

# **HIF1 $\alpha$ drives chemokine factor pro-tumoral signaling pathways in acute myeloid leukemia**

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**Running Title:** Hypoxia drives AML microenvironment

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## **Abstract**

Approximately 80% of patients diagnosed with acute myeloid leukemia (AML) die as a consequence of failure to eradicate the tumor from the bone marrow microenvironment. We have recently shown that stroma derived interleukin-8 (IL-8) promotes AML growth and survival in the bone marrow in response to AML derived macrophage migration inhibitory factor (MIF). In the present study we show that high constitutive expression of MIF in AML blasts in the bone marrow is hypoxia-driven and, through knockdown of MIF, HIF1 $\alpha$  and HIF2 $\alpha$ , establish that hypoxia supports AML tumor proliferation through HIF1 $\alpha$  signalling. In-vivo targeting of leukemic cell HIF1 $\alpha$ , inhibits AML proliferation in the tumor microenvironment, through transcriptional regulation of MIF but inhibition of HIF2 $\alpha$  had no measurable effect on AML blast survival. Functionally, targeted inhibition of MIF in-vivo improves survival in models of AML. Here we present a mechanism linking HIF1 $\alpha$  to a pro-tumoral chemokine factor signaling pathway and in doing so, we establish a potential strategy to target AML.

## Introduction

Survival of patients with acute myeloid leukemia (AML) is poor; two-thirds of young adults and 90% of older adults die of their disease (1). Even in patients fit enough to be treated with intensive cytotoxic chemotherapies, despite high initial response rates, relapse is common and occurs from minimal residual disease harbored within the bone marrow microenvironment (2). Improved outcomes require novel treatment strategies derived from a better understanding of the biology of the tumor and its microenvironment.

Hypoxia is a key microenvironmental factor that influences the biology of the hematopoietic stem cell (HSC), as well as leukemic cells within the bone marrow (3-6). The bone marrow microenvironment is hypoxic and targeting hypoxia inducible genes can eliminate cancer stem cells in hematological malignancies (7). The hypoxic state is principally maintained by members of the hypoxia-inducible factor (HIF) family. Both HIF1 $\alpha$  and HIF2 $\alpha$  respond to hypoxia; HIF1 $\alpha$  responds to acute hypoxia and HIF2 $\alpha$  to a chronic hypoxic state (8). Despite an increase in the understanding of the different pathways regulated by HIF1 $\alpha$  and HIF2 $\alpha$ , their role in HSC maintenance and leukemogenesis remains to be delineated (9, 10). Constitutive HIF1- $\alpha$  expression has been detected in normal karyotype AML and was found to be associated with poor prognosis (11), moreover, targeting HIF1 $\alpha$  in AML cancer stem cells abrogated their colony forming activity. Recent data showed that HIF2 $\alpha$  silencing impairs long term engraftment of HSC and also inhibits proliferation of primary AML in vitro (12). Others have shown that HIF2 $\alpha$  is high in subsets of both human AML and mouse models of AML, and overexpression of HIF2 $\alpha$  accelerated disease progression, however this study also highlights that patients with high HIF2 $\alpha$  expression trend toward disease free survival (13). Together these data describe a complex interplay between hypoxia regulated transcription factors HIF1 $\alpha$  and HIF2 $\alpha$  and their regulatory role in normal HSC and AML.

We have previously shown that AML cells constitutively express high levels of macrophage migration inhibitory factor (MIF) which drives Interleukin-8 (IL-8) expression by the bone marrow mesenchymal stromal cells (BM-MS), that in turn supports AML cell survival and proliferation (14). Furthermore, hypoxia has been identified as a potent inducer of the pro-inflammatory cytokine MIF in inflammatory

diseases (15, 16) . Specifically, a single nucleotide polymorphism (SNP) mapping to a functional hypoxia response element in the MIF locus, prevents induction of MIF by hypoxia (17). Together these studies lead us to hypothesize a role for hypoxia in regulating MIF survival signals in AML.

