Macrophage migration inhibitory factor promotes Warburg effect via activation of the NF-κB/HIF-1α pathway in lung cancer

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Abstract. Macrophage migration inhibitory factor (MIF) is upregulated in various solid tumors, a process that is associated with tumor progression and metastasis. The present study aimed to investigate the role and the underlying mechanism of MIF in human lung cancer. Human lung cancer H358, H460, H524, H1650, H838, H1975 and A549 cell lines were used to examine the expression of MIF by real time-quantitative polymerase chain reaction and western blotting. The lentivirus was used to over-express MIF and the expression of MIF and hypoxia-inducible factor 1-α (HIF-1α) were knocked down by shRNA or siRNA. The proliferation of cell lines was detected by MTT assay. Glucose uptake, adenosine 5'-triphosphate (ATP) production, the glycolytic rate and lactate production were used to examine the Warburg effect in cells. BAY 11-7082 (BAY) was used to inhibit the nuclear translocation of nuclear factor-κB (NF-κB), which was detected using immunofluorescence. It was revealed that overexpression of MIF promoted cell proliferation and the Warburg effect in lung cancer, whereas knockdown of MIF inhibited cell proliferation and the Warburg effect. Mechanistically, MIF promoted the Warburg effect by upregulating HIF-1α. Knockdown of HIF-1α largely abolished the promotional effect of MIF on the Warburg effect. Additionally, the results in the current study provided evidence that MIF regulates HIF-1α through NF-κB. In conclusion, the findings of the present study demonstrated that MIF is a key component in lung cancer progression through promoting the Warburg effect, and that the novel MIF/NF-κB/HIF-1α axis may prove to be useful for the development of new strategies for treating patients with lung cancer.

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Introduction

Lung cancer is the most common cancer and the leading cause of cancer-associated mortality in the world (1). It is estimated that the 5-year survival rate is <16% (1,2). However, the underlying molecular mechanisms of lung cancer pathogenesis are poorly understood. Therefore, novel therapeutic targets for human lung cancer are urgently warranted.

Macrophage migration inhibitory factor (MIF) is a pro-inflammatory cytokine produced by a variety of cell types (3-5), which has been involved in numerous inflammatory diseases, including rheumatoid arthritis (6), atherosclerosis (7) and hepatitis (8). In addition, MIF is overexpressed in various solid tumors (9-11), and regulates autonomous properties of tumor cells, including proliferation, apoptosis, and chemo-resistance. Furthermore, MIF is involved in the metastatic potential of tumors, including hepatoma, gastric cancer and lung cancer (12-15). Overwhelming evidence has been produced to highlight the essential role of MIF in tumor progression and tumorigenesis. MIF promotes sustained extracellular signal-regulated kinase activation, which is a major outcome of mutations in Ras, occurring in a third of human tumors (16,17). MIF signal transduction is necessary for maximal tumor-associated evasion from cell senescence (18) and inhibition of the tumor suppressor, p53 (19,20). MIF has been shown to support hypoxic adaptation by inducing stabilization of hypoxia-inducible factor-1α (HIF-1α) (21). In a hypoxic environment, MIF may also promote tumor growth through a marked enhancement of angiogenesis (16,21). Recently, an expanding body of studies on lung cancer have identified MIF as a key factor of tumor growth. MIF has also been identified in high quantities in the non-small cell lung cancer cells that subsequently serve to promote neovascularization (22,23). MIF overexpression promoted lung tumor growth and progression (24), whereas specific knockdown of MIF expression or functional inhibition significantly reduced migration and invasion of lung adenocarcinoma cells (25,26). The present study will provide persuasive evidence that MIF is a promising therapeutic target in human lung cancer.

The majority of cancer cells preferentially use aerobic glycolysis instead of oxidative phosphorylation to meet their increased energetic and biosynthetic demands, despite the presence of oxygen, which has long been known as the Warburg effect (27,28). The Warburg effect serves a critical role in tumorigenesis, and can be targeted for cancer therapy (29) MIF has been shown to exert an important role in the control of glucose disposal and carbohydrate metabolism (30,31). However, the role of MIF on the Warburg effect in lung cancer has yet to be elucidated.

