LETTERS

Macrophage migration inhibitory factor stimulates AMP-activated protein kinase in the ischaemic heart

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Understanding cellular response to environmental stress has broad implications for human disease. AMP-activated protein kinase (AMPK) orchestrates the regulation of energy-generating and -consuming pathways, and protects the heart against ischaemic injury and apoptosis[1]. A role for circulating hormones such as adiponectin[2] and leptin[3] in the activation of AMPK has received recent attention. Whether local autocrine and paracrine factors within target organs such as the heart modulate AMPK is unknown. Here we show that macrophage migration inhibitory factor (MIF), an upstream regulator of inflammation[4], is released in the ischaemic heart, where it stimulates AMPK activation through CD74, promotes glucose uptake and protects the heart during ischaemia-reperfusion injury. Germline deletion of the *Mif* gene impairs ischaemic AMPK signalling in the mouse heart. Human fibroblasts with a low-activity *MIF* promoter polymorphism[5] have diminished MIF release and AMPK activation during hypoxia. Thus, MIF modulates the activation of the cardioprotective AMPK pathway during ischaemia, functionally linking inflammation and metabolism in the heart. We anticipate that genetic variation in *MIF* expression may impact on the response of the human heart to ischaemia by the AMPK pathway, and that diagnostic *MIF* genotyping might predict risk in patients with coronary artery disease.

Macrophage migration inhibitory factor (MIF) is a pleiotropic cytokine that controls the inflammatory 'set point' by regulating the release of other pro-inflammatory cytokines[6]. MIF is expressed in several cell types, including monocytes/macrophages[7], vascular smooth muscle[8] and cardiomyocytes[9], and is released on stimulation from pre-formed storage pools. MIF is involved in the pathogenesis of inflammatory diseases, such as atherosclerosis[8,10], rheumatoid arthritis[5], sepsis[4], asthma[11] and acute respiratory distress syndrome[12]. Human *MIF* gene expression is determined by promoter polymorphisms, including a tetra-nucleotide CATT repeat at position –794 (ref. 5). MIF signalling is known to activate ERK1/2 MAPK (ref. 13) through a receptor complex comprising CD74 (ref. 14) and CD44 (ref. 15). In contrast, the chemokine receptors CXCR2 and CXCR4 participate in MIF-mediated migratory function[10].

MIF also stimulates glycolysis during sepsis, increasing the synthesis of fructose 2,6-bisphosphate and cellular glucose uptake[16]. The signalling pathways by which MIF exerts its metabolic effects are unknown, but one candidate is the AMP-activated protein kinase (AMPK)—an important regulator of both glycolysis and glucose uptake during cellular stress[1]. AMPK senses the cellular energy state and affects diverse pathways to increase cellular ATP production and limit energy consumption. AMPK activity is regulated by AMP binding to its regulatory γ-subunit[17] and by phosphorylation of the catalytic α-subunit by upstream kinases, including LKB1 (ref. 18) and CaMKKβ (ref. 19). In the heart, AMPK stimulates 6-phosphofructo-2-kinase activity and glycolysis[20], induces glucose transporter-4 (GLUT4, encoded by the *SLC2A4* gene) translocation[21], increases ischaemic glucose uptake[1,22] and limits myocardial injury and apoptosis[1].

AMPK phosphorylation is also modulated by the adipocyte-derived circulating hormones leptin[3] and adiponectin[23], raising the possibility that cytokines might also activate AMPK. We hypothesized that AMPK might be activated in an autocrine/paracrine fashion by MIF in the heart during ischaemia, linking the regulatory control of inflammation and metabolism.

Initial experiments examined whether MIF has a role in the stimulation of the AMPK pathway during hypoxia in rat heart muscles. Hypoxic activation of AMPK (Fig. 1a) was associated with a twofold increase in muscle MIF release (Fig. 1b), the latter consistent with previous results in cardiomyocytes[24]. Pre-treatment with anti-MIF antibody reduced hypoxic AMPK activation by 67% (Fig. 1c). One of the important AMPK actions during hypoxia and ischaemia is to increase glucose transport[1,22]. Hypoxic glucose transport was inhibited 38% by anti-MIF antibody (Fig. 1d), indicating that secreted extracellular MIF modulates downstream AMPK action.

