Molecular and Cellular Mechanisms of Increased Angiogenesis in Multiple Myeloma: A Role for CXCL12

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Chapter 4:

THE ROLE OF HYPOXIA ON CXCL12 AND CXCR4 EXPRESSION: *IN VITRO* STUDIES

4.1 Introduction

The term hypoxia defines a state in which cells or tissues are deprived of sufficient oxygen to maintain homeostatic ATP production [270]. Due to the fundamental importance of oxygen for cellular metabolism, energy production and survival, cells have evolved intricate response mechanisms to detect even the slightest decrease in local oxygen tension and counteract it through the activation of cell-specific transcription programs involving HIF-1 [278], HIF-2 [279], ETS-1 [280], CREB [281], AP-1 [282-284], and NFκB [285]. This ability to sense and respond to changes in oxygen availability represents a fundamental property of all metazoan cells [269,437]. The activation of transcription programs under hypoxic conditions co-ordinates the expression of hundreds of gene products which mount a variety of adaptive cellular responses including erythropoiesis, angiogenesis, vascular remodelling, increased iron transport, cell proliferation and a switch to glycolytic metabolism [272-275].

The HIF-1 and HIF-2 transcription factors are the principal mediators of cellular adaptation to hypoxia [275,438]. Both HIF-1 and HIF-2 are heterodimeric complexes composed of an oxygen-sensitive, inducible α-subunit and a constitutively-expressed β-subunit [279,286-288]. As a consequence of the high sequence homology in the ODD domain of HIF-1α and HIF-2α, the same proteasomal degradative mechanisms control protein levels of both factors [279,439]. It is well established that protein expression of the HIF α-subunits is tightly regulated under normal oxygen tensions and both are rapidly degraded, with a half-life of less than 5 minutes [278,286,329,440]. HIF-1 and HIF-2 also share similar mechanisms of activation and bind to the same DNA consensus sequence [293,299]. However, despite their similarity, they exhibit discrete expression patterns and have overlapping but distinct target gene specificities which vary greatly between cell types [441].

Hypoxia is associated with a number of both short- and long-term pathologies, including inflammation and cardiovascular disease [271]. Hypoxia also has particular relevance in many aspects of cancer biology. In solid tumours, the cellular expansion of malignant cells progressively distances the innermost cells from the host vasculature, thus depriving them of oxygen and nutrients and creating a hypoxic core. Consequently, while solid tumours contain a heterogeneous range of oxygen pressures, ranging from approximately 5% O_2 in well-vascularised areas to complete anoxia (no oxygen) in necrotic regions, the average oxygen pressure generally lies in the hypoxic range at approximately 1% O_2 [270,442,443]. In cancer, both hypoxia and genetic alterations affecting hypoxic signalling cascades lead to

constitutive HIF expression in tumour cells. These circumstances apply strong selective pressures to tumour cell subsets that are able to adapt quickly to reduced oxygen availability, thus selecting for a highly aggressive tumour phenotype. Given the key role of hypoxia in disease pathogenesis, it is not surprising that HIF-1 and HIF-2 have been implicated in numerous aspects of tumour progression and are over-expressed in a number of human tumours including cancers of the brain, colon, lung, breast, prostate, kidney, pancreas, cervix, bladder, and ovary [295,349-353].

The role of hypoxia and the HIF transcription factors in the pathogenesis of haematological malignancies is less defined, but in order to support normal BM haematopoiesis, the BM microenvironment is physiologically hypoxic compared to most other tissues and organs [366,367]. Several recent immunohistochemical studies have demonstrated that HIF-1 and HIF-2 are over-expressed in haematological cancers such as diffuse large B-cell lymphoma [444,445], CLL [446] and ALL [447]. In the context of MM, there remains a paucity of published data relating to the expression of these molecules in samples from patients. However, preliminary findings from our laboratory suggest that MM PCs express strong levels of both HIF-1 and HIF-2 protein. While HIF-1 staining was specific to MM PCs, HIF-2 was also expressed by other cells, particularly tumour-associated macrophages, within the BM (Dr. Hampton-Smith, unpublished observations).

As discussed previously (see Section 1.2.9), CXCL12 is an important chemokine involved in several key aspects of MM pathogenesis, including transendothelial migration [164,264,265], MM PC migration and retention within the BM [168,266], osteoclastogenesis and osteolytic bone disease [9] and, as demonstrated in the previous chapter, angiogenesis. Studies from our laboratory have shown that MM PCs aberrantly express CXCL12 at high levels [9]. The following chapter describes studies designed to identify the mechanism by which MM PCs aberrantly express CXCL12, the reason for which remains unknown.

In 2002, Hitchon *et al* demonstrated that CXCL12 expression is up-regulated by hypoxia in cultured human synovial fibroblasts from patients with rheumatoid arthritis and osteoarthritis [387]. This provided the first evidence that hypoxia is a regulatory stimulus for CXCL12 expression. However, while the authors noted the existence of a candidate HIF-1 consensus binding motif within the CXCL12 promoter, they did not confirm whether HIF-1 was indeed the mediator of this response. Direct evidence for the

mechanism by which hypoxia regulates CXCL12 expression was subsequently provided by Ceradini *et al* [15]. In a murine model of ischemia, they observed that elevated levels of CXCL12 expression were directly proportional to reduced local oxygen levels. Using RNAi technology and chromatin immunoprecipitation techniques on cultured HUVECs, they conclusively showed that HIF-1 mediates the up-regulation of CXCL12 expression under hypoxic conditions via binding to the HRE region of the CXCL12 promoter. Subsequent studies have demonstrated that hypoxia up-regulates CXCL12 expression in a variety of other cell types, including primary ovarian tumour cells [247], renal cell carcinomas [389], melanoma cell lines [390], retinal glial cells [388], and glioma cell lines [391]. However, one group has also reported that CXCL12 expression is strongly down-regulated by hypoxia in the MCF-7 breast cancer cell line, which suggests that hypoxia-induced changes in CXCL12 expression are cell-specific [392].

Interestingly, the expression of the CXCR4 receptor is also up-regulated by hypoxia. First reported in 2003, Staller *et al* [393] showed that CXCR4 expression is negatively regulated by VHL through its degradation of HIF. They associated the mutation of VHL in clear cell renal carcinoma with strong CXCR4 expression and poor survival, thus implicating the hypoxic up-regulation of CXCR4 with tumorigenesis. In the same year, Schioppa *et al* [394] showed that hypoxia induces a HIF-1-mediated up-regulation of CXCR4 in monocytes, monocyte-derived macrophages, tumour-associated macrophages, ECs, and cancer cells. Subsequent studies have demonstrated hypoxic up-regulation of CXCR4 in HUVECs [395], pancreatic tumour cell lines [229], clear cell renal carcinoma [389], haemangioblastoma [389], renal cell carcinoma [396], glioblastoma [397,398], lymphatic ECs [399], normal and malignant B-cells [400], microglia [401], mesenchymal stem cells [402] and non-small cell lung cancer cells [232,403].

To date, the effect of hypoxia on CXCL12 and CXCR4 expression in MM PCs has not been investigated. This chapter outlines studies that were performed to investigate the hypothesis that the high CXCL12 expression by MM PCs is due to an adaptive cellular response to hypoxic conditions within hypoxic BM niches. Using MM cell lines, the hypoxic regulation of CXCL12 and CXCR4 in MM PCs was examined. Moreover, the effect of either over-expressing or knocking down HIF-1α and HIF-2α expression was examined using retroviral transduction and lentiviral RNAi technologies, respectively.

4.2 Results

In all of the *in vitro* studies presented in this chapter, the term "normoxia" refers to atmospheric oxygen concentrations, which equates to approximately 21% O_2, and "hypoxia" refers to an extremely O_2-deprived environment containing less than 1% O_2. Considering the end capillary O_2 pressure in many normal tissues is estimated to be in the 5-6% range [270], 21% O_2 could be considered a grossly unphysiological "normoxic" oxygen concentration. However, previous *in vitro* studies have reported that the overall phenotypic difference between cells cultured at 21% versus 5% O_2 is small and as such, 21% O_2 is suitable for normoxic studies [328].

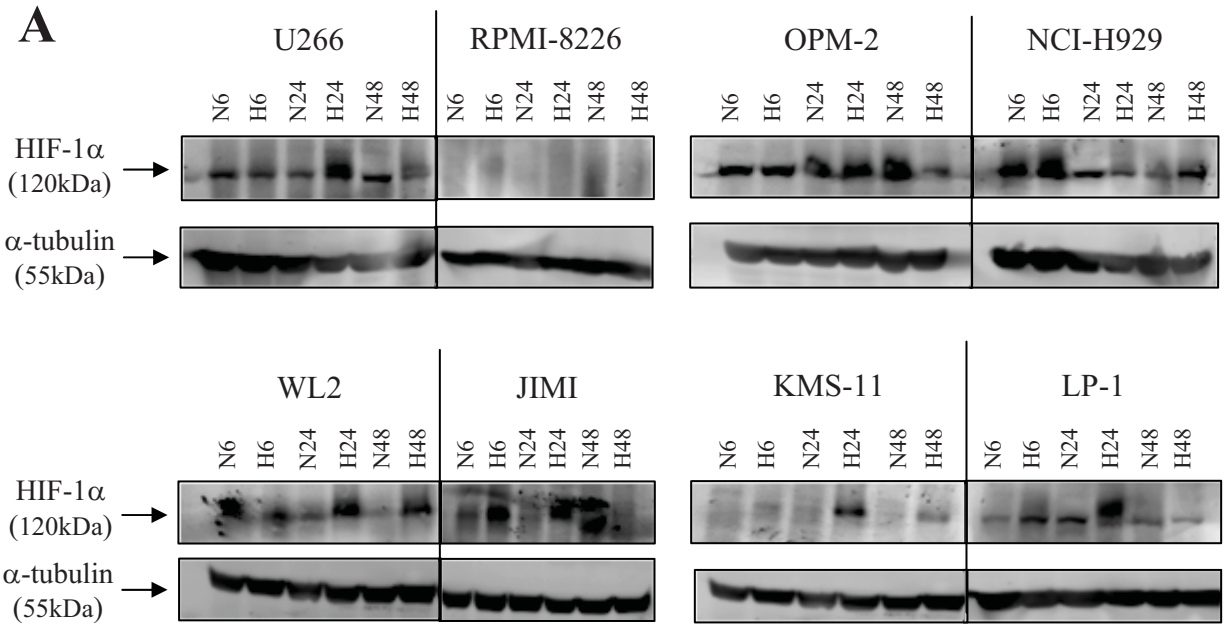
4.2.1 Profiling of MM PC lines for hypoxic regulation of HIF-1α and HIF-2α and their target genes.

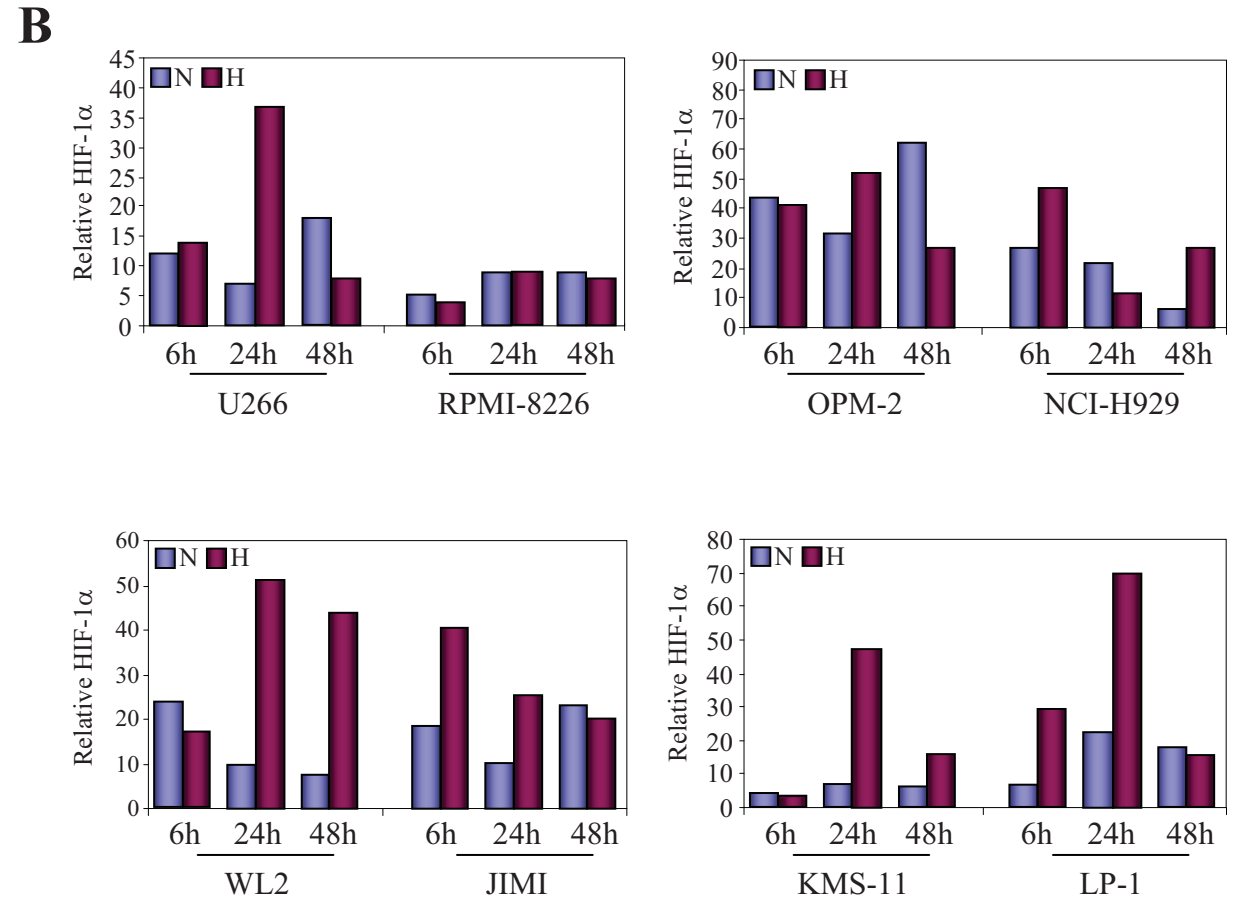
Due to the limited number of primary MM PCs which can be recovered from MM patients and the difficulty associated with their *in vitro* culture, MM PC lines were used to investigate the hypothesis that hypoxia regulates the expression of CXCL12 and/or its receptor, CXCR4, in MM PCs. To select a suitable cell line, the effect of hypoxic culture conditions on the expression of HIF-1α and HIF-2α was examined in eight human MM PC lines: U266, RPMI-8226, OPM-2, NCI-H929, WL2, JIMI, KMS-11 and LP-1. In these studies, cells were cultured under normoxic or hypoxic culture conditions for 48 hours, and whole cell lysates were prepared at 6, 24 and 48 hour time points. The extracts were then examined for HIF-1α and HIF-2α protein expression using Western immunoblotting and anti-HIF-1α (Figure 4.1) or anti-HIF-2α (Figure 4.2) antibodies.

4.2.1.1 The hypoxic regulation of HIF-1α protein expression in MM cell lines.

HIF-1α Western immunoblots were performed using lysates collected from the eight human MM PC lines cultured under either normoxic or hypoxic culture conditions for 6, 24 and 48 hours (Figure 4.1A). Levels of HIF-1α protein expression in lysates prepared from RPMI-8226, WL2, JIMI, KMS-11 and LP-1 cell lines cultured under normoxic oxygen conditions exhibited minimal HIF-1α expression at all three time points examined. In contrast, the U266, OPM-2 and NCI-H929 cell lines exhibited relatively strong levels of endogenous HIF-1α protein under normoxic oxygen conditions, suggesting that HIF-1α may be constitutively expressed in these cell lines. As indicated by the intensity of the

Figure 4.1. Screening of MM Cell Lines for HIF-1α Protein Induction by Hypoxia. (**A**) Eight human MM cell lines (U266, RPMI-8226, OPM-2, NCI-H929, WL2, JIMI, KMS-11 and LP-1) were cultured under normoxic (N) or hypoxic (H) culture conditions for 48 hours and whole cell lysates were prepared at 6, 24 and 48 hour time points. Lysates were resolved on 10% SDS-PAGE gels under reducing conditions and levels of HIF-1α protein expression measured using Western immunoblotting. An RCDC protein assay was performed to ensure that an equal amount of protein was loaded in each lane. Representative immunoblots show the levels of HIF-1α protein expression (≈120kDa doublet) in each cell line under normoxic and hypoxic culture conditions at the three time points examined. Membranes were also probed with anti-α-tubulin (≈55kDa band) to confirm equal protein loading. (**B**) ImageQuant image analysis software was used to quantitate the intensity of the HIF-1α bands relative to the α-tubulin loading control bands. Graphical representation of these data highlights the differences in HIF-1α protein expression under normoxic ( ) and hypoxic ( ) conditions in each cell line at the three time points examined. Results are representative of three individual experiments.


A
U266
RPMI-8226
OPM-2
NCI-H929
N6 H6 N24 H24 N48 H48
HIF-1α (120kDa)
α-tubulin (55kDa)
WL2
JIMI
KMS-11
LP-1


B
Relative HIF-1α
N H
6h 24h 48h
U266
RPMI-8226
OPM-2
NCI-H929
WL2
JIMI
KMS-11
LP-1

HIF-1α protein band in the normoxic lysates, the basal level of expression of HIF-1α protein under normoxic culture conditions varied significantly between the cell lines tested.

Using image analysis software, the intensity of the HIF-1α protein bands were measured, normalised to the α-tubulin loading control and plotted in Figure 4.1B. In the U266 and OPM-2 cell lines, HIF-1α expression was elevated in hypoxic lysates from the 24 hour time point but not at 6 and 48 hours. These were two of the lines which displayed higher endogenous levels of HIF-1α under normoxic conditions. Surprisingly, HIF-1α protein was not detected in any of the lysates prepared from RPMI-8226 cells. In NCI-H929 cells, HIF-1α was up-regulated by hypoxia at the 6 and 48 hour time points, but not at 24 hours, whereas in WL2 cells, HIF-1α was strongly up-regulated by hypoxia at all time points examined. In both the JIMI and LP-1 cell lines, elevated HIF-1α levels were detected in hypoxic lysates at the 6 and 24 hour time points, but not at 48 hours, and HIF-1α expression was up-regulated by hypoxia in KMS-11 cells at the 24 and 48 hour time points, but not at 6 hours.

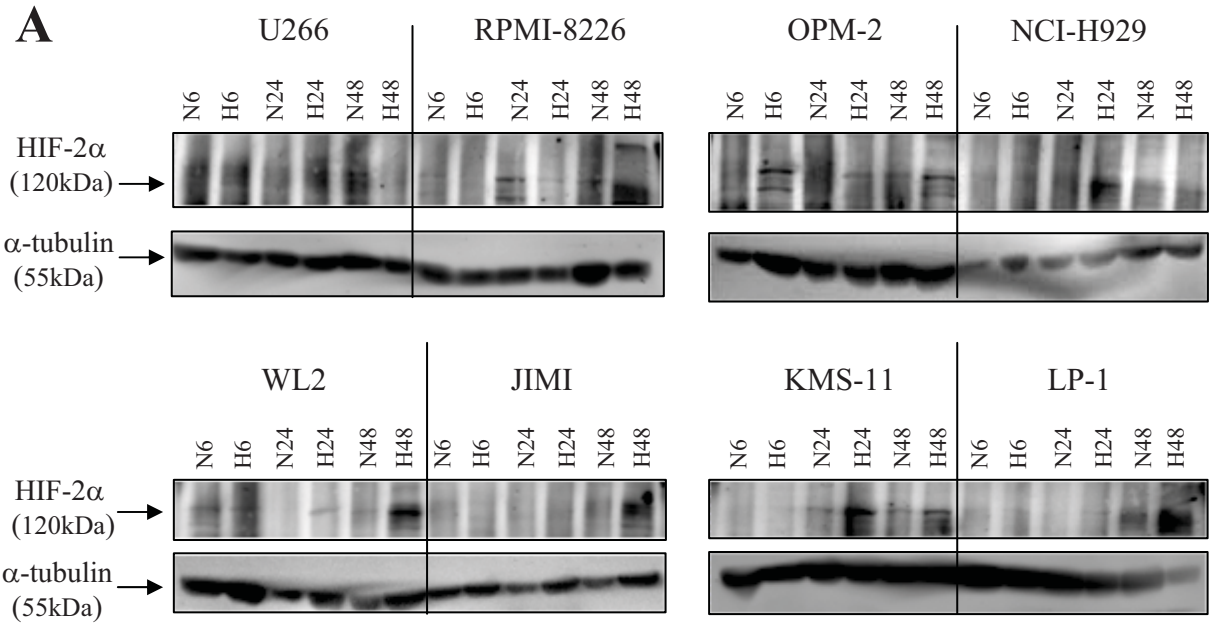
4.2.1.2 The hypoxic regulation of HIF-2α protein expression in MM cell lines.

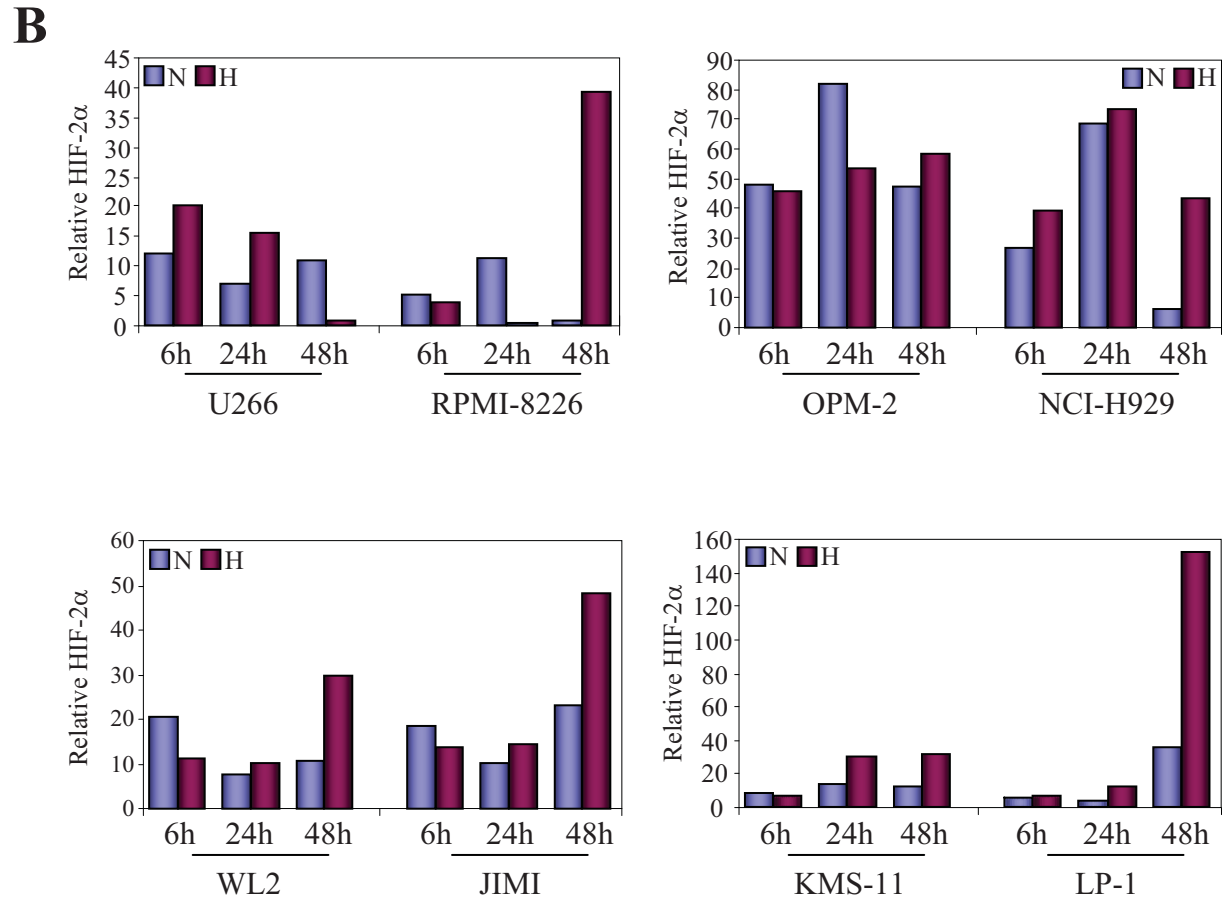
In parallel studies, HIF-2α Western immunoblots were performed using lysates collected from the eight human MM PC lines cultured under either normoxic or hypoxic culture conditions for 6, 24 and 48 hours (Figure 4.2A). Using image analysis software, the intensity of the HIF-2α bands were measured, normalised to the α-tubulin loading control and the data plotted in Figure 4.2B. In the RPMI-8226, NCI-H929, WL2 and JIMI cell lines, HIF-2α protein expression was strongly up-regulated by hypoxia at the 48 hour time point alone. In contrast, in the U266 cell line, HIF-2α was up-regulated by hypoxia at the 6 and 24 hour time points but not at 48 hours, and in OPM-2 cells, a hypoxic regulation of HIF-2α was not detected at any of the time points examined. Lysates from the KMS-11 and LP-1 cell lines exhibited strong hypoxic induction of HIF-2α at the 24 and 48 hour time points.

