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Secreted and O-GlcNAcylated MIF binds to the human EGF receptor and inhibits its activation

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Abstract

Activation of epidermal growth factor receptor (EGFR), which occurs in many types of tumour, promotes tumour progression^{1,2}. However, no extracellular antagonist of human EGFR has been identified. We found that human macrophage migration inhibitory factor (MIF) is *O*-GlcNAcylated at Ser 112/Thr 113 at its carboxy terminus. The naturally secreted and *O*-GlcNAcylated MIF binds to EGFR, thereby inhibiting the binding of EGF to EGFR and EGF-induced EGFR activation, phosphorylation of ERK and c-Jun, cell invasion, proliferation and brain tumour formation. Activation of EGFR through mutation or its ligand binding enhances the secretion of MMP13, which degrades extracellular MIF, and results in abrogation of the negative regulation of MIF on EGFR. The finding that EGFR activation downregulates its antagonist in the tumour microenvironment represents an important feedforward mechanism for human tumour cells to enhance EGFR signalling and promote tumorigenesis.

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AUTHOR CONTRIBUTIONS

This study was conceived by Z.L.; Y.Z. and Z.L. designed research, Y.Z., X.L., X.Q., Y.W., J.-H.L., Y.X., D.H.H., G.Z. and J.L. performed experiments; Z.L. wrote the paper with comments from all authors.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Overexpression of epidermal growth factor (EGF) receptor (EGFR) and EGFR-activating mutations have been reported in many human tumours and are associated with a poor clinical prognosis¹⁻³. Activation of EGFR promotes tumour cell proliferation, migration and invasion^{1,2,4} and results in immediate and late regulatory loops that either positively or negatively mediate the activity of EGFR (ref. 5). In *Drosophila melanogaster*, the naturally secreted protein Argos functions as the only known extracellular inhibitor of EGFR (refs 6,7). Whether there is an extracellular antagonist for EGFR in human cells is still unknown.

Macrophage migration inhibitory factor (MIF), which is an immunostimulatory cytokine, is expressed in monocytes, macrophages, T and B lymphocytes, eosinophils, mast cells, basophils and neutrophils^{8,9}. MIF is a functional non-cognate ligand for the chemokine receptor CXCR4 and promotes the recruitment of both monocytes and T cells by interacting with CXCR2 and CXCR4 (ref. 8). MIF also interacts with CD74 (ref. 9). CD74 can form functional complexes with CXCR4 that mediate MIF-specific signalling¹⁰.

Overexpression of MIF has been detected in several types of human cancer¹¹. However, reports of the role of MIF expression in tumour progression are contradictory. For instance, breast cancer patients with high MIF expression in tumour tissues had poorer disease-free survival rates than those with low MIF expression, and exogenous MIF induced an increase in vascular endothelial growth factor and interleukin-8 secretion in breast cancer cells^{12,13}. In contrast, another study showed that MIF was more abundantly expressed in non-invasive breast cancer cells than in invasive breast cancer cells. MIF expression was positively correlated with progesterone and oestrogen receptor expression, both markers of a favourable prognosis, and was negatively correlated with tumour size. In addition, overall and recurrence-free survival rates were significantly higher in breast cancer patients with abundant cytosolic MIF expression than in patients with low MIF expression¹⁴. These findings suggest that the regulation of MIF expression during tumour development depends on the cellular signalling context and that the function of intracellular MIF might be different from that of extracellular MIF. However, the mechanism underlying the regulation of intra- or extracellular MIF expression remains unclear.

In this report, we show that MIF was modified at Ser 112/Thr 113 by *O*-linked β -*N*-acetylglucosamine (*O*-GlcNAc). Secreted *O*-GlcNAcylated MIF bound to the extracellular domain of human EGFR and competitively inhibited EGF-induced EGFR activation. EGFR activation in human cancer cells results in the degradation of MIF, which is mediated by EGFR activation-enhanced matrix metalloproteinase (MMP)13 secretion. MIF degradation promotes EGFR-induced tumour cell invasion and brain tumorigenesis.

RESULTS

EGFR activation results in MMP13-dependent degradation of secreted MIF

To determine whether EGFR activation regulates intra- and extracellular MIF expression, we treated A431 human epidermoid carcinoma cells with EGF. Prolonged EGF treatment, which did not alter intracellular MIF expression, decreased the secreted MIF in medium in a time-dependent manner (Fig. 1a). Pretreating the cells with AG1478, an EGFR inhibitor,

which blocked EGF-induced EGFR phosphorylation, blocked EGF-induced downregulation of extracellular MIF (Fig. 1b). This EGFR activation-dependent extracellular MIF downregulation was also observed in MDA-MB-231 human breast cancer cells, DU145 human prostate cancer cells, and U251 and U87 human glioblastoma cells (Fig. 1c). In addition, expression of a constitutively active EGFRvIII mutant¹⁵ resulted in downregulation of extracellular MIF (Fig. 1d). These results indicate that reduced extracellular MIF is a general response to EGFR activation in various types of human tumour cell.