To investigate whether MIF modulates AMPK, we added MIF to normoxic heart muscles. MIF caused time- and dose-dependent increases in AMPK phosphorylation (Fig. 1e and f), and increased heart muscle glucose uptake (Fig. 1g). Hypoxia and insulin-stimulated glucose uptake in the heart are mediated by translocation of the glucose transporter GLUT4 to the cell surface where it is physiologically active[21]. We used a cell-membrane impermeant photolabel compound and found significant translocation of GLUT4 to the cell surface (Fig. 1h), elucidating the mechanism through which MIF increases glucose uptake.

We next examined whether MIF modulates AMPK signalling in the ischaemic heart. MIF is expressed by cardiomyocytes[9,24], endothelial cells, monocytes and macrophages[7]. We used the isolated mouse heart perfused with crystalloid buffer, eliminating the potential contribution of MIF from circulating cells. MIF was highly expressed in cardiomyocytes, according to immunohistochemical data (Fig. 2a). Ischaemia triggered cardiac MIF release into the coronary venous effluent and decreased heart MIF content after ischaemia-reperfusion (Fig. 2b).

To determine whether MIF plays a part in ischaemic AMPK activation, we used hearts from $Mif^{-/-}$ mice[25] and compared them to wild-type controls. $Mif^{-/-}$ mice demonstrated a normal baseline cardiac phenotype with respect to left ventricular size and function, histology and the expression of AMPK and glucose transporter proteins

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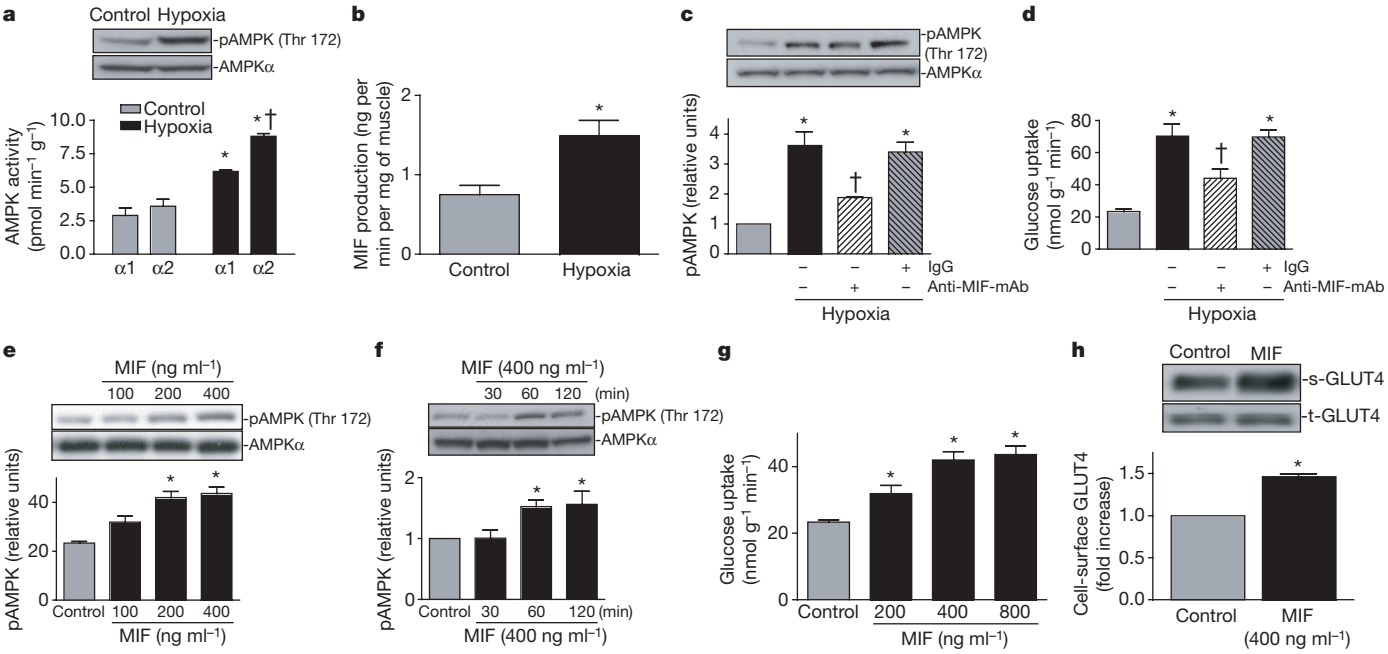

