Macrophage migration inhibitory factor (MIF) is expressed and secreted in response to mitogens and integrin-dependent cell adhesion. Once released, autocrine MIF promotes the activation of RhoA GTPase leading to cell cycle progression in rodent fibroblasts. We now report that small interfering RNA-mediated knockdown of MIF and MIF small molecule antagonist results in a greater than 90% loss of both the migratory and invasive potential of human lung adenocarcinoma cells. Correlating with these phenotypes is a substantial reduction in steady state as well as serum-induced effector binding activity of the Rho GTPase family member, Rac1, in MIF-deficient cells. Conversely, MIF overexpression by adenovirus in human lung adenocarcinoma cells induces a dramatic enhancement of cell migration, and co-expression of a dominant interfering mutant of Rac1 (Rac1N17) completely abrogates this effect. Finally, our results indicate that MIF depletion results in defective partitioning of Rac1 to caveolin-containing membrane microdomains, raising the possibility that MIF promotes Rac1 activity and subsequent tumor cell motility through lipid raft stabilization.

The acquisition of migratory and invasive properties by tumor cells is a central and often fatal step in neoplastic disease progression. Although normal, nontransformed cells have strict growth factor and adhesive requirements for motility, malignant cells have overcome these requirements through multiple mechanisms including gain of function oncogene mutations, growth factor receptor overexpression, and/or constitutive deregulation of extracellular matrix degrading enzymes (1, 2). A critical group of effectors downstream of mutated oncogenes or constitutively active growth factor receptors is the family of Rho GTPase enzymes (3). Of the three main family members, Rac, Rho, and Cdc42, Rac is arguably the most important in promoting and maintaining an invasive phenotype (3–5). For example, in addition to promoting actin cytoskeletal reorganization, Rac is also necessary for nonsmall cell lung cancer matrix metalloprotease expression and subsequent invasive behavior (4).

Among the described Rac effectors, p21-activated kinase (PAK)2 family members are considered to be responsible for many Rac-dependent cytoskeletal effects (6–8). In addition to the well described properties of GTP-loaded Rac and/or Cdc42 in PAK effector binding and activation, del Pozo et al. (9) recently described an additional requirement for Rac-mediated PAK activation. Integrin-dependent membrane translocation of GTP-loaded Rac was shown to be necessary for Rac-dependent PAK activation. Serum-induced PAK activation was shown to be defective in cells held in suspension, and this defect could not be rescued by the introduction of constitutively active mutants of RacV12 (9). Importantly, the requirement for cell adhesion in Rac membrane localization and subsequent PAK activation was independent of GTP-loading of Rac consistent with the inability of serum or RacV12 mutants to activate PAK in suspension cells. Rather, suspension cells were found to contain higher levels of GTP-loaded Rac bound to Rho-GDI in the cytoplasm, thus conferring spatially constricted Rac effector activation (6). Subsequent studies by the same group demonstrated that the requirement for cell adhesion in Rac-dependent PAK activation was due to the stabilization of caveolin-containing, cholesterol-enriched membrane microdomains and that these “lipid rafts” are necessary for Rac-GTP-mediated PAK activation (10, 11).

Many studies have reported that MIF expression is increased in premalignant, malignant, and metastatic tumors (12, 13). Breast-, prostate-, colon-, brain-, skin-, and lung-derived tumors have all been shown to contain significantly higher levels of MIF message and protein than their noncancerous cell counterparts (14–16). Several reports also indicate that MIF expression closely correlates with tumor aggressiveness and metastatic potential, possibly suggesting an important contribution to disease severity by MIF (12, 17, 18). MIF has been
indirectly implicated in tumor growth and progression by stimulating tumor-dependent stromal processes such as neovascularization (16, 19, 20) as well as macrophage and lymphocyte activation and survival (21, 22). In fact, certain tumors possess an important functional requirement for MIF in maintaining optimal growth and progression (16, 23, 24). However, the mechanistic processes induced by MIF to achieve these protumorigenic functions are still largely unresolved.