The aim of the present study is to determine if there is a connection between the hypoxic bone marrow microenvironment and MIF in regulating AML survival. If so, to further determine the role of HIF1 $\alpha$  and HIF2 $\alpha$  in this response and to evaluate the functional consequences and potential therapeutic effect of inhibiting such a pathway in vivo in AML patient-derived xenograft models.

## Results

### **AML blasts derived from the bone marrow express higher levels of MIF compared to blasts in the systemic circulation and spleen**

We have previously shown that primary AML cells from the bone marrow constitutively express high levels of MIF which subsequently drives survival signals within the BM microenvironment (14). To determine if this is a function of the BM environment, we looked at the expression levels of MIF in AML BM samples compared to AML peripheral blood (PB) samples (GEO accession number GSE49642) (18). These data show that MIF gene expression was significantly higher in AML samples from the BM compared to those from PB (Figure 1A). We next carried out qRT-PCR of five patient matched primary AML samples (both BM and PB) to show in both samples that AML cells from the BM expressed higher levels of MIF than those taken from the PB (Figure 1B). We then used a patient-derived xenograft (PDX) model, in which 9 primary AML patient samples were injected into NSG mice, in order to compare MIF gene expression between AML blasts that engrafted in the BM to blasts engrafted in the spleen (Figure 1C). Figure 1D confirms that the primary AML reliably engrafted the BM and spleen of the NSG mice. Figure 1E shows that MIF had a lower deltaCT or dCT (the lower the dCT value the higher the gene expression level) value in the AML blasts from the BM compared to blasts from the spleen.

### **Hypoxia induces MIF in primary AML blasts**

As the BM has been shown to be hypoxic (19-22) and hypoxia has been shown to regulate the expression of MIF in various cells (7, 16), we hypothesized that hypoxia was responsible for the higher expression of MIF in AML cells located in the BM. In 2014 Wierenga and colleagues, were able to identify common hypoxia-HIF1 $\alpha$ -HIF2 $\alpha$  gene signatures through the overexpression of HIF1 $\alpha$  and HIF2 $\alpha$  in CD34+ cord blood cells (23), we used the generated HIF1 $\alpha$ , HIF2 $\alpha$  and hypoxia specific gene lists to determine the gene signature in the data set used in figure 1A. Figure 2A and supplementary data 1 show that a set of hypoxia-related genes are preferentially expressed in AML BM compared to PB, with MIF being a significant gene in this list. To determine whether hypoxic conditions could induce expression of MIF in AML blasts in vitro we established hypoxic culture conditions either by using the hypoxia-mimetic agents cobalt chloride (CoCl<sub>2</sub>) and desferrioxamine (DFO) (which induce molecular hypoxic responses with similar kinetics to hypoxia) or by using a hypoxic

chamber (1% O<sub>2</sub>). Figure 2B shows the induction of HIF1 $\alpha$  protein in AML cells by hypoxia or hypoxia mimic reagents CoCl<sub>2</sub> and DFO. MIF mRNA and protein secretion were then assayed in experiments on five primary BM-derived AML patient samples. Figures 2C and Figure 2D show that CoCl<sub>2</sub>, DFO and 1% O<sub>2</sub> all significantly induced MIF mRNA expression and cytokine secretion. Next we sought to determine the contribution of HIF1 $\alpha$  or HIF2 $\alpha$  in regulating the expression of MIF in BM. We determined the significantly upregulated/enriched hypoxia, HIF1 $\alpha$  and HIF2 $\alpha$  genes in AML BM samples compared to AML peripheral blood (PB) samples (GEO accession number GSE49642). The analysis showed that MIF expression is part of the HIF1 $\alpha$  signature but not HIF2 $\alpha$ , the VENN diagram (Figure 2E) shows the distribution and overlap of the differentially expressed genes identified, there are 57 genes including MIF that are regulated by Hypoxia and HIF1 $\alpha$  but not HIF2 $\alpha$ , based on these findings we hypothesize that HIF1 $\alpha$  plays a significant role in upregulating MIF in primary AML. Figure 2F shows that MIF mRNA expression and protein secretion was not induced in non-malignant CD34<sup>+</sup> cells under hypoxia. Together these results demonstrate that hypoxic regulation of MIF is a tumour specific event.

