

**Role of the ROS/HIF/HMHA1 axis
activated by reoxygenation after irradiation
in the invasion of ex-hypoxic cancer cells**

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Abstract

In solid tumours, rapid cancer cell proliferation and aberrant blood vessel vasculatures give rise to regions with insufficient supply of oxygen. Tumour cells in these low-oxygen (hypoxic) regions are known to acquire malignant properties like invasiveness and therapy resistance, contributing to the poor prognosis of cancer patients. Previously, it has been reported that after the severely hypoxic tumour cells in the perinecrotic regions of tumour tissues survive radiotherapy, they are reoxygenated and translocate towards tumour blood vessels in a hypoxia-inducible factor (HIF)-dependent manner, and thus contribute to post-radiotherapy recurrence and distant metastasis. However, the detailed molecular mechanism underlying HIF-dependent post-irradiation invasion of hypoxic cancer cells was not fully explored. In this study, genome-wide microarray analysis and RNA-Seq analysis were performed to identify genes related to both hypoxia and invasion and identified *HMHA1* (human minor histocompatibility antigen 1) as a potential responsible factor. It was found that the expression of *HMHA1* gene was induced at the transcription level under hypoxic conditions in a HIF-dependent manner. Moreover, transwell invasion assay revealed that the induction of *HMHA1* expression under hypoxia is required for the enhanced invasion activity of hypoxic cancer cells. It was shown from a gain-of-function approach that *HMHA1*, apart from regulating actin filament organisation, enhances extracellular MMP (matrix metalloproteinase) activities to promote cancer cell invasion. In addition, analysis using The Cancer Genome Atlas (TCGA) database demonstrated that *HMHA1* expression is associated with the high expression of hypoxia-inducible genes, which predicts poor survival outcomes of cancer patients. Furthermore, post-radiotherapy reoxygenation was recapitulated *in vitro* by re-exposing irradiated hypoxic cells to oxygen, and this caused a further increase in the expression levels of *HMHA1* in reactive oxygen species (ROS)- and HIF-dependent manners. This increase in turn augmented the invasiveness of the irradiated ex-hypoxic cancer cells. Taken together, these results depicted a potential molecular mechanism *via* which ex-hypoxic cancer cells enhance invasive potential after radiotherapy and reoxygenation by activating the ROS/HIF/*HMHA1* axis.

List of Abbreviations

Ab	Antibody
ACTB	β -actin
ANGPTL4	Angiopoietin Like 4
ARNT	Aryl hydrocarbon receptor nuclear translocator
bHLH	Basic helix-loop-helix
BSA	Bovine serum albumin
CA9	Carbonic anhydrase 9
CBP	CREB-binding protein
ChIP	Chromatin immunoprecipitation
CREB	cAMP response element-binding protein
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CTC	Circulating tumour cell
DFO	Deferoxamine
DMEM	Dulbecco's Modified Eagle Medium
DMOG	Dimethyloxalylglycine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EMT	Epithelial-mesenchymal transition
EV	Empty vector
EPAS1	Endothelial PAS domain protein 1
Epo	Erythropoietin
FBS	Fetal bovine serum
FFPE	Formalin-fixed paraffin-embedded
FIH	Factor Inhibiting HIF
GAP	GTPase-activating protein
GDP	Guanosine diphosphate
GEO	Gene Expression Omnibus
γ H2AX	Phosphorylated H2A histone family member X
GTP	Guanosine triphosphate
HCl	Hydrochloric acid
H & E	Hematoxylin and Eosin
HGF	Hepatocyte growth factor
HIF	Hypoxia-inducible factor
HMHA1	Human minor histocompatibility antigen 1
HRE	Hypoxia response element
IgG	Immunoglobulin G
IHC	Immunohistochemical
IP	Intraperitoneal
IR	Ionising radiation
IRES	Internal ribosome entry site
KD	Knockdown
KO	Knockout
LET	Low-linear energy transfer

LOX	Lysyl oxidase
LZIP	Leucine zipper
MET	HGF receptor
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
NAC	N-acetyl-L-cysteine
NaCl	Sodium chloride
NaHCO ₃	Sodium hydrogen carbonate
NLS	Nuclear localisation signal
ODD	Oxygen-dependent degradation
OE	Overexpression
2-OGDD	O ₂ /Fe ²⁺ /2-oxoglutarate-dependent dioxygenase
PAS	Per-Arnt-Sim
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PHD	Prolyl-4-hydroxylase
PHZ	Phenylhydrazine
PVDF	Polyvinylidene difluoride
pVHL	von Hippel-Lindau protein
qPCR	Quantitative PCR
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
sgRNA	Single guide RNA
siRNA	Small interfering RNA
SV40	Simian virus 40
TAD	Transactivation domain
TBS-T	Tris-buffered saline with Tween
TCGA	The Cancer Genome Atlas
TE	Tris-EDTA
Tris	Tris(hydroxymethyl)aminomethane
VEGF	Vascular endothelial growth factor
WT	Wild-type

Chapter 1

Introduction

1.1. Tumour hypoxia and HIF

In solid tumours, cells locating in close proximity (approximately 70 to 100 μm) to the blood vessels gain access to sufficient nutrients and oxygen. These tumour cells around the blood vessels actively grow and proliferate, and thus consume oxygen at very high rate. In addition, malformed tumour blood vessels, being leaky and fragile, generate high interstitial pressure within tumour tissue and impede the diffusion of oxygen. These together create a highly heterogeneous oxygen microenvironment within the tumour tissue, and cause oxygen consumption exceeding the supply in regions distant from tumour blood vessels (Figure 1.1).

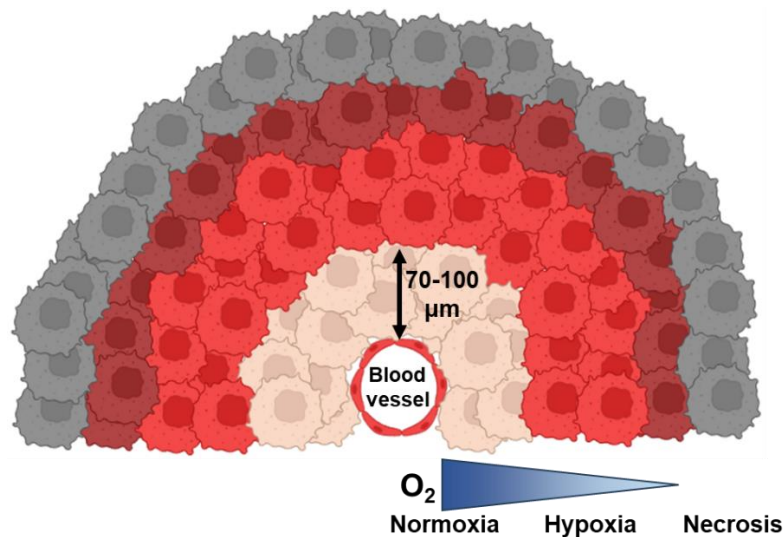


Figure 1.1 Schematic diagram of oxygen gradient in tumour. Cells in the proximal region around the functional tumour blood vessel have rapid access to oxygen, so these cells are under normoxic conditions. As the distance from blood vessel increases, availability of oxygen gradually drops. This generates an oxygen gradient ranging from mildly hypoxic to near anoxic conditions. Cells in these regions of low oxygen content are under hypoxic stress, and thus hypoxic response is triggered. Further away from the blood vessel lies the necrotic region, wherein cells cannot survive and undergo necrosis.

Such persistent shortage of oxygen, or tumour hypoxia, is known to confer malignant properties, like metabolic reprogramming, invasiveness, and therapy resistance, on cancer cells [1-3]. Moreover, clinical data have shown that patients with higher hypoxic fraction within tumour tissues have worse survival outcomes, meaning that tumour hypoxia predicts poor prognosis of patients [4-6]. Hence, tremendous effort has been devoted in an attempt to elucidate the molecular mechanisms of hypoxia responses in cancer cells.

Hypoxia-inducible factor, HIF, is the master transcription factor that regulates cellular responses to hypoxia stimuli. Comprising one α subunit and one β subunit, HIF functions as a heterodimer to control the expressions of a multitude of downstream genes. There are 3 isoforms of the HIF- α subunit - HIF-1 α , HIF-2 α and HIF-3 α . The human HIF-1 α subunit, which is the most well-studied amongst the three isoforms, is an 826-amino acid protein which consists of several functionally important domains. The basic helix-loop-helix (bHLH) domain and the two following Per-Arnt-Sim (PAS) domains mediate DNA binding and subunit interaction [7]. These domains at the N-terminus together form the interface for HIF subunit dimerisation and function in its recruitment to the promoter regions in target gene loci. The oxygen-dependent degradation (ODD) domain contains the key amino acid residues which govern the degradation of HIF-1 α protein in the presence of oxygen, and thus is important for regulating the stability of HIF-1 α . In addition, the N-terminal transactivation domain (N-TAD), which overlaps the ODD domain, and the C-terminal transactivation domain (C-TAD) are responsible for the protein-protein interaction between HIF-1 α and other co-activators, thereby allowing the regulation of HIF transactivation activity. Between the two TADs, there is also a bipartite nuclear localisation signal (NLS) sequence that directs the transport of HIF-1 α to the nucleus. Human HIF-2 α , also called EPAS1 (endothelial PAS domain protein 1), is an 870-amino acid protein that shares high sequence homology with HIF-1 α and exhibits similar functional domain arrangement as described above. Such homology renders HIF-2 α its resemblance to HIF-1 α in both biochemical properties and biological functions. HIF-3 α is much less explored. Unlike HIF-1 α and HIF-2 α , the HIF-3 α (*HIF3A*) gene produces a wide range of mRNA variants due to the extensive transcriptional regulations and alternative splicing [8]. Structurally, HIF-3 α also differs from HIF-1 α and HIF-2 α in that it lacks the NLS and C-TAD sequences locating at the C-terminus side of the ODD domain, but instead has a distinctive leucine zipper (LZIP) domain. Apart from the α subunits, the HIF heterodimer also contains one β subunit - HIF-1 β , or ARNT (aryl hydrocarbon receptor nuclear translocator). Human HIF-1 β , with a total of 789 amino acid residues, has the bHLH and PAS domains as the HIF- α subunits do. However, HIF-1 β does not possess the ODD domain and the N-TAD therein (Figure 1.2). Due to the lack of ODD domain, HIF-1 β proteins are not under the control of the oxygen-dependent proteolysis.

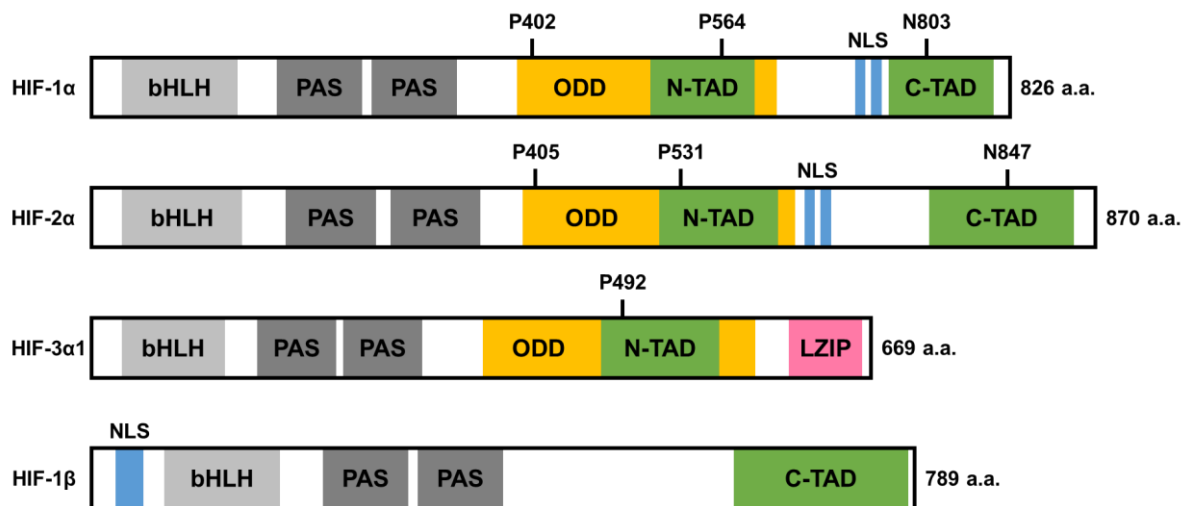


Figure 1.2 Schematic diagram of the protein domain structures of the human hypoxia-inducible factors (HIF). The major domains and motifs of the HIF- α subunits and HIF- β subunit are shown. Key amino acid residues in the α subunits are as indicated. HIF-3 α is represented by its longest, major isoform (HIF-3 α 1).

All three of the HIF- α subunits can bind with HIF-1 β , respectively, *via* inter-subunit interaction between the bHLH and PAS domains to form heterodimers. For HIF-1 and HIF-2, it is well established that they recognise and bind to specific sequences in the genome, called hypoxia response element (HRE; 5'-RCGTG-3', R: A or G), and subsequently recruit co-activators like p300/CBP to promote the transcription of downstream genes. On the other hand, the function of HIF-3 is less clear. Studies have shown that one of the HIF-3 α isoforms suppresses the transcription activity of HIF-1 and HIF-2 in reporter assays [9,10], and data from a more recent report demonstrated HIF-3 α reduces HIF-1 α and HIF-2 α protein expressions [11], suggesting that HIF-3 α may be a repressor of HIF-1 and HIF-2.

The regulation of HIF expression and activity largely depends on the oxygen-responsive post-translational modification of the α subunit. Under normoxic conditions, proline residues in the ODD domain (for human, P402 and P564 of HIF-1 α ; P405 and P531 of HIF-2 α ; P492 of HIF-3 α) are hydroxylated by the prolyl-4-hydroxylases (PHDs). These hydroxylated proline residues are then recognised by the von Hippel-Lindau protein (pVHL), which is an E3 ubiquitin ligase, resulting in the polyubiquitination of the HIF- α subunit and eventually its degradation *via* the 26S proteasome pathway (Figure 1.3). In addition, an asparagine residue in the C-TAD (N803 of HIF-1 α ; N847 of HIF-2 α) is hydroxylated by the Factor Inhibiting HIF-1 (FIH-1) under normoxia as well. This hydroxylated asparagine residue interferes with the interaction of the transcription co-factors, p300/CBP with the HIF- α subunits, and in turn suppresses the transactivating activity of HIF. Importantly, PHDs and FIH-1 belong to the

$O_2/Fe^{2+}/2$ -oxoglutarate-dependent dioxygenase (2-OGDD) protein family, and oxygen is indispensable for their enzymatic activities. Under mild hypoxic conditions, PHDs lose activities and cannot hydroxylate the HIF- α subunits. As a result, the HIF- α subunit is no longer degraded and the stabilised HIF- α subunit dimerises with HIF-1 β , which is constitutively expressed regardless of oxygen conditions due to the absence of ODD domain. The heterodimer then enters the nucleus and binds to HREs. However, FIH-1 has a much lower K_m value for oxygen molecules than PHDs, so only under severe hypoxia or anoxia would FIH-1 become inactive. The inactivation of FIH-1 allows HIF to recruit the co-activators CBP/p300, and thus to exhibit its full potential to activate the transcription of various hypoxia-responsive genes (Figure 1.3).

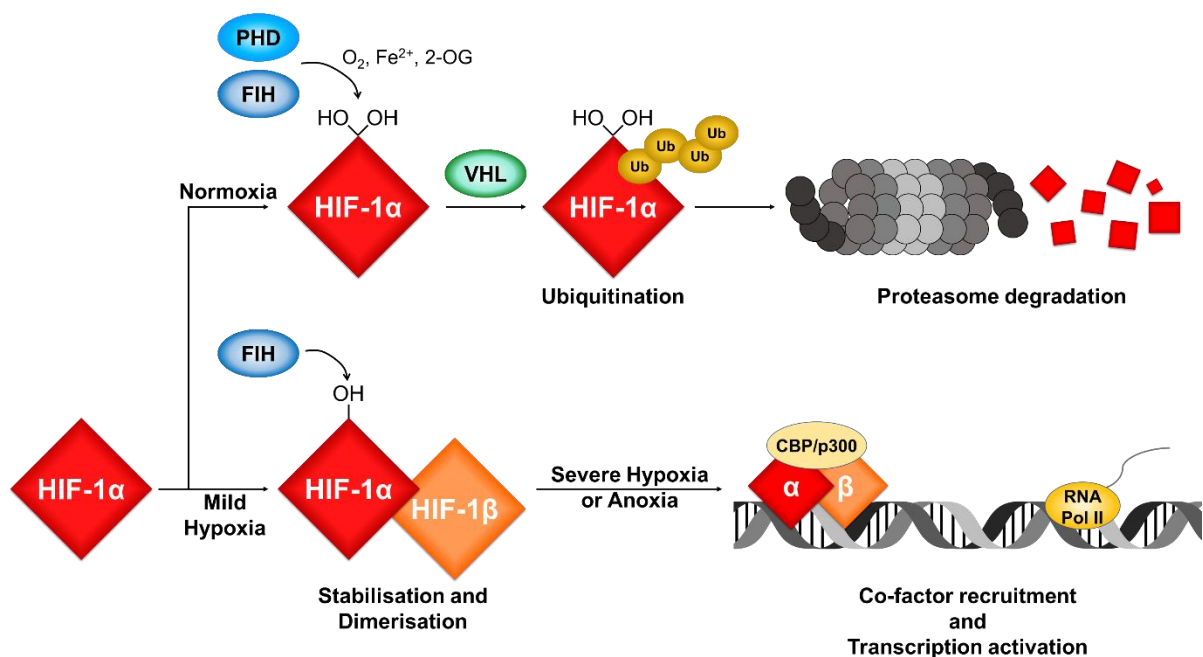


Figure 1.3 Canonical oxygen-dependent regulation of HIF. Under normoxia, the α subunit of HIF is hydroxylated by the oxygen-dependent 2-OGDDs, PHD and FIH-1. The E3 ubiquitin ligase pVHL then recognises and promotes the proteasomal degradation of the hydroxylated α subunit. Under mild hypoxia, PHD is not functional, the α subunit is thus retained and can interact with HIF-1 β , whereas FIH remains active and prevents HIF from being fully activated. As oxygen level further decreases, FIH loses activity, allowing HIF's recruitment of the co-factors CBP/p300 to activate the transcription of downstream genes.

