Rho GTPase-dependent Signaling Is Required for Macrophage Migration Inhibitory Factor-mediated Expression of Cyclin D1*

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Our previous studies demonstrated that the proinflammatory peptide, macrophage migration inhibitory factor (MIF), functions as an autocrine mediator of both growth factor- and integrin-dependent sustained ERK MAPK activation, cyclin D1 expression, and cell cycle progression. We now report that MIF promotes the activation of the canonical ERK MAPK cascade and cyclin D1 expression by stimulating the activity of the Rho GTPase and downstream signaling to stress fiber formation. Rho-dependent stress fiber accumulation promotes the sustained activation of ERK and subsequent cyclin D1 expression during G1-S phase cell cycle progression. This pathway is reported to be dependent upon myosin light chain (MLC) kinase, integrin clustering, and subsequent activation of focal adhesion kinase, leading to sustained MAPK activity. Our studies reveal that recombinant MIF induces cyclin D1 expression in a Rho-, Rho kinase-, MLC kinase-, and ERK-dependent manner in asynchronous NIH 3T3 fibroblasts. Moreover, MIF-/- murine embryonic fibroblasts display aberrant cyclin D1 expression that is linked to defective Rho activity, stress fiber formation, and MLC phosphorylation. These results suggest that MIF is an integral autocrine mediator of Rho GTPase-dependent signaling events and provide mechanistic insight into how MIF regulates proliferative, migratory, and oncogenic processes.

Despite being one of the earliest cytokines discovered, macrophage migration inhibitory factor (MIF) is arguably the least understood. MIF has been shown to exert effects on a variety of cell types and influence the regulatory functions of many diverse biological processes. These include inflammation, immune regulation, physiologic and pathophysiologic neovascularization, and cell replication (1–3). This broad array of effects of MIF on cellular functions is difficult to understand. Perhaps one unifying interpretation might be that MIF is elaborated in response to tissue wounding. MIF affects both ends of the complex cascade of reactions caused by injury, from inflammation to reparative cell replication. In this same vein, MIF production following growth factor or extracellular matrix stimulation would also be in accord with its involvement in cell replication. In the case of neoplastic cells, internal (oncogenic) or external (growth factors, extracellular matrix) signals could serve to increase MIF production that, in turn, may facilitate anchorage independence and loss of contact inhibition.

Our previous studies have established that MIF, a protein historically associated with inflammation and immune regulation, stimulates the proliferation of mouse fibroblasts (4). This response is associated with the activation of the p44/p42 extracellular signal-regulated kinase (ERK) MAPKs. We further demonstrated that growth factors stimulate the rapid release of preformed MIF from adherent, quiescent fibroblasts. Importantly, the sustained activation of MAPK in serum-stimulated fibroblasts is dependent upon MIF autocrine action (4).

In addition to the discovery that MIF participates in growth factor signaling to ERK, we recently completed a study that describes a critical role for MIF in the modulation of adhesion-dependent activation of ERK, also in a sustained fashion (5). This finding is particularly important in light of the fact that both growth factors and adhesion are required for efficient signaling to sustained ERK activation and subsequent cell cycle progression (6). The requirement for sustained ERK activity in growth factor plus adhesion-dependent cell cycle control is now known to be at the level of cyclin D1 transcription and the subsequent activation of specific cyclin-dependent kinases (Cdks) (7–9). The regulation of G1 phase progression relies on cyclins, Cdks, and Cdk inhibitors (10, 11). Cyclin D1-Cdk4/6 and cyclin E-Cdk2 activities phosphorylate the retinoblastoma (Rb) tumor suppressor, which in turn releases free E2F transcription factors, resulting in the transcription of critical S phase enzymes and regulators (12).

A central role for RhoA GTPase in modulating sustained ERK activation and cyclin D1 expression in fibroblasts was recently described (7, 13). The activation of Rho by integrins and growth factors is essential for modulating the sustained activation of ERK and subsequent cyclin D1 transcription (7). Interestingly, Rho is also responsible for maintaining the correct timing of cyclin D1 expression in mid G1 phase of the cell cycle, in part by negatively regulating Rac1 activity (13, 14). Further studies have revealed that Rho-dependent Rho kinase activation contributes to adhesion/growth factor-dependent regulation of sustained ERK MAPK activity by promoting the formation of stress fibers and the consequent recruitment and clustering of integrins, leading to focal adhesion formation (13). In this model, Rho, Rho kinase, and myosin light chain kinase...
enhance stress fiber formation/integrin clustering and, hence, the sustained activation of the canonical Ras, Raf-1, MEK, and ERK MAPK pathway.