We previously demonstrated that murine fibroblasts secrete MIF in response to growth factor stimulation of quiescent cells (25). Both growth factor-induced and exogenously added MIF activate the ERK subfamily of mitogen-activated protein (MAP) kinases in a sustained fashion. MIF activation of ERK MAP kinase was subsequently shown to contribute to growth factor-stimulated cell cycle progression (25). In addition to the discovery that MIF participates in growth factor signaling to MAP kinase, later studies revealed a critical role for MIF in the modulation of adhesion-dependent activation of MAP kinase, also in a sustained fashion (26). Subsequent studies found that MIF is both necessary and sufficient for growth factor plus adhesion-induced sustained MAP kinase activation leading to cyclin D1 expression (26, 27). MIF-dependent cyclin D1 expression was found to be dependent upon the activity of RhoA GTPase-mediated stress fiber formation and subsequent focal adhesion kinase activation leading to the sustained stimulation of ERK MAP kinases (27). Because Rho GTPase family members are central regulators of actin cytoskeletal dynamics leading to proliferative, migratory, and invasive properties, we sought to investigate whether MIF might functionally contribute to human lung adenocarcinoma growth and metastatic potential.

EXPERIMENTAL PROCEDURES

Cell Culture—A549 cells were grown in DMEM, 1% fetal calf serum, 2 mM glutamine, and 50 μg/ml gentamicin. pLXSN-A549 and pLXSN-RacV12-A549 cells were grown in the same medium supplemented with 400 μg/ml Geneticin (Invitrogen). Primary normal human bronchial epithelial cells (NHBE; Cambrex, Baltimore, MD), T antigen NHBE, and T antigen/RasV12 NHBE cells (generous gifts of Dr. Barrett Rollins, Dana Farber Cancer Institute, Boston, MA) (28) were cultured in bronchial epithelial growth medium with supplied supplements (Cambrex). For secreted MIF studies, semi-confluent cells were cultured in Opti-MEM serum-free medium for 48 h, concentrated in Microcon-10s (Amicon), and immunoblotted for MIF.

RNA Interference—Two independent MIF siRNAs were used for silencing of MIF in these studies. The targeted base sequences for human MIF were 5′-CCCTTCTGGTGGG-GAGAAT-3′ (corresponding to the 3′-untranslated region of human MIF mRNA) and 5′-CAACTCCACCTGGCTCAA-3′ (corresponding to the 3′ end of the open reading frame) (Dharmacon, Lafayette, CO). As negative controls, both a commercially available siRNA and a scrambled siRNA based on the sequence of siRNA 1 above were used interchangeably (both referred to as nonspecific, NS). In all cases, the cells were transfected with 50 nM annealed siRNA oligonucleotides using Oligofectamine reagent (Invitrogen) following the manufacturer’s protocol. The cells were incubated at 37 °C for 48 or 72 h and subjected to further analysis. For extracellular MIF reconstitution experiments, parallel control siRNA (NS) transfected cell supernatants were collected 36 h after transfection, mixed 1:1 with fresh medium, and placed on MIF siRNA transfected cells (transfected at the same time as control). For the nonreconstituted plates, parallel MIF siRNA transfected cell supernatants were collected 48 h after transfection, mixed 1:1 with fresh medium, and placed on MIF siRNA transfected cells.

Adenovirus Preparation and Cell Infection—Rac1N17 recombinant adenovirus was purchased from Cell Biolabs (San Diego, CA) and was used to infect cells at 5 × 10⁶ virus particles/ml. Murine MIF cDNA was PCR-amplified using GGTTAACCATT-GCCTATGTTCTACGTG (forward) and GCTCGAGTCGAAGC-GAAGGTGGAACC (reverse) oligonucleotides. The PCR fragment was cloned in the pGEM(R)-T Easy vector (Promega), sequenced, and subcloned into the pAdTrack-CMV shuttle vector and was then co-transformed into Escherichia coli BJ5I83 cells with a pAdEasy-1 adenoviral backbone plasmid. Recombinant virus was produced in 293A cells and purified by CsCl gradient. Viral titer was determined by multiplicity of infection testing of 293A cells and was used to infect cells at 5 × 10⁶ virus particles/ml.

