIF-p53 and MIF(C60S)-p53 complexes. HEK293 cells were transfected with expression vectors for wild-type MIF (left panel, 1.5 μg) or MIF(C60S) (right panel, 1.5 μg), together with p53 (2 μg), in the presence or absence of wild-type or mutant forms of NM23-H1 (2 μg each). GST-MIF was purified using glutathione-Sepharose beads (GST purification), and complex formation between MIF and p53 (left top panel) or MIF(C60S) and p53 (right top panel), as well as the amount of FLAG-MIF-NM23-H1 and p53 in cell lysates (bottom panels, Lysate), was determined by immunoblot using an anti-FLAG antibody. The relative levels of MIF-p53 or MIF(C60S)-p53 complexes were quantitated by densitometry, and fold increase relative to control cells expressing p53 and either wild-type MIF or MIF(C60S) in the absence of NM23-H1 is presented. The data are representative of at least three independent experiments. WB, Western blot.

p53-induced G0/G1 arrest (Fig. 5B, upper, lanes 3 and 8 versus lanes 7 and 12). Consistent with these results, the knockdown of NM23-H1 using siRNAs decreased p53-induced apoptosis and cell cycle arrest in a dose-dependent manner as compared with control cells that expressed p53 and MIF alone (Fig. 5, A and B, lower, lane 3 versus lanes 5–8). These results suggested that the association between NM23-H1 and MIF plays a critical role in the ability of NM23-H1 to alleviate the MIF-mediated suppression of p53-induced apoptosis and cell cycle arrest, but that a direct interaction between NM23-H1 and p53 partially regulates this process as well.

NM23-H1 Stimulates the Removal of MDM2 from p53-MDM2 Complexes—To determine whether the alleviation of the MIF-mediated suppression of p53-induced apoptosis and cell cycle arrest by NM23-H1 correlated with the dissociation of MDM2 from p53-MDM2 complexes, we carried out an in vivo binding assay to determine the effect of wild-type and mutant forms of NM23-H1 on the association of p53 and MDM2 in the presence of wild-type MIF. Compared with control HCT116 or MCF7 cells that expressed MIF alone, the expression of wild-type NM23-H1, NM23-H1(C4S), or NM23-H1(C109S) significantly decreased the association between p53 and MDM2, whereas NM23-H1(C145S) had no effect (Fig. 6A). These results suggested that the direct association of NM23-H1 and MIF, as well as the association between NM23-H1 and p53, plays a role in the ability of NM23-H1 to alleviate the MIF-mediated suppression of p53-induced apoptosis and cell cycle arrest. We next investigated the role of NM23-H1 in MDM2-mediated p53 ubiquitination. The coexpression of wild-type NM23-H1, NM23-H1(C4S), or NM23-H1(C109S) considerably decreased p53 ubiquitination as compared with control cells that expressed MIF alone, whereas p53 ubiquitination was unaffected by
Negative Regulation of MIF by NM23-H1

the expression of NM23-H1(C145S) (Fig. 6B). These results suggested that NM23-H1 physically interacts with MIF and diminishes the MIF-induced stabilization of p53-MDM2 complexes, leading to p53 stabilization. To further characterize the role of NM23-H1 in the rescue of p53-induced apoptosis and cell cycle arrest in the presence of MIF, we carried out an in vivo binding assay to examine the level of association of endogenous p53 and MDM2 in the presence of wild-type NM23-H1 or NM23-H1(C145S), and wild-type MIF or MIF(C60S). The expression of wild-type NM23-H1 decreased the association between endogenous p53 and MDM2 in a dose-dependent manner in cells that expressed wild-type MIF, whereas complex formation was less affected by wild-type NM23-H1 in the presence of MIF(C60S), most likely due to the failure of NM23-H1(C145S) to associate with each other (Fig. 6C, left). To determine whether the direct interaction between NM23-H1 and p53 was also important for the alleviation of MIF-mediated suppression of p53-induced apoptosis and cell cycle arrest by NM23-H1, we examined the effect of NM23-H1(C145S) on the association between endogenous p53 and MDM2. The coexpression of NM23-H1(C145S) had no effect on the association of p53 and MDM2 in the presence of either wild-type MIF or MIF(C60S) (Fig. 6C, right), which indicated that in addition to the association between NM23-H1 and MIF, the association of NM23-H1 with p53 plays a partial role in the ability of NM23-H1 to alleviate the MIF-mediated suppression of p53 signaling. This was confirmed by analyzing the stability of p53 in HCT116 and MCF7 cells. The expression of wild-type NM23-H1 considerably increased p53 stability in the presence of wild-type MIF as compared with control cells that expressed MIF alone (Fig. 6D, lanes 1 and 2 versus lanes 3 and 4). However, the expression of wild-type NM23-H1 increased p53 stability to a lesser extent in the presence of MIF(C60S) as compared with wild-type MIF (Fig. 6D, lanes 3 and 4 versus lanes 5 and 6). In addition, coexpression of NM23-H1(C145S) had no effect on the MIF-mediated suppression of p53 stability (Fig. 6D, lanes 1 and 2 versus lanes 7–10). These results suggested that NM23-H1 physically interacts with both MIF and p53, and stimulates the dissociation of MDM2 from p53-MDM2 complexes, resulting in the enhancement of p53 signaling.