We now report that MIF positively induces cyclin D1 expression through a pathway dependent upon the activities of Rho and Rho kinase and the phosphorylation of myosin light chain. Moreover, this MIF-mediated pathway of activation results in the sustained stimulation of ERK MAPKs, leading to efficient cyclin D1 transcription and Rb inactivation. These findings are the first to demonstrate a requirement for Rho GTPase family members in the regulation of MIF-dependent cell cycle regulation and provide a long sought after link between MIF and its downstream signaling effectors.

EXPERIMENTAL PROCEDURES

Cell Culture and Drug Treatment—MIF−/− mice and their wild-type littermates were maintained on a mixed 129Sv × C57Bl/6 background (F3). Mouse embryonic fibroblasts (MEFs) were generated from embryos at day 14.5 and grown in DMEM with 10% FCS and antibiotics (5). NIH 3T3 fibroblasts were maintained in low glucose DMEM with 10% FCS and antibiotics. For recombinant MIF (15) stimulation and inhibition studies, NIH 3T3 cells were plated density-dependently (5 × 104 cells/6-cm plate) and allowed to grow for 48–72 h in low glucose DMEM with 10% FCS and antibiotics. Without a medium change, the cells were then treated with either 10 μM ML-7 (Biomol), 10 μM Y27632 (EMD Biosciences), or 50 μM U0126 (Promega). After incubation with the specified inhibitor for 30 min, recombinant MIF (rMIF) was added at 50 ng/ml unless otherwise specified. For transient transfection experiments, NIH 3T3 cells were plated as above, and 24 h later, cells were transfected with the indicated amounts of either pDNA1.1 GS or pcDNA3.1 GS/MIF using Fugene 6 Transfection Reagent (Roche Applied Science) as described previously (5, 16).

For cell proliferation experiments, 1 × 105 NIH 3T3 fibroblasts were plated in each well of a 96-well plate in 0.5% serum containing low glucose media for 48 h. Without a media change, cells were treated with either 10 μM ML-7, 10 μM Y27632, or 50 μM U0126. After incubation, with the specified inhibitor for 30 min, rMIF was added at 50 ng/ml and allowed to sit for 30 h. 0.5 μCi/well [3H]thymidine (PerkinElmer Life Sciences) was added to each well for an additional 4 h, at which time the cells were harvested and the incorporation of [3H]thymidine into DNA was quantified by liquid scintillation counting (Packard Instrument Co.).

For Rho inactivation experiments, pEXV-RhoN19 or pEXV vector alone were transfected into 1 × 105 NIH 3T3 cells/well of a 6-well plate at 4 μg/well using Fugene 6. rMIF was added to transfected cells 48 h later as indicated.

For adhesion/growth factor induction of Rho activation assays, 2 × 105 MIF−/− or MIF−/−/p53H175 immortalized cells were serum-starved for 4 h. Cells were lifted and plated onto 10-cm fibronectin-coated dishes (BD Biosciences) in the presence of 10% FCS for the indicated times.

Immunoblotting Studies—Whole cell extracts were prepared from cells after the indicated treatments for the indicated times. Cells first were lysed in ice-cold radioimmune precipitation buffer (containing 1 mM NaVO3, 2 mM NaF, and a protease inhibitor mixture (Roche Applied Science)). The cells were disrupted by repeated aspiration through a 21-gauge needle. After microcentrifugation at 3000 rpm for 10 min (4 °C), the supernatants were removed, and equal amounts of cellular proteins were fractionated on SDS-polyacrylamide gels (Bio-Rad) and transferred to polyvinylidene difluoride membranes (Millipore Corp.). Immunoblotting was performed with antibodies directed against phospho-ERK MAPK, phospho-MLC (Cell Signaling Technology), cyclin D1, Cdc42, Myc epitope 9E10, HA tag (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and phospho-FAK (Tyr397) (BIOSOURCE International).