Rho and Rac Effector Binding Assay—The rhotein-binding domain (RBD) and PAK-binding domain (PBD)-GST plasmids were kindly provided by Dr. Keith Burridge (University of North Carolina). The plasmids were transformed into BL-21 E. coli and GST-PBD (for Rac) and GST-RBD (for Rho) were purified as described (29). 2 × 10⁶ cells/sample were lysed in 10-cm dishes with lysis buffer (50 mM Tris, pH 7.5, 500 mM NaCl, 0.1% SDS, 0.5% DOC, 1% Triton X-100, and 0.5 mM MgCl₂, followed by centrifugation at 14,000 rpm for 10 min at 4 °C. For GTPγS experiments, the cells were lysed in lysis buffer (above) supplemented with 20 mM EDTA (for all samples) and 100 μM GTPγS (for the + GTPγS samples) followed by incubation for 10 min at 30 °C (30). Lysates were incubated with 60 μl of a 50% slurry of GST-RBD beads or 30 μl of GST-PBD beads pre-equilibrated with lysis buffer. After rotating for 30 min at 4 °C the beads were collected by centrifugation, washed once in lysis buffer, and released by boiling in 1× Laem ml sample buffer. Rac1 and RhoA effector binding determination and total Rac1 and RhoA from lysates were assessed by Western blot analyses using RhoA (Santa Cruz), and Rac1 (Upstate Biotechnology, Inc.) antibodies.

Migration and Invasion Assays—For migration assays, modified Boyden chambers (Millipore-PCF, 8-μm pore size; Millipore, Bedford, MA) were placed in a 24-well plate and coated with 10 μg/ml rat tail collagen (Roche Applied Science) for 16 h at 37 °C. After removal of collagen and washing with PBS, migration medium (DMEM with 0.5% bovine serum albumin) was added to lower chamber in 0.4 ml. For the invasion assays, growth factor-reduced Matrigel matrix-coated transwell chambers (Becton Dickinson Lab Ware, Two Oak Park, Bedford, MA) were placed in a 24-well plate containing invasion medium (DMEM with 10% fetal bovine serum). Transfected or treated A549, pLXSN-A549, or pLXSN-RacV12-A549 cells were added to the upper compartment (2 × 10⁵ in 0.3 ml of migration or invasion medium, respectively). The plates were incubated at 37 °C for 16 h for migration and 36 h for invasion. The
Increased MIF secretion from premalignant and malignant bronchial epithelial cells contributes to migration of lung adenocarcinoma cell lines. A, normal (lane 1), immortalized (lane 2, T-Ag), and H-rasV12 transformed (lane 3, T-Ag/ras) normal human bronchial epithelial cells were cultured for 48 h, and supernatant was collected, normalized against cell number, concentrated, and then assayed for MIF by Western blot. The data shown are the results from two independent experiments (Expts). B, A549 human lung adenocarcinoma cells were transfected in duplicate with either a scrambled nonsense (NS) or MIF-specific siRNA for 48 h. Corresponding cell lysates were subjected to immunoblotting for MIF and β-actin. C, A549 human lung adenocarcinoma cells were transfected with either a scrambled nonsense or MIF-specific siRNA for 48 h. MIF-conditioned supernatant from NS siRNA cells was added at 1:1 with fresh medium for MIF add back. The results are representative of three independent experiments and were performed in duplicate. For migration assay: *p < 0.05; **p < 0.01; ***p < 0.001 by Student's t test (two-tailed).

FIGURE 1. Increased MIF secretion from premalignant and malignant bronchial epithelial cells contributes to migration of lung adenocarcinoma cell lines. A, normal (lane 1), immortalized (lane 2, T-Ag), and H-rasV12 transformed (lane 3, T-Ag/ras) normal human bronchial epithelial cells were cultured for 48 h, and supernatant was collected, normalized against cell number, concentrated, and then assayed for MIF by Western blot. The data shown are the results from two independent experiments (Expts). B, A549 human lung adenocarcinoma cells were transfected in duplicate with either a scrambled nonsense (NS) or MIF-specific siRNA for 48 h. Corresponding cell lysates were subjected to immunoblotting for MIF and β-actin. C, A549 human lung adenocarcinoma cells were transfected with either a scrambled nonsense or MIF-specific siRNA for 48 h. MIF-conditioned supernatant from NS siRNA cells was added at 1:1 with fresh medium for MIF add back. The results are representative of three independent experiments and were performed in duplicate. For migration assay: *p < 0.05; **p < 0.01; ***p < 0.001 by Student’s t test (two-tailed).

