Hypoxia-inducible factor-2 is a novel regulator of aberrant CXCL12 expression in multiple myeloma plasma cells

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ABSTRACT

Background
Multiple myeloma is an incurable malignancy of bone marrow plasma cells. Progression of multiple myeloma is accompanied by an increase in bone marrow angiogenesis. Studies from our laboratory suggest a role for the CXCL12 chemokine in this process, with circulating levels of CXCL12 correlating with bone marrow angiogenesis in patients with multiple myeloma. While the mechanisms responsible for aberrant plasma cell expression of CXCL12 remain to be determined, studies in other systems suggest a role for hypoxia and hypoxia-inducible transcription factors.

Design and Methods
The expression of hypoxia-inducible factor protein was examined in patients' bone marrow biopsy specimens using immunohistochemistry. The hypoxic regulation of CXCL12 was examined in multiple myeloma plasma cell lines using polymerase chain reaction and western blotting. The role of hypoxia-inducible factors-1 and -2 in the regulation of CXCL12 expression was examined using over-expression and short hairpin RNA knockdown constructs, electrophoretic mobility shift assays and chromatin immunoprecipitation. The contribution of CXCL12 to hypoxia-induced angiogenesis was examined *in vivo* using a subcutaneous murine model of neovascularization.

Results
Strong hypoxia-inducible factor-2 protein expression was detected in $CD138^+$ multiple myeloma plasma cells in patients' biopsy specimens. Prolonged exposure to hypoxia strongly up-regulated CXCL12 expression in multiple myeloma plasma cells and hypoxia-inducible factor-2 was found to play a key role in this response. Promoter analyses revealed increased hypoxia-inducible factor-2 binding to the CXCL12 promoter under hypoxic conditions. Over-expression of hypoxia-inducible factor in multiple myeloma plasma cells strongly induced *in vivo* angiogenesis, and administration of a CXCL12 antagonist decreased hypoxia-inducible factor-induced angiogenesis.

Conclusions
Hypoxia-inducible factor-2 is a newly identified regulator of CXCL12 expression in multiple myeloma plasma cells and a major contributor to multiple myeloma plasma cell-induced angiogenesis. Targeting the hypoxic niche, and more specifically hypoxia-inducible factor-2, may represent a viable strategy to inhibit angiogenesis in multiple myeloma and progression of this disease.

Key words: multiple myeloma, CXCL12, hypoxia, HIF-2.

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The Online version of this article has a Supplementary Appendix.

Introduction

Multiple myeloma (MM) is an incurable hematologic malignancy characterized by the clonal proliferation of malignant plasma cells in the bone marrow. Accounting for approximately 1% of all cancers, MM is the second most common hematologic malignancy after non-Hodgkin's lymphoma. As for all tumors, the survival and expansion of MM plasma cells is dependent upon an adequate supply of oxygen and nutrients, and the acquisition of an angiogenic phenotype is a key event in the progression from monoclonal gammopathy of undetermined significance (MGUS) and indolent MM to active MM.[1,2]

CXCL12 is a constitutively expressed chemokine which binds primarily to the CXCR4 receptor and regulates cell growth, chemotaxis, myelopoiesis, lymphopoiesis, and development of the nervous and cardiovascular systems.[3-5] CXCL12 is highly expressed by MM plasma cells,[6] and circulating levels of CXCL12 are higher in the peripheral blood of MM patients than in age-matched normal donors and MGUS patients.[6,7] CXCL12 is an important mediator of several aspects of MM biology including transendothelial migration,[8,9] MM plasma cell migration and retention within the bone marrow,[10,11] angiogenesis,[7] and osteoclastic bone resorption.[6] Recent animal studies involving systemic injection of labeled MM plasma cells have also demonstrated that blocking the CXCL12/CXCR4 axis leads to a 20% reduction in bone marrow tumor burden.[11]

Unlike most other organs, the bone marrow microenvironment is physiologically hypoxic, a pre-requisite for normal bone marrow hematopoiesis.[12] It is well established that hypoxia is an important selective force in the evolution of tumor cells,[13] and elevated expression of the hypoxia-inducible transcription factors HIF-1 and HIF-2 has been documented in several human cancers.[14,15] HIF-1 and HIF-2 mediate adaptive responses to hypoxia by inducing the transcription of genes associated with erythropoiesis, glycolytic metabolism, cell survival and angiogenesis. While the role of hypoxia in the pathogenesis of hematologic malignancies has yet to be elucidated, recent animal studies have shown that changes in oxygen levels within the bone marrow microenvironment support the survival and expansion of MM plasma cells.[16] Furthermore, some drugs active in MM, such as bortezomib and lenalidomide, are believed to exert their effects, in part, by interfering with hypoxia-induced signaling cascades.[17,18]

HIF-1 and HIF-2 are heterodimers composed of an inducible α-subunit and a constitutively-expressed β-subunit called aryl hydrocarbon receptor nuclear translocator (ARNT). Under normoxic conditions, HIF-α subunits are functionally repressed and undergo rapid proteosomal degradation ($t_{1/2}$=5 min).[19] However under hypoxic conditions, these processes are abrogated and the stabilized HIF-α translocates to the nucleus to dimerize with ARNT. HIF-1 and HIF-2 bind to the same DNA consensus motif; however, they have overlapping but distinct target gene specificities and distinct, non-redundant physiological roles.[20,21]

In 2002, Hitchon *et al.* showed for the first time that CXCL12 expression is up-regulated by hypoxia in human synovial fibroblasts.[22] While subsequent studies extended this finding to other cell types,[23-26] the effect of hypoxia on CXCL12 expression in MM plasma cells has not been investigated. We, therefore, studied the expression of HIF-1, HIF-2 and CXCL12 in relation to hypoxia in MM and the contribution of CXCL12 to hypoxia-induced angiogenesis.