1.2. Hypoxia and metastasis

One of the major causes of cancer patient mortality is metastasis. Its development involves a series of biological events. First, cancer cells have to acquire motility and migratory phenotypes *via* processes like epithelial-mesenchymal transition (EMT) in order to disseminate from the primary tumour mass. These cells then break through the local confinement by the basement membrane, and invade through the adjacent tissue whilst actively interacting with and modifying the extracellular matrix (ECM). After the invading cells reach a blood vessel and penetrate the blood vessel wall (intravasation), the cells enter the circulatory system and become circulating tumour cells (CTCs). Despite the hostile intravascular environment, a small portion of the CTCs can survive in the blood circulation and eventually exit the blood vessel (extravasation), arriving at secondary organs. These cancer cells usually remain in a dormant state or proliferate very slowly to form micrometastases until successful adaptation to the microenvironment in the secondary organ (e.g. by the acquisition of stem cell properties). Once adapted, the cancer cells/micrometastases can profoundly grow in the metastatic niche and develop into clinically significant metastases (Figure 1.4).

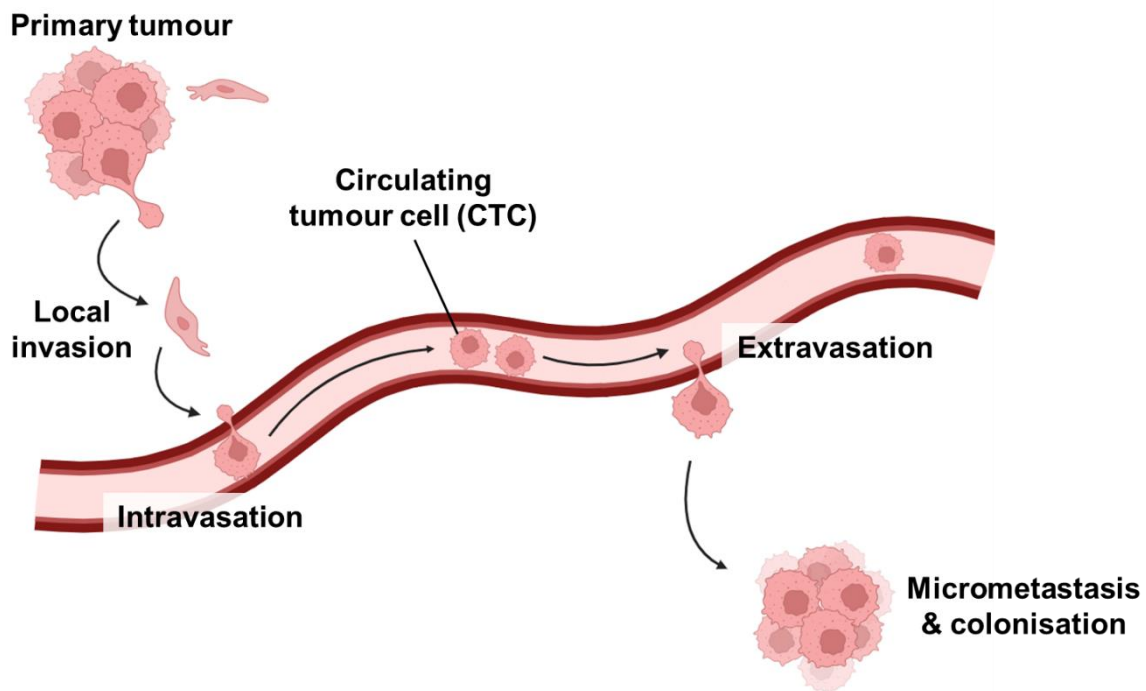


Figure 1.4 Schematic diagram showing the major events of the metastatic cascade. Cancer cells disseminated from the primary tumour invade locally and enter a blood vessel. After exiting the circulatory system, cancer cells gradually develop metastases at the secondary site. Hypoxia and HIF have been shown to contribute to each of these biological processes to promote metastasis.

Noteworthy, there have been accumulating research showing the involvement of hypoxia and HIF in each of these steps of the metastatic cascade. For instance, hypoxia increases cell motility by inducing lysyl oxidase (LOX) expression as well as by activating the HIF-HGF-MET axis. In addition, hypoxia also upregulates VEGF and ANGPTL4 expression to promote intravasation and extravasation, respectively [12,13]. Studies have also shown that HIF inhibition can potentially block metastasis [14,15]. Concordantly, clinical data have demonstrated the association between HIF-1 expression and incidence of metastasis [16-19]. It is therefore important to deepen our understanding of the role of HIF-1 in relation to the stimulatory effect of hypoxia on metastasis.

1.3. Hypoxia leads to radioresistance and promotes post-radiotherapy recurrence

Radiotherapy is a major treatment modality for cancer. When exposed to ionising radiation (IR), cancer cells are killed by the following mechanisms: (1) the high-energy particles directly create DNA strand breaks; and (2) the radiolysis of intracellular water molecules generates various free radicals and reactive oxygen species (ROS) like peroxide, superoxides, and hydroxyl radical, etc., which can chemically react with the DNA sugar-phosphate backbone and the nitrogenous base (Figure 1.5). Such DNA damages, when non-repairable, cause tumour cell death. These mechanisms, however, become less efficient when oxygen concentration is low, especially when low-linear energy transfer (LET) radiation like X-ray and γ -ray are used [20].

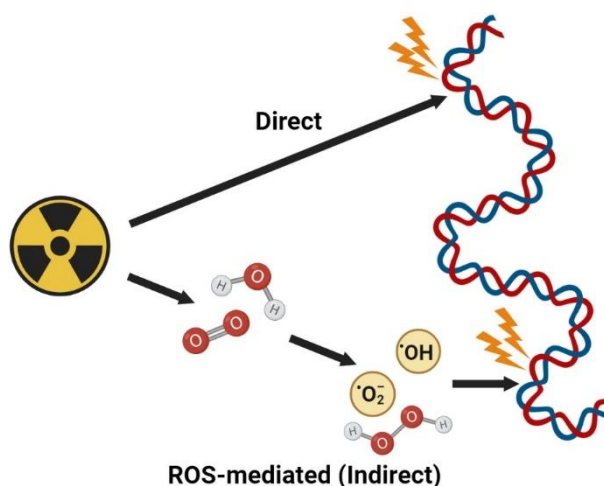


Figure 1.5 Ionising radiation induces DNA damages. During radiotherapy, DNA damages are generated in cancer cells by ionising radiation *via* two mechanisms. Ionising radiation can directly introduce breaks in DNA strands, or alternatively, cause the radiolysis of water molecules to generate reactive oxygen species (ROS) and indirectly introduce DNA strand breaks.

In the presence of oxygen, DNA strand breaks are stabilised by oxidation, and this prevents the DNA damage from being repaired. Moreover, the generation of free radicals and ROS requires oxygen, so under hypoxic conditions, ROS production is greatly suppressed and less cancer cells can be killed with the same dose of irradiation when compared to normoxia (Figure 1.6). In addition, radiosensitivity is dependent on cell cycle phase. Whilst cells in G₂/M phase are the most sensitive to radiation treatment, hypoxic cells are known to undergo cell cycle arrest and do not proceed through S phase. These hypoxic cells halted in G₁ or S phase have higher tolerance to DNA damage, and thus are more likely to survive irradiation treatment. These together cause normoxic cells being approximately 2-3 times more radiosensitive than hypoxic cells [20,21].

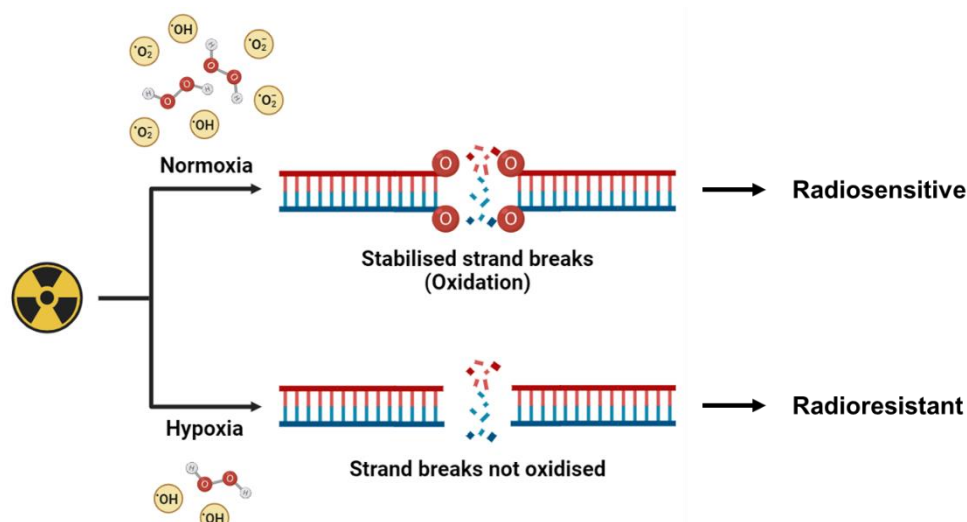


Figure 1.6 Contribution of oxygen to radiosensitivity. In the presence of oxygen, there are more free radicals and ROS generated after irradiation. Also, DNA strand breaks are stabilised by oxidation, and are thus less likely to be repaired, causing the irradiated cells more prone to cell death (radiosensitive). Contrarily, under hypoxic conditions, due to the limited availability of oxygen, free radical and ROS generation is reduced, and DNA strand breaks are not oxidised. Irradiated cells are thus less likely to undergo cell death (radioresistant).

Indeed, it has been demonstrated that hypoxic tumour cells locating in the perinecrotic regions form significantly less γ H2AX foci after irradiation than the non-hypoxic cells, indicating a lower degree of DNA damage [22]. Furthermore, after radiation treatment, because the radiosensitive normoxic/mildly hypoxic cells are killed, oxygen can diffuse to the radioresistant, surviving severely hypoxic/anoxic cells. These cells, re-gaining access to oxygen, undergo the process of reoxygenation after radiotherapy. Importantly, it has been reported that after irradiation treatment, the reoxygenated (or “ex-hypoxic”) cells had enhanced HIF activity [23]. This can be explained by that upon the re-exposure to oxygen, large amount of reactive oxygen species (ROS) is generated, which in turn oxidises Fe^{2+} ions into Fe^{3+} ions. The depletion of Fe^{2+} ion causes the inactivation of the 2-OGDDs, PHDs and FIH-1, and thereby promotes HIF activation upon reoxygenation. Of note, the post-irradiation activation of HIF has been shown to contribute to the translocation of surviving cells towards the tumour blood vessels, and thus potentiate distant metastasis and recurrence after radiotherapy [24].

Clinically, corroborating reports have also shown that HIF expression is associated with poor response to radiotherapy, relapse risks, as well as metastasis after radiation therapy [25-27]. Understanding the underlying molecular mechanisms may provide insights into better post-radiotherapy palliative treatment strategies.

1.4. Aim and Summary

Based on previous findings from our group, hypoxic tumour cells in the perinecrotic regions can survive radiotherapy and translocate towards blood vessels afterwards in a hypoxia-inducible factor (HIF)-dependent manner. However, how HIF promoted such process remains unclear. The aim of this study is to identify the downstream gene regulated by HIF and to elucidate the underlying molecular mechanism.

In Chapter 3, our group's previous data on the identification of genes responsive to hypoxic stimuli is first collated. The *HMHA1* gene is characterised as a potential candidate based on its reported biological functions. Then, using a variety of human cancer cell lines, the hypoxic induction of HMHA1 expression *in vitro* is verified. And, in a xenograft tumour mouse model, the expression of HMHA1 in the hypoxic regions of tumour *in vivo* is also observed. Next, in order to investigate the regulatory mechanism and the involvement of HIF, knockout cell lines are used. The induction of HMHA1 expression under hypoxic conditions is found to be dependent on both HIF-1 and HIF-2. Moreover, by performing chromatin immunoprecipitation (ChIP)-qPCR assay, the putative binding site of HIF-1 is further narrowed down.

Having shown that HMHA1 is induced by hypoxic stimuli under the regulation of HIF, its role in cancer cell invasion is next interrogated. In the latter part of Chapter 3, the transwell invasion assay is employed to assess the invasion activities of cancer cells. It is first confirmed that hypoxic treatment augments cancer cell invasiveness. And, from a loss-of-function approach, it is shown that HMHA1 is indeed required for cancer cell invasion under hypoxia. Next, to study how HMHA1 enhances cancer cell invasion activity in more detail, a stable HMHA1 overexpression cell line is established and applied for function analyses. It is revealed that HMHA1 not only can alter the organisation of actin filaments, but also promotes extracellular matrix degradation by regulating matrix metalloproteinases (MMPs). And transwell invasion assay corroborates the results, showing that HMHA1 promotes cancer cell invasion in a MMP-dependent manner. Finally, HMHA1 expression in cancer patients is analysed in relation to tumour hypoxia and patient prognosis using the dataset from The Cancer Genome Atlas (TCGA).

Chapter 4 focuses on the post-irradiation changes that induce hypoxic cancer cell invasion. When hypoxic cells are re-exposed to oxygen, ROS generation promotes HIF activation. It is found that HMHA1 expression is further increased after irradiation and reoxygenation treatment in the “ex-hypoxic” cancer cells, and such increase is dependent on ROS generation

and HIF activity. Moreover, by knockout experiments, HMHA1 is confirmed to contribute to the invasion of “ex-hypoxic” cancer cells after irradiation and reoxygenation treatment.

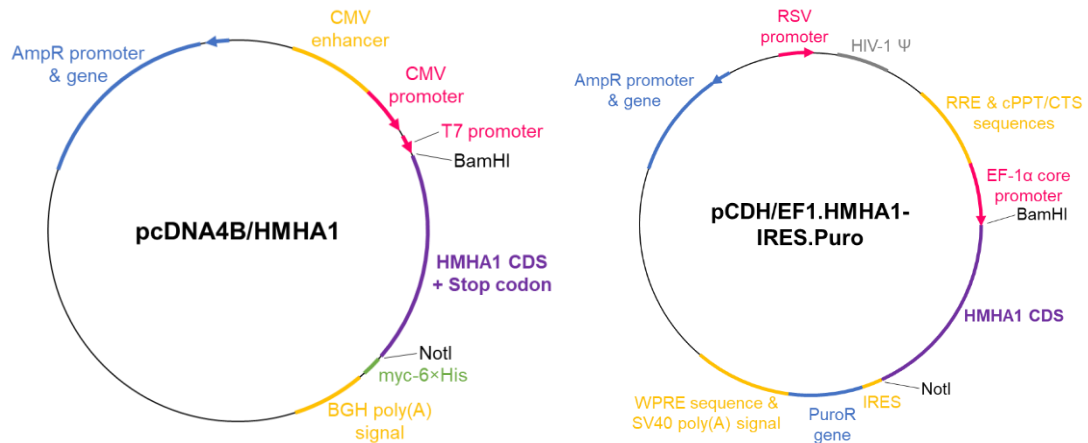
Together, this study delineates the mechanism of hypoxia-induced and post-irradiation reoxygenation-induced cancer cell invasion *via* the activation of the ROS-HIF-HMHA1 axis.

Chapter 2

Materials and Methods

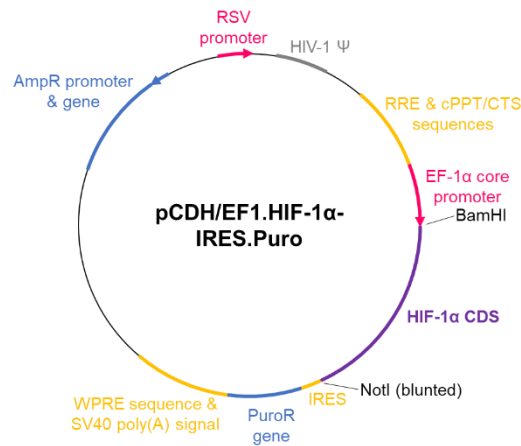
2.1. Plasmids

- pcDNA4B/HMHA1 and pCDH/EF1.HMHA1-IRES.Puro



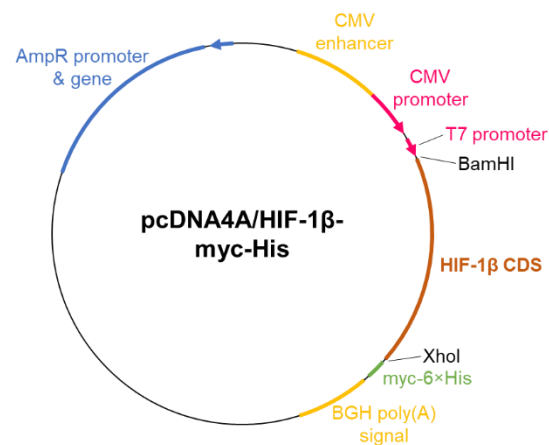
Total RNA from HeLa, a human cervical cancer cell line, was extracted after lysing cells with Sepasol-RNA I Super G (Nacalai tesque, #09379-84). Total cDNA was generated by reverse transcription using RNA LA PCR™ Kit (AMV) Ver.1.1 (Takara, #RR012A), and the resultant reaction mixture was used as the template for amplifying the DNA fragment encoding HMHA1 coding sequence by PCR reaction with 5'-ATAGGATCCGCCATGAGTCGGGGGCAAAG-3' as the forward primer and 5'-ATAGCGGCCGCTCACACGAATTCCGGCTG-3' as the reverse primer. After digestion with the restriction enzymes, BamHI and NotI, the DNA fragment was inserted into the pcDNA4/myc-His B plasmid and the pCDH/EF1-MCS-IRES.Puro plasmid digested with BamHI and NotI in order to generate the HMHA1 overexpression vectors, pcDNA4B/HMHA1 and pCDH/EF1.HMHA1-IRES.Puro, respectively. The HMHA1 coding sequence was confirmed by Sanger sequencing after insertion into the plasmids. The pcDNA4B/HMHA1 plasmid was used for transient overexpression of HMHA1 and the pCDH/EF1.HMHA1-IRES.Puro plasmid was used for the lentivirus-mediated establishment of stable HMHA1 overexpression cell lines.