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MIF Is Overexpressed in Premalignant and Malignant Bronchial Epithelial Cells and Is Necessary for Human Lung Adenocarcinoma Cell Migration—Prior studies reported an inherent requirement for MIF in oncogenic ras mediated malignant transformation of rodent fibroblasts in defined settings (31, 32). Furthermore, MIF is overexpressed in human lung adenocarcinomas (33, 34), and mutations of ras are estimated to occur in up to 50% of lung cancers (35). We determined whether there was a positive level of regulation of MIF expression and secretion by immortalizing and transforming oncogenes in lung bronchioalveolar epithelial cells. Three independent cell types representing three stages of malignant transformation of human lung epithelial cells were evaluated (28). Levels of secreted MIF from normal (primary), immortalized (SV40 large T-antigen), and transformed (T-Ag/rasV12) human bronchial epithelial cells (28) were determined by culturing equal numbers of each cell type for 48 h, normalizing supernatant to cell number, and then assessing by Western blotting. As shown in Fig. 1A, two independent experiments demonstrate that both human lung epithelial cell immortalization and human lung epithelial cell immortalization/ transformation results in a significant increase in secreted MIF, suggesting that immortaliz-
ing and transforming oncoproteins induce the expression and/or secretion of MIF (by densitometry, average increase MIF secretion over NHBE was: T-Ag = 2.02-fold, T-Ag/ras<sup>121</sup> = 2.58-fold). These results, coupled with the high expression of MIF previously observed in human lung adenocarcinoma cell lines and tumors (20, 33, 36), suggest that bronchial epithelial cell MIF expression may reflect the relative transformation state of nonsmall cell lung cancers (NSCLC).

We recently described a central role for MIF in the regulation of steady state RhoA activity in mesenchymal cell signaling to cyclin D1 expression. Additionally, Sun et al. (37) showed that MIF contributes to mitogen-induced migration and cell invasion in a RhoA-dependent manner. To determine whether human NSCLC cells were similarly dependent upon MIF for motility, we inhibited the expression of MIF by siRNA oligonucleotides in the human nonsmall cell lung adenocarcinoma cell line, A549. As shown in Fig. 1B, transfection of siRNA oligonucleotides directed against human MIF consistently resulted in the loss of MIF expression when compared with scrambled siRNA oligonucleotides (NS) or mock transfection in the A549 cell line. Importantly and consistent with prior observations (37), siRNA-mediated MIF depletion resulted in a profound inhibition of human lung cancer cell migration on collagen-coated transwell chambers (Fig. 1C, top panel). Reconstitution of MIF by MIF-containing conditioned supernatants restored, by greater than 70%, the ability of the MIF-depleted cells to migrate (Fig. 1C, bottom panel).

**Silencing or Inhibition of MIF Results in a Loss of Cell Adhesion and Invasive Potential in Human Lung Adenocarcinoma—** Because integrin-dependent binding to extracellular matrices is necessary for actin reorganization and subsequent motility, we next evaluated whether siRNA-mediated depletion of MIF influenced NSCLC cell adhesion and spreading on collagen. As shown in Fig. 2A, depletion of MIF by siRNA greatly reduced the adhesive ability of NSCLC cells. This loss of adhesion associated with MIF deficiency was likely due to the observed inability of these cells to adequately and appropriately spread when plated onto their normal extracellular matrix substrate (Fig. 2A, top panels). Importantly, when MIF-depleted cells were cultured in MIF-containing conditioned medium, the cells spread and adhered much more efficiently, suggesting that this defect is specifically due to loss of extracellular MIF (Fig. 2A).

The observed loss of adhesive and migratory properties in MIF-depleted cells would predict that these cells would be similarly defective in invasive potential. As such, we next investigated whether MIF was necessary for NSCLC cell invasion. As shown in Fig. 2B, cells (NS siRNA versus MIF siRNA) lacking MIF were consistently more than 80% defective in migrating through a basement membrane (Matrigel). In parallel, we also tested whether treatment of A549 cells with a well-characterized small molecule inhibitor of MIF, ISO-1 (38–40), reduced the invasive capacity of these cells. As shown in Fig. 2B, inhibition of MIF by small molecule antagonist results in a loss of invasive potential that is nearly identical to that observed with MIF depletion with siRNA. As such, using two independent means of MIF inhibition, our results strongly indicate that there is an inherent requirement for MIF for NSCLC motility and metastatic potential. Moreover, these findings are consistent with the notion that endogenous, cellular MIF is an important participant in adhesion-dependent processes (26, 27).

