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Cooperative Regulation of Non-Small Cell Lung Carcinoma Angiogenic Potential by Macrophage Migration Inhibitory Factor and Its Homolog, D-Dopachrome Tautomerase[1]

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Tumor-derived growth factors and cytokines stimulate neoangiogenesis from surrounding capillaries to support tumor growth. Recent studies have revealed that macrophage migration inhibitory factor (MIF) expression is increased in lung cancer, particularly non-small cell lung carcinomas (NSCLC). Because MIF has important autocrine effects on normal and transformed cells, we investigated whether autocrine MIF and its only known family member, D-dopachrome tautomerase (D-DT), promote the expression of proangiogenic factors CXCL8 and vascular endothelial growth factor in NSCLC cells. Our results demonstrate that the expression of CXCL8 and vascular endothelial growth factor are strongly reliant upon both the individual and cooperative activities of the two family members. CXCL8 transcriptional regulation by MIF and D-DT appears to involve a signaling pathway that includes the activation of JNK, c-jun phosphorylation, and subsequent AP-1 transcription factor activity. Importantly, HUVEC migration and tube formation induced by supernatants from lung adenocarcinoma cells lacking either or both MIF and D-DT are substantially reduced when compared with normal supernatants. Finally, we demonstrate that the cognate MIF receptor, CD74, is necessary for both MIF- and D-DT-induced JNK activation and CXCL8 expression, suggesting its potential involvement in angiogenic growth factor expression. This is the first demonstration of a biological role for D-DT, and its synergism with MIF suggests that the combined therapeutic targeting of both family members may enhance current anti-MIF-based therapies. ***The Journal of Immunology,*** **2008, 181: 2330–2337.**

Lung cancer is one of the leading causes of cancer-related deaths in the United States. Overall, survival is poor and has not improved substantially during the last half century (1, 2). Of the three types of lung cancer, the most common are non-small cell lung carcinoma (NSCLC)[3] and small cell lung carcinoma (SCLC), which together account for >90% of all pulmonary malignant diseases. Tumor growth, invasion, and metastasis are dependent on a proangiogenic environment. In normal tissues, angiogenesis is carefully regulated by maintaining an even balance between pro- and antiangiogenic factors. However, within a tumor microenvironment, the balance is tipped in favor of proangiogenic growth factors that serve to promote a constant, disorganized state of blood vessel formation (3).

Vascular endothelial growth factor (VEGF) is a key mediator of tumor-associated angiogenesis and is thought to support neovascularization by inducing endothelial cell migration and proliferation leading to vascular permeability (4). In addition to VEGF, CXCL8 (formerly referred to as IL-8) supports angiogenesis directly through the induction of endothelial cell chemotaxis and proliferation and indirectly by recruiting inflammatory effector cells to tumor tissue where they secrete additional proangiogenic factors (5). A member of the CXC chemokine family, CXCL8 binds to and signals from CXCR2 receptors, which are reportedly the primary functional chemokine receptors in endothelial cell chemotaxis (6). As with VEGF, inflammatory cell-derived CXCL8 has been shown to be associated with promoting angiogenesis in NSCLC (7–9).

One of the first cytokine activities ever described, macrophage migration inhibitory factor (MIF) is a proinflammatory cytokine that is released from activated macrophages and other immune effector cells (10, 11). Several recent studies have shown that MIF is overexpressed in a variety of human tumors, most notably non-small cell lung cancers (7, 12–15).

From crystallographic studies, MIF exists as a homotrimer and shares structural homology with two bacterial enzymes (16). Extracellular MIF signals through a CD74/CD44 cell-surface receptor complex that serves to initiate MIF-dependent activation of several signaling pathways (17, 18). Interestingly, a recent study revealed that MIF is also a noncognate ligand for both CXCR2 and CXCR4 chemokine receptors (19).

Unlike other cytokines, MIF has the unique ability to catalyze a nonphysiologic enzymatic reaction. MIF converts D-dopachrome into 5,6-dihydroxyindole-2-carboxylic acid. The only known MIF homolog, D-dopachrome tautomerase (D-DT), retains this tautomerase activity but also decarboxylates the D-dopachrome substrate to give a 5,6-dihydroxyindole product (20). While D-DT

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[3] Abbreviations used in this paper: NSCLC, non-small cell lung carcinoma; D-DT, D-dopachrome tautomerase; HIF, hypoxia-inducible factor; MIF, migration inhibitory factor; SCLC, small cell lung carcinoma; siRNA, small interfering RNA; VEGF, vascular endothelial growth factor.

retains only 38% identity and 49% homology to MIF, the tertiary structure of D-DT is remarkably similar (21). Despite these intriguing similarities to the very well-studied MIF, there have been no reports to date on the biologic functions of D-DT.

Several studies have shown that MIF promotes both the expression and secretion of CXCL8 and VEGF from an array of different cell types (22–25). Interestingly, one such study reported that lung adenocarcinoma-derived MIF induces the expression of stromal macrophage CXCL8 (26). A recent investigation from our laboratory revealed that in human lung adenocarcinoma cells, autocrine-acting MIF is necessary for Rho GTPase Rac1 effector binding, JNK activation, and subsequent cell migratory and invasive properties (12). Not coincidentally, the JNK activation pathway has been demonstrated to be responsible for the transcription of several gene products induced by MIF (27–29, 30).

Because the importance of contributions of MIFs to many tumor-associated processes is generally accepted, we sought to determine whether the only known MIF homolog functionally regulates, or similarly contributes to, MIF-dependent tumor angiogenesis. We now present evidence that MIF and D-DT, individually and additively, promote VEGF and CXCL8 expression in human lung adenocarcinoma cell lines. Moreover, both MIF family members are required for maximal NSCLC-induced endothelial cell migration and vascular tube formation. Finally, our data indicate that MIF and D-DT signaling to angiogenic growth factor production is initiated by the MIF receptor CD74 and converges upon JNK activation and AP-1-dependent transcription.