- pCDH/EF1.HIF-1 α -IRES.Puro



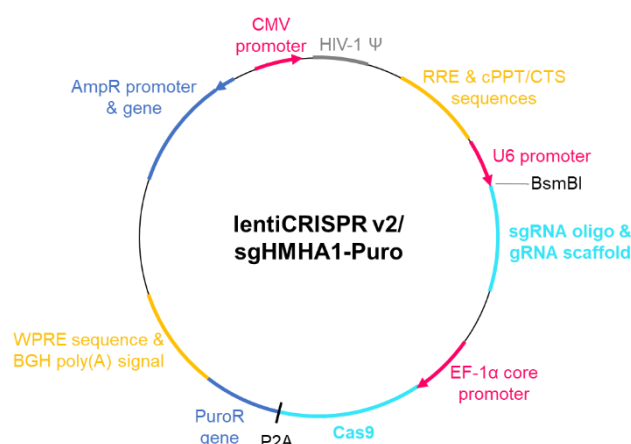
This HIF-1 α overexpression vector was created by our group member, Mr. Satoshi Takeuchi. Briefly, the pCDH/EF1-MCS-IRES.Puro plasmid was digested with NotI and subjected to blunting treatment before the second digestion with BamHI. DNA fragment encoding HIF1A coding sequence was obtained by digesting the previously generated pcDNA4/HIF1A overexpression vector with BamHI and EcoRV. The fragment was then inserted into the pre-digested pCDH/EF1-MCS-IRES.Puro plasmid. This vector was used for the stable reconstitution of HIF-1 α in HIF-1 α knockout cells.

- pcDNA4A/HIF-1 β -myc-His



This HIF-1 β overexpression vector was created by our group member, Dr. Christalle C. T. Chow. Briefly, the DNA fragment encoding HIF-1 β (ARNT) gene was amplified from the total cDNA of HeLa and then inserted into the pcDNA4/myc-His A plasmid between the BamHI and XhoI sites. This vector was used for the reconstitution of HIF-1 β in HIF-1 β knockout cells.

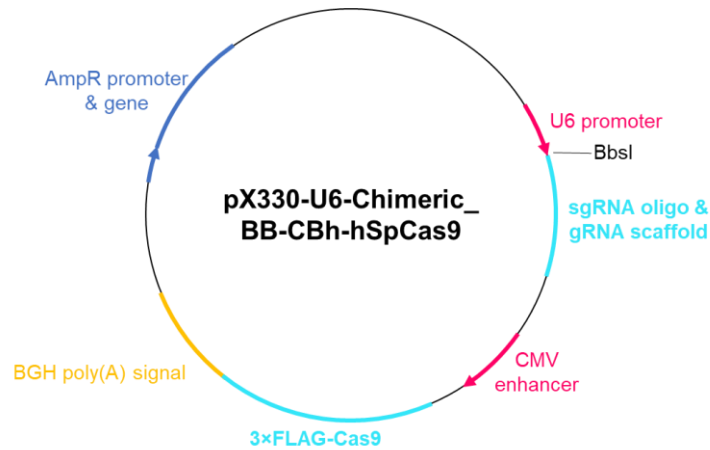
- lentiCRISPRv2/sgHMHA1-EF1.Cas9



Customised oligos were designed for the expression of single guide RNAs (sgRNAs) that target the HMHA1 coding sequence. Complementary oligo pairs were annealed and phosphorylated by incubating with T4 polynucleotide kinase, 10 × Buffer (Takara, #2021) and ATP (final concentration 100 μM) for 30 min at 37°C. The oligos were then inserted into the lentiCRISPRv2-puro plasmid (Addgene, #52961) digested with BsmBI. The resultant vector was used for the simultaneous expression of sgRNA targeting HMHA1 (sgHMHA1), Cas9, and puromycin resistance gene to establish HMHA1 knockout cells. The sequences of oligos used are listed below (target sequences bolded).

Oligos	Sequences
sgHMHA1 #1-1	5'-CACC GGCGTCTGCGAGATCGAGCGC -3'
sgHMHA1 #1-2	5'-AAACCC GCTCGATCTCGCAGACGCC -3'
sgHMHA1 #2-1	5'-CACC GGCCTCTACCACGAGCTCGTA -3'
sgHMHA1 #2-2	5'-AAACTAC GAGCTCGTGGTAGAGGCC -3'

- pX330/sgARNT-Cas9, pX330/sgHIF1A-Cas9, and pX330/sgEPAS1-Cas9



This series of vectors were created by our group members, Ms. Kumi Johchi and Ms. Lina R. Koseki. Briefly, oligo sets designed for the expression of sgRNAs respective targeting the upstream of exon 1 and inside intron 1 of the gene of interest were inserted into the BbsI-digested pX330-U6-Chimeric_BB-CBh-hSpCas9 plasmid (Addgene, #42230). These vectors were used for establishing HIF-1 β , HIF-1 α and HIF-2 α knockout cells. The sequences of oligos used are listed below (target sequences bolded).

Oligos	Sequences
For HIF-1 β (<i>ARNT</i>) knockout	
sgARNT_upstream-1	5'-CACCGACGCAAGGAGGGCGAGATTA-3'
sgARNT_upstream-2	5'-AAACTAATCTCGCCCTCCTTGCGTC-3'
sgARNT_intron#1-1	5'-CACCGAAGTTTCAAAGTATAGTCCG-3'
sgARNT_intron#1-2	5'-AAACCGGACTATACTTTGAAACTTC-3'
sgARNT_intron#2-1	5'-CACCGACTGGCTGCCGCTCCAACGG-3'
sgARNT_intron#2-2	5'-AAACCCGTTGGAGCGGCAGCCAGTC-3'
For HIF-1 α (<i>HIF1A</i>) knockout	
sgHIF1A_upstream#1-1	5'-CACCGTCGCTCGCCATTGGATCTCG-3'
sgHIF1A_upstream#1-2	5'-AAACCGAGATCCAATGGCGAGCGAC-3'
sgHIF1A_intron#1-1	5'-CACCGTCTAATGGTGTACAGGCTCA-3'
sgHIF1A_intron#1-2	5'-AAACTGAGCCGTGACACCATTAGAC-3'
sgHIF1A_upstream#2-1	5'-CACCGTGATTGGCTGAGAGCGGCGT-3'
sgHIF1A_upstream#2-2	5'-AAACACGCCGCTCTCAGCCAATCAC-3'
sgHIF1A_intron#2-1	5'-CACCGGTGCTCTGCAGTGGACCGT-3'
sgHIF1A_intron#2-2	5'-AAACACGGTCCACTGCAGAGCACC-3'
For HIF-2 α (<i>EPAS1</i>) knockout	
sgEPAS1_upstream-1	5'-CACCGACAGGCAACGGTTAGCGCTC-3'
sgEPAS1_upstream-2	5'-AAACGAGCGCTAACCGTTGCCTGTC-3'
sgEPAS1_intron-1	5'-CACCGAGCGGGCGTCCGGGCCCAGTC-3'
sgEPAS1_intron-2	5'-AAACGATCGGCCCGGACGCCCGCTC-3'

2.2. Cell culture

Cell lines, including HeLa, HepG2, SBC-3, HT1080, U251, H4, U-87 MG, HCT-116, LoVo, LS180, SaOS-2, EBC-1, PC-9, AOI, RMG-1, PC-3, DU145, KK47, Caki-1, MeWo, M-14, MKN45, MKN7, Mia-PaCa-2, BxPC3, MCF-7, MDA-MB-231, and HEK293 stably expressing SV40 large T antigen and neomycin resistance genes (HEK293TN), were purchased from American Type Culture Collection (ATCC), JCRB cell bank and RIKEN BRC cell bank. All cells were maintained using Dulbecco's Modified Eagle Medium (DMEM) with high glucose (4.5 g/L Glucose), L-Glutamine, and sodium pyruvate (Nacalai Tesque, #08458-16) supplemented with 10% v/v FBS (EQUITECH-BIO, #268-1), penicillin (final concentration 100 units/mL), and streptomycin (final concentration 100 µg/mL), and cultured in a 95% air/5% CO₂ humidified incubator set at 37°C. For hypoxic treatment, cells were incubated in workstation chambers set at 37°C, < 0.1% O₂/5% CO₂/4% H₂/91% N₂ (INVIVO₂ 500, Ruskinn), at 37°C, 1% O₂/5% CO₂/94% N₂ (INVIVO₂ 400, Ruskinn), or at 37°C, 3% O₂/5% CO₂/92% N₂ (INVIVO₂ 400, Ruskinn).

2.3. CRISPR/Cas9-mediated knockout

HIF-1β (*ARNT*) knockout HeLa cells were established as previously described [28]. Briefly, HeLa cells were transfected with the two pX330/sgARNT-Cas9 vectors to express sgRNAs that respectively target the upstream of exon 1 and inside intron 1 in the ARNT locus. This would excise the start codon from the gene locus and thus abrogate HIF-1β expression. After transfection, cells were sparsely seeded in order to isolate single-cell clones. Clones were screened by genotyping PCR and candidate cells were subjected to Western blot analysis. The two clones selected for experiments were generated from different combinations of sgRNA to minimise off-target effects.

With the same approach, HIF-1α (*HIF1A*) knockout and HIF-2α (*EPAS1*) knockout HeLa cell lines were respectively established using the pX330/sgHIF1A-Cas9 and pX330/sgEPAS1-Cas9 vectors.

2.4. Lentiviral transduction and the establishment of stable overexpression/knockout cell lines

To produce lentivirus for infection, HEK293TN cells (1.5×10^6 cells per 10 cm dish) were transfected with the lentiviral packaging plasmids pVSV-G, pPACKH1/GAG, and pPACKH1/REV, in addition to the pCDH lentiviral vector containing the desired coding sequence or the empty pCDH lentiviral vector (EV, negative control). Or in case of knockout, lentiCRISPRv2/sgHMHA1-EF1.Cas9 or the corresponding empty vector were used. Volume of plasmids and transfection reagent added (per 10cm dish) are listed below:

	Amount
Plasmid	
pVSV-G	2.5 μ g
pPACKH1/GAG	2.5 μ g
pPACKH1/REV	2.5 μ g
pCDH/lentiCRISPR v2 vector	7.5 μ g
Transfection reagent	
PEI MAX	45 μ L

After transfection, HEK293TN cells were incubated in 7 mL of fresh DMEM (10% FBS) for 2 days. Lentivirus medium was collected using a syringe and then passed through a 0.22 μ m filter (Millipore, #SLGSV255F). Recipient cells were sparsely seeded (2×10^5 cells per 10 cm dish), allowed to adhere overnight and then treated with polybrene solution (8 μ g/mL) for 30 min before infection. To infect cells, approximately 2 mL of fresh DMEM (10% FBS) was added to the filtered lentivirus medium (~ 6 mL), and the entire volume was directly applied to the recipient cells. After 24 h of incubation, cells would be allowed to recover for one day and then subjected to puromycin selection for 4 to 7 days. To obtain single-cell colonies, the bulk of puromycin-selected cells were re-seeded sparsely. After the colonies reached appropriate sizes, they would be isolated and separately cultured. Plasmids and cell lines used are listed below:

Cell line used for infection	Plasmid	Transduced cell line
HIF-1 α knockout HeLa (clone)	pCDH/EF1-IRES.Puro (EV)	HeLa/HIF1A-KO/EV
	pCDH/EF1.HIF-1 α -IRES.Puro	HeLa/HIF1A-KO/HIF1A
HeLa (wild-type)	pCDH/EF1-IRES.Puro (EV)	HeLa/EV
	pCDH/EF1.HIF-1 α -IRES.Puro	HeLa/HIF1A
HeLa (wild-type)	pCDH/EF1-IRES.Puro (EV)	HeLa/EV
	pCDH/EF1.HMHA1-IRES.Puro	HeLa/HMHA1 (OE)
HeLa (wild-type)	lentiCRISPRv2 (EV)	HeLa/lentiEV
	lentiCRISPRv2/sgHMHA1-EF1.Cas9	HeLa/HMHA1-KO (clone)

For reconstitution or overexpression, successful transduction was confirmed by detecting the corresponding protein in Western blotting. For knockout, all isolated clones were screened

by Western blotting, and candidate clones without protein expression were subjected to genome sequencing analysis for verification.

2.5. Plasmid transfection

For transient transfection, 2 µg of plasmid was mixed with 6 µL of PEI MAX reagent (Polysciences, #24765) in 100 µL DMEM, gently vortexed, and incubated at room temperature for 10 minutes. The reaction mixture would then be added to cells (1.25×10^5 cells per 35mm dish) seeded 16 hours beforehand. After overnight incubation, transfection was stopped by changing the medium to fresh serum-supplemented DMEM, and the transfected cells were used for subsequent assays or analysis.

2.6. Small interfering RNA (siRNA)-mediated knockdown

To transfect siRNA into cells, 2 µL of siRNA (50 nM) in 100 µL DMEM was mixed with 2 µL of Lipofectamine™ RNAiMAX Transfection Reagent (Invitrogen, #13778150) in 100 µL DMEM, gently vortexed, and incubated at room temperature for 10 minutes. The reaction mixture would then be added to cells (1.25×10^5 cells per 35mm dish) seeded 16 hours beforehand. After overnight incubation with serum-supplemented antibiotics-free DMEM, transfection was stopped by changing the medium to serum- and antibiotics-supplemented DMEM, and the transfected cells were used for subsequent assays or analysis. siRNA against HIF1A (HSS104775 and HSS179231), EPAS1/HIF2A (HSS176568 and HSS176569), and HMHA1 (HSS118888 and HSS118890) were purchased from Invitrogen. For negative control, Stealth RNAi™ siRNA Negative Control, Med GC (Invitrogen, Cat. #12935300) was used.

2.7. Irradiation

Cells (1.5×10^5 cells per 60mm collagen-coated dish) cultured under normoxic or hypoxic ($O_2 < 0.1\%$) conditions for 24 hours were irradiated with the indicated dose of ^{137}Cs γ -rays using the Gammacell 40 Exactor (MDS Nordion International Inc.). For irradiation under hypoxia, cells were transferred from the hypoxia workstation chambers to a rectangular jar of AnaeroPack (Mitsubishi Gas Chemical), irradiated, and then reoxygenated. For N-acetyl-L-cysteine (NAC; Sigma-Aldrich, #A9165) treatment, cells were incubated in NAC-containing DMEM (final concentration: 5 mM) for 1 hour and then irradiated.

2.8. Reverse transcription and quantitative real-time PCR (qPCR)

To collect total RNA, cells were lysed with Sepasol-RNA I Super G (Nacalai tesque, #09379-84) and RNA was extracted and purified in accordance with manufacturer's instructions. RNA sample was then reverse-transcribed using the PrimeScript RT Reagent Kit (Takara, #RR037A). The resultant complementary DNA (cDNA) was used for real-time qPCR analysis with TB Green Premix Ex Taq II (Takara #RR820) and the Thermal Cycler Dice Real Time System (Takara). β -actin (*ACTB*) was used as housekeeping gene to assess the mRNA expression levels of target genes. Sequences of the primers used are listed below.

