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Macrophage migration inhibitory factor produced by the tumour stroma but not by tumour cells regulates angiogenesis in the B16-F10 melanoma model

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BACKGROUND: Macrophage migration inhibitory factor (MIF) has been proposed as a link between inflammation and tumorigenesis. Despite its potentially broad influence in tumour biology and prevalent expression, the value of MIF as a therapeutic target in cancer remains unclear. We sought to validate MIF in tumour models by achieving a complete inhibition of its expression in tumour cells and in the tumour stroma.

METHODS: We used MIF shRNA-transduced B16-F10 melanoma cells implanted in wild-type and MIF − / − C57Bl6 mice to investigate the effect of loss of MIF on tumour growth. Cytokine detection and immunohistochemistry (IHC) were used to evaluate tumours *ex vivo*.

RESULTS: Macrophage migration inhibitory factor shRNA inhibited expression of MIF protein by B16-F10 melanoma cells *in vitro* and *in vivo*. *In vitro*, the loss of MIF in this cell line resulted in a decreased response to hypoxia as indicated by reduced expression of VEGF. *In vivo* the growth of B16-F10 tumours was inhibited by an average of 47% in the MIF − / − mice compared with wild-type but was unaffected by loss of MIF expression by the tumour cells. Immunohistochemistry analysis revealed that microvessel density was decreased in tumours implanted in the MIF − / − mice. Profiling of serum cytokines showed a decrease in pro-angiogenic cytokines in MIF − / − mice.

CONCLUSION: We report that the absence of MIF in the host resulted in slower tumour growth, which was associated with reduced vascularity. While the major contribution of MIF appeared to be in the regulation of angiogenesis, tumour cell-derived MIF played a negligible role in this process.

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Translational Therapeutics

Macrophage migration inhibitory factor (MIF) is a multifunctional cytokine, with complex and wide reaching activities, which has been proposed as a bridge between inflammation and tumorigenesis (Bucala and Donnelly, 2007). Macrophage migration inhibitory factor has been implicated in many aspects of tumour cell biology. Macrophage migration inhibitory factor was shown to control autonomous properties of tumour cells, such as the regulation of proliferation, apoptosis, DNA-damage response, senescence and invasion (Petrenko and Moll, 2005; Welford *et al*, 2006; Bucala and Donnelly, 2007; Fingerle-Rowson and Petrenko, 2007; Lue *et al*, 2007; Nemajerova *et al*, 2007b; Bifulco *et al*, 2008; Verjans *et al*, 2009; Dessein *et al*, 2010). Macrophage migration inhibitory factor also acts within the tumour stoma to promote angiogenesis and immune escape (Abe *et al*, 2001; Krockenberger *et al*, 2008; Zhou *et al*, 2008; Rendon *et al*, 2009; Mittelbronn *et al*, 2011). In addition, a high level of circulating MIF in serum has been reported in many cancer settings highlighting its potential as a detection biomarker (Grieb *et al*, 2010).

Most cells of the immune system as well as endothelial, endocrine, and epithelial cells produce MIF (Calandra and Roger, 2003). Macrophage migration inhibitory factor is predominantly localised in the cytoplasm and released by a non-conventional secretion pathway (Merk *et al*, 2009). Upon release, MIF triggers a number of signalling pathways utilising membrane-associated and intracellular binding partners. Macrophage migration inhibitory factor binds its best-characterised extracellular receptor CD74 in a complex with CD44. This binding triggers the sustained activation of the MAPK pathway, delivering a proliferative signal and the activation of Akt leading to inhibition of apoptosis (Leng *et al*, 2003; Meyer-Siegler *et al*, 2004; Lue *et al*, 2006, 2007, 2011; Shi *et al*, 2006; Li *et al*, 2009). The CXCR2 and CXCR4 chemokine receptors also bind to MIF, an interaction that may contribute to the recruitment of inflammatory cells (Bernhagen *et al*, 2007; Schwartz *et al*, 2009). Through physical interaction and inhibition of the cytosolic Jab1/CSN5 subunit of the COP9 signalosome, MIF inhibits AP-1 activity and the ubiquitin-mediated degradation of a number of proteins that play important roles in cell-cycle control and DNA-damage checkpoints (Kleemann *et al*, 2000; Nemajerova *et al*, 2007a, b). Jab1/CSN5-dependent stabilisation of HIF-1α is enhanced by MIF, leading to the subsequent activation of hypoxia-responsive genes (Bozza *et al*, 1999; Honma *et al*, 2000; Gregory *et al*, 2006; Winner *et al*, 2007; Rendon *et al*, 2009). Functional screens and the use of fibroblasts and macrophages derived from MIF − / − mice have

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unravelled a role for MIF as an antagonist of p53 tumour suppressor protein (Hudson *et al*, 1999; Mitchell *et al*, 2002; Fingerle-Rowson *et al*, 2003). Macrophage migration inhibitory factor deletion resulted in premature growth arrest, resistance to H-Ras^{v12}-induced transformation and to p53-dependent apoptosis.