Materials and Methods

Cell culture

Murine embryonic fibroblast and A549 lung adenocarcinoma (American Type Culture Collection) cell lines were maintained in DMEM, and H23 was cultured in RPMI 1640 media (Invitrogen). All media were supplemented with 10% FBS, L-glutamate, and gentamicin. HUVECs (Cambrex) were maintained in EGM media (Cambrex) supplemented with growth factors and gentamicin-amphotericin B and passaged on gelatin-coated plates.

Small interfering RNA (siRNA)

A549 and H23 lung cancer cells were plated at ~20% confluency and incubated overnight at 37°C in a 5% CO_2 incubator. Cells were transfected with 50 nM annealed siRNA oligos using Oligofectamine reagent (Invitrogen) following the manufacturer's protocol. The targeted base sequence for human MIF was 5′-CCTTCTGGTGGGGAGAAAT-3′ (Dharmacon). The targeted base sequence for human D-DT was 5′-CTGGCAGATTGGCA AGATA-3′ (Dharmacon). Scrambled oligos for MIF and D-DT were 5′-ACGATCCGGATGTGAGTGT-3′ and 5′-TGACGCAGTATCGATGC CA-3′, respectively. The targeted base sequence for human CD74 was 5′-AAACTGACAGTCACCTCCCAG-3′. A commercially available siRNA referred to as nonspecific oligo (Dharmacon) was used as an internal siRNA control for CD74 experiments.

ELISAs

Cytokines were measured by ELISA from supernatants of lung adenocarcinoma cells that were subjected to siRNA and allowed to incubate 3–6 days depending on the experiment. ELISA kits used were the human CXCL8 ELISA DuoSet system development kit and the human VEGF ELISA kit (R&D Systems). Assays and measurements were performed according to the manufacturer's protocols.

Human umbilical vein endothelial cell migration assay

Modified Boyden chambers (Millicell PCF, 8 μm pore size; Millipore) were placed in a 24-well plate and coated with 10 μg/ml rat tail collagen (Roche Diagnostics) for 16 h at 37°C. After removal of collagen and washing with PBS, 0.4 ml of conditioned media was placed in the bottom chamber. In some cases, neutralizing mAbs against human CXCL8 and VEGF (R&D Systems) were added to conditioned supernatants before coculture with HUVECs. HUVECs (2×10^5) were resuspended in 0.3 ml serum-free media, plated in the top of the transwell chambers, and incubated for 24 h at 37°C with 5% CO_2. Cells were removed from the upper membrane surface with a cotton tip applicator, washed with PBS, and cells on the lower membrane surface were fixed with 4% formaldehyde. HUVEC migration was quantified by manually counting the number of cells on the inserts under low power at ×40 magnification.

Human umbilical vein endothelial tube formation

Plates (48-well) were coated with 200 μl of an equal mixture of growth factor-depleted Matrigel plus serum-free DMEM (BD Biosciences) and incubated overnight at 37°C. The following day, 7×10^5 HUVECs were resuspended in 0.3 ml of the appropriate conditioned media, dispensed onto growth factor-depleted Matrigel-coated wells, and incubated for 24 h. Tubes were quantified by counting the number of connecting branches between discrete endothelial cells. Individual wells were photographed with a Nikon Act 1 phase-contrast microscope at ×10 magnification.

Luciferase assay

CXCL8 wild-type and mutant promoter luciferase reporter plasmids were kindly provided by Dr. Allan Brasier (University of Texas Medical Branch). Cells (0.4×10^5/ml) were plated in a 24-well plates and transfected with siRNA the following day for 48 h. Cells were then transiently cotransfected with 0.125 μg/well of CXCL8 wild-type or CXCL8 mutant luciferase promoter plasmid together with 0.0125 μg/well *Renilla* pRL-null plasmid (Promega) using Lipofectamine (Invitrogen) transfection reagent. After 24 h, firefly and *Renilla* luciferase activities were measured by the dual-luciferase reporter assay system (Promega) on a TD-20/20 luminometer (Turner Designs).

Adenovirus

Adenoviruses for MIF and D-DT were generated using the Gateway cloning system (Invitrogen). Briefly, human MIF and D-DT were PCR amplified and TOPO cloned into the pENTR/D-TOPO plasmid. LR recombinase (Invitrogen) was used to shuttle inserts into the pAd/CMV/V5-DEST destination vector, and subclones were confirmed by sequencing. Adenoviral vectors were digested with *Pac*I, ethanol precipitated, and transfected into 293A adenoviral packaging cells using Lipofectamine. After amplifying viral supernatants, virus was purified using ViraBind purification columns (Cell Biolabs) and tested for expression efficiency vs toxicity.

Quantitative PCR analysis

Total RNA was extracted from the cells using a RNeasy Mini kit (Qiagen). The RNA was transcribed into the first-strand cDNA with the Omniscript RT kit (Qiagen) according to the manufacturer's description. Reactions were performed using a DNA Engine Opticon (Bio-Rad), and amplifications were conducted at a final volume of 25 μl containing 1.5 μl of cDNA, 5 μl of Takara PCR mix 5× (Takara Bio), forward and reverse primers at a 0.3 μM final concentration, and SYBR Green (Molecular Probes) at a final dilution ratio of 25,000. The specific primers were forward 5′-AGAA CCGCTCCTACAGCAAG-3′ and reverse 5′-TAGGCGAAGGTGGAGT TGTT-3′ for MIF; forward 5′-TCTCCTCCATCGGCGTAGTG-3′ and reverse 5′-AATCTGCCAGGACTCCAAG-3′ for D-DT; forward 5′-ATGA CTTCCAAGCTGGCCGTGGCT and reverse 5′-TCTCAGCCCTCTTC AAAAACTTCTC for CXCL8; and forward 5′-CAAGGCCAACCGCGAG AAGA-3′ and reverse 5′-GGATAGCACAGCCTGGATAG-3′ for β-actin. Relative expression was determined using the ΔC_t method.