4.2.1.3 The downstream regulation of the HIF target genes: GLUT-1, CXCR4 and CXCL12.

Having demonstrated that HIF-1α and HIF-2α protein expression is differentially up-regulated by hypoxia in the eight MM PC lines, the modulation of downstream HIF target

Figure 4.2. Screening of MM Cell Lines for HIF-2α Protein Induction by Hypoxia. (**A**) Eight human MM cell lines (U266, RPMI-8226, OPM-2, NCI-H929, WL2, JIMI, KMS-11 and LP-1) were cultured under normoxic (N) or hypoxic (H) culture conditions for 48 hours and whole cell lysates were prepared at 6, 24 and 48 hour time points. Lysates were resolved on 8% SDS-PAGE gels under reducing conditions and levels of HIF-2α protein expression measured using Western immunoblotting. An RCDC protein assay was performed to ensure that an equal amount of protein was loaded in each lane. Representative immunoblots show the levels of HIF-2α protein expression (≈120kDa doublet) in each cell line under normoxic and hypoxic culture conditions at the three time points examined. Membranes were also probed with anti-α-tubulin (≈55kDa band) to confirm equal protein loading. (**B**) ImageQuant image analysis software was used to quantitate the intensity of the HIF-2α bands relative to the α-tubulin loading control bands. Graphical representation of these data highlights the differences in HIF-2α protein expression under normoxic ( ) and hypoxic ( ) conditions in each cell line at the three time points examined. Results are representative of three individual experiments.


A
U266
RPMI-8226
OPM-2
NCI-H929
N6 H6 N24 H24 N48 H48
HIF-2α (120kDa)
α-tubulin (55kDa)
WL2
JIMI
KMS-11
LP-1


B
Relative HIF-2α
N H
6h 24h 48h
U266
RPMI-8226
OPM-2
NCI-H929
WL2
JIMI
KMS-11
LP-1

genes was examined. Each of the eight human MM cell lines were cultured under either normoxic or hypoxic culture conditions over a period of 48 hours and total RNA was harvested at 6, 24 and 48 hour time points. Using real-time PCR, changes in mRNA expression of the glucose transporter gene, *GLUT-1* [448,449] in response to hypoxia was examined, and data normalised to the expression of the standard housekeeping gene, *β2-microglobulin* (Figure 4.3). With the exception of the JIMI and KMS-11 cell lines, *GLUT-1* expression was significantly up-regulated under hypoxic culture conditions at all time points examined (*$p<0.05$ and **$p<0.005$, one-way ANOVA). In the JIMI cell line, *GLUT-1* expression was significantly up-regulated by hypoxia at the 6 and 24 hour time points ($p<0.005$, one-way ANOVA), but not at 48 hours, and in the KMS-11 cell line, a significant up-regulation of *GLUT-1* expression was only observed at 24 and 48 hours ($p<0.05$, one-way ANOVA).

Previous studies have shown that CXCL12 [15,247,387-391] and CXCR4 [229,232,389,393-403] are both up-regulated by hypoxia in several cell types. To examine whether hypoxia regulates CXCR4 and CXCL12 expression in the eight MM PCs lines, real-time PCR was used to measure changes in mRNA expression of these genes under normoxic and hypoxic culture conditions. In contrast to the consistent hypoxic up-regulation of *GLUT-1* mRNA expression observed in the previous experiment, changes in *CXCR4* mRNA expression were much more varied between the MM PC lines (Figure 4.4). In the U266, JIMI and LP-1 cell lines, *CXCR4* expression was significantly up-regulated under hypoxic culture conditions at all three time points examined (*$p<0.05$ and **$p<0.005$, one-way ANOVA). *CXCR4* expression was also induced by hypoxia at all three time points in the NCI-H929 cell line, however this was only significant at the 24 and 48 hour time points (*$p<0.05$ and **$p<0.005$, one-way ANOVA). Similarly, *CXCR4* expression was modestly up-regulated by hypoxia at each of the three time points examined in the OPM-2 and WL2 cell lines. In contrast, *CXCR4* was unaffected by hypoxia at 6 hours and was down-regulated at 24 and 48 hours in RPMI-8226 cells.

The hypoxic modulation of *CXCL12* expression was also highly variable between the eight MM cell lines (Figure 4.5). In the U266 and LP-1 cell lines, *CXCL12* was strongly induced under hypoxic conditions at 24 hours and 48 hours, but was unaffected at the 6 hour time point (*$p<0.05$ and **$p<0.005$, one-way ANOVA). In RPMI-8226 and OPM-2 cells, no significant change in *CXCL12* expression under hypoxic conditions was observed at any of

Figure 4.3. Changes in *GLUT-1* mRNA Expression in Response to Hypoxia in MM Cell Lines. Each of the eight human MM cell lines (**A**) U266, (**B**) RPMI-8226, (**C**) OPM-2, (**D**) NCI-H929, (**E**) WL2, (**F**) JIMI, (**G**) KMS-11 and (**H**) LP-1 were cultured under normoxic ( ) or hypoxic ( ) culture conditions for 48 hours. Total RNA was harvested at 6, 24 and 48 hour time points and the level of *GLUT-1* mRNA expression in these normoxic and hypoxic samples examined using real-time PCR. Data were normalised to the standard housekeeping gene, *β2-microglobulin*. Graphical representation of these data demonstrates relative levels of *GLUT-1* mRNA expression under normoxic and hypoxic conditions in each cell line at each of the time points examined. Data are expressed as mean ± standard deviation from replicate samples and are representative of three individual experiments.

*$p<0.05$ and **$p<0.005$, one-way ANOVA.

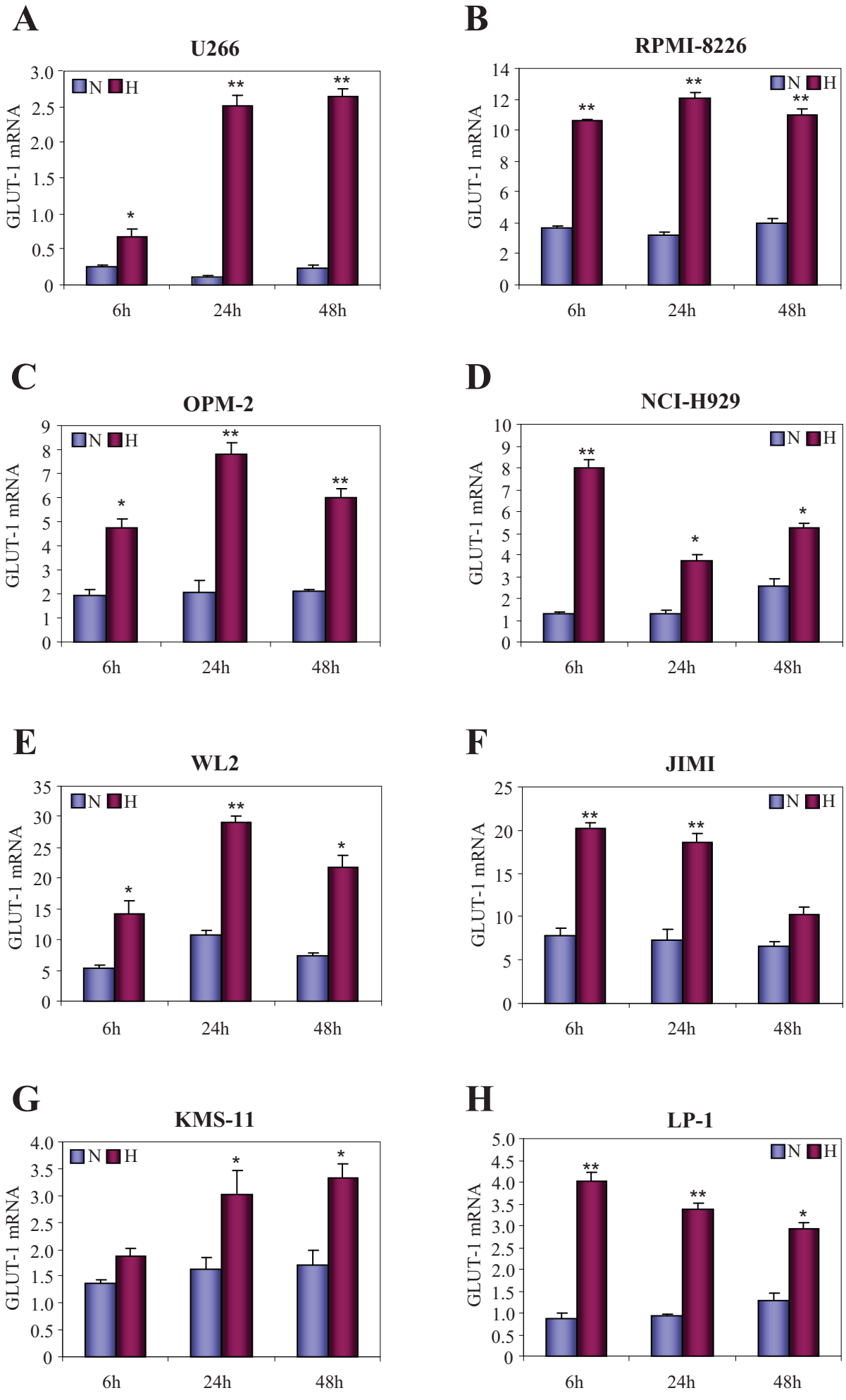

A
U266
B
RPMI-8226
C
OPM-2
D
NCI-H929
E
WL2
F
JIMI
G
KMS-11
H
LP-1
GLUT-1 mRNA
N
H
6h
24h
48h

Figure 4.4. Changes in *CXCR4* mRNA Expression in Response to Hypoxia in MM Cell Lines. Each of the eight human MM cell lines (**A**) U266, (**B**) RPMI-8226, (**C**) OPM-2, (**D**) NCI-H929, (**E**) WL2, (**F**) JIMI, (**G**) KMS-11 and (**H**) LP-1 were cultured under normoxic ( ) or hypoxic ( ) culture conditions for 48 hours. Total RNA was harvested at 6, 24 and 48 hour time points and the level of *CXCR4* mRNA expression in these normoxic and hypoxic samples examined using real-time PCR. Data were normalised to the standard housekeeping gene, *β2-microglobulin*. Graphical representation of these data demonstrates relative levels of *CXCR4* mRNA expression under normoxic and hypoxic conditions in each cell line at each of the time points examined. Data are expressed as mean ± standard deviation from replicate samples and are representative of three individual experiments.

*$p<0.05$ and **$p<0.005$, one-way ANOVA.

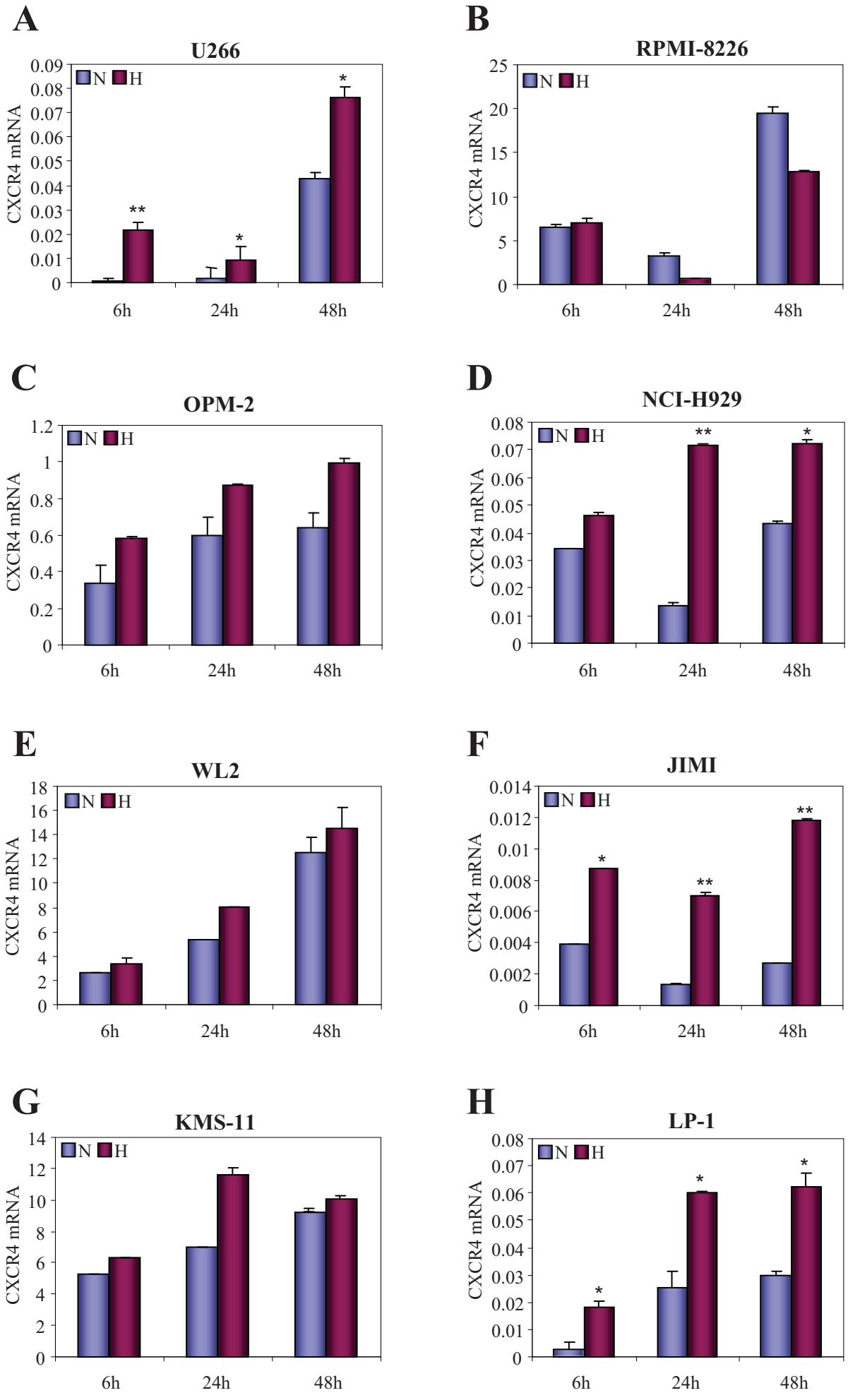
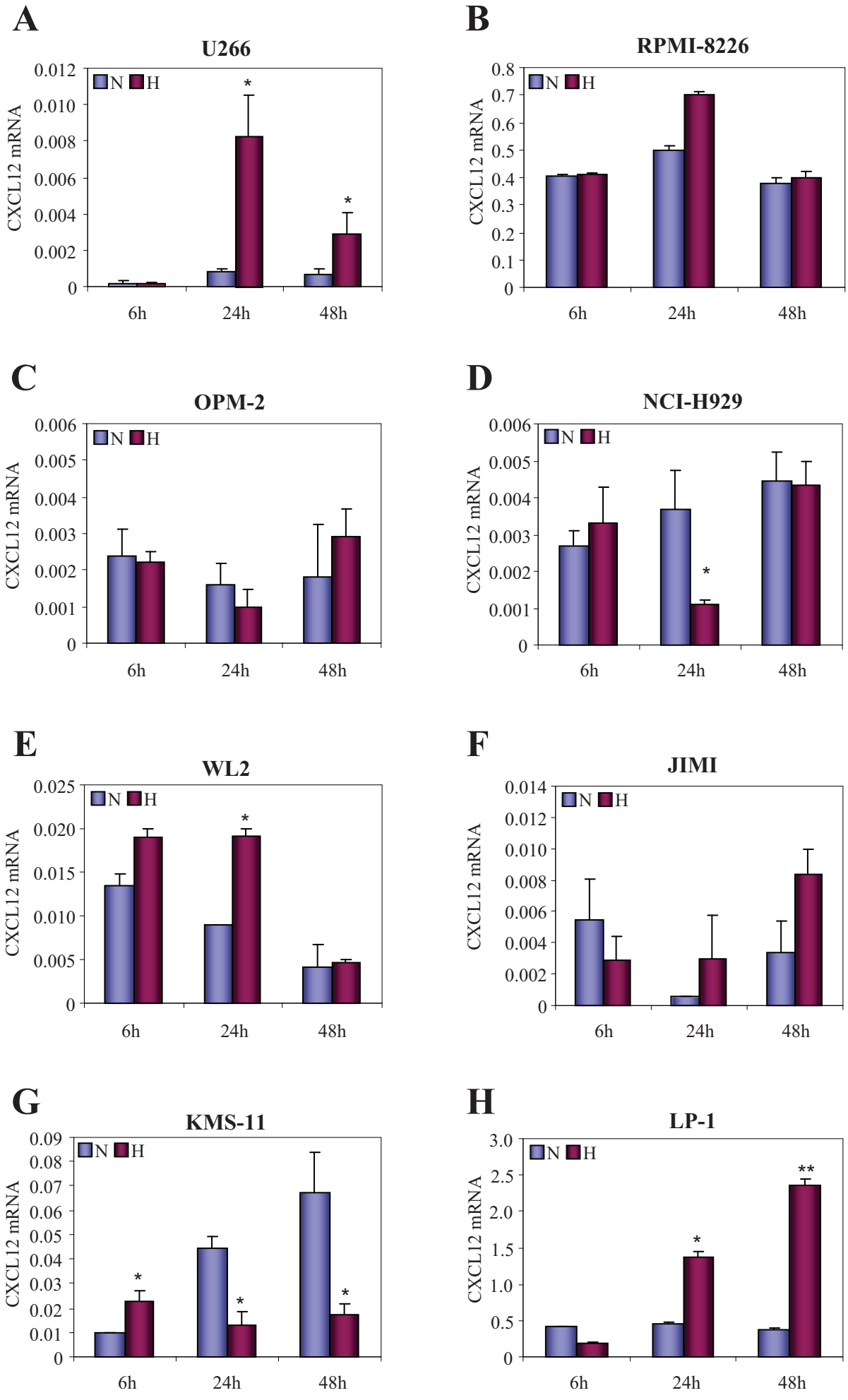

A
U266
CXCR4 mRNA
N H
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
**
*
*
6h
24h
48h
B
RPMI-8226
CXCR4 mRNA
N H
25
20
15
10
5
0
6h
24h
48h
C
OPM-2
CXCR4 mRNA
N H
1.2
1
0.8
0.6
0.4
0.2
0
6h
24h
48h
D
NCI-H929
CXCR4 mRNA
N H
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
**
*
6h
24h
48h
E
WL2
CXCR4 mRNA
N H
18
16
14
12
10
8
6
4
2
0
6h
24h
48h
F
JIMI
CXCR4 mRNA
N H
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
*
**
**
6h
24h
48h
G
KMS-11
CXCR4 mRNA
N H
14
12
10
8
6
4
2
0
6h
24h
48h
H
LP-1
CXCR4 mRNA
N H
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
*
*
*
6h
24h
48h

Figure 4.5. Changes in *CXCL12* mRNA Expression in Response to Hypoxia in MM Cell Lines. Each of the eight human MM cell lines (**A**) U266, (**B**) RPMI-8226, (**C**) OPM-2, (**D**) NCI-H929, (**E**) WL2, (**F**) JIMI, (**G**) KMS-11 and (**H**) LP-1 were cultured under normoxic ( ) or hypoxic ( ) culture conditions for 48 hours. Total RNA was harvested at 6, 24 and 48 hour time points and the level of *CXCL12* mRNA expression in these normoxic and hypoxic samples examined using real-time PCR. Data were normalised to the standard housekeeping gene, *β2-microglobulin*. Graphical representation of these data demonstrates relative levels of *CXCL12* mRNA expression under normoxic and hypoxic conditions in each cell line at each of the time points examined. Data are expressed as mean ± standard deviation from replicate samples and are representative of three individual experiments.

*$p<0.05$, one-way ANOVA.


A
U266
CXCL12 mRNA
N H
0.012
0.010
0.008
0.006
0.004
0.002
0
*
*
6h
24h
48h
B
RPMI-8226
CXCL12 mRNA
N H
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
6h
24h
48h
C
OPM-2
CXCL12 mRNA
N H
0.006
0.005
0.004
0.003
0.002
0.001
0
6h
24h
48h
D
NCI-H929
CXCL12 mRNA
N H
0.006
0.005
0.004
0.003
0.002
0.001
0
*
6h
24h
48h
E
WL2
CXCL12 mRNA
N H
0.025
0.020
0.015
0.010
0.005
0
*
6h
24h
48h
F
JIMI
CXCL12 mRNA
N H
0.014
0.012
0.010
0.008
0.006
0.004
0.002
0
6h
24h
48h
G
KMS-11
CXCL12 mRNA
N H
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
*
*
*
6h
24h
48h
H
LP-1
CXCL12 mRNA
N H
3.0
2.5
2.0
1.5
1.0
0.5
0
*
**
6h
24h
48h

the time points examined. Whilst unaffected by hypoxia at the 6 and 48 hour time points, *CXCL12* expression was significantly down-regulated at the 24 hour time point in NCI-H929 cells ($p<0.05$, one-way ANOVA). In the WL2 cell line, *CXCL12* expression was modestly up-regulated at 6 hours and significantly up-regulated at 24 hours ($p<0.05$, one-way ANOVA), but was unaffected by hypoxia at the 48 hour time point. *CXCL12* expression was down-regulated by hypoxia at 6 hours in the JIMI cell line, but up-regulated at the 24 and 48 hour time points, however these trends were not statistically significant. And finally, *CXCL12* expression was significantly up-regulated by hypoxia at 6 hours in the KMS-11 cell line, but strongly down-regulated at both 24 and 48 hours ($p<0.05$, one-way ANOVA).

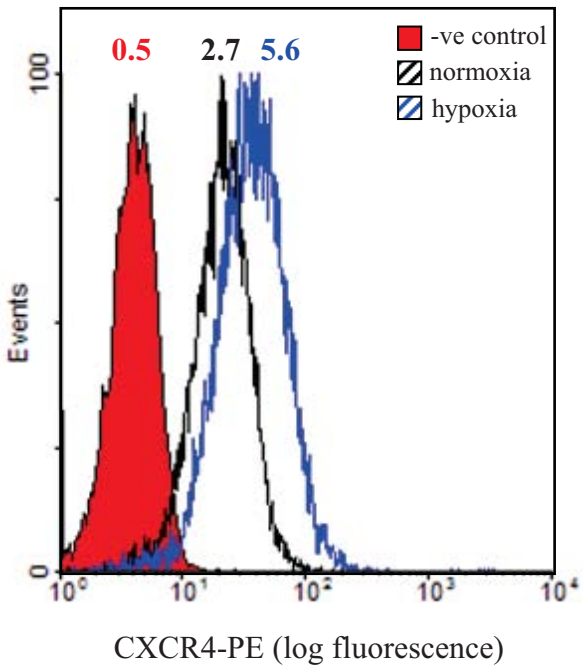
The studies described thus far were designed to identify a candidate MM cell line with which to investigate the hypoxic regulation of HIF-1α and HIF-2α and their downstream HIF target genes. The variation in expression profiles between the eight MM cell lines, displayed in the preceding figures, is likely to be attributed to the genetic background of each cell line. Throughout these preliminary studies, the LP-1 cell line displayed a consistently strong hypoxic induction of both HIF-1α (Figure 4.1) and HIF-2α (Figure 4.2) protein expression, and of downstream HIF target genes *GLUT-1* (Figure 4.3H), *CXCR4* (Figure 4.4H) and *CXCL12* (Figure 4.5H) at the mRNA level. From these studies, the LP-1 cell line was chosen to further investigate the hypoxic regulation of CXCL12 and CXCR4 in MM PCs.

4.2.2 Hypoxia up-regulates CXCR4 and CXCL12 protein expression in LP-1 cells.

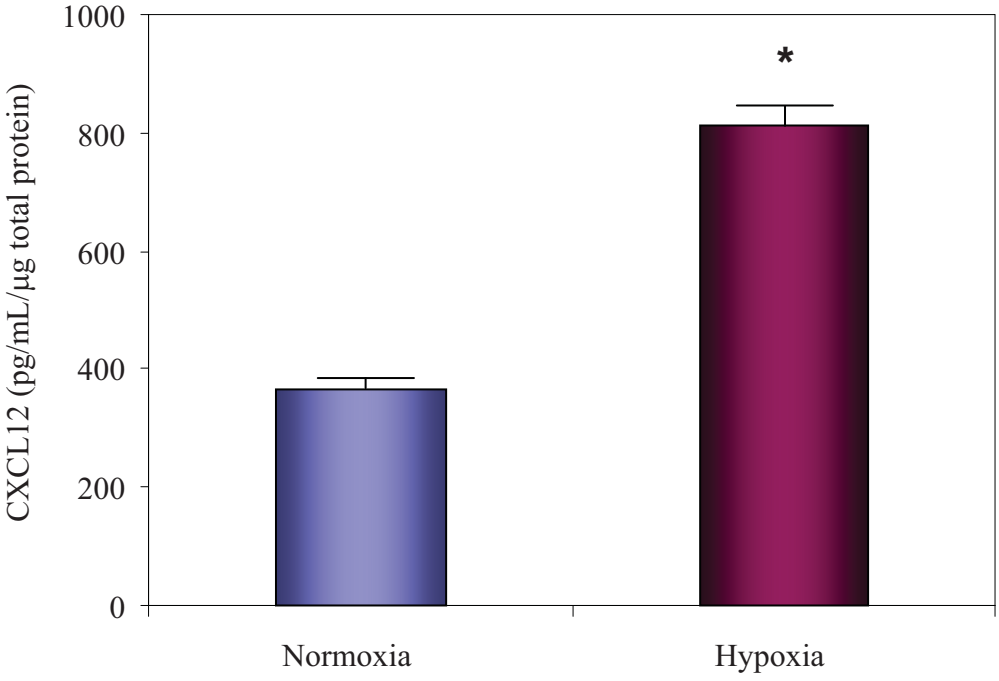
To examine whether hypoxia up-regulates CXCR4 protein expression MM PCs, LP-1 cells were cultured in either normoxic or hypoxic oxygen conditions for 24 hours. The hypoxic induction of CXCR4 protein expression was examined after 24 hours based on the earlier data showing that hypoxia up-regulates *CXCR4* mRNA expression in these cells after 6 hours (Figure 4.4H). After 24 hours, the cells were collected and stained with an anti-CXCR4 antibody to measure CXCR4 protein expression using flow cytometry. As shown in Figure 4.6A, LP-1 cells cultured under normoxic oxygen conditions expressed moderate levels of CXCR4 protein (mean fluorescence intensity [MFI] =2.7, black line). When cultured under hypoxic conditions, a 2-fold increase in CXCR4 protein expression was observed (blue line, MFI=5.6).