In the present study, it is demonstrated that MIF promotes cell proliferation and the Warburg effect in lung cancer. The study also demonstrates that blocking the nuclear factor-κB (NF-κB)/HIF-1α signaling pathway largely abolishes the promotional effects of MIF on the Warburg effect. This novel MIF/NF-κB/HIF-1α axis may be valuable in the development of novel strategies for treating patients with lung cancer.

Materials and methods

Cells and reagents. Procedures concerning cell culture were performed as previously described (32). Seven human lung cancer cell lines (H358, H460, H524, H1650, H838, H1975 and A549) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured according to their protocol. All the cell lines used in the present study were authenticated through short-tandem repeat profiling less than 6 months prior to the initiation of this project, and the cells had not been in culture for more than 2 months.

RNA interference. These procedures were also performed as previously described (32). Lentiviral plasmids expressing MIF, short hairpin (sh)MIF and negative control were purchased from Cyagen Biosciences, Inc. (Shanghai, China). The specific sequences against MIF were as follows: 5'-TCATCGTAAA CACCAACGT-3' and 5'-GCGCAGAACCGCTCCTACA-3'). Short interfering RNAs (siRNAs) against HIF-1α (the specific sequences, 5'-AGUUAGUUCAAACUGAGUUAAUCCC-3') were purchased from Ruibo Biotech Co., Ltd. (Guangzhou, China). Scrambled siRNA (Ruibo Biotech Co., Ltd.) was used as a non-specific siRNA control. Approximately $2x10^5$ cells/well were seeded in a 60-mm culture dish on the day prior to transfection. Transfection with 50 nmol siRNA was performed according to the manufacturer's protocol using the Lipofectamine™ RNAiMAX transfection reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA). After transfection for 48 h in a incubator with 5% CO_2 at 37˚C, real time-quantitative polymerase chain reaction (RT-qPCR) and western blot assays were performed.

RNA extraction and RT-qPCR. Total RNA was extracted from cells and isolated using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. First-strand cDNA was synthesized from 1 μg total RNA using a RevertAid™ First Strand cDNA Synthesis kit (MBI Fermentas, Burlington, ON, Canada). The RT-qPCR was performed using SYBR-Green Master mix (LightCycler 480; Roche, Basel, Switzerland). The primers used in the present study were as follows: MIF forward, 5'-ACTAAGAAAGACCCGAGGC-3' and reverse, 5'-GGGGCACGTTGGTGTTTAC-3'; GAPDH forward, 5'-AGCCACATCGCTCAGACA-3' and reverse, 5'-TGGACTCCACGACGTACT-3'. The reaction conditions included the denaturation at 95˚C for 15 sec, renaturation at 60˚C for 60 sec, with 40 cycles in total. The results were quantified with $2^{-\Delta\Delta CT}$.

Western blot analysis. Total proteins were obtained from the cultured cells. The bicinchoninic acid (BCA) method was used to determine the protein concentrations. Equal amounts of samples (4 samples per group) were loaded onto 10% SDS-PAGE gels and the proteins were transferred to polyvinyl difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% skimmed milk at room temperature for 1 h, and then incubated overnight at 4˚C with the primary antibodies [rabbit monoclonal antibody against MIF (1:1,000, ab175189); mouse monoclonal antibody against HIF-1α (1:1,000, ab16066) (both from Abcam, Cambridge, MA, USA); rabbit monoclonal antibody against NF-κB (1:1,000, #8242); mouse monoclonal antibody against β-actin (1:1,000, #3700) (both from Cell Signaling Technology, Inc., Danvers, MA, USA)]. The blots were detected using a SuperSignal ECL kit (Invitrogen; Thermo Fisher Scientific, Inc.) in a western blotting detection system (Kodak Digital Science, Rochester, NY, USA) and quantified by densitometry using ImageJ analysis software (National Institutes of Health, Bethesda, MD, USA).

Cell proliferation. For the cell counting kit-8 (CCK-8) assay, ~$5x10^3$ cells/well were seeded into 96-well plates. CCK-8 solution (10 μl) was added to each well at the indicated time-points of 1, 3, 5 and 7 days post-transfection, and the mixture was incubated for 2 h at 37˚C. Subsequently, the absorbance was measured at 450 nm using a spectrophotometer.