Design and Methods

Cell cultures

LP-1 cells (a human myeloma cell line) were cultured in RPMI medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 15 mM HEPES and 50 IU/mL penicillin-streptomycin in a humidified atmosphere at 37°C. Where specified, hypoxic culture conditions were established (less than 1% oxygen) using an anaerobic sachet (Oxoid, UK).

Immunohistochemical staining

Paraffin-embedded sections (5 μm) of MGUS (n=8) or MM (n=7) trephine specimens collected at the time of diagnosis at the Royal Adelaide Hospital were immunostained with antibodies against CXCL12 (sc-6193, Santa Cruz, CA, USA), HIF-1α (NB100-449, Novus Biologicals, CO, USA), HIF-2α (NB100-132, Novus Biologicals) and CD138 (M7228, Dako, Denmark) as previously described.[6] Studies were performed with Institutional Ethics approval following written, informed consent. Images were captured using a NanoZoomer Slide Scanner (Hamamatsu, Japan).

Stable transduction of LP-1 cells

To generate stable over-expressing cell lines, full-length cDNA encoding human CXCL12,[27] HIF-1α,[28] and HIF-2α[29] were cloned into pRUF-IRES-GFP to generate pRUF-IRES-GFP-HIF-1α, pRUF-IRES-GFP-HIF-2α and pRUF-IRES-GFP-CXCL12. Following retroviral infection of LP-1 cells, pooled cell lines were established from the top 30% of GFP-expressing cells as previously described.[27] To generate stable knock-downs, RNA duplexes targeting human HIF-1α (CCATGAGGAAATGAGAGAAATGCTT), human HIF 2α (GGGGGCTGTGTCTGAGAAGAGT) or a scrambled control (CCAAGGAGTAAGAGATAAAGGTC)[30,31] were cloned into the pFIV-H1-copGFP lentiviral vector (System Biosciences, CA, USA), and clonal cell lines generated from the top 10% of GFP-expressing cells using preparative cell sorting and single-cell deposition. For *in vivo* studies, cells were co-transduced with the SFG-$_{NES}$-TGL luciferase vector[32] to enable bioluminescent detection of these cells.

Real-time polymerase chain reaction

RNA was reverse transcribed from 1 μg of total RNA using Superscript III according to the manufacturer's instructions (Invitrogen, CA, USA) and real-time polymerase chain reaction (PCR) was performed on a Rotor-Gene 3000 instrument (Corbett Life Science, Australia) using the following primers: β_2-microglobulin Fwd 5'-aggctatccagcgtactcca-3' and Rev 5'-tcaatgtcggatggatgaaa-3'; human CXCL12 Fwd 5'-atgcccatgccgattcttcg-3' and Rev 5'-gtctgttgttgttcttcagcc-3'; human HIF-1α Fwd 5'-ccacctatgacctgcttggt-3' and Rev 5'-tgtcctgtggtgacttgtcc-3'; human HIF-2α Fwd 5'- ctctcctcagtttgctctgaaaa-3' and Rev 5'-gtcgcagggatgagtgaagt-3'; human vascular endothelial growth factor (VEGF) Fwd 5'- atgccaagtggtcccagg-3' and Rev 5'-cacacaggatggcttgaaga-3'; human GLUT-1 Fwd 5'- ggccaagagtgtgctaaagaa-3' and Rev 5'-cagcgttgatgccagaca-3'; human CXCR4 Fwd 5'- cagcaggtagcaaagtgacg-3' and Rev 5'-gtagatggtgggcaggaaga-3', as previously described.[27] Changes in gene expression were calculated relative to β2-microglobulin using the $2^{-\Delta Ct}$ method.[33]

Western immunoblotting

Whole cell extracts (100 μg) were separated by 8-10% sodium

dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and transferred to a polyvinylidene fluoride membrane. Immunoblotting was performed using antibodies to HIF-1α (610959, BD Bioscience, CA, USA), HIF-2α (NB100-122, Novus Biologicals), and α-tubulin (ab6160, Abcam, MA, USA). Following incubation with the appropriate alkaline phosphatase-conjugated secondary antibodies, membranes were developed with ECF (GE Healthcare, UK).

CXCL12 enzyme-linked immunosorbent assay

CXCL12 protein levels in culture media were measured using a commercial immunoassay (R&D Systems, MN, USA) as previously described,[6,7] and data were normalized to the total protein content of the cells from which the supernatant was collected.

Luciferase assay

The proximal CXCL12 promoter was amplified from LP-1 genomic DNA (Fwd: 5'-gcgctcgagccatctaacggccaaagtgg-3' and Rev: 5'-gcgaagcttggctgacggagagtgaaagtg-3') using Pfu turbo (Stratagene, CA, USA) according to the manufacturer's instructions, and ligated into the pGL3-basic vector (E1751, Promega, WI, USA) to generate pGL3b-CXCL12. LP-1 cells (4.5×10^6) were electroporated (Bio-Rad Gene Pulser, 270V/960μF) in 500 μL in RPMI medium (supplemented with 20% fetal calf serum) with 5 μg of reporter plasmid and 10 μg of expression plasmid. Twenty-four hours after transfection, cells were cultured under normoxic or hypoxic conditions for 48 h, and their luciferase activity was assayed as previously described.[34]

Chromatin immunoprecipitation

Chromatin immunoprecipitation assays were performed using the EZ-Magna ChIP kit (Millipore, MA, USA) according to the manufacturer's instructions. Briefly, LP-1 cells (1×10^7/treatment) were cultured under normoxic or hypoxic conditions for 48 h, then cross-linked in 1% formaldehyde for 10 min at room temperature and quenched with 125 mM glycine for 5 min. Cells were washed five times in chilled phosphate-buffered saline, sonicated for 30 min (Diagenode, Belgium, 30 s pulses, 30 s rests) and immunoprecipated with magnetic beads and 5 μg primary antibody (ab199, Abcam) overnight at 4°C with rotation. The next day, complexes were eluted, sequentially washed in low salt buffer, high salt buffer, LiC buffer and TE buffer (5 min each with rotation), then eluted with 100 μg/mL proteinase K at 62°C for 2 h with rotation. Eluted DNA was purified and subjected to PCR analysis using primers directed against the HBS1 region of the CXCL12 promoter: Fwd 5'-tctaacggccaaagtggttt-3' and Rev 5'-gccacctctctgtgtccttc-3'.