Despite the tantalizing rationale linking MIF and tumour development, the value of inhibiting MIF has not been convincingly validated in preclinical models of cancer *in vivo*. Although neuroblastoma SK-N-DZ cells stably transfected with MIF antisense vector failed to grow in mice, other studies targeting MIF have shown only a modest effect on tumour growth (Ren *et al*, 2006). Inhibition of tumour growth by $\leqslant$40% was observed after treatment of Colon 26 or 38C13 syngeneic tumour models with MIF-neutralising antibodies (Chesney *et al*, 1999; Ogawa *et al*, 2000). Similarly treatment with ISO-1, a small molecule inhibitor of MIF activity led to 40% tumour growth inhibition in the DU-145 prostate tumour model (Meyer-Siegler *et al*, 2006).

We reasoned that the modest activity generated by MIF inhibitors *in vivo* might be the result of incomplete inhibition of MIF. Indeed, antibody treatment may not neutralise MIF-dependent activities in the cytoplasm. To the same extent, ISO-1 dosing regimen may not lead to a complete and sustained inhibition of MIF. In order to resolve this question, we used a combination of shRNA targeting MIF in tumour cells and implantation of these tumour cells in wild-type and MIF $-/-$ mice. In a B16-F10 melanoma model, we found that MIF produced by tumour cells is dispensable and that most of the tumour-promoting activity is provided by host-derived MIF, through the stimulation of angiogenesis. We also report on the effect of systemic MIF inactivation in the tumour stroma in additional *in vivo* models derived from mouse and human cell lines.

MATERIALS AND METHODS

Cell culture and reagents

Mouse cell lines B16-F10, CT26, 4T1, and Lewis Lung (LL/2) as well as human cell lines Raji, HT29, and HCT116 were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were routinely cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (Hyclone, Logan, UT, USA), penicillin with streptomycin and L-Glutamine (Invitrogen, Grand Island, NY, USA). Cultures were grown at 37°C in 5% CO_2.

Generation of MIF shRNA B16-F10

The lentiviral vector backbone has been described by Lippa *et al* (2007) and was modified for improved expression in murine cell types by incorporating a T-Rex tetracycline repressor gene (Invitrogen) expressed from the strong MSCVLTR promoter, as well as a puromycin-resistance gene expressed from the human PGK promoter and bovine growth hormone polyadenylation signal (Invivogen, Grand Island, NY, USA) (Lippa *et al*, 2007). The shRNA expression components were incorporated in a double-copy format as a Gateway cassette in the SIN-LTR of the vector backbone, such that two expressed copies were generated following incorporation into the genome of the target cell. Gene-specific shRNA triggers were designed and cloned into the pENTR/hH1/TO T-Rex expression vector according to the manufacturer's protocols (Invitrogen). The sense-strand sequence of each shRNA trigger was as follows: shMIF-361, 5′-CCGGGTCTACATCAACTAT-TACGA-3′; shCTRL, 5′-GTCTCCACGCGCAGTACATTA-3′. Lentiviral vector stocks were generated according to the manufacturer's protocols (Invitrogen). B16-F10 cells were transduced with specific lentiviral vectors by incubating 10^6 cells with 10^7 TU of vector in a single well of a 12-well tissue culture dish in 500 μl of RPMI containing 10 μg ml^{-1} DEAE Dextran for 6 h. Two days after transduction, cells were selected in media containing 2.5 μg ml^{-1} puromycin (Invivogen). To test induction of the shRNA, cells were cultured in standard DMEM with the addition of 1 μg ml^{-1} doxycycline for 24 h.

Tumour growth in wild-type and MIF $-/-$ mice

Tumour cell lines were implanted subcutaneously in the right flank of wild-type and MIF $-/-$ mice in 100 μl of Matrigel (BD, San Jose, CA, USA) diluted 1 : 1 with PBS. The optimal density of tumour cells to implant was determined experimentally in wild-type mice. The following amounts of tumour cells were implanted: 5×10^5 B16-F10, CT26, 4T1, or LL/2 cells, 1×10^6 HT29 cells, or 5×10^6 HCT116 or Raji cells. MIF $-/-$ mice were generated by Honma *et al* (2000) and bred for >10 generations to C57Bl/6 or Balb/c mice. Balb/c MIF $-/-$ mice were also intercrossed to CB17-Prkdcscid to generate the MIF mutation in an immune-deficient background to allow for implantation of human tumour cell lines. Colonies of wild-type and MIF $-/-$ mice were bred at Charles River (Wilmington, MA, USA). The mice used in each experiment were a mix of males and females ranging in age from 6 to 10 weeks. An equal distribution of age and sex was targeted for each experimental group. When tumours transduced with control or MIF shRNA were implanted into wild-type or MIF $-/-$ mice, subjects were distributed into groups for doxyxyline treatment when the average tumour volume reached 100 mm^3. Doxycycline was administered (2 mg ml^{-1} with 5% sucrose in drinking water *ad libidum*) until the termination of the study. Tumour volumes were measured with a digital caliper and body weights were recorded three times per week. Tumour volumes were approximated using the formula(width^2 $\times$ length)/2. All continuous measurements were expressed as mean $\pm$ s.e. Statistical analysis consisted of RM-ANOVA on log-transformed volume with Dunnett's test for significance. All experimental procedures were approved by the Institutional Animal Care and Use Committee and in accordance with the standards of the Association for Assessment and Accreditation of Laboratory Animal Care.