NM23-H1 Inhibits MIF-induced Cell Proliferation in Quiescent NIH 3T3 Fibroblasts—It was previously shown that MIF stimulates the proliferation of quiescent NIH 3T3 fibroblasts (32). To determine whether NM23-H1 affected cell proliferation induced by MIF, we analyzed the induction of proliferation of quiescent NIH 3T3 cells by MIF in the presence of wild-type and mutant forms of NM23-H1. Compared with parental NIH 3T3 cells, the expression of MIF significantly increased cell proliferation in a dose-dependent manner (Fig. 7A, left), in agreement with previous results (32). The opposite trend was observed in MIF-knockdown cells (Fig. 7A, right). To investigate the importance of the direct interaction between
NM23-H1 and MIF in the regulation of MIF-induced cell proliferation, we analyzed the effect of wild-type and mutant forms of NM23-H1 on MIF-induced cell proliferation of quiescent NIH 3T3 cells. The coexpression of wild-type NM23-H1, NM23-H1(C4S), or NM23-H1(C109S) inhibited MIF-induced cell proliferation, whereas NM23-H1(C145S) had no effect (Fig. 7B). These results suggested that the physical association between NM23-H1 and MIF plays a critical role in the negative regulation of MIF-induced cell proliferation by NM23-H1. To elucidate the mechanism of inhibition of MIF-induced cell prolifera-

FIGURE 6. Effect of NM23-H1 on the MIF-mediated suppression of p53 stability. A, effect of NM23-H1 on the MIF-induced stabilization of p53-MDM2 complexes. HCT116 and MCF7 cells were transiently transfected with the indicated expression plasmids, and then cell lysates were subjected to immunoprecipitation (IP) using an anti-FLAG antibody, followed by immunoblot analysis using an anti-p53 antibody (top panels). The amount of MDM2 in the immune complexes and the levels of FLAG-NM23-H1 and MIF in total cell lysate were analyzed by immunoblot using an anti-FLAG antibody (second panels) and an anti-FLAG antibody (bottom panels, Lysate), respectively. B, effect of NM23-H1 on MIF-induced p53 ubiquitination. p53-null HCT116 cells (16) were transfected with expression vectors for hemagglutinin-ubiquitin (Ub, 0.6 μg), p53 (1 μg), wild-type or mutant forms of NM23-H1 (1.5 μg each), and wild-type MIF (1.5 μg), as indicated. Cell lysates were subjected to immunoprecipitation (IP) using an anti-p53 antibody, followed by immunoblot analysis using an anti-hemagglutinin antibody to determine the level of p53 ubiquitination (top panel). C, effect of the direct interaction between NM23-H1 and p53 on MIF-induced stabilization of p53-MDM2 complexes. MCF7 cells were transiently transfected with expression vectors for GST-MIF or GST-MIF(C60S) (2 μg each), along with increasing amounts (1 and 2 μg) of FLAG-NM23-H1 or -NM23-H1(C145S), as indicated. Cell lysates were subjected to immunoprecipitation using an anti-FLAG antibody, followed by immunoblot analysis using an anti-p53 antibody to determine the level of association between endogenous p53 and MDM2 (top panels). The expression levels of wild-type (WT) and mutant MIF and NM23-H1 in total cell lysate were analyzed by immunoblot using anti-GST and anti-FLAG antibodies, respectively (third and fourth panels, Lysate). The relative levels of p53-MDM2 complexes were quantitated by densitometry, and -fold increase relative to control parental cells is presented. D, analysis of p53 stability. HCT116 and MCF7 cells were transiently transfected with expression vectors for wild-type or mutant forms of NM23-H1 and MIF (2 μg each). Cells were treated with cycloheximide (CHX) (20 μg/ml) for the indicated time intervals (min), and p53 stability was analyzed by immunoblot using an anti-p53 antibody. The levels of p53 were quantitated by densitometry, and -fold increase relative to untreated cells that expressed wild-type MIF alone is presented. Data are representative of at least three independent experiments. WB, Western blot.
liferation of quiescent NIH 3T3 cells by NM23-H1, we first examined the effect of NM23-H1 on the modulation of the PI3K/PDK1 signaling pathway, which is known to be crucial for many cellular responses, such as cell growth, cell survival, and protein synthesis (17, 33). The coexpression of wild-type NM23-H1, NM23-H1(C4S), or NM23-H1(C109S) with MIF resulted in a significant decrease in several markers of MIF-induced PI3K/PDK1 signaling, including PDK1 kinase activity.