Analysis of glucose uptake, the glycolytic rate and lactate production in cells. These procedures were performed as previously described (32). Glucose uptake levels were determined by measuring the uptake of [^{3}H]2-deoxyglucose. Briefly, cells cultured in 12-well plates were pre-incubated in glucose-free media for 30 min prior to the addition of [^{3}H]2-deoxyglucose (1 μCi/well) to the cells. After incubation for 30 min, the cells were washed with phosphate-buffered saline (PBS) and lysed in 1% SDS. The radioactivity of the cell lysates was determined in a liquid scintillation counter, and normalized to the protein concentrations of the cell lysates. Cellular glycolytic rates were measured by monitoring the conversion of [5-^{3}H]glucose into ^3H_2O. Briefly, the cells ($1x10^6$) were collected and washed once in PBS prior to resuspension in 1 ml Krebs buffer without glucose for 30 min at 37˚C. The cells were collected and resuspended in 0.5 ml Krebs buffer containing 10 mM glucose and 5 μCi [5-^{3}H]glucose for 1 h at 37˚C. Triplicate 100 μl aliquots were transferred to uncapped PCR tubes containing 100 μl 0.2 N HCl, and the tubes were transferred to scintillation vials containing 0.5 ml H_2O. The scintillation vials were then sealed and left for 48 h. The quantities of diffused and undiffused ^{3}H were subsequently determined in a liquid scintillation counter. Lactate production levels were measured using a Lactate Fluorimetric/Colorimetric Assay kit (BioVision, Inc., Milpitas, CA, USA). Cells were plated in 100-mm culture dishes at a density of $1x10^6$ cells/plate. After incubation for 24 h at 37˚C, the culture medium