Primers	Sequences
ACTB Forward	5'-TGGCACCCAGCACAATGAA-3'
ACTB Reverse	5'-CTAAGTCATAGTCCGCCTAGAAGC-3'
HMHA1/ARHGAP45 Forward	5'-GTGCAACAGCTACGTCTACTTCCAG-3'
HMHA1/ARHGAP45 Reverse	5'-TGTATGGCCAGCGTCTCCAG-3'
HIF1A Forward	5'-TCATCCAAGAAGCCCTAACGTG-3'
HIF1A Reverse	5'-TTTCGCTTTCTCTGAGCATTCTG-3'
HIF2A/EPAS1 Forward	5'-CACTGCAGACTTGTCCAGTGCTC-3'
HIF2A/EPAS1 Reverse	5'-CACTGCTCGGATTGTCACACCTA-3'
ARNT1 (HIF-1 β) Forward	5'-CTACCCGCTCAGGCTTTTC-3'
ARNT1 (HIF-1 β) Reverse	5'-CACCAAAGTGGGAAGTACGAG-3'
CA9 Forward	5'-ACCAGACAGTGATGCTGAGTGCTAA-3'
CA9 Reverse	5'-TCAGCTGTAGCCGAGAGTCACC-3'
VEGFA Forward	5'-TCACAGGTACAGGGATGAGGACAC-3'
VEGFA Reverse	5'-CAAAGCACAGCAATGTCCTGAAG-3'

2.9. Western blotting

Cells were washed with 1× PBS, harvested in CellLytic M reagent (Sigma-Aldrich, #C2978), and then snap-frozen at -80°C . Samples were sonicated (310 W output, 30 sec on/30 sec off, 8 cycles on ice) using the Bioruptor UCW-310 (Cosmo Bio) for cell disruption, and then centrifuged at $15000 \times g$ (15 min, 4°C) to remove cell debris. The supernatant was collected, and protein concentration was measured using the NanoDrop 2000 (Thermo Scientific). In each experiment, same amount of protein (at least 50 μg) were loaded to each lane of the 5% polyacrylamide stacking gel (pH 6.8) laid on top of the 7.5% polyacrylamide resolving gel (pH 8.8). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by applying a constant electric current of 40 mA. Proteins were transferred to a PVDF membrane (0.45 μm pore size; Cytiva, #10600023) using the Trans-blot SD semi-dry electrophoretic transfer cell (Bio-rad, #1703940) by applying a constant electric current of 2.5 mA/cm^2 for 30 min. The membrane was briefly rinsed once with TBS-T (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% v/v Tween-20) before blocking in skim milk solution (5% w/v in TBS-T) for 2 hours at room temperature. After washing in TBS-T (5 min $\times$ 3), the membrane was cropped according to the positions of protein standards (Bio-rad, #1610374) and incubated with the appropriate primary antibodies diluted in 5% skim milk solution for 16 hours at 4°C . After washing in TBS-T (5 min $\times$ 4), the membrane was incubated in corresponding secondary antibodies diluted in 1% w/v skim milk for 2 hours at room temperature. For signal development, membrane was washed with TBS-T (5 min $\times$ 4), and then incubated in ECL reagents (Cytiva, #RPN2232) for 5 min at room temperature. Bands were visualised using the AmershamTM Imager 680 system (Cytiva). Antibodies and dilution ratios are as follow:

Antibody	Manufacturer	Dilution
Primary antibodies		
Anti-HMHA1/ARHGAP45 rabbit polyclonal	Atlas antibodies, #HPA019816	1:300
Anti-HIF-1 α mouse monoclonal	BD Biosciences, #610959	1:250
Anti-EPAS1/HIF-2 α mouse monoclonal	Santa Cruz, #sc-13596	1:200
Anti-ARNT1/HIF-1 β mouse monoclonal	Novus Biologicals, #NB100-124	1:1000
Secondary antibodies		
HRP-conjugated anti-mouse IgG	Cytiva, #NA931	1:5000
HRP-conjugated anti-rabbit IgG	Cytiva, #NA934	1:5000

2.10. Chromatin immunoprecipitation (ChIP)-qPCR

ChIP was performed using the Dynabeads Protein G Immunoprecipitation Kit (Invitrogen, #10007D). For each reaction, 50 μ L magnetic beads were incubated with 200 μ L antibody on a rotator for 1 hour at room temperature. Antibodies and quantity used are as follow:

Antibody	Manufacturer	Amount per reaction
Anti-HIF-1 α mouse monoclonal	Abcam, #ab1	5 μ g
Purified Mouse IgG1 κ Isotype Control	BD Biosciences, #554121	5 μ g

To prepare ChIP samples, HeLa cells (1.5×10^6 cells per 10cm dish) cultured under normoxic (20% O₂) or hypoxic (< 0.1% O₂) conditions for 24 hours were fixed by adding formaldehyde solution (final concentration 0.9% v/v) and incubated for 2 min at room temperature, before quenching with glycine solution (final concentration 125 mM) for 5 min at room temperature. Cells were washed twice with ice-cold PBS, harvested by scraping in PBS containing 1 $\times$ Protease Inhibitor (Takara, #635673), and centrifuging at 1000 $\times$ g (5 min, 4°C). Cell pellet was lysed by resuspending in 300 μ L SDS Lysis Buffer (1% w/v SDS, 10 mM EDTA, 50 mM Tris pH 8.0, 1 $\times$ Protease Inhibitor), and then sonicated (310 W output, 30 sec on/30 sec off, 8 cycles $\times$ 4) on ice for chromatin shearing. Cell debris was removed by centrifugation at 15,000 $\times$ g (15 min, 4°C), and protein concentration of the supernatant was quantified using the Lowry method-based DC Protein Assay (Bio-rad, #5000112). Cell lysate samples containing equal amount of protein were diluted 10 times in ChIP Dilution Buffer (0.01% w/v SDS, 1.1% v/v Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.0, 167 mM NaCl, 1 $\times$ Protease Inhibitor), and 2 mL of the diluted lysate was applied to the antibody-bound magnetic beads for immunoprecipitation. After incubation on a rotator overnight at 4°C, magnetic beads were sequentially washed at 4°C with Low Salt Wash Buffer (0.1% w/v SDS, 1% v/v Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.0, 150 mM NaCl) for once, High Salt Wash Buffer (0.1% w/v SDS, 1% v/v Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.0, 500 mM NaCl) for once, LiCl Wash Buffer (0.25 M LiCl, 1% v/v IGEPAL-CA630, 1% w/v deoxycholic acid, 1 mM EDTA, 10 mM Tris pH 8.0) for once, and TE buffer for twice. Magnetic beads were incubated with 200 μ L Elution Buffer (1% w/v SDS, 100 mM NaHCO₃) on a rotator for 30 min at room temperature for elution. To reverse DNA-protein crosslinks, NaCl (final concentration 200 mM) was added and samples were incubated for at least 5 hours at 65°C. Samples were then treated with RNase A for 30 min at 37°C, followed by proteinase digestion (4 μ L 0.5 M EDTA, 8 μ L 1 M Tris-HCl pH 8.0 per μ L of 20 mg/mL Proteinase K) for 1 hour at 45°C. Chromatin DNA sample was purified using the QIAquick PCR Purification Kit

(QIAGEN, #28106) in accordance with manufacturer's protocol. The eluate was subjected to qPCR analysis using the primers listed below.

Primers	Sequences
CA9 TSS Forward	5'-TCTCGTTTCCAATGCACGTACAGC-3'
CA9 TSS Reverse	5'-AGTGACAGCAGCAGTTGCACAGT-3'
5' upstream Forward	5'-ATTCATAGGACACAGGTGTG-3'
5' upstream Reverse	5'-ATTCCTGAGTCCTGACCTC-3'
Intron 1 Region 1 Forward	5'-TAGGAGGCTGGGATCTGTATG-3'
Intron 1 Region 1 Reverse	5'-AAGCTGCATTCCAGTGA CTC-3'
Intron 1 Region 2 Forward	5'-AGTCACTGGAATGCAGCTTG-3'
Intron 1 Region 2 Reverse	5'-CGTCCAGGAAGTCAGAACAAATC-3'
Intron 1 Region 3 Forward	5'-GCTTGCCTTCAGGGAAAAG-3'
Intron 1 Region 3 Reverse	5'-TGACTCTCACTCCCGGAC-3'

2.11. Transwell invasion assay

Cells (3×10^5 cells per 10 cm collagen-coated dish) were seeded in serum-reduced DMEM (1% v/v FBS) and allow to adhere for 16 hours before starvation treatment using serum-free DMEM. Matrigel-coated invasion device (Corning, #354480) was pre-hydrated with serum-free DMEM in accordance with manufacturer's instruction before use. After 16 hours of starvation, 2×10^4 cells were re-seeded in serum-reduced DMEM (0.1% v/v FBS) to the upper chamber of the device and allowed to adhere for 12 hours. For knockdown experiment, siRNA-transfected HT1080 cells were allowed to recover in fresh serum-supplemented DMEM (10% v/v FBS) for 9 hours before starvation treatment overnight. The starved siRNA-transfected cells (2×10^4) were re-seeded to pre-hydrated invasion device and allow to adhere for 18 hours. For irradiation experiments, wild-type or HMHA1 knockout HeLa cells were seeded and starved as aforementioned before re-seeding to pre-hydrated invasion device (8×10^4 cells per chamber). The cells were allowed to adhere for 12 hours and then incubated in hypoxia ($< 0.1\%$ O₂) for 24 hours. To maintain hypoxic conditions during irradiation, the entire plate with invasion chamber inserts was transferred to the AnaeroPouch Zipper Pouch-Bag (Mitsubishi Gas Chemical) inside the hypoxia workstation chamber. To initiate invasion, medium in the bottom chamber was replenished with serum-supplemented DMEM (10% v/v FBS). The cells are then incubated under normoxic (20% O₂) or hypoxic (1% O₂) conditions for 24 hours. Invading cells were fixed with 4% paraformaldehyde solution for at least 30 min, and then stained with crystal violet solution (0.5% w/v in 20% methanol) for at least 1 hour. Non-invading cells in the upper chamber were wiped away with clean cotton buds. Stained cells were imaged using the Keyence BZ-9000 system and quantified by the ImageJ software.

2.12. Colorimetric cell viability assay

Cell viability was assessed using the Cell Count Reagent SF kit (Nacalai Tesque, #07553-44) in accordance with manufacturer's instruction. Briefly, cells (8×10^3 cells/well) were seeded to a 96-well plate in triplicates (as technical replicate) and allowed to adhere for 16 hours before subsequent hypoxic treatment. To take measurements, SF reagent diluted in DMEM (1:1) to a final volume of 20 μ L was added to each well. Absorbance values at 450 nm (developed from SF reagent) and 655 nm (background absorbance) were measured at regular time intervals using the iMark Microplate Absorbance Reader (Bio-Rad) until the values reached the reliable range. For each experiment, two 96-well plates were prepared; one plate would be used for measurement just before treatment (0 h) for normalisation and the other would be used for measurement after treatment (24 h). The fold change in absorbance (24 h divided by 0 h) reflected the change in cell number after treatment.

2.13. Phalloidin staining

Cells were serum-starved with the same procedures as detailed in the transwell invasion assay. The starved cells (8×10^3 cells/dish) were re-seeded to collagen-coated glass bottom dishes (Matsunami Glass, #D11134H) and allowed to adhere for 12 hours. After changing the culture medium to fresh serum-supplemented DMEM (10% FBS), cells were incubated for 24 hours, fixed with 4% paraformaldehyde phosphate buffer solution at room temperature for 30 min, and then washed twice with PBS. Green fluorescein-conjugated Phalloidin, Phalloidin-iFluor 488 (AAT Bioquest, #23115), was diluted in 1% BSA (v/v in PBS) and used for actin filament staining. Cells were incubated in Phalloidin solution for 90 min at room temperature, and then washed twice with PBS. Nucleus was counterstained using Hoechst 33342 diluted in PBS (1:1000) and washed thrice with PBS before mounting in ProLong Glass Antifade Mountant with NucBlue Stain (Invitrogen #P36981). Cells were imaged with the FLUOVIEW FV10i confocal laser scanning microscope (Evident, Olympus Life Science) at high magnification using a 60 $\times$ objective lens. For each sample, at least 5 random fields were imaged. Counting of stress fibre-positive cells was independently performed by a blinded individual.

2.14. Gelatin zymography

HT1080 cells (8×10^5 cells per 35mm collagen-coated dish) transfected with pcDNA4B/HMHA1 or with pcDNA4B/myc-His (empty vector) were seeded and allowed to adhere for 12 hours. Cells were incubated in serum-reduced DMEM (5% v/v FBS) for 48 hours under normoxia. Afterwards, the culture medium was collected for further assay and the cells were harvested for quantification of protein amount in whole cell lysate. Culture medium was centrifuged at $6000 \times g$ (5 min, 4°C) to remove dead cells and debris. Amount of medium used for electrophoresis was normalised according to the cellular protein concentration. Medium sample was mixed with 10 $\times$ non-reducing sample buffer (250 mM Tris-HCl pH 6.8, 8% w/v SDS, 40% v/v glycerol, 0.02% w/v bromophenol blue) and then electrophoresed in 7.5% polyacrylamide gel (pH 8.8) containing 1.25 mg/mL gelatin by applying a constant voltage of 250V. The gel was then rinsed with 2.5% v/v Triton X-100 (in 10 mM Tris-HCl pH 8.0; 30 min $\times$ 2) to remove SDS, followed by washing with 10 mM Tris-HCl pH 8.0 (30 min $\times$ 1) to remove Triton X-100. For enzymatic reaction to occur, the gel was incubated in reaction buffer (50 mM Tris-HCl pH 8.0, 0.5 mM CaCl_2 , 1.0 μM ZnCl_2) at 37°C for 18-24 hours. To visualise gelatinolytic activity, the gel was stained with Coomassie Blue solution (0.2% w/v Coomassie Blue G-250, 10% v/v acetic acid, 50% v/v methanol) and washed with 10% v/v acetic acid/30% v/v methanol solution. Colorimetric images (trans-illumination) of transparent bands were captured using the AmershamTM Imager 680 system (Cytiva). Densitometric analysis was performed using ImageJ software.

2.15. *In vivo* experiment

All animal experiments were approved by the Animal Research Committee of Kyoto University, and conducted in accordance with the guidelines governing animal care in Japan. Eight-week-old female BALB/c *nu/nu* mice were purchased from SLC Inc. To generate xenografts, wild-type HeLa cells or HMHA1 knockout HeLa cells (1×10^6 cells in 100 μ L ice-cold PBS per mouse) were transplanted into the right hind leg of mice by subcutaneous injection. Tumour size was measured using a calliper, and tumour volume was calculated by the following formula: $0.5 \times \text{width}^2 \times \text{length}$ (mm^3). Mice were subjected to anaemia treatment to artificially reduce oxygen supply to the xenografted tumour by intraperitoneal injection of a hemolytic agent, phenylhydrazine hydrochloride (dissolved in PBS, 60 μ g/g of body weight) for three times at 1-day intervals. One day after the last injection, all mice were euthanised by cervical dislocation. The entire tumour and the left kidney were immediately excised and then homogenised in Sepasol RNA I Super G (Nacalai tesque, #09379-84) using the TissueLyser LT (QIAGEN). Total RNA was then extracted for qPCR analysis as previously described. To prepare tumour specimen samples for immunohistochemical analysis, the tumour-bearing mice were intraperitoneally injected with pimonidazole (Hypoxypore, Inc., #HP1), a hypoxia marker, at a dose of 60 μ g/g of body weight. Mice were euthanised by cervical dislocation 60 min after pimonidazole injection, and the tumour was immediately excised and fixed by immersion in 10% formalin solution at room temperature.

2.16. Immunohistochemical (IHC) analysis

Xenograft tumours excised from mice were fixed in 10% formalin solution at room temperature for at least 24 hours. The preparation of formalin-fixed paraffin-embedded (FFPE) tissue blocks and sections was performed by the Center for Anatomical Studies, Graduate School of Medicine, Kyoto University. Tumour sections were deparaffinised and rehydrated by sequential washes in xylene (5 min $\times$ 3), 100% ethanol (5 min $\times$ 2), 90% ethanol (5 min $\times$ 1), 80% ethanol (5 min $\times$ 1), 70% ethanol (5 min $\times$ 1), and PBS (5 min $\times$ 3) at room temperature. For antigen retrieval, slides were incubated in Histofine buffer (pH 6.0; Nichirei #415281) heated to 75°C in water bath for 45 min. After PBS wash (5 min $\times$ 3), samples were permeabilised by incubating in PBS-T (0.05% v/v Tween-20 in PBS) for 5 min, and then washed with PBS (5 min $\times$ 3). Next, the samples were blocked for 1 hour at room temperature using the Protein Block Serum-Free Ready-to-use reagent (Agilent, Dako #X0909), washed with PBS (5 min $\times$ 3), and incubated with the anti-HMHA1 antibody diluted in PBS-T for 16 hours in a humidified box at 4°C. After washing with PBS-T (5 min $\times$ 3), samples were incubated with the corresponding fluorophore-conjugated secondary antibody and anti-pimonidazole antibody diluted in blocking solution, shielded from light, for 90 min at room temperature, and then washed with PBS (5 min $\times$ 3). For nucleus staining, samples were incubated in Hoechst 33342 diluted in PBS (1:1000) for 5 min at room temperature, and then washed with PBS (5 min $\times$ 3). Finally, the sections were mounted with ProLong Glass Antifade Mountant with NucBlue Stain (Invitrogen #P36981) and imaged with the Olympus IX71 fluorescence microscope (Evident, Olympus Life Science). HMHA1 knockout HeLa xenograft tumour was used as negative control. Antibodies and dilution ratios are as follow:

Antibody	Manufacturer	Dilution
Primary antibodies		
Anti-HMHA1/ARHGAP45 rabbit polyclonal	Atlas antibodies, #HPA019816	1:100
FITC-conjugated anti-pimonidazole mouse monoclonal	Hypoxypore, Inc., #HP2-100	1:200
Secondary antibodies		
Alexa Flour 594 goat anti-rabbit IgG	Invitrogen, #A-11072	1:1000

2.17. Patient data analysis

Patient data analysis was performed by Prof. Yasuhara Takaaki. Briefly, the datasets of patient cohorts of ovarian cancer, gastric cancer, bladder cancer, low-grade glioma, and glioblastoma were extracted from The Cancer Genome Atlas (TCGA) database *via* the GDC Data Portal. Patients in each cohort were ranked according to their expression levels of the previously established hypoxia signature which comprises 24 hypoxia-responsive genes [29]. Patients ranked in the highest 10% of the cohort for 6 or more of the 24 hypoxia-responsive genes were considered having high expression of hypoxia signature (“Hypoxia” group), whereas patients ranked in the lowest 10% of the cohort for 6 or more of the 24 hypoxia-responsive genes were considered having low expression of hypoxia signature (“Normoxia” group). Patient overall survival in each group was estimated by the Kaplan-Meier method. The overall survival of “Hypoxia” group and “Normoxia” group of each cancer type were compared by the log-rank test, and the hazard ratio was calculated using the GraphPad Prism software.

2.18. Statistical analysis

All statistical analyses were performed with the GraphPad Prism software. Error bars show the standard deviation (s.d.). Statistical significance was calculated using the Student's *t*-test or Log-rank test. Difference with a *p*-value of 0.05 or smaller was considered statistically significant. (ns: not significant, **p* < 0.05, ** *p* < 0.01, *** *p* < 0.001, **** *p* < 0.0001). For comparisons using patient datasets, the variance was confirmed to be similar between the statistically compared groups.