Figure 4.6. Hypoxic Induction of CXCR4 and CXCL12 Protein Expression in LP-1 Cells. (**A**) LP-1 cells were cultured under normoxic or hypoxic culture conditions for 8 hours, then stained with an anti-CXCR4 antibody or an isotype-matched negative control and levels of CXCR4 expression assessed using flow cytometry. The filled histogram (■) indicates background fluorescence following staining with an isotype-matched negative control antibody. The black unfilled histogram (▨) represents CXCR4 expression in LP-1 cells cultured under normoxic conditions, and the blue unfilled histogram (▨) represents CXCR4 expression in LP-1 cells cultured under hypoxic conditions. The mean fluorescence intensity for each stain is displayed above each histogram in the corresponding colour. Data from a representative experiment of three is shown. (**B**) Levels of CXCL12 protein expression were measured in conditioned media collected from LP-1 cells cultured under normoxic or hypoxic culture conditions for 72 hours using a CXCL12-specific ELISA. Graphical representation of these data illustrates the difference in CXCL12 protein concentration in the conditioned media from LP-1 cells cultured in normoxic (■) and hypoxic (■) conditions, normalised to total cellular protein concentration. Data are expressed as mean ± standard deviation from replicate samples from three individual experiments. *$p<0.001$, one-way ANOVA.

A


0.5
2.7
5.6
-ve control
normoxia
hypoxia
100
0
Events
10^0
10^1
10^2
10^3
10^4
CXCR4-PE (log fluorescence)

B


1000
800
600
400
200
0
CXCL12 (pg/mL/µg total protein)
*
Normoxia
Hypoxia

To investigate whether hypoxia affects CXCL12 protein expression in MM PCs, LP-1 cells were cultured in either normoxic or hypoxic culture conditions for 72 hours. Hypoxic induction of CXCL12 protein expression was assessed after 72 hours to maximise the time for CXCL12 "conditioning" of the medium. After 72 hours, the culture supernatants were harvested and CXCL12 levels measured using an ELISA. In order to account for decreased cell growth under hypoxic conditions (and therefore less cells present in hypoxic cultures compared to normoxic cultures at the time of collection), data were normalised to the total protein content of the cells from which the supernatant was collected.

The supernatant collected from LP-1 cells cultured under normoxic conditions contained an average of 364 ± 7.52pg/mL CXCL12, compared to 812 ± 5.52pg/mL CXCL12 under hypoxic conditions (Figure 4.6B). These data demonstrate that hypoxia is able to induce a 2.3-fold increase in CXCL12 protein expression compared to normoxic conditions ($p<0.001$, one-way ANOVA).

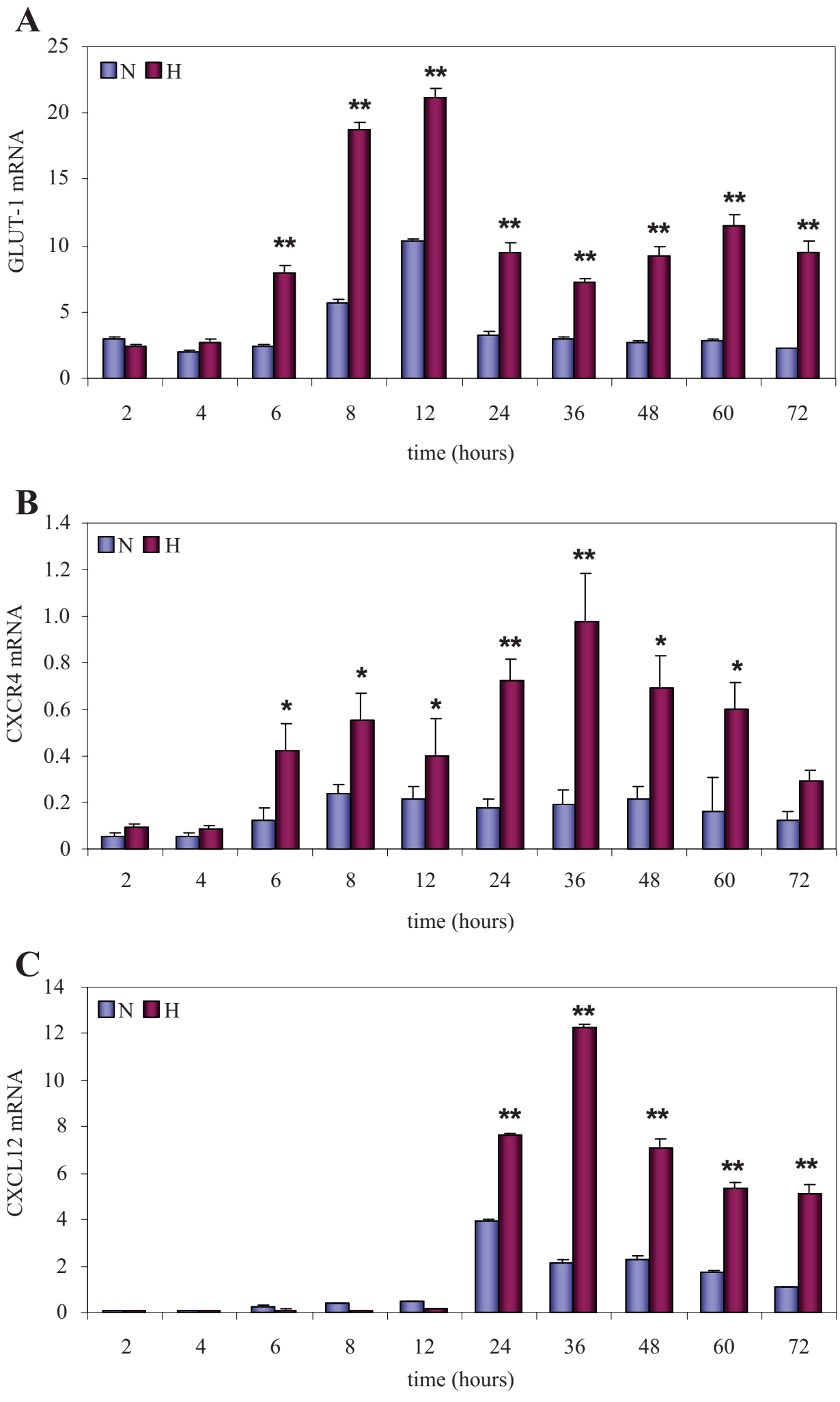
4.2.3 Detailed characterisation of the cellular response to hypoxia in LP-1 cells.

4.2.3.1 The hypoxic regulation of GLUT-1, CXCR4 and CXCL12 mRNA expression in LP-1 cells.

While the studies thus far suggest that hypoxia can induce both CXCR4 and CXCL12 protein expression in LP-1 cells, further studies were performed to better characterise the magnitude and kinetics of this response. To do this, LP-1 cells were cultured under either normoxic or hypoxic culture conditions over a period of 72 hours, and total RNA harvested at 2, 4, 6, 8, 12, 24, 36, 48, 60 and 72 hour time points. Using real-time PCR, changes in the mRNA expression of *GLUT-1* (Figure 4.7A), *CXCR4* (Figure 4.7B), and *CXCL12* (Figure 4.7C) were examined.

Relative *GLUT-1* mRNA expression was unaffected by hypoxia at the 2 and 4 hour time points in these cells, however a significant and sustained up-regulation of *GLUT-1* expression was observed after 6 hours of hypoxia compared to the normoxic conditions (Figure 4.7A, $p<0.001$, one-way ANOVA). *GLUT-1* expression was found to be highest at the 8 and 12 hour time points in both normoxic and hypoxic conditions. Similarly, the expression of *CXCR4* mRNA was significantly up-regulated following 6 hours of hypoxia compared to normoxic culture (Figure 4.7B, $*p<0.05$ and $**p<0.001$, one-way ANOVA).

Figure 4.7. A Time Course of *GLUT-1*, *CXCR4* and *CXCL12* mRNA Expression in Response to Hypoxia in LP-1 Cells. LP-1 cells were cultured under normoxic ( ) or hypoxic ( ) culture conditions for 72 hours and total RNA was harvested at 2, 4, 6, 8, 12, 24, 36, 48, 60 and 72 hour time points. Levels of (**A**) *GLUT-1*, (**B**) *CXCR4*, and (**C**) *CXCL12* mRNA expression were examined using real-time PCR, and data were normalised to the standard housekeeping gene, *β2-microglobulin*. Graphical representation of these data shows the relative levels of mRNA expression under normoxic and hypoxic conditions at each of the time points examined. Data are expressed as mean ± standard deviation from replicate samples and are representative of three individual experiments. *$p<0.05$ and **$p<0.001$, one-way ANOVA.


A
25
20
15
10
5
0
N H
GLUT-1 mRNA
**
2 4 6 8 12 24 36 48 60 72
time (hours)
B
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
N H
CXCR4 mRNA
* **
2 4 6 8 12 24 36 48 60 72
time (hours)
C
14
12
10
8
6
4
2
0
N H
CXCL12 mRNA
**
2 4 6 8 12 24 36 48 60 72
time (hours)

Interestingly, the expression profile for *CXCL12* (Figure 4.7C) was strikingly different to that of *GLUT-1* (Figure 4.7A) and *CXCR4* (Figure 4.7B). Although the culture of LP-1 cells in hypoxia resulted in a significant increase in *CXCL12* mRNA expression, this up-regulation was not observed until the 24 hour time point ($p<0.001$, one-way ANOVA). The hypoxic induction of *CXCL12* mRNA remained consistently and strongly up-regulated at all time points examined beyond 24 hours ($p<0.001$, one-way ANOVA).

4.2.3.2 The hypoxic regulation of HIF-1α and HIF-2α mRNA and protein expression in LP-1 cells.

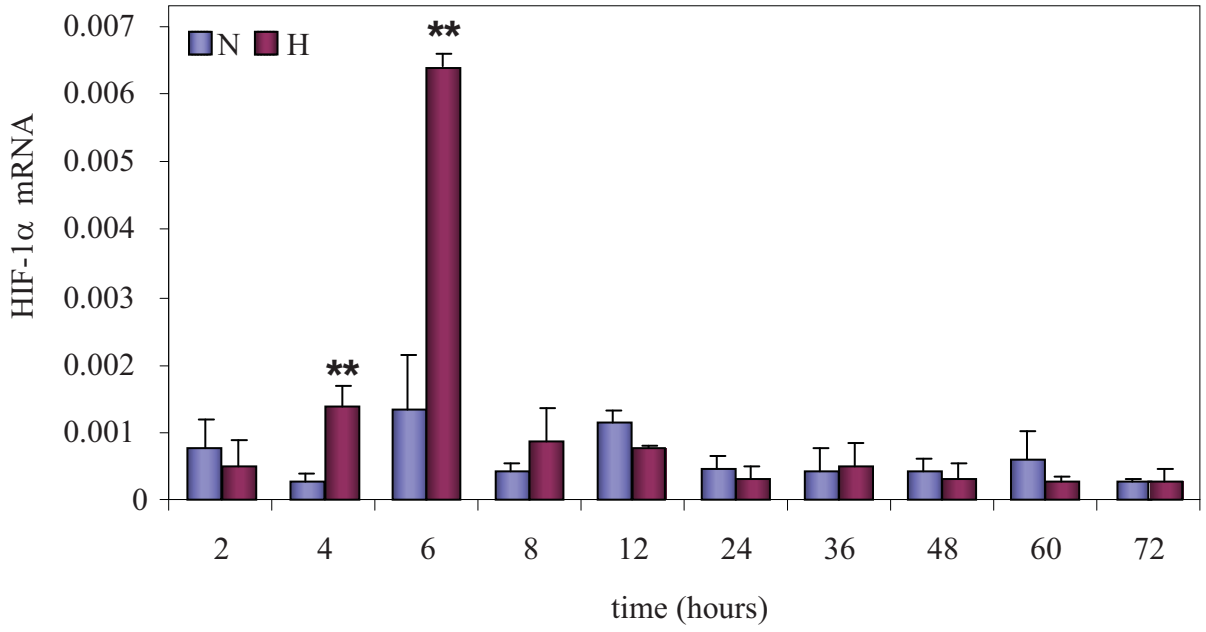
Previous studies have demonstrated that, in certain cell types, HIF-1 and HIF-2 expression display distinct induction kinetics [327,328]. To further investigate the mechanism associated with the delayed up-regulation of *CXCL12* mRNA expression under hypoxic conditions in LP-1 cells, detailed studies were performed to characterise the hypoxic induction of *HIF-1α* and *HIF-2α* mRNA expression in these cells. LP-1 cells were cultured under either normoxic or hypoxic conditions over a period of 72 hours and total RNA harvested at 2, 4, 6, 8, 12, 24, 36, 48, 60 and 72 hour time points. Levels of *HIF-1α* (Figure 4.8A) and *HIF-2α* (Figure 4.8B) mRNA expression were examined using real-time PCR.

The relative level of *HIF-1α* mRNA expression was significantly up-regulated following 4-6 hours of culture under hypoxic oxygen conditions ($p<0.001$, one-way ANOVA), but returned to basal levels after 8 hours (Figure 4.8A). In contrast, *HIF-2α* mRNA expression was not induced until 24 hours of continuous hypoxic exposure and, rather than being a transient response, the up-regulation of *HIF-2α* was observed at all subsequent time points examined (Figure 4.8B, $p<0.05$, one-way ANOVA).

To extend these studies to the protein level, LP-1 cells were cultured in normoxic or hypoxic conditions over a period of 48 hours and whole cell lysates prepared from cells harvested at 4, 6, 8, 12, 24 and 48 hour time points and HIF-1α and HIF-2α protein expression examined using Western immunoblotting. As indicated by the changing intensity of the HIF-1α protein band in hypoxic lysates compared to the respective normoxic lysates at each time point, a hypoxic up-regulation of HIF-1α protein was observed at all time points examined (Figure 4.9A). Quantitative analysis of these data confirmed that HIF-1α was consistently up-regulated by hypoxia at all time points examined over the 48 hour time course (Figure 4.9B).

Figure 4.8. A Time Course of *HIF-1α* and *HIF-2α* mRNA Expression in Response to Hypoxia in LP-1 Cells. LP-1 cells were cultured under normoxic ( ) or hypoxic ( ) culture conditions for 72 hours and total RNA was harvested at 2, 4, 6, 8, 12, 24, 36, 48, 60 and 72 hour time points. Levels of (**A**) *HIF-1α*, and (**B**) *HIF-2α* mRNA expression were examined using real-time PCR, and data were normalised to the standard housekeeping gene, *β2-microglobulin*. Graphical representation of these data shows the relative levels of mRNA expression under normoxic and hypoxic conditions at each of the time points examined. Data are expressed as mean ± standard deviation from replicate samples and are representative of three individual experiments. *$p<0.05$ and **$p<0.001$, one-way ANOVA.

A


N
H
HIF-1α mRNA
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0
**
**
2
4
6
8
12
24
36
48
60
72
time (hours)

B

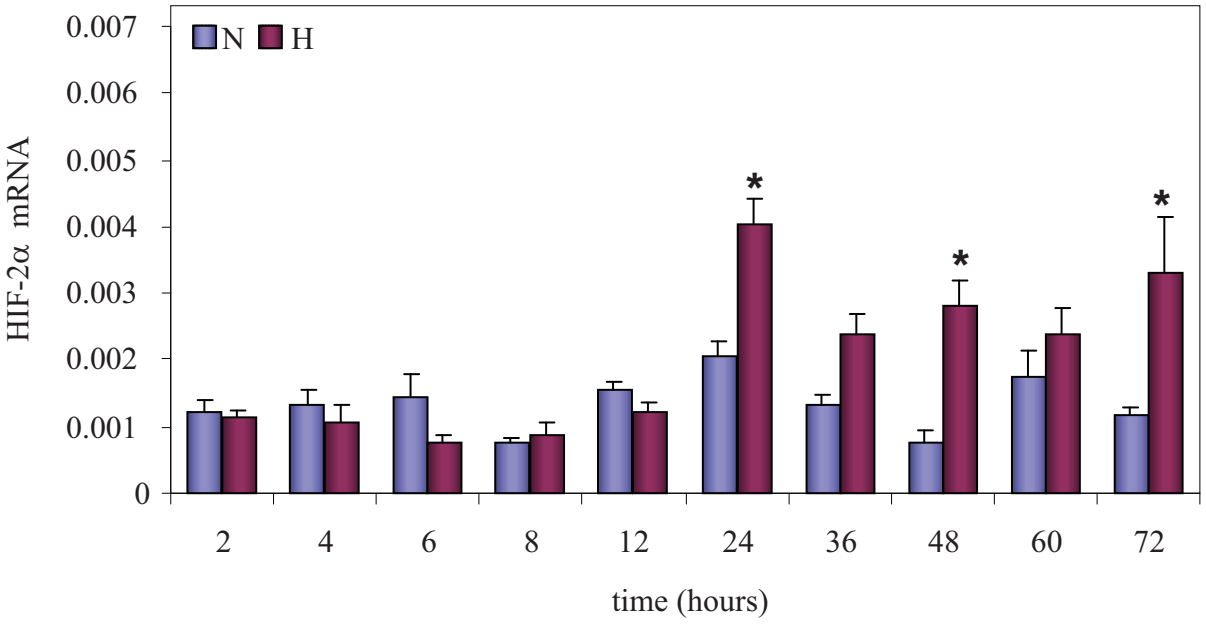
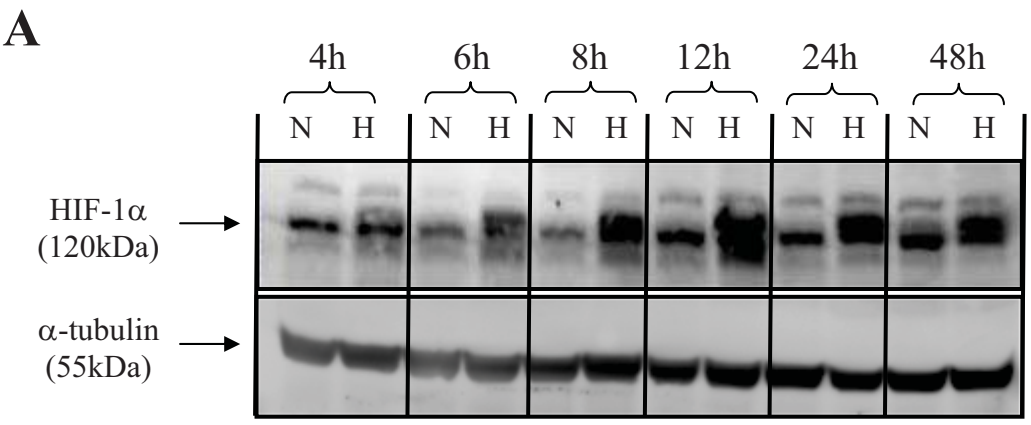
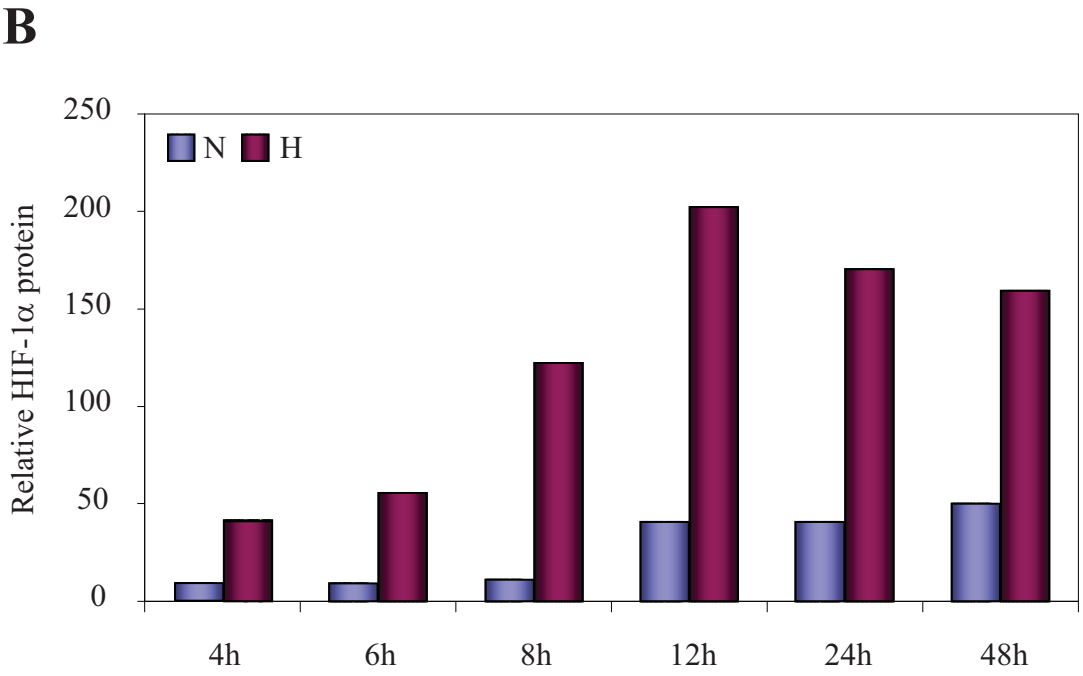

N
H
HIF-2α mRNA
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0
*
*
*
2
4
6
8
12
24
36
48
60
72
time (hours)

Figure 4.9. A Time Course of HIF-1α Protein Induction in Response to Hypoxia in LP-1 Cells. (**A**) LP-1 cells were cultured under normoxic or hypoxic culture conditions for 48 hours and whole cell lysates were prepared at 4, 6, 8, 12, 24 and 48 hour time points. An RCDC protein assay was performed to ensure that an equal amount of protein was loaded in each lane. Lysates were resolved on 10% SDS-PAGE gels under reducing conditions and levels of HIF-1α protein expression measured using Western immunoblotting. This representative immunoblot illustrates the levels of HIF-1α protein expression (≈120kDa doublet) under normoxic and hypoxic culture conditions at each of the time points examined. Membranes were also probed with anti-α-tubulin (≈55kDa band) to confirm equal protein loading. (**B**) ImageQuant image analysis software was used to quantitate the intensity of the HIF-1α bands relative to the α-tubulin loading control bands. The graphical representation of these data highlights the differences in HIF-1α protein expression under normoxic ( ) and hypoxic ( ) conditions at each of the time points examined. Results are representative of three individual experiments.


A
4h
6h
8h
12h
24h
48h
N H N H N H N H N H N H
HIF-1α
(120kDa)
α-tubulin
(55kDa)


B
250
200
150
100
50
0
N H
Relative HIF-1α protein
4h
6h
8h
12h
24h
48h

In parallel studies, a detailed examination of the hypoxic regulation of HIF-2α protein expression was also performed over a period of 72 hours. As indicated by the changing intensity of the HIF-2α protein bands in these immunoblots, endogenous levels of HIF-2α steadily accumulated over time under normal oxygen conditions, and were up-regulated in response to prolonged exposure to hypoxic conditions (Figure 4.10A). Quantitative analysis of these immunoblots demonstrated that, despite an initial spike in HIF-2α levels after 4 hours of hypoxic exposure, HIF-2α is predominantly induced by prolonged exposure to hypoxia, with increased levels of HIF-2α observed in protein lysates at 12, 24, 48 and 72 hours (Figure 4.10B).

4.2.4 The creation and characterisation of HIF-1α- and HIF-2α- over-expressing LP-1 cells.

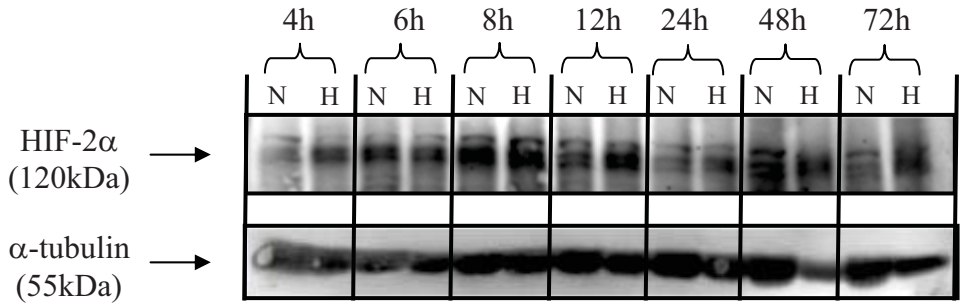
In order to further examine the relative contribution of HIF-1α and HIF-2α to the hypoxic induction of CXCR4 and CXCL12 expression in LP-1 cells, LP-1 cells were engineered to stably over-express HIF-1α or HIF-2α via retroviral infection with a bi-cistronic GFP vector, pRUF-IRES-GFP (abbreviated to pRUF) harbouring the relevant HIF over-expression construct (as described in Section 2.7, Materials and Methods). Transduced cells were subsequently sorted on the basis of GFP expression, with the top 30% of GFP-expressing cells harbouring each of the over-expression constructs, or the pRUF vector alone, selected (Figure 4.11A).

Using real-time PCR, relative levels of *HIF-1α* and *HIF-2α* mRNA expression were measured in the HIF-1α-over-expressing LP-1 cells (abbreviated to LP-1-HIF-1α) and the HIF-2α-over-expressing LP-1 cells (abbreviated to LP-1-HIF-2α), compared to the pRUF vector control (abbreviated to LP-1-pRUF). Constitutive over-expression of HIF-1α was confirmed in LP-1-HIF-1α cell line, with a 45-fold increase in relative *HIF-1α* mRNA expression compared to both LP-1-pRUF and LP-1-HIF-2α (Figure 4.11B, $p<0.001$, one-way ANOVA). Similarly, a 30-fold increase in relative *HIF-2α* mRNA expression was observed in the LP-1-HIF-2α cell line compared to both LP-1-pRUF and LP-1-HIF-1α (Figure 4.11C, $p<0.001$, one-way ANOVA).

In parallel studies, relative levels of HIF-1α and HIF-2α protein expression were also examined in the transduced HIF-over-expressing cell lines using Western immunoblotting.