**FIGURE 7.** Effect of NM23-H1 on the MIF-mediated stimulation of proliferation of quiescent NIH 3T3 cells. A, MIF stimulates the proliferation of quiescent NIH 3T3 cells in a dose-dependent manner. NIH 3T3 cells were transfected with increasing amounts of an expression vector for MIF, or 200 nM of an MIF-specific siRNA (1 or 2). Cells were incubated in DMEM supplemented with 0.5% FBS, and then the viable cell number at the indicated times, based on trypan blue exclusion, was determined by counting with a hemocytometer. B, effect of the direct binding of NM23-H1 to MIF on the MIF-mediated stimulation of proliferation of quiescent NIH 3T3 cells. NIH 3T3 cells were transiently transfected with the indicated expression vectors for MIF and wild-type or mutant forms of NM23-H1 (2 μg each), and then incubated in DMEM supplemented with 0.5% FBS. Proliferation was assessed by trypan blue exclusion at the indicated times. Data represents the mean (±S.E.) of three independent experiments. *p < 0.05 relative to control NIH 3T3 cells; significance was calculated by Student’s *t* test (A and B). C, NM23-H1 suppresses MIF-induced PDK1 and p44/p42 ERK activation in quiescent NIH 3T3 cells. NIH 3T3 cells were transfected with the indicated expression vectors for MIF and wild-type or mutant forms of NM23-H1 (2 μg each), and then incubated as described in B. At day 4, cell lysates were analyzed for PDK1 kinase activity (upper left), Akt phosphorylation (upper right), Bad phosphorylation (lower left), and p44/p42 ERK phosphorylation (lower right). The data are representative of three independent experiments. WB, Western blot; IP, immunoprecipitation.
NEGATIVE REGULATION OF MIF BY NM23-H1

(Fig. 7C, upper left), PKB/Akt phosphorylation (Fig. 7C, upper right), and Bad phosphorylation (Fig. 7C, lower left), whereas NM23-H1(C145S), which is unable to bind to MIF (see Fig. 2), had no effect. We next examined whether the inhibition of MIF-induced cell proliferation by NM23-H1 was associated with the activation of p44/p42 ERK (32). In cells that expressed wild-type NM23-H1, NM23-H1(C4S), or NM23-H1(C109S), there was a significant decrease in MIF-induced p44/p42 ERK phosphorylation as compared with control cells that expressed MIF alone, whereas NM23-H1(C145S) had no effect (Fig. 7C, lower right). Together, these results suggested that the direct interaction of NM23-H1 with MIF is also critical for the negative regulation of MIF-induced cell proliferation in quiescent NIH 3T3 cells.

DISCUSSION

We previously identified proliferation-associated gene (PAG) as an interacting partner of MIF and demonstrated that the binding of PAG to MIF could repress the p-dopachrome tautomerase activity of MIF (27). These results suggested that there might be other cellular proteins that interact with and regulate MIF activity. In the current study, we demonstrated that NM23-H1 physically associates with MIF in vivo and negatively regulates several MIF activities, including the suppression of p53-induced apoptosis and cell cycle arrest (Figs. 4–6), the stimulation of cell proliferation in quiescent NIH 3T3 fibroblasts (Fig. 7), and the suppression of JAB1-induced AP-1 transactivation (see supplemental data).