**Silencing or Inhibition of MIF in NSCLC Cells Results in Loss of Anchorage-independent Growth—** Because MIF participates in adhesion-dependent signaling processes, we hypothesized that the increased MIF expression observed in NSCLC cell lines and tumors (20, 33, 36) may be permissive to adhesion-independent signaling processes. To test this, we evaluated whether the targeted depletion of MIF in lung adenocarcinoma cells conferred resistance to anchorage-independent growth. Mock transfected, nonsense siRNA (NS siRNA) transfected and MIF siRNA transfected cells were placed into soft agar to determine
whether cells lacking MIF could divide and form colonies. As shown in Fig. 3A, cells lacking MIF were refractory to soft agar colony growth. Of perhaps greater importance, soft agar co-cultivation of lung adenocarcinoma cells with the ISO-1 MIF inhibitor reduced anchorage-independent growth by roughly 50% (Fig. 3B). Although these studies support the conclusion that MIF is an important endogenous mediator of anchorage-independent growth, further studies were needed to investigate the mechanism behind both the adhesion-dependent and adhesion-independent phenotypes conferred by MIF.

**Active Rac1-mediated Migration and Effector Binding**—Based on these findings we suspected that MIF-dependent modulation of Rac1 effector binding/activation was likely involved in MIFs contribution to observed NSCLC malignant phenotypes (Figs. 1–4). To further explore this possibility, we sought to restore the defective migratory phenotype of MIF-depleted cells with a constitutively active allele of Rac1 (Rac1V12). Stable cell lines containing pLXSN vector alone or pLXSN-Rac1V12 (44) were generated in A549 cells by retroviral infection and were tested for rescue of the loss of NSCLC migration associated with loss of MIF. As would be expected, A549-Rac1V12-expressing cells were significantly more motile than vector control cells (Fig. 5A). We were very surprised to see, however, that MIF-depleted A549-Rac1V12 cells exhibited no more migration than MIF-depleted A549 vector control cells (Fig. 5A).

We next tested whether Rac1V12 effector binding was compromised in MIF-deficient cells as we had previously found with serum-induced Rac1 binding to PBD-GST beads. As shown in Fig. 5B, MIF depletion in both vector control and Rac1V12-expressing cells were significantly more motile than vector control cells (Fig. 5A). We were very surprised to see, however, that MIF-depleted A549-Rac1V12 cells exhibited no more migration than MIF-depleted A549 vector control cells (Fig. 5A).

Surprisingly, lysates from cells depleted in MIF had a moderate increase in the relative amount of RhoA binding to RBD compared with a significant decrease in the amount of Rac1 capable of binding to PBD (Fig. 4A). MIF-depleted A549 cells also contained a corresponding decrease in phosphorylated c-Jun N-terminal kinase (JNK) that corresponded with substantially reduced steady state c-Jun phosphorylation. Note that the decreased JNK and c-Jun phosphorylation levels closely correlated with the observed loss of Rac1 binding to PBD beads (Fig. 4A). These results are consistent with the well documented effector status of JNK in Rac1-dependent signal transduction (42, 43).

We previously reported that endogenous MIF is necessary for serum + integrin-induced RhoA-dependent signaling to cyclin D1 expression (26, 27). To investigate whether MIF was similarly required for serum-induced Rac1 activation, MIF-depleted cells were stimulated with or without 20% serum to evaluate Rac1 effector binding to PBD beads. Interestingly, not only was basal Rac1 binding to PBD compromised in MIF-deficient cells, serum induction resulted in virtually no increase in Rac1-PBD binding in MIF-depleted cells, whereas control cells showed a 2.5-fold increase (Fig. 4B).

Because these findings suggested a requirement for MIF in maximal steady state and growth factor-induced Rac1 activation levels and migratory processes, we next tested whether MIF was sufficient for Rac1 activation and subsequent NSCLC migration. Adenovirus was used to ectopically overexpress MIF in A549 lung adenocarcinoma cells in the presence or absence of an interfering mutant of Rac1 (Rac1N17). As shown in Fig. 4C, MIF overexpression strongly induced A549 cell migration, whereas Rac1N17 very efficiently inhibited both basal and enhanced migration observed with Ad-MIF alone, even though increased expression of MIF was observed in parallel cell lysates (supplemental Fig. S1).