Figure 4.10. A Time Course of HIF-2α Protein Induction in Response to Hypoxia in LP-1 Cells. (**A**) LP-1 cells were cultured under normoxic or hypoxic culture conditions for 48 hours and whole cell lysates were prepared at 4, 6, 8, 12, 24 and 48 hour time points. An RCDC protein assay was performed to ensure that an equal amount of protein was loaded in each lane. Lysates were resolved on 8% SDS-PAGE gels under reducing conditions and levels of HIF-2α protein expression measured using Western immunoblotting. This representative immunoblot shows the levels of HIF-2α protein expression (≈120kDa doublet) under normoxic and hypoxic culture conditions at each of the time points examined. Membranes were also probed with anti-α-tubulin (≈55kDa band) to confirm equal protein loading. (**B**) ImageQuant image analysis software was used to quantitate the intensity of the HIF-2α bands relative to the α-tubulin loading control bands. The graphical representation of these data highlights the differences in HIF-2α protein expression under normoxic ( ) and hypoxic ( ) conditions at each of the time points examined. Results are representative of three individual experiments.

A


4h
6h
8h
12h
24h
48h
72h
N H N H N H N H N H N H N H
HIF-2α
(120kDa)
α-tubulin
(55kDa)

B

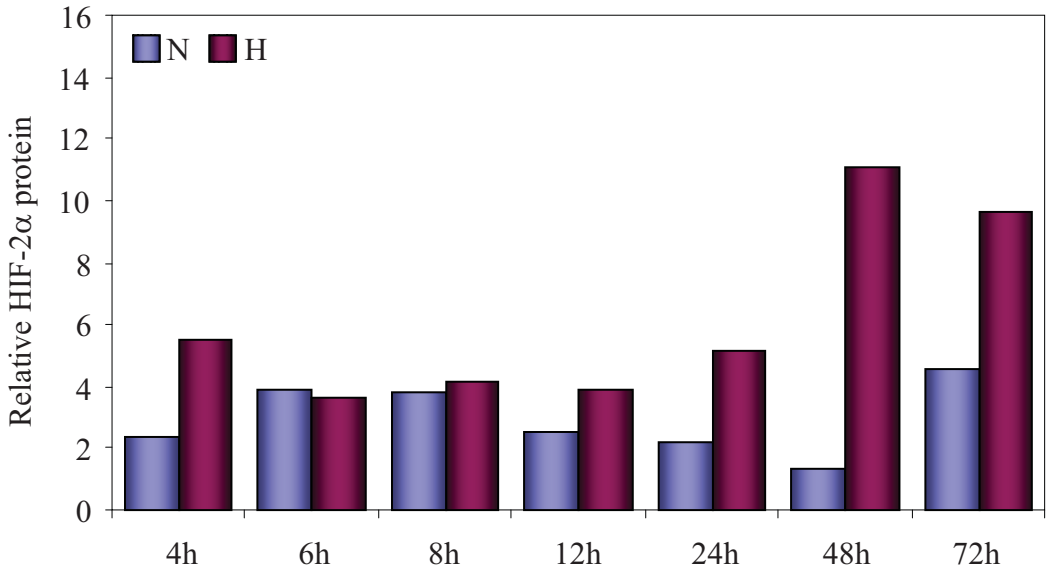
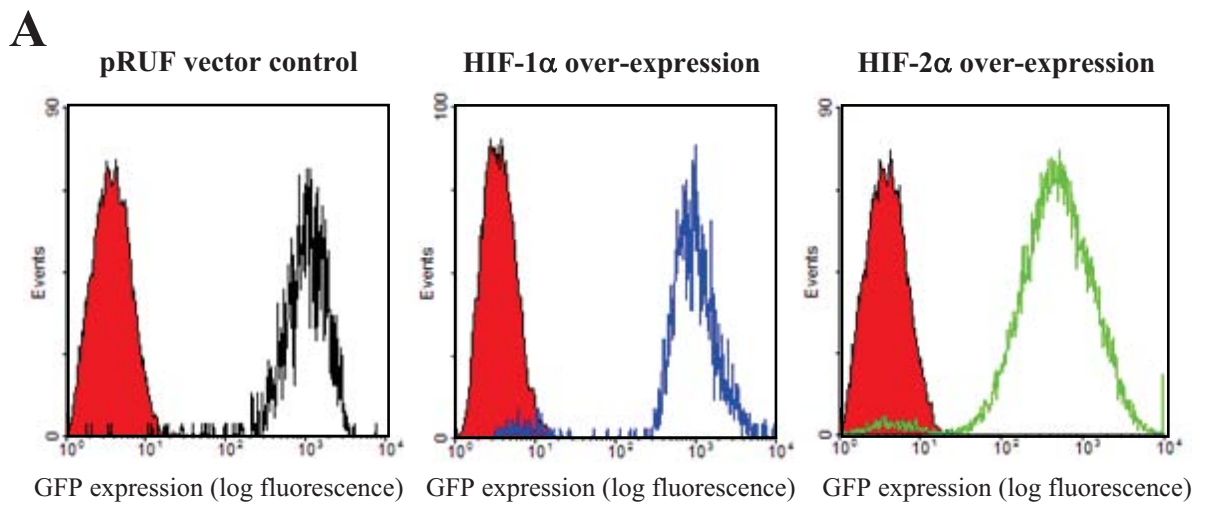
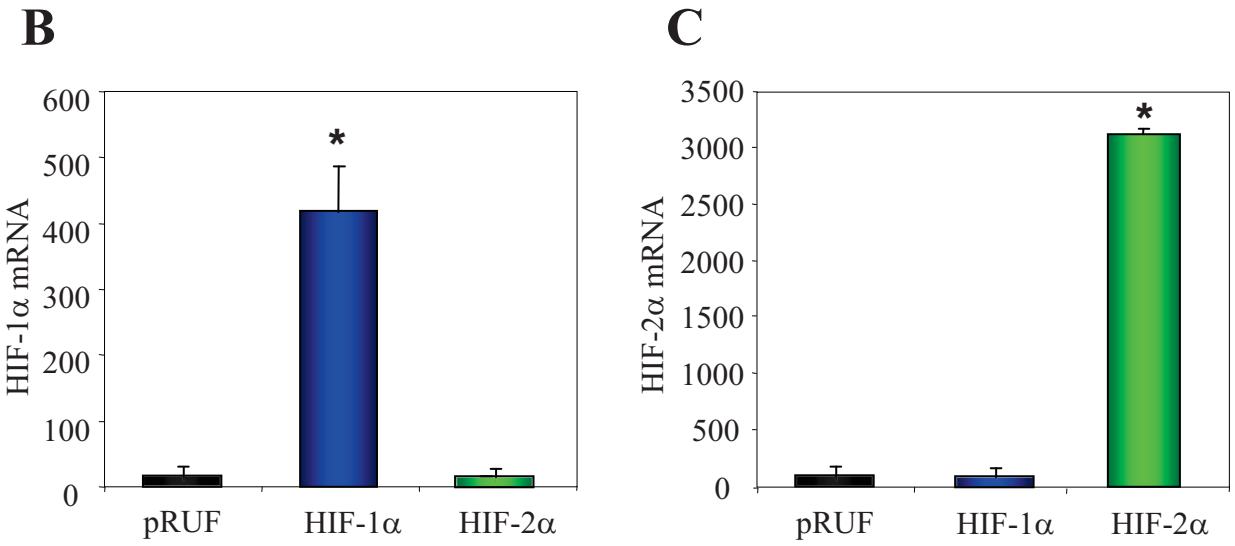
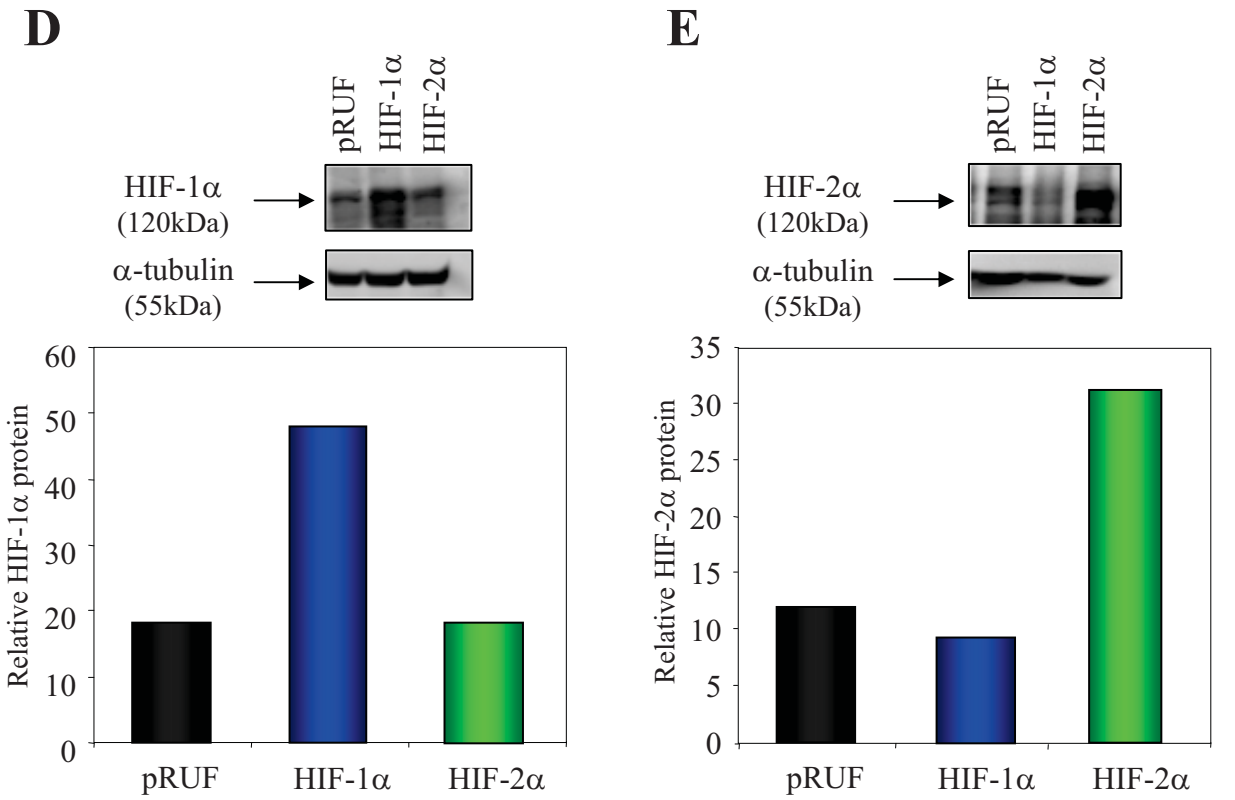

Relative HIF-2α protein
16
14
12
10
8
6
4
2
0
N
H
4h
6h
8h
12h
24h
48h
72h

Figure 4.11. The Creation of HIF-1α- and HIF-2α- Over-Expressing LP-1 Cell Lines. (**A**) LP-1 cells were engineered to stably over-express HIF-1α or HIF-2α via retroviral infection and the top 30% of transduced cells were selected based on the level of GFP expression. (**B** and **C**) Stable over-expression of *HIF-1α* and *HIF-2α* mRNA in these sorted cell populations was determined using real-time PCR, and data were normalised to the standard housekeeping gene, *β2-microglobulin*. Graphical representation of these data demonstrates relative levels of *HIF-1α* and *HIF-2α* mRNA expression in the vector control and HIF-over-expressing cell lines. Data are expressed as mean ± standard deviation from replicate samples and are representative of three individual experiments. *$p<0.001$, one-way ANOVA. (**D** and **E**) To confirm successful over-expression of HIF-1α and HIF-2α protein, whole cell lysates were resolved on 10% and 8% SDS-PAGE gels respectively under reducing conditions and Western immunoblotting performed. Membranes were also probed with anti-α-tubulin as a loading control. ImageQuant image analysis software was used to quantitate the intensity of the HIF-1α and HIF-2α bands relative to the α-tubulin loading control bands. Graphical representation of these data illustrates the differences in HIF-1α and HIF-2α protein expression in each transduced cell line compared to each other and to the pRUF vector control. Results are representative of three individual experiments.


A
pRUF vector control
HIF-1α over-expression
HIF-2α over-expression
Events
GFP expression (log fluorescence)
GFP expression (log fluorescence)
GFP expression (log fluorescence)


B
HIF-1α mRNA
600
500
400
300
200
100
0
*
pRUF
HIF-1α
HIF-2α
C
HIF-2α mRNA
3500
3000
2500
2000
1500
1000
500
0
*
pRUF
HIF-1α
HIF-2α

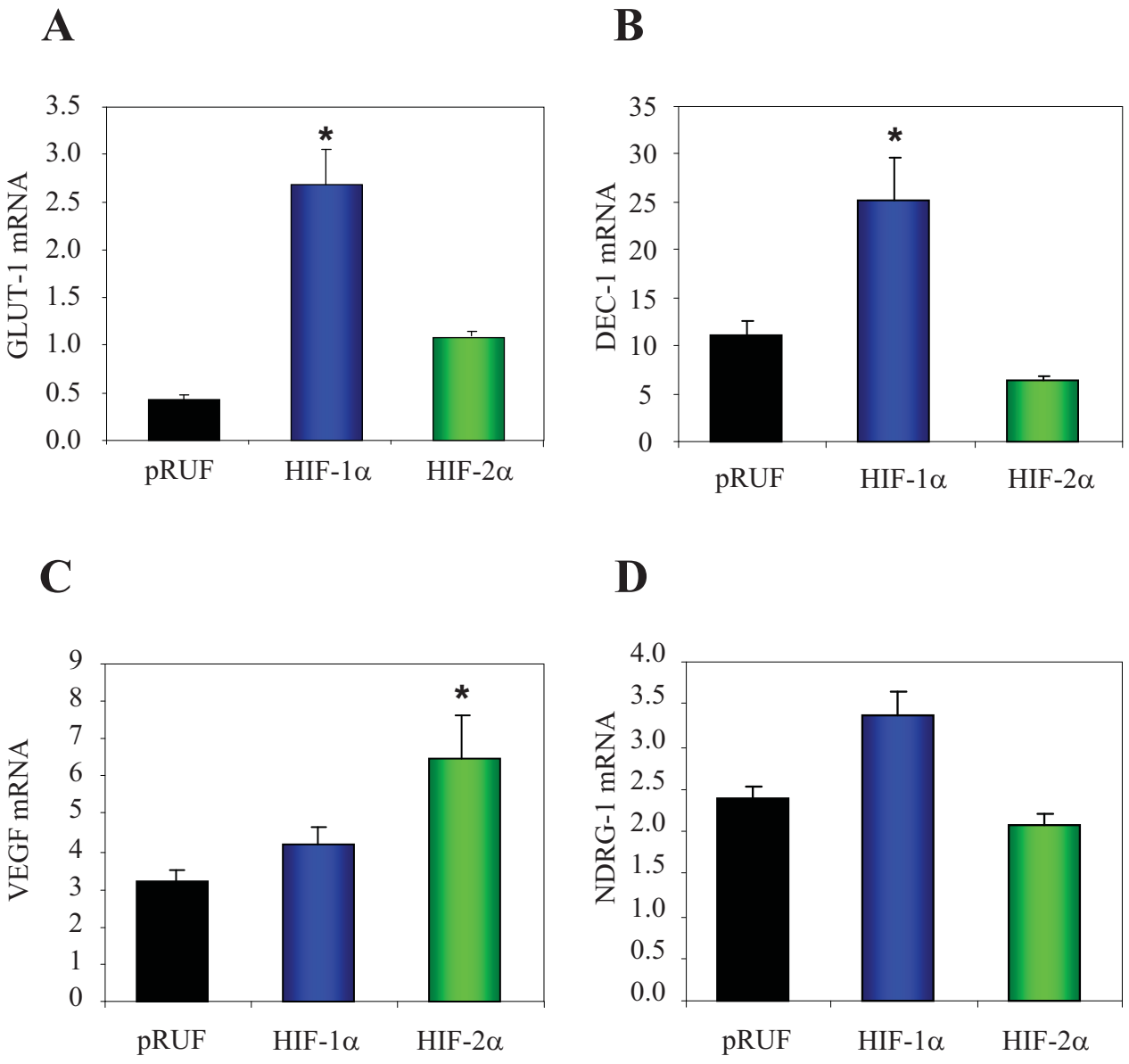

D
pRUF
HIF-1α
HIF-2α
HIF-1α
(120kDa)
α-tubulin
(55kDa)
Relative HIF-1α protein
60
50
40
30
20
10
0
pRUF
HIF-1α
HIF-2α
E
pRUF
HIF-1α
HIF-2α
HIF-2α
(120kDa)
α-tubulin
(55kDa)
Relative HIF-2α protein
35
30
25
20
15
10
5
0
pRUF
HIF-1α
HIF-2α

Surprisingly, despite the strong over-expression of *HIF-1α* and *HIF-2α* mRNA in the respective cell lines, little or no over-expression of HIF-1α or HIF-2α protein was detected under normoxic conditions. Subsequently, the cells were cultured under hypoxic conditions for 8 hours prior to the preparation of lysates. Under hypoxic conditions, the intensity of the HIF-1α protein bands showed a 2.5-fold increase in HIF-1α protein in the LP-1-HIF-1α cell line compared to both LP-1-pRUF and LP-1-HIF-2α (Figure 4.11D). No change in HIF-1α protein expression was detected in the LP-1-HIF-2α cells. Similarly, under hypoxic conditions, the intensity of the HIF-2α bands demonstrated a 2.5-fold increase in HIF-2α protein in the LP-1-HIF-2α cell line, compared to both LP-1-pRUF and LP-1-HIF-1α (Figure 4.11E). Again, no change in HIF-2α protein expression was detected in the LP-1-HIF-1α cell line.

Having demonstrated constitutive over-expression of HIF-1α and HIF-2α in these transduced cell lines, the modulation of downstream HIF target genes was examined using real-time PCR. Four established HIF target genes were examined: *GLUT-1* [448,449], *DEC-1* (a bHLH transcription factor also known as Stra13/Sharp2/BHLHB2 involved in controlling the proliferation/differentiation of some cell lineages) [450-452], *VEGF* [373,374,453,454], and *NDRG*-1 (N-myc downstream regulated-1, also known as Cap43, Drg 1, RTP and rit42, a putative differentiation-related gene) [455-457].

HIF-1 was identified as the predominant regulator of GLUT-1 expression in LP-1 cells, with a 5.5-fold elevation in *GLUT-1* mRNA expression detected in the LP-1-HIF-1α cell line compared to LP-1-pRUF (Figure 4.12A, $p<0.05$, one-way ANOVA). A 2-fold increase in *GLUT-1* mRNA expression was also observed in the LP-1-HIF-2α cell line compared to the vector control. Similarly, mRNA levels of *DEC-1* (Figure 4.12B) and *NDRG-1* (Figure 4.12D) were highest in the LP-1-HIF-1α cell line and, rather surprisingly, were slightly decreased in the LP-1-HIF-2α cell line compared to LP-1-pRUF. In contrast to the predominantly HIF-1α-mediated up-regulation of *GLUT-1*, *DEC-1* and *NDRG-1*, VEGF appeared to be predominantly regulated by HIF-2 in LP-1 cells, with a 2.1-fold increase in relative *VEGF* mRNA expression detected in the LP-1-HIF-2α cell line compared to LP-1-pRUF (Figure 4.12C, $p<0.05$, one-way ANOVA). A marginal elevation in *VEGF* expression was also observed in LP-1-HIF-1α cells, however this was not significant ($p=0.1$, one-way ANOVA).

Figure 4.12. Changes in *GLUT-1*, *DEC-1*, *VEGF* and *NDRG-1* mRNA Expression in Response to HIF Over-Expression in LP-1 Cells. Using real-time PCR, levels of (**A**) *GLUT-1*, (**B**) *DEC-1*, (**C**) *VEGF*, and (**D**) *NDRG-1* mRNA expression were examined in LP-1 cells engineered to stably over-express either HIF-1α or HIF-2α and the pRUF vector control. Data were normalised to the standard housekeeping gene, *β2-microglobulin*. Graphical representation of these data illustrates relative mRNA expression in each HIF- over-expressing cell line and the corresponding pRUF vector control. Data are expressed as mean ± standard deviation from replicate samples and are representative of three individual experiments. *$p<0.05$, one-way ANOVA.


A
GLUT-1 mRNA
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
pRUF
HIF-1α
HIF-2α
B
DEC-1 mRNA
35
30
25
20
15
10
5
0
*
pRUF
HIF-1α
HIF-2α
C
VEGF mRNA
9
8
7
6
5
4
3
2
1
0
*
pRUF
HIF-1α
HIF-2α
D
NDRG-1 mRNA
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
pRUF
HIF-1α
HIF-2α

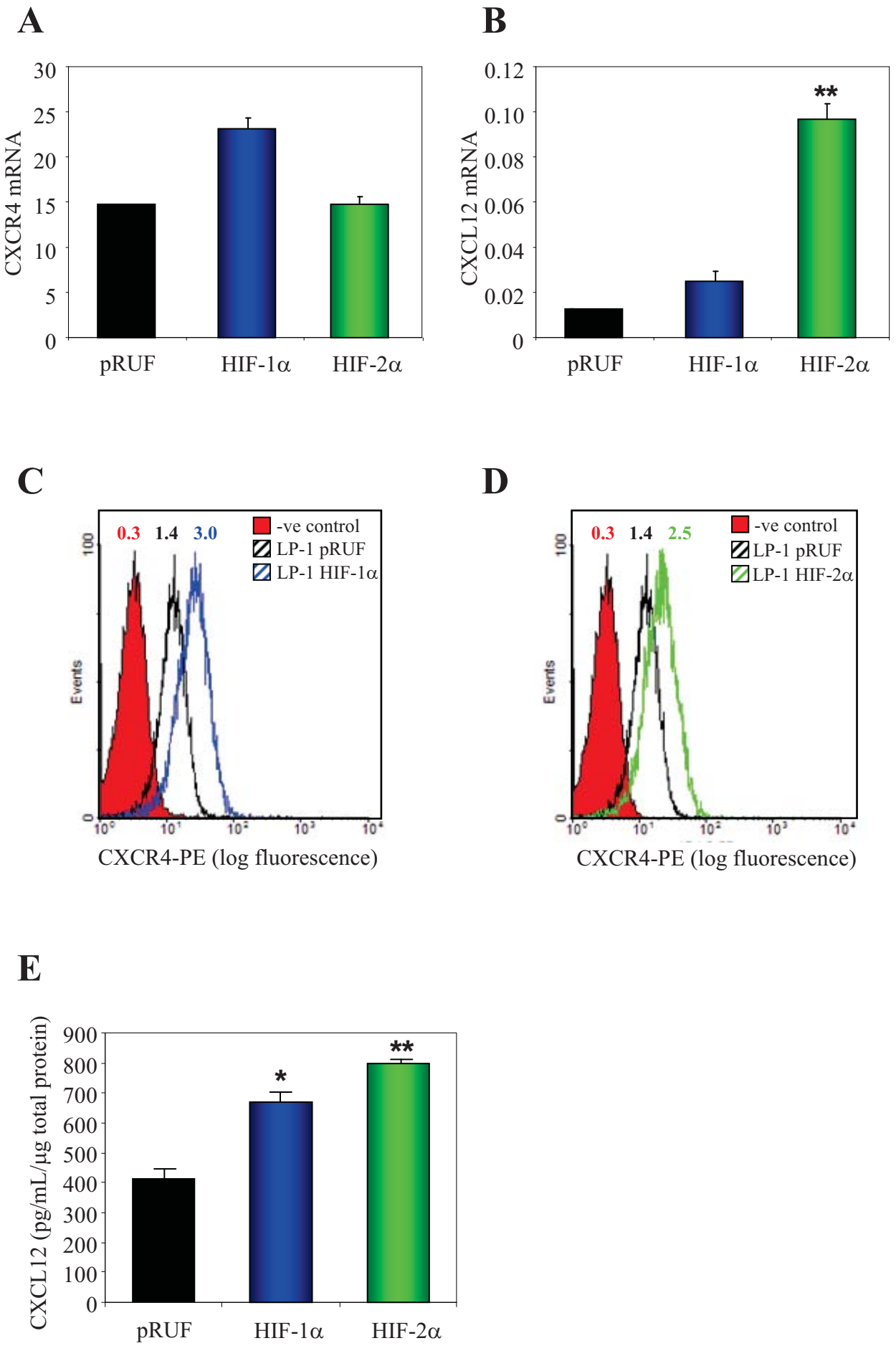
4.2.4.1 The effect of HIF-1α or HIF-2α over-expression on CXCL12 and CXCR4 expression in LP-1 cells.

To examine the effect of HIF-1α and HIF-2α over-expression on CXCL12 and CXCR4 expression in the transduced cell lines, total RNA was collected from each line and relative levels of *CXCR4* (Figure 4.13A) and *CXCL12* (Figure 4.13B) mRNA expression measured using real-time PCR. HIF-1 was identified as the sole regulator of CXCR4 expression in LP-1 cells, with a 1.5-fold increase in *CXCR4* mRNA detected in the LP-1-HIF-1α cell line compared to LP-1-pRUF (Figure 4.13A). While the over-expression of HIF-1α in LP-1 cells also mediated a 2.2-fold increase in *CXCL12* mRNA expression, HIF-2 was identified as the predominant regulator of CXCL12 expression in LP-1 cells with a 9.5-fold increase in *CXCL12* mRNA expression detected in LP-1-HIF-2α cells compared to LP-1-pRUF (Figure 4.13B, $p<0.001$, one-way ANOVA).

To extend these real-time PCR studies to the protein level, levels of CXCR4 protein expression in each of the transduced cell lines was assessed using flow cytometry. As shown in Figure 4.13C, LP-1 cells harbouring the empty pRUF vector expressed moderate levels of CXCR4 protein (black line, MFI=1.4). A 2-fold increase in CXCR4 protein was observed in the LP-1-HIF-1α cell line (blue line, MFI=3.0) compared to the LP-1-pRUF vector control. Similarly, as shown in Figure 4.12D, a 1.7-fold increase in CXCR4 expression was observed in LP-1-HIF-2α cells (green line, MFI=2.5) compared to the LP-1-pRUF control (black line, MFI=1.4).