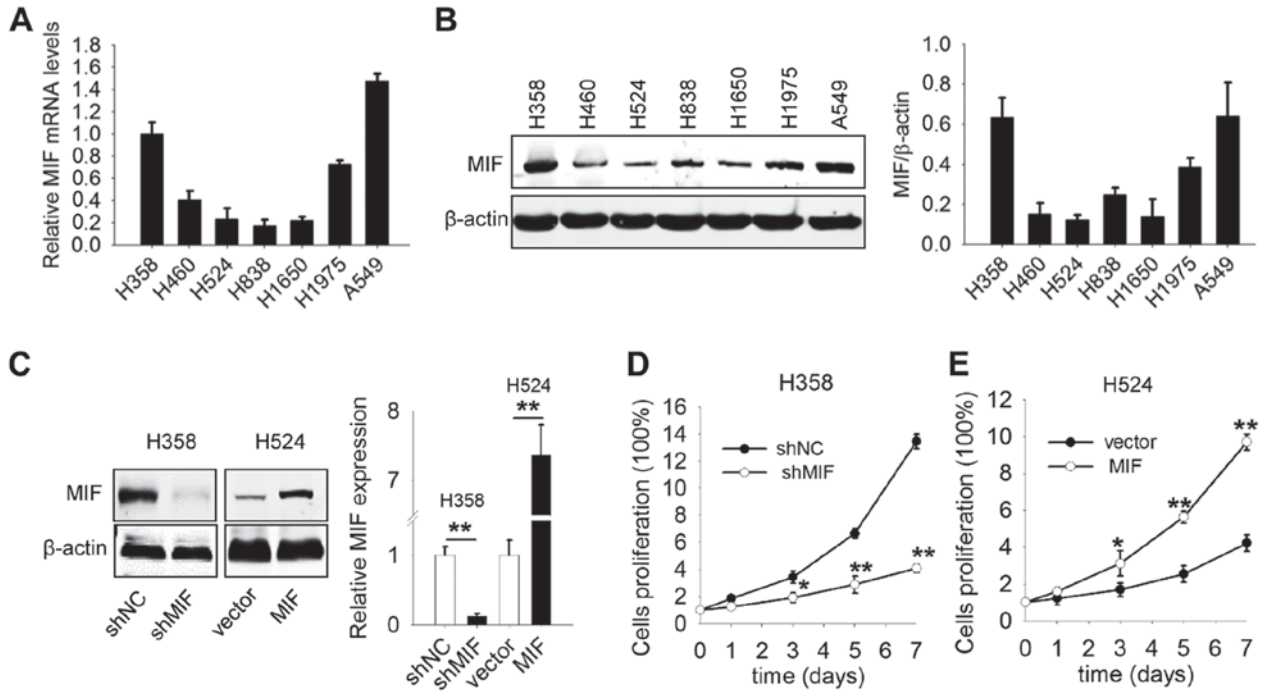

Figure 1. MIF promotes lung cancer cell proliferation. The mRNA and protein levels of MIF were determined in 7 lung cancer cell lines through RT-qPCR (A) and western blotting (B), respectively. GAPDH was used as an internal control in (A). (C) The generation of stable cell lines in H358 and H524 cells in which MIF was silenced or overexpressed was confirmed through western blotting. The bars correspond to the mean ± standard error of the mean. (D and E) The cell proliferation of the indicated stable cell lines *in vitro* was measured at different time points, as indicated by the cell counting kit-8 assay. The bars correspond to the mean ± standard error, and the P-value was calculated using Student's t-test. *P<0.05 and **P<0.01 vs. shNC (D) or vector (E), respectively. MIF, macrophage migration inhibitory factor;siNC, negative control.

was replaced with fetal bovine serum (FBS)-free DMEM. The lactate levels in the culture medium were examined with the Lactate Fluorimetric/Colorimetric Assay kit, and normalized against the number of cells.

Adenosine 5'-triphosphate (ATP) measurements. ATP measurements were obtained using an ATP determination kit (Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's protocol. The ATP content was determined based on comparison with a concurrent standard curve.

Immunofluorescence. The cells were fixed using 4% paraformaldehyde for 15 min. After washing and permeabilization with 0.1% Triton X-100 for 5 min, the cells were blocked with a 5% solution of BSA (for 1 h at room temperature). Primary antibodies of NF-κB (rabbit monoclonal antibody, 1:200, \$8242; Cell Signaling Technology, Inc.) were applied overnight at 4˚C. The corresponding secondary antibodies [goat anti-rabbit IgG (H+L), Alexa Fluor Plus 647 (#A32733; Invitrogen; Thermo Fisher Scientific, Inc.)] were added for a further incubation for 30 min at room temperature. To visualize the nuclei, fixed cells were incubated with 4',6-diamidino-2-phenylindole for 10 min.

Statistical analysis. Data are expressed as the means ± standard deviation of at least three separate experiments. A one-way ANOVA and Student-Newman-Keuls tests were used for statistical analysis, and a value of P<0.05 was considered to indicate a statistically significant difference. All analyses were performed using SPSS version 13.0 (Statistical Software for Social Sciences, Chicago, IL, USA).

Results

MIF promotes lung cancer cell proliferation. First, the expression of MIF in seven lung cancer cell lines was detected using RT-qPCR and western blotting (Fig. 1A and B). High expression of MIF was observed in the H358 and A549 cell lines, whereas low expression of MIF was found in the H524 and H460 cell lines (Fig. 1A and B). Based on the expression level of MIF in these lung cancer cells, stable cell lines were constructed either with silenced MIF in H358 cells, or ectopic expression of MIF in H524 cells (Fig. 1C). Subsequently, the effect of MIF on the biological behaviors of these cell lines was investigated. Cell proliferation assays indicated that silencing MIF significantly inhibited cell proliferation (Fig. 1D); by contrast, the ectopic expression of MIF increased the cell proliferative ability of H524 cells (Fig. 1E).

MIF promotes the Warburg effect in lung cancer. The majority of cancer cells preferentially use aerobic glycolysis instead of oxidative phosphorylation to meet their increased energetic and biosynthetic demands, a phenomenon known as the Warburg effect (27). The Warburg effect is involved in tumor development, and is able to fulfill the energetic demands of the membrane transport activities required for proliferation and migration (33). Since MIF has previously been reported to regulate glucose uptake and catabolism (30,31), the issue of