Figure 1 | Role of MIF in heart muscle AMPK signalling during hypoxia. **a**, Immunoblots show phosphorylated and total AMPK, bars show α2 or α1 AMPK activity. *$P = 0.001$, versus control; †$P = 0.012$, α1 versus α2. **b**, Muscle MIF release. *$P = 0.03$, versus control. **c**, **d**, Inhibition of AMPK activation and downstream glucose transport by anti-MIF antibody (100 μg ml^{-1}). *$P = 0.02$, versus control; †$P = 0.04$, versus hypoxia alone. **e**, AMPK activation by recombinant MIF (60 min). *$P < 0.02$, versus control. **f**, AMPK activation by recombinant MIF (400 ng ml^{-1}). *$P < 0.05$, versus control. **g**, Glucose uptake during incubation with recombinant MIF. *$P < 0.05$, versus control. **h**, Immunoblots of cell-surface (s-GLUT4) and total (t-GLUT4) GLUT4 in muscles incubated without or with 400 ng ml^{-1} rat MIF. *$P < 0.001$ versus control, $n = 3$–6 per group. Values are means ± s.e.m.

(Supplementary Fig. 1). When perfused with mixed-substrate buffer and subjected to 15 min of global ischaemia, AMPK activation was significantly blunted in the *Mif*$^{-/-}$ hearts owing to decreased phosphorylation of the activating Thr 172 site (Fig. 3a). The tumour-suppressor kinase LKB1 (also known as SKT11) has an important role in mediating AMPK phosphorylation during ischaemia[18]. However, we observed no change in the expression of LKB1, or CaMKKβ (also known as Camkk2), another potential upstream kinase (Supplementary Fig. 1).

Because AMPK mediates glucose uptake during ischaemia[1], we examined whether the defect in AMPK signalling in the *Mif*$^{-/-}$ hearts also diminished downstream glucose uptake. Although glucose uptake was normal in *Mif*$^{-/-}$ hearts during control perfusions, the stimulation of glucose uptake during ischaemia-reperfusion was significantly blunted compared to wild-type hearts (Fig. 3b). This was associated with impaired glycogen synthesis in *Mif*$^{-/-}$ hearts during post-ischaemic reperfusion, despite a comparable amount of glycogen breakdown during ischaemia (Supplementary Fig. 2).

Consistent with prior observations that AMPK deficiency is functionally deleterious to the heart during ischaemia-reperfusion[1], *Mif*$^{-/-}$ hearts also demonstrated impaired ischaemic tolerance (Fig. 3c). *Mif*$^{-/-}$ hearts subjected to *ex vivo* ischaemia had decreased post-ischaemic left ventricular function (Fig. 3c) as well as increased ischaemic diastolic pressure and reduced contractility during reperfusion (Supplementary Fig. 3). *Mif*$^{-/-}$ hearts subjected to *in vivo* left coronary occlusion/reperfusion showed 2.3-fold greater infarct size compared to wild-type controls (Fig. 3d). These results indicate that MIF promotes early adaptive responses in the heart during ischaemia-reperfusion.

Human gene mutations influence AMPK signalling and MIF expression. Individuals with rare mutations in the AMPK γ2 subunit