Electrophoretic mobility shift assay

The oligonucleotide for HBS1 of the CXCL12 promoter (5'-gggacagggacgtgtccccaggg-3') was purchased from Geneworks (Australia) and the full-length product purified from non-denaturing polyacrylamide gels following established protocols.[35] Single-stranded DNA probes were prepared by end-labeling 100ng of oligonucleotide with T4 polynucleotide kinase and [γ-^{32}P] ATP (Geneworks) followed by gel purification.

Nuclear extracts were prepared from LP-1 cells following 48 h of normoxic or hypoxic culture as previously described.[36] Gel retardations were performed using 0.25 ng double-stranded ^{32}P-labeled oligonucleotide probe in a 10 μL reaction mix containing 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, 5% glycerol, 0.25 μg poly dIdC and 5 μg nuclear extract. Reaction mixes were incubated at 4°C for 30 min and resolved on 4% non-denaturing polyacrylamide gels run in 0.5x TBE buffer. For antibody blocking experiments, protein and antibody (5 μg or 10 μg) were incubated for 5 min at room temperature before adding ^{32}P-labeled probe for 20 min prior to gel loading. Gels were visualized using a Storage Phosphor Screen (GE Healthcare).

Implantation of modified LP-1 cell lines into mice

Cells (5×10^6/implant) were suspended in 200 μL chilled serum-free RPMI 1640 medium, mixed with 200 μL chilled Matrigel matrix (BD Bioscience) and subcutaneously injected into the right ventral flank of 6-week old female BALB/c nude mice. An equivalent implant containing no cells was injected into the left ventral flank. After 14 days, mice were killed humanely and Matrigel plugs were photographed and removed, and the hemoglobin content determined. Where specified, osmotic pumps (DURECT Corporation, CA, USA) containing 100 μL of the CXCR4 antagonist, T140 (4F-benzoyl-TN14003, 80 mg/mL in phosphate-buffered saline), were subcutaneously implanted in the upper dorsum 2 days prior to cell implantation.

In vivo bioluminescence imaging

Ongoing assessment of tumor growth was performed using bioluminescence imaging as previously described.[27]

Hemoglobin assessment

Excised implants were sonicated for 15 min (Diagenode, 30 s pulses, 30 s rests) in 300 μL water and centrifuged at 16,000 x g for 90 min at 4°C to remove debris. The hemoglobin content was then assessed according to instructions from Sigma (MO, USA). Briefly, homogenised tumor supernatants (50 μL/well) were added to Drabkin's solution (200 μL/well), and absorbance was read at 540 nm after 15 min. A standard curve was generated using bovine hemoglobin (Sigma).

The hemoglobin content of each cell-containing implant was initially normalized to that of the corresponding empty implant from each mouse. This value was then normalized to the bioluminescence reading obtained at the time of sacrifice, to relate the angiogenesis assessment to the number of viable cells present in the implant.

Statistical analyses

Experiments were performed in triplicate, and data are presented as mean ± standard error of measurement (SEM). Statistical analyses were performed using a one-way ANOVA with Dunnett's post-hoc test using SigmaStat® 3.0 software (Systat, IL, USA). In all cases, *P* values less than 0.05 were considered statistically significant.

Results

Expression of hypoxia-inducible factor and CXCL12 proteins in the bone marrow

Sections of bone marrow trephine specimens from patients with MGUS or MM were co-immunostained with an antibody to CD138 (a marker of MM plasma cells) and antibodies to HIF-1α, HIF-2α or CXCL12. In keeping with the findings of others,[15] weak HIF-1α expression was detected in numerous cells throughout the bone marrow (Figures 1A and 1B, panels *c* and *d*). In contrast, HIF-2α expression was restricted to CD68$^+$ macrophages (*data not shown*) and CD138$^+$ MM plasma cells (Figure 1B, panels *e* and *f*). Importantly, HIF-2α and CXCL12 proteins were both expressed in CD138$^+$ MM plasma cells (Figure 1B, panels *a*, *b*, *e* and *f*).

The hypoxic regulation of CXCL12 expression in the multiple myeloma cell line, LP-1

To examine the effect of hypoxia on CXCL12 expression in MM plasma cells, levels of *CXCL12* mRNA were measured in the MM plasma cell line, LP-1, following 6, 24 and 48 h of normoxic or hypoxic culture. Strong up-regulation of *CXCL12* mRNA was observed in response to 24 and 48 h of hypoxia (Figure 2A). This delayed hypoxic induction of CXCL12 expression was observed in three of four MM cell lines tested (U266, JIMI, and LP-1, *Online Supplementary Figure S1*), with one cell line (RPMI-8226) exhibiting no response. Using enzyme-linked immunosorbent assays, levels of CXCL12 protein were measured in LP-1 culture media following 72 h of normoxic or hypoxic culture (Figure 2B), and higher levels of CXCL12 protein were detected in hypoxic culture media (812±15.52 pg/mL) than in normoxic culture media (364±17.52 pg/mL). While hypoxic up-regulation of CXCL12 protein expression was observed in the U266 and JIMI cell lines (*data not shown*), the LP-1 cell line was selected for all the subsequent studies outlined below.