Western blotting

A 1× RIPA buffer containing Na_3VO_4, NaF, and a protease inhibitor cocktail was used to lyse cells. Adherent NSCLC cells were scraped and homogenized with a 23-gauge needle. Protein concentration was measured using the DC protein assay (Bio-Rad). Equal amounts of Laemmli sample buffer and protein lysate were added together, boiled for 10 min, and loaded on 4–20% SDS-polyacrylamide gel and blotted to polyvinylidene difluoride. Blots were probed with Abs that recognize MIF (pAb, Santa Cruz Biotechnology), D-DT (rabbit polyclonal; see below), total JNK (pAb, Cell Signaling Technology), phospho-JNK (pAb, Cell Signaling Technology), and phospho-c-jun (pAb, Cell Signaling) followed by HRP-conjugated anti-rabbit Ab and detected using ECL. β-actin immunoblotting (mAb, Sigma-Aldrich) was used to ensure equal protein loading.

Recombinant D-DT and D-DT Ab production

Recombinant His-tagged D-DT fusion protein was prepared by PCR amplifying the open reading frame of human D-DT using *Bam*H1- and *Xho*I-linked primers, respectively (5′-CACCATGCCGTTCCTGGAGCTGGA C-3′ and 5′-TAAAAAAGTCATGACCGTC-3′). PCR amplicon and pET 21b vector (Novagen) were digested with *Bam*H1 and *Xho*I, purified by

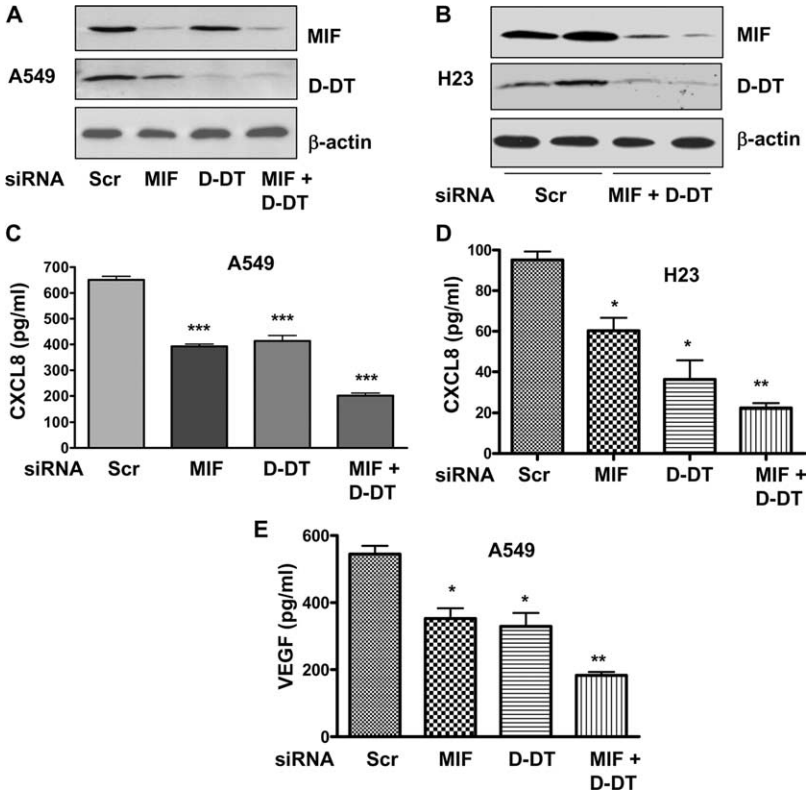

FIGURE 1. Additive contributions by MIF family members to lung adenocarcinoma cell angiogenic growth factor expression. *A*, A549 lung adenocarcinoma cells were oligo-transfected with 50 nM scrambled MIF + 50 nM scrambled D-DT siRNA (Scr), 50 nM MIF + 50 nM Scr MIF siRNA (MIF), 50 nM D-DT + 50 nM Scr D-DT siRNA (D-DT), and 50 nM MIF + 50 nM D-DT siRNA (MIF + D-DT) or (*B*) 50 nM scrambled MIF + 50 nM scrambled D-DT siRNA (Scr) and 50 nM MIF + 50 nM D-DT siRNA (MIF + D-DT) in duplicate for H23 cells. Cells were harvested, lysed, and immunoblotted for MIF, D-DT, and β-actin as indicated. A549 cells (*C* and *E*) and H23 cells (*D*) were oligofected as in *A*. Forty-eight hours later, media were changed and supernatants were collected the next day and analyzed by ELISA for CXCL8 (*C* and *D*) or VEGF (*E*). ELISA results are expressed $\pm$SD of the average of data from duplicate siRNA-transfected samples and are representative of at least two independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ by one-way ANOVA analysis are shown for control and individual group comparisons, while statistical significance between relevant groups was found by Tukey's post hoc test.

GeneClean II (Qbiogene), and ligated. pET21b/D-DT subclones were confirmed by sequencing and transformed into BL21(DE3) competent bacteria (Novagen) and rD-DT was induced by 0.4 mM isopropyl β-D-thiogalactoside for 4 h followed by bacterial disruption and purification using Ni-NTA Sepharose (Novagen). Rabbit polyclonal anti-human D-DT was prepared by immunizing a New Zealand White rabbit with 200 μg recombinant D-DT in suspension with CFA (Sigma-Aldrich) followed by boosts at weeks 2 and 4 with 200 μg recombinant D-DT suspended in IFA (Sigma-Aldrich). Blood was collected by ear bleeds at week 6 and tested for reactivity of human rD-DT by Western blotting.

Statistical analyses

Results are expressed as means $\pm$ SD. Multiple data comparisons were derived by one-way ANOVA followed by Tukey's post hoc test using GraphPad Prism 4.1 statistical program. p values <0.05 were considered significant.