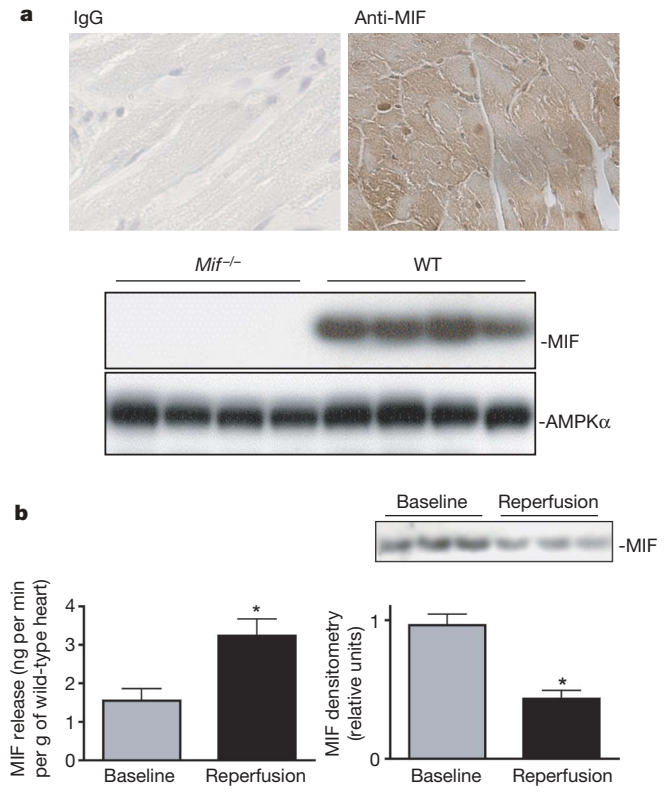

Figure 2 | Heart MIF expression and release triggered by ischaemia. **a**, Immunohistochemistry of wild-type (WT) mouse hearts with MIF antibody or non-immune immunoglobulin G (IgG). Immunoblots of heart lysates confirm the lack of immunoreactivity of the MIF antibody in *Mif*$^{-/-}$ hearts. Total AMPK is shown for loading comparison. **b**, Coronary effluent MIF production from wild-type hearts during baseline normal perfusion or during reperfusion after 10 min of ischaemia. MIF concentration was multiplied by the coronary flow to calculate the production rate. *$P = 0.01$, versus baseline by unpaired *t*-test comparing means of MIF concentration at five baseline and five reperfusion time points. MIF immunoblots of heart homogenates quantified by densitometry. *$P = 0.003$ versus control perfusions, $n = 2$–3 hearts each. Values are means ± s.e.m.

(PRKAG2, GeneID 51422) develop glycogen overload cardiomyopathy and Wolf–Parkinson–White syndrome[26]. A common polymorphism in the human *MIF* promoter, containing 5, 6, 7 or 8 CATT tetra-nucleotide repeat units (–794 $CATT_{5–8}$), also has functional consequences on *MIF* expression[5]. The $CATT_5$ allele demonstrates low *MIF* promoter activity compared to the others[5] and has been associated with less severe clinical manifestations of inflammatory diseases such as asthma[11], cystic fibrosis[27] and rheumatoid arthritis[5], presumably owing to decreased MIF signalling. The *MIF* promoter genotype varies in the population according to ethnicity, but the low expression genotype is relatively common with 6% of Caucasians and 14.5% of African-Americans homozygous for the –794 $CATT_5$ allele[28]. Despite demonstrable changes in *MIF* promoter activity, there are few data demonstrating the influence of the low expression genotype on the level of cellular MIF release.

Thus, we examined whether polymorphisms in the human *MIF* promoter might lead to functional differences in MIF secretion and cellular AMPK activation, using early passage human dermal fibroblasts. Cells from three of seven subjects were homozygous for the low expression −794 $CATT_5$ allele ('5/5' genotype) and the remainder had at least one high expression 6-, 7- or 8-CATT repeat allele ('non-5/5' genotype). The 5/5 cells had significantly less MIF release into the culture media, during both normal and hypoxic incubations, when compared to non-5/5 cells (Fig. 4a). Reduced MIF release from the 5/5 cells was associated with less AMPK phosphorylation during hypoxic stress (Fig. 4b). To determine whether the relative MIF deficiency in the 5/5 cells was responsible for the impaired AMPK activation during hypoxic stress, MIF (10 ng ml^{-1}) was added to the media during hypoxic incubation. Exogenous MIF restored hypoxic AMPK activation in the 5/5 cells to levels that were equivalent to the non-5/5 cells (Fig. 4b). In contrast, MIF did not augment AMPK activation in hypoxic non-5/5 fibroblasts (Fig. 4b). Similarly, the addition of exogenous MIF to hypoxic rat heart muscles did not augment AMPK activation (Supplementary Fig. 4). These data indicate that endogenous MIF release maximally modulates AMPK phosphorylation during hypoxia in normal heart tissue and cells. However, in relatively MIF-deficient cells (that is the 5/5 *MIF* promoter genotype), which have diminished MIF secretion during hypoxia, exogenous MIF augmented AMPK activation. The results indicate that recombinant MIF (or MIF agonists) might have a therapeutic effect by increasing AMPK activation during ischaemia or hypoxia in selected individuals with the low-expression 5/5 *MIF* promoter genotype. Thus, these experiments demonstrate that a common polymorphism in the *MIF* promoter leads to differential MIF release, which has consequences in cellular stress signalling in human cells. They also imply that exogenous MIF might have a beneficial effect in hypoxic tissues, specifically in patients with the 5/5 genotype.