To examine the kinetics of the hypoxic induction of CXCL12 in MM plasma cells, LP-1 *CXCL12* mRNA expression was measured following 2, 4, 6, 8, 12, 24, 36, 48, 60 and 72 h of normoxic or hypoxic culture. A minimum of 24 h of continuous hypoxic exposure was required to up-regulate *CXCL12* mRNA (Figure 2C). The hypoxic induction of other HIF target genes (*GLUT1*, *CXCR4* and *VEGF*) was also examined: unlike CXCL12, their induction occurred in response to 4 - 6 h of hypoxic culture (*Online Supplementary Figure S2*). To examine the kinetics of HIF-1 and HIF-2 induction in LP-1 cells under hypoxic conditions, levels of HIF-1α and HIF-2α protein expression were measured. While HIF-1α expression was rapidly induced in response to 4-6 h of exposure to hypoxia, the induction of HIF-2α expression was delayed, requiring more than 24 h of continuous exposure to hypoxia (Figure 2C).

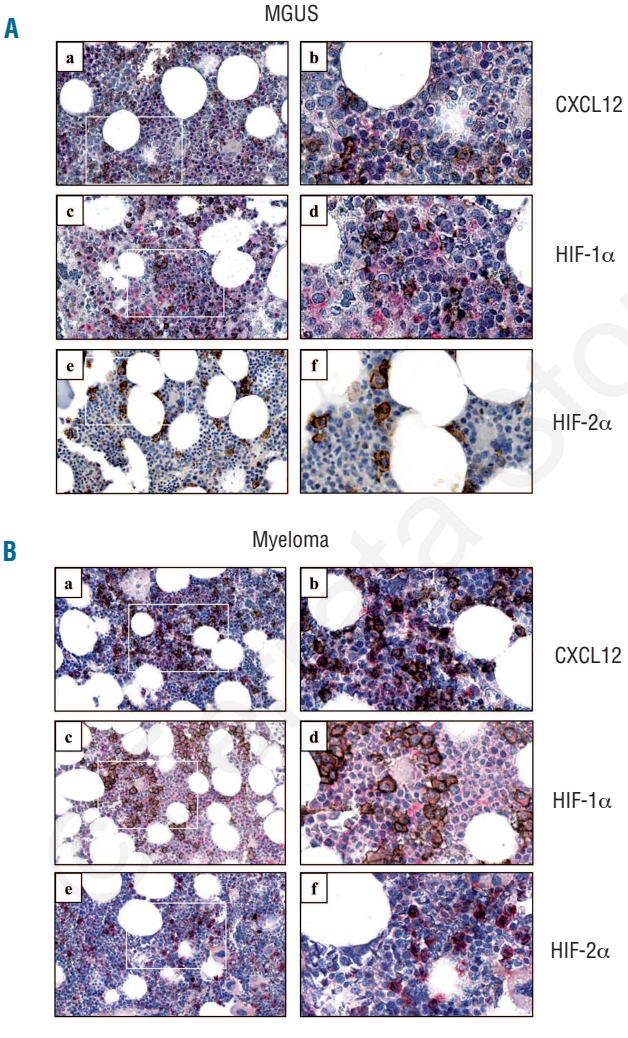

Figure 1. Expression of CXCL12, HIF-1α and HIF-2α in patients' trephine specimens. Bone marrow trephine sections from (A) MGUS and (B) MM patients at diagnosis were co-stained with CXCL12 (a and b, pink), HIF-1α (c and d, pink) or HIF-2α (e and f, pink) and CD138 (all sections, brown). Original magnifications x40 (a, c and e) and x200 (b, d and f); colors corrected after acquisition with Adobe Photoshop.

Over-expression and knockdown of hypoxia-inducible factors 1α and 2α in LP-1 multiple myeloma plasma cells: the effect on CXCL12

To further examine the contributions of HIF-1 and HIF-2 to the induction of MM plasma cell CXCL12 expression, LP-1 cells were engineered to stably over-express HIF-1α (LP-1-HIF-1α) or HIF-2α (LP-1-HIF-2α), and HIF over-expression confirmed by western immunoblotting (Figure 3A). Levels of *CXCL12* mRNA were then measured in LP-1-HIF-1α and LP-1-HIF-2α: it was found that *CXCL12* mRNA expression was higher in both cell lines than in the vector control (Figure 3B). Levels of CXCL12 protein were also measured in culture media collected from these transduced cell lines, and increased levels of CXCL12 protein were detected in LP-1-HIF-1α (685.0±17.05 pg/mL) and LP-1-HIF-2α culture media (805.1±20.3 pg/mL), compared to in the vector control culture media (410±28.8 pg/mL) (Figure 3C).

RNA interference (RNAi) was also used to knockdown HIF-1α or HIF-2α expression in LP-1 cells, and reduced HIF expression was confirmed by western immunoblotting (Figure 4A). Using PCR, levels of *CXCL12* mRNA were measured in these transduced cell lines, and a marked reduction in the hypoxic up-regulation of *CXCL12* was observed in response to the HIF-1α RNAi, compared to the vector and scrambled controls (Figure 4B). Strikingly, CXCL12 expression was strongly down-regulated under both normoxic and hypoxic conditions in response to the HIF-2α RNAi. CXCL12 protein levels were also measured in culture media from each of these cell lines following normoxic or hypoxic culture (Figure 4C). Importantly, the hypoxic induction of CXCL12 protein was markedly reduced in response to both the HIF-1α RNAi (653.7±53 pg/mL) and HIF-2α RNAi (520.7±43 pg/mL), compared to the vector (1079±59 pg/mL) and scrambled (936.6±21 pg/mL) controls. Considering the degree of down-regulation of CXCL12 mRNA in response to HIF-2α knockdown, the lack of a similar response at the protein level suggests that the hypoxic induction of CXCL12 involves translational and/or post-translational regulation in these cells.