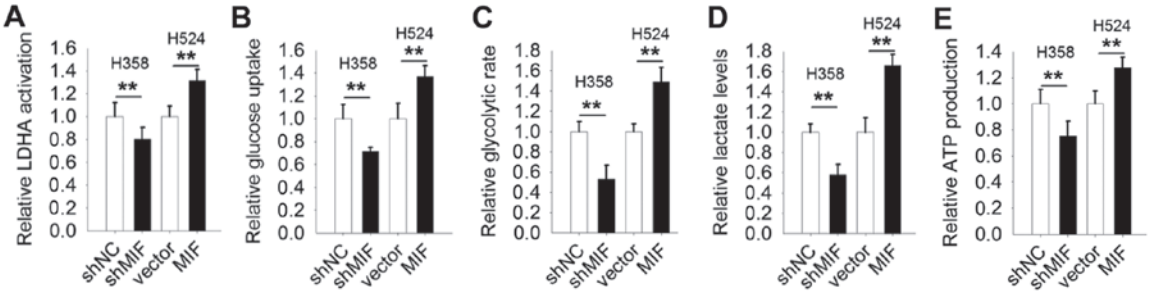

Figure 2. Macrophage migration inhibitory factor (MIF) promotes the Warburg effect in lung cancer. (A) LDHA activation was determined in the stable cell lines in which MIF was overexpressed or knocked down. (B-E) Glucose uptake levels, glycolytic rate, lactate production and ATP concentrations were measured in the stable cell lines in which MIF was overexpressed or knocked down, as described in the Materials and methods section. The data are presented as the mean ± standard error (n=3). **P<0.01 (Student's t-test). MIF, macrophage migration inhibitory factor; LDHA, lactate dehydrogenase ATP, adenosine 5'-triphosphate; siNC, negative control.