Results

MIF and D-DT expression in NSCLC cells is attenuated by siRNA

MIF is overexpressed in non-small cell lung cancers and modulates tumor progression by stimulating both autocrine- and paracrine-associated processes (7, 12, 30). However, very little is known about D-DT, particularly its biological functions. Similar to MIF, D-DT was expressed at relatively high levels in both human lung adenocarcinoma cell lines that we examined (Fig. 1, *A* and *B*). Because of the structural and enzymatic similarities between MIF and D-DT, we hypothesized that D-DT also participates in tumorigenic processes and may act additively with MIF. Therefore, we silenced both MIF and D-DT, individually and in combination, using siRNA transfection. siRNA effectively abrogated the protein expression of both MIF and D-DT in A549 (Fig. 1*A*) and H23 (Fig. 1*B*) lung adenocarcinoma cells.

Loss of MIF and D-DT reduces basal CXCL8 and VEGF levels in NSCLC cells

We next sought to determine whether there was a functional correlation between MIF, D-DT, and CXCL8 levels in NSCLC cells. We consistently observed a 40–50% reduction of basal CXCL8 levels in both A549 (Fig. 1*C*) and H23 (Fig. 1*D*) cells subjected to either MIF or D-DT siRNA alone. Intriguingly, combined MIF and D-DT deficiency resulted in ~70–80% reductions in basal CXCL8 levels as compared with the control (Fig. 1, *C* and *D*). These results suggest that MIF and D-DT play an important and additive functional role in modulating steady-state CXCL8 expression in human NSCLC cell lines.

Because VEGF is an important regulator of both normal and dysregulated angiogenesis, and MIF has been reported to modulate its expression (31–33), we sought to determine whether MIF and D-DT functionally regulate VEGF in lung adenocarcinoma cells. Similar to CXCL8, basal VEGF levels were reduced when MIF and D-DT levels were attenuated individually and, in an additive fashion, when depleted together (Fig. 1*E*). Combined with results demonstrating both individual and additive requirements for MIF and D-DT in CXCL8 production (Fig. 1, *C* and *D*), our results support the conclusion that both of these family members are important autocrine contributors to angiogenic growth factor expression in lung adenocarcinoma cells.

MIF and D-DT depletion results in the loss of CXCL8 transcription corresponding to decreased JNK activation, c-jun phosphorylation, and AP-1 activity

We next sought to determine whether the loss of CXCL8 expression/secretion was due to defective CXCL8 transcription in cells lacking MIF and D-DT. From Fig. 2*A*, quantitative PCR analysis of MIF- and D-DT-deficient cells resulted in a nearly complete loss of both MIF and D-DT message, consistent with the previously observed decreases in protein expression in A549 cells (Fig. 1*A*). Accompanying decreased MIF and D-DT mRNA was a nearly uniform loss of CXCL8 mRNA, consistent with the reduction of CXCL8 protein secretion observed in MIF- and D-DT-deficient cells (Fig. 1, *C* and *D*).

Several studies have demonstrated an important role for JNK-dependent AP-1 activation in the contribution of MIFs to inflammatory and mesenchymal cell matrix metalloprotease production

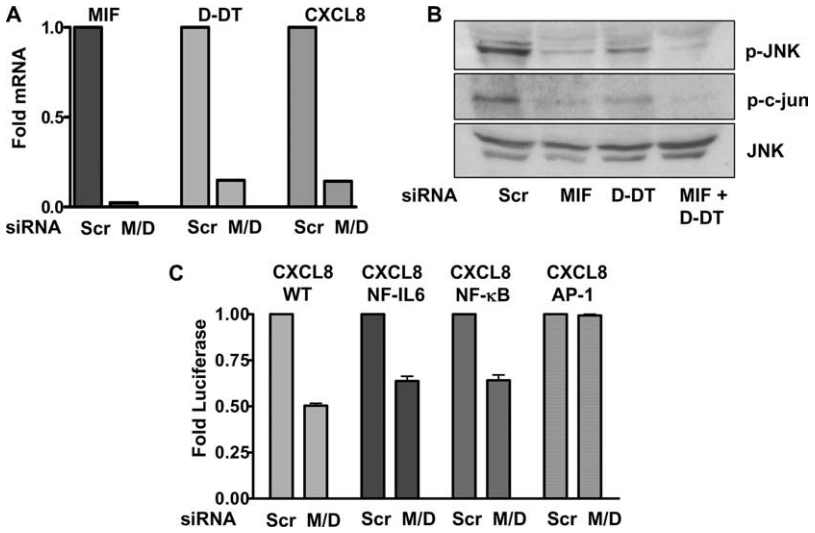

FIGURE 2. MIF and D-DT additively regulate CXCL8 transcription and JNK activation. *A*, MIF, D-DT, IL-8, and β-actin mRNA from A549 cells transfected for 48 h with either scrambled RNA oligos or MIF + D-DT siRNA oligos were analyzed by quantitative PCR (qPCR). Data shown represent the ΔC_t of the average of duplicate reactions for each condition between target mRNA (MIF, D-DT, or CXCL8) and β-actin and are representative of two independent experiments. *B*, A549 lung adenocarcinoma cells were oligo-transfected as indicated, and 48 h later cells were harvested, lysed, and immunoblotted for phospho-JNK, phospho-c-jun, and total JNK. *C*, A549 cells were oligofected for 48 h with either scrambled RNA oligos or MIF + D-DT siRNA oligos and then cotransfected with null *Renilla* luciferase plasmid (pRL-null *Renilla*) and either wild-type (IL-8-WT) or mutant CXCL8 firefly luciferase promoter constructs. Twenty hours post-transfection, firefly and *Renilla* luciferase activities were measured by the dual-luciferase reporter assay system. Results are expressed as fold increase/decrease over control after normalizing ratios of luciferase/*Renilla* luciferase from triplicate samples.