Chapter 3

Characterisation of *HMHA1* as a hypoxia-responsive gene and its role in the regulation of cancer cell invasiveness

3.1. Screening for a hypoxia-responsive candidate gene responsible for the enhanced invasiveness of hypoxic cancer cells

Under hypoxia, tumour cells acquire malignant properties to promote cancer progression. It is widely recognised that hypoxic cancer cells are more prone to invade and thus contribute to metastasis and post-therapy recurrence. To explore the underlying molecular mechanism, we attempted to pursue a responsible gene that is induced by hypoxia and is related to invasion. Previously, our group performed a genome-wide cDNA microarray analysis to identify the genes that are differentially expressed when HeLa cells were cultured under 20% and < 0.1% O₂ conditions (GEO accession number GSE161393). There were 77 genes that exhibited over 5-fold induction upon hypoxia treatment (Table 1), amongst which we characterised the *HMHA1* gene, with approximately 12.5-fold induction. We decided to focus on it as a plausible candidate because it has been reported to regulate actin cytoskeleton dynamics and naïve T cell migration, as well as to be associated with the *in vitro* migration and invasion activities of melanoma cells [30-33].

To verify the microarray analysis results, we additionally extracted a list of hypoxia-responsive genes from the dataset of an RNA-Seq analysis that our group conducted separately (GEO accession number GSE254480). In line with the microarray dataset, RNA-Seq results also showed that *HMHA1* is upregulated by approximately 9.5-fold in HeLa cells cultured under < 0.1% O₂ (hypoxia) when compared to the 20% O₂ (normoxia) control (Table 2).

Together, these screening analyses reveal that the expression of *HMHA1* gene is enhanced in response to hypoxia and may potentially be the gene contributing to hypoxic cancer cell invasion.

Table 1 List of genes with over 5-fold induction under hypoxia in microarray analysis.

Rank	Gene name	Fold change (H / N)	Rank	Gene name	Fold change (H / N)
1	NDUFA4L2	1619.51	40	ADFP	8.54
2	CA9	538.79	41	PDK1	8.36
3	PPFIA4	190.31	42	ERO1L	7.85
4	FBXL8	72.73	43	TCP11L2	7.83
5	PFKFB4	70.31	44	PAQR6	7.73
6	ANKRD37	35.20	45	CHAC1	7.70
7	PAG1	31.95	46	FOS	7.70
8	LOX	30.37	47	VLDLR	7.55
9	ANGPTL4	28.99	48	INHBE	7.47
10	DDIT3	25.27	49	VEGFA	7.35
11	SLC6A8	24.57	50	MAFB	7.30
12	EGLN3	23.00	51	EFNA3	7.07
13	GAL3ST1	22.56	52	C1QL1	7.06
14	EDN2	21.26	53	P4HA1	7.05
15	ALDOC	19.75	54	SLC6A6	6.84
16	BHLHE40	18.82	55	MUC1	6.74
17	DDA1	18.65	56	JUN	6.50
18	SPINK1	18.09	57	SERPINE1	6.47
19	PIK3IP1	16.84	58	GPR155	6.38
20	KCNK3	14.71	59	WDR54	6.27
21	NDRG1	12.54	60	ORAI3	6.13
22	HMHA1	12.47	61	DOK3	6.07
23	ISG20	11.92	62	SLCO1B3	5.95
24	BNIP3L	11.00	63	QSOX1	5.92
25	GPR146	10.92	64	FOXD1	5.91
26	FUT11	10.86	65	DUSP5	5.87
27	SLC2A3	10.26	66	HOXA13	5.87
28	INSIG1	10.14	67	NIACR2	5.76
29	PPP1R3B	9.94	68	KDM3A	5.69
30	CCNG2	9.88	69	HK1	5.58
31	INSIG2	9.52	70	HEY1	5.51
32	KCTD11	9.35	71	HK2	5.47
33	ITGA5	9.32	72	GPR160	5.35
34	ERRFI1	9.20	73	KIAA1715	5.27
35	AK3L1	9.18	74	SFXN3	5.15
36	PDK3	9.10	75	IER3	5.15
37	IGFBP3	9.10	76	TMEM45A	5.09
38	BNIP3	8.78	77	SPRY1	5.06
39	LOXL2	8.67			

Table 2 List of top 80 genes with highest induction ratio in RNA-Seq analysis.

Rank	Gene name	Fold change (H / N)	Rank	Gene name	Fold change (H / N)
1	CA9	N / A *	36	UPP1	18.40
	SERPINE1		37	DNAJB9	18.17
	EDN2		38	IL32	17.38
	RNF223		39	ASNS	17.04
	BIK		40	SHB	16.60
	FAM115C		41	SLC2A3	16.32
	IL1A		42	HERPUD1	15.95
	SLX1B		43	BIRC3	15.75
	INHBE		44	ISG20	15.01
	DMBT1		45	ADM	14.64
	NKG2-E		46	PDK3	14.52
	CNTD2		47	HSPA5	14.43
	HNRNPA1P48		48	SESN2	14.34
2	SPRR2D	185.82	49	ZNF503	14.28
3	NDUFA4L2	137.06	50	BNIP3	13.83
4	DDIT3	128.58	51	HKDC1	13.66
5	TRAF1	114.72	52	PPP1R15A	13.64
6	UPK3BL	65.41	53	BNIP3L	12.83
7	JUN	56.45	54	IER3	12.60
8	ANGPTL4	52.99	55	TM4SF19_2	12.53
9	PPFIA4	47.47	56	GJB3	12.47
10	EGLN3	46.76	57	DOK3	12.43
11	ERRFI1	45.41	58	TRIB3	12.33
12	DUSP5	44.25	59	LOX	12.25
13	NDRG1	44.21	60	BHLHE40	12.07
14	SLC6A8	43.41	61	PTPRH	12.06
15	LUCAT1	41.78	62	ARID5A	11.86
16	PPP2R5B	36.20	63	TNFAIP3	11.40
17	IGFBP3	32.29	64	ATF3	11.13
18	KCTD11	31.21	65	SLC2A1	10.92
19	ANKRD37	31.15	66	TMPRSS3	10.71
20	RABGEF1	29.80	67	GSTT2B	10.68
21	ALDOC	28.86	68	CSRNP1	10.37
22	MAFF	28.32	69	ITGA5	10.06
23	RNF144B	28.21	70	RPPH1	10.04
24	TMEM158	26.95	71	FAM110C	9.98
25	PFKFB4	25.76	72	LIF	9.85
26	SH3D21	23.03	73	HMHA1	9.68
27	INSIG2	22.88	74	MC1R	9.51
28	PLIN2	22.13	75	EDEM1	9.34
29	TCP11L2	19.62	76	LOXL2	9.34
30	GDF15	19.46	77	HILPDA	9.30
31	CHAC1	19.13	78	PTTG1IP	9.26
32	KRT16	19.09	79	GPT2	9.18
33	RN7SL2	19.08	80	SGK223 (PRAG1)	9.18
34	FICD	19.01			
35	HK2	18.60			

* Gene expression level below detection level (value = 0) under normoxia

3.2. HMHA1 expression is induced under hypoxia both *in vitro* and *in vivo*

In order to examine the generality of hypoxia-induced HMHA1 expression, a panel of human cancer cell lines were used for qPCR analysis. After incubating cells at < 0.1% O₂ hypoxic conditions for 24 hours, it was found that although the basal expression levels varied between different cell lines, the mRNA level of HMHA1 was significantly higher than the normoxia control in all tested cell lines (Figure 2.1). These results mean that the hypoxic induction of HMHA1 is not cell line-specific, but common to different types of cancer cells.

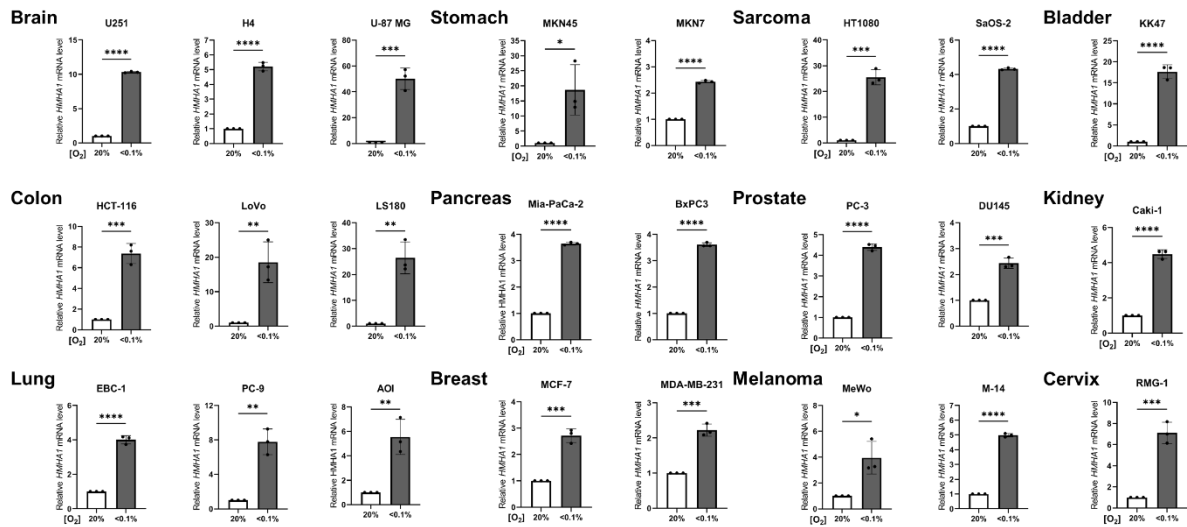


Figure 2.1 Hypoxia increases HMHA1 expression in a variety of cancer cell lines. qPCR was performed using the indicated cell lines after incubation under normoxic (20% O₂) or hypoxic (< 0.1% O₂) conditions to quantify HMHA1 mRNA expression. Results are shown as mean $\pm$ s.d. ($n = 3$); * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ (Student's t -test).

Next, to analyse the HMHA1 expression levels under different oxygen, three different cell lines, HeLa (cervical cancer), SBC-3 (small cell lung cancer) and HepG2 (hepatoma), were selected and incubated under normoxic (20% O₂), moderately hypoxic (1% O₂), and severely hypoxic (< 0.1% O₂) for 24 hours. The hypoxic induction of HMHA1 was first analysed by qPCR. In all three cell lines, both moderate and severe hypoxic conditions led to increased HMHA1 mRNA levels when compared to the normoxia control (Figure 2.2, upper panel). Of note, in HeLa and SBC-3 cells, HMHA1 mRNA level increased to a much smaller extent at 1% O₂ than at < 0.1% O₂, whereas in HepG2 cells, the HMHA1 mRNA levels at 1% and < 0.1% O₂ conditions were similarly high (Figure 2.2). This indicates that although different cell lines may have different sensitivities to hypoxia stimuli and may respond differently under the same culture condition, hypoxia enhances the mRNA expression levels of HMHA1. Alongside the increase in HMHA1 mRNA levels, Western blotting results show that both moderate and severe hypoxia treatment significantly enhanced HMHA1 protein expression as well (Figure 2.2, bottom panel).

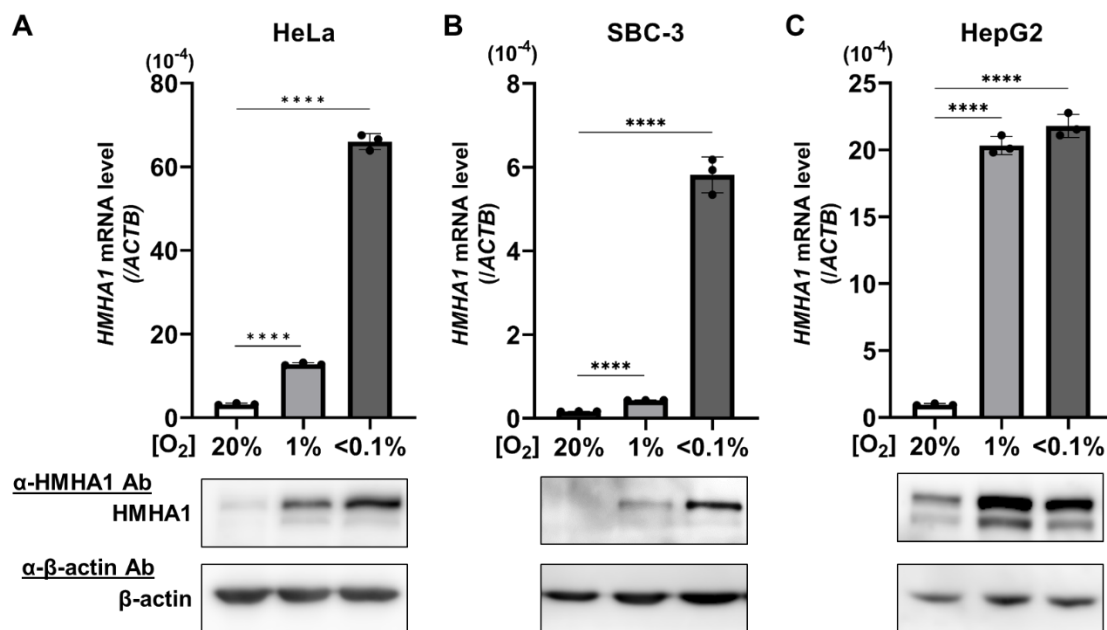


Figure 2.2 Hypoxia increases both mRNA and protein levels of HMHA1. (A) HeLa cells were cultured under normoxic (20% O₂), moderately hypoxic (1% O₂), or severely hypoxic (< 0.1% O₂) conditions. qPCR (upper panel) and Western blotting (bottom panel) were performed to determine HMHA1 mRNA and protein expression levels, respectively. (B-C) The same experiments were repeated using SBC-3 small cell lung cancer cells (B) and HepG2 hepatoma cells (C). Results are shown as mean $\pm$ s.d. ($n = 3$); **** $P < 0.0001$ (Student's t -test).

Interestingly, when HeLa cells were cultured under milder hypoxic condition (3% O₂), an oxygen concentration that is below the oxygen content of normal cervical tissue but above that of cervical cancer tissue [34], mRNA level of HMHA1 was not elevated compared to the normoxia control, and protein expression was also not detected (Figure 2.3).

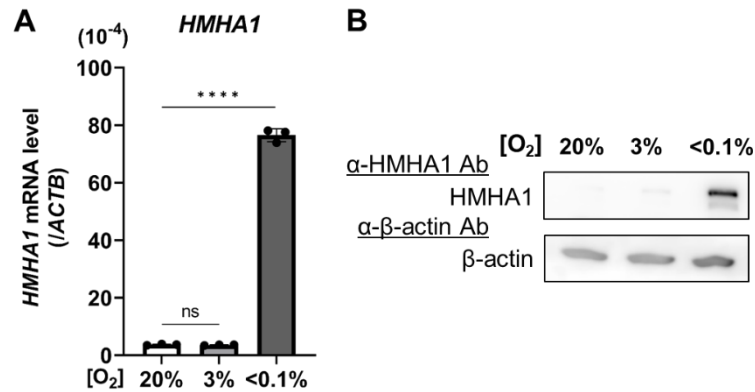


Figure 2.3 Mild hypoxia does not induce HMHA1 expression. (A-B) HeLa cells were cultured under normoxic (20% O₂), mildly hypoxic (3% O₂), or severely hypoxic (< 0.1% O₂) conditions. qPCR (A) and Western blotting (B) were performed to determine HMHA1 mRNA and protein expression levels, respectively. Results are shown as mean ± s.d. (*n* = 3); *****P* < 0.0001 (Student's *t*-test).

Having shown that hypoxia induces HMHA1 *in vitro*, we then investigated whether the induction can also be observed *in vivo*. To this end, xenograft tumour mouse model was employed. HeLa cells were injected subcutaneously to athymic nude mice to generate xenograft tumours. As the tumour volume exceeded 1,000 mm³, the tumour-bearing mice were subjected to anaemia treatment by intraperitoneal (IP) administration of phenylhydrazine, which allowed for the pharmacological induction of hypoxia, and subsequently sacrificed for nephrectomy and tumour excision (Figure 2.4A).

As expected, qPCR analysis showed that Epo (erythropoietin) mRNA levels in kidneys of phenylhydrazine-treated mice were significantly upregulated when compared to that of PBS-treated mice (negative control). These results indicate that IP injection of phenylhydrazine successfully induced anaemia and hypoxia response in the treated mice (Figure 2.4B). Concordantly, qPCR analysis using the xenograft tumour samples found that tumour HMHA1 mRNA level was significantly higher in the phenylhydrazine-treated group than the PBS-treated group (Figure 2.4C). These data indicate that tumour hypoxia can enhance HMHA1 expression.

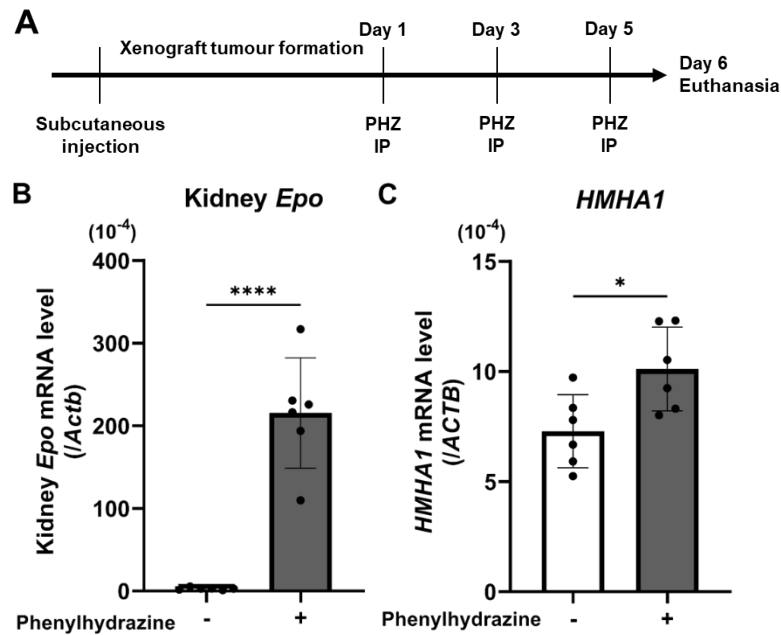


Figure 2.4 Phenylhydrazine-induced anaemia increases HMHA1 expression in xenografted tumour. (A) Schedule of anaemia treatment. Mice were treated with phenylhydrazine (PHZ) by intraperitoneal (IP) injection on Day 1, Day 3 and Day 5 after the volume of xenografted tumour exceeded 1,000 mm³. On Day 6, mice were sacrificed for sample collection. (B) Kidney RNA samples from PHZ-treated and control mice were subjected to qPCR analysis to assess *Epo* mRNA expression level. (C) Xenografted tumour RNA samples from the same mice in (B) were subjected to qPCR analysis to assess HMHA1 mRNA expression level. Results are shown as mean $\pm$ s.d. ($n = 6$); * $P < 0.05$, **** $P < 0.0001$ (Student's t -test).