Fluorescence Microscopy—5 × 104 cells/ml were plated onto fibronectin-coated (10 μg/ml in phosphate-buffered saline) chamber slides (BD Biosciences) for 2 h, washed, and then fixed with 4% formaldehyde in phosphate-buffered saline. After washing and permeabilization, fluorescein isothiocyanate-labeled phalloidin (Sigma) was added at a final concentration of 1 μg/ml for 1 h. After staining, cells were washed five times with phosphate-buffered saline, fixed with Vectastain (Vector Laboratories), and observed/photographed by fluorescence microscopy at ×20 magnification (Nikon).

Retroviral Plasmid Constructs and Infections—The p53H175 (a gift of Dr. Oleksi Petrenko, SUNY Stonybrook, Stonybrook, NY) retroviral expression vector is described elsewhere (20). 1 × 105 fibroblasts were transfected with viral supernatant, and refed normal media for the indicated times postinfection. The expression of p53H175 in primary fibroblasts resulted in efficient immortalization of both MIF−/− and MIF−/− cells as evidenced by passageing for greater than 25 times with no evidence of cell senescence.

RESULTS

Exogenously Added Recombinant and Ectopically Expressed MIF Induce Cyclin D1 Expression in Fibroblasts—Our previous studies revealed that both endogenous and exogenously added rMIF induce the sustained activation of ERK MAPK in NIH 3T3 and mouse embryonic fibroblasts (4, 5). We have additionally found that MIF-deficient cells display defective growth factor/adhesion-induced sustained ERK activation, leading to cyclin D1 expression, Rb inactivation, and phase cell cycle progression (5). Despite these findings, very little is known about how proximal signaling by extracellular MIF promotes the sustained activation of ERK, leading to cyclin D1 expression.

To determine whether exposure of cells to exogenously added recombinant MIF was sufficient to induce cyclin D1 transcription/translation that is reportedly linked to the sustained activation of ERK (7–9), NIH 3T3 cells were treated with rMIF (15) at various concentrations. As shown in Fig. 1A, cyclin D1 protein expression was dose-dependently induced by treatment with rMIF. To verify this finding and to determine whether forced overexpression of MIF was also capable of inducing cyclin D1 expression, MIF was transiently transfected into NIH 3T3 fibroblasts. As shown in Fig. 1B, a transiently transfected MIF mammalian expression plasmid (16) dose-dependently induced the expression of cyclin D1 (top panel) concordant with increased intra- and extracellular expression of MIF (data not shown).

Because the sustained activation of ERK promotes the expression of cyclin D1 through a transcription-dependent process (7, 8), we then determined whether transfection of MIF was
Recent studies describe a link between Rho GTPase-dependent activation of ERK MAPK, cyclin D1 expression, and G1/S phase cell cycle progression in fibroblasts (7). GTPase-dependent activation of ERK MAPK, cyclin D1 expression, and an intact G1/S cell cycle result in enhanced myosin light chain phosphorylation and subsequent stress fiber formation by two distinct mechanisms. The first involves a direct Rho kinase-mediated phosphorylation of MLC (22), and the second requires a Rho kinase-dependent inactivating phosphorylation of myosin light chain phosphatase (MLCP) (23). Regardless of the mechanism, enhanced phosphorylation of MLC is thought to lead to stress fiber formation, enhanced integrin clustering, and the subsequent focal adhesion kinase (FAK)-dependent sustained activation of ERK MAPK (13). To determine whether MIF-induced Rho-GTPase activation results in enhanced myosin light chain phosphorylation, immunoblot analysis of MLC using phosphorylation-specific antibodies revealed that MLC phosphorylation increases in direct proportion to the MIF-induced stimulation of GTP-bound Rho (Fig. 2A). These data demonstrate that MIF induces ERK activation concomitant with Rho GTPase activation and enhanced myosin light chain phosphorylation.

Inhibition of Rho, Rho Kinase, Myosin Light Chain Kinase, and MEK1 Prevents the Induction of MAPK, Cyclin D1, and DNA Synthesis by MIF in NIH 3T3 Fibroblasts—To investigate in more detail the precise requirements for this hypothesized pathway of cyclin D1 expression induced by MIF, we next sought to inhibit the key enzymes thought to be involved in this pathway. Because we hypothesize that MIF-dependent induction of cyclin D1 expression in fibroblasts is reliant upon sustained ERK MAPK activation, a well characterized inhibitor of the upstream activator of ERK1/2 was used. As shown in Fig. 3A, pretreatment of NIH 3T3 fibroblasts with the MEK1 inhibitor U0126, prevented the induction of cyclin D1 expression by MIF.