**MIF Is Necessary for Both Endogenous and Constitutively Active Rac1-mediated Migration and Effector Binding**—Based on these findings we suspected that MIF-dependent modulation of Rac1 effector binding/activation was likely involved in MIFs contribution to observed NSCLC malignant phenotypes (Figs. 1–4). To further explore this possibility, we sought to restore the defective migratory phenotype of MIF-depleted cells with a constitutively active allele of Rac1 (Rac1V12). Stable cell lines containing pLXSN vector alone or pLXSN-Rac1V12 (44) were generated in A549 cells by retroviral infection and were tested for rescue of the loss of NSCLC migration associated with loss of MIF. As would be expected, A549-Rac1V12-expressing cells were significantly more motile than vector control cells (Fig. 5A). We were very surprised to see, however, that MIF-depleted A549-Rac1V12 cells exhibited no more migration than MIF-depleted A549 vector control cells (Fig. 5A).

We next tested whether Rac1V12 effector binding was compromised in MIF-deficient cells as we had previously found with serum-induced Rac1 binding to PBD-GST beads. As shown in Fig. 5B, MIF depletion in both vector control and Rac1V12-expressing cells rendered both endogenous and constitutively active Rac1 proteins defective in binding to PBD-GST beads (by densitometry of PBD bound/total Rac: V/NS = 1, V/MIF = 0.35, RacV12/NS = 3.46, RacV12/MIF = 0.89).

**FIGURE 3.** siRNA-mediated silencing of MIF expression results in a loss of anchorage-independent growth. A, A549 cells were transfected as described or left untreated. 48 h later cells were lifted and plated in soft agar at 5,000 cells/6-cm dish in duplicate. 18 days later colonies were counted under low power magnification (40×). The results shown are the means ± S.D. of the averages of two experiments each using the averages from five low powered field counts. B, A549 human lung adenocarcinoma cells were pre-treated with vehicle (0.1% dimethyl sulfoxide (DMSO)), 10 μM or 100 μM ISO-1 for 24 h before plating 5 × 10^4 cells in soft agar containing in vehicle or indicated concentration of ISO-1. 20 days later, the colonies were counted under low power magnification (40×). The results shown are the means ± S.D. of the averages of duplicate plates and are representative of two independent experiments. For A and B: *, p < 0.05; **, p < 0.01; ***, p < 0.001 by Student’s t test (two-tailed).
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Importantly, this loss of GST-PBD binding correlates almost exactly with the defect observed in migration upon loss of MIF (Fig. 5A), suggesting that GST-PBD binding likely reflects Rac1 effector activation and subsequent migration.

To determine whether this apparent requirement for MIF in both endogenous and constitutively active Rac1 effector binding and activation was, in fact, due to loss of MIF, we reconstituted MIF expression by adenoviral MIF. Because our siRNA target sequence is based on the mRNA of human MIF, we used a murine MIF adenovirus for these studies. Importantly, murine MIF is not affected by the human MIF siRNA oligonucleotides used for these experiments. Similar to Fig. 5B, MIF depletion significantly reduced both the basal and constitutively active Rac1 binding to PBD-GST beads, and Ad-MIF very efficiently rescued both the binding of Rac1 to PBD beads (Fig. 5C) (by densitometry of PBD bound/total Rac; NS/- = 1, NS/Ad-MIF = 1.53, MIF/- = 0.28, MIF-Ad-MIF = 0.63) and the defective migratory phenotype associated with loss of MIF in the presence of Rac1V12 (supplemental Fig. S1).

Combined, these results were extremely surprising to us given the fact that the gain of function activity of this Rac1 mutant is due to its inability to hydrolyze GTP and should therefore have GTP constitutively loaded in its nucleotide binding cleft. This, coupled with the fact that serum was also unable to rescue Rac1 binding to PBD-GST beads from MIF-deficient cell lysates, suggested to us that GTP loading of both endogenous and constitutively active Rac1 might be compromised in the absence of MIF. To investigate whether there was a defect in Rac1 GTP loading in MIF-deficient cells, GTPγS was added to control and MIF-depleted cell lysates, and PBD-GST pulldown assays were performed. As shown in Fig. 5D, in control cell lysates, GTPγS dramatically enhanced Rac1 binding to PBD-GST beads, pre-
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FIGURE 6. MIF depletion disrupts lipid raft marker partitioning. pLXSN-A549 or pLXSN-RacV12-A549 cells were transfected with either NS or MIF siRNA oligonucleotides. A, 48 h later transfected cells were lysed in 0.5% Triton X-100, and Triton X-100-soluble and insoluble fractions were analyzed for caveolin by immunoblotting. B, sucrose gradient fractionation of pLXSN-A549 NS or MIF siRNA transfected cells followed by caveolin and flotillin-2 immunoblotting. C, sucrose gradient fractionation of pLXSN-RacV12/A549 NS or MIF siRNA transfected cells and subsequent caveolin and Rac1 immunoblotting. D, densitometric analysis of relative ratios of band intensities of raft-associated Rac1 over nonraft-associated Rac1 using Scion Image software. The data shown are representative of four independent experiments, except for the Rac1 targeting, which is representative of two experiments.