The MMPs, a family of 23 proteolytic enzymes that are either membrane-anchored or secreted, degrade extracellular matrix proteins and are involved in many phases of cancer progression^{16,17}. Pretreating cells with GM6001, a MMP inhibitor for MMP1-3, MMP8 and MMP9 (ref. 18), or CL-82198, a potent selective inhibitor for MMP13 (ref. 19), showed that only CL-82198 inhibited EGF-induced extracellular MIF degradation (Fig. 1e). These results suggest that MMP13, a member of the matrix MMPs, is involved in MIF degradation. In line with these results, EGF treatment enhanced the MMP13 secretion in medium with correlated MIF degradation, and these effects were blocked by AG1478 in A431 and U87 cells (Fig. 1f). In addition, MMP13 depletion largely reduced EGF-induced MMP13 accumulation in medium and blocked EGF-enhanced MIF degradation (Fig. 1g). These results indicate that EGFR activation-induced MMP13 secretion results in MIF degradation.

MIF binds to the extracellular domain of EGFR and inhibits EGF-induced EGFR activation

To determine the cellular functions of MIF, we overexpressed Flag-MIF in A431 cells (Fig. 2a, left panel). Flag-MIF expression largely reduced EGF-induced phosphorylation of EGFR, ERK1/2 and c-Jun (Fig. 2a, right panel), indicating that MIF suppresses EGFR activation and its downstream signalling. To illustrate the role of extracellular MIF in the regulation of EGFR, we incubated the purified Flag-MIF (Fig. 2b, left panel) with A431 cells for 30 min before EGF treatment. Purified Flag-MIF reduced the activation of EGFR, ERK1/2 and c-Jun (Fig. 2b, right panel).

To define the mechanism underlying MIF-regulated EGFR inhibition, we incubated immunoprecipitated Flag-MIF with purified recombinant EGF and found that MIF does not bind to EGF directly (Fig. 2c), whereas the recombinant EGF was able to bind to EGFR (Supplementary Fig. 1). In contrast, incubation of immunoprecipitated EGFR with purified Flag-MIF revealed an interaction between EGFR and MIF (Fig. 2d, left panel). This finding was further supported by showing that immobilized Flag-MIF interacted with EGFR from the cell lysate (Fig. 2d, right panel). Furthermore, incubation of purified His-EGFR extracellular domain with purified Flag-MIF demonstrated that MIF directly bound to the extracellular domain of His-EGFR (Fig. 2e). Notably incubation of A431 cells with purified Flag-MIF before adding Texas-Red-labelled EGF largely reduced the binding of EGF to EGFR (Fig. 2f). In line with the finding that MMP13 degrades MIF, CL-82198 treatment or expression of MMP13 short hairpin RNA (shRNA), which inhibited EGF-induced degradation of extracellular MIF, blocked EGF-induced EGFR phosphorylation (Fig. 2g). These results indicate that extracellular MIF directly binds to the extracellular domain of

EGFR and blocks the binding of EGF to EGFR, thereby inhibiting EGF-induced EGFR activation.

MIF is O-GlcNAcylated at Ser 112 and Thr 113

We purified bacterially expressed His–MIF. However, purified His–MIF was unable to block EGF-induced EGFR activation (Supplementary Fig. 2a), suggesting that bacterially expressed His–MIF may not have the same structure or post-translational modification as its mammalian homologue from cells. Liquid chromatography-coupled ion trap/orbital trap mass spectrometry (LC-MS/MS) analyses of purified immunoprecipitated Flag–MIF from 293T cells revealed that MIF was modified at Ser 112 and/or Thr 113 by *O*-GlcNAc (Fig. 3a), a carbohydrate post-translational modification on hydroxyl groups of serine and/or threonine residues of proteins²⁰. Immunoblotting analyses of purified His–MIF from bacteria and purified Flag–MIF from 293T cells showed that only Flag–MIF was *O*-GlcNAcylated (Fig. 3b).

We next labelled *O*-GlcNAc-modified proteins from 293T cell lysates with a non-natural azido sugar through exposure to an exogenous galactosyltransferase enzyme that specifically glycosylates terminal GlcNAc sugars. Pulldown of the biotin-labelled *O*-GlcNAcylated proteins with streptavidin resin showed that overexpression of Myc-tagged *O*-GlcNAc transferase (OGT; Fig. 3c, left panel) or treatment of 293T (Fig. 3c, right panel), U251 and DU145 (Supplementary Fig. 2b) cells with the *O*-(2-acetamido-2-deoxy-*D*-glucopyranosylidenamino) *N*-phenylcarbamate (PUGNAc), an inhibitor of the *O*-GlcNAcase (OGA), enhanced *O*-GlcNAcylation of MIF. In addition, mutations of Ser 112 and Thr 113 of MIF into alanine showed that MIF S112/T113A with double mutations, but not MIF S112A or MIF T113A, largely reduced MIF *O*-GlcNAcylation (Fig. 3d), indicating that MIF is *O*-GlcNAcylated at Ser 112 and Thr 113.