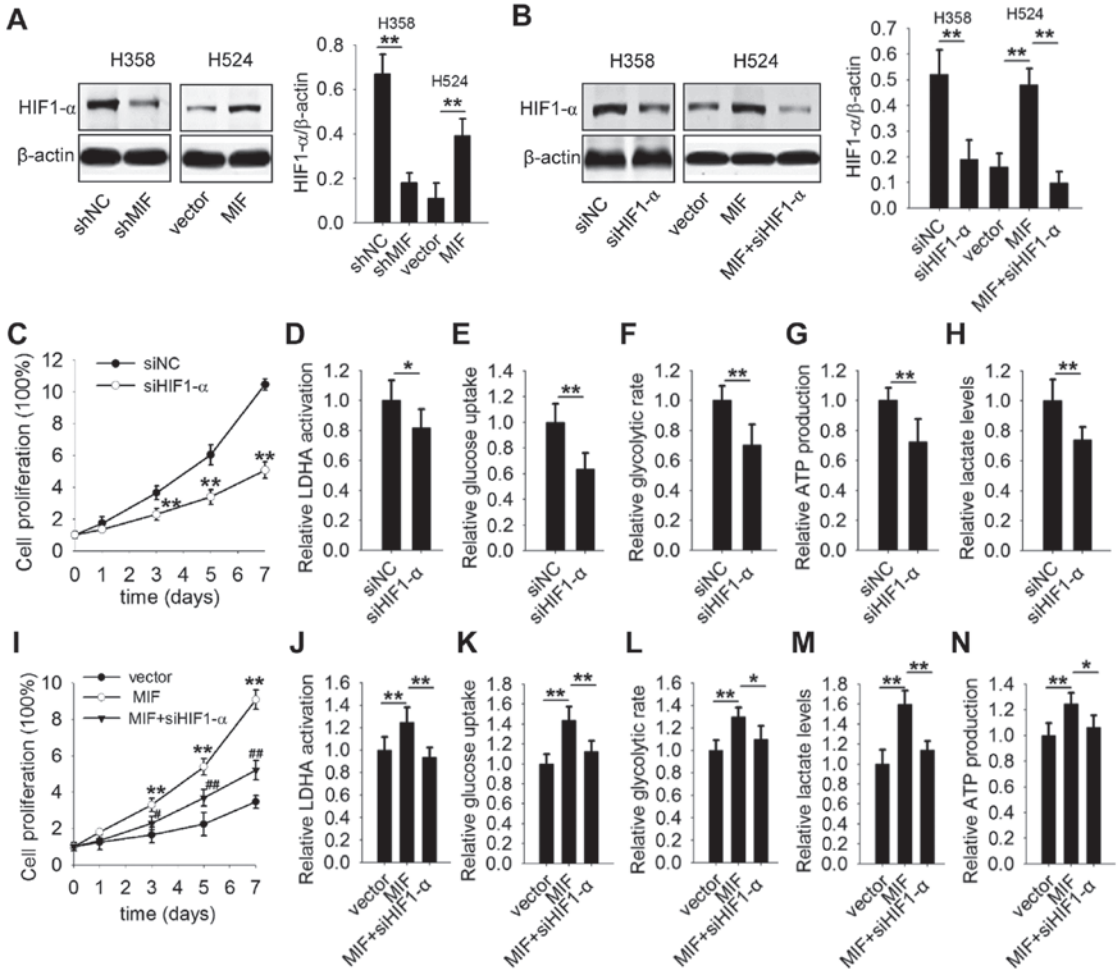

Figure 3. MIF regulates the Warburg effect through activation of HIF-1α. (A) Protein levels of HIF-1α were detected by western blotting in H358 and H524 cells in which MIF was silenced or overexpressed. (B) H358 cells were transfected with siNC or siRNA targeting HIF-1α for 48 h. H524 cells were transfected with control vector, ectopic MIF expression (MIF), or MIF with siRNA targeting HIF-1α for 48 h. The cells were then harvested to determine the protein levels of HIF-1α through western blotting. (C-H) Cell proliferation, LDHA activation, glucose uptake levels, glycolytic rate, lactate production, and ATP concentrations were measured in H358 cells in which HIF-1α was knocked down, as described in the Materials and methods section. (I-N) Cell proliferation, LDHA activation, glucose uptake levels, glycolytic rate, lactate production and ATP concentrations were measured in H524 cells transfected with control vector, ectopic MIF expression (MIF), or MIF with siRNA targeting HIF-1α, as described in the Materials and methods section. Data are presented as the mean ± standard error (n=3). *P<0.05; **P<0.01 (according to Student's t-test). MIF, macrophage migration inhibitory factor; HIF 1-α, hypoxia inducible factor-1α; LDHA, lactate dehydrogenase A; siNC, negative control; ATP, adenosine 5'-triphosphate

whether MIF may regulate the Warburg effect in lung cancer cells was investigated. Lactate dehydrogenase A (LDHA) is required for the conversion of pyruvate into lactate, which is associated with the Warburg effect. As predicted, overexpression or knockdown of MIF increased or decreased LDHA activation (Fig. 2A). Furthermore, overexpression of MIF