MIF has been shown to have diverse enzymatic activities, including p-dopachrome tautomerase (34), phenylpyruvate tautomerase (35), and thiol protein oxidoreductase (36) activities. Thus, whereas it is not unreasonable to hypothesize that the enzymatic activities of MIF reflect its various functions in cells, the mechanism(s) by which MIF exerts its biological functions is far from clear. As the function of MIF appears to involve interactions with cellular proteins, the identification and characterization of the binding partners of MIF will be helpful in the elucidation of the molecular mechanisms of MIF function. Recently, several proteins, including JAB1 (9), PAG (27), heparin-binding EGF-like growth factor-2 (37), and BCL2/adenovirus E1B 19-kDa interacting protein 2-like (38) have been identified as interacting partners of MIF. However, the role of these MIF-interacting proteins in the diverse cellular actions of MIF is still the object of extensive study. Here we provide evidence that NM23-H1 binds to MIF in vivo through cysteine residues (Fig. 2), and that this association is important in modulating the activity of MIF (Figs. 4–7, and see supplemental data). As shown in Fig. 2, MIF physically interacted with wild-type NM23-H1 as well as NM23-H1(C4S) and NM23-H1(C109S) in mammalian cells, but failed to interact with NM23-H1(C145S), which indicates that Cys145 of NM23-H1 plays a critical role in complex formation between NM23-H1 and MIF. We also identified Cys60 of the redox-active conserved sequence motif (Cys57-Ala-Leu-Cys60) of MIF as a key cysteine residue for complex formation. The association of NM23-H1 with MIF was dependent on redox status (Fig. 2C), similar to previous reports showing the redox-dependent binding of MIF to PAG or p53 (26, 27). MIF has been shown to be a negative regulator of JAB1-induced AP-1 transscription and JNK activity through its ability to interact with JAB1 (9). However, to date, there has been no evidence that the suppression of AP-1 and JNK activity by MIF is regulated by other cellular proteins, i.e. MIF-interacting proteins. Here, we provide evidence that NM23-H1, which interacts with MIF, is a potential negative regulator of the suppression of p53-mediated apoptosis and cell cycle arrest and AP-1 transcriptional activity by MIF (Figs. 4 and 5 and supplemental Fig. S1). However, we cannot exclude the possibility that additional intracellular proteins that interact with MIF are also involved in regulating the suppressive effects of MIF on p53 activity and AP-1 transactivation, because several cellular proteins have recently been identified that interact with MIF (9, 27, 37, 38). Of note, when we investigated the mechanism of regulation of MIF-mediated suppression of JAB1-induced AP-1 transcriptional activity by NM23-H1, we discovered that the substitution mutant NM23-H1(C145S), which is unable to bind to MIF, exerted a partial effect on JAB1-mediated AP-1 transactivation in the presence MIF (see supplemental Fig. S1). Based on these results, we speculate that NM23-H1 exerts its effects on MIF-mediated suppression of AP-1 transcriptional activity in part through its association with JAB1, because wild-type NM23-H1 and all three Cys to Ser substitution mutants of NM23-H1 (C4S, C109S, and C145S) directly interacted with JAB1 and stimulated JAB1-induced AP-1 transcription to a similar extent (see supplemental Fig. S2). Moreover, we observed that NM23-H1 rescued JAB1-induced AP-1 transactivation by destabilizing MIF-JAB1 complexes and counteracting the stabilizing effect of MIF on p27kip1 levels through a direct interaction with MIF (see supplemental Figs. S2 and S3). Similar trends were also observed in the regulation of MIF-mediated suppression of p53-induced transcription, apoptosis, and cell cycle arrest by NM23-H1 (Figs. 4 and 5), which again suggests that in addition to the critical role of the direct interaction between NM23-H1 and MIF, the direct interaction of NM23-H1 and p53 plays a partial role in the alleviation of MIF-mediated suppression of p53 signaling by NM23-H1. We also demonstrated that the physical association of NM23-H1 with MIF inhibits MIF-induced cell proliferation in quiescent NIH 3T3 fibroblasts (Fig. 7). Collectively, these results strongly suggest that the direct interaction between NM23-H1 and MIF plays a key role in the negative regulation of MIF function.

In conclusion, our results provide evidence for a putative mechanism of regulation of the MIF-mediated suppression of p53-induced apoptosis and cell cycle arrest and JAB1-induced AP-1 transcriptional activity by NM23-H1. They also suggest that the ability of NM23-H1 to modulate MIF-induced cell proliferation may underlie its function as a potential tumor suppressor. We suggest that the relative levels of binding of NM23-H1 and MIF, as well as the direct association of NM23-H1 with either p53 or JAB1, play an important role in modulating the MIF-mediated suppression of p53 and JAB1 activity.

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