As mentioned above, one pathway by which Rho could promote signaling to ERK, leading to cyclin D1 expression, is through the inactivation of MLCP by Rho kinase (13, 24). The net result of MLCP inactivation by Rho kinase would be an enhancement of steady state MLC phosphorylation that is dependent upon myosin light chain kinase activity (13). We therefore tested whether inhibitors of myosin light chain kinase could prevent MIF-mediated FAK and ERK activation, leading to cyclin D1 expression. As depicted in Fig. 3B, pretreatment of cells with the myosin light chain kinase inhibitor ML-7,
after plating, U0126 (cyclin D1 expression, and cell proliferation. MIF was added to cells at 50 ng/ml and allowed to incubate overnight.

**Fig. 3.** Preferential inhibition of MEK1, myosin light chain kinase, Rho kinase, or Rho blocks MIF-induced MAPK activation, cyclin D1 expression, and cell proliferation. 3 days prior to the experiment, NIH 3T3 fibroblasts were plated as in Fig. 1. On the third day after plating, U0126 (A) ML-7 (B), or Y27632 (C) was added at 10 μM, or 0.1% Me2SO was added as a vehicle control. 30 min later, recombinant MIF was added to cells at 50 ng/ml and allowed to incubate overnight. D, 2 days after plating, 4 μg of either pEXV plasmid alone or pEXV-RhoN19 were transfected into NIH 3T3 cells for 30 h, at which time rMIF was added at 50 ng/ml and allowed to incubate overnight. Lysates were prepared as described under “Experimental Procedures,” and normalized samples were analyzed for the indicated proteins or phosphoproteins by Western blotting as described under “Experimental Procedures.” E, NIH 3T3 cells were plated in 0.5% FCS media, and 2 days later inhibitors were added as indicated for 30 min prior to the addition of 50 ng/ml rMIF. 30 h later, cells were pulsed for 4 h with [3H]thymidine, cells were harvested, and [3H]thymidine incorporation was assessed by liquid scintillation counting. Data shown for A–C are representative of at least two independent experiments, and data shown for D are representative of three experiments.

blocked the induction/activation of these proposed MIF effectors, suggesting a strict requirement for MLC-dependent stress fiber formation and subsequent FA$k$ activation in carrying out MIF signaling to cyclin D1 expression.

To verify the importance of Rho kinase as a mediator of MIF-stimulated signaling to focal adhesion formation, sustained MAPK activation, and cyclin D1 expression, the Rho kinase inhibitor, Y27632, was tested for its ability to block MIF signaling events. As shown in Fig. 3C, inhibition of Rho kinase blocks MIF-induced MLC phosphorylation, FA$k$ phosphorylation, and sustained ERK activation and, to a slightly lesser extent, inhibits the relative-fold induction of cyclin D1 induced by MIF. Finally, to verify that RhoA itself was required for MIF-mediated signaling to cyclin D1 expression, forced overexpression of RhoN19 was found to very effectively block the ability of exogenously added rMIF to induce MLC, FA$k$, and ERK MAPK phosphorylation and cyclin D1 expression. Together, these findings indicate that Rho, Rho kinase, MLC kinase, and ERK MAPK are all part of an MIF-induced signaling program that serves to enhance cyclin D1 expression in mesenchymal cell types.

Because we hypothesized that this Rho-dependent signaling pathway leading to cyclin D1 expression is responsible for the previously observed induction of mesenchymal cell proliferation initiated by MIF, we next sought to determine whether blockade of the individual components of this signaling cascade could prevent MIF-induced S phase cell cycle progression. As shown in Fig. 3E, inhibition of Rho kinase, myosin light chain kinase, or MEK1 all prevent G, S phase progression induced by rMIF.