Hypoxia-inducible factor-2 binds to the CXCL12 promoter under hypoxic conditions

Previous studies by Ceradini *et al.* showed that the prox-

imal CXCL12 promoter harbors two putative HIF binding sites (HBS1 and HBS2, situated at nucleotides -1,238 and -783 respectively, Figure 5A) and that the hypoxic induction of CXCL12 expression in human umbilical vein endothelial cells is mediated by the binding of HIF-1 to HBS1.[23] While the binding of HIF-1 to the CXCL12 promoter has been demonstrated previously,[23] the role of HIF-2 in the regulation of CXCL12 expression has not been examined. In this study, promoter analyses were performed to examine whether HIF-2 binds to and activates the CXCL12 promoter in MM plasma cells.

To measure changes in CXCL12 promoter activity in response to hypoxia, LP-1 cells were transiently transfected with a luciferase reporter plasmid containing the proximal CXCL12 promoter (pGL3b-CXCL12), and reporter assays performed (Figure 5B). These studies revealed that luciferase activity was increased (2-fold) under hypoxic conditions (96.8±7.2 cps) compared to normoxic conditions (53.4±10.8 cps). To delineate the role of HIF-1 and HIF-2α in the activation of the CXCL12 promoter, the pGL3b-CXCL12 construct was then transfected into the HIF-over-expressing LP-1 cell lines (Figure 5C), and increased luciferase activity was observed in LP-1-HIF-1α cells (122.4±6.3 cps) and LP-1-HIF-2α cells (211.6±36.5 cps), compared to the vector control (63.5 ±5.9 cps).

To examine the binding of HIF-2 to the CXCL12 promoter in LP-1 cells, electromobility shift assays were performed. These studies revealed strong binding of a hypoxia-inducible complex to the CXCL12 promoter (Figure 5D, *lane 2*). The presence of HIF-2 within this complex was confirmed using a HIF-2α antibody (Figure 5D, *lanes 3 and 4*). Chromatin immunoprecipitation assays were then performed to assess the level of HIF-2α binding to the CXCL12 promoter under normoxic and hypoxic conditions. Importantly, a 6-fold increase in the level of HIF-2α binding to the CXCL12 promoter was observed under hypoxic conditions (Figure 5E).

Hypoxia-inducible factor-induced CXCL12 stimulates angiogenesis in vivo

Hypoxia is a major physiological cue for triggering angiogenesis and initiates the transcription of angiogenic genes, such as those coding for VEGF,[37,38] basic fibroblast growth factor (bFGF),[39] and platelet-derived growth factor (PDGF)[40] to activate the "angiogenic switch". To examine the effect of HIF-1α and HIF-2α over-expression on MM-

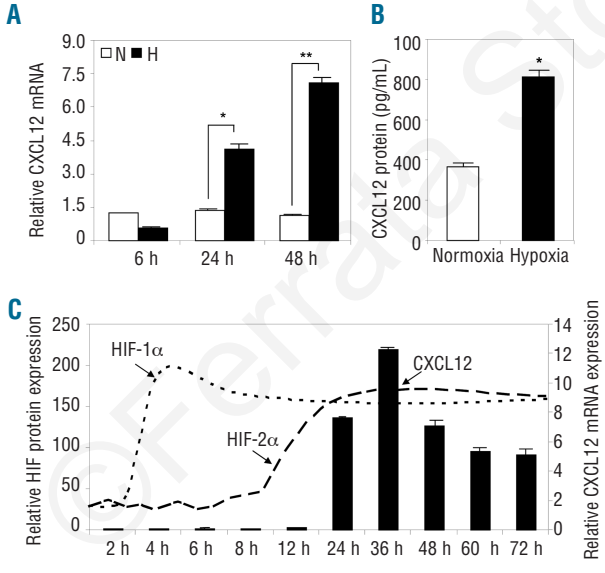

Figure 2. Hypoxic regulation of CXCL12 expression in LP-1 cells. (A) Levels of *CXCL12* mRNA expression were assessed in LP-1 cells following 6, 24 and 48 h of normoxic (white bars) or hypoxic (black bars) culture. Columns, mean (n=3); bars, SEM. **P*<0.05, ***P*<0.001, compared to normoxia. (B) Levels of CXCL12 protein were measured in LP-1 conditioned medium following 72 h of normoxic or hypoxic culture. Columns, mean (n=3); bars, SEM. **P*<0.001, compared to normoxia. (C) The hypoxic induction of *CXCL12* (black bars) mRNA, and HIF-1α (dotted line) and HIF-2α (dashed line) protein expression was examined in LP-1 cells over 72 h. Columns and dashed lines, mean (n=3); bars, SEM.