Western blotting analysis

For western blot analysis cells were lysed directly on the tissue culture plate in RIPA buffer with protease and phosphatase inhibitors (Sigma, St. Louis, MO, USA) for 30 min at 4 °C. Lysates were quantified by Bradford analysis (Thermo Scientific, Rockville, MD, USA). In all, 40 μg of total protein was subjected to electrophoresis on Novex 4–20% Tris-Glycine Gels (Invitrogen), and blotted to PVDF membranes. Antibodies used against mouse target proteins were MIF (Abcam ab7207, 1 : 1000, Cambridge, MA, USA), VEGF (Santa Cruz Biotechnology sc507, Santa Cruz, CA, USA, 1 : 100), and β-actin (Sigma A2066, 1 : 500).

Quantitative reverse transcription PCR

RNA was extracted from frozen B16-F10 cells using Qiagen mini-prep RNA extraction kit as directed (Qiagen RNeasy mini, Valencia, CA, USA). First-strand cDNA synthesis was performed using the Invitrogen SuperScript III First-Strand Synthesis System for RT–PCR using Oligo(dT) primers. Quantitative PCR was conducted in a 384-well format using Taqman Master Mix (Invitrogen) under standard conditions with probes against mouse MIF (Invitrogen #Mm01611157_gH) and GAPDH (Invitrogen #4352339E).

Immunohistochemistry

Tumour samples were harvested on day 13 after implantation and at the time of study termination on day 20. Tumour samples were fixed in formalin, embedded in paraffin and 4 mm histologic

sections were prepared. Sections were de-paraffinized and subjected to aDecloaker Pressure Instrument (Biocare, Concord, CA, USA) for antigen retrieval. Each tumour was probed for CD31 (BD Bioscience #553370, 1 μg ml^{-1}), phospho-histone H3 (Millipore, Billerica, MA, USA/Upstate #06-570, 1 μg ml^{-1}), cleaved caspase-3 (Cell Signaling Technology, Danvers, MA, USA; #9661) and F4/80 (Abcam, #ab6640, 2 μg ml^{-1}). Briefly, each slide was blocked for 15 min with 3% Peroxidase Block and an additional 10 min with Protein Block (Dako, Carpinteria, CA, USA). The primary antibodies were incubated for 60 min followed by incubation with the secondary Mach3 Rabbit Probeand Mach3 Rabbit Alkaline Phosphatase (Biocare). Finally, slides were treated with Perm Red (Dako) for 10 min and Hematoxylin (Dako) for 1 min. A xylene-based mountant was used.

For morphometric analysis digital images of three representative $\times 10$ microscopic fields were taken using a QImaging Micropublisher 5.0 digital camera attached to a Nikon E600 microscope. Fields were selected to avoid areas of necrosis; however, the photographer was blinded to the mouse genotype and tumour group. MetaVue version 6.2r6 (Universal Imaging Corp., Downingtown, PA, USA) imaging software was used to establish a threshold on the red chromogen labelling the CD31-positive endothelial cells lining vessel walls and to calculate the area thresholded. The result for each image was expressed as vessel area in μm^2 per mm^2 of tumour and the average of the three images provided the value for each tumour. An unpaired Student's *t*-test was used to compare the mean vessel area.

Cytokine analysis

Serum samples were harvested for mouse cytokine/chemokine analysis on day 13 and at termination of the study on day 20. The Linco pre-mixed cytokine/chemokine 22 multiplex kit (Millipore) was used as directed for cytokine profiling on a luminex instrument (Millipore). Mouse VEGF was analysed by ELISA (R&D Systems, Minneapolis, MN, USA). An unpaired Student's *t*-test was used to compare group means.

RESULTS

shRNA knockdown of MIF expression

To test the influence of MIF produced by tumour cells on tumour growth *in vitro* and *in vivo*, we transduced the B16-F10 mouse melanoma cell line with a lentivirus expressing either control or MIF targeting shRNA. B16-F10 cells express MIF mRNA and protein; treatment with doxycycline did not change the level of expression in cells transduced with the control shRNA (Figure 1A and B). In the absence of doxycycline, a 75% decrease in MIF mRNA compared with control B16-F10 was observed by quantitative RT–PCR, indicating that our vector enabled the transcription of MIF shRNA in basal condition (Figure 1A). In presence of doxycycline, MIF shRNA elicited a 90% decrease in MIF mRNA expression. Macrophage migration inhibitory factor protein was barely detectable by western blot in the absence of doxycycline and not detectable after the addition of doxycycline to the MIF shRNA-transduced cells compared with control (Figure 1B). Based on these results, MIF expression appeared to be constitutively knocked down by MIF shRNA at both the mRNA and protein level. In agreement with this observation, we did not observe any phenotypic differences *in vitro* or *in vivo* as a consequence of doxycycline treatment (data not shown and Figure 3B).