To assess the induction of HMHA1 in relation to intratumoral hypoxia more directly, HeLa xenograft tumours were generated in another group of nude mice separately, and the mice were injected with Pimonidazole, a hypoxia marker, before euthanasia. The resultant tumours were used to prepare formalin-fixed paraffin-embedded (FFPE) serial sections for immunohistochemical (IHC) analysis in addition to H&E staining. Pimonidazole-stained section, when apposed to the H&E section, shows that pimonidazole-positive hypoxic regions were indeed situated distant from blood vessels in the tumour (Figure 2.5A,D). Besides, co-staining of the tumour sections with anti-pimonidazole antibody and anti-HMHA1 antibody illustrates that HMHA1 was expressed in the pimonidazole-positive regions (Figure 2.5A-C).

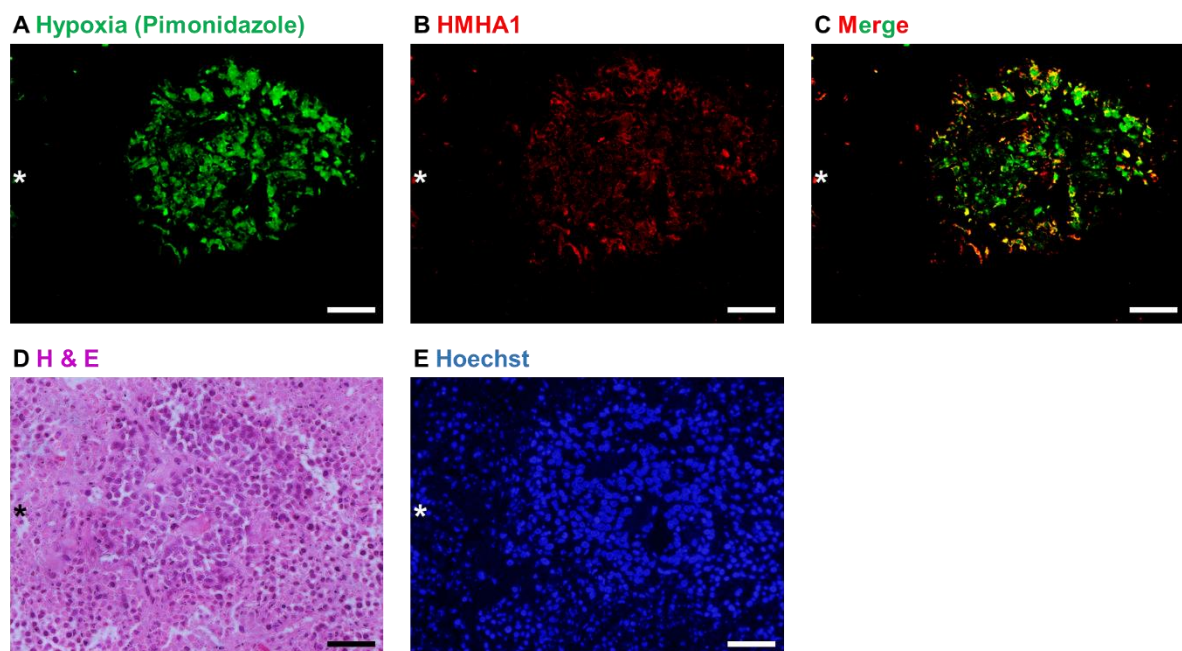


Figure 2.5 Immunohistochemical (IHC) staining of formalin-fixed paraffin-embedded (FFPE) serial sections of HeLa xenograft tumour. (A-B) Tumour section was co-stained with FITC-conjugated anti-pimonidazole antibody (A) and anti-HMHA1 antibody (B). (C) Merged image of (A) and (B) shows the colocalisation of HMHA1 and hypoxia marker. (D) H & E staining for histopathological analysis of the serial section from the same tumour. (E) Same section as (A) and (B) was counterstained with Hoechst 33342 to visualise the nucleus. The asterisk symbol (*) denotes the position of blood vessel. Scale bar: 50 μ m.

For negative control, HMHA1 knockout HeLa cells were used to generate xenograft tumours. As expected, when double staining with anti-pimonidazole antibody and anti-HMHA1 antibody was performed, HMHA1 could not be detected in the pimonidazole-positive regions, indicating that the anti-HMHA1 antibody specifically recognises and detects HMHA1 protein expression in this setting of IHC staining (Figure 2.6). Thus, these IHC data reflect that HMHA1 expression is induced in the intratumoral hypoxic regions.

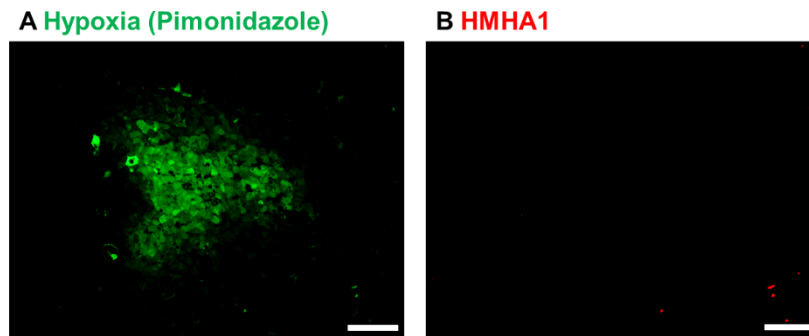


Figure 2.6 Immunohistochemical (IHC) staining of formalin-fixed paraffin-embedded (FFPE) serial sections of HMHA1 knockout HeLa xenograft tumour. (A-B) Tumour section was co-stained with FITC-conjugated anti-pimonidazole antibody (A) and anti-HMHA1 antibody (B).

Taken all together, these results demonstrate that hypoxia induces HMHA1 expression both *in vitro* and *in vivo*.

3.3. Hypoxic induction of HMHA1 is regulated by HIF at the transcription step

To elucidate the regulatory mechanism of HMHA1 induction under hypoxia, we started by examining whether HMHA1 is regulated transcriptionally. Actinomycin D, which binds DNA to stall RNA polymerase activity, was used to inhibit transcription. Cells were incubated in the presence or absence of actinomycin D under normoxic and hypoxic conditions, and then harvested for qPCR analysis. The mRNA levels of representative hypoxia-inducible genes, *CA9* and *VEGFA*, were significantly increased by hypoxia treatment in the ethylene glycol-treated (solvent control) group. The increase was abrogated in the actinomycin D-treated group, indicating that actinomycin D treatment indeed obstructed transcription activity (Figure 3.1A,B). Similarly, hypoxia treatment upregulated HMHA1 mRNA expression in ethylene glycol-treated cells, but there were no changes between normoxia and hypoxia upon actinomycin D treatment (Figure 3.1C). These results demonstrate that the hypoxic induction of HMHA1 is regulated at the transcription step.

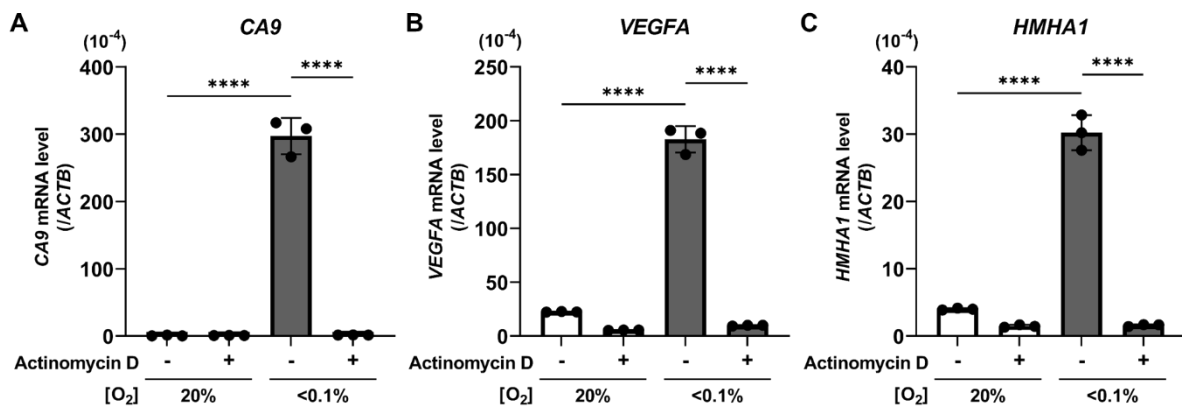


Figure 3.1 Hypoxic induction of HMHA1 is regulated at the transcription initiation stage. (A-C) HeLa cells treated with actinomycin D or the solvent control were incubated under normoxic (20% O_2) or hypoxic (< 0.1% O_2) conditions. qPCR was performed to assess the mRNA expression levels of CA9 (A), VEGFA (B) and HMHA1 (C). Results are shown as mean $\pm$ s.d. ($n = 3$); **** $P < 0.0001$ (Student's t -test).

Hypoxia-inducible factor (HIF) is the major transcription factor that governs the expressions of thousands of hypoxia-responsive genes. Therefore, we sought to test whether HIF also regulates the hypoxic induction of HMHA1 expression. Under normoxia, the α -subunit of HIF is hydroxylated by the O_2/Fe^{2+} ion/2-oxoglutarate-dependent dioxygenases (2-OGDDs), propyl-4-hydroxylase domain protein (PHD) and factor inhibiting HIF (FIH), which respectively cause the degradation of the HIF α -subunit and suppression of HIF transactivation activity. Thus, we first used deferoxamine (DFO), a chelator of Fe^{2+} ions, to inhibit 2-OGDD activities and analyse the involvement of HIF. As expected, cells treated with DFO retained the expression of HIF-1 α under normoxic conditions (Figure 3.2A), and the mRNA expression of CA9, a representative HIF downstream gene, was also significantly increased in the DFO-treated group when compared to the untreated control (Figure 3.2B). These data show that DFO treatment could indeed induce HIF activity even under normoxia. Next, qPCR and Western blotting were performed to assess HMHA1 expression. Compared to the untreated control, it was found that both mRNA and protein levels of HMHA1 were significantly higher upon DFO treatment (Figure 3.2A,C).

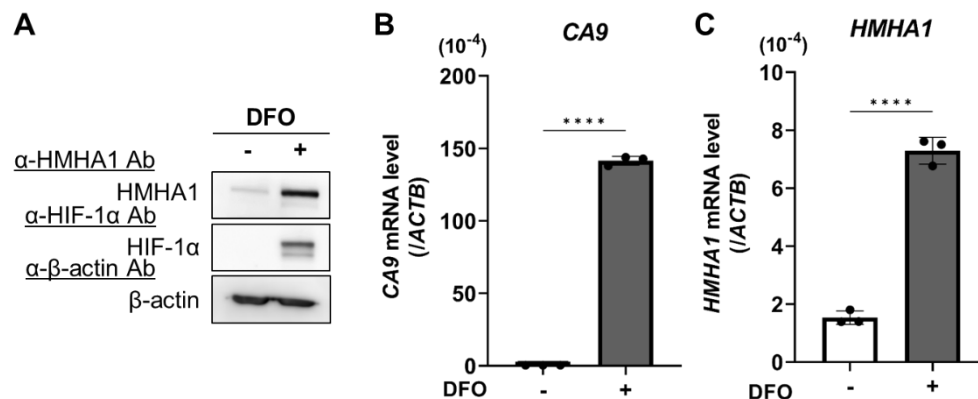


Figure 3.2 Deferoxamine (DFO) treatment increases HMHA1 expression under normoxia. (A) HeLa cells were cultured under normoxic conditions with or without DFO treatment. Western blotting was performed to determine HIF-1 α and HMHA1 protein expression levels. (B-C) qPCR was performed to assess the mRNA expression levels of CA9 (B) and HMHA1 (C) in HeLa cells subjected to the same treatment in (A). Results are shown as mean $\pm$ s.d. ($n = 3$); **** $P < 0.0001$ (Student's t -test).

Similarly, when the cells were treated with dimethyloxallylglycine (DMOG), a 2-oxoglutarate analogue, HIF-1 α protein could be detected under normoxia, and the expression of the HIF downstream gene CA9 was upregulated, indicating that DMOG can also activate HIF even under normoxia (Figure 3.3A,B). And, as expected, both the mRNA and protein expression of HMHA1 was increased in DMOG-treated cells when compared with the untreated control (Figure 3.3A,C).

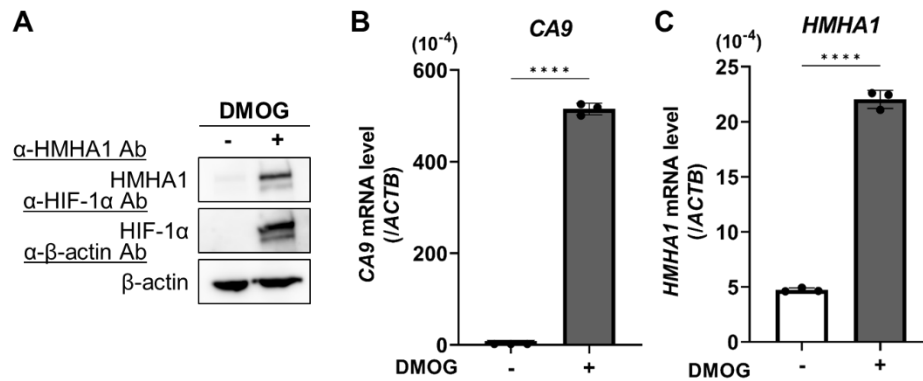


Figure 3.3 Dimethyloxallylglycine (DMOG) treatment increases HMHA1 expression under normoxia. (A) HeLa cells were cultured under normoxic conditions with or without DMOG treatment. Western blotting was performed to determine HIF-1 α and HMHA1 protein expression levels. (B-C) qPCR was performed to assess the mRNA expression levels of CA9 (B) and HMHA1 (C) in HeLa cells subjected to the same treatment in (A). Results are shown as mean $\pm$ s.d. ($n = 3$); **** $P < 0.0001$ (Student's t -test).

These results together show that the induction of HMHA1 is dependent on 2-OGDD activities, supporting that HIF is likely involved in the regulation of HMHA1 expression.

There exists more than 60 members in the 2-OGDD protein family, and neither DFO nor DMOG is specific to PHD and FIH inhibition. Therefore, the involvement of HIF in HMHA1 expression under hypoxia was directly investigated by adopting the knockout strategy. The CRISPR/Cas9 system was employed to respectively establish HIF-1 α (*HIF1A*) knockout HeLa cells and HIF-1 β (*ARNT*) knockout HeLa cells. For both HIF-1 α knockout and HIF-1 β knockout, two individual clones generated using different combinations of guide RNAs were selected for subsequent experiments in order to cope with clonal variation and non-specific off-target effects (Figure 3.4).

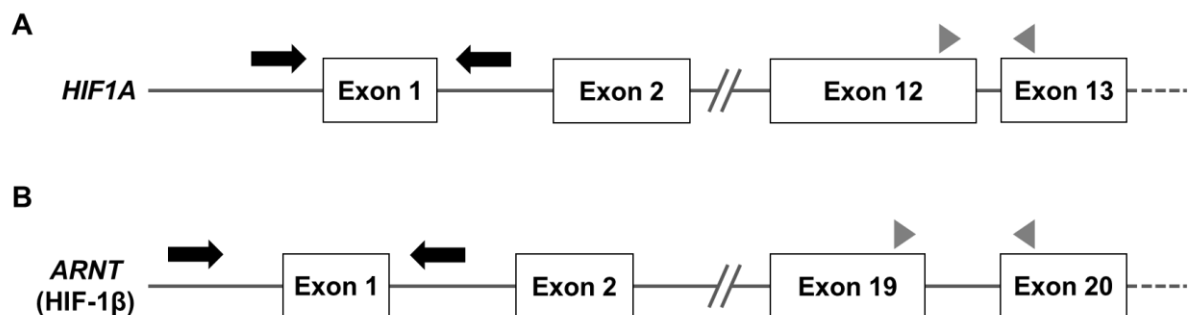


Figure 3.4 Schematic diagram of HIF1A and ARNT knockout. (A-B) The guide RNAs were designed to target sequences in the upstream of Exon 1 and in the Intron 1 (black arrows) of *HIF1A* gene (A) or *ARNT* (HIF-1 β) gene (B). Annealing sites of the primer sets used for qPCR are indicated by the grey arrowhead.

Successful knockout has previously been confirmed by genome sequencing our group members performed. Additionally, qPCR results verified that the mRNA expressions of both HIF1A and ARNT (HIF-1 β) dramatically decreased, likely because the excision of the first exon and its upstream region which contains the transcription start site abrogated the initiation of transcription (Figure 3.5A,C). Moreover, the representative downstream gene of HIF1A and ARNT (HIF-1 β), CA9, was completely abrogated in the corresponding knockout cells (Figure 3.5B,D).