### **HIF1 $\alpha$ but not HIF2 $\alpha$ is stabilized and induces MIF in primary AML blasts in response to hypoxia**

To further characterize the role of HIF1 $\alpha$  and HIF2 $\alpha$  in primary AML blasts, we assayed for HIF1 $\alpha$  and HIF2 $\alpha$  protein expression in AML blasts cultured under normoxic and hypoxic conditions. Figure 3A confirms that HIF1 $\alpha$  is stabilized under hypoxic conditions in primary AML blasts, however, we found that HIF2 $\alpha$  was detected in under normoxic conditions in U937, OCI-AML3 and 5/6 primary AML tested (Figure 3A, 3B). Moreover, only U937 and 1/6 primary sample AML samples showed an increase in HIF2 $\alpha$  under hypoxic conditions. To further characterize the role of HIF1 $\alpha$  in regulating MIF and confirm the specificity of the response to HIF1 $\alpha$  and not HIF2 $\alpha$ , we used lentiviral-mediated knockdown (KD) of HIF1 $\alpha$  or HIF2 $\alpha$  in AML patient blasts. Figure 3C confirms reduced mRNA expression of HIF1 $\alpha$  and HIF2 $\alpha$  after infection with HIF1 $\alpha$ -KD and HIF2 $\alpha$ -KD lentivirus. Figure 3D shows that HIF1 $\alpha$  or HIF2 $\alpha$  stabilization is reduced in AML blasts under hypoxic conditions in KD cells when compared to control-KD (ShE-KD) cells. Figures 3E and Figure 3F show that under hypoxic conditions, HIF1 $\alpha$ -KD, but not HIF2 $\alpha$ -KD, reduced basal levels of secreted MIF in five primary AML patient cells and reduced leukemic survival by 65% compared to control cells.

However, since the knockdown between HIF1 $\alpha$ -KD and HIF2 $\alpha$ -KD are not comparable we cannot rule out HIF2 $\alpha$  involvement in the up-regulation of MIF. Figure 3G shows that when we treated HIF1 $\alpha$ -KD AML cells with MIF this prevented cell death. Together these results demonstrate that HIF1 $\alpha$  regulates MIF secretion in AML blasts under hypoxic conditions.

### **HIF1 $\alpha$ -regulated MIF functions to promote AML tumor proliferation in vitro**

To understand the importance of HIF1 $\alpha$ -regulated MIF for the survival of AML cells within the bone marrow microenvironment, we used a lentiviral-mediated knockdown (KD) of MIF in our in vitro and in vivo AML assays. Reduced mRNA and protein expression of MIF after lentiviral targeting was confirmed prior to functional analysis (Figures 4A and 4B). MIF-KD in AML significantly reduced IL-8 mRNA expression from BMSC (figure 4C), reduced survival of primary AML cells in vitro, and compromised leukemic colony-forming ability in methylcellulose media assays (Figures 4D and 4E). Moreover, knockdown of MIF in AML induced apoptosis (Figure 4F).

### **Leukemic cell HIF1 $\alpha$ functions to promote tumor proliferation in vivo**

In order to track AML disease progression in vivo, we transduced OCI-AML3 cells with a luciferase construct that is detectible by bioluminescence (BLI) in live animals. OCI-AML3-luc cells were then infected with control lentivirus (ShE-KD) or HIF1 $\alpha$ -KD or MIF-KD lentivirus. NSG mice (6-8 weeks) were injected with  $0.5 \times 10^6$  modified OCI-AML3-luc cells from ShE-KD or HIF1 $\alpha$ -KD or MIF-KD cultures. Animals were imaged at day 21 and 28 post injection. At both time points mice transplanted with HIF1 $\alpha$ -KD and MIF-KD cells had lower BLI detection compared to control ShE-KD transplant animals (Figures 5A and 5B). Figure 5C shows that NSG mice engrafted with HIF1 $\alpha$ -KD or MIF-KD OCI-AML3-luc cells had significantly increased survival compared to control animals (n=4). To show that AML engrafted the bone marrow of NSG mice in ShE-KD or HIF1 $\alpha$ -KD or MIF-KD we analysed human CD45 expression, the results show that in all treatment groups AML was present in the bone marrow after the end point of the experiment was reached (Figure 5D).