In vitro characterisation of MIF-deficient B16-F10 melanoma cells

We characterised the effect of inhibiting MIF expression on B16-F10 cell viability and proliferation. A kinetic analysis revealed

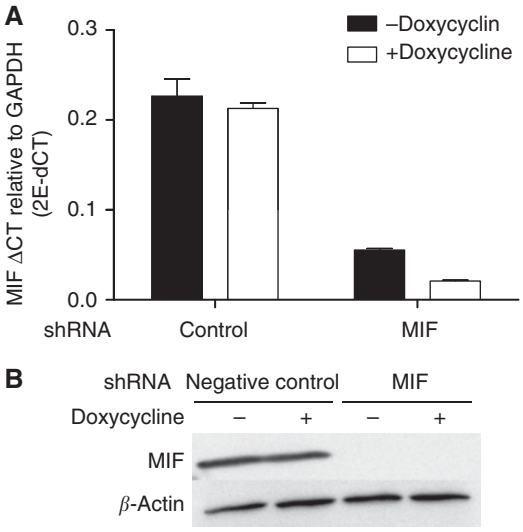

Figure 1 Inhibition of MIF mRNA and protein expression in B16-F10 mouse melanoma cells transduced a lentiviral construct expressing MIF-targeting shRNA. Pools of B16-F10 expressing a control or MIF shRNA were grown under standard conditions with or without 2.5 μg ml^{-1} doxycyclin for 48 h. (**A**) RNA was isolated and cDNA was synthesised. Primers against MIF and GAPDH (control) were used in quantitative PCR. Data are shown as ΔC_t for each condition between MIF and GAPDH. (**B**) Protein was harvested from cell lysates and western blot was performed on 40 μg total protein. Blots were probed with an anti-MIF rabbit polyclonal antibody and an anti-β actin antibody.

a faster rate of proliferation upon MIF inhibition (Supplementary Figure S1A and B). The increased proliferation was accompanied by a higher level of cyclin D1, phosphorylated retinoblastoma protein and phosphorylated p42/44 MAPK (Supplementary Figure S1C). The cause for this phenotype was not further investigated as we did not find any consequence of MIF inhibition on cell proliferation as assessed by phospho-histone H3 staining *in vivo* (Supplementary Figure S2A).

We also characterised the consequence of reducing MIF expression in B16-F10 cells *in vitro* in response to hypoxia, where MIF was proposed to play a role. Macrophage migration inhibitory factor was reported to be upregulated by hypoxia, to be necessary for the full activity of HIF-1α, and to be required for the full induction of hypoxia-inducible genes such as VEGF (Winner *et al*, 2007). We therefore investigated VEGF expression in B16-F10 cells. Under normoxic conditions expression of both MIF and VEGF was inhibited by MIF-targeted shRNA (Figure 2A). In support of a role for MIF in response to hypoxia, expression of secreted VEGF protein was lower in MIF shRNA-transduced B16-F10 cells than in control cells under both normoxic and hypoxic (0.5% O_2) conditions (Figure 2B). In normoxic growth conditions VEGF secretion by MIF shRNA-transduced cells was 63% lower than in control cells. After 24 h of hypoxic treatment VEGF secretion was 50% lower in MIF shRNA transduced than in control B16-F10 cells (Figure 2B).

Growth inhibition of B16-F10 syngeneic tumours by loss of host-derived MIF

To test the role of MIF on tumour growth *in vivo*, pools of B16-F10 cells selected to express a nonsense control shRNA or anti-MIF shRNA were implanted subcutaneously in syngeneic C57BL/6 mice. In addition, we tested the role of MIF produced by the host by implanting the same cell pools in MIF $-/-$ mice (Honma *et al*, 2000). Figure 3A is an example of three *in vivo* studies. A 47% reduction in tumour growth was observed when comparing tumours grown in wild-type *vs* MIF $-/-$ mice. This difference

was statistically significant ($P<0.05$). The average tumour growth inhibition of the three studies was 43% ± 7. In contrast, the loss of MIF expression in the B16-F10 melanoma cell line did not affect tumour growth. This result was not altered by the addition of doxycycline in the drinking water, indicating that the lack of effect is not due to regaining MIF expression *in vivo* in the absence of doxycycline (Figure 3A). The knockdown of MIF expression was confirmed by quantitative RT–PCR (Figure 3B) and ELISA (Figure 3C) in tumour lysates at termination of the study. Both quantitative RT–PCR and ELISA showed that the major contribution of total MIF in the control B16-F10 tumours originated from the tumour cells and not from the host (Figure 3B and C).