(27–29). Because AP-1 is also found to be an important contributor to both basal and induced CXCL8 in normal (34, 35) and transformed cells (36, 37), we investigated whether JNK and/or AP-1 was involved in the regulation of CXCL8 by MIF and D-DT. As shown in Fig. 2*B*, analysis of phosphorylated JNK in lysates from cells deficient in either MIF or D-DT alone revealed significant reductions in each. More importantly, MIF plus D-DT depletion in A549 cells resulted in a synergistic and nearly complete loss of JNK phosphorylation. Consistent with a loss of JNK phosphorylation/activation, we observed a very similar pattern of defective c-jun phosphorylation with individual MIF or D-DT knockdown and a nearly complete loss of steady-state c-jun phosphorylation with the combined loss of MIF/D-DT (Fig. 2*B*, *bottom panel*).

We next used CXCL8 promoter-driven luciferase constructs to further analyze the transcriptional contribution of MIF family members to CXCL8 expression. Using these CXCL8 reporter plasmids, we tested MIF/D-DT-deficient cells for their relative CXCL8-driven transcription from wild-type promoters compared with promoters harboring mutated binding sites for NF-IL-6, NF-κB, and AP-1. As shown in Fig. 2*C*, luciferase activity from wild-type CXCL8 promoter was ~50% reduced in cells lacking both MIF and D-DT when compared with cells transfected with scrambled oligos only. Interestingly, relative differences in CXCL8 promoter-driven luciferase between MIF/D-DT-containing and MIF/D-DT-deficient cells were relatively unchanged by mutation of either NF-IL-6 or NF-κB binding sites when compared with wild-type promoters, suggesting that these transcription factors are unaffected by loss of MIF and D-DT (Fig. 2*C*). In contrast, when the consensus sequence for AP-1 was mutated in the CXCL8 promoter, reduction of wild-type luciferase was decreased to the levels of MIF/D-DT knockdown cells, indicating that AP-1 activity is necessary for maximal CXCL8 transcription in these cells and that AP-1 activity is defective in MIF/D-DT-deficient cells. Combined with the requirements for both MIF family members in steady-state JNK and c-jun phosphorylation, these results suggest that MIF and D-DT may functionally regulate CXCL8 transcription, at least in part, through modulation of the JNK pathway.

Adenoviral delivery of MIF family members rescues JNK activation and CXCL8 expression in MIF/D-DT-deficient cells

To test for specificity of MIF and/or D-DT in defective CXCL8 expression, we asked whether forced overexpression of MIF family members can rescue CXCL8 levels in their respective siRNA-knocked down cells. To achieve this, we made adenoviral constructs of both MIF and D-DT and generated high-titer adenoviral supernatants of both. To accomplish efficient rescue in cells transfected with MIF and D-DT siRNA, we utilized siRNA that targets the 3′-untranslated region of MIF mRNA, allowing for efficient expression of the MIF open reading frame. Because D-DT siRNA targets sequences within the open reading frame of human D-DT (see *Materials and Methods*), we used the murine homolog of D-DT for adenoviral expression because the sequence is not conserved within this siRNA-targeting domain.

As shown in Fig. 3*B*, combined adenoviral transduction of MIF and D-DT efficiently rescues defective JNK phosphorylation concomitant with the restoration of MIF and D-DT expression. To determine whether adenoviral MIF family members rescue CXCL8 expression in a manner consistent with their rescue of JNK activation, scrambled and MIF siRNA-transfected cells were infected with MIF adenovirus, D-DT siRNA-transfected cells were infected with D-DT adenovirus, and MIF + D-DT siRNA-transfected cells received both adenoviruses. As shown in Fig. 3*B*, defective CXCL8 expression associated with either loss of MIF or D-DT was fully rescued by MIF and D-DT adenoviruses, respectively, while combined MIF + D-DT adenoviruses fully rescued CXCL8 in cells lacking both MIF family members.

Maximal angiogenic potential of lung adenocarcinoma cells requires both MIF and D-DT

Tumor-initiated angiogenesis arises as a result of secreted, paracrine-acting angiogenic growth factors eliciting responses from stromal endothelial cells and surrounding vasculature. To further

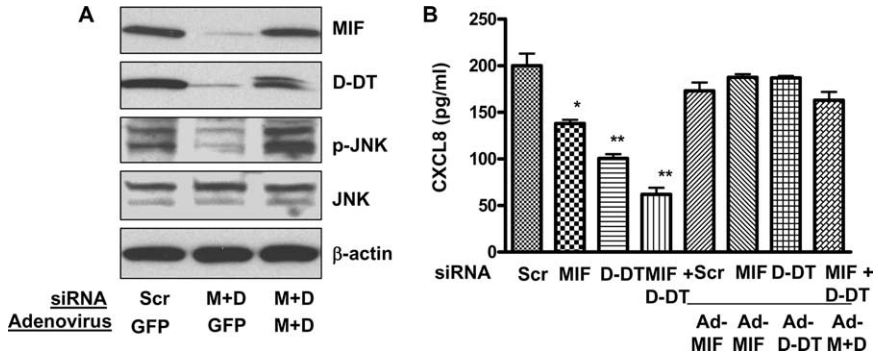

FIGURE 3. Rescue of defective JNK activation and CXCL8 expression in MIF/D-DT-deficient cells by adenoviral reconstitution of MIF and D-DT. *A*, A549 lung adenocarcinoma cells were oligo-transfected and 48 h later GFP adenovirus or MIF + D-DT adenovirus was added as indicated. Twenty-four hours after adenovirus addition, cells were lysed and immunoblotted for MIF, D-DT, p-JNK, total JNK, and β-actin. *B*, A549 cells were transfected with scrambled, MIF, D-DT, or MIF + D-DT siRNA oligos for 48 h and adenoviral MIF and/or D-DT was added to cells. Twenty-four hours later, media were replaced and collected the following day for CXCL8 ELISA measurements. ELISA results are expressed $\pm$SD of the average of data from two samples and are representative of two independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ by one-way ANOVA analysis are shown for control and individual group comparisons while statistical significance between relevant groups was found by Tukey's post hoc test.