Taken together with the results implicating MIF in the activation of AMPK in the ischaemic heart, these data raise the possibility that a common polymorphism in the *MIF* promoter influences the susceptibility of patients with coronary artery disease to ischaemic injury. AMPK is under current investigation as a potential target molecule for the treatment of type 2 diabetes, because of its metabolic actions that increase skeletal muscle glucose uptake and suppress hepatic glucose production. AMPK is also a potential target in ischaemic heart disease, because of its cardioprotective effects[1] and potential role in ischaemic preconditioning[29]. Treatment with MIF or MIF agonists warrants further study as an adjunctive therapy targeted at

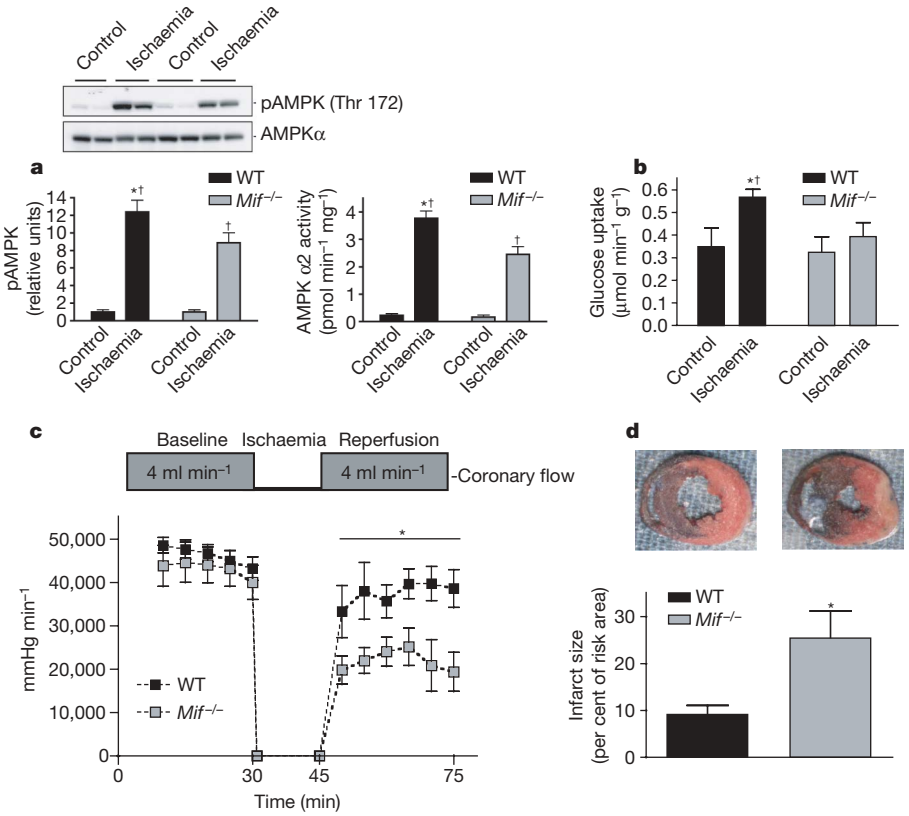

Figure 3 | Genetic MIF deletion impairs ischaemic heart AMPK activation and glucose uptake, and exacerbates post-ischaemic cardiac dysfunction and injury. a, AMPK phosphorylation and activity after ischaemic or control perfusions. $*P < 0.05$, versus $Mif^{-/-}$ ischaemic hearts; $\dagger P < 0.05$, ischaemic versus control, $n = 3$–4 hearts for each genotype. **b**, Glucose uptake during control perfusion and during reperfusion after ischaemia ($n = 5$ for each genotype). $*P = 0.01$, versus wild-type baseline, $\dagger P = 0.04$, versus $Mif^{-/-}$ reperfusion. **c**, Heart-rate–left-ventricular-developed pressure product during control perfusion and post-ischaemic reperfusion. $n = 6$–7 hearts for each genotype. $*P = 0.03$, by repeated measures ANOVA during reperfusion. **d**, Myocardial infarction induced by 15 min of left coronary occlusion *in vivo* followed by 4 h of reperfusion. Viable myocardium stained red with TTC; infarcted tissue, white; and normal non-ischaemic tissue, blue. The infarct area was quantified and expressed as a per cent of the ischaemic area at risk. $*P = 0.04$ versus wild type. $n = 5$–6 hearts per genotype. Values are means ± s.e.m.