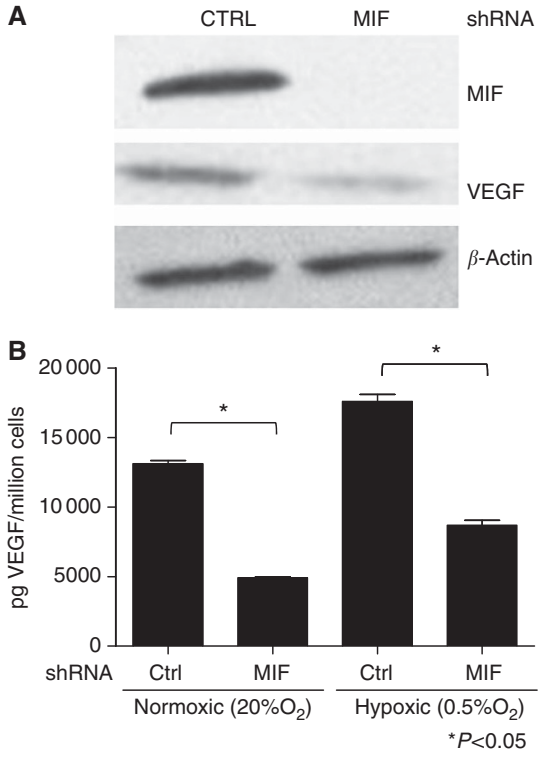

Figure 2 MIF-regulated VEGF expression *in vitro*. (**A**) B16-F10 cells were grown 48 h in a normoxic (20% O_2) environment with or without Doxycyclin. Protein was harvested from cell lysates and western blot was performed on 40 μg total protein. Blots were probed with antibodies against MIF, VEGF, or β-actin. (**B**) B16-F10 cells transfected with shRNA were grown 24 h in normoxic conditions after which plates were grown an additional 24 h under normoxic or hypoxic (0.5% O_2) conditions. Cell supernatant was collected and VEGF secretion was evaluated by ELISA. (*$P<0.05$).

Reduction of angiogenesis in B16-F10 tumours growing in MIF −/− mice

To explore the mechanism of tumour growth inhibition in MIF −/− mice immunohistochemistry (IHC) analysis of tumours was used to evaluate tumour cell proliferation, apoptosis, vascularisation and the presence of tumour-associated macrophages. Two time points were compared, an intermediate time point at day 13 after tumour implant and the terminal time point at day 20. Tumour samples were collected at both time points to ensure that we were capturing early events leading to tumour growth inhibition. Cell proliferation (as measured by phospho Histone H3 staining) and apoptosis (as assessed by cleaved Caspase-3 staining) showed no significant difference between tumours grown in wild-type and MIF −/− mice (Supplementary Figure S2A and data not shown). In contrast, we consistently observed a decrease in the vessel area as assessed by CD31 staining in tumours growing in the MIF −/− mice compared with wild-type mice (Figure 4A and B). A 40% decrease in CD31 staining ($P<0.05$) was evident at the two time points in the analysis (Figure 4B). The number of F4/80 stained cells were highly variable across tumours. We could not detect a significant difference between groups, although there was a trend ($P=0.09$ at day 20) toward an increased number of macrophages in tumours of MIF −/− mice (Supplementary Figure S2B).

Cytokine analysis

To further define the cause of the decrease in vascular density in B16-F10 tumours in MIF −/− mice, we investigated the level of VEGF in serum and in the B16-F10 tumour lysates. Despite the decrease in VEGF mediated by MIF shRNA *in vitro* in B16-F10 compared with control shRNA, no statistically significant differences could be observed at the day 13 or day 20 time points *in vivo* (Figure 5A). The lack of significant change in circulating and local tumour VEGF *in vivo* suggested that altered expression of this particular factor was not the reason for the observed reduction in vascularity.

The expression level of additional cytokines and chemokines potentially involved in MIF-mediated effects was then evaluated. In serum, four cytokines showed a statistically significant

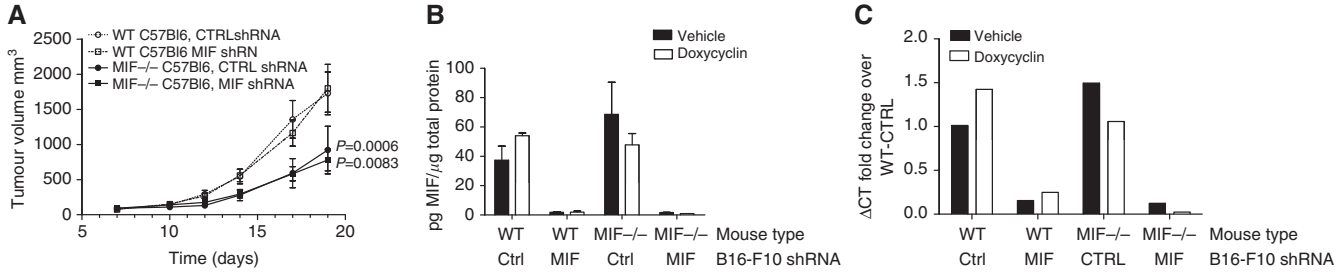

Figure 3 Growth inhibition of B16-F10 subcutaneous syngeneic tumours in MIF −/− C57Bl6 mice. (**A**) In all, 1×10^5 B16-F10 cells transduced with control or MIF shRNA in 50% matrigel matrix solution were implanted subcutaneously in the right flank of age-matched wild-type C57Bl/6 or MIF −/− C57Bl/6 congenic strain. After 7 days of tumour growth, mice were randomized by tumour size into treatment groups of either standard drinking water or water with 2 mg ml^{-1} doxycyclin with 5% sucrose. Groups not treated with doxycyclin are not shown. Tumours were measured with digital calipers three times per week for 2 weeks. (**B**) Protein lysates were made from tumours harvested at the termination of the study. Lysates were evaluated for MIF expression by ELISA. (**C**) A portion of each tumour harvested at the termination of the study was preserved in RNALater. RNA was isolated and cDNA was synthesised. Primers against MIF, VEGF, and GAPDH were used in quantitative PCR. Data are shown as ΔC_t comparing the control tumours from wild-type mice to MIF shRNA tumours from wild-type mice and control or MIF shRNA tumours from MIF −/− mice.