**MIF-deficient Mouse Embryonic Fibroblasts Are Defective in Rho GTPase Activation**—Given the fact that exogenously added rMIF activates Rho, leading to the sustained activation of ERK and cyclin D1 expression (Fig. 3), we would predict that cells defective in MIF would possess less active Rho. To test this, quiescent p53H175 immortalized MIF+/− and MIF−/− MEFs (5) were plated onto fibronectin-coated dishes in the presence of growth factors for varying times. Welsh et al. (7) previously reported that under these conditions, Rho is responsible for the sustained activation of ERK that had previously been shown to promote mid-G1 phase cyclin D1 expression (6, 13). As shown in Fig. 4, A and B, immortalized MIF−/− fibroblasts are strongly impaired in adhesion/growth factor-induced Rho activation when compared with the levels of Rho activity in MIF+/− cells. Similar to our earlier findings with ERK (5), the requirement for MIF in modulating adhesion/growth factor-dependent Rho activation appears to be highest during the sustained phase (i.e. 1–4.5 h) of cell activation as opposed to the acute phase (i.e. 0.2 h). This finding is also consistent with our prior observation that MIF is required for maximal cyclin D1 expression in response to adhesion/growth factor stimulation (5).

**Active RhoA GTPase, Stress Fiber Formation, Phosphorylated ERK, and Cyclin D1 Are Defective in MIF-deficient Fibroblasts**—To investigate in greater detail the relative requirements for endogenous MIF in maintaining Rho activity and downstream effector activation in murine embryonic fibroblasts, we next determined the extent of stress fiber formation in the absence and presence of MIF. Normally cycling, asynchronous MIF+/− and MIF−/− p53H175 immortalized MEFs were plated onto fibronectin and assessed for their relative abilities to form stress fibers. As expected, MIF-deficient cells display significantly fewer and less organized stress fiber formation than their wild type counterparts (Fig. 5A). Importantly, and consistent with this result, we also find that Rho activity is substantially reduced in normally cycling, asynchronous MIF−/− cells, and this decreased Rho activity correlates closely with decreased cyclin D1 expression and sustained ERK activity in these cells (Fig. 5B). Together, these findings sug-
suggest that both induced (Fig. 4) and steady state (Fig. 5) Rho activity and downstream signaling to cyclin D1 are, at least in part, dependent upon functional MIF.

**Rescue of Defective Cyclin D1 Transcription in MIF$^{-/-}$ Cells by Rho GTPase and MLC Phosphatase Inhibition**—In order to establish a functional link between decreased Rho activity and defective cyclin D1 expression observed in MIF$^{-/-}$ cells, we next sought to determine whether restoration of Rho-dependent signal transduction could rescue MIF-stimulated cyclin D1 transcription and Rb inactivation (5). Rho$^{14,14}$, a constitutively active mutant of Rho, was co-transfected into p53$^{H175}$ immortalized MIF$^{-/-}$ cells along with a SRF, cyclin D1, or Rb-sensitive luciferase reporter plasmid. SRF is a well characterized downstream transcriptional effector of Rho activity (25, 26), and, in line with the decrease in active Rho observed in MIF$^{-/-}$ fibroblasts (Figs. 4 and 5), these cells contain nearly 75% less active SRF than MIF$^{+/+}$ cells (Fig. 6A). Importantly, reconstitution of constitutively active Rho restores defective SRF activity in MIF$^{-/-}$ cells well above and beyond that of the MIF$^{+/+}$ cells.

Consistent with our hypothesis that MIF-mediated Rho activity promotes steady state cyclin D1 expression and Rb inactivation in rodent fibroblasts, transcription from both the cyclin D1 promoter and associated Rb inactivation are both significantly reduced in MIF-deficient cells versus MIF-containing cells (Fig. 6A). Importantly, both of these defects are overcome by the reintroduction of constitutively active Rho into MIF$^{-/-}$ cells (Fig. 6A). Combined, these data suggest that the observed decrease in Rho activity associated with the loss of MIF is at least partially responsible for the defective cyclin D1 transcription and Rb inactivation also found in these cells (Fig. 5) (5).

To further investigate whether Rho-dependent enhancement of myosin light chain phosphorylation leading to cyclin D1 expression is part of an effector pathway utilized by MIF to promote cyclin D1 transcription, we attempted to recapitulate MLCP inhibition by CPI-17. CPI-17 is an endogenous inhibitor of MLCP and is also reported to be a substrate of Rho kinase (27–29). When phosphorylated at Thr$^{38}$ by Rho kinase or a variety of other kinases, the inhibitory activity of CPI-17 toward MLCP is increased nearly 1000-fold (28).