AMPK activation during acute myocardial ischaemia or infarction. To the extent that MIF is released from the heart during ischaemic preconditioning, MIF agonists might also augment preconditioning by increasing AMPK activation during ischaemia. Therapy directed at AMPK might prove most effective in patients with low-expression *MIF* promoter polymorphisms. These hypotheses deserve further investigation and might also be addressed by analysis of gene banks from large cardiovascular clinical trials.

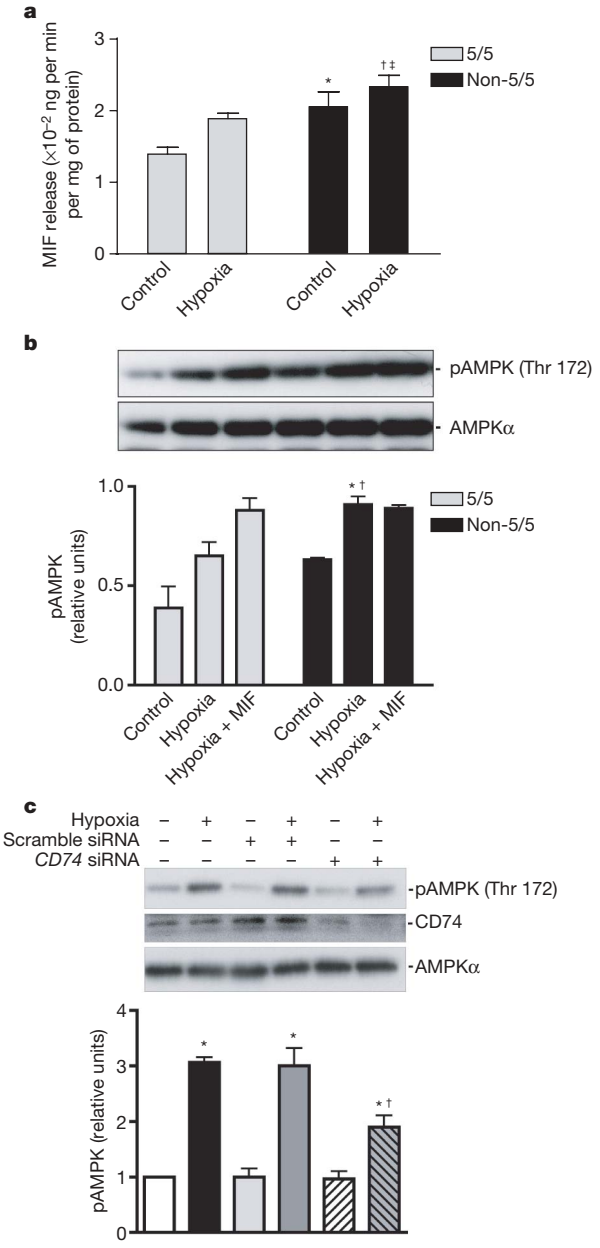

Figure 4 | Human *MIF* promoter genotype determines MIF secretion and AMPK activation during hypoxia. a, MIF secretion from human fibroblasts homozygous for the *MIF* promoter allele containing five CATT repeats (5/5 CATT, $n = 3$) or 6, 7 or 8 CATT repeat alleles (non-5/5 genotype, $n = 4$). $*P = 0.03$, versus 5/5 control cells, $\dagger P = 0.05$, versus 5/5 hypoxic cells, $\ddagger P = 0.03$ versus non-5/5 control cells. **b**, AMPK activation under control or hypoxic conditions with or without 10 ng ml^{-1} MIF. $*P = 0.01$, versus 5/5 and non-5/5 control cells; $\dagger P = 0.04$, versus 5/5 hypoxic cells. **c**, Human fibroblasts (non-5/5 genotype, $n = 4$) treated with MIF receptor *CD74* siRNA or control siRNA before hypoxia. Immunoblots show AMPK phosphorylation, CD74 and AMPK expression. The ratios of phosphorylated to total AMPK are expressed relative to control muscles. $*P = 0.01$, versus control; $\dagger P = 0.01$, versus control siRNA hypoxia. $n = 3$ experiments.Values are means ± s.e.m.