study the impact of MIF and D-DT on lung adenocarcinoma-initiated angiogenesis we tested the effects of MIF and/or D-DT depletion on the potential of NSCLC-conditioned supernatants to induce HUVEC migration and vascular tube formation. Endothelial cells cocultured with supernatants from control siRNA-transfected NSCLC cells were 40–50% more efficient at migrating than those cells cocultured with supernatants from MIF or D-DT siRNA-transfected cells. Additionally, consistent with an additive function for these family members, supernatants from cells deficient in both MIF and D-DT were >70% defective in inducing HUVEC migration compared with control cells (Fig. 4*A*). Mirroring almost exactly the results from endothelial cell migration, loss of MIF and/or

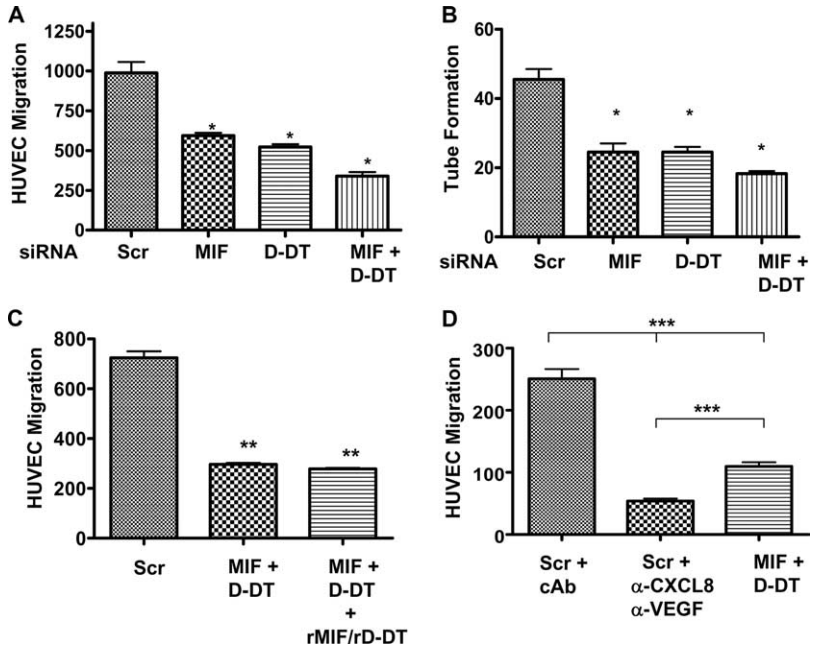

FIGURE 4. MIF and/or D-DT deficiency renders lung adenocarcinoma supernatants defective in induced endothelial cell migration and tube formation. *A*, A549 cells were transfected with scrambled, MIF, D-DT, or MIF + D-DT siRNA for 48 h, replaced with fresh media, and supernatants were collected 48 h later. HUVECs (2×10^5) were plated in serum-free media in the top chamber, and cell-free supernatants were added to the bottom chamber of collagen-coated transwell chambers. Twenty-four hours later cells were fixed, stained, and enumerated. Results represent $\pm$SD of the average of parallel coculture assays and are representative of three independent experiments. *B*, Cell-free supernatants from *A* were used to resuspend 7×10^5 HUVECs, which were plated onto growth factor-depleted Matrigel-coated 48-well plates. Twenty-four hours later, tubes were quantified by counting the number of connecting branches between discrete endothelial cells. Results represent $\pm$SD of the average of parallel coculture assays and are representative of four independent experiments. *C*, HUVEC migration was determined as in *A* using cell-free supernatants from cells transfected with scrambled and MIF + D-DT siRNAs. In one set of supernatants from MIF + D-DT siRNA-transfected lung adenocarcinoma cells, rMIF and rD-DT were added at a final concentration of 50 ng/ml each. Results represent $\pm$SD of the average of parallel coculture assays and are representative of three independent experiments. *D*, HUVEC migration was determined as in *A* using cell-free supernatants from cells transfected with scrambled and MIF + D-DT siRNAs. Neutralizing mAbs against CXCL8 and VEGF were added at a final concentration of 10 μg/ml each to supernatants from scrambled siRNA-transfected A549 cells. For control, 20 μg/ml isotype control Ab was added (cAb) to supernatants from scrambled siRNA-transfected A549 cells. Results represent $\pm$SD of the average of parallel coculture assays and are representative of two independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ by one-way ANOVA analysis are shown for control and individual group comparisons, while statistical significance between relevant groups was found by Tukey's post hoc test.

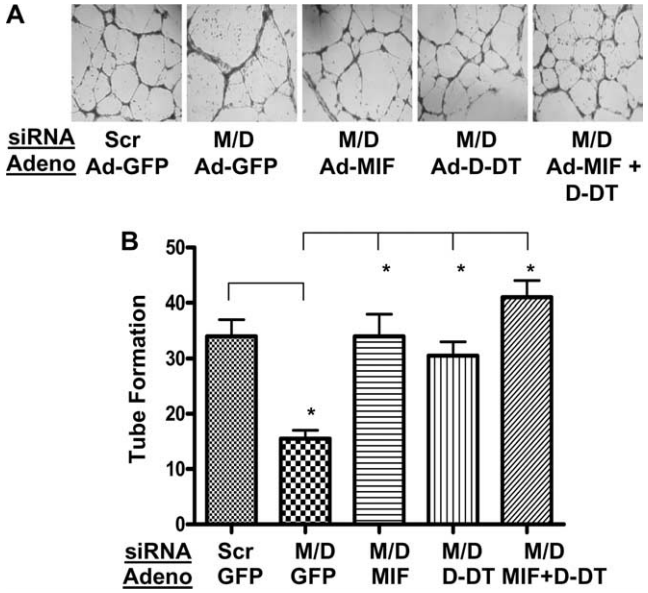

FIGURE 5. Adenoviral reconstitution of MIF and/or D-DT rescues defective tube formation from supernatants derived from MIF + D-DT-deficient cells. A549 lung adenocarcinoma cells were oligo-transfected and 48 h later GFP, MIF, D-DT, or MIF + D-DT adenoviruses were added as indicated. After overnight incubation, media were replaced and 48 h later conditioned media were collected for HUVEC tube formation. Representative growth factor-depleted Matrigel coated wells were photographed (*A*) and enumerated (*B*). Results from panel *B* are tubes/transwell and represent ±SD of the average of parallel coculture assays and are representative of three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ by one-way ANOVA analysis are shown for control and individual group comparisons, while statistical significance between relevant groups was found by Tukey's post hoc test.