To rescue the defective Rho-dependent MLCP inhibition in immortalized MIF$^{-/-}$ fibroblasts, we overexpressed CPI-17 or a vector control along with a cyclin D1 promoter-driven luciferase construct. CPI-17 overexpression in MIF$^{+/+}$/p53$^{H175}$ immortalized cells had only a very modest effect on transcription from the cyclin D1 promoter, suggesting that the requirement for MLCP inhibition on cyclin D1 promoter activity in MIF-containing cells was nearly maximal at the time of analysis (Fig. 6B). In contrast, however, transcription from the cyclin D1 promoter in MIF$^{-/-}$ cells was only half that of MIF$^{+/+}$ cells (Fig. 6, A and B), and restoration of MLC phosphorylation (not shown) through MLCP inhibition by CPI-17 fully restored cyclin D1 transcription from these cells (Fig. 6B). It is important to note that several other enzymes, in addition to Rho kinase, are capable of stimulating the activity of CPI-17 (30–32). Because we anticipate that Rho kinase activity is reduced in MIF-deficient cells, the activation of CPI-17 is probably induced by one of these other enzymes in a MIF-deficient setting. Together, these data imply that both Rho activity and myosin light chain phosphorylation are important effectors of MIF-dependent cyclin D1 expression in fibroblasts.

In summary, these results provide a mechanistic link between MIF and cyclin D1 expression in rodent fibroblasts. This pathway includes the MIF-dependent activation of Rho, Rho kinase, MLC phosphorylation, FAK, and ERK MAPK (Fig. 7).
**FIG. 6.** Constitutively active Rho and inhibition of MLC phosphatase by CPI-17 restores cyclin D1 expression in MIF-deficient cells. A, subconfluent MIF+/+ and MIF−/− immortalized fibroblasts were transiently transfected with either the indicated transcription factor-responsive luciferase plasmids or a −1745 cyclin D1 promoter luciferase construct. pRL-null plasmid with or without 1 μg/ml pEXV-RhoV14 was additionally used in the co-transfections. Results are expressed as −fold increase/decrease over control after normalizing ratios of luciferase/Renilla luciferase from quadruplicate samples and are representative of two independent experiments. Results are expressed as −fold promoter activity after normalizing ratios of luciferase/Renilla luciferase and represent the mean ± S.D. from three experiments. B, cells were plated as in A. Transient co-transfections consisted of the −1745 cyclin D1 promoter luciferase construct, pRL-null plasmid, pK.HA vector alone, or pK.HA-CPI-17. For A and B, 30 h after transfections, firefly luciferase and Renilla luciferase activities were measured by the Dual Luciferase reporter assay system. For B, parallel transfections were performed with pK.HA vector alone or pK.HA-CPI-17, and expression of CPI-17 was assessed by Western blotting for HA epitope tag. Results are expressed as the relative light units (RLUs) after normalizing ratios of luciferase/Renilla luciferase and represent the mean ± S.D. from two independent experiments.

Because Rho activation impinges upon several parallel effector pathways (14), it is likely that MIF-deficient modulation of steady state Rho activity leads to phenotypic changes other than those examined in this study. Further investigation is warranted to evaluate the relative role of MIF in the modulation of these and other signaling intermediates.

**DISCUSSION**

We have demonstrated that MIF is a central participant in the maintenance of steady state cyclin D1 expression in immortalized rodent fibroblasts. Moreover, we identified a number of signaling elements that mediate MIF-induced cyclin D1 expression, comprising Rho, Rho kinase, myosin light chain phosphorylation, and FAK-mediated ERK MAPK activation.

Our findings are in line with results obtained by Roovers and colleagues (6, 13, 24), who established the involvement of Rho kinase and MLC in the activation of mid-G1 phase cyclin D1 expression induced by the synergistic actions of growth factors and adhesion to extracellular matrices in quiescent fibroblasts.

**Our earlier studies revealed that MIF secretion is induced by both growth factors and cell adhesion and that, through its autocrine action, MIF is partially responsible for the sustained ERK MAPK activation and cyclin D1 expression induced by these mitogenic stimuli (4, 5). Our current results strongly indicate that MIF is also partially responsible for the previously observed growth factor/adhesion-dependent activation of Rho- and Rho kinase-dependent signaling to sustained ERK activation and subsequent cyclin D1 expression.