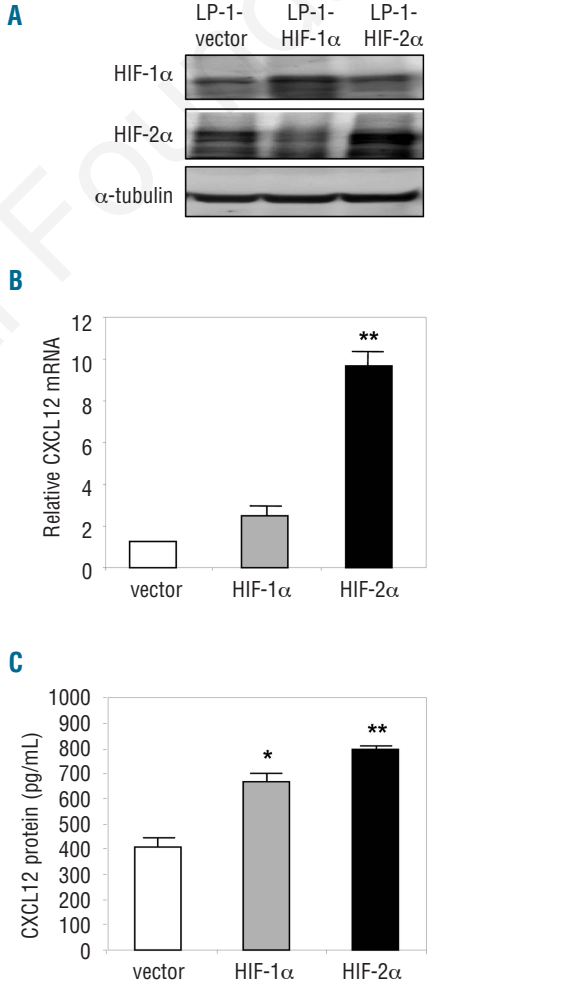

Figure 3. Stable over-expression of HIF-1α and HIF-2α in LP-1 cells. (A) LP-1 cells were engineered to stably over-express HIF-1α or HIF-2α and up-regulation of HIF protein confirmed by western immunoblotting. (B) Levels of *CXCL12* mRNA expression were examined in the HIF over-expressing LP-1 cell lines. Columns, mean (n=3); bars, SEM. ***P*<0.001, compared to vector control. (C) Levels of CXCL12 protein were measured in conditioned media from the HIF over-expressing cell lines. Columns, mean (n=3); bars, SEM. **P*<0.05, ***P*<0.001, compared to vector control.

induced angiogenesis and the contribution of CXCL12 to this process, LP-1-HIF-1α and LP-1-HIF-2α cells were implanted subcutaneously (ventral groin region) into nude mice and half of the mice were administered T140, a potent CXCR4 antagonist[41,42] (Figure 6A). In these studies, LP-1 cells stably over-expressing CXCL12 (LP-1-CXCL12) were used as a positive control. Tumor growth was monitored using bioluminescence imaging and a progressive increase in signal intensity and diameter was observed in all mice not receiving T140 (*Online Supplementary Figure S3A*). While progressive increases in signal intensity and diameter were also observed in mice receiving LP-1-pRUF cells + T140, mice implanted with LP-1-CXCL12, LP-1-HIF-1α and LP-1-HIF-2α and receiving T140 exhibited a marked decrease in signal intensity. The amount of vessel infiltration induced by each cell line after 2 weeks was determined by measuring implant hemoglobin content; vessel infiltration was significantly increased in implants containing LP-1-CXCL12, LP-1-HIF-1α and LP-1-HIF-2α cells, compared to vector control cells (Figure 6B). Furthermore, a significant reduction in vessel infiltration was observed in implants containing LP-1-CXCL12 cells in mice which had been systemically administered T140, compared to the vessel infiltration in mice that had not received T140 (Figure 6B). While decreased implant vascularization was also observed in T140-treated mice harboring LP-1-HIF-1α and LP-1-HIF-2α cell implants compared to that in mice that did not received T140, these differences were not statistically significant. Importantly, the observed differences in *in vivo* growth and angiogenesis were not attributable to differences in the rate of proliferation of the cell lines (*Online Supplementary Figure S3B*).

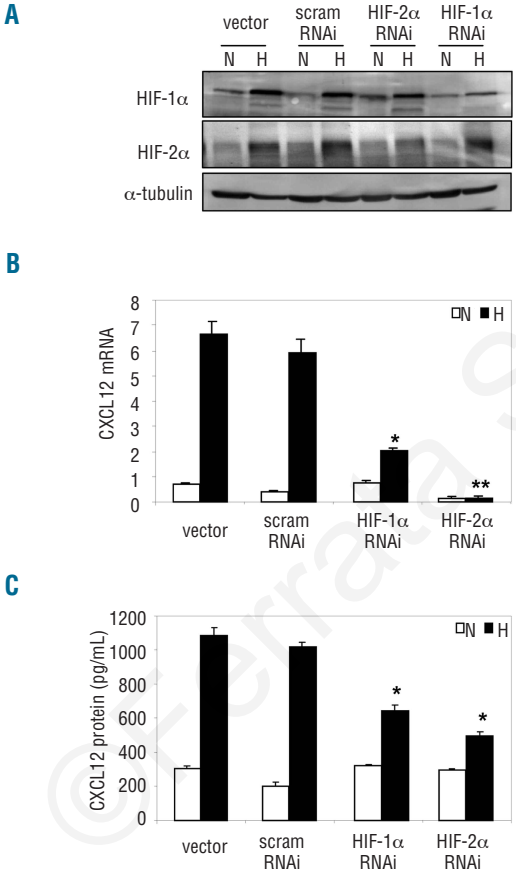

Figure 4. Stable knockdown of HIF-1α and HIF-2α in LP-1 cells. (A) RNA interference was used to knock down endogenous HIF-1α or HIF-2α expression in LP-1 cells, and down-regulation of HIF protein confirmed by western immunoblotting. (B) Levels of *CXCL12* mRNA expression were measured in the HIF knockdowns in response to normoxic (white bars) or hypoxic (black bars) culture. Columns, mean (n=3); bars, SEM. **P*<0.05, ***P*<0.001, compared to vector control. (C) Levels of CXCL12 protein were measured in conditioned media from the HIF knockdowns following 72 h of normoxic (white bars) or hypoxic (black bars) culture. Columns, mean (n=3); bars, SEM. **P*<0.05, compared to vector control.