To investigate the effect of HIF-1α and HIF-2α over-expression on CXCL12 protein expression in these cells, an ELISA was performed to measure levels of CXCL12 in conditioned media collected from the LP-1-pRUF, LP-1-HIF-1α and LP-1-HIF-2α cell lines. In order to take into account the any differences in the rate of cell growth over the 72 hour period, the CXCL12 data were normalised to the total number of cells from which the supernatant was collected. The culture supernatant of the LP-1-pRUF cells contained an average of 410 ± 28.8pg/mL CXCL12, and the supernatant from the LP-1-HIF-1α cell line contained 685.0 ± 17.05pg/mL CXCL12, equating to a 1.7-fold increase in CXCL12 protein compared to the vector control (Figure 4.13E, $p<0.05$, one-way ANOVA). Importantly, the culture supernatant collected from LP-1-HIF-2α cells contained the

Figure 4.13. The Effect of HIF Over-expression on CXCL12 and CXCR4 Expression in LP-1 Cells. Using real-time PCR, levels of (**A**) *CXCR4*, and (**B**) *CXCL12* mRNA expression in LP-1 cells engineered to stably over-express either HIF-1α or HIF-2α and the pRUF vector control were examined, and data were normalised to the standard housekeeping gene, *β2-microglobulin*. Data are expressed as mean ± standard deviation from replicate samples and are representative of three individual experiments. **p<0.001, one-way ANOVA. (**C** and **D**) LP-1 cells engineered to stably over-express either HIF-1α or HIF-2α and the vector control were stained with an anti-CXCR4 antibody or an isotype-matched negative control and levels of CXCR4 protein expression assessed using flow cytometry. The filled histograms (■) indicate background fluorescence following staining with the isotype-matched negative control antibody. The black unfilled histograms (▨) represent CXCR4 expression in LP-1 cells harbouring the empty pRUF vector alone, and the blue and green unfilled histograms (▨ and ▨) represent CXCR4 expression in LP-1 cells over-expressing HIF-1α and HIF-2α, respectively. The mean fluorescence intensity for each stain is displayed above each histogram in the corresponding colour. Data from a representative experiment of three is shown. (**E**) Levels of CXCL12 protein expression were measured in conditioned media collected from cultures of LP-1 cells engineered to stably over-express either HIF-1α or HIF-2α and the pRUF vector control using a CXCL12-specific ELISA. Graphical representation of these data illustrates changes in CXCL12 protein concentration in the conditioned media collected from each of these cell lines, normalised to total cellular protein concentration. Data are expressed as mean ± standard deviation from replicate samples of a representative experiment of three. *p<0.05 and **p<0.001, one-way ANOVA.


A
CXCR4 mRNA
30
25
20
15
10
5
0
pRUF
HIF-1α
HIF-2α
B
CXCL12 mRNA
0.12
0.10
0.08
0.06
0.04
0.02
0
**
pRUF
HIF-1α
HIF-2α
C
0.3
1.4
3.0
-ve control
LP-1 pRUF
LP-1 HIF-1α
Events
100
0
CXCR4-PE (log fluorescence)
D
0.3
1.4
2.5
-ve control
LP-1 pRUF
LP-1 HIF-2α
Events
100
0
CXCR4-PE (log fluorescence)
E
CXCL12 (pg/mL/μg total protein)
900
800
700
600
500
400
300
200
100
0
*
**
pRUF
HIF-1α
HIF-2α

highest level of CXCL12 with an average of 805.1 ± 20.3pg/mL, which was 2-fold higher than the LP-1-pRUF control (Figure 4.13E, $p<0.001$, one-way ANOVA).

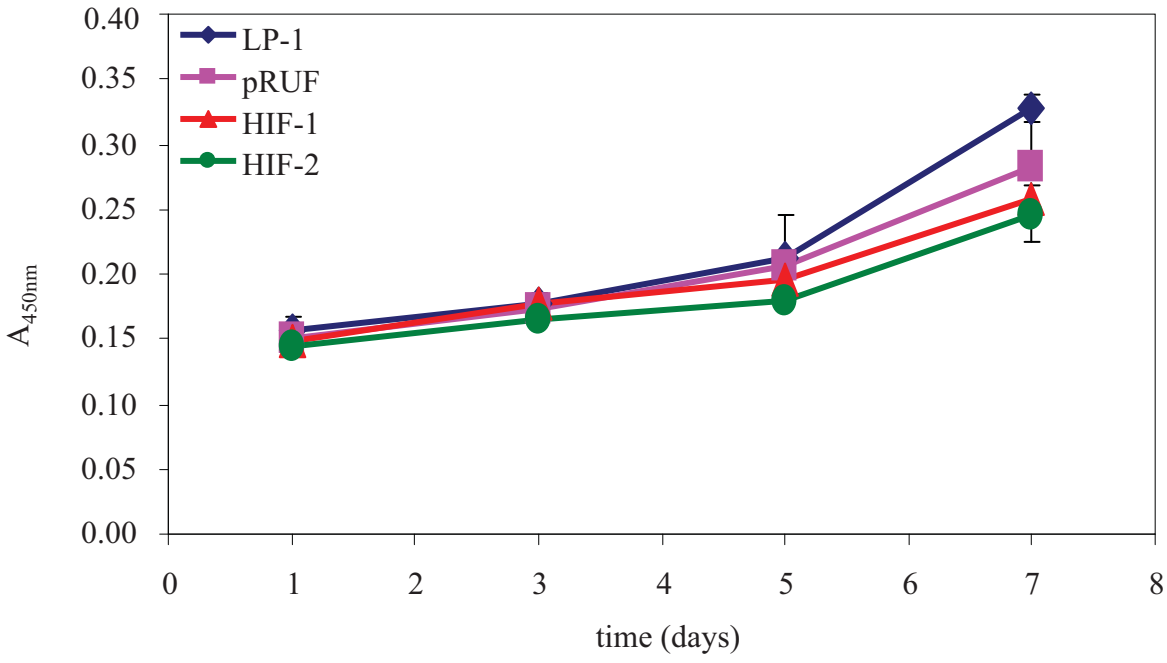
4.2.4.2 The effect of HIF-1α or HIF-2α over-expression on cell proliferation in LP-1 cells.

Given that the HIF transcription factors mediate the expression of at least 100 target genes required for cellular adaptation to hypoxia, the over-expression of HIF-1α and HIF-2α is likely to impart numerous changes to cellular behaviour. To investigate whether the over-expression of HIF-1α or HIF-2α in LP-1 cells caused any change in the rate of proliferation, a proliferation assay was performed. Both of the HIF over-expressing cell lines, the pRUF vector control and the unmodified parental LP-1 cell line were seeded at a constant density using preparative cell sorting, cultured for 1, 3, 5, and 7 days and the relative numbers of viable cells assessed using the colorimetric assay reagent, WST-1. All three of the engineered cell lines (ie. LP-1-pRUF, LP-1-HIF-1α and LP-1-HIF-2α) displayed a lower rate of proliferation compared to the parental, unmodified LP-1 cell line (Figure 4.14). Whilst not statistically significant for the pRUF vector control cell line ($p=0.172$, Mann-Whitney Rank Sum), this reduction in proliferation was statistically significant for both of the HIF over-expressing cell lines compared to the unmodified parental line ($p<0.01$, one-way ANOVA). As the introduction of the empty vector alone was shown to cause a minor decrease in cell proliferation, it was concluded that any changes in proliferation of the HIF over-expressing lines should be compared with the vector control rather than the unmodified LP-1 cell line. Compared to LP-1-pRUF, the HIF over-expressing cell lines both displayed a marginal reduction in the rate of proliferation, ($p=0.376$ and $p=0.228$, one-way ANOVA, for LP-1-HIF-1α and LP-1-HIF-2α respectively).

4.2.5 The creation and characterisation of HIF-1α-knockdown in LP-1 cells.

It has been suggested that constitutive over-expression of HIF-1α or HIF-2α may lead to a loss of HIF target gene specificity, thereby compromising the validity of any findings arising from over-expression studies [458,459]. To address this, HIF-1α or HIF-2α RNAi sequences were introduced into LP-1 cells by lentiviral-mediated gene transfer (as described in Section 2.7, Materials and Methods). Briefly, LP-1 cells were transduced with the pFIV-H1-copGFP (abbreviated to pFIV) lentiviral vector containing a HIF-1α RNAi construct, a scrambled RNAi control construct or the empty pFIV vector alone. Cells were

Figure 4.14. The Effect of HIF Over-Expression on the Rate of Proliferation of LP-1 Cells. Unmodified parental LP-1 cells and LP-1 cells engineered to stably over-express either HIF-1α or HIF-2α and the pRUF vector control were seeded at 4000 cells/well in 96-well tissue culture plates using preparative cell sorting and cultured for 7 days. At day 1, 3, 5, and 7 time points, the number of viable, proliferating cells was assessed using WST-1, and cumulative data were plotted as shown. Data are expressed as mean ± standard deviation from replicate samples and are representative of three individual experiments.

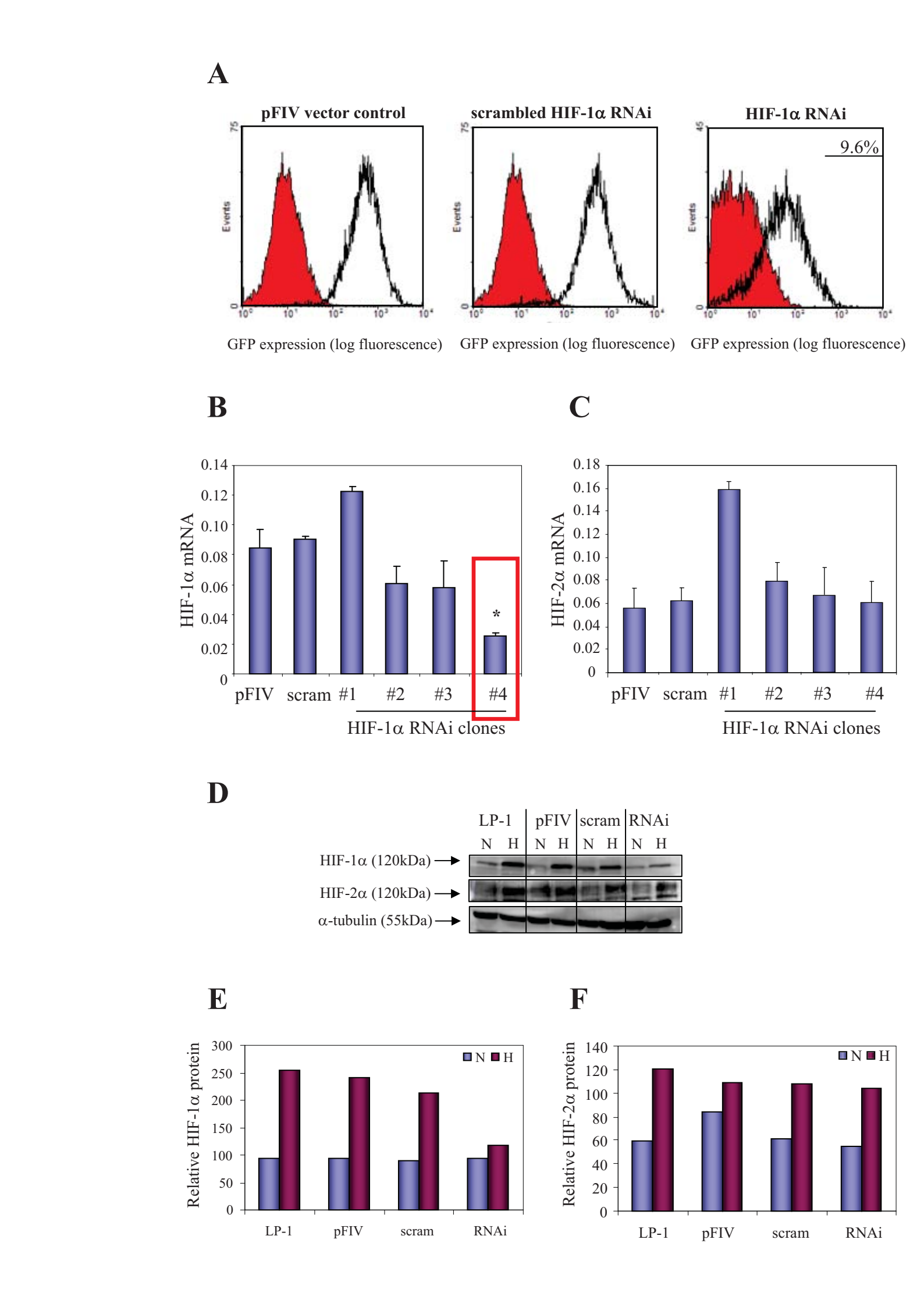

0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
LP-1
pRUF
HIF-1
HIF-2
A450nm
0
1
2
3
4
5
6
7
8
time (days)

subsequently sorted on the basis of GFP expression, with the top 30% of GFP-expressing cells collected for each transduced cell line (Figure 4.15A). In order to create a cell line harbouring the greatest knockdown of HIF-1α, clonal populations were subsequently created from the top 9.6% of sorted cells containing the HIF-1α RNAi sequence using preparative cell sorting and single cell deposition.

Using real-time PCR, relative levels of *HIF-1α* mRNA expression in several LP-1-HIF-1α RNAi clones were assessed and compared to the pFIV vector control (abbreviated to LP-1-pFIV) and scrambled RNAi control (abbreviated to LP-1-scramRNAi) cell lines to determine the level of HIF-1α knockdown (Figure 4.15B). As expected, these data demonstrated varying degrees of HIF-1α suppression amongst the RNAi clones (designated #1, #2, #3 and #4) compared to LP-1-pFIV and LP-1-scramRNAi. Of the HIF-1α RNAi clones tested, "Clone #4" exhibited the greatest level of HIF-1α knockdown, with an approximately 65% reduction in *HIF-1α* mRNA expression compared to LP-1-pFIV ($p<0.001$, one-way ANOVA). To ensure that there was no non-specific silencing of HIF-2α by the HIF-1α RNAi, *HIF-2α* mRNA expression was also assessed by real-time PCR. With the exception of "Clone #1", *HIF-2α* mRNA expression was unaffected by the introduction of the HIF-1α RNAi construct (Figure 4.15C).

In parallel studies, changes in HIF-1α and HIF-2α protein expression under hypoxic conditions were also measured. The parental (unmodified) LP-1 cell line, and the transduced LP-1-pFIV, LP-1-scramRNAi and LP-1-HIF-1α RNAi ("Clone #4") cell lines were cultured under normoxic or hypoxic culture conditions for 12 hours, and whole cell lysates were prepared. These lysates were subsequently examined for HIF-1α and HIF-2α protein expression using Western immunoblotting (Figure 4.15D). Increased HIF-1α protein was evident in hypoxic lysates from the LP-1, LP-1-pFIV and LP-1-scramRNAi cell lines, and this was markedly reduced in cells harbouring the HIF-1α RNAi. Quantitative analysis of these data confirmed a strong hypoxic induction of HIF-1α protein in the LP-1, LP-1-pFIV and LP-1-scramRNAi cell lines (Figure 4.15E). Importantly, the hypoxic induction of HIF-1α protein was strongly reduced in the HIF-1α RNAi cell line, with an approximately 60% reduction in HIF-1α protein compared to vector control. Quantitative analysis of the HIF-2α protein bands showed that HIF-2α was strongly

Figure 4.15. The Creation of HIF-1α Knockdown in LP-1 Cells. (**A**) RNA interference technology was used to knock down endogenous HIF-1α expression in LP-1 cells, and the top 30% of lentivirally transduced cells were selected based on the level of GFP expression. Single cell clones were then created from the top 9.6% of these sorted GFP-expressing cells. Clones were screened for levels of (**B**) *HIF-1α,* and (**C**) *HIF-2α* mRNA expression using real-time PCR, and data were normalised to the standard housekeeping gene, *β2-microglobulin*. Data are expressed as mean ± standard deviation from replicate samples and are representative of three individual experiments. *$p<0.001$, one-way ANOVA. (**D**) To confirm successful knockdown of HIF-1α protein, whole cell lysates were prepared from the parental LP-1 cell line, the pFIV vector control, the scrambled RNAi control and HIF-1α RNAi clone #4, cultured under normoxic or hypoxic conditions for 12 hours. An RCDC protein assay was performed to ensure that an equal amount of protein was loaded in each lane. Lysates were resolved on 10% SDS-PAGE gels under reducing conditions and HIF-1α protein detected using Western immunoblotting. Membranes were also probed with anti-HIF-2α to determine whether there was any concomitant change in HIF-2α protein expression, and anti-α-tubulin to confirm equal protein loading in each lane. (**E** and **F**) ImageQuant image analysis software was used to quantitate the intensity of the HIF-1α and HIF-2α bands relative to the α-tubulin loading control bands. Graphical representation of these data highlights the differences in HIF-1α and HIF-2α under normoxic (■) and hypoxic (■) conditions in each cell line.


A
pFIV vector control
scrambled HIF-1α RNAi
HIF-1α RNAi
9.6%
Events
GFP expression (log fluorescence)
GFP expression (log fluorescence)
GFP expression (log fluorescence)
B
HIF-1α mRNA
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0
*
pFIV
scram
#1
#2
#3
#4
HIF-1α RNAi clones
C
HIF-2α mRNA
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0
pFIV
scram
#1
#2
#3
#4
HIF-1α RNAi clones
D
LP-1
pFIV
scram
RNAi
N H N H N H N H
HIF-1α (120kDa)
HIF-2α (120kDa)
α-tubulin (55kDa)
E
Relative HIF-1α protein
300
250
200
150
100
50
0
N H
LP-1
pFIV
scram
RNAi
F
Relative HIF-2α protein
140
120
100
80
60
40
20
0
N H
LP-1
pFIV
scram
RNAi

induced under hypoxic conditions in all four cell lines (Figure 4.15F), indicating that the hypoxic induction of HIF-2α protein was not affected by the introduction of the HIF-1α RNAi, thereby confirming the specificity of the HIF-1α RNAi.

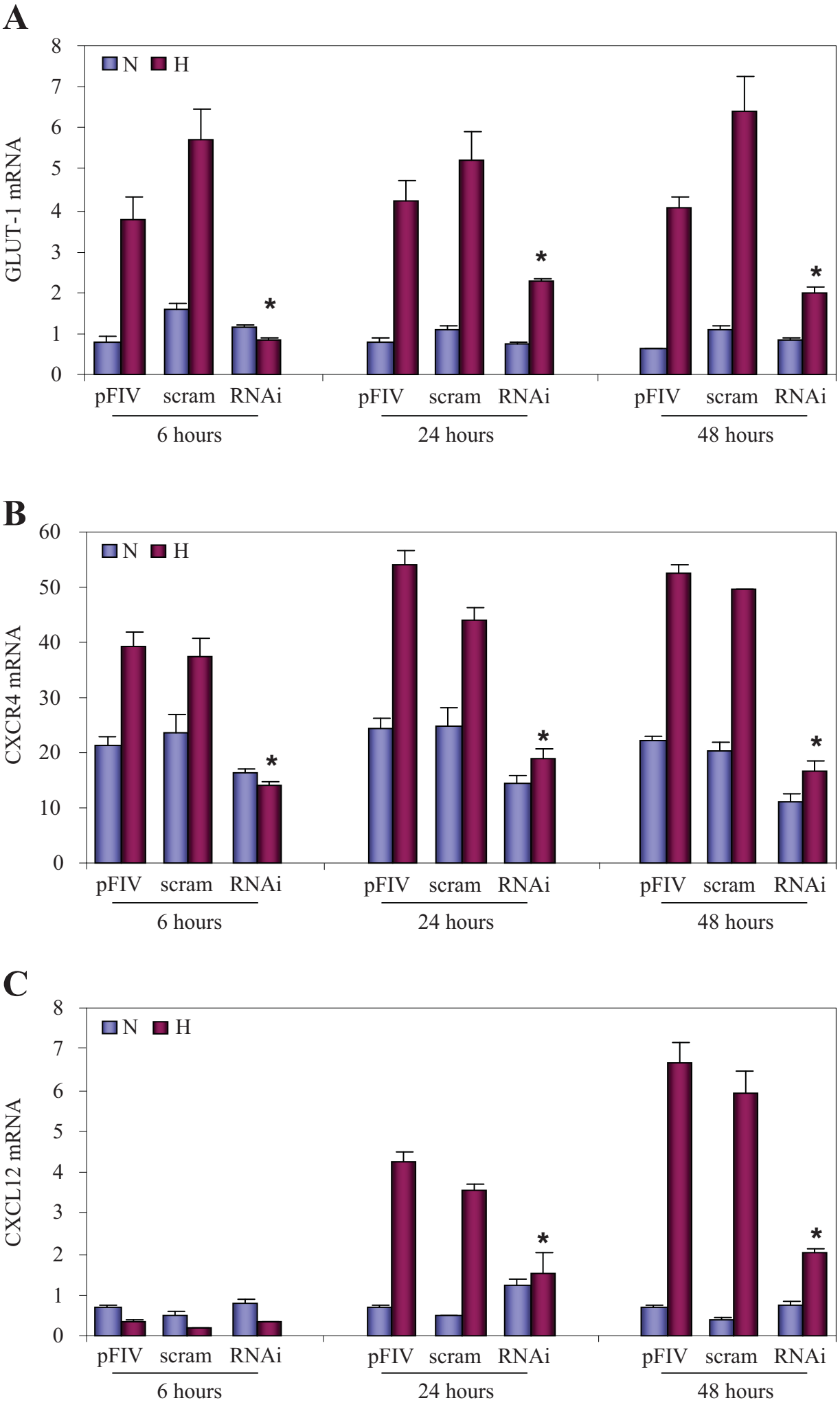
On the basis of these real-time PCR and Western immunoblot data, "Clone #4" was identified as containing a strong, specific knockdown of HIF-1α expression and was used for all subsequent experiments. Hereafter, the HIF-1α RNAi "Clone 4" cell line is referred to as LP-1-HIF-1α-KD.

4.2.5.1 The effect of HIF-1α knockdown on GLUT-1, CXCR4 and CXCL12 mRNA expression in LP-1 cells.

To investigate the effect of HIF-1α knockdown on downstream HIF target gene expression, the LP-1-pFIV LP-1-scramRNAi, and LP-1-HIF-1α-KD cell lines were cultured under normoxic or hypoxic culture conditions for 48 hours, and total RNA harvested at 6, 24, and 48 hour time points. Real-time PCR was then used to examine changes in the mRNA expression of *GLUT-1* (Figure 4.16A), *CXCR4* (Figure 4.16B), and *CXCL12* (Figure 4.16C).

GLUT-1 mRNA expression was strongly up-regulated by hypoxia in the LP-1-pFIV and LP-1-scramRNAi control cell lines at all time points examined (Figure 4.16A). Importantly, the hypoxic induction of *GLUT-1* mRNA expression was significantly reduced in the cells harbouring the HIF-1α RNAi ($p<0.05$, one-way ANOVA). While not of the same magnitude of induction as *GLUT-1*, similar mRNA expression profiles were also observed for *CXCR4* (Figure 4.16B). *CXCR4* mRNA expression was up-regulated by hypoxia in the LP-1-pFIV and LP-1-scramRNAi control cell lines at all time points examined, and this hypoxic induction was significantly reduced in the LP-1-HIF-1α-KD cell line ($p<0.05$, one-way ANOVA). In accordance with previous findings, *CXCL12* mRNA expression was not affected by hypoxia at the 6 hour time point (see Figure 4.7C). However, at both the 24 and 48 hour time points, *CXCL12* mRNA expression was strongly induced by hypoxia in LP-1-pFIV and LP-1-scramRNAi cells (Figure 4.16C). Importantly, this hypoxic induction of *CXCL12* expression was significantly reduced in LP-1 cells harbouring the HIF-1α RNAi ($p<0.05$, one-way ANOVA).

Figure 4.16. Changes in *GLUT-1*, *CXCR4* and *CXCL12* mRNA Expression in Response to HIF-1α Knockdown in LP-1 Cells. LP-1 cells transduced with the pFIV vector control, scrambled RNAi control or the HIF-1α RNAi were cultured under normoxic ( ) or hypoxic ( ) culture conditions for 48 hours and RNA was harvested at 6, 24, and 48 hour time points. Levels of (**A**) *GLUT-1*, (**B**) *CXCR4,* and (**C**) *CXCL12* mRNA expression were examined using real-time PCR, and data were normalised to the standard housekeeping gene, *β2-microglobulin*. Graphical representation of these data illustrates relative mRNA expression under normoxic and hypoxic culture conditions at each of the time points examined. Data are expressed as mean ± standard deviation from replicate samples and are representative of three individual experiments. *$p<0.05$, one-way ANOVA.


A
GLUT-1 mRNA
N
H
pFIV
scram
RNAi
6 hours
24 hours
48 hours
B
CXCR4 mRNA
C
CXCL12 mRNA

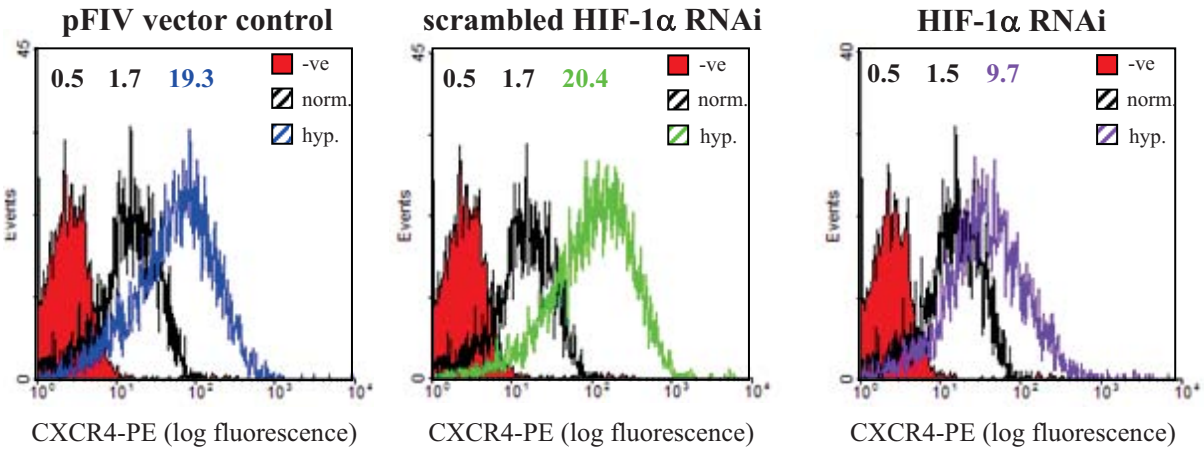
4.2.5.2 The effect of HIF-1α knockdown on CXCR4 and CXCL12 protein expression in LP-1 cells.