## Discussion

We have previously shown that MIF is an important mediator of AML survival. Here we report that hypoxia acting through HIF1 $\alpha$  is responsible for the up-regulation of MIF and subsequent proliferation and survival of AML in the tumor microenvironment. We find that AML in the BM has increased levels of MIF compared to AML in the PB or spleen. We show that under hypoxic conditions MIF is up-regulated at both the transcriptional and protein levels and functionally the hypoxia/HIF1 $\alpha$ /MIF axis promotes tumor survival and proliferation of AML within the BM microenvironment *in vivo*.

AML arises from malignant transformation and proliferation of hematopoietic progenitor cells in the BM. Under physiologic non-malignant conditions the BM has been identified as a hypoxic environment, Fiegl et al. have established that the AML BM is also hypoxic (24). In addition, tumor-specific transcriptional programs in AML patient samples include an up-regulation of the hypoxic response gene signature (25). Moreover, hypoxia has as broader malignant phenotype in the proliferation of solid tumor cells in studies of breast, prostate, ovarian and pancreatic cancers (26-28). Here, we describe a significant mechanism by which hypoxia is pro-tumoral in the AML microenvironment.

We report that a functional consequence of the hypoxic tumor microenvironment in AML is the up-regulation of the chemokine MIF. Previously, we have shown that AML-derived MIF functions to re-program the tumor microenvironment by inducing BM-MSC derived IL-8 (29). Interestingly, others have shown that hypoxia regulates MIF expression in endothelial cells (30) and vascular smooth muscle cells (15). Moreover, hypoxia-induced MIF expression is dependent upon a hypoxia response element (HRE) in the 5'UTR of the MIF gene (31). In addition, MIF has recently been shown to be up-regulated by hypoxia in several tumor cell types *in vitro* including breast carcinoma cells (31). Interestingly, a number of studies have shown a complex relationship between HIF1 $\alpha$  and MIF exists in chronic lymphocytic leukemia (CLL) (37). It has been shown that MIF can regulate and be regulated by HIF1 $\alpha$ . Moreover, MIF regulation has been shown to be both dependent and independent of HIF1 $\alpha$  (32). In our work on AML, we demonstrate that silencing HIF1 $\alpha$  inhibits transcriptional regulation of MIF under hypoxia.



In our model, knockdown of HIF1 $\alpha$  in primary AML cells significantly reduces survival of AML cells in culture and significantly increases survival of NSG mice engrafted with HIF1 $\alpha$ -KD OCI-AML3-luc cells. Previously published results using a murine AML model show that genetic deletion of HIF1 $\alpha$  has no effect on mouse AML maintenance and may accelerate disease development (10). The same group further show that loss of HIF1 $\alpha$  accelerates murine FLT3-ITD induced myeloproliferation (33). However, others have shown that deleting HIF1 $\alpha$  in a subset of primary AML leukemic stem cells (LSCs) leads to a decrease in sensitivity of these cells to HIF1 $\alpha$  inhibition (7). Moreover, in their work, Wang and colleagues demonstrated an important role for HIF1 $\alpha$  in the in vitro colony forming activity of their 7 clinical AML samples with experiments using treatment with echinomycin (a HIF1 $\alpha$  inhibitor) (7). Echinomycin has also been shown to inhibit growth and induce apoptosis of AML cell lines (34). Vukovic et al. reported that HIF1 $\alpha$  and HIF2 $\alpha$  are not required for leukemia stem cell maintenance and AML propagation, but they act synergistically to suppress leukemia development in mice, in their models, knockout of HIF2 $\alpha$  or pharmacological inhibition of the HIF pathway in human AML cells has no impact on their survival and proliferation under hypoxic conditions (35). In our studies, we show that HIF2 $\alpha$  was detected in the majority of AML samples and cell lines under normoxic conditions. Moreover, this was not increased under hypoxic condition. We used U937 as a positive control and showed that this cell line did increase HIF2 $\alpha$  under hypoxia. These results suggest that HIF2 $\alpha$  is regulated in AML without the need for hypoxia as is the case for other tumours (36, 37). Together, there is a lot of conflicting data regarding the regulatory role of HIF1 $\alpha$  in normal HSC and malignant AML cells. In this manuscript we describe a HIF1 $\alpha$  driven mechanism which results in up-regulation of MIF in a tumour specific manner. Moreover, in-vivo knockdown of HIF1 $\alpha$  in AML showed a decreased tumour burden and had an increased survival compared to control AML, thus suggesting that human AML benefits from the hypoxic bone marrow microenvironment.