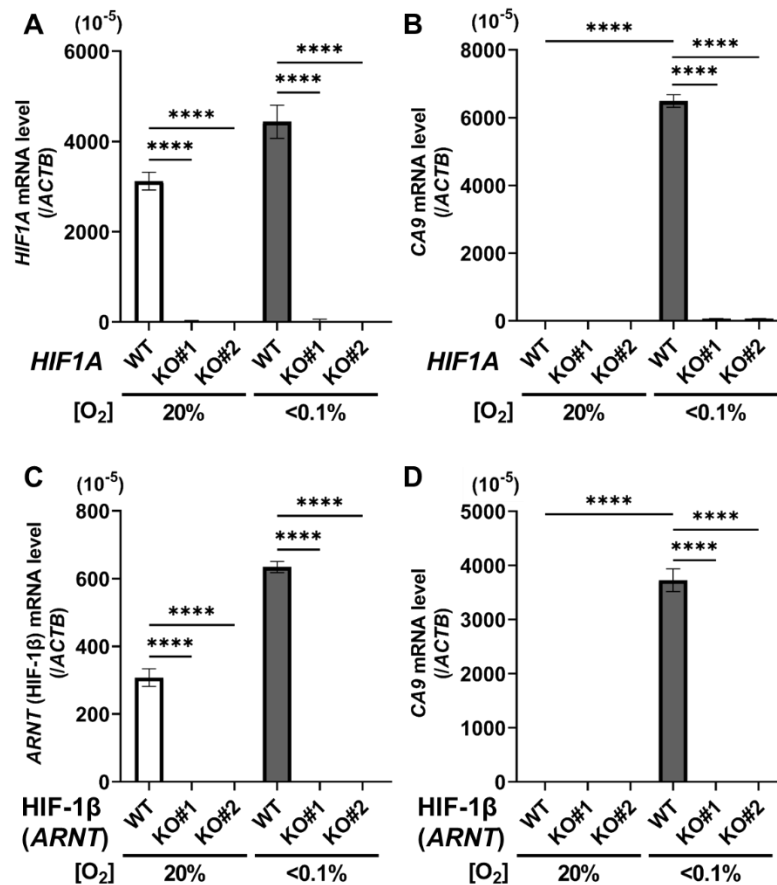


Figure 3.5 HIF-1 α and HIF-1 β knockout confirmation. (A-B) HIF-1 α wild-type (WT) parent HeLa cells and HIF-1 α knockout clones (KO#1 and KO#2) were cultured under normoxic (20% O₂) or hypoxic (< 0.1% O₂) conditions. qPCR was performed to assess the mRNA expression levels of HIF1A (A) and CA9 (B). (C-D) HIF-1 β wild-type (WT) parent HeLa cells and HIF-1 β knockout clones (KO#1 and KO#2) were cultured under normoxic (20% O₂) or hypoxic (< 0.1% O₂) conditions. qPCR was performed to assess ARNT (HIF-1 β) mRNA (C) and CA9 mRNA (D) expression levels. Results are shown as mean $\pm$ s.d. ($n = 3$); **** $P < 0.0001$ (Student's t -test).

The expression of HMHA1 was then examined using the knockout cell lines. Under hypoxia, HMHA1 expression was significantly enhanced in parental HeLa cells that constitutively expresses HIF-1 β . On the other hand, in the two clones of HIF-1 β knockout cells, HMHA1 mRNA expression showed no differences between the hypoxia-treated cells and the normoxia control (Figure 3.6A). In line with this, HMHA1 protein expression was also not detected under hypoxic conditions in Western blotting (Figure 3.6B). These data demonstrate that HIF-1 β knockout completely cancelled HMHA1 induction under hypoxia.

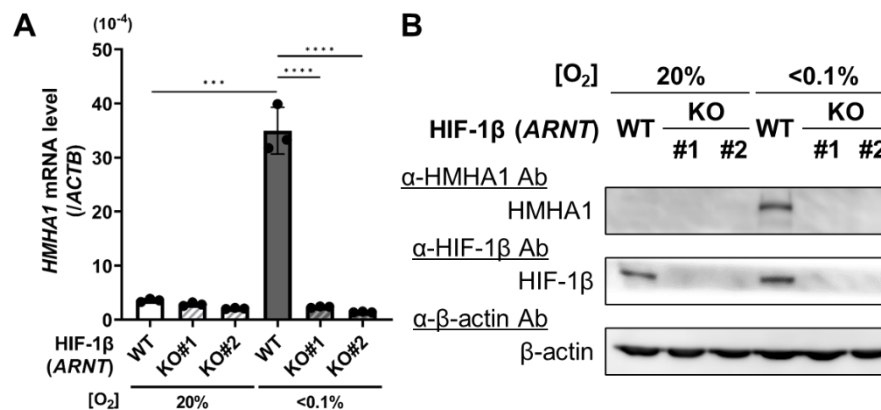


Figure 3.6 Knockout of HIF-1 β abolishes HMHA1 induction under hypoxia. (A-B) HIF-1 β wild-type (WT) parent HeLa cells and HIF-1 β knockout clones (KO#1 and KO#2) were cultured under normoxic (20% O₂) or hypoxic (< 0.1% O₂) conditions. qPCR was performed to determine HMHA1 mRNA expression levels (A) and Western blotting was performed to assess the protein expression levels of HMHA1 and HIF-1 β (B). Results are shown as mean $\pm$ s.d. ($n = 3$); *** $P < 0.001$, **** $P < 0.0001$ (Student's t -test).

Moreover, when HIF-1 β expression was reconstituted by transfecting the HIF-1 β knockout cells with the pcDNA4/HIF-1 β overexpression vector, the induction of HMHA1 by hypoxia treatment was restored (Figure 3.7). These data together showed that the hypoxic induction of HMHA1 was dependent on HIF-1 β .

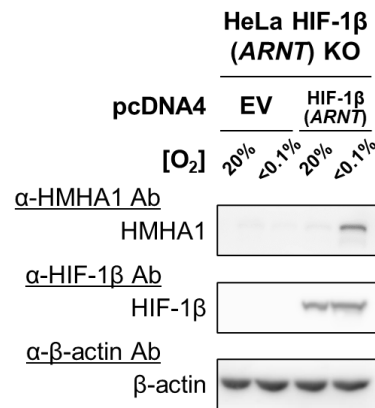


Figure 3.7 Reconstitution of HIF-1 β to knockout cells rescued HMHA1 expression. HIF-1 β knockout HeLa cells were transfected with the pcDNA4A/HIF-1 β -myc-His vector or the corresponding empty vector (EV) and then cultured under normoxic (20% O₂) or hypoxic (< 0.1% O₂) conditions. Western blotting was performed to assess the protein expression levels of HMHA1 and HIF-1 β .

Surprisingly, in HIF-1 α knockout cells, HMHA1 expression was, despite to a lesser extent than the parental HeLa cells, significantly upregulated under hypoxic conditions (Figure 3.8A). Western blotting results also showed that, unlike HIF-1 β knockout which completely abrogated HMHA1 induction, substantially higher expression of HMHA1 protein could be detected under hypoxia than normoxia even in the absence of HIF-1 α , although the extent of increase was milder than the parent cells (Figure 3.8B).

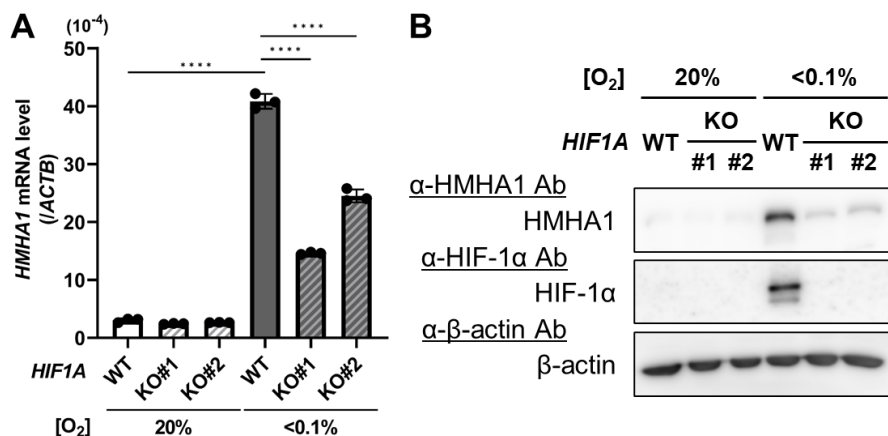


Figure 3.8 Knockout of HIF-1 α partially cancelled HMHA1 induction under hypoxia. (A-B) HIF-1 α wild-type (WT) parent HeLa cells and HIF-1 α knockout clones (KO#1 and KO#2) were cultured under normoxic (20% O₂) or hypoxic (< 0.1% O₂) conditions. qPCR was performed to determine HMHA1 mRNA expression levels (A) and Western blotting was performed to assess the protein expression levels of HMHA1 and HIF-1 α (B). Results are shown as mean $\pm$ s.d. ($n = 3$); **** $P < 0.0001$ (Student's t -test).

In addition, HIF-1 α reconstitution reversed such decrease. The forced expression of HIF-1 α in knockout cells resulted in a comparable level of HMHA1 expression as in the control cells overexpressing HIF-1 α (Figure 3.9). Together, these results showed that the hypoxic induction of HMHA1, albeit partially, was dependent on HIF-1 α .

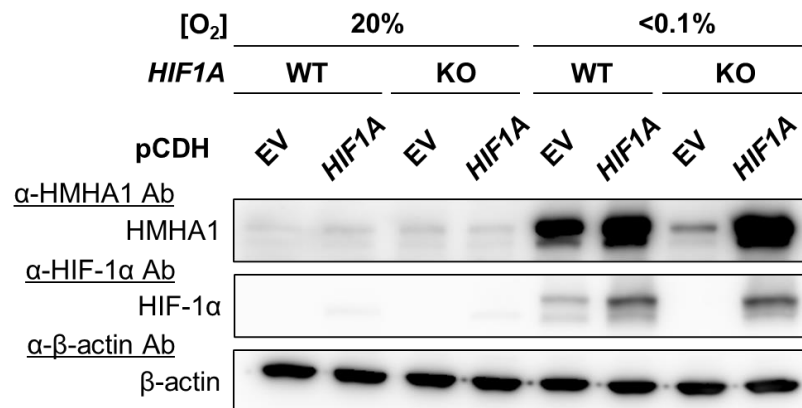


Figure 3.9 Reconstitution of HIF-1 α to knockout cells rescued HMHA1 expression. Wild-type (WT) parent HeLa cells and HIF-1 α knockout (KO) HeLa cells stably transfected with the pCDH/EF1.HIF-1 α -IRES-Puro vector (HIF1A) or the corresponding empty vector (EV) were cultured under normoxic (20% O₂) or hypoxic (< 0.1% O₂) conditions. Western blotting was performed to assess the protein expression levels of HMHA1 and HIF-1 α .

Whilst HIF-1 β knockout totally cancelled HMHA1 induction, knockout of its major binding partner, HIF-1 α , only partially reduced HMHA1 expression, indicating that HIF-1 α is not the only factor that function conjunctively with HIF-1 β . Thus, we explored the contribution of another binding partner of HIF-1 β - HIF-2 α (*EPAS1*), an isoform of the HIF-1 α protein.

siRNA-mediated knockdown of HIF-2 α (siHIF2A) was first confirmed by qPCR analysis (Figure 3.10A). Suppression of HIF-2 α expression decreased HMHA1 induction under hypoxia modestly in parental HeLa cells. Meanwhile, in HIF-1 α knockout cells, siHIF2A led to a further reduction in HMHA1 expression when compared to the treatment with control siRNA (Figure 3.10B), demonstrating that in addition to HIF-1 α , HIF-2 α is also involved in the regulation of HMHA1 induction under hypoxia.

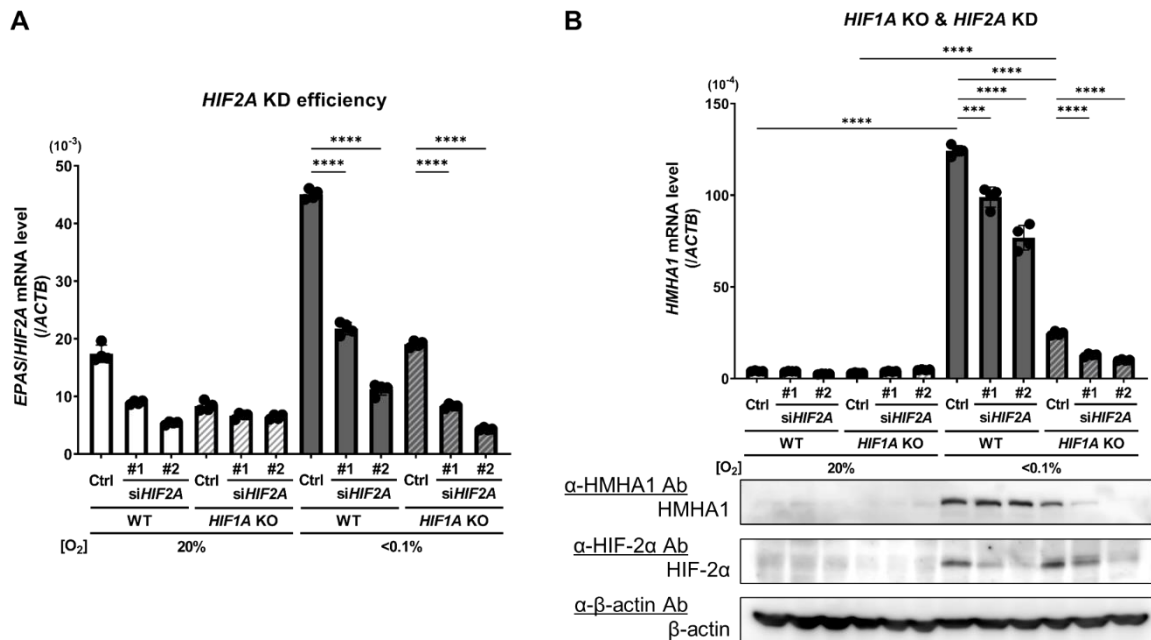


Figure 3.10 Suppression of HIF-2 α in addition to HIF-1 α knockout further reduced HMHA1 expression. (A) Wild-type (WT) parent HeLa cells and HIF-1 α knockout HeLa cells were transfected with siRNA targeting HIF-2 α (siHIF2A) or the control siRNA (Ctrl), and then incubated under normoxic (20% O₂) or hypoxic (< 0.1% O₂) conditions. qPCR was performed to assess HIF2A (EPAS) mRNA levels. (B) Cells subjected to the same treatment as in (A) were analysed for HMHA1 mRNA and protein expression levels by qPCR and Western blotting, respectively. Results are shown as mean $\pm$ s.d. ($n = 4$); *** $P < 0.001$, **** $P < 0.0001$ (Student's t -test).

Likewise, siRNA-mediated knockdown of HIF-1 α (siHIF1A) was also performed and the knockdown efficiency was confirmed by qPCR analysis (Figure 3.11A). When HIF-1 α was knocked down in parental HeLa cells, HMHA1 induction was substantially subdued, with low level of expression remaining. HIF-2 α knockout alone only partially reduced HMHA1 expression. But the combination of HIF-1 α knockdown and HIF-2 α knockout completely abrogated the hypoxic induction of HMHA1 (Figure 3.11B). This substantiates that HMHA1 expression is under the regulation of both HIF α -subunits.

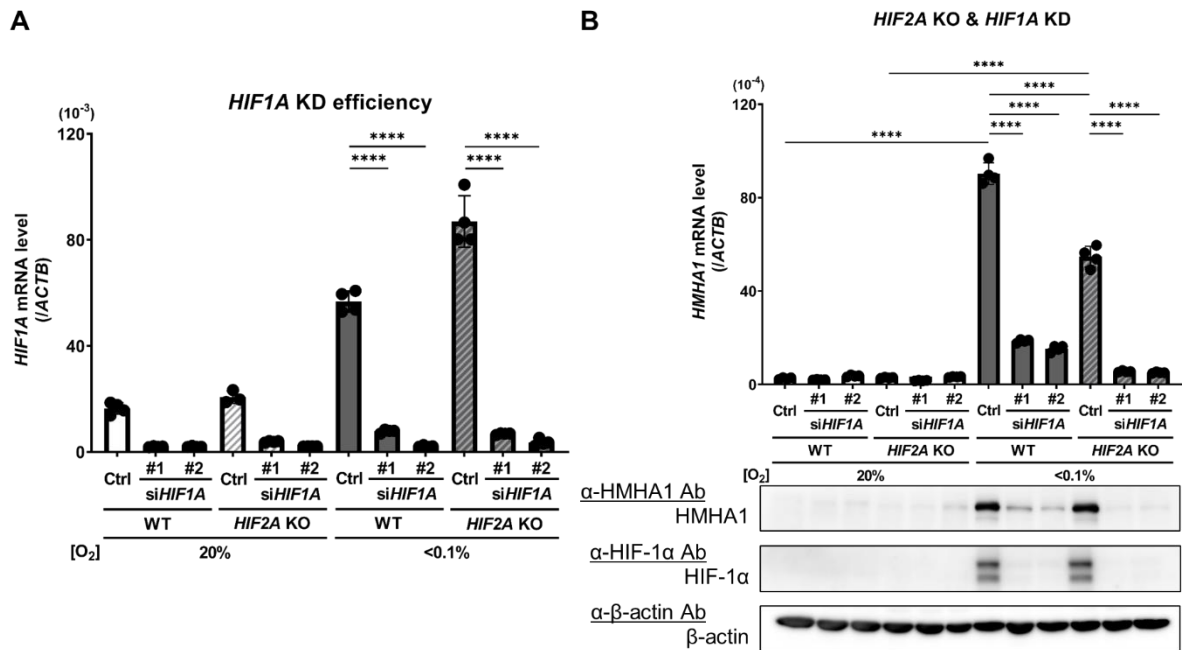


Figure 3.11 Suppression of HIF-1 α in addition to HIF-2 α knockout abrogated HMHA1 expression. (A) Wild-type (WT) parent HeLa cells and HIF-2 α knockout HeLa cells were transfected with siRNA targeting HIF-1 α (siHIF1A) or the control siRNA (Ctrl), and then incubated under normoxic (20% O₂) or hypoxic (< 0.1% O₂) conditions. qPCR was performed to assess HIF1A mRNA levels. (B) Cells subjected to the same treatment as in (A) were analysed for HMHA1 mRNA and protein expression levels by qPCR and Western blotting, respectively. Results are shown as mean $\pm$ s.d. ($n = 4$); **** $P < 0.0001$ (Student's t -test).