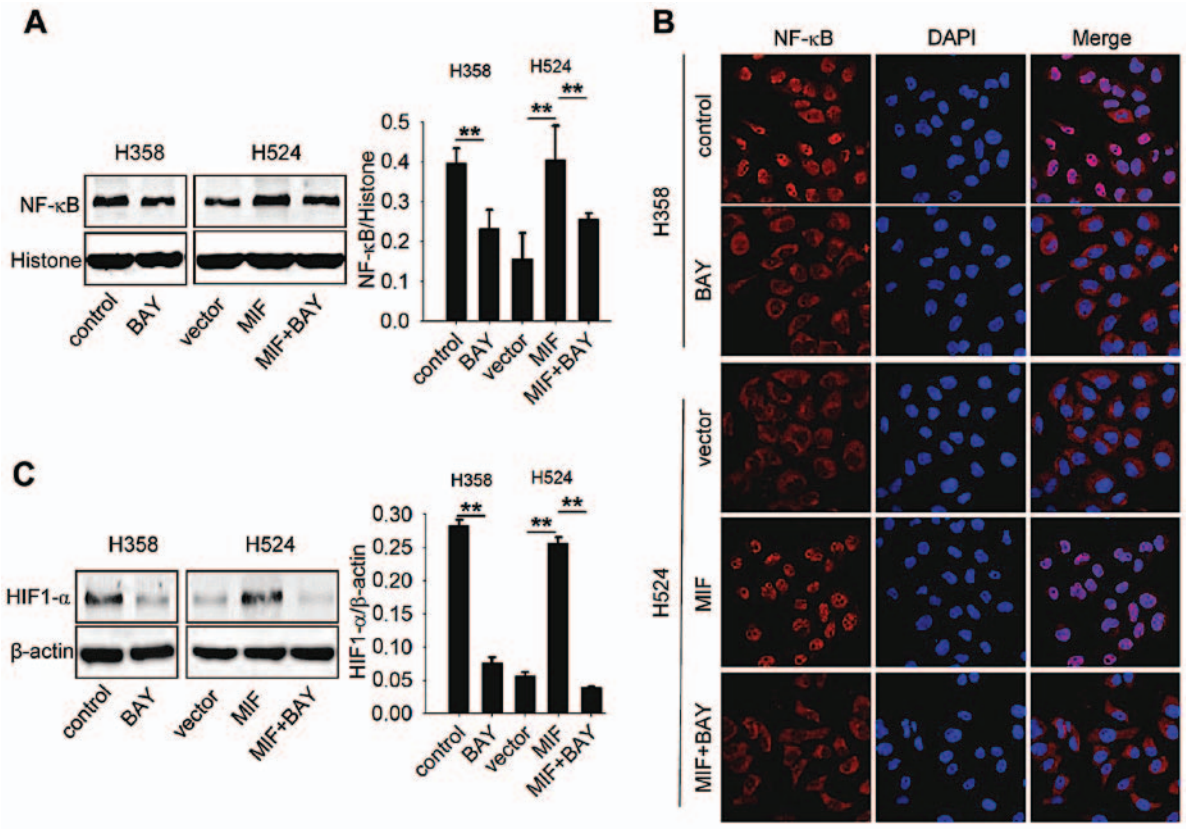

Figure 4. MIF regulates HIF-1α through activation of NF-κB. (A) Western blotting analysis of NF-κB in H358 and H524 cells. (B) Immunofluorescence images of co-localization of NF-κB and DAPI in the cytoplasm of H358 and H524 cells. (C) Western blotting analysis of HIF-1α in H358 and H524 cells. Data are presented as the mean±standard error (n=3). **P<0.01 (according to Student's t-test). MIF, macrophage migration inhibitory factor; HIF-1α, hypoxia inducible factor-1α; NF-κB, nuclear factor-κB; DAPI, 4',6-diamidino-2-phenylindole.

significantly promoted the Warburg effect in H524 cells, whereas knockdown of MIF significantly inhibited the Warburg effect in H358 cells (Fig. 2B-E). These findings indicated that MIF promotes the Warburg effect in lung cancer cells.

MIF promotes the Warburg effect through activation of HIF-1α. How MIF regulates the Warburg effect in lung cancer was subsequently investigated. HIF-1α-induced transcriptional regulation controls several key genes that are crucial to deregulated glucose metabolism in cancers; that is, the Warburg effect (34). Furthermore, MIF has been shown to support hypoxic adaptation by inducing stabilization of HIF-1α (21). Consequently, the aim was to determine the association between MIF and HIF-1α on the Warburg effect in lung cancer. Knockdown of MIF decreased the protein expression of HIF-1α in H358 cells, whereas ectopic expression of MIF significantly upregulated HIF-1α in H524 cells (Fig. 3A). H358 cells were transfected with non-targeting negative control RNA and HIF-1α-specific siRNA. The specificity of siRNA was examined with western blotting, which confirmed that the siHIF-1α reduced the expression of HIF-1α (Fig. 3B). In addition, knockdown of HIF-1α expression significantly inhibited HIF-1α upregulation induced by MIF overexpression in H524 cells (Fig. 3B). Subsequently, it was shown that knockdown of HIF-1α expression significantly inhibited cell proliferation, LDHA activation and the Warburg effect in H358 cells (Fig. 3C-H). By contrast, MIF overexpression promoted cell proliferation, LDHA activation, and the Warburg effect in H524 cells, and silencing HIF-1α clearly abolished the promotional effect of MIF on cell proliferation, LDHA activation, and the Warburg effect (Fig. 3I-N). Taken together, these results strongly suggested that MIF promotes the Warburg effect through activation of HIF-1α.

MIF regulates HIF-1α through activation of NF-κB. Subsequently, the molecular mechanisms underlying MIF regulation of the Warburg effect were investigated. NF-κB and its associated signaling pathway, which is frequently activated in various types of human cancers, serves a pivotal role in tumorigenesis (27). The activated NF-κB signaling pathway has been shown to promote the Warburg effect in cancer cells (35). In the present study, H358 cells were treated with BAY 11-7082 (BAY; an NF-κB inhibitor) to block NF-κB activation. Nuclear translocation of NF-κB was significantly reduced, a result that was confirmed by western blotting and immunofluorescence (Fig. 4A and B). Ectopic expression of MIF significantly increased the translocation of NF-κB to the nucleus in H524 cells, whereas BAY largely inhibited the translocation of NF-κB to the nucleus induced by MIF overexpression, as determined by western blotting and the immunofluorescence experiments (Fig. 4A and B). Blocking NF-κB activation with BAY significantly reduced the expression of HIF-1α in H358 cells (Fig. 4C). MIF overexpression upregulated HIF-1α expression in H524 cells, whereas blocking NF-κB activation with BAY clearly inhibited the HIF-1α upregulation induced by MIF (Fig. 4C). These data suggested that MIF regulates HIF-1α through activation of NF-κB.