The *O*-GlcNAcylation of MIF is required for MIF to bind to EGFR and to inhibit EGF-induced EGFR activation

O-GlcNAcylation regulates the functions of substrate proteins, such as protein–protein interactions, protein stability and protein activity²¹. To determine whether the *O*-GlcNAcylation of MIF regulates the binding of MIF to EGFR, we incubated purified wild-type (WT) Flag–MIF or Flag–MIF S112/T113A with the cell lysate or purified His–EGFR extracellular domain and showed that MIF S112/T113A lost its ability to bind to endogenous EGFR and the EGFR extracellular domain (Fig. 4a). In line with these findings, purified Flag–MIF S112/T113A failed to inhibit EGF-induced EGFR activation in U87 (Fig. 4b) and A431 cells (Supplementary Fig. 2c).

Notably, incubation of purified Flag–MIF with purified GST–OGA, which resulted in the removal of MIF *O*-GlcNAcylation (Fig. 4c), reduced the binding of MIF to EGFR (Fig. 4d) and failed to block the binding of EGF to EGFR (Fig. 4e) and EGF-induced EGFR phosphorylation (Fig. 4f). These results strongly suggest that *O*-GlcNAcylation of MIF at Ser 112/Thr 113, which are the residues in the C-terminal β -strand and part of a looped structure composed of two β -strands²², is required for MIF to bind to the extracellular domain of EGFR and to inhibit EGF-induced EGFR activation.

LC-MS/MS analyses revealed that MIF was modified at Ser 112 and/or Thr 113 by *O*-GlcNAc, but not by any other post-translation modifications. In line with this finding, immunoblotting analysis of purified Flag–MIF with a phospho-Ser/Thr antibody did not detect any phosphorylation of MIF whereas immunoblotting with the same antibody showed that calf intestinal alkaline phosphatase (CIP) largely dephosphorylated cellular proteins (Supplementary Fig. 3a). CIP treatment, which did not affect the binding of MIF to immunoprecipitated endogenous EGFR and purified His–EGFR extracellular domain (Supplementary Fig. 3b), had no effect on MIF-inhibited EGF binding to EGFR (Supplementary Fig. 3c) and EGF-induced EGFR phosphorylation (Supplementary Fig. 3d). These results strongly suggest that MIF is not phosphorylated at Ser 112/Thr 113.

The tumour microenvironment contains not only tumour cells but also immune cells, and both groups of cells express MIF (refs 8,9). Immunoblotting analyses showed that THP1 human monocytic cells, which were treated with phorbol ester to induce their differentiation into macrophages, and BV2 mouse microglia, which are the resident macrophages of the central nervous system, secreted amounts of MIF comparable to the amount secreted by U87 cells (Fig. 4g). Considering that most cells in tumours are tumour cells, most of the MIF in the tumour microenvironment should be from tumour cells. Notably, the *O*-GlcNAc level of secreted MIF from U87 cells was significantly higher than that from THP1 and BV2 cells (Fig. 4g), suggesting that tumour cells contribute most of the *O*-GlcNAcylated MIF in the tumour microenvironment.

The *O*-GlcNAcylation of MIF inhibits EGF-induced tumour cell invasion and brain tumorigenesis

EGFR activation promotes tumour cell invasion⁴. Overexpression of WT Flag–MIF, but not Flag–MIF S112/T113A, in U87 (Fig. 5a) or A431 (Supplementary Fig. 4a) cells significantly inhibited EGF-induced tumour cell invasion, suggesting that the binding of *O*-GlcNAcylated MIF to EGFR inhibits EGFR activation-induced tumour cell migration. Similarly, when U87 (Fig. 5b) or A431 (Supplementary Fig. 4b) cells were incubated with EGF in the presence or absence of purified WT Flag–MIF or Flag–MIF S112/T113A, only WT Flag–MIF largely blocked EGF-induced tumour cell invasion. Similarly, the deglycosylated Flag–MIF, but not dephosphorylated Flag–MIF, lost the effect of MIF on EGFR-promoted tumour cell invasion (Fig. 5c). Of note, WT Flag–MIF exhibited no effect on the invasion of U87 cells overexpressing constitutively activate EGFRvIII (Supplementary Fig. 4c). In line with these findings, overexpression of WT Flag–MIF, but not Flag–MIF S112/T113A, largely inhibited EGF-promoted U87 cell proliferation (Fig. 5d).

To determine the role of MIF *O*-GlcNAcylation in tumorigenesis, we intracranially injected endogenous MIF-depleted U87 cells with reconstituted expression of RNAi-resistant WT rMIF or rMIF S112/T113A into athymic nude mice. MIF-depleted U87 cells elicited rapid tumorigenesis (Fig. 5e) and shorter survival (Fig. 5f), and this enhanced tumour growth was alleviated by the expression of EGFR shRNA (Supplementary Fig. 5a). In contrast, reconstituted expression of WT rMIF, but not rMIF S112/T113A expression, significantly inhibited tumour growth (Fig. 5e) and prolonged the survival of the mice (Fig. 5f). Similar

results were also observed by overexpression of WT MIF or MIF S112/T113A in U87 cells (Supplementary Fig. 5b), whereas overexpression of WT MIF lost its inhibitory effect on EGFRvIII-induced tumour growth (Supplementary Fig. 5c). These results indicated that the regulation of *O*-GlcNAcylated MIF expression plays an instrumental role in brain tumour development.