sumably by loading Rac1 with this unhydrolyzable GTP analog. In stark contrast, however, neither endogenous nor constitutively active Rac1 from MIF-deficient cell lysates displayed an increase in PBD-GST binding when exposed to GTPγS (Fig. 5D). Combined, these results suggested to us that there was an inherent defect in the ability of Rac1 from MIF-deficient cell lysates to be loaded with GTP. Moreover, the failure of Rac1 to be loaded with GTP could conceivably account for the inability of MIF-deficient cells to carry out Rac1-dependent cytoskeletal processes.

MIF Regulates Caveolin-1 Partitioning to Cholesterol-enriched Membrane Microdomains—The observed loss of MIF-associated defects in Rac1 GTP loading and effector binding/activation are consistent with an inability of Rac1 to dissociate from the Rho GDP dissociation inhibitor (RhoGDI) (45). RhoGDI acts to maintain Rho family members in a GDP-bound state, and when bound to RhoGDI these proteins are unable to be loaded with either GTP or GTPγS (46, 47). A similar inability of RhoGDI to uncouple from Rac thus preventing effector activation has been described in nontransformed cells held in suspension (6). Studies by del Pozo et al. (9) demonstrated that disruption of integrin-dependent signaling renders cells resistant to both serum and RacV12-induced activation of the Rac1 effector, PAK. Subsequent studies revealed that the requirement for cell adhesion in Rac and RacV12 effector activation lies in the ability of Rac to localize to caveolin-containing lipid raft membrane microdomains, the partitioning of which is governed by the phosphorylation state of caveolin (10, 11). These similarities to MIF-deficient cells suggested to us that Rac targeting to caveolin-containing lipid rafts might represent a point of control by MIF in its modulation of NSCLC Rac1 effector binding and activation.

We first investigated whether MIF expression influenced targeting of the lipid raft marker, caveolin, to cholesterol-enriched membrane microdomains (lipid rafts) (10, 11). As an initial approach, we used the classical lipid raft separation technique of Triton X-100 membrane insolubility to determine whether there were any differences in caveolin partitioning associated with loss of MIF. Triton X-100-soluble and insoluble fractions from control siRNA transfected and MIF siRNA transfected cells were obtained by high speed centrifugation and then analyzed for caveolin by Western blotting. As shown in Fig. 6A, MIF-depleted cells contained significantly reduced caveolin in detergent-resistant fractions and slightly more in soluble fractions (by densitometry: soluble: V/NS = 1, V/MIF = 1.14, RacV12/NS = 0.84, RacV12/MIF = 1.09; insoluble: V/NS = 1, V/MIF = 0.41, RacV12/NS = 1.16, RacV12/MIF = 0.31). These findings were validated with a more detailed analysis of membrane fractionation by sucrose gradients. In parental cells or pLXSN vector-containing A549 cells, we found that lipid raft fractions consistently corresponded to the light sucrose fractions 2 and 3 and sometimes fraction 4, and nonraft and cytosolic fractions concentrated in fractions 7 and 8. As shown in Fig. 6B, our results indicate that knockdown of MIF results in a significant loss of lipid raft-associated caveolin and flotillin-2 with a corresponding increase in nonlipid raft partitioning of these markers. Importantly, we also find that the loss of caveolin in lipid rafts is associated with a corresponding decrease in Rac1 partitioning to these lipid raft-enriched fractions. Although cytosolic Rac1 levels were slightly less in MIF-depleted cells, the relative ratio of raft versus nonraft Rac1 was more than 50% less in MIF-deficient cells. The fact that this is observed in Rac1V12-A549 cells suggests that defective partitioning of Rac1V12 to lipid rafts in MIF-depleted cells may be responsible for the observed loss of enhanced effector binding and migration found in MIF-deficient cells (Fig. 5).