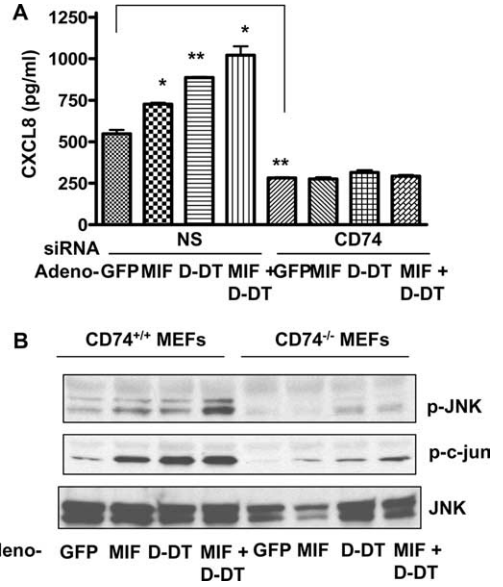

FIGURE 6. MIF and D-DT are individually and additively sufficient to induce CXCL8 from lung adenocarcinoma cells, and CD74 is necessary for maximal JNK activation in mouse fibroblasts. *A*, A549 lung adenocarcinoma cells were oligo-transfected with either control siRNA (nonspecific oligo, NS) or CD74 siRNA, and 48 h later GFP, MIF, D-DT, or MIF + D-DT adenoviruses were added for 16 h. After media were replaced, cells were incubated for an additional 24 h and supernatants were collected and analyzed by CXCL8 ELISA. Results are ±SD of the average of CXCL8 ELISA readings from supernatants from two parallel infections and are representative of two independent experiments. *B*, $CD74^{+/+}$ and $CD74^{-/-}$ murine embryonic fibroblasts were infected with GFP, MIF, D-DT, or MIF + D-DT for 48 h. Cell lysates were examined for phospho-JNK, phospho-c-jun, and total JNK. Results are representative of two independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ by one-way ANOVA analysis are shown for control and individual group comparisons, while statistical significance between relevant groups was found by Tukey's post hoc test.

D-DT individually and additively resulted in significant reductions in NSCLC supernatant-induced HUVEC tube formation (Fig. 4*B*). Note also that defects observed in MIF- and/or D-DT-deficient cell supernatants correlate closely with defective CXCL8 and VEGF levels in the same supernatants (Fig. 1).

To determine whether decreased MIF and/or D-DT levels in the NSCLC supernatants were partially or wholly responsible for the defective phenotypes observed in endothelial cell migration, we added back recombinant MIF and D-DT to the endothelial cell/MIF + D-DT-depleted supernatant coculture experiment. As shown in Fig. 4*C*, HUVEC migration was unaffected by the reconstitution of MIF and D-DT in lung adenocarcinoma supernatants, suggesting that the defective HUVEC migration observed in these coculture experiments was likely due to loss of angiogenic growth factors. Finally, because we hypothesized that loss of CXCL8 and VEGF in MIF/D-DT-deficient NSCLC supernatants is responsible for the defects observed in migration and tube formation, we assessed the relative contribution of CXCL8 and VEGF to NSCLC supernatant-induced endothelial cell migration. As shown in Fig. 4*C*, supernatants from control siRNA-transfected A549 cells coincubated with neutralizing Abs to CXCL8 and VEGF resulted in the reduction of NSCLC supernatant-induced EC migration by nearly 80%, while supernatants from MIF/D-DT-deficient cells were ~60% less efficient than controls. These findings are consistent with our findings that CXCL8 and VEGF levels are only ~60% reduced in MIF/D-DT-deficient cells (Fig. 1), while neutralizing Abs should effectively render all CXCL8 and VEGF inactive. Collectively, these results imply that CXCL8 and VEGF secretion from NSCLC cells account for the vast majority of the soluble angiogenic inducing capacity of these cells and suggest that MIF- and D-DT-dependent regulation of CXCL8 and VEGF expression is of central importance in NSCLC-associated angiogenesis.

Crossover rescue by MIF family members of HUVEC tube formation

Data from Fig. 4 suggested to us that the requirement for MIF family members in NSCLC supernatant-induced HUVEC migration and tube formation was at the level of CXCL8 and VEGF expression. If this were the case, then adenoviral rescue of MIF family members resulting in rescue of CXCL8 (from Fig. 3*B*) should result in the rescue of HUVEC angiogenic phenotypes. To investigate this possibility, we tested supernatants from combined MIF/D-DT siRNA-transfected cells that had been adenovirally infected with either MIF, D-DT, or both. Interestingly, both MIF and D-DT were fully capable of rescuing defective tube formation from supernatants lacking both MIF family members (Fig. 5). Although cocultures with supernatants from cells with add-back of both family members resulted in higher levels of tube formation than did those from control cells (Fig. 5), our findings that one family member can fully compensate for the loss of both indicate that there is a common mechanism of action shared by these two proteins.