DISCUSSION

Activation of EGFR promotes tumour progression^{1,2,4,23}. The ligand binding or mutation of EGFR initiates many EGFR activation-induced cellular activities. We demonstrated here that MIF is *O*-GlcNAcylated at Ser 112/Thr 113. The naturally secreted and *O*-GlcNAcylated MIF binds to EGFR, thereby inhibiting the binding of EGF to EGFR and subsequent EGF-induced EGFR activation. EGFR activation enhanced the secretion of MMP13, which degraded extracellular MIF, thereby alleviating the negative regulation of EGFR by MIF and promoting EGFR activation (Fig. 5g). Given the heterogeneous nature of human tumours and the fact that individual glioblastoma tumours express both amplified EGFR and EGFRvIII and undergo tumour-promoting effects from EGFRvIII-expressing cells through paracrine mechanisms²⁴, EGFRvIII expression in some tumour cells downregulates extracellular MIF, which, in turn, is likely to promote EGF-induced WT EGFR activation in the tumour cells expressing WT EGFR. Our finding demonstrates an important mechanism underlying amplified EGFR activation in tumours, which is mediated by downregulation of its antagonist MIF in the tumour microenvironment.

The *Drosophila* protein Argos binds to EGF, thereby sequestering EGF and inhibiting its bindings to EGFR (refs 6,25). In contrast, MIF, which is *O*-GlcNAcylated at its C-terminal Ser 112/Thr 113 on a β -strand²², binds to EGFR directly and competitively inhibits the binding of EGF to EGFR. The *O*-GlcNAcylated looped structure at the C terminus of MIF (ref. 22) may directly interact with the extracellular domain of EGFR, which physically hinders the access of EGF to EGFR.

MIF has been reported to bind to the extracellular domain of CD74 and CXCR4 and to activate ERK1/2 and AKT. In previous studies, anti-CXCR4 and anti-CD74 antibodies blocked MIF-induced activation of ERK1/2 and AKT, and cell proliferation^{9,10}. In contrast, extracellular *O*-GlcNAcylated MIF did not induce a regulatory effect on ERK and AKT in tested tumour cells. Instead, it blocked EGF-induced signalling and tumour cell invasion. Given that intracellular MIF may have distinct functions according to the cell type and the nature of the extracellular stimulus, our results in combination with previous findings strongly suggest that the cellular activities of MIF can vary based on differences in cell types and cellular microenvironments. That the *O*-GlcNAcylation-deficient MIF mutant failed to block EGF-induced tumour cell invasion and inhibit tumour growth reveals the importance of post-translational modification of MIF in its involvement of tumour progression. Our finding of EGFR-induced and MMP13-dependent MIF degradation and subsequent amplification of EGFR activation provides an instrumental insight into tumour progression mediated by the regulation of the tumour microenvironment and may provide an alternative approach for treating human cancer by intervening in this auto-regulation loop.

METHODS

Cells and cell culture conditions

A431 human epidermoid carcinoma cells, MDA-MB-231 human breast adenocarcinoma cells, DU145 human prostate carcinoma cells, U251 and U87 human glioblastoma cells, and BV2 mouse microglia cells were all maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (HyClone) and penicillin/streptomycin (Sigma). THP-1 monocytes were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (HyClone) and penicillin/streptomycin (Sigma). All of the cell lines were from ATCC and are routinely tested for mycoplasma. None of the cell lines are listed in the database of commonly misidentified cell lines maintained by ICLAC and NCBI Biosample.

Materials

Rabbit polyclonal antibodies against MIF (sc-20121, 1:500 for immunoblotting), ERK2 (sc-154, 1:1,000 for immunoblotting) and Myc (sc-40, 1:1,000 for immunoblotting), goat polyclonal antibody against MMP13 (sc-12363, 1:500 for immunoblotting), and mouse monoclonal antibodies against phospho-ERK (sc-7383, clone E-4, 1:1,000 for immunoblotting), phospho-c-Jun (sc-822, clone KM-1, 1:1,000 for immunoblotting) and EGF (sc-101477, clone KT2, 1:1,000 for immunoblotting) were from Santa Cruz Biotechnology. The selective inhibitor of MMP13 CL-82198 (C9498), the OGA inhibitor PUGNAc (A7229), Flag peptide (F4799), and monoclonal antibodies against Flag (F3165, clone M2, 1:5,000 for immunoblotting, 1:1,000 for immunoprecipitation), His (H1029, clone HIS-1, 1:5,000 for immunoblotting) and tubulin (T6074, clone B-5-1-2, 1:5,000 for immunoblotting) were from Sigma. The EGFR pY869 (no. 11229, 1:1,000 for immunoblotting) and EGFR (no. 21222, 1:1,000 for immunoblotting, 1:400 for immunofluorescence) rabbit polyclonal antibodies were obtained from Signalway Biotechnology and Signalway Antibody. Monoclonal antibody against c-Jun (no. 610327, clone 3, 1:1,000 for immunoblotting), phosphoserine/threonine (no. 612548, clone 22A/pSer/Thr, 1:1,000 for immunoblotting) and Matrigel (no. 354234) were from BD Biosciences. The *O*-GlcNAc (no. 9875S, clone CTD110.6, 1:1,000 for immunoblotting) mouse monoclonal antibody was from Cell Signaling Technology. The EGFR inhibitor AG1478 (no. 658552) and the MMP inhibitor GM6001 (no. 364205) were from Calbiochem. Human recombinant EGF (01-407) was obtained from Millipore. Texas-Red-labelled EGF (E3480), Click-iT *O*-GlcNAc enzymatic labelling system (C33368), and Click-iT protein analysis detection kits (C33372) were purchased from Life Technologies.