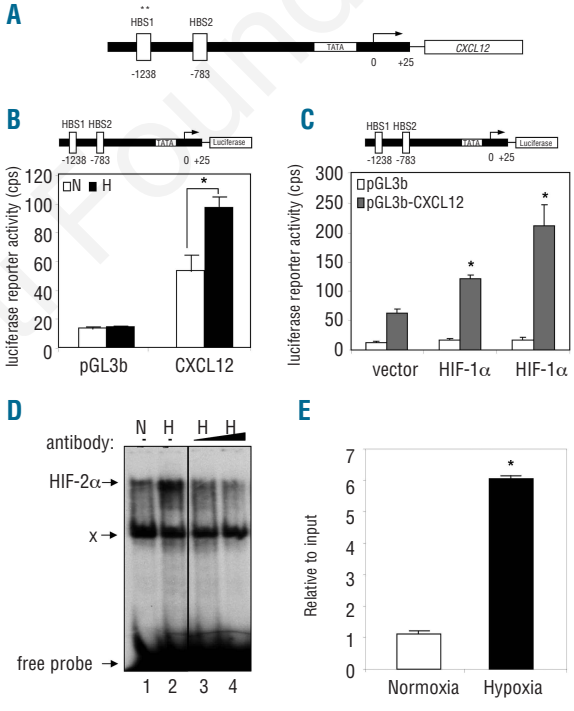

Figure 5. Hypoxia induces HIF-2α binding to the CXCL12 promoter in LP-1 cells. (A) Human CXCL12 locus containing two HIF binding site (HBS) sequences and transcriptional start site. Hypoxic induction of CXCL12 is primarily mediated via HBS1. (B) LP-1 cells were transiently transfected with a luciferase plasmid containing the proximal CXCL12 promoter (top, pGL3b-CXCL12). Twenty-four hours post-transfection, cells were cultured for 48 h under normoxic (white bars) or hypoxic (black bars) conditions, and luciferase assays performed. Columns, mean (n=3); bars, SEM. **P*<0.05, compared to normoxia. (C) The pGL3b-CXCL12 construct (top) was transiently transfected into the HIF-over-expressing LP-1 cell lines and luciferase assays performed. Columns, mean (n=3); bars, SEM. **P*<0.05, compared to vector control. (D) LP-1 cells were cultured under normoxic (N, lane 1) or hypoxic (H, lane 2) conditions for 48 h and DNA binding activity to HBS1 examined by electromobility shift assay. To determine the contribution of HIF-2 to the hypoxia-inducible complex formation, extracts were pre-incubated with HIF-2α antibody (lanes 3 and 4). (E) Chromatin immunoprecipitation was performed on LP-1 cells cultured under normoxic (white bars) or hypoxic (black bars) conditions for 48 h, and the level of HIF-2α binding to HBS1 of the CXCL12 promoter assessed by PCR. Columns, mean; bars, SEM. **P*<0.05, compared to normoxia.

Discussion

Hypoxia is an important selective force in the evolution of tumor cells[13] and aberrant HIF expression is associated with a highly aggressive disease phenotype.[43,44] While hypoxia is known to be involved in the pathogenesis of solid tumors, there is a paucity of published data regarding the role of hypoxia and the HIF transcription factors in the pathogenesis of hematologic malignancies such as MM. In this study, we examined HIF-1α and HIF-2α expression in bone marrow trephine specimens from patients with MGUS or MM. While HIF-1α was found to be widely expressed throughout the bone marrow, HIF-2α expression was restricted to macrophages and CD138$^+$ MM plasma cells. These findings suggest that the induction of aberrant HIF-2α expression is associated with the malignant transformation of MM cells, and are in keeping with previous studies showing that aberrant HIF-2α expression in tumor cells is associated with a poorer prognosis than that conferred by HIF-1α.[45,46]

Of note, our immunohistochemical analyses also revealed strong CXCL12 protein expression in CD138$^+$ MM plasma cells. CXCL12 is involved in several aspects of MM pathogenesis, including transendothelial migration, bone marrow retention of plasma cells, angiogenesis and osteoclastic bone resorption.[6-11] Based on previous studies showing that hypoxia regulates CXCL12 expression in other cell systems,[22-26] we examined the role of hypoxia in inducing aberrant CXCL12 expression in MM plasma cells. Given the limited number of primary MM plasma cells that can be recovered from MM patients and the difficulty associated with their *in vitro* culture, MM plasma cell lines were used in this study. We showed, for the first time, that CXCL12 expression is up-regulated in MM plasma cells in response to prolonged hypoxia and that the HIF-2 transcription factor is a key mediator of this response. These findings contrast with those of previous studies which showed that CXCL12 is rapidly up-regulated in response to brief exposure to hypoxia (3 - 4 h) and that this is mediated by HIF-1.[22-24] Given the distinct induction kinetics of HIF-1 *versus* HIF-2 in response to acute and chronic hypoxia, respectively, these findings likely reflect the varying requirements for CXCL12 in adapting to hypoxia - in MM plasma cells, the induction of CXCL12 expression is required for more "long-term" adaptive responses to hypoxia such as angiogenesis rather than immediate, "acute responses" such as cellular conversion to glycolytic metabolism. These data are in keeping with the notion that progressive tumor cell proliferation creates a sustained hypoxic environment and results in the induction of HIF-2α.