What is less clear is what role, if any, this pathway plays in malignant cell transformation. Similar to the requirements for Rho and ERK MAPK activity in H-ras V12-mediated fibroblast transformation (19, 34), MIF is also a required factor for H-ras V12-induced transformation (20). Moreover, our data reveal that this observed resistance to oncogenic cell transformation is most evident in cells lacking a functional tumor suppressor p53 and appears to be due to defective cyclin D1 transcription and resultant Rb inactivation (3, 5, 20) (data not shown).

Guo et al. (35–37) recently reported that Rho GTPase activities are markedly increased in fibroblasts with defective p53 or mediators of p53. The authors report that loss of functional p53 results in increased phosphoinositide 3-kinase activity that is ultimately responsible for the activation of Rho proteins (36, 37). Interestingly, the enhanced RhoA activation observed in p53−/− mouse embryonic fibroblasts was found to be responsible for a strong up-regulation in cyclin D1 expression and a corresponding increase in cell cycle progression (36). Coupled with our findings, it is likely that MIF contributes to the pathway described above in settings with dysfunctional p53 and serves to promote Rho activation that then leads to cyclin D1 expression, Rb inactivation, and cell cycle progression. Moreover, because MIF−/− mice are developmentally normal yet display markedly resistant or altered cell growth phenotypes in differently immortalized and transformed cells (4, 5, 20), it is likely that the relative contribution of MIF to normal cell growth and division is minimal, but pathological cell hyperplasia and/or malignant cell growth processes are reliant upon functional MIF. This scenario is in line with the hypoth-
esion that the enhanced growth properties and Rho-dependent signaling observed in p53-deficient settings (36) may require functional MIF. Studies are currently under way to evaluate these possibilities.

The identification of Rho as a key mediator of MIF bioactivities is important for the understanding of the many biological functions of MIF. This cytokine is reported to contribute to the pathologies of many clinically important human disease states, including cancer, and understanding the downstream signaling intermediates involved in relaying its signal into a cell is an important first step in targeting this molecule for therapeutic purposes. In particular, MIF has been shown to promote tumor growth and viability by supporting tumor-associated angiogenesis (38, 39). Notably, Rho and Rho kinase-dependent stress fiber formation have been tightly linked to endothelial cell reorganization and mature blood vessel formation (40–42). Indeed, endostatin, a potent antiangiogenic fragment of collagen XVIII, has been shown to inhibit neovascularization in part by inhibiting endogenous endothelial cell Rho activity (43). It is not unreasonable to speculate that the reported proangiogenic activities of MIF are, at least in part, mediated through Rho GTPase-associated stress fiber formation.

The knowledge that signaling initiated at the plasma membrane by MIF is linked to Rho activation should additionally provide important clues as to how the recently described cell surface receptor of MIF, CD74, relays MIF-dependent signals to proximal effectors (44). Because binding of CD74 by MIF reportedly stimulates the sustained activation of MAPK (44), our results suggest that Rho probably participates in relaying this signal.

What is less clear is how CD74 may be linked to Rho GTPase activation. CD74 is a type II integral membrane protein whose intracellular portion lacks any obvious signal-initiating domains (44). One mechanism proposed for CD74-dependent signaling is the previously reported heterotypic binding of cell surface CD74 to CD44 in a chondroitin sulfate-dependent manner that may trigger CD44-mediated signal transduction (44–46). CD44 is a widely expressed family of cell surface receptors best known for its importance in promoting innate and adaptive immunity as well as contributing to neoplasm-associated disease progression (47, 48). Interestingly, activation of CD44-dependent signaling by its major ligand, hyaluronic, induces p115RhoGEF-dependent Rho GTPase activation (49). Although the signaling events proximal to Rho activation by MIF are beyond the scope of this report, the identification of these mediators and associated effectors will yield significant insight into MIF biology.

We now demonstrate that MIF, a cytokine/growth factor overexpressed in most human cancers tested (33, 50–53), is critically important in maintaining cyclin D1 expression in cells. Overexpression of cyclin D1 transcription. Although this is compelling evidence that MIF is an essential component of immortalized mesenchymal cell growth and tumorigenesis, data linking functional MIF to the growth of most clinically relevant human cancers are lacking. Because the vast majority of prognostically unfavorable human malignancies derive from epithelial tissues, future studies designed to investigate the importance of this growth factor in epithelial cell-derived cancers are critical.

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