Previously published studies observed that HIF2 $\alpha$  is constitutively expressed in a number of AML subtypes and overexpression studies of HIF2 $\alpha$  accelerated myeloid leukemia in mice (13). Rouault-Pierre and colleagues show that cells from primary AML samples are dependent on the level of HIF2 $\alpha$  for their survival and protection from apoptosis induced by ER stress. The degradation kinetics of HIF1 $\alpha$  and HIF2 $\alpha$

also differ although they are ~60% homologous. HIF2 $\alpha$  has been shown to be detectable in normoxic condition while HIF1 $\alpha$  has not (38). Although the similarity in homology contributes to overlapping genes/targets regulated by both HIF1 $\alpha$  and HIF2 $\alpha$ , each also regulate a distinctive set of genes in their own right and which is supported by our in silico analysis. Moreover, our results also show that HIF2 $\alpha$  is constitutively expressed under normoxia and hypoxic conditions had little or no effect on its expression. Knockdown of HIF2 $\alpha$  in primary AML cells did not reduce MIF expression or secretion from primary AML. Thus suggesting that MIF is not regulated by HIF2 $\alpha$ . Our results however, do support studies showing HIF2 $\alpha$  expression is heterogeneous in AML subtypes (13).

Finally, we found that knocking down MIF significantly reduces AML survival and colony forming ability in vitro, moreover, animals transplanted with AML cells post MIF-KD had significantly improved survival compared to controls, these results in the context of recent clinical observations of the use of novel MIF inhibitors in colorectal cancer and in solid tumors early phase trials is effective and well tolerated (39, 40), suggests the plausibility of a MIF inhibitor trial in patients with AML. In conclusion, our study presents a mechanism linking hypoxia to a chemokine factor pro-tumoral signaling pathway in AML microenvironment. In doing so we establish a potential strategy to target AML, a hypoxia-driven malignancy.

## **Materials and Methods**

### **Materials**

Anti-HIF1 $\alpha$  antibody was purchased from BD Biosciences (Oxford, UK). Anti-HIF2 $\alpha$  was purchased from (Novus Bio, Cambridge, UK Cat: NB100-122SS) IgG-FITC, IgG-PE, IgG-APC, anti-CD34-PE, anti-CD33-APC and anti-CD45-FITC antibodies were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany Cat. 130098847, 130098845, 130092214, 130098139, 130098043, 130098864). Recombinant human MIF, and MIF ELISA were purchased from R&D Systems (Abingdon, UK). All other reagents were obtained from Sigma-Aldrich (St Louis, MO, USA), unless otherwise indicated

### **Methods**

#### **Cell cultures**

The AML derived cell lines were obtained from the European Collection of Cell Cultures (ECCC) where they are authenticated by DNA fingerprinting. All cell lines are tested for mycoplasma contamination. Primary AML blasts were obtained from patient bone marrow or blood following informed consent and under approval from the United Kingdom Health Research Authority (LRECref07/H0310/146). For primary AML and BMSC cell isolation, heparinized bone marrow and blood were isolated by density centrifugation using Histopaque which has been previously described (41). Primary AML and BMSC cells and cell lines were cultured in growth medium comprising DMEM and 10% FBS and 1% L-glutamine at 5% CO<sub>2</sub> at 37 °C.