Trichloroacetic acid precipitation

Trichloroacetic acid (TCA) precipitation was used to concentrate the secreted MIF protein in the cell culture medium. We first cleared 1 ml of medium by centrifugation at 13,000 *g* for 10 min at 4 °C and then added 250 µl 100% (w/v) TCA into the separated supernatant. After mixing, the solution was incubated on ice for 10 min. Then, the precipitate was collected by centrifugation (15,000 *g* for 5 min at 4 °C). The supernatant was carefully removed, and the pellet was washed twice with 200 µl cold acetone. The tubes were placed in a 95 °C heat block for 5–10 min to drive off acetone. Finally, the pellet was redissolved in 20 µl 2×

sample buffer, and the samples were boiled for 10 min at 95 °C before being loaded onto SDS–polyacrylamide gels.

DNA constructs and mutagenesis

The cDNA corresponding to the extracellular domain (ECD) of EGFR was cloned into the pCold vector. A polymerase chain reaction-amplified human MIF cDNA was cloned into the pCold and pcDNA3.1-Flag vector. Flag–MIF S112A, Flag–MIF T113A and Flag–MIF S112/T113A mutants were generated using the QuikChange site-directed mutagenesis kit (Stratagene). pGIPZ-MMP13 shRNA was generated using oligonucleotides 5'-ATTATGGA GGAGATGCCCATT-3'. pGIPZ-MIF shRNA was generated using oligonucleotides 5'-ACCGCTCCTACAGCAAGCTG-3'. pGIPZ-EGFR shRNA was generated using oligonucleotides 5'-TTGCGATCTGCACACACCA-3'.

Transfection

Cells were plated at a density of 4×10^5 per 60-mm-diameter dish 18 h before transfection. Transfection was performed using HyFect reagents (Denville Scientific) according to the vendor's instructions²⁶. Stable cell lines were selected with hygromycin ($100 \mu\text{g ml}^{-1}$) or puromycin ($5 \mu\text{g ml}^{-1}$) for 10–14 days at 37 °C. The antibiotic-resistant colonies were picked, pooled, and expanded for further analysis under selective conditions.

Immunoprecipitation and immunoblotting analysis

Extraction of proteins from cultured cells using a modified lysis buffer (50 mM Tris-HCl (pH 7.5), 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 0.5 mM EDTA, 1 mM dithiothreitol, and protease inhibitor cocktail (Calbiochem)) was followed by immunoprecipitation and immunoblotting with the corresponding antibodies. The immunoprecipitated Flag-tagged proteins were eluted from the resin with $100 \mu\text{g ml}^{-1}$ Flag peptide.

Purification of recombinant proteins

His–EGFR ECD and His–MIF were expressed in bacteria and purified as described previously²⁶. Briefly, the pCold His–EGFR ECD and pCold His–MIF were transformed into BL21/DE3 bacteria. Transformants were used to inoculate 50 ml cultures of LB/ampicillin, which were grown overnight at 37 °C to stationary phase. A measure of 5 ml preculture was then used to inoculate 200 ml LB/ampicillin. The cultures were grown at 37 °C to an attenuation of ~0.4–0.6 at 600 nm before inducing with 0.5 mM IPTG at 16 °C for 24 h. Cell pellets were collected, resuspended in 10 ml Bugbuster protein extraction reagent (EMD) with the addition of 20 μl protease cocktail inhibitor (EMD), and incubated at room temperature for 20 min, before centrifuging at 10,000 r.p.m. for 10 min at 4 °C. Cleared lysates were then bound to Ni-NTA His bind resin (EMD) for 3 h, with rolling at 4 °C. Beads were washed extensively with the extraction buffer before eluting for 1 h in extraction buffer (pH 7.5) plus 500 mM imidazole. Eluted proteins were then dialysed extensively against 20 mM Tris-HCl pH 8.0, 50 mM NaCl, 10% glycerol and 1 mM dithiothreitol.

His pulldown assay

Cell lysates (250 µg per sample) were incubated with 100 ng of His fusion proteins on Ni-NTA His-binding beads overnight at 4 °C. The beads were then washed three times with lysis buffer, and the bound proteins were eluted with sodium dodecyl sulphate (SDS) sample buffer before electrophoresis on SDS–polyacrylamide gels.