To extend these real-time PCR studies, the effect of HIF-1α knockdown on CXCR4 protein expression was examined. LP-1-pFIV, LP-1-scramRNAi and LP-1-HIF-1α-KD cells were cultured under normoxic or hypoxic culture conditions for 12 hours, and levels of CXCR4 expression measured using flow cytometry. As shown in Figure 4.17A, the staining of LP-1-pFIV cells revealed an 11-fold increase in CXCR4 expression under hypoxic conditions (blue line, MFI=19.3) compared to normoxic conditions (black line, MFI=1.7). Similarly in the LP-1-scramRNAi cell line, a 12-fold increase in CXCR4 expression was observed under hypoxic conditions (green line, MFI=20.4) when compared to normoxic culture conditions (black line, MFI=1.7). The staining of LP-1-HIF-1α-KD cells revealed a 6-fold increase in CXCR4 expression under hypoxic conditions (purple line, MFI=9.7) compared to normoxic conditions (black line, MFI=1.5), equating to an approximately 50% reduction in the hypoxic up-regulation of CXCR4 protein compared to LP-1-pFIV and LP-1-scramRNAi.

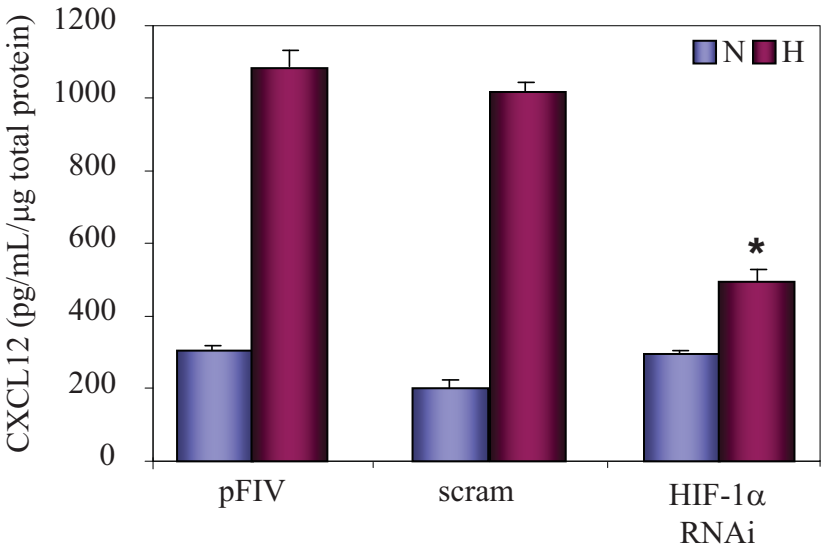
To examine the effect of HIF-1α knockdown on CXCL12 protein expression in MM PCs, LP-1-pFIV, LP-1-scramRNAi and LP-1-HIF-1α-KD cells were cultured under normoxic or hypoxic culture conditions for 72 hours. Using a CXCL12-specific ELISA, levels of CXCL12 were measured in the conditioned media collected from each of these cultures. While the amount of CXCL12 protein was comparable between the LP-1-pFIV, LP-1-scramRNAi and LP-1-HIF-1α-KD cell lines under normoxic conditions (Figure 4.17B), when placed under hypoxic culture conditions, the effect of HIF-1α knockdown on CXCL12 protein expression was revealed. In the LP-1-pFIV vector control, CXCL12 levels were 3.6-fold higher under hypoxic conditions (1079 ± 59 pg/mL), compared to normoxic culture conditions (305.9 ± 27 pg/mL). Similarly, CXCL12 protein expression was 3.2-fold higher in the LP-1-scramRNAi control under hypoxic culture conditions (636.6 ± 21 pg/mL), compared to normoxic conditions (198.9 ± 42 pg/mL). Importantly, a 1.6-fold induction of CXCL12 levels under hypoxic conditions was detected in LP-1-HIF-1α-KD cells (493.7 ± 53 pg/mL) compared to normoxic culture conditions (296.4 ± 38 pg/mL). Compared to the vector control, the hypoxic induction of CXCL12 protein was significantly reduced in LP-1-HIF-1α-KD cells ($p<0.05$, one-way ANOVA).

Figure 4.17. Changes in CXCR4 and CXCL12 Protein Expression in Response to HIF-1α Knockdown in LP-1 Cells. (**A**) LP-1 transduced with the pFIV vector control, scrambled RNAi control or the HIF-1α RNAi were cultured under normoxic or hypoxic conditions for 12 hours, stained with an anti-CXCR4 antibody or an isotype-matched negative control and levels of CXCR4 expression analysed using flow cytometry. The filled histograms ( ) indicate background fluorescence following staining with the isotype-matched negative control antibody. The black unfilled histograms ( ) represent CXCR4 expression in each respective cell line under normoxic conditions, and the blue, green and purple unfilled histograms (, and) represent CXCR4 expression in each respective cell line under hypoxic conditions. The mean fluorescence intensity for each stain is displayed above each histogram in the corresponding colour. Data from a representative experiment of three is shown. (**B**) Using a CXCL12-specific ELISA, levels of CXCL12 protein expression were measured in conditioned media collected from LP-1 cells containing the HIF-1α RNAi, the pFIV vector control, or scrambled RNAi sequence cultured under normoxic ( ) or hypoxic ( ) culture conditions for 72 hours. Graphical representation of these data illustrates changes in CXCL12 protein concentration in the culture supernatant collected from each cell line, normalised to total cellular protein concentration. Data are expressed as mean ± standard deviation from replicate samples of a representative experiment of three. *$p<0.05$, one-way ANOVA.

A


pFIV vector control
scrambled HIF-1α RNAi
HIF-1α RNAi
0.5 1.7 19.3
0.5 1.7 20.4
0.5 1.5 9.7
-ve
norm.
hyp.
Events
CXCR4-PE (log fluorescence)
CXCR4-PE (log fluorescence)
CXCR4-PE (log fluorescence)

B


CXCL12 (pg/mL/µg total protein)
1200
1000
800
600
400
200
0
N
H
*
pFIV
scram
HIF-1α RNAi

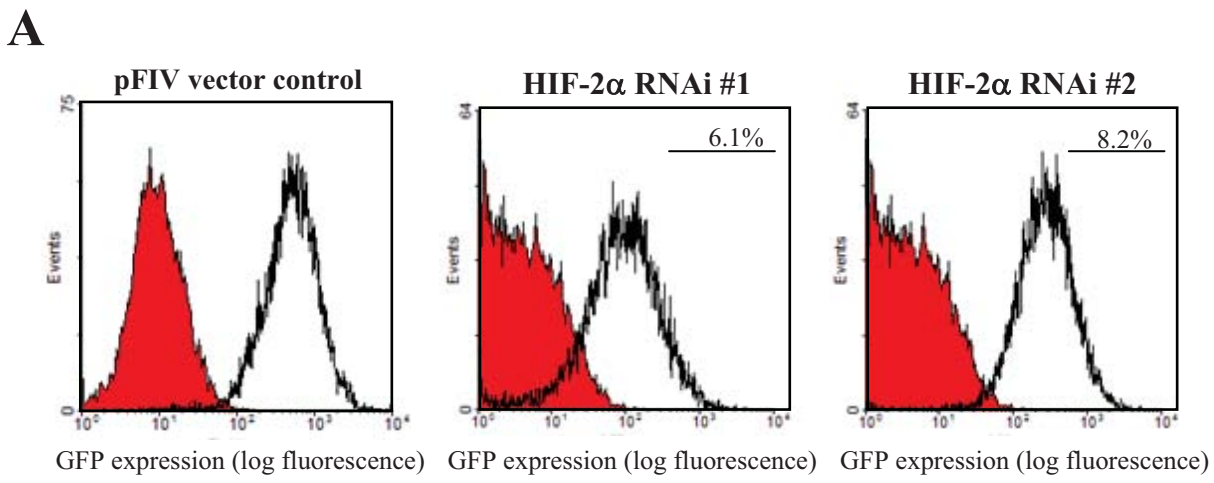
4.2.6 The creation and characterisation of HIF-2α-knockdown in LP-1 cells.

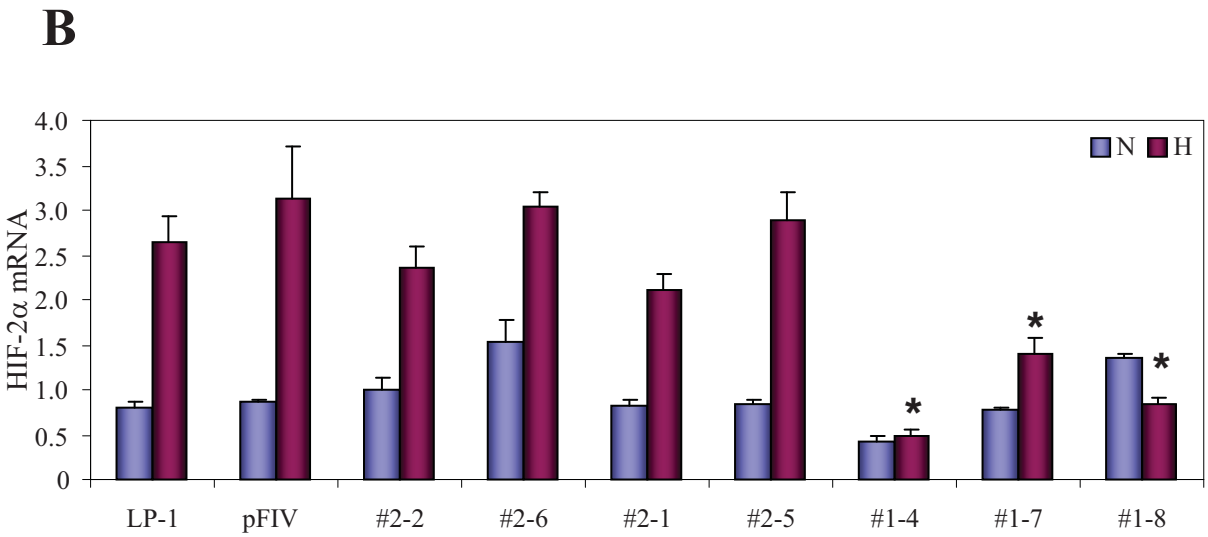
A stable knockdown of HIF-2α in LP-1 cells was created with the pFIV lentiviral vector containing one of two candidate HIF-2α RNAi sequences (designated "#1" and "#2"). Transduced cells were subsequently sorted on the basis of GFP expression, with the top 30% of GFP-expressing cells collected for each cell line (Figure 4.18A). In order to create cell lines harbouring the strongest knockdown of HIF-2α, preparative cell sorting and single cell deposition was used to create clonal populations from the top 6.1% and 8.2% of these sorted HIF-2α #1 and HIF-2α #2 RNAi populations respectively.

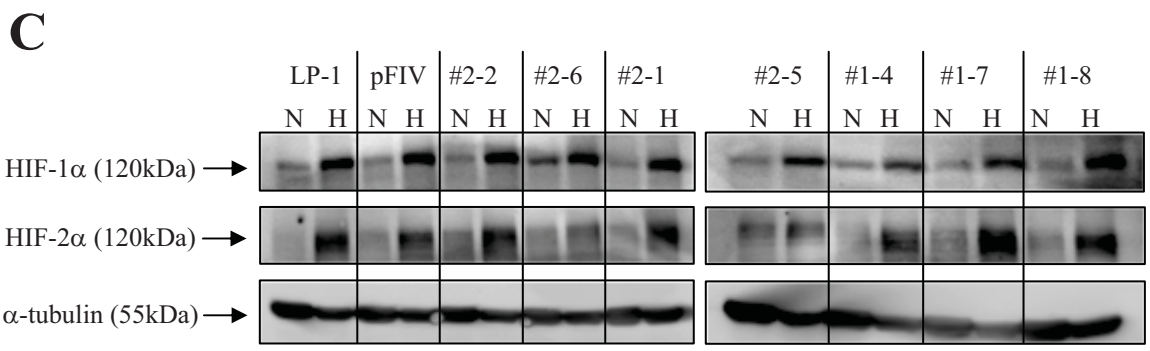
To assess the level of HIF-2α knockdown in these clones, levels of *HIF-2α* mRNA expression were measured using real-time PCR and compared to unmodified LP-1 cells and transduced LP-1-pFIV cells (Figure 4.18B). As expected, these data demonstrated varying degrees of HIF-2α knockdown amongst the HIF-2α RNAi clones (designated #2-2, #2-6, #2-1, #2-5, #1-4, #1-7 and #1-8) compared to LP-1-pFIV. HIF-2α RNAi clones "#2-6" and "#2-5" displayed no difference in HIF-2α expression under normoxic or hypoxic conditions compared to the vector control, while clones "#2-2" and "#2-1" displayed a slight reduction in HIF-2α expression under hypoxic conditions. In contrast, clones "#1-4", "#1-7" and "#1-8" displayed a strong knockdown of *HIF-2α* mRNA expression under hypoxic conditions, with a 50-80% reduction in HIF-2α expression compared to the vector control (Figure 4.18B, $p<0.05$, one-way ANOVA).

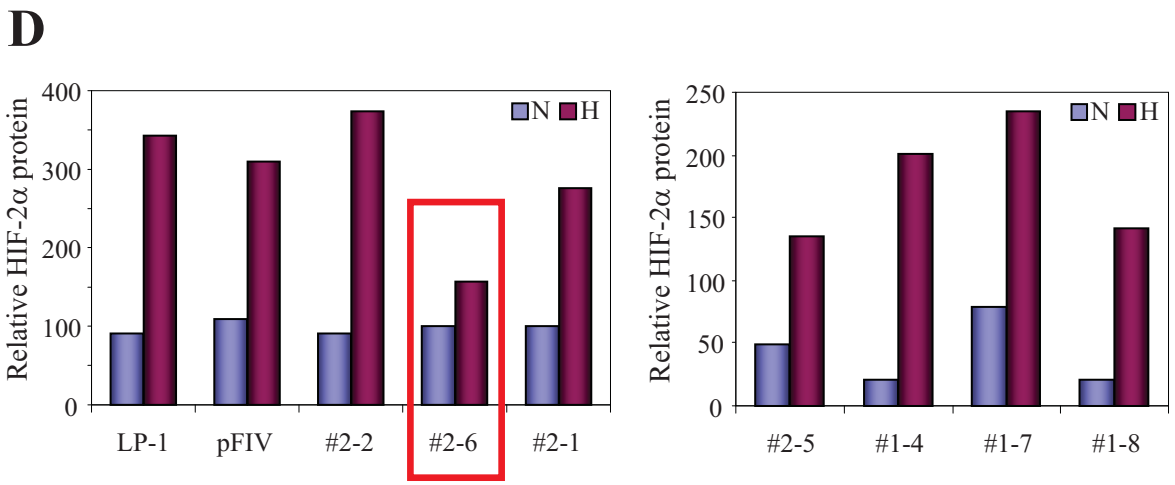
The generation of the HIF-2α knockdown cell line proved somewhat problematic. In order to avoid incorrectly excluding any of the clones based on the mRNA expression data alone, the level of HIF-1α and HIF-2α protein expression under normoxic and hypoxic conditions was assessed in these seven HIF-2α RNAi clones using Western immunoblotting. The unmodified parental LP-1 cell line, the LP-1-pFIV vector control and the seven LP-1-HIF-2α RNAi clones were cultured under normoxic or hypoxic culture conditions for 48 hours, and whole cell lysates were prepared. The 48 hour time point was selected in these studies on the basis of previous detailed time courses which showed that HIF-2α was strongly induced after 48 hours of continuous exposure to hypoxia (see Figure 4.10).

Figure 4.18. The Creation of HIF-2α Knockdown in LP-1 Cells. (**A**) RNA interference technology was used to knock down endogenous HIF-2α expression in LP-1 cells, and the top 30% of lentivirally transduced cells were selected based on the level of GFP expression. Two separate HIF-2α RNAi sequences were used (designated #1 and #2). Single cell clones were then created from the top 6.1% and 8.2% of these sorted GFP-expressing cells respectively. (**B**) Clones were screened for knockdown of *HIF-2α* mRNA expression using real-time PCR, and data normalised to standard housekeeping gene, *β2-microglobulin*. Data are expressed as mean ± standard deviation from replicate samples and are representative of three individual experiments. *$p<0.05$, one-way ANOVA. **(C)** To examine knockdown of HIF-2α protein in these clones, whole cell lysates were prepared from the parental LP-1 cell line, the pFIV vector control and each of the HIF-2α RNAi clones cultured under normoxic or hypoxic conditions for 48 hours. An RCDC protein assay was performed to ensure than an equal amount of protein was loaded in each lane. Lysates were resolved on 8% SDS-PAGE gels under reducing conditions and levels of HIF-2α protein expression measured using Western immunoblotting. Membranes were also probed with anti-HIF-1α to detect any concomitant change in HIF-1α expression, and anti-α-tubulin to confirm equal protein loading in each lane. (**D**) ImageQuant image analysis software was used to quantitate the intensity of the HIF-2α bands relative to the α-tubulin loading control bands. Graphical representation of these data demonstrates differences in HIF-2α protein expression under normoxic (■) and hypoxic (■) conditions in each of the transduced cell lines.


A
pFIV vector control
HIF-2α RNAi #1
HIF-2α RNAi #2
6.1%
8.2%
Events
GFP expression (log fluorescence)
GFP expression (log fluorescence)
GFP expression (log fluorescence)


B
HIF-2α mRNA
N H
LP-1
pFIV
#2-2
#2-6
#2-1
#2-5
#1-4
#1-7
#1-8


C
LP-1
pFIV
#2-2
#2-6
#2-1
#2-5
#1-4
#1-7
#1-8
HIF-1α (120kDa)
HIF-2α (120kDa)
α-tubulin (55kDa)


D
Relative HIF-2α protein
N H
LP-1
pFIV
#2-2
#2-6
#2-1
#2-5
#1-4
#1-7
#1-8

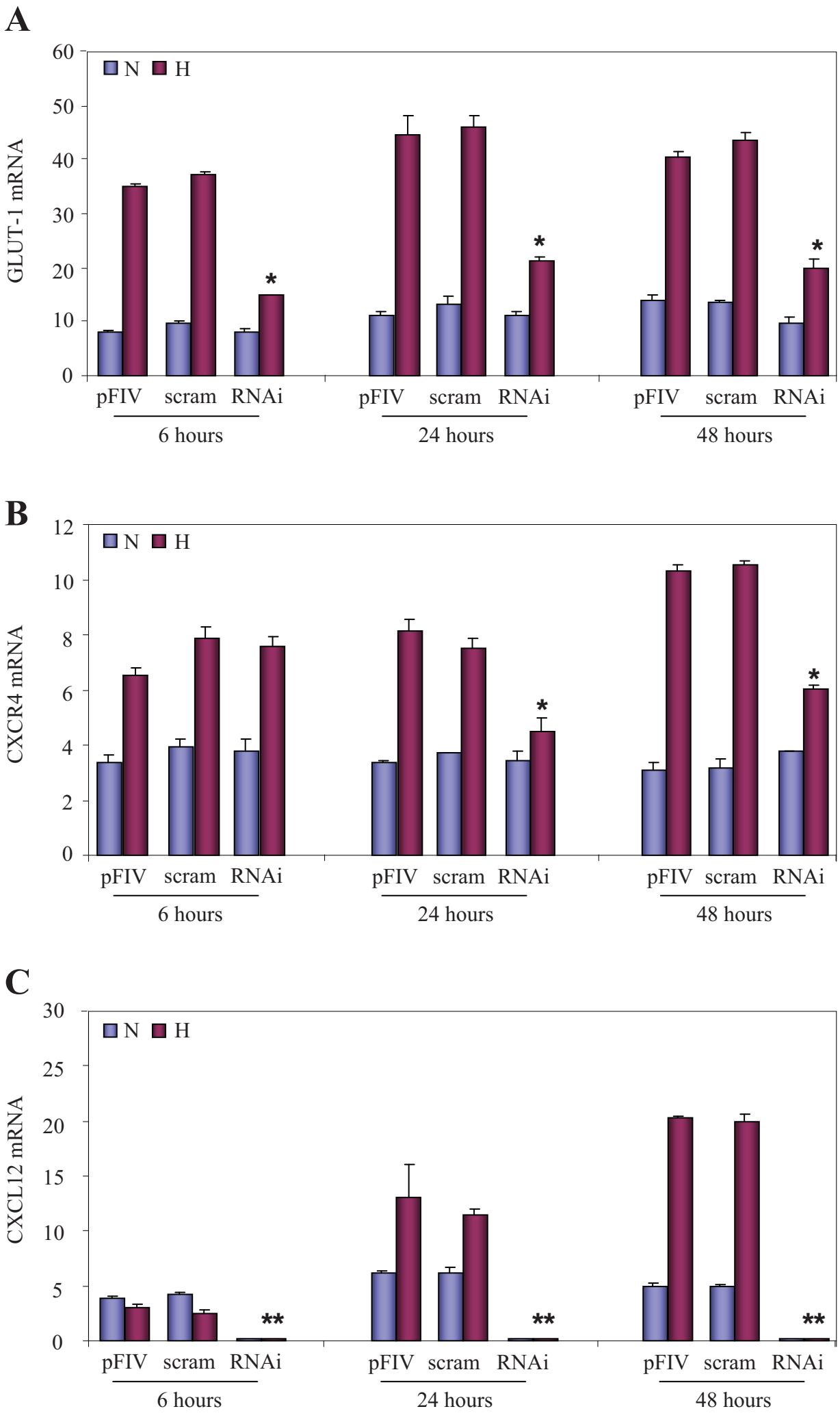
In these Western immunoblots, a strong hypoxic induction of HIF-1α protein was observed in hypoxic lysates prepared from unmodified LP-1 cells, the LP-1-pFIV vector control and each of the seven HIF-2α RNAi clones, compared to their respective normoxic lysates. These findings confirmed that the hypoxic induction of HIF-1α protein unaffected by the introduction of the HIF-2α RNAi, thereby confirming the specificity of the HIF-2α RNAi (Figure 4.18C). Furthermore, a strong hypoxic induction of HIF-2α protein was observed in LP-1, LP-1-pFIV, and HIF-2α RNAi clones #2-2, #2-1, #2-5, #1-4, #1-7 and #1-8 (Figure 4.18C). In contrast, markedly lower levels of HIF-2α protein were observed in HIF-2α RNAi clone"#2-6" under hypoxic conditions (Figure 4.18C). Quantitative analysis of these data confirmed a strong hypoxic induction of HIF-2α protein in the parental LP-1 cell line, LP-1-pFIV, and HIF-2α RNAi clones #2-2, #2-1, #2-5, #1-4, #1-7, #1-8, and a marked reduction in hypoxic HIF-2α protein induction in clone "#2-6" (Figure 4.18D). Despite no evidence of the knockdown of *HIF-2α* mRNA expression in this clone using real-time PCR, clone "#2-6" was selected as the clone harbouring the strongest, specific knockdown of HIF-2α based on the protein expression data. HIF-2α RNAi "Clone #2-6" was used for all subsequent experiments. Hereafter, the HIF-2α RNAi "Clone #2-6" cell line is referred to as LP-1-HIF-2α-KD.

4.2.6.1 The effect of HIF-2α knockdown on GLUT-1, CXCR4 and CXCL12 mRNA expression in LP-1 cells.

To examine the effect of HIF-2α knockdown on downstream HIF target gene expression, LP-1-pFIV, LP-1-scramRNAi and LP-1-HIF-2α-KD cells were cultured under normoxic or hypoxic culture conditions for 48 hours, and cells harvested for total RNA at 6, 24, and 48 hour time points. Using real-time PCR, changes in the mRNA expression of *GLUT-1* (Figure 4.19A), *CXCR4* (Figure 4.19B), and *CXCL12* (Figure 4.19C) were examined.

GLUT-1 mRNA expression was strongly up-regulated by hypoxia in the LP-1-pFIV and LP-1-scramRNAi control cell lines at all time points examined (Figure 4.19A). While a significant reduction in the hypoxic induction of *GLUT-1* expression was observed in LP-1-HIF-2α-KD cells line at each of these time points ($p<0.05$ one-way ANOVA), this reduction was not as marked as that seen in the LP-1-HIF-1α-KD cell line (see Figure 4.16A).

Figure 4.19. Changes in *GLUT-1*, *CXCR4* and *CXCL12* mRNA Expression in Response to HIF-2α Knockdown in LP-1 Cells. LP-1 cells transduced with the pFIV vector control, scrambled RNAi control or the HIF-2α RNAi were cultured in normoxic (■) or hypoxic (■) culture conditions for 48 hours and total RNA was harvested at 6, 24, and 48 hour time points. Levels of (**A**) *GLUT-1*, (**B**) *CXCR4*, and (**C**) *CXCL12* mRNA expression were examined using real-time PCR, and data were normalised to the standard housekeeping gene, *β2-microglobulin*. Graphical representation of the data demonstrates changes in relative levels of mRNA expression under normoxic and hypoxic conditions at each of the time points examined. Data are expressed as mean ± standard deviation from replicate samples and are representative of three individual experiments. *$p<0.05$ and **$p<0.001$, one-way ANOVA.