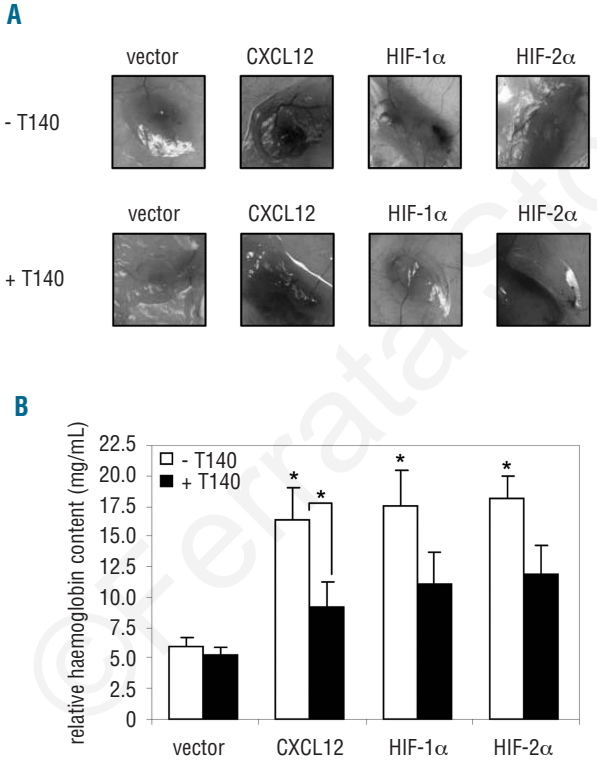

Figure 6. HIF-induced CXCL12 stimulates *in vivo* angiogenesis. (A) CXCL12-, HIF-1α- or HIF-2α-over-expressing LP-1 cells were injected subcutaneously in a Matrigel plug into mice (n=12/group), and half of the mice were administered the CXCR4 antagonist, T140. After 2 weeks, mice were euthanized and macroscopic photographs were taken of the implants. (B) The hemoglobin content of implants from untreated (white bars) and T140-treated (black bars) mice were measured and normalized to cell burden as described in the Design and Methods sections. Columns, mean (n=6/group); bars, SEM. *P<0.05, compared to vector control.

As for all tumor cells, MM plasma cell growth is dependent upon an adequate supply of oxygen and nutrients, and in response to increased metabolic demand, MM plasma cells induce the formation of an additional blood supply. In this study, we examined the contribution of HIF-induced CXCL12 to *in vivo* angiogenesis using an established subcutaneous Matrigel xenograft model of human MM.[47-49] As an extension of our previous studies showing that MM-derived CXCL12 is a potent inducer of angiogenesis *in vitro*,[7] we now show that CXCL12 is a potent pro-angiogenic agent in this *in vivo* model. These findings complement those of previous studies showing that tumor-derived CXCL12 stimulates angiogenesis in other human cancers.[24,50,51] However, in the context of MM, our data directly contradict findings published by Menu *et al.*,[11] who showed that the administration of T140 had no effect on bone marrow microvessel density in myelomatous 5T33MM mice. This may, in part, be explained by the fact that 5T33MM cells do not express CXCL12 and as such, the ability of 5T33MM-derived CXCL12 to stimulate angiogenesis was not under direct investigation.

The implantation of HIF over-expressing MM cells in this *in vivo* model stimulated a marked increase in MM-induced angiogenesis, and using the CXCR4 inhibitor, T140, we showed that CXCL12 is an important contributor to HIF-induced angiogenesis. Given that hypoxia and HIF are known to up-regulate the expression of other angiogenic growth factors such as VEGF,[37,38] bFGF,[39] and PDGF,[40] the fact that T140 was unable to reduce HIF-induced vascularization significantly most likely reflects the important contribution of other angiogenic factors to this response.

The molecular mechanisms underlying angiogenesis in MM are complex and involve angiogenic factors secreted by both MM plasma cells themselves and microenvironmental cells such as stromal cells, osteoclasts and endothelial cells.[52,53] The subcutaneous Matrigel xenograft model is a surrogate *in vivo* assay which is used to examine the pro-

vascular properties of MM plasma cells, independently of bone marrow microenvironmental influences.[47-49,54] As a result, further studies are required to examine the role of hypoxia and the HIF transcription factors on angiogenesis in the bone marrow setting and to examine the contribution of microenvironmental factors to angiogenesis in MM.

With respect to the wider implications of hypoxia on MM biology, previous studies by Asosingh *et al*. showed that MM plasma cell survival and expansion are supported by the hypoxic bone marrow environment.[16] Using transduced HIF over-expressing MM cell lines, we now show that aberrant HIF expression stimulates a marked increase in MM-induced angiogenesis. Collectively, these findings suggest that hypoxia may play an important role in the transition from indolent MM to active, aggressive MM. This notion is supported by the immunohistochemical staining presented in this study showing that HIF-2α is aberrantly expressed by MM plasma cells in patients' bone marrow biopsies. Further studies are required to examine the effect of hypoxia on the expression of other cytokines and growth factors known to be important in the pathogenesis of MM.

In summary, we have shown that hypoxic regulation of aberrant CXCL12 expression in MM plasma cells is mediated by the HIF-2 transcription factor. To the best of our knowledge, this is the first study to delineate the role of HIF-1 and HIF-2 in MM, and highlights the importance of assessing whether current and future anti-MM strategies modulate HIF-2 expression and/or activity. These findings also add to our understanding of the CXCL12/CXCR4 axis in MM and will, it is to be hoped, aid the development of future therapeutic strategies directed at blocking the actions of CXCL12 and/or hypoxia in MM.

Authorship and Disclosures

SKM, PD, SAW and ACWZ contributed to the conception and design of the study, data analysis and interpretation, and manuscript writing; LBT contributed to the final approval of the manuscript; DJP, NF and ALH provided study material; SG contributed to the conception and design of the study, data analysis and interpretation, and final approval of manuscript. All authors were involved in the discussion and interpretation of data and all approved the final version.

The authors reported no potential conflicts of interest.

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