Immunofluorescence analysis

Cells were incubated with Texas-Red-labelled EGF (50 ng ml⁻¹) on ice for 10 min and then fixed and incubated with an anti-EGFR antibody, Alexa Fluor dye-conjugated secondary antibody, and Hoechst 33342, according to standard protocols. Cells were examined using a deconvolutional microscope (Zeiss) with a 40-Å oil immersion objective. Axio Vision 4 software from Zeiss was used to deconvolve Z-series images. The relative fluorescence intensity of Texas-Red-labelled EGF of the whole-cell images was compared and quantified using image analysis software.

Mass spectrometry analysis

GelCode blue-stained gel pieces were washed destained and digested in-gel with 200 ng of trypsin or chymotrypsin (sequencing grade, Promega) for 18 h at 37 °C. Resulting peptides were extracted and analysed by high-sensitivity LC-MS/MS on an Orbitrap Elite or Orbitrap Fusion mass spectrometer (Thermo Scientific) using CID, HCD or ETD. Proteins were identified by database searching of the fragment spectra against the SwissProt (EBI) protein database using Mascot (v 2.3, Matrix Science). Typical search settings were: mass tolerances, 10 ppm precursor, 0.8d fragments; variable modifications, methionine sulfoxide, pyro-glutamate formation; up to 2 missed cleavages for trypsin, 6 for chymotrypsin.

MIF glycosylation analysis

Chemoenzymatic labelling and biotinylation of proteins in cell lysates were carried out as described previously²⁷. Briefly, cells were lysed in lysis buffer (50 mM Tris-HCl (pH 7.5), 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 0.5 mM EDTA, 1 mM dithiothreitol, 10 µM PUGNAc, and protease inhibitor cocktail (Calbiochem)). The cell lysate (400 µg) was enzymatically labelled with an azido-containing nucleotide sugar analogue (UDP-GalNAz) using an engineered β(1,4)-galactosyltransferase according to the Click-iT *O*-GlcNAc enzymatic labelling system protocol (Invitrogen) and conjugated with an alkyne-biotin compound as per the Click-iT protein analysis detection kit protocol (Invitrogen). Biotinylation enabled the capture of *O*-GlcNAc-modified proteins from the lysate using streptavidin resin. The glycoproteins were eluted by boiling the resin in loading buffer for 10 min, and subsequent immunoblotting with an antibody against MIF was performed. Control experiments were carried out in parallel in the absence of the labelling enzyme.

In vitro invasion assay

Matrigel Transwell assays were performed as described previously²⁸. Briefly, Transwell inserts with 8-µm pores were coated with 100 µl of Matrigel in cold serum-free medium at a final concentration of 1 mg ml⁻¹. Cells were then trypsinized and resuspended in serum-free

medium. A cell suspension (1×10^5 cells in 100 μ l of medium) was added to the Transwell inserts. After 24 h of incubation, cells that invaded the Matrigel and passed through the filters were stained with 0.1% crystal violet for at least 5 min. The non-invading cells were then wiped from the inside of the inserts with a cotton swab. The membrane was photographed using a digital camera mounted onto a microscope, dissolved in 4% deoxycholic acid, and read colorimetrically at 590 nm.

Cell proliferation assay

A total of 2×10^4 cells were plated and counted 7 days after seeding in DMEM with 0.5% bovine calf serum. Data represent the mean $\pm$ s.d. of three independent experiments.

Intracranial injection

We intracranially injected 5×10^5 indicated cells (in 5 μ l of DMEM per mouse) into 4-week-old female athymic nude mice. The intracranial injections were carried out as described in a previous publication²⁹. Eight mice per group were used in each experiment. Animals were euthanized 2 weeks after the glioma cell injection. The brain of each mouse was collected, fixed in 4% formaldehyde and embedded in paraffin. Tumour formation and the phenotype were determined by histologic analysis of haematoxylin and eosin-stained sections. Tumour volumes were measured by using length (*a*) and width (*b*) and calculated using the following equation: $V = ab^2/2$. Data represent the means $\pm$ s.d. of 8 mice. The use of athymic nude mice was approved by the institutional review boards at MD Anderson Cancer Center.

Statistical analysis

The mean values obtained in the control and experimental groups were analysed for significant differences. Pair-wise comparisons were performed using a two-tailed Student's *t*-test. *P* values < 0.05 were considered significant. The sample size and the group size of animals chosen are based on the numbers we used for previous publications, which is most optimal to generate statistically significant results. The cells for immunofluorescence studies were randomly examined. The mice were randomly put into separate groups/cages for experiments. The investigators were divided into two groups. One group was blinded to allocation during experiments and outcome assessment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