DISCUSSION

Our findings that MIF is necessary for human lung adenocarcinoma cell migration, invasion, and anchorage-independent growth are further evidence that MIF plays a central role in human neoplastic disease. We now report that the Rho GTPase family member, Rac1, is a likely mediator of MIF actions on cell migration and metastatic invasion in human NSCLC cells. Importantly, our results indicate that MIF is both sufficient and necessary for both endogenous Rac1 and ectopically expressed Rac1V12 distal signaling leading to lung adenocarcinoma cell migration.

This defect in Rac1 activity is remarkably similar to studies describing a requirement for cell adhesion in Rac1 membrane translocation and effector activation (6, 9). Intriguingly, like cells held in suspension, MIF-deficient cells exhibit defective membrane partitioning of caveolin and Rac1 to lipid rafts.
Because the internalization state of caveolin dictates Rac membrane localization and effector activation (6, 9–11), it is possible that defective caveolin partitioning to lipid rafts in MIF-deficient cells is causal to the observed defects in migration, invasion, and anchorage-independent phenotypes in MIF-depleted cells. Although more work is needed to definitively make any conclusions linking MIF-dependent changes in lipid raft dynamics to Rac1 activity, our results are suggestive of a potential regulatory role for MIF in this pathway (9, 10).

This possibility is especially intriguing given the fact that integrin-dependent adhesion induces the rapid secretion of preformed MIF from cells (26). Prior studies from our laboratory demonstrated that integrin-mediated MIF secretion participates in adhesion-dependent signal transduction in rodent fibroblasts (26, 27). It is therefore possible that both oncogene-induced (Fig. 1) and integrin-dependent (26) MIF expression and secretion contribute to lipid raft partitioning and serve to influence specific membrane-initiated signaling events. This supposition is consistent with the idea that malignant cells possess mechanisms that serve to override suppressive signals (i.e. lipid raft internalization leading to defective Rac1-dependent effector activation), thus allowing for anchorage-independent growth and invasive behavior. It is not unreasonable to speculate that MIF serves as just such a mechanism, elaborated by tumor cells and used to enhance both adhesion-dependent and adhesion-independent Rac1 membrane localization and effector activation. Although more work is needed to elucidate the mechanism behind MIF-dependent lipid raft dynamics and its effects on Rac1 activity, these results provide the first description of a requirement for MIF in Rac1 activation and lipid raft organization.

Recent studies have described important roles for MIF in contributing to human brain and prostate and murine colon tumor growth and invasive properties (37, 48, 49). One of these studies concluded that the requirement for MIF in promoting mitogen-induced migration and invasion was at the level of RhoA activity (37). These data support our earlier findings in murine fibroblasts that MIF predominantly affects RhoA activity and has little to no effect on Rac1 (27). Combined with our current findings, we hypothesize that there is a cell type dependence for whether MIF contributes primarily to RhoA or Rac1 activation. This supposition is supported by the fact that MIF is classically defined as a migration inhibiting cytokine in monocytes/macrophages (50) but is clearly promigratory and proinvasive in several tumor cell lines (37, 48, 49) including NSCLC. This apparent paradox is also consistent with the conflicting contributions of different Rho family members to migration states of cells. That is to say, anti-migratory signals have been associated with increased RhoA activity (51), whereas promigratory signals are frequently the result of increased Rac1 activity (52). This rationalization also supports our observations that loss of MIF in murine fibroblasts results in increased migration3 consistent with decreased Rho activity (27). Whereas this explanation may account for these differences in Rho family activation and migration patterns mediated by MIF, the regulation of Rho family members and their relative roles in directing cell migration/invasion is complicated by other variables such as cross-talk among family members and downstream effector activation patterns (51, 52).

Small molecule antagonists rationally designed against MIF have been shown to neutralize MIF-dependent bioactivities (38, 53, 54). Our current studies reveal that one of these compounds, ISO-1, is almost as effective as MIF siRNA in blocking NSCLC invasive and anchorage-independent phenotypes consistent with a recent study in human prostate cancer (49). Because of the potent anti-tumor effects associated with loss or inhibition of MIF, we and others are currently evaluating the viability of treating established tumors in several mouse models of cancer. Our findings, coupled with earlier studies reporting MIF-dependent effects on tumor-associated angiogenesis (16, 19, 20, 55) and Th1 tumor response inhibition (41), suggest that small molecule targeting of MIF may represent a unique and clinically viable approach to lung cancer disease intervention.

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