#### **Hypoxic assays**

For hypoxic cultures, cells were incubated in a hypoxia chamber (Billups-Rothenberg Inc, Del Mar, USA) with 1% O<sub>2</sub>/ 5% CO<sub>2</sub>/ N<sub>2</sub> 200 bar. The chamber was incubated at 37 °C for indicated times. Control cell cultures, not deprived of oxygen, were incubated under normal conditions. Cobalt chloride (CoCl<sub>2</sub>) and desferrioxamine (DFO) were added to cell cultures at indicated concentrations and time points.

#### **Clonogenic methylcellulose assay**

AML blasts were plated in complete methylcellulose medium supplemented with cytokines (R&D Systems, Abingdon, UK) at a density of 1x10<sup>3</sup>. Light microscopy was used to visualize and count colonies at days 10-14.

### **Flow cytometry**

Cells were collected by centrifugation for 3 minutes at 2,000 rpm, then washed and resuspended in phosphate-buffered saline (PBS), incubated for 5 minutes with the FcR blocking reagent and then stained with anti-CD45, anti-CD34 or anti-CD33 or isotype control. Analysis was performed on a CyFlow® Cube 6 flow cytometer (Sysmex, Milton Keynes, UK).

### **Cell viability assay**

Cell viability was measured using the Cell Titer-Glo Luminescent Cell Viability Assay kit (Promega, Southampton, UK) according to the manufacturer's instructions.

### **RNA extraction and real-time PCR**

ReliaPrep RNA extraction kit from Promega (Southampton, UK) was used to extract total RNA. PCR BIO cDNA synthesis kit (PCR Biosystems, London, UK) was used to generate cDNA from total RNA. Relative quantitative real-time PCR using qPCR BIO SyGreen Mix (PCR Biosystems) was performed as previously described (41). mRNA expression was normalized against B-actin mRNA expression.

### **Western immunoblotting and ELISAs**

Briefly, whole cell lysates were extracted and SDS PAGE gel electrophoresis separation performed as previously described (42). Western blot analysis performed with anti-HIF1 $\alpha$  and anti-HIF2 $\alpha$  antibody, membranes were re-probed for B-actin as a loading control. MIF chemokine expression in the media we used target-specific MIF ELISA (R&D Systems).

### **shRNA silencing of MIF, HIF1 $\alpha$ and HIF2 $\alpha$**

Mission shRNA targeted lentivirus plasmids (Sigma-Aldrich) were used to generate lentivirus as previously described (43). OCI-AML3 cells were infected with pCDH-luciferase-T2A-mCherry (44). OCI-AML3 cells expressing mCherry (OCI-AML3-luc) were sorted on a FACSAria (BD Biosciences). The MIF-KD and the HIF1 $\alpha$ -KD in vivo experiments were carried simultaneously, therefore the same control-KD (ShE-KD) mice were used for both MIF-KD and HIF1 $\alpha$ -KD mice (Figure 2G-I and Figure 3E-G).

### **OCI-AML3-luc xenograft model**

NOD.Cg-Prkdcscid IL2rgtm1Wjl/SzJ (NSG) mice from The Jackson Laboratory (Bar Harbor, ME, USA) were used for this study. All animal experiments were performed in accordance with UK Home Office approvals and regulations and with approval from the Animal Welfare and Ethical Review Board of the University of East Anglia.  $0.5 \times 10^6$  OCI-AML3-luc cells were intravenously injected into the tail vein of non-irradiated 6-8-week-old NSG mice. When clinical signs of illness became apparent, mice were sacrificed. Bone marrow (BM) and spleen were harvested and analyzed for human CD45 expression. No animals were excluded from experiments and no randomization was used. No blinding was used.

### **Patient-derived xenograft model**

Primary AML were cultured in vitro as previously described (45).  $2 \times 10^6$  viable AML cells were injected into the tail vein of non-irradiated 6-8-week-old female NSG mice. When clinical signs of illness became apparent, mice were sacrificed. BM and spleen were harvested and analyzed for human CD33 and CD45.