EGFR activation results in MMP13-dependent MIF degradation. **(a)** A431 cells were treated with or without EGF (100 ng ml⁻¹) for the indicated periods. **(b)** A431 cells were treated with or without AG1478 (1 μM) for 30 min before being treated with or without EGF (100 ng ml⁻¹) for 24 h. **(c)** The indicated cells (MDA-MB-231, DU145, U251 or U87) were treated with or without AG1478 (1 μM) for 30 min before being treated with or without EGF (100 ng ml⁻¹) for 24 h. **(d)** U87 cells were stably transfected with plasmids expressing EGFRvIII. **(e)** A431 cells were treated with or without GM6001 (10 μM) or CL-82198 (50 μM) for 30 min before being treated with or without EGF (100 ng ml⁻¹) for 24 h. **(f)** A431 or U87 cells were treated with or without AG1478 (1 μM) for 30 min before being treated with or without EGF (100 ng ml⁻¹) for 24 h. **(g)** A431 cells stably transfected with pGIPZ expressing a control or a MMP13 shRNA (top panel) were treated with or without EGF (100 ng ml⁻¹) for 24 h (bottom panel). Protein levels were quantified through densitometry. Data represent the mean ± s.d. (*n* = 3 independent experiments). Cell lysate and TCA-precipitated proteins from conditioned medium were subjected to western blot analysis using the indicated antibodies. WB, western blot. Data represent 1 out of 3 experiments. Unprocessed original scans of blots are shown in Supplementary Fig. 6.

Figure 2.

MIF binds to the extracellular domain of EGFR and inhibits EGF-induced EGFR activation.

(a) A431 cells stably expressing an empty vector or pcDNA3.1-Flag-MIF (left panel) were treated with or without EGF (50 ng ml⁻¹) for 30 min (right panel). (b) Immunoprecipitated Flag-MIF from 293T cells overexpressing Flag-MIF was eluted from the resin with 100 µg ml⁻¹ Flag peptide (GelCode blue-stained gel, left panel). A431 cells were incubated with or without Flag-MIF proteins for 30 min before being treated with or without EGF (50 ng ml⁻¹) for 30 min (right panel). (c) Immunoprecipitated Flag-MIF was incubated with purified EGF *in vitro*. Purified EGF was loaded as a positive control (right lane). (d) Immunoprecipitated EGFR from A431 cells was incubated with purified Flag-MIF or Flag peptide (left panel). 293T cells were transiently transfected with a plasmid expressing Flag-MIF or control vector. Immunoprecipitated Flag-MIF or the proteins immunoprecipitated by normal IgG on the agarose beads were incubated with A431 cell lysates (right panel). (e) His pull-down assay was performed by incubating purified His-EGFR extracellular domain (ECD) with or without purified Flag-MIF. (f) A431 cells were incubated with purified Flag-MIF proteins (500 ng ml⁻¹) or Flag peptide (500 ng ml⁻¹) for 30 min before being treated with Texas-Red-labelled EGF (50 ng ml⁻¹) for 10 min. Immunofluorescence analysis was

performed with an anti-EGFR antibody. Scale bar, 10 μm . The relative fluorescence intensity of Texas-Red-labelled EGF was quantified. The data represent the mean $\pm$ s.d. ($n = 30$ cells, 3 independent experiments). Source data are provided in Supplementary Table 1. A two-tailed Student's t test was used. *, $P < 0.05$; NS, not significant. (g) A431 cells treated with or without CL-82198 (50 μM) for 24 h (left panel) or expressing or not expressing MMP13 shRNA (right panel) were stimulated with EGF (50 ng ml^{-1}) for 30 min (for EGFR phosphorylation) or 24 h (for MIF degradation). In **a–e,g**, western blotting and immunoprecipitation analyses were performed with the indicated antibodies. WB, western blot; IP, immunoprecipitation. Data represent 1 out of 3 experiments. Unprocessed original scans of blots are shown in Supplementary Fig. 6.

Figure 3.

MIF is *O*-GlcNAcylated at Ser 112 and Thr 113. **(a)** Purified Flag–MIF from 293T cells was subjected to LC-MS/MS analyses. The MS/MS spectrum (CID) shows matched ions for the peptide DMNAANVGWNNSTFA, which was modified by oxidation at the methionine residue. A Hex-NAc in the C-terminal region (S or T) was identified. The mass error for the parent ion was 3.8 ppm, the Mascot score was 45, expectation value 0.0028. **(b)** Purified His–MIF from bacteria and purified Flag–MIF from 293T cells were subjected to western blot analysis using the indicated antibodies. **(c)** Flag–MIF was co-expressed with or without Myc–OGT in 293T cells (left panel), or 293T cells, which were transiently transfected with empty vector or Flag–MIF expressing vector, were subsequently treated with or without PUGNAc (10 μ M) for 24 h (right panel). The cell lysates were treated without (as a control) or with the permissive mutant β -1,4-galactosyltransferase (Gal-T1 Y289L), which modified *O*-GlcNAc residues of cellular proteins with azido-modified galactose. The azide-modified proteins were then labelled with biotin and incubated with streptavidin beads. **(d)** 293T cells were transiently transfected with vectors expressing Flag–MIF or the Flag–MIF mutants. The *O*-GlcNAc-modified proteins were modified by azide and labelled with biotin and incubated with streptavidin beads. In **b–d**, western blotting and immunoprecipitation analyses were performed with the indicated antibodies. WB, western blot; IP,

immunoprecipitation. Data represent 1 out of 3 experiments. Unprocessed original scans of blots are shown in Supplementary Fig. 6.