Discussion

An improved understanding of the mechanism underlying human lung cancer and novel therapeutic targets is urgently required for this deadly cancer. In the present study, it has been demonstrated that MIF is a key player in lung cancer progression via promoting the Warburg effect. It was also shown that MIF regulates the Warburg effect through the NF-κB/HIF-1α pathway. This novel MIF/NF-κB/HIF-1α axis may be useful for development of novel strategies for treating patients with lung cancer.

MIF, a pro-inflammatory cytokine, has been highlighted as a key factor in lung cancer. High expression of MIF in patients with lung cancer has been shown to lead to a worse prognosis (23,36), and it has been suggested as a potential biomarker in non-small cell lung cancer (37). It has been reported that MIF overexpression directly enhanced lung cancer growth and metastasis (24). In addition, functional inhibition of MIF in lung cancer cell lines suppressed cell proliferation and numerous hallmarks of tumor development, including angiogenesis and metastasis (25,26,38). In agreement with a previous study on cell proliferation (24), the present study has confirmed that MIF overexpression promotes cell proliferation, whereas silencing MIF significantly inhibited proliferation in human lung cancer cells. Our data indicate that MIF promotes cell proliferation and tumor growth in lung cancer.

The Warburg effect is one of the characteristics of cancer cells (28,39). The switch from oxidative phosphorylation to glycolysis provides an essential mechanism that enables cancer cells to meet their significantly increased energetic and biosynthetic demands to support their rapid growth and proliferation (27). Understanding the mechanism underlying the Warburg effect is important for the development of novel strategies for cancer therapy. MIF has been reported to exert an important role in the control of glucose disposal and carbohydrate metabolism (30,31). Brock *et al* (40) revealed that MIF promotes glucose uptake and ATP generation in non-small cell lung carcinomas. Consistent with these discoveries (27,40), our group has observed that overexpression of MIF significantly promotes the Warburg effect in H524 cells, whereas knockdown of MIF significantly inhibited the Warburg effect in H358 cells. The results of the present study suggest that MIF promotes the Warburg effect in lung cancer, indicating that MIF inhibition represents a potentially important therapeutic strategy for the treatment of human lung cancer.

How does MIF regulate the Warburg effect in lung cancer? It is essential to understand its mechanism. HIF-1 activation has been associated with angiogenesis, erythropoiesis and modulation of key enzymes involved in aerobic glycolysis, thereby modulating key processes required for the Warburg effect (41). The Warburg effect is due, at least in part, to the failure of cancer cells to appropriately downregulate HIF under well-oxygenated conditions (42). Furthermore, MIF has been shown to support hypoxic adaptation by inducing stabilization of HIF-1α (21). The results in the current study have provided evidence that MIF regulates the Warburg effect through activation of HIF-1α. NF-κB and its associated signaling pathway, which is frequently activated in various types of human cancers, exerts a pivotal role in tumorigenesis (27). Activated NF-κB signaling has been shown to promote the Warburg effect in cancer cells (35). MIF has been reported to activate NF-κB to promote breast cancer metastasis (12). Furthermore, emerging evidence indicates cross-talk between the HIF and NF-κB pathways. For example, inflammatory stimuli, such as tumor necrosis factor-α, activate HIF-1α in an NF-κB-dependent manner (43). Furthermore, NF-κB increases HIF-1β stabilization (44), suggesting a potential role of NF-κB in regulation of the HIF pathways. The results in the present study have demonstrated that MIF regulates HIF-1α through activation of NF-κB in lung cancer. Taken together, these results suggest that MIF regulates the Warburg effect through activation of the NF-κB/HIF-1α pathway.

In conclusion, the findings of the current study have demonstrated that MIF promotes the Warburg effect through the NF-κB/HIF-1α pathway in lung cancer. These results contribute towards an improved understanding of the role of MIF in human lung cancer, and provide evidence that MIF inhibition may be therapeutically beneficial. However, additional studies are required; for example, a further assessment of the functions of MIF is required to investigate its biological significance in lung cancer *in vivo*. Mechanistic details underlying the promotion of the Warburg effect by MIF in lung cancer also require further characterization.

Acknowledgements

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