A
GLUT-1 mRNA
60
50
40
30
20
10
0
N
H
*
*
*
pFIV
scram
RNAi
6 hours
24 hours
48 hours
B
CXCR4 mRNA
12
10
8
6
4
2
0
N
H
*
*
pFIV
scram
RNAi
6 hours
24 hours
48 hours
C
CXCL12 mRNA
30
25
20
15
10
5
0
N
H
**
**
**
pFIV
scram
RNAi
6 hours
24 hours
48 hours

Similarly, *CXCR4* mRNA expression was also up-regulated by hypoxia in LP-1-pFIV and LP-1-scramRNAi cells at all time points examined (Figure 4.19B). Unlike the consistent reduction in the hypoxic up-regulation of *CXCR4* expression previously observed in LP-1-HIF-1α-KD cells (see Figure 4.16B), the hypoxic induction of *CXCR4* was only affected by the HIF-2α RNAi construct at the 24 and 48 hour time points (Figure 4.19B, $p<0.05$, one-way ANOVA).

In accord with earlier studies, a strong induction of *CXCL12* mRNA expression was observed in the LP-1-pFIV and LP-1-scramRNAi cell lines following 24 and 48 hours of hypoxic culture (Figure 4.19C). Strikingly, *CXCL12* expression was almost completely ablated in LP-1-HIF-2α-KD cells under both normoxic and hypoxic conditions at all time points examined (Figure 4.19C, $p<0.001$, one-way ANOVA).

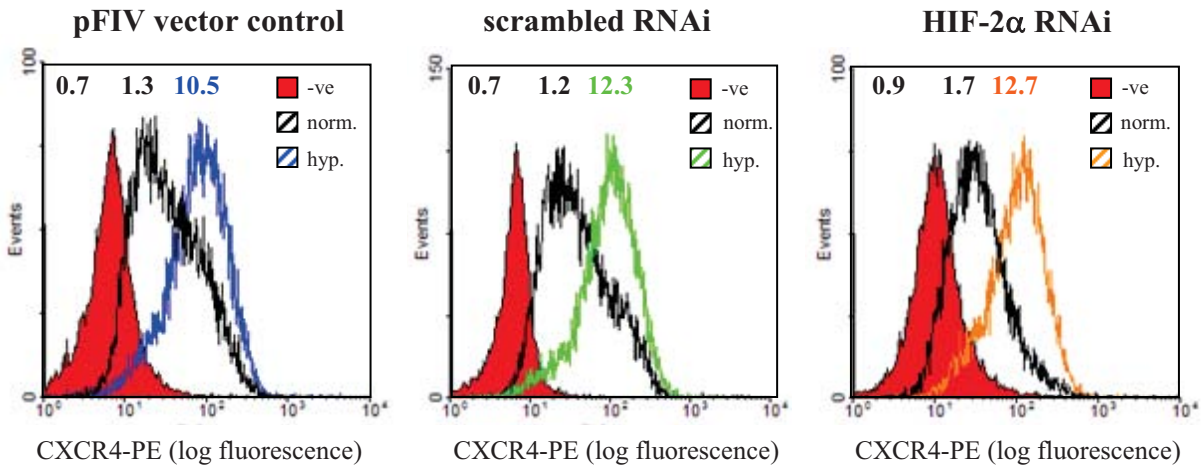
4.2.6.2 The effect of HIF-2α knockdown on CXCR4 and CXCL12 protein expression in LP-1 cells.

Flow cytometry was employed to examine the effect of HIF-2α knockdown on CXCR4 protein expression. LP-1-pFIV, LP-1-scramRNAi and LP-1-HIF-2α-KD cells were cultured under normoxic or hypoxic culture conditions for 24 hours, and levels of CXCR4 protein expression examined. As shown in Figure 4.20A, an 8-fold increase in CXCR4 expression was observed in LP-1-pFIV cells cultured under hypoxic conditions (blue line, MFI=10.5) compared to normoxic conditions (black line, MFI=1.3). Similarly, a 10-fold increase in CXCR4 expression was observed in LP-1-scramRNAi cells under hypoxic conditions (green line, MFI=12.3) compared to normoxic conditions (black line, MFI=1.2). The staining of LP-1-HIF-2α-KD cells revealed a 7.5-fold increase in CXCR4 expression under hypoxic conditions (orange line, MFI=12.7) compared to normoxic conditions (black line MFI=1.7).

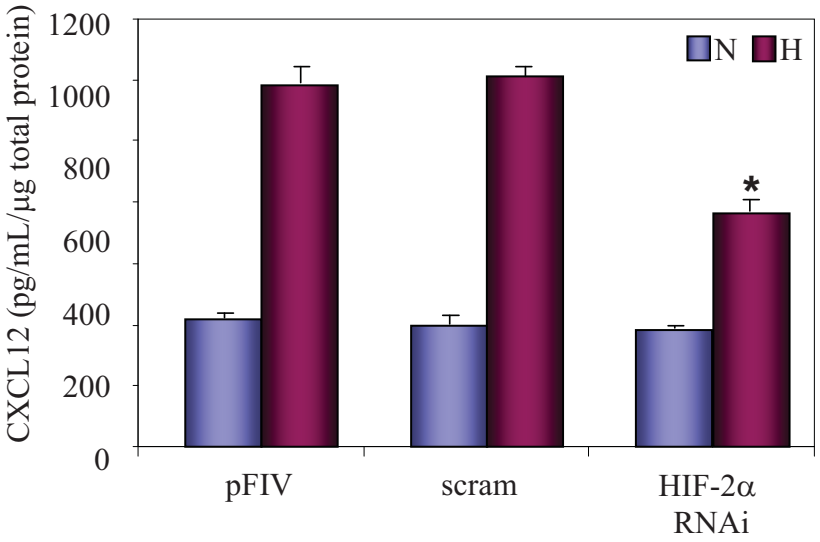
To examine the effect of HIF-2α knockdown on CXCL12 protein expression in MM PCs, the LP-1-pFIV, LP-1-scramRNAi and LP-1-HIF-2α-KD cell lines were cultured for 72 hours under normoxic or hypoxic culture conditions and levels of CXCL12 were measured in the resultant conditioned media using a CXCL12-specific ELISA. Under hypoxic culture conditions, CXCL12 levels were 2.5-fold higher in the LP-1-pFIV vector control (998.5 ± 33 pg/mL) compared to normoxic conditions (401.9 ± 19 pg/mL). Similarly, CXCL12 protein levels were 2.5-fold higher in LP-1-scramRNAi cells under hypoxic

Figure 4.20. Changes in CXCR4 and CXCL12 Protein Expression in Response to HIF-2α Knockdown in LP-1 Cells. (**A**) LP-1 cells transduced with the pFIV vector control, scrambled RNAi control or HIF-2α RNAi were cultured under normoxic or hypoxic conditions for 48 hours, stained with an anti-CXCR4 antibody or an isotype-matched negative control and levels of CXCR4 expression analysed using flow cytometry. The filled histograms ( ) indicate background fluorescence following staining with the isotype-matched negative control antibody. The black unfilled histograms ( ) represent CXCR4 expression in each cell line under normoxic conditions, and the blue, green and orange unfilled histograms (, and) represent CXCR4 expression in each respective cell line under hypoxic conditions. The mean fluorescence intensity for each stain is displayed above each histogram in the corresponding colour. Data from a representative experiment of three is shown. (**B**) Using a CXCL12-specific ELISA, levels of CXCL12 protein expression were measured in conditioned media collected from LP-1 cells containing the pFIV vector control, scrambled RNAi control or the HIF-2α RNAi cultured under normoxic ( ) or hypoxic ( ) culture conditions for 72 hours. Graphical representation of these data shows the changes in CXCL12 protein concentration in the culture supernatant collected from each cell line, normalised to total cellular protein concentration. Data are expressed as mean ± standard deviation from replicate samples of a representative experiment of three. *$p<0.05$, one-way ANOVA.

A


pFIV vector control
scrambled RNAi
HIF-2α RNAi
0.7 1.3 10.5
0.7 1.2 12.3
0.9 1.7 12.7
-ve
norm.
hyp.
Events
CXCR4-PE (log fluorescence)
CXCR4-PE (log fluorescence)
CXCR4-PE (log fluorescence)

B


CXCL12 (pg/mL/µg total protein)
1200
1000
800
600
400
200
0
N
H
*
pFIV
scram
HIF-2α RNAi

conditions (1006.5 ± 29 pg/mL) compared to normoxic conditions (395.7 ± 39 pg/mL). Importantly, a 1.6-fold induction of CXCL12 protein was detected in LP-1-HIF-2α-KD cells under hypoxic conditions (690.7 ± 43 pg/mL) compared to normoxic conditions (389.5 ± 18 pg/mL). Compared to the pFIV vector control, the hypoxic induction of CXCL12 protein in LP-1-HIF-2α-KD cells was significantly reduced ($p<0.05$, one-way ANOVA).

4.2.7 Detailed promoter analysis of the hypoxic induction of CXCL12 in LP-1 cells.

Studies by Ceradini *et al* showed that the CXCL12 proximal promoter harbours two putative HIF binding sites and that the hypoxic induction of CXCL12 expression in HUVECs is mediated by HIF-1 [15]. To date, the role of HIF-2 in the hypoxic regulation of CXCL12 expression has not been examined. In light of the data presented in this Chapter, detailed promoter studies were performed to determine the contribution of HIF-2α in the regulation of CXCL12 in MM PCs.

4.2.7.1 Luciferase reporter assays.

The full proximal CXCL12 promoter (≈1435bp), containing the two putative HIF binding sites as defined by Ceradini *et al* [15], was cloned into the pGL3 basic luciferase reporter vector (abbreviated to pGL3b) to create the CXCL12 promoter construct, pGL3b-CXCL12 (as described in Section 2.7.1, Materials and Methods and Appendix 1). LP-1 cells were transiently transfected with pGL3b-CXCL12 or the empty pGL3b vector alone. Twenty four hours post-transfection, cells were cultured under normoxic or hypoxic culture conditions for a further 48 hours and whole cell extracts were prepared. Using luciferase reporter assays, changes in the activity of the CXCL12 promoter under normoxic and hypoxic conditions were examined.

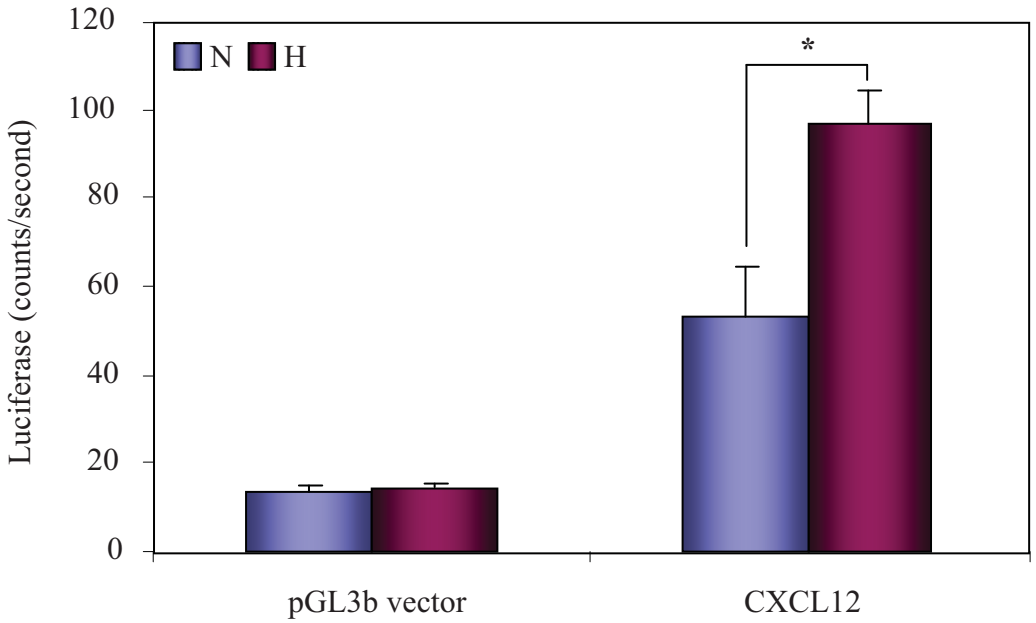
Following the transfection of LP-1 cells with pGL3b-CXCL12, an average luciferase activity of 53.4 ± 10.8 counts/second was detected under normoxic conditions (Figure 4.21A). A 2-fold increase in promoter activity was observed under hypoxic culture conditions (96.8 ± 7.2 counts/second, Figure 4.21A, $p<0.001$, one-way ANOVA).

To further investigate the role of HIF-1 and HIF-2 in the transcriptional regulation of CXCL12 in LP-1 cells, the pGL3b and pGL3b-CXCL12 constructs were transfected into the HIF-over-expressing LP-1 cell lines (LP-1-HIF-1α and LP-1-HIF-2α and the

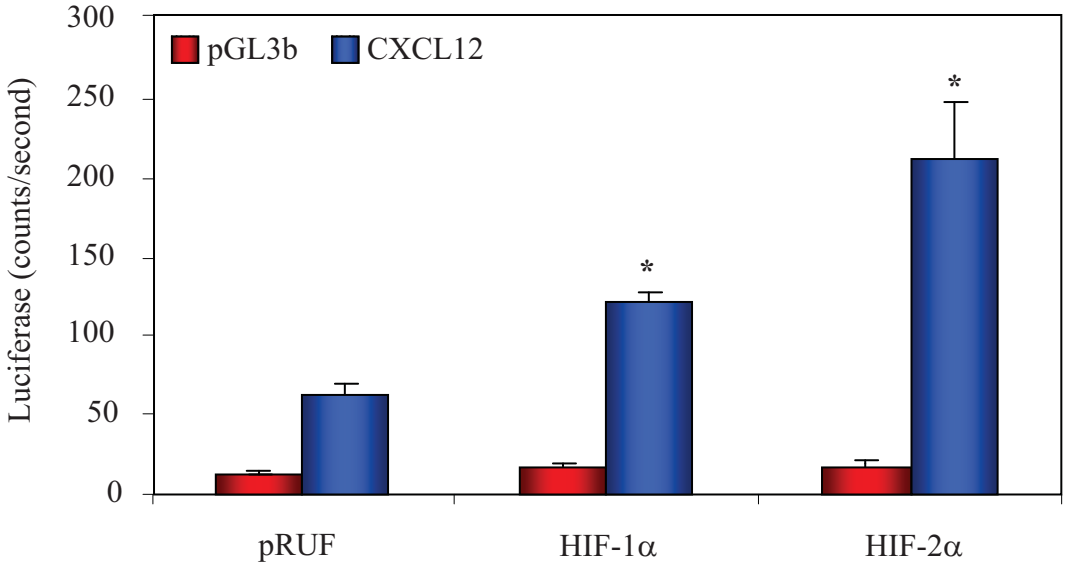
Figure 4.21. Activation of the CXCL12 Promoter Under Hypoxic Conditions. (**A**) LP-1 cells were transiently transfected with a luciferase reporter construct (pGL3b) containing the CXCL12 proximal promoter, or the empty construct alone. Twenty four hours post-transfection, these cells were cultured under normoxic or hypoxic culture conditions for 48 hours and whole cell extracts were prepared. The level of luciferase reporter activity in these normoxic and hypoxic extracts (20μg) was measured using a luciferase assay. Graphical representation of the data illustrates levels of luciferase activity under normoxic ( ) and hypoxic ( ) culture conditions in LP-1 cells harbouring either the pGL3b vector control or the vector containing the CXCL12 promoter. Data are expressed as mean ± standard deviation from quadruplicate samples of a representative experiment of three. (**B**) The HIF-1α- and HIF-2α- over-expressing LP-1 cell lines and the pRUF vector control were transiently transfected with the luciferase reporter construct containing the CXCL12 promoter or the empty reporter construct alone. Twenty four hours post-transfection, these cells were cultured under hypoxic culture conditions for 48 hours and whole cell extracts were prepared. The level of luciferase reporter activity in these hypoxic extracts (20μg) was measured in a luciferase assay. Graphical representation of the data illustrates levels of luciferase activity in cell line harbouring either the vector control ( ) or the CXCL12 promoter ( ). Data are expressed as mean ± standard deviation from quadruplicate samples of a representative experiment of three.

*$p<0.001$, one-way ANOVA.

A


120
100
80
60
40
20
0
Luciferase (counts/second)
N
H
*
pGL3b vector
CXCL12

B


300
250
200
150
100
50
0
Luciferase (counts/second)
pGL3b
CXCL12
*
*
*
pRUF
HIF-1α
HIF-2α

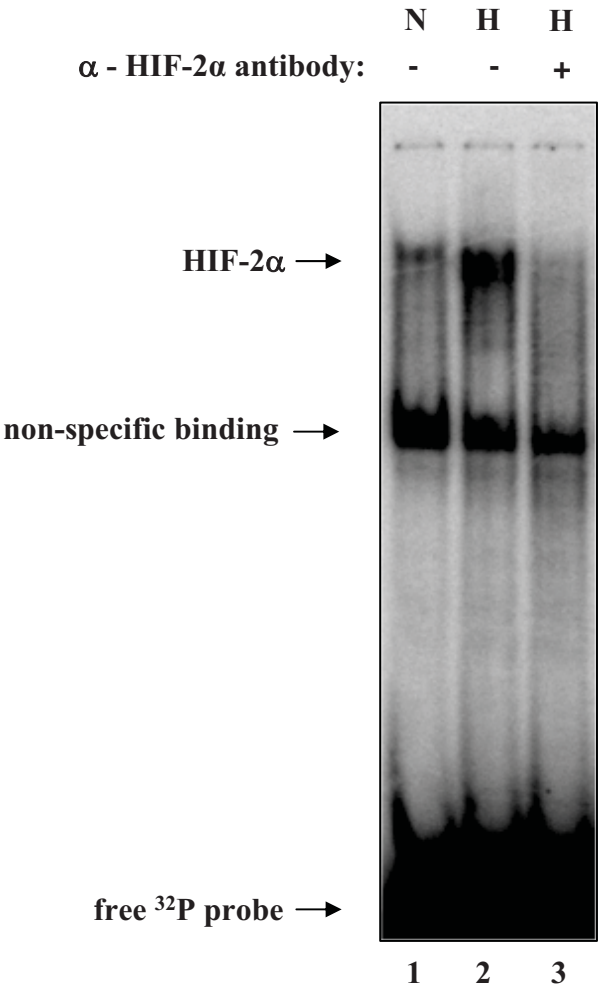
corresponding vector control (LP-1-pRUF). Twenty four hours post-transfection, cells were subjected to hypoxic culture conditions for an additional 48 hours, based on previous data demonstrating that HIF over-expression was only detectable in these cells under hypoxic conditions (see Figure 4.11). As displayed in Figure 4.21B, a 1.9-fold increase in luciferase activity was observed in LP-1-HIF-1α cells (122.4 ± 6.3 counts/second) compared to LP-1-pRUF (63.5 ± 5.9 counts/second), whereas a 3.3-fold increase in luciferase activity was detected in the LP-1-HIF-2α cell line (211.6 ± 36.5 counts/second) compared to LP-1-pRUF. These co-transfection experiments show that, while both HIF-1α and HIF-2α activate the CXCL12 promoter, HIF-2α is a stronger mediator of this response (3.3-fold) than HIF-1α (1.9-fold, $p<0.001$, one-way ANOVA).

4.2.7.2 HIF-2α binds to the CXCL12 promoter.

Having confirmed that HIF-2 mediates the transcriptional regulation of CXCL12 under hypoxic conditions, further experiments were performed to determine whether HIF-2α binds directly to the CXCL12 promoter. The CXCL12 promoter contains two putative HIF binding sites, designated HBS1 and HBS2 and situated at nucleotides -1,238 and -783 respectively [15]. Using serial deletions and mutational analyses, Ceradini *et al* showed that the hypoxic regulation of CXCL12 occurs solely via HBS1, and that HBS2 plays a minimal role in this process [15]. To examine this further in the context of MM PCs, the contribution of both HBS1 and HBS2 in the hypoxic regulation of CXCL12 in LP-1 cells was examined. These experiments confirmed that HBS2 is not involved in this process (data not shown) and therefore was not examined further. All subsequent promoter analyses presented here solely focussed on the activity of HBS1 in the CXCL12 promoter.

The HIF-1 and HIF-2 transcription factors both bind to the same DNA consensus sequence [293,299]. To specifically examine the binding of HIF-2α to the CXCL12 promoter in MM cells, LP-1 cells were cultured under normoxic or hypoxic culture conditions for 48 hours and nuclear extracts prepared for analysis by electromobility shift analysis (EMSA). This time point was selected based on previous Western immunoblot data which showed that HIF-2α is strongly up-regulated by hypoxia at the 48 hour time point (see Figure 4.2). The nuclear extracts were incubated with a P^{32}-labelled oligonucleotide encompassing the HBS1 region of the CXCL12 promoter and analysed by EMSA. In these assays, strong binding of a hypoxia-inducible complex to the HBS1 oligonucleotide under hypoxic conditions was observed (Figure 4.22). HIF-2α was identified within this complex by pre-

Figure 4.22. The Role of HIF-2α in Hypoxia-Inducible Binding to the CXCL12 Promoter. LP-1 cells were cultured under normoxic (N) or hypoxic (H) culture conditions for 48 hours and nuclear extracts prepared. Nuclear extracts (5μg) were bound to a P^{32}-labelled oligonucleotide encompassing HIF Binding Site #1 of the CXCL12 promoter and analysed by EMSA. This representative gel illustrates strong levels of hypoxia-inducible binding (Lane 2), compared to normoxic culture conditions (Lane 1). To detect the presence of HIF-2α within this hypoxia-inducible complex, hypoxic extracts were pre-incubated with a HIF-2α antibody (15μg, Lane 3). No antibody was added to the reactions (denoted by a minus (-) sign) in Lanes 1 and 2.


N H H
α - HIF-2α antibody: - - +
HIF-2α
non-specific binding
free 32P probe
1 2 3

incubating the hypoxic extracts with a HIF-2α antibody, which resulted in a marked reduction in complex formation. To demonstrate the specificity of the HIF-2α antibody, these experiments were repeated with titrating amounts of antibody. Increasing amounts of HIF-2α antibody inhibited complex formation confirming the presence of HIF-2α within the hypoxia-inducible complex (Figure 4.23).

To obtain final, conclusive evidence for the interaction between HIF-2α and the CXCL12 promoter in LP-1 cells, chromatin immunoprecipitation (ChIP) assays were performed. In these assays, LP-1 cells were cultured under normoxic or hypoxic conditions for 48 hours to promote maximal HIF-2α induction, and nuclear extracts prepared. The immunoprecipitation of cross-linked DNA-protein complexes was performed using three different anti-HIF-2α antibodies and their respective isotype-matched negative controls. Using specific primers encompassing the HBS1 region of the CXCL12 promoter, real-time PCR was used to determine the level of HIF-2α binding under normoxic and hypoxic conditions. Data were normalised to the levels of expression in the "pre-IP input" samples and plotted as the fold induction of expression relative to the respective isotype-matched negative control antibodies (Figure 4.24). These ChIP/PCR analyses demonstrated increased HIF-2α binding to the CXCL12 promoter under hypoxic conditions compared to the normoxic conditions using all three of the anti-HIF-2α antibodies tested (*$p<0.05$ and **$p<0.001$, one-way ANOVA). As a control, these experiments were repeated using PCR primers flanking the CXCL12 HBS2, and hypoxia-inducible binding of HIF-2α was not observed (data not shown).

Figure 4.23. The Role of HIF-2α in Hypoxia-Inducible Binding to the CXCL12 Promoter. LP-1 cells were cultured under normoxic (N) or hypoxic (H) culture conditions for 48 hours and nuclear extracts prepared. Nuclear extracts (5μg) were bound to a P^{32}-labelled oligonucleotide encompassing HIF Binding Site #1 of the CXCL12 promoter and analysed by EMSA. This representative gel illustrates strong levels of hypoxia-inducible binding (Lane 2), compared to normoxic culture conditions (Lane 1). To demonstrate the specificity of the HIF-2α antibody in detecting HIF-2α within the hypoxia-inducible complex, hypoxic extracts were pre-incubated with increasing suboptimal concentrations of HIF-2α antibody (5μg and 10μg, Lanes 3 and 4 respectively). No antibody was added to the reactions (denoted by a minus (-) sign) in Lanes 1 and 2.