### **Bioinformatic analysis**

Publicly available RNA sequencing data were downloaded for a panel of 43 AML patients, which comprised 22 AML samples obtained from peripheral blood and 21 AML samples obtained from BM aspirate (Gene Expression Omnibus Accession ID: GSE49642). RPKM data for MIF were extracted and processed further by first replacing zero-valued entries with one followed by logarithmic transformation to the base 2.21. MIF RPKM values for blood and bone marrow samples were compared with a Wilcoxon rank-sum test. Data were extracted for genes that have been shown to be upregulated as a result of HIF1 $\alpha$  overexpression, HIF2 $\alpha$  overexpression and hypoxia (23). These data were processed further by first replacing zero-valued entries with one, and followed by logarithmic transformation to the base 2.

### **Statistical analyses**

For Western blotting, data are representative images of three independent experiments. Calculations were made using the software G\*Power. The Mann-Whitney U test was used to compare test groups where stated in the legends. Results where  $p < 0.05$  were considered statistically significant and denoted by \*. Results

represent the mean  $\pm$  standard deviation of four or more independent experiments. We generated statistics with Graphpad Prism5 software (Graphpad, San Diego, CA, USA).

## **Authors declare no conflict of interest**

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### **Author Contribution**

A.A.A., M.S.S., K.M.B and S.A.R designed the research; A.A.A., C.R.M., R.E.P and M.S.S., performed the research; S.A.R., C.R.M., S.D.R., and R.E.P. carried out in vivo work; D.R.E., Z.Z., A.C. and K.M.B. provided essential reagents and knowledge. A.A.A., M.S.S, K.M.B., and S.A.R. wrote the paper.

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## Figure legends:

**Figure 1. Bone marrow derived AML express higher level of MIF.** (A) MIF gene expression (expressed in log<sub>2</sub> RPKM values) was obtained from GSE49642 for 22 peripheral blood (PB) and 21 bone marrow (BM) AML patient samples. *p*-value was obtained by Wilcoxon rank-sum test. Line denotes the median value. (B) RNA was extracted from AML derived from the BM and PB from matched patients. MIF mRNA expression was determined by qPCR and normalized to B-actin. (C) Primary AML blasts were isolated from BM and injected into the tail vein of NSG mice. (D) and (E) 2x10<sup>6</sup> primary AML cells (9 patient AML) were injected into NSG mice. (D) Engraftment was measured using human CD33 and human CD45, in the dot plot each AML engraftment into NSG mice is shown for bone marrow and spleen. (E) RNA was extracted from AML from the BM and spleen. RNA was analysed for MIF mRNA using human specific primers. mRNA differences are presented as delta cycle threshold (dCT), normalized to human B-actin.

**Figure 2. Hypoxia induces MIF expression in Primary AML blasts.** (A) Heatmap of genes that are differentially expressed between bone marrow and peripheral blood AML samples (adjusted *P* < 0.05, Significance Analysis of Microarrays) (data from GSE49642). Only genes that are upregulated under hypoxia are displayed (23). Samples (columns) are grouped according to the type of sample: bone marrow (black) or peripheral blood (grey). The expression of individual genes (rows) across the dataset is represented by mean-centred log<sub>2</sub> expression values. The genes are ordered from top to bottom by descending fold change. (B) AML cells were treated with CoCl<sub>2</sub> (100uM), DFO (150uM) or cultured under hypoxic conditions (1% O<sub>2</sub>) for 4 hours, then protein was extracted and Western blotting performed. (C) Primary AML cells were treated with CoCl<sub>2</sub> (100uM) or DFO (150uM) for 4 hours or cultured under hypoxic conditions for 24 hours, RNA was extracted and MIF mRNA expression was determined with RT-PCR. Data represented as mean ± standard deviation (n=5). (p<0.05) (D) Media was collected from the respective conditions described in (B) and MIF protein secretion was determined by target-specific ELISA, Data represented as mean ± standard deviation (n=5). (p<0.05) (E) Venn diagram showing overlaps in genes up regulated upon induction of hypoxia, overexpression of HIF1α and HIF2α. (F) Non-malignant CD34+ cells were cultured under hypoxic conditions for 24 hours.