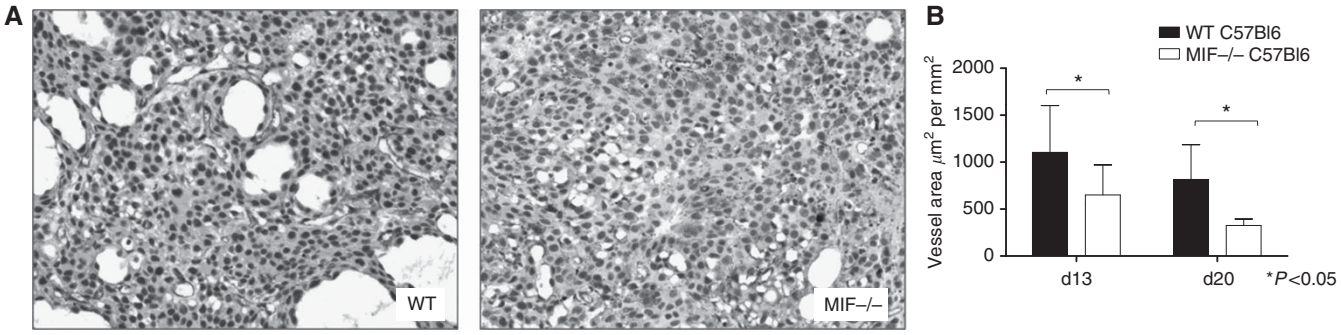

Figure 4 Reduced vascular density of B16-F10 tumours growing in MIF − / − mice. (**A**) B16-F10 tumour grown in wild-type or MIF − / − mice were collected at termination of the *in vivo* study and preserved in formalin. The proportion of endothelial cells present in the tumour was evaluated by IHC staining of CD31. Positive staining is visualised as red chromogen. Note the brown colour is melanin pigment inherent in the tumour and phagocytised (SP) by tumour-associated macrophages (melanophages). This pigment tended to be more prominent in the tumours of MIF KO mice. (**B**) Quantitated vessel area of B16-F10 control tumours in wild-type and MIF − / − mice at the intermediate time point (day 13) and terminal time point (day 20). The area of CD31 positivity (vessel area) is expressed in microns per mm^2 of tumour area (*$P<0.05$).