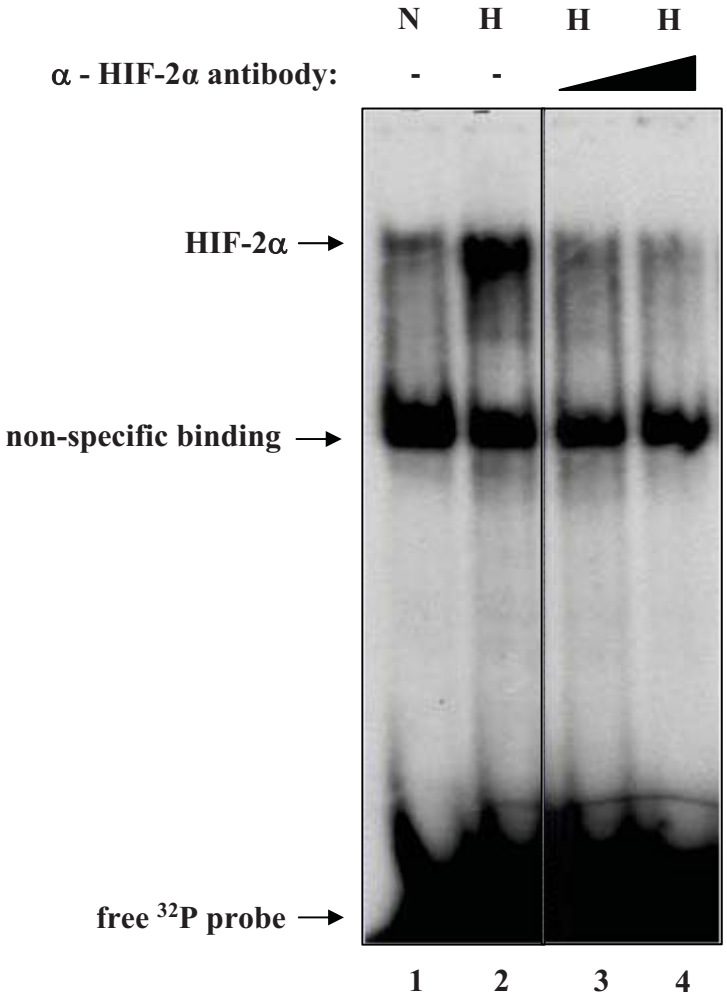
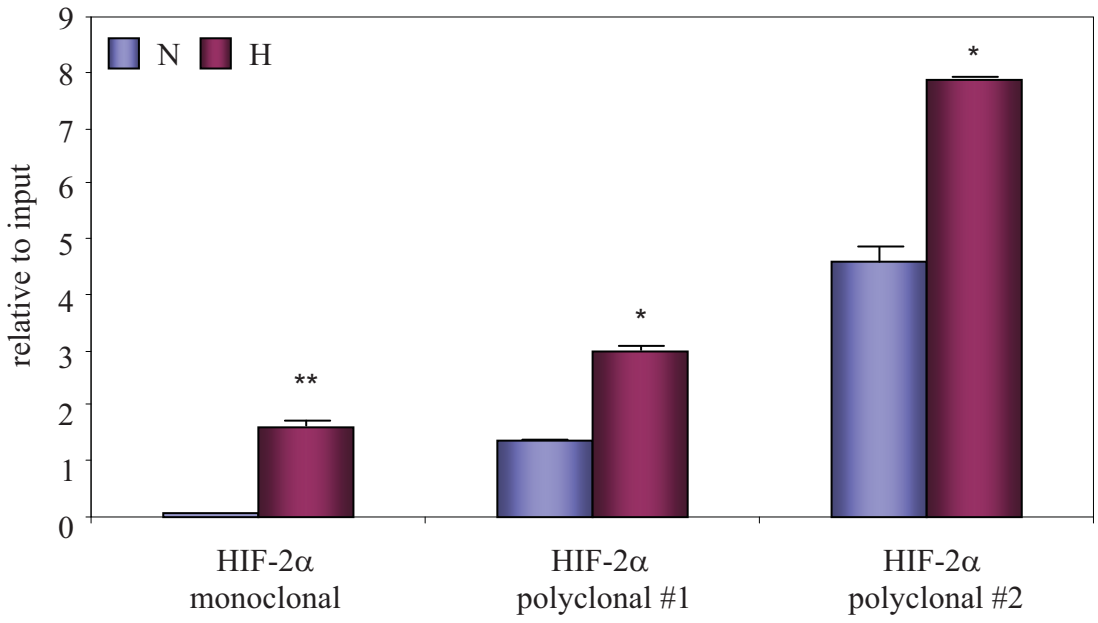

N
H
H
H
α - HIF-2α antibody:
-
-
HIF-2α
non-specific binding
free 32P probe
1
2
3
4

Figure 4.24. HIF-2α Binds to the CXCL12 Promoter in LP-1 Cells Under Hypoxic Conditions. The human MM cell line LP-1 was cultured in normoxic ( ) or hypoxic ( ) culture conditions for 48 hours and cross-linked nuclear DNA-protein complexes prepared using a commercial ChIP kit. Immunoprecipitations of the resultant complexes were performed using various antibodies directed against HIF-2α, and their isotype-matched negative controls. Co-precipitated DNA fragments were detected using real-time PCR with primers specific for the HIF Binding Site #1 region of the CXCL12 promoter, and data were normalised to the endogenous expression of this region in the respective "pre-IP" input samples. Data are expressed as fold induction of the normalised HRE signal compared to the respective isotype-matched negative control ± standard deviation from replicate samples.

*$p<0.05$ and **$p<0.001$, one-way ANOVA.


9
8
7
6
5
4
3
2
1
0
relative to input
N
H
**
*
*
HIF-2α
monoclonal
HIF-2α
polyclonal #1
HIF-2α
polyclonal #2

4.3 Summary and Discussion

As demonstrated in Chapter 3, CXCL12 is associated with increased BM angiogenesis in MM patients and strongly induces EC tube formation *in vitro*. A recent pioneering publication by Ceradini *et al* [15] demonstrated that CXCL12 expression is up-regulated by hypoxia, a finding which has particular relevance to MM given the hypoxic nature of the BM microenvironment [366,367]. Subsequent studies by others have demonstrated that hypoxia regulates CXCL12 in numerous cell types [247,387-391] and that distinct hypoxic niches throughout the BM are associated with increased levels of CXCL12 [163]. Interestingly, the expression of the CXCL12 receptor, CXCR4, is also regulated by hypoxia in many different cell types [229,232,389,393-403]. Given the key role of the CXCL12/CXCR4 axis in MM biology, we investigated whether hypoxia regulates CXCL12 and/or CXCR4 expression in MM PCs, and examined the relative roles of the HIF-1 and HIF-2 transcription factors in mediating these responses.

To date, the expression of HIF-1 and HIF-2 and the relative contribution of these proteins to hypoxia-induced gene expression have not been studied in the context of MM. In preliminary screens of eight widely-used MM PC lines, variable HIF-1α and HIF-2α protein expression profiles were observed. This was most evident with respect to HIF-1α expression, with 3/8 cell lines exhibiting constitutively high endogenous levels of HIF-1α and 1/8 displaying a total absence of HIF-1α protein expression. In addition, the temporal regulation of HIF-1α induction under hypoxic conditions varied considerably between the cell lines, with 3/8 displaying an early induction at the 6 hour time point and 5/8 displaying a delayed induction at the 24 and/or 48 hour time points. In contrast, the hypoxic induction of HIF-2α protein expression was more consistent between the MM cell lines, and was generally up-regulated in response to prolonged exposure to hypoxia. This was demonstrated by the fact that 6/8 cell lines displayed a strong hypoxic up-regulation of HIF-2α protein after 48 hours.

The hypoxic regulation of *CXCR4* and *CXCL12* mRNA expression in these MM PC lines was also examined. With the exception of the RPMI-8226 cell line, *CXCR4* expression was up-regulated by hypoxia in all MM PC lines. In contrast, the regulation of *CXCL12* expression was much more varied, with 2/8 displaying an early up-regulation at 6 hours and 4/8 exhibiting a delayed up-regulation at 24 and/or 48 hours. Furthermore, 3/8 cell

lines displayed a down-regulation of *CXCL12* expression in at least one of the three time points examined.

Considering the marked heterogeneity in MM PC morphology, immunophenotype and cytogenetics between patients [17-20], it was not surprising that differences in the expression and hypoxic regulation of the HIFs and their downstream target genes were observed in these cell lines. The main aim of these preliminary screens was to identify a suitable cell line with which to investigate the mechanisms of hypoxic regulation of CXCL12 expression in MM PCs. Based on the strong and consistent hypoxic regulation of HIF-1α and HIF-2α protein and their downstream target genes, the LP-1 cell line was selected.

Previous studies examining the hypoxic up-regulation of CXCL12 have shown this to occur relatively quickly, with an increase in CXCL12 mRNA and protein expression observed in response to as little as three or four hours of hypoxic exposure [15,247,387,460]. In contrast, studies presented here show that at least 24 hours of continuous exposure to hypoxic conditions was required to induce *CXCL12* mRNA expression in LP-1 cells. There are many possible explanations for this delayed response, the most obvious of which is that the hypoxic induction of CXCL12 occurs indirectly via an unknown, secondary factor. Alternatively, the delayed response may also reflect differences in the relative contribution of the HIF-1 and HIF-2 transcription factors. Given that previous studies have demonstrated that HIF binds directly to the CXCL12 promoter to mediate the up-regulation of this protein [15,391], we examined the relative contribution of the HIF-1 and HIF-2 transcription factors. Closer investigation of the kinetics by which hypoxia modulates HIF-1α and HIF-2α expression in LP-1 cells revealed that while HIF-1α is rapidly induced by exposure to hypoxic conditions, the induction of HIF-2α is a delayed response. These data suggest that in LP-1 cells, HIF-1 is predominantly responsible for mediating immediate, acute responses to hypoxia, while HIF-2 is responsible for mediating adaptive responses to prolonged, chronic hypoxic exposure. This phenomenon has been previously reported in the PC12 rat adrenal pheochromocytoma cell line [327], HeLa human cervical cancer cell line [327], and the SK-N-BE(2)C and KCN-69n human neuroblastoma cell lines [328].

The distinct kinetics of HIF-1α and HIF-2α protein expression under hypoxic conditions may relate to differences in the presence and/or activity of the enzymes that control HIF

degradation. The regulation of cellular HIF-1α and HIF-2α protein levels is controlled by PHD enzymes which, under normoxic conditions, hydroxylate specific prolyl residues within the ODD domain of the HIF α-subunits [317,319,320]. This facilitates the binding of VHL and the subsequent proteasomal degradation of the HIF-α protein [312,321,322]. Each of the three human PHD isoforms identified to date (PHD1, PHD2, and PHD3) display varying affinities for the hydroxylation of target proteins, with PHD2 displaying the highest specificity for hydroxylation of HIF-1α under normoxic conditions [316]. Furthermore, the expression of PHD1, PHD2 and PHD3 has been shown to be differentially induced by exposure to varying degrees of hypoxia [328,461]. PHD1 expression is unaffected by oxygen concentration, whereas PHD2 and PHD3 are progressively induced under hypoxic conditions over time. The different induction kinetics of HIF-1α and HIF-2α protein expression following hypoxic exposure might therefore reflect the expression levels and relative affinities of these HIF-regulating PHD enzymes. However, further studies are required to investigate this hypothesis.

The contrasting induction patterns of HIF-1α and HIF-2α under hypoxic conditions raised the possibility that the delayed induction of CXCL12 expression in LP-1 cells may be mediated by HIF-2, which is induced in response to prolonged hypoxic exposure. Of the reports showing that CXCL12 expression is regulated by hypoxia, only studies by Ceradini *et al* [15] and Tabatabai *et al* [391] examined the mechanism by which this occurs and both identified HIF-1 as the predominant mediator of this response. In these studies, a HIF-1α RNAi construct was used to demonstrate that the increased activity of the HRE regions of the CXCL12 promoter under hypoxic conditions was attributable to HIF-1. However neither group directly examined the contribution of HIF-2 to this process, nor did they consider the fact that HIF-1 and HIF-2 bind to the same consensus sequence [293,299]. In light of our data showing that HIF-1α and HIF-2α are differentially regulated by hypoxia in LP-1 cells, we examined the role of both HIF-1 and HIF-2 in the hypoxic regulation of CXCL12.

To do this, we generated stable LP-1 cell lines in which HIF-1α or HIF-2α were either constitutively over-expressed or knocked down. Surprisingly, while constitutive HIF over-expression was readily detectable at the mRNA level in both cell lines under normoxic conditions, we were unable to detect this at the protein level. Instead, short-term culture under hypoxic conditions was necessary to observe elevated HIF-1α or HIF-2α protein

expression, relative to the LP-1-pRUF vector control. These findings suggested that the level of HIF over-expression in these cells was insufficient to saturate the endogenous degradative hydroxylation machinery. Considering that cells expressing the highest level of GFP (and therefore the transgene) were selected during the generation of these cell lines, the inability to detect abundant HIF protein under normoxic conditions was somewhat surprising. Stable, constitutive over-expression of HIF-1α and HIF-2α might not be tolerated by LP-1 cells and therefore, while the highest expressing cells were initially selected, perhaps only cells in which HIF was moderately over-expressed survived. However, it should be noted that routine flow cytometric analyses of these cells did not reveal any substantial decrease in GFP expression and more importantly, routine PCR analyses did not detect any change in the level of *HIF* mRNA expression in these cells.

An alternative and more plausible explanation for the inability to detect HIF over-expression under normoxic conditions is the knowledge that the hydroxylase enzymes which target HIFs for degradation under normoxic conditions are themselves HIF targets, and are up-regulated by hypoxia [462]. The forced over-expression of the HIFs in LP-1 cells may initiate a negative feedback loop, whereby the increased HIF expression also up-regulates the expression of the degradative machinery, which in turn decreases HIF expression via enhanced proteasomal degradation. Unfortunately, even if transient HIF over-expression was a potential solution to these problems, this was not a feasible solution because (a) LP-1 are refractory to conventional transfection methods (maximum transfection frequency of 20% achievable via electroporation), and (b) CXCL12 expression is up-regulated in response to long-term exposure to hypoxia, making transient transfection less useful. Despite the inability to detect HIF over-expression at the protein level in these transduced cell lines under normoxic conditions, downstream HIF target genes were consistently activated, indicating that the levels of HIF over-expression were sufficient for the induction of downstream target genes.

Given the vast number of downstream HIF target genes, it was anticipated that constitutive over-expression of HIF-1α and HIF-2α in LP-1 cells would exert significant changes to cellular behaviour. Surprisingly however, no significant difference was observed in terms of cell viability or rate of proliferation in LP-1-HIF-1α and LP-1-HIF-2α, compared to the LP-1-pRUF vector control. Interestingly however, the over-expression of HIF-2α did have

a marked effect on cell morphology: the cells appeared noticeably larger and displayed a propensity to grow in clumps rather than as a single-cell suspension, suggesting that HIF-2 may regulate cell surface adhesion molecule expression. In contrast, no visible morphological difference was observed in the HIF-1α over-expressing LP-1 cells. The ability of HIF-2α over-expression to promote cellular hypertrophy has been previously reported in transfected neuroblastoma cells [463], however the precise mechanisms responsible for this remain unknown.

Detailed studies examining the effect of HIF-1α- or HIF-2α- over-expression on downstream target gene activation in LP-1 cells revealed that the hypoxic up-regulation of CXCR4 and CXCL12 in these cells is predominantly mediated by HIF-1 and HIF-2, respectively. The role of HIF-1 in the hypoxic up-regulation of CXCR4 expression has been previously reported in a number of different cell types, including mouse embryonic fibroblasts [394], haemangioblastomas [389], clear cell renal cell carcinoma [389], non-small cell lung cancer [403], proximal renal tubular epithelia [393], and human embryonic kidney cells [393]. However, the identification of HIF-2 as the predominant mediator of CXCL12 regulation under hypoxic conditions is a novel, un-published observation.

To complement the data provided by the HIF over-expression studies, RNAi studies were performed to examine the effect of knocking down the expression of HIF-1α or HIF-2α in LP-1 cells. These studies were particularly pertinent given that previous studies have shown that HIF-1α and HIF-2α can interact reciprocally, with the over-expression or suppression of one HIF isoform mediating a concomitant down-regulation or up-regulation in the expression of the other [458,459]. This reciprocality has the potential to confound issues when individual HIF isoforms are over-expressed or suppressed in a particular cell, as it is difficult to ascertain whether the resultant effects are actually due to the over-expression/suppression itself, or due to compensatory changes in the expression of the other isoform. In this study, several attempts were made to create an LP-1 cell line in which both HIF-1 and HIF-2 were stably over-expressed or knocked down, however due to technical difficulties associated with the sequential retroviral infection of LP-1 cells with three vectors (all cell lines created for this project were transduced with the SFG-$_{NES}$-TGL luciferase vector for *in vivo* bioluminescence monitoring and either a pRUF-IRES-GFP over-expression vector or pFIV-H1-cop-GFP RNAi vector), these attempts were abandoned. Instead, RNAi was used to knock down the expression of HIF-1α or HIF-2α

individually in the LP-1 cell line and the downstream effects of these interventions examined.

In order to achieve optimal levels of each HIF knock down, clonal cell lines were created from the highest GFP-expressing cells. Due to the vast number of validated HIF-1α-specific target genes, it was relatively simple to validate HIF-1α RNAi clones and identify those exhibiting the greatest knockdown. However, the creation and validation of HIF-2α RNAi clones was problematic, and required several attempts using different RNAi constructs. A number of recent studies have indicated that genes such as VEGF [459], adrenomedullin [300], myomesin-2 [464], IL-6 [300], lysyl oxidase [464], erythropoietin [298,465,466], growth-related oncogene-2 (GRO-2) [300], transforming growth factor-α (TGF-α) [459,467], PHD3 [468], DEC-1 [328], and NDRG-1 [300] may be HIF-2α-specific target genes. However subsequent studies have found that this specificity is largely cell-type related and to date, no universally exclusive HIF-2 target gene has been identified. Unfortunately, when the hypoxic regulation of each of these candidate genes was tested in the LP-1 cell line, none were found to be HIF-2α-specific targets. Therefore, without a positive control gene to assess changes in downstream HIF-2α target gene expression, it was extremely difficult to conclusively identify suitable HIF-2α RNAi clones. In addition, discrepancies between levels of *HIF-2α* mRNA expression and HIF-2α protein expression were encountered during the validation process. Therefore, the HIF-2α RNAi clone chosen for further study were selected on the basis of a knockdown in HIF-2α protein expression as this was deemed the most functionally-relevant evidence.

The introduction of the HIF-1α RNAi into LP-1 cells mediated a marked reduction in the hypoxic up-regulation of *CXCR4* mRNA, and reduced the hypoxic induction of CXCR4 protein expression by approximately 50%. These data, in conjunction with the previous over-expression data, confirm the role of HIF-1 in regulating CXCR4 expression. While the introduction of the HIF-2α RNAi into LP-1 cells decreased the hypoxic induction of *CXCR4* mRNA expression at some time points, this was not observed at the protein level and therefore we concluded that HIF-2 is not involved in the hypoxic up-regulation of CXCR4 expression in LP-1 cells.

The RNAi-mediated suppression of HIF-1α or HIF-2α in LP-1 cells each resulted in a marked decrease in the hypoxic up-regulation of *CXCL12* mRNA expression, and an approximate 50% decrease in the hypoxic up-regulation of CXCL12 protein expression. These findings support earlier observations which suggest that while both HIF-1 and HIF-2 are capable of regulating CXCL12 expression under hypoxic conditions, HIF-2 is the predominant HIF mediating this response. Considering that the suppression of HIF-2α in LP-1 cells almost completely ablated endogenous *CXCL12* mRNA expression, it was surprising that the reduction of CXCL12 protein expression was not more pronounced. This finding suggests that the hypoxic induction of CXCL12 may involve translational and post-translational mechanisms of CXCL12 regulation. Enzymes such as MMP-2 [469], MMP-9 [469], CD26/dipeptidyl peptidase IV [470,471], cathepsin [472], and leukocyte elastase [473] cleave CXCL12 *in vitro* to generate distinct, N-terminally truncated isoforms. However, relatively little is known about the physiological post-translational processing of CXCL12 *in vivo*, and further studies are required to investigate this.

Using a luciferase reporter assay system, changes in the activity of the CXCL12 promoter activity under hypoxic conditions were measured in LP-1 cells. A 2-fold increase in the activity of the CXCL12 promoter was detected under hypoxic conditions. Providing further evidence of the importance of HIF-2 in mediating the hypoxic up-regulation of CXCL12, luciferase assays performed using the HIF over-expressing cell lines revealed a 3.3-fold increase in activity of the CXCL12 promoter in LP-1-HIF-2α cells compared to LP-1-pRUF, and a 1.9-fold increase in promoter activity in LP-1-HIF-1α cells. These luciferase reporter assays showed that the hypoxic regulation of CXCL12, mediated predominantly by HIF-2, occurs at the transcriptional level.

The HIF-1 and HIF-2 transcription factors bind to the same DNA consensus sequence [293,299]. To investigate whether HIF-2 binds to the CXCL12 promoter in MM cells, EMSAs were performed. Strong binding of a hypoxia-inducible complex to the HBS1 oligonucleotide under hypoxic conditions was observed, and a reduction in this binding was observed following the addition of HIF-2α antibody, thereby confirming the presence of HIF-2α within this complex. These data showed that HIF-2α is able to bind to the CXCL12 promoter. Conclusive evidence of HIF-2α binding to the CXCL12 promoter in LP-1 cells was provided by ChIP analyses, which showed that HIF-2α mediates the up-regulation of CXCL12 expression under hypoxic conditions in LP-1 cells via binding to

the HBS1 region of the promoter. Parallel ChIP assays also demonstrated that HIF-2α does not bind to the HBS2 region of the CXCL12 promoter. These studies show, for the first time, that HIF-2α binds directly to the CXCL12 promoter and that HBS1 is the primary HIF-2α binding site.

As summarised in Figure 4.25, the studies presented in this chapter extend the findings of the previous chapter and show that hypoxia is a strong regulatory stimulus for aberrant CXCL12 expression in MM PCs. In agreement with observations previously reported in other cell systems, HIF-1 and HIF-2 was found to activate the same gene (in this case, CXCL12) but at different times, corresponding to the differential induction of HIF-1 and HIF-2 by acute or chronic exposure to hypoxia, respectively [328]. Furthermore, these studies demonstrate for the first time that HIF-2 is the predominant regulator of hypoxic induction of CXCL12 expression in MM PCs, and occurs in response to prolonged exposure to low oxygen concentrations.

At present, the biological significance of hypoxic up-regulation of both a ligand and its receptor in the same cell is unknown. Studies by Zagzag *et al* [389] demonstrated that both CXCR4 and CXCL12 are also aberrantly expressed in haemangioblastoma and clear-cell renal carcinoma due to a loss of the VHL tumour suppressor gene which, incidentally, is required for oxygen-dependent degradation of the HIFs. In any case, given the importance of the CXCL12/CXCR4 axis in MM biology and disease pathogenesis, the potentiation of these autocrine signalling pathways in MM PCs could have important implications in the pathogenesis of MM. In relation to this project, the most relevant of these implications is the effect on angiogenesis: does the increased CXCL12 production by MM PCs stimulate an increase in angiogenesis by acting on ECs within nearby vasculature? To investigate this, studies presented in the next chapter were performed to examine the role of CXCL12 in mediating angiogenesis in an *in vivo* mouse model. Furthermore, the contribution of HIF-1 and HIF-2 to this process was also investigated using the HIF over-expressing LP-1 cell lines created in this chapter.

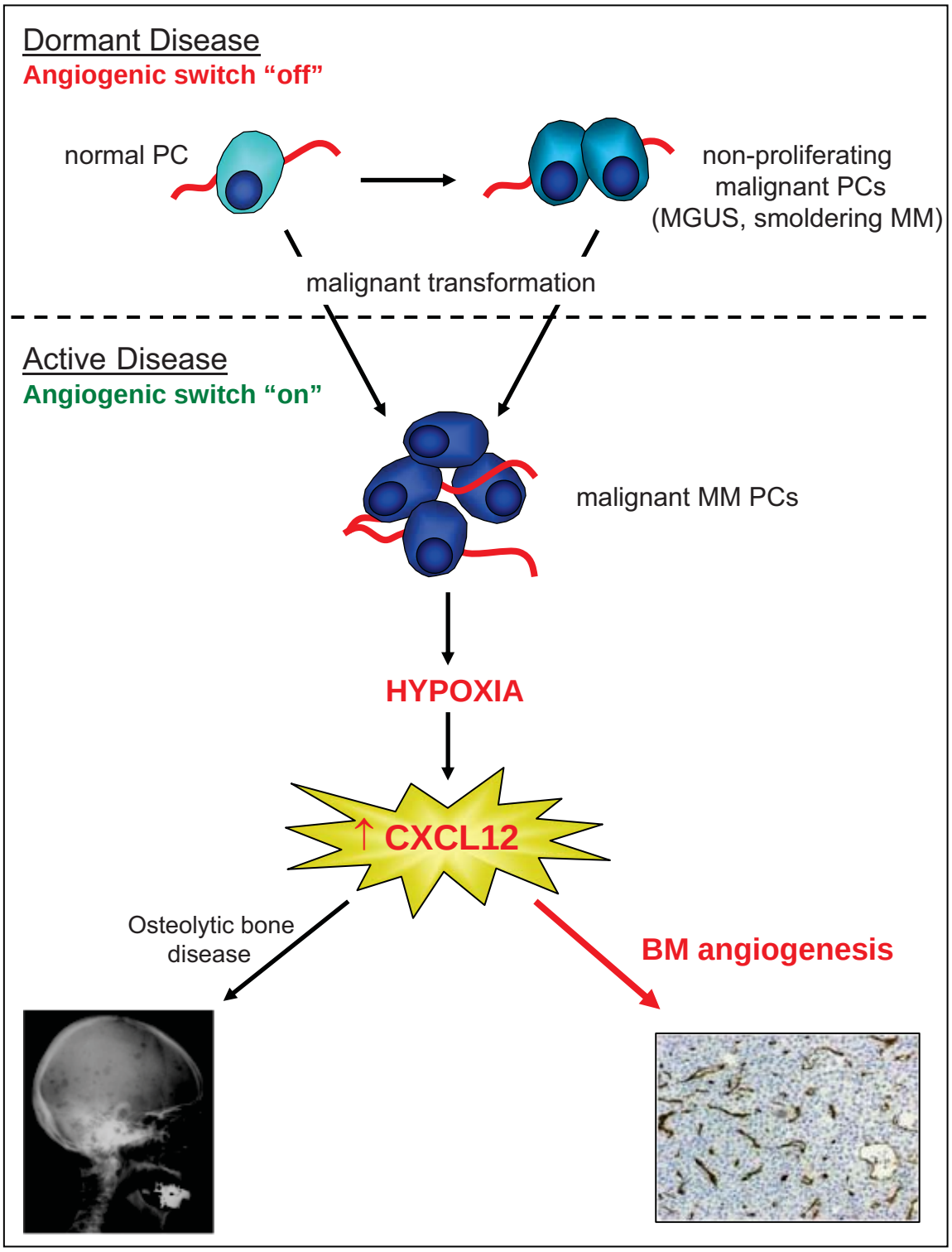


Figure 4.25. Key Findings Arising From This Chapter. As was demonstrated in the previous chapter, aberrant MM PC expression of CXCL12 is associated with BM angiogenesis in MM patients. In the current chapter, the hypothesis that the hypoxic nature of the BM microenvironment mediates this aberrant CXCL12 expression was examined. These studies revealed that MM PC expression of CXCL12 is up-regulated by hypoxia. In contrast to previous findings which have implicated HIF-1 as the mediator of this effect, HIF-2 was found to be the predominant regulator of CXCL12 expression in MM PCs.