To define the proximal mechanisms linking MIF and AMPK activation better, we next examined whether components of MIF cell-surface receptor complex, which is comprised of the ligand-binding component, CD74 (ref. 14), and the signal-transducing component, CD44 (ref. 15), is involved in AMPK activation during hypoxia. Treatment of human fibroblasts with a CD74-specific short interfering RNA (siRNA) decreased MIF receptor CD74 protein expression and blunted hypoxia-stimulated AMPK phosphorylation (Fig. 4c). We also studied MIF-induced AMPK phosphorylation in $CD74^{null}/CD44^{null}$ COS-7/M6 cells that were stably transfected with either *CD74* alone, *CD44* alone, or *CD74* together with *CD44* (ref. 15). COS-7/M6 cells that expressed *CD74* or *CD44* alone showed no AMPK response to either hypoxia or exogenously added MIF. In contrast, COS-7/M6 cells expressing both transmembrane proteins showed significant AMPK phosphorylation responses (Supplementary Fig. 5). These results support an important role for the two-component receptor complex, consisting of the MIF binding CD74 protein and the signal-transducing CD44 protein, in MIF-mediated AMPK signalling during cellular hypoxia in the heart. A CD74-dependent interaction between MIF and CXCR2 also has been reported and has a role in inflammatory cell recruitment[10]. Whether MIF activation of CXCR2 also has a role in the cellular response to hypoxic injury beyond its migratory function is worthy of additional investigation.

In conclusion, these results define new models of both MIF action and AMPK activation, establishing a link between pathways central to inflammation and metabolism. MIF release leads to autocrine/paracrine activation of the AMPK-signalling pathway in the ischaemic heart. In other inflammatory disease states, high levels of MIF signalling, potentially activating additional pathways, might be deleterious. A common polymorphism in the human *MIF* promoter influences AMPK activation, and might predispose susceptible individuals to ischaemic injury and provide a potential new risk marker for patients with coronary artery disease.

METHODS SUMMARY

Rat heart muscles were incubated *in vitro*[21] to assess the effects of exogenous MIF on AMPK activation and downstream signalling, as well as to determine the role of endogenous MIF on the AMPK pathway during hypoxia. Isolated hearts from wild-type or $Mif^{-/-}$ mice were retrogradely perfused *in vitro*[1], as well as subjected to *in vivo* coronary occlusion/reperfusion, to examine the role of endogenous MIF in AMPK activation and myocardial injury during ischaemia. Human fibroblasts with various polymorphisms in the *MIF* promoter were incubated under hypoxic conditions to determine the role of *MIF* genotype on hypoxic AMPK activation, as well as the mechanism of AMPK activation by siRNA knockdown of the CD74 receptor. CD74 and/or CD44 were expressed in COS cells to assess the role of these receptors in the MIF response.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Information The authors declare competing financial interests: details accompany the HTML version of this paper at www.nature.com/nature. Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to L.H.Y. (lawrence.young@yale.edu).

METHODS

Antibodies. Murine polyclonal and monoclonal MIF antibodies were used for immunostaining/immunoblotting and neutralization studies, respectively (Torrey Pines Biolabs). Rabbit anti-phospho-Thr 172 AMPK (Cell Signaling), anti-AMPK α subunit (Cell Signaling), anti-GLUT4 (gift from S. Cushman), and anti-CaMKKβ (gift from A. Edelman) were used as described.

Animals. Animal procedures were approved by the Yale Animal Care Committee. Male Sprague-Dawley rats and wild-type BALB/c mice were purchased from Charles River Laboratories. *Mif*$^{-/-}$ mice were backcrossed onto the BALB/c genetic background (generation N9)[25].

Human fibroblasts. Human dermal samples were obtained from the Yale Human Cell Resource Center in accordance with the regulations of the Yale Human Investigation Committee. Fibroblasts were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), penicillin (100 IU ml^{-1}) and streptomycin (100 μg ml^{-1}) at 37 °C, and screened for the *MIF* genotype[5]. Early-passage fibroblasts were incubated either for 9 h of hypoxia (95% nitrogen/5% CO_2) or normoxia (room air/5% CO_2), with or without MIF (10 ng ml^{-1}).