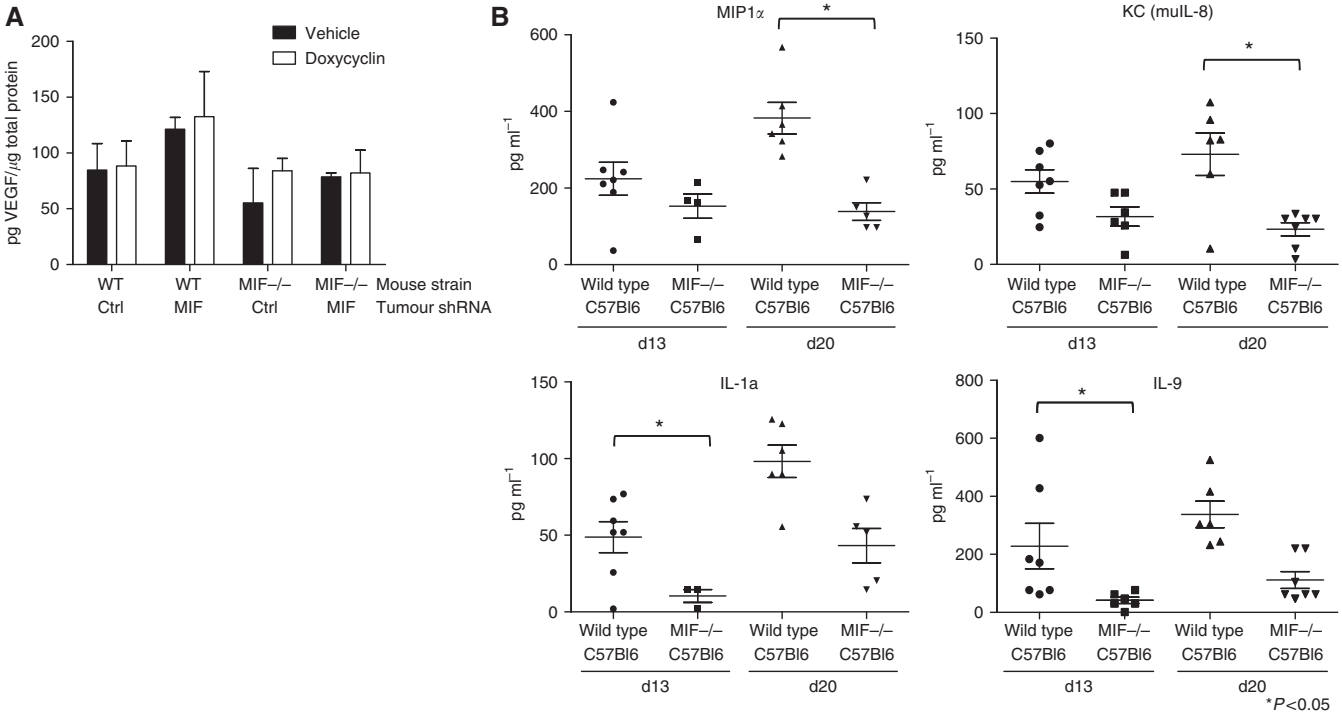

Figure 5 Expression of serum cytokines. (**A**) Serum was collected from all mice at termination of the study. Expression of VEGF in 50 μl undiluted mouse serum was evaluated by ELISA. (**B**) Serum collected at the intermediate and terminal time points was evaluated undiluted by Cytokine/chemokine luminex bead assay. The circulating levels of 4 of the 22 cytokines evaluated MIP-1α, KC, IL-1α, and IL-9 were found to be reduced in the serum of MIF − / − mice compared with wild-type mice bearing B16-F10 tumours (*$P<0.05$).

differential expression level based on the MIF status of the mouse. At day 13, IL-1α and IL-9 were decreased in tumour bearing MIF − / − mice compared with wild-type mice; the same trend was perpetuated at day 20 (Figure 5B). At day 13 and 20, MIP1α (CCL3) and KC (CXCL1) were significantly reduced in the serum from MIF − / − mice, the difference being only significant ($P<0.05$) at day 20. MCP-1 (CCL2) showed a consistent trend at days 13 and 20 for a lower expression level in MIF − / − bearing B16-F10 tumours (data not shown). These differences were only observable in the circulation, but not in tumour lysates. G-CSF, GM-CSF, IFNγ, IP-10, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12, IL-13, IL-15, IL-17, and RANTES while within the detection limits of the assay did not show any significant difference in expression between wild-type and MIF − / − mice or tumours (data not shown). The modulation of cytokine levels appeared to be elicited by the host response to the B16-F10 tumour as we were not able to detect any significant difference in any cytokine between non-tumour bearing wild-type and MIF − / − mice (data not shown).

Growth of additional tumour models in MIF − / − mice

To evaluate the broader relevance of MIF inhibition, additional tumour cell lines of mouse origin (LL/2, 4T1, CT26) or of human origin (HT-29, HCT116, Raji) were introduced in wild-type and MIF − / − mice in a suitable syngeneic or SCID genetic background. Macrophage migration inhibitory factor shRNA were

tested in B16-F10 (see above) and in HT-29 (data not shown). Given that we did not detect any change in growth rate between control and MIF shRNA-transduced tumour cells *in vitro* and *in vivo*, the effect of MIF shRNA was not evaluated in the other tumour models. No anti-tumour efficacy was observed in any of the models, with the exception of the Raji tumour model that showed a trend for a reduced growth rate in the MIF $-/-$ mice (Supplementary Figure S3). A significant decrease (30%, $P=0.015$) in blood vessel density, as assessed by CD31, and in proliferation (28% $P=0.05$) by pH3 staining was observed in Raji Xenografts in MIF $-/-$ mice. Histological analysis of CD31 staining in the LL/2 model did not reveal any significant inhibition of angiogenesis (data not shown). The other models were not assessed by IHC. Cytokine evaluation of the CT26 and 4T1 tumour bearing mice did not reveal any significant changes in circulating cytokine profile, in contrast to our observation in the B16-F10 studies (data not shown).

DISCUSSION

Despite its broad potential influence, the importance of MIF as a therapeutic target in cancer remains to be established (Bucala and Donnelly, 2007; Rendon *et al*, 2009). Pharmacological inhibition of MIF activity, using antibodies that neutralises MIF binding to CD74, or small molecules such as ISO-1 than inhibit MIF tautomerase activity and binding to CD74 have not yielded profound activity in preclinical models *in vivo* (Chesney *et al*, 1999; Ogawa *et al*, 2000; Meyer-Siegler *et al*, 2006; Leng *et al*, 2011). These two therapeutic approaches have a limitation: antibodies may not be able to inhibit intracellular MIF function mediated by its interaction with Jab1/CSN5 and one can speculate that ISO-1 dosed twice a week may not lead to sustained inhibition of MIF function (Kleemann *et al*, 2000; Lue *et al*, 2006, 2007; Meyer-Siegler *et al*, 2006; Nemajerova *et al*, 2007a, b). In our experience, despite achieving a near complete removal of MIF from both the B16-F10 tumour cells and the host mouse, we inhibited tumour growth by 43%. This result failed to improve upon the reported 40% inhibition achieved with neutralising MIF antibodies or ISO-1 (Chesney *et al*, 1999; Ogawa *et al*, 2000; Meyer-Siegler *et al*, 2006). Targeting MIF expression in B16-F10 mouse melanoma cells had no measurable consequence on tumour growth *in vivo*. By contrast, the absence of MIF in the host stroma fully accounted for the reduced tumour growth rate observed in the MIF $-/-$ mice. The most likely cause for this reduced tumour growth was a decrease in tumour angiogenesis.