Culture media was assayed for MIF cytokine secretion and RNA was extracted and MIF mRNA expression was determined with RT-PCR. Data represented as mean  $\pm$  standard deviation (n=5) (p<0.05).

**Figure 3. HIF1 $\alpha$  but not HIF2 $\alpha$  regulates MIF mRNA and protein expression in AML blasts.** (A) OCI-AML3 and primary AML blasts were cultured under hypoxic conditions for 4 hours to detect HIF1 $\alpha$  and 12 hours to detect HIF2 $\alpha$ , protein was extracted and Western blotting performed. (B) OCI-AML3, U937 and primary AML blasts were cultured under hypoxic conditions for 6 hours to detect HIF2 $\alpha$ , protein was extracted and Western blotting performed. Lentiviral knockdown (KD) of HIF1 $\alpha$  or HIF2 $\alpha$  in AML blasts was determined by RT-PCR for mRNA expression. Data represented as mean  $\pm$  standard deviation (n=5) (C) and Western blotting for protein expression (D) under hypoxic conditions. (E) MIF protein expression in culture media from HIF1 $\alpha$  or HIF2 $\alpha$ -KD blasts compared to control-KD (ShE) cells was determined by ELISA. Data represented as mean  $\pm$  standard deviation (n=5). (F) Control-KD (ShE) and HIF1 $\alpha$  or HIF2 $\alpha$  KD AML blasts were cultured for 10 days post-infection in hypoxia; cell survival was measured using CellTiter-Glo Luminescent Cell Viability Assay. Data represented as mean  $\pm$  standard deviation (n=5). (G) Control-KD (ShE) and HIF1 $\alpha$  or HIF2 $\alpha$  KD primary AML blasts were cultured for 24 hours in hypoxia while treated with MIF (100ng/ml); cell survival was measured using CellTiter-Glo Luminescent Cell Viability Assay. Data represented as mean  $\pm$  standard deviation (n=5) (p<0.05).

**Figure 4. MIF regulates AML survival and colony formation.** Lentiviral KD of MIF in OCI-AML3-luc cell line and primary AML blast was confirmed by RT-PCR (A) and protein by ELISA (B). (C) ShE-KD and MIF-KD Primary AML blasts were cultured with BMSC for 4 hours, then IL-8 mRNA expression in BMSC was confirmed by RT-PCR. (D) ShE-KD and MIF-KD cells were cultured in basal media for 10 days post infection in hypoxia; cell survival was measured using CellTiter-Glo Luminescent Cell Viability Assay. (E) Colony-forming assays of ShE-KD and MIF-KD cells were performed in methylcellulose media. (F) Apoptosis assays of ShE-KD and MIF-KD cells were performed using annexin V and propidium iodide. Data represented as mean  $\pm$  standard deviation (n=5) (p<0.05).

**Figure 5. Targeting HIF1 $\alpha$  and MIF in AML xenograft model.** (A-D)  $0.5 \times 10^6$  control-KD (ShE) OCI-AML3-luc and HIF1 $\alpha$ -KD or MIF-KD OCI-AML3-luc cells were injected into NSG mice (n=4 for each KD). NSG mice were monitored for disease progression using bioluminescence and sacrificed upon signs of disease. (A) Bioluminescence images of recipient mice, control-KD (ShE) and HIF1 $\alpha$ -KD (HF) OCI-AML3-luc cells on day 21 and day 28 respectively. (B) The mean bioluminescence intensity of each image was determined with Image J software. (C) Kaplan-Meier survival curves for NSG mice injected with OCI-AML3-luc HIF1 $\alpha$ -KD cells or OCI-AML3-luc control-KD cells. (D) Chimerism data showing engraftment of OCI-AML3 which was measured using human CD45. In the dot plot each AML engraftment into NSG mice is shown for control-KD (ShE) and HIF1 $\alpha$ -KD.