A role for the MIF receptor, CD74, in MIF- and D-DT-induced JNK activation

Thus far our findings had suggested that MIF and D-DT are necessary for maximal CXCL8 expression and that they likely activate signaling to CXCL8 expression in a mechanistically similar fashion. To investigate whether the MIF cell-surface receptor CD74 is involved in this shared signaling pathway, A549 cells were transfected with CD74 siRNA and evaluated for CXCL8 induction by MIF and D-DT overexpression. As shown in Fig. 6*A*, significant increases in CXCL8 secretion were observed in both MIF- and D-DT-infected cells, and a corresponding additive induction was found with MIF and D-DT combined expression in control siRNA-transfected cells (nonspecific oligo). In contrast, CD74 siRNA-transfected cells produced ~50% less steady-state CXCL8 and were completely resistant to MIF-, D-DT-, and MIF/D-DT-induced CXCL8 expression. To confirm and extend the observed requirements for CD74 in MIF- and D-DT-induced CXCL8 expression, we next tested whether CD74 was necessary for MIF family member-induced JNK pathway of signal transduction using $CD74^{+/+}$ and $CD74^{-/-}$ murine embryonic fibroblasts. As shown in Fig. 6*B*, JNK activation in GFP-, MIF-, D-DT-, or MIF/D-DT-infected lysates revealed that MIF family members, alone or combined, strongly induced JNK and c-jun phosphorylation in $CD74^{+/+}$ cells but not in $CD74^{-/-}$ cells. Collectively, these studies suggest that MIF and D-DT are both sufficient and necessary for maximal CXCL8 expression in NSCLC cell lines and that CD74 is likely responsible for initiating signaling by both MIF family members resulting in JNK activation and subsequent CXCL8 expression.

Discussion

We report for the first time that the MIF homolog, D-dopachrome tautomerase, supports CXCL8 expression and secretion from lung adenocarcinoma cells. Moreover, D-DT functionally cooperates with MIF in promoting angiogenic growth factor expression and subsequent paracrine-induced endothelial cell angiogenic phenotypes. Several studies have demonstrated an important contribution by MIF to CXCL8 and VEGF expression and maintenance of angiogenic phenotypes in malignant cells and tissue (7, 22, 24, 38–40). However, this is the first demonstration of a functional overlap between MIF and its only known homolog, D-DT.

Our results suggest that MIF and D-DT modulate JNK-dependent AP-1 transactivation and subsequent CXCL8 transcription in lung adenocarcinoma cells. Perhaps more importantly, the cognate MIF receptor, CD74, is necessary for both CXCL8 expression and maximal JNK and c-jun phosphorylation induced by MIF and D-DT alone and in combination. These findings are consistent with a recent study demonstrating that CD74 is necessary for MIF-dependent contributions to prostatic adenocarcinoma cell invasion, anchorage-independence, and tumor-associated neovascularization (40). What is less clear is how JNK is activated by CD74. An earlier study from our laboratory revealed that MIF functionally regulates Rac1 effector binding by stabilizing cholesterol-enriched membrane microdomains (12). Although JNK is a well-known effector of Rac1, we have no evidence as yet that the defective JNK observed with loss of MIF is linked to Rac1. Studies are currently underway to determine whether Rac1 is necessary for MIF- and D-DT-dependent, CD74-mediated JNK activation. It is also interesting that JNK activation has been linked to MIF activity by virtue of MIF binding to, and regulating the activity of, Jab1/CSN5 (41). It is unknown at present whether D-DT functionally interacts with Jab1/CSN5, but this pathway may represent an alternative pathway for MIF family member-dependent regulation of JNK activity.

Although our results indicate that AP-1 activity is important for MIF and D-DT contributions to CXCL8 expression, it is possible that other signaling pathways may be involved. Of note, MIF/CD74 signaling has recently been suggested to modulate CXCL8 expression in an NF-κB-dependent manner (38). Although we have not observed any correlation between MIF or D-DT and NF-κB activation (Fig. 2*B* and our unpublished observations), we cannot rule out the possible contribution of these family members to other pathways reported to be important for CXCL8 and VEGF expression (25, 35). Nonetheless, our studies do reveal an important and previously undescribed requirement by CD74 for both MIF- and D-DT-induced JNK activation and c-jun phosphorylation.

Our studies with HUVECs reveal that supernatants from lung adenocarcinoma cells deficient in MIF and/or D-DT result in significant reductions in both endothelial cell migration and tube formation. While our findings indicate that the depleted CXCL8 and VEGF levels present in these supernatants are responsible for this defective angiogenic phenotype, we cannot rule out the possibility that endothelial cell-derived MIF and/or D-DT may influence endothelial cell activation in an autocrine manner, as has been reported (42). This possibility, coupled with the fact that tumor-derived MIF stimulates CXCL8 and VEGF from tumor stromal macrophages (26), suggests that MIF family members modulate intratumoral neoangiogenesis on several different levels.

Our laboratory recently discovered a novel role for MIF in pancreatic ductal adenocarcinoma cell hypoxic adaptation (43, 44). Specifically, endogenous pancreatic ductal adenocarcinoma MIF was found to be necessary for maximal hypoxia-inducible factor 1α (HIF-1α) stabilization and subsequent transcription of VEGF. Intriguingly, CXCL8 is also a transcriptional target of hypoxia and HIF-1α (45–47) and, as such, it is likely that MIF is similarly necessary for HIF-dependent CXCL8 expression in hypoxic regions of tumors. Because our current studies indicate that both MIF and D-DT functionally regulate both CXCL8 and VEGF in normoxic conditions, it is not unreasonable to conclude that MIF family members are necessary for both HIF-dependent and HIF-independent maximal expression of these angiogenic growth factors. This is an important distinction, as HIF-independent CXCL8 was recently shown to compensate for loss of HIF-1α in tumor-associated angiogenesis, and co-neutralization of HIF-dependent and HIF-independent CXCL8 and VEGF markedly reduces tumor angiogenesis and corresponding tumor burdens (48). Although studies are ongoing to determine whether D-DT is similarly important for HIF-dependent regulation of angiogenic growth factor expression, our present findings support a strategy of dual targeting of MIF and D-DT in patients with established tumors. Further in vivo studies to evaluate the individual vs combined influence of MIF and D-DT in intratumoral angiogenesis and malignant disease progression may well lead to a reassessment of current MIF therapeutic targeting strategies for lung cancer.

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Disclosures

R.A.M. and R.B. are coinventors on patents and patent applications describing the therapeutic value of MIF antagonists.

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