In the B16-F10 melanoma cell line MIF was induced by hypoxia and in turn modulated the downstream response to hypoxia, as exemplified by the reduction in VEGF expression level elicited by MIF shRNA (Figure 2). In B16-F10 subcutaneous tumours however, we were not able to demonstrate a contribution of the inhibition of MIF to the overall production of VEGF (Figure 5A). The VEGF levels were not found to be different in B16-F10 tumours transduced with MIF or control shRNA whether these tumours were implanted in wild-type or MIF $-/-$ mice. Despite the lack of significant changes in VEGF expression, the most profound effect of MIF deficiency was the modulation of angiogenesis in the B16-F10 and Raji models. A reduction of angiogenesis in MIF $-/-$ mice was also observed in the $APC^{min/+}$ mouse model of colorectal cancer (Wilson *et al*, 2005). In this model, as in our experience with the B16-F10, no alteration in tumour cell proliferation and apoptosis could be observed *in vivo*. However, our results differ from previously published results in which B16-F10 transfected with MIF siRNA exhibited a delayed onset of tumour growth in wild-type mice attributed to a reduction of blood vessel density and an increase in the expression of thrombospondin-1 (Culp *et al*, 2007). In our experiment, thrombospondin-1 protein was not detectable by western blot in B16-F10 tumours (data not shown). The identity of the MIF producing cells in the tumour stroma is not clear, although MIF is known to be produced at a high level by many cell types including macrophages, neutrophils and endothelial cells (Calandra and Roger, 2003). Four cytokines were consistently found to be expressed at reduced level in the serum of MIF $-/-$ growing B16-F10 tumours. While we cannot exclude the possibility of a relationship between the smaller tumour size and the reduced cytokine level in the MIF $-/-$ mice, the observation that, particularly for KC and MIP-1α, the level found in serum of MIF $-/-$ at day 20 (average tumour volume 800 mm^3) does not rise above the level of the wild-type mice at day 13 (average tumour volume 500 mm^3) makes this possibility less likely. Among the cytokines that we found decreased in the serum of MIF $-/-$ mice, KC (a functional homologue of human IL-8), MIP-1α and IL-1α have been reported to directly or indirectly promote angiogenesis and to be regulated by MIF (White *et al*, 2001; Payne and Cornelius, 2002; Apte *et al*, 2006; Kudrin *et al*, 2006; Wu, 2008). These cytokines may account for the observed inhibition of vascularisation in the absence of any change in VEGF. Alternatively, MIF may also directly promote the proliferation of endothelial cells. Macrophage migration inhibitory factor-neutralising mAbs have been indeed shown to inhibit human endothelial cells proliferation *in vitro* (Chesney *et al*, 1999; Ogawa *et al*, 2000).

MIF involvement in the regulation of angiogenesis appears to be tumour specific. No defect in the vasculature in the MIF $-/-$ mice was reported (Bozza *et al*, 1999; Honma *et al*, 2000; Gregory *et al*, 2006). In addition, the decrease in serum cytokine expression between wild-type and MIF $-/-$ mice was not observed in the absence of tumour or with tumours insensitive to MIF knockout. The assessment of the circulating level of these cytokines may offer a biomarker to evaluate the activity of potential MIF-targeting therapeutics.

CONCLUSIONS

Understanding the role of MIF in neoplastic development and progression is complicated by the pleiotropic influences that MIF plays in many tumorigenic processes. This study was conducted to understand the full contribution of MIF activities by combining its inactivation in tumour cells by shRNA and in the tumour stroma by using MIF $-/-$ mice. Among the models we tested, we found the B16-F10 melanoma to be the most sensitive to loss of MIF activity. *In vitro*, the loss of MIF in this cell line resulted in a decreased response to hypoxia based upon reduced expression of VEGF. The latter however had little influence on the growth of B16-F10 *in vivo*. While the major contribution of MIF appears to be in the regulation of angiogenesis, the tumour cell-derived MIF played a negligible role in this process. We show that MIF produced by the host was a critical factor in the regulation of angiogenesis. We speculate that MIF regulation of tumour vascularisation occurs through its ability to regulate the expression of pro-angiogenic chemokines and the modulation of the recruitment and/or the activity of pro-inflammatory cells.

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Conflict of interest

All authors are or were Amgen Inc. employees and shareholders.

Author contributions

EG participated in the design of the studies, performed or supervised the experiment with the exception of the immunohistochemical analysis, and contributed to writing this report. CS generated the lentiviral constructs and the transduced cell lines. ET performed the immunohistochemical analysis. CQ led the research and contributed to the study design and to the redaction of the manuscript.

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