***CD74* knock-down by siRNA.** Small interfering RNA (siRNA) was used to knock-down cellular *CD74* levels. The *CD74*-specific sequence was 5′-CAUGGGAUGAGGUACAGGGUdTdT-3′ (corresponding to *CD74* nucleotides 622–641; GenBank accession number NM_001025158) and the scrambled control sequence was 5′-CAGAAGCUAGUUACGAGAUdTdT-3′. Human fibroblast cells ('non-5/5' *MIF* gene promoter genotype) were transfected with siRNA with RNAiFECT (Qiagen) for 24 h. The cells then were subjected either to 9 h of hypoxia or normoxia.

Heart-muscle incubations. Rat heart left ventricular papillary muscles were incubated in oxygenated or hypoxic buffer at 37 °C[21]. MIF (0–800 ng ml^{-1}), anti-MIF monoclonal antibody (100 μg ml^{-1}), or isotypic IgG1 control (100 μg ml^{-1}) were added for 30–120 min as designated.

Heart-muscle glucose transport. Following pre-incubation with MIF, or hypoxic incubations, 2-deoxy-[1-^{3}H]glucose was added to measure the rates of glucose transport and phosphorylation[21].

Cell-surface GLUT4 photolabelling. Heart muscles were incubated in ice-cold glucose-free Krebs–Henseleit buffer containing 400 μmol l^{-1} bio-LC-ATB-BGPA (bis-D-glucose photolabel), a gift from G. Holman. Following UV cross-linking of bio-LC-ATB-BGPA to cell-surface glucose transporters, GLUT4 was isolated on Streptavidin-agarose, identified by immunoblotting and quantified by densitometry[31].

Mouse heart perfusions. Hearts from male *Mif*$^{-/-}$ and age-matched wild-type BALB/c mice were retrogradely perfused in the Langendorff mode with KHB containing 7 mmol l^{-1} glucose, 0.4 mmol l^{-1} oleate, 1% BSA and insulin (10 μU ml^{-1})[1]. Hearts were perfused for 30 min at a flow of 4 ml min^{-1}, followed by either: (1) 15 min of global ischaemia; (2) 15 min of global ischaemia followed by 30 min of reperfusion; or (3) additional control perfusion. The left-ventricular balloon inflated to achieve a diastolic pressure of 5 mm Hg during baseline perfusion and its volume kept constant during ischaemia and reperfusion. Hearts were freeze-clamped in liquid nitrogen at the end of the perfusions.

***In vivo* coronary occlusion/reperfusion.** Mice were anaesthetized, intubated and ventilated with oxygen (Harvard Rodent Ventilator; Harvard). The core temperature was maintained at 37 °C with a heating pad. After left lateral thoracotomy, the LAD was occluded for 15 min with an 8–0 nylon suture and polyethylene tubing to prevent arterial injury, and then reperfused for 4 h. Electrocardiograms confirmed ischaemic repolarization changes (ST-segment elevation) during coronary occlusion (AD Instruments).

The heart was then excised and perfusion stained to delineate the extent of myocardial necrosis as a per cent of non-perfused ischaemic area at risk (AAR). Viable tissue in the ischaemic region was stained red by TTC and the non-ischaemic region was stained blue with Evan's blue dye. Hearts were fixed and sectioned into 1-mm slices, photographed using a Leica microscope and analysed using NIH Image software.

Immunohistochemistry. Hearts were fixed by formalin perfusion, paraffin embedded, and cut into 3-μm sections. Sections were incubated with primary antibody at a dilution of 1:1,000, incubated for 4 h and immunostained (DakoCytomation). Negative controls for each heart included non-specific rabbit immunoglobulin G and secondary antibody alone.

AMPK assay. AMPK activity was determined by measuring [^{32}P]ATP incorporation into the synthetic SAMS peptide after immunoprecipitation[1,31,32].

MIF assay. MIF concentration was measured by a one-step sandwich enzyme-linked immunosorbent assay (detection limit, 0.16 ng ml^{-1})[11]

Recombinant MIF. Human or mouse recombinant MIF was prepared from an *Escherichia coli* expression system, purified by sequential column chromatography and re-natured under endotoxin free conditions[30]. Recombinant proteins contained < 10 ng LPS per mg protein.

Immunoblotting. Proteins were resolved by SDS–PAGE and detected with enhanced chemiluminesence[1,31,32].

Statistics. Data were means ± s.e.m. Significance was tested by Student two-tail *t*-tests or two-way repeated measures ANOVA with Bonferroni correction for multiple comparisons when appropriate.

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