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Figure 4.

The *O*-GlcNAcylation of MIF is required for MIF to bind to EGFR and to inhibit EGF-induced EGFR activation. **(a)** Immunoprecipitated and purified Flag-MIF or Flag-MIF S112/T113A on the agarose beads was incubated with A431 cell lysates (left panel). A pull-down assay was performed by mixing purified His-EGFR ECD on the agarose beads with purified WT Flag-MIF or Flag-MIF S112/T113A protein (right panel). **(b)** 500 ng ml⁻¹ purified WT Flag-MIF or Flag-MIF S112/T113A protein was incubated with U87 cells for 30 min before EGF (50 ng ml⁻¹) treatment for 30 min. **(c)** Immunoprecipitated and purified Flag-MIF was incubated with or without purified GST-OGA. **(d)** Immunoprecipitated and purified Flag-MIF on agarose beads in the presence or absence of purified GST-OGA was incubated with A431 cell lysates (left panel). Immunoprecipitated and purified Flag-MIF in the presence or absence of purified GST-OGA was incubated with immobilized purified His-EGFR ECD. A pull-down assay was performed (right panel). **(e)** A431 cells were incubated with GST-OGA-treated or -untreated purified 500 ng ml⁻¹ Flag-MIF proteins or Flag peptide for 30 min before being treated with Texas-Red-labelled EGF (50 ng ml⁻¹) for 10 min. Immunofluorescence analysis was performed with an anti-EGFR antibody. Scale bar, 10 μm. The relative fluorescence intensity of Texas-Red-labelled EGF was quantified. The data represent the mean ± s.d. (*n* = 30 cells, 3 independent experiments). Source data are provided in Supplementary Table 1. A two-tailed Student's *t* test was used. *, *P* < 0.05; NS, not significant. **(f)** U87 cells were incubated with GST-OGA-treated or -untreated purified 500 ng ml⁻¹ Flag-MIF proteins or Flag peptide for 30 min before

being treated with EGF (50 ng ml⁻¹) for 30 min. (g) A total of 5×10^6 U87, BV2 and PMA-treated (50 ng ml⁻¹, 24 h) THP1 cells were seeded. MIF was precipitated from the medium. The *O*-glycosylated MIF was detected by the same method as described in Fig. 3c. In **a–d,f,g**, western blotting and immunoprecipitation analyses were performed with the indicated antibodies. WB, western blot; IP, immunoprecipitation. Data represent 1 out of 3 experiments. Unprocessed original scans of blots are shown in Supplementary Fig. 6.

Figure 5.

The *O*-GlcNAcylation of MIF inhibits EGF-induced tumour cell invasion and brain tumorigenesis. **(a)** U87 cells with or without expression of WT Flag-MIF or Flag-MIF S112/T113A were plated on the top surface of Matrigel inserts and treated with or without EGF (50 ng ml⁻¹) for 24 h. **(b)** 500 ng ml⁻¹ purified WT Flag-MIF or Flag-MIF S112/T113A protein in the presence or absence of EGF (50 ng ml⁻¹) was incubated with U87 cells on the top surface of Matrigel inserts for 24 h. **(c)** U87 cells, which were incubated with purified Flag-MIF (500 ng ml⁻¹) that was untreated or treated with GST-OGA or CIP, were plated on the top surface of Matrigel inserts and treated with or without EGF (50 ng ml⁻¹) for 24 h. **(d)** A total of 2×10^4 U87 cells with or without expression of WT Flag-MIF

or Flag-MIF S112/T113A were treated with or without EGF (100 ng ml^{-1}) for 7 days. The cells were collected and counted. (e) A total of 5×10^5 U87 cells with or without MIF shRNA and with or without reconstituted expression of rMIF WT or rMIF S112/T113A (left panel) were intracranially injected into athymic nude mice ($n = 8$ mice per group). Haematoxylin and eosin-stained coronal brain sections show representative tumour xenografts (right upper panel). Tumour volumes were measured. Data represent the means $\pm$ s.d. ($n = 8$ mice per group). Source data are provided in Supplementary Table 1. *, $P < 0.05$; NS, not significant (right bottom panel). (f) A total of 5×10^5 U87 cells with or without MIF shRNA and with or without reconstituted expression of rMIF WT or rMIF S112/T113A were intracranially injected into athymic nude mice ($n = 8$ mice per group). The survival times of the mice were recorded. (g) A model of cellular activity of secreted MIF. Unprocessed original scans of blots are shown in Supplementary Fig. 6. In a–c, images represent one out of three experiments. Scale bar, $100 \mu\text{m}$. Column data represent the mean $\pm$ s.d. ($n = 3$ independent experiments). Source data are provided in Supplementary Table 1. A two-tailed Student's t test was used. *, $P < 0.05$; NS, not significant.