Collectively, these data attested the hypoxic induction of HMHA1 being regulated at transcription level by both HIF-1 and HIF-2.

Last, in order to investigate whether *HMHA1* is a direct target gene of HIF, chromatin immunoprecipitation (ChIP)-qPCR was performed to evaluate the recruitment of HIF-1 α to the *HMHA1* gene locus. HeLa cells incubated under normoxic or hypoxic (< 0.1% O₂) conditions for 24 hours were fixed by formalin so that DNA-protein crosslinks were created. After cell lysis and shearing of chromatin by sonication, anti-HIF-1 α antibody or mouse IgG antibody (negative control) was used for the immunoprecipitation of chromatin fragments. Purified DNA was obtained after crosslink reversal and RNase digestion, and was then subjected to qPCR analysis.

Using a primer set that flanks the transcription start site in the promoter region (hereafter, TSS region) of the *CA9* gene locus, which is known to contain HRE sequence that HIF binds to, qPCR was performed to confirm the recruitment of HIF-1 α to the loci of its downstream genes (Figure 3.12A). As expected, results showed that the TSS region was not immunoprecipitated by IgG antibody in both normoxia and hypoxia samples, indicating that there were no non-specific pulldown of chromatin DNA (Figure 3.12B). On the other hand, with the anti-HIF-1 α antibody, whilst the *CA9* TSS region was not detected in the normoxia sample, significantly increased amount was immunoprecipitated from the hypoxia sample (Figure 3.12B). This verifies that the recruitment of HIF-1 α protein to the loci of its downstream genes, like *CA9*, could be specifically detected in this ChIP-qPCR setting.

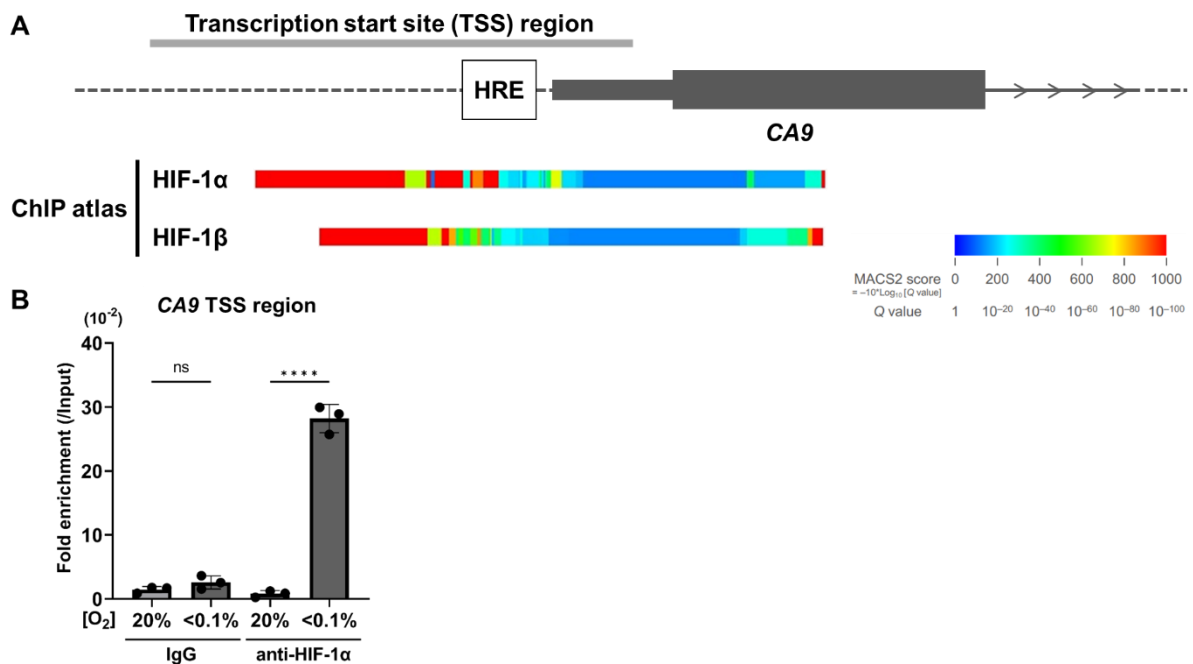


Figure 3.12 HIF-1 α is recruited to the transcription start site (TSS) region in the *CA9* gene locus under hypoxia. (A) Schematic diagram showing the *CA9* gene locus. Primers for ChIP-qPCR were designed to amplify the transcription start site (TSS) region as indicated by the bold grey line on top. Putative binding regions of HIF-1 α and HIF-1 β were retrieved from the ChIP atlas database. Statistical

significance values (MACS2 scores) are shown by the colour code as indicated. **(B)** HeLa cells were incubated under normoxic (20% O₂) or hypoxic (< 0.1% O₂) conditions and subjected to ChIP-qPCR analysis. Chromatin immunoprecipitation was performed using anti-HIF-1 α antibody or mouse IgG (negative control). Results are shown as mean $\pm$ s.d. ($n = 3$); *ns*: not significant, **** $P < 0.0001$ (Student's *t*-test).

According to the ChIP atlas database (<https://chip-atlas.org/>), it is suggested that HIF binds to the intron 1 of *HMHA1* gene in various cell lines. Hence, different sets of primers amplifying three consecutive regions (Region 1-3) in the *HMHA1* intron 1 locus and a primer set flanking the 5' upstream region of exon 1 (negative control) were designed for ChIP-qPCR (Figure 3.13A). From the results, it was found that the amount of immunoprecipitated chromatin DNA containing Region 1 was slightly increased under hypoxia (Figure 3.13B). And that containing Region 2 was considerably enhanced to a similar extent as that observed in the CA9 positive control (Figure 3.12B,3.13C). This indicates that hypoxia treatment promoted the recruitment of HIF-1 α to these regions in the intron 1 of *HMHA1* gene locus. Conversely, HIF-1 α recruitment to Region 3, akin to the case of the 5' upstream control region, remained unchanged from the background levels and showed no upregulation under hypoxia, suggesting that HIF-1 α was not recruited therein (Figure 3.13D,E).

Together, these data demonstrated that HIF-1 α was recruited to the *HMHA1* gene locus at the intron 1, particularly Region 2, under hypoxia, supporting that the hypoxic induction of HMHA1 expression is under the direct regulation by HIF.

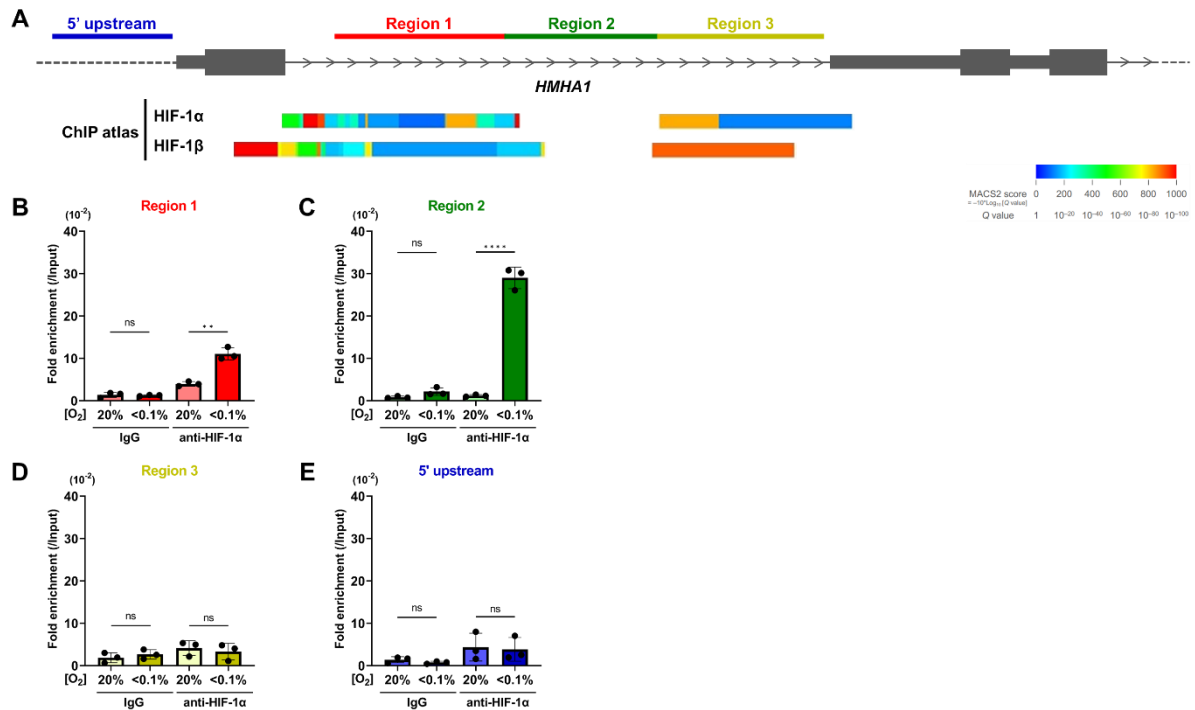


Figure 3.13 HIF-1α is recruited to the intron 1 of *HMHA1* gene locus under hypoxia. (A) Schematic diagram showing the *HMHA1* gene locus. Primer sets for ChIP-qPCR were designed to amplify the various regions as indicated. Putative binding regions of HIF-1α and HIF-1β were retrieved from the ChIP atlas database. Statistical significance values (MACS2 scores) are shown by the colour code as indicated. (B-E) HeLa cells were incubated under normoxic (20% O₂) or hypoxic (< 0.1% O₂) conditions and subjected to ChIP-qPCR analysis. Chromatin immunoprecipitation was performed using anti-HIF-1α antibody or mouse IgG (negative control). Primers specifically amplifying Region 1 (B), Region 2 (C), Region 3 (D), and the 5' upstream promoter region (E) were used for qPCR, respectively. Results are shown as mean ± s.d. (n = 3); ns: not significant, **P < 0.01, ****P < 0.0001 (Student's *t*-test).

3.4. HMHA1 expression is required for the augmented invasiveness of cancer cells under hypoxia

Having shown that HMHA1 expression is induced by hypoxia in a HIF-dependent manner, we next sought to investigate whether such induction effectuates changes in the invasiveness of hypoxic cancer cells. To study cancer cell invasion *in vitro*, the standard transwell invasion assay was conducted. Cells were primed for invasion by starvation with serum-reduced medium, and then seeded to the upper chamber of the device (Figure 4.1A). Following 24 hours of culture under normoxia (20% O₂) or hypoxia (1% O₂), it was found that invading cells mounted by over 2 times under hypoxic conditions when compared to the normoxia control (Figure 4.1B,C), indicating that the stimulatory effect of hypoxia on cancer cell invasion could be aptly captured and appraised by this experiment setting.

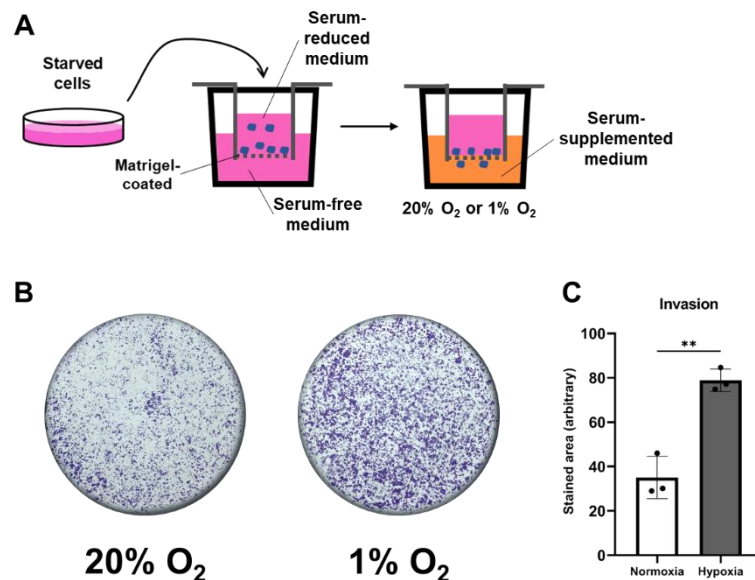


Figure 4.1 Hypoxia augments cancer cell invasion. (A) Schematic diagram illustrating the design of transwell invasion assay. (B) HeLa cells were seeded to the upper chamber of the transwell invasion device and incubated under normoxic (20% O₂) or hypoxic (1% O₂) conditions to determine invasion activities. (C) Number of invading cells in (B) was quantified as the stained area. Results are shown as mean $\pm$ s.d. ($n = 3$); ** $P < 0.01$ (Student's t -test).

To assess the involvement of HMHA1 in hypoxic cancer cell invasion, the loss-of-function approach was adopted. For this purpose, a highly invasive fibrosarcoma cell line, HT1080, was selected, and knockdown using siRNA was performed. After transfecting HT1080 cells with siRNA targeting HMHA1 (siHMHA1) or with the control siRNA, the knockdown efficiency was confirmed by qPCR. Results showed that mRNA levels of HMHA1 were greatly reduced in siHMHA1-treated cells when compared to the control siRNA-treated cells, with knockdown efficiencies reaching approximately 80% or higher (Figure 4.2).

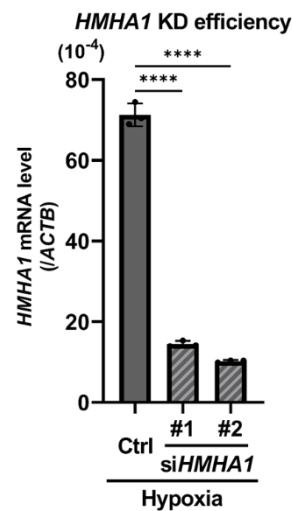


Figure 4.2 Efficiency of siRNA-mediated HMHA1 knockdown (KD). HT1080 cells transfected with siRNA targeting HMHA1 (siHMHA1 #1 and #2) or with control siRNA (Ctrl) were incubated under hypoxic ($< 0.1\% \text{ O}_2$) conditions. qPCR was performed to assess HMHA1 mRNA expression levels. Results are shown as mean $\pm$ s.d. ($n = 3$); **** $P < 0.0001$ (Student's t -test).

Next, siRNA-transfected cells were starved and subjected to transwell invasion assay under hypoxia. Results showed that whilst cells transfected with the control siRNA displayed remarkable invasion activity, siHMHA1-transfected cells had significantly fewer invading cells. This result means that HMHA1 functions in invasion of cancer cells under hypoxia (Figure 4.3).

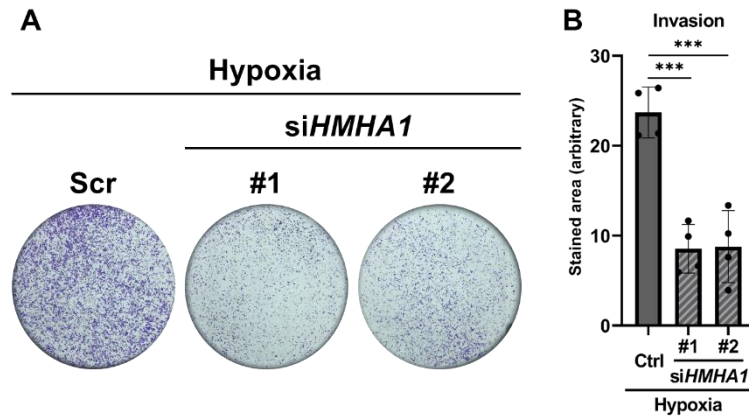


Figure 4.3 Knockdown of HMHA1 suppresses cancer cell invasion under hypoxia. (A) HT1080 cells were transfected with siRNA targeting HMHA1 (siHMHA1 #1 and #2) or with control siRNA (Ctrl) and then subjected to the transwell invasion assay under hypoxic (1% O₂) conditions. (B) Number of invading cells in (A) was quantified as the stained area. Results are shown as mean $\pm$ s.d. ($n = 4$); *** $P < 0.001$ (Student's t -test).

In order to examine whether the forced depletion of HMHA1 by siRNA affected cell survival, which might be fallaciously interpreted as changes in invasion activity, a mitochondrial activity-based colorimetric cell viability assay was performed. Cells were pre-treated and incubated under the same conditions as in the transwell invasion assay, and measurements were taken just before (0h) and after 24 hours (24h) of hypoxia treatment. As expected, mitochondrial activity of siHMHA1-treated cells and the control siRNA-treated cells increased by the same extent after 24 hours, suggesting that there were no differences in proliferation and cell death between the treated groups and control group (Figure 4.4). Hence, siHMHA1 did not affect cell viability and the transwell invasion assay results truly reflected the changes in invasion activity upon HMHA1 knockdown.

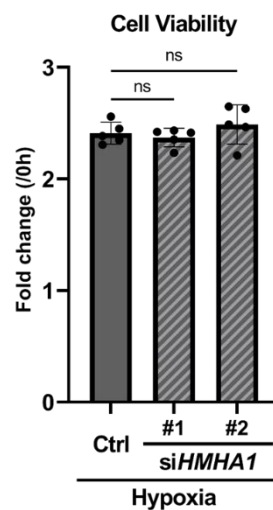


Figure 4.4 HMHA1 knockdown does not affect cell viability. HT1080 cells transfected with siRNA targeting HMHA1 (siHMHA1 #1 and #2) or with control siRNA (Ctrl) were cultured under hypoxic (1% O₂) conditions for 24 hours and then subjected to a colorimetric cell viability assay. Results are shown as mean $\pm$ s.d. ($n = 5$); ns: not significant (Student's t -test).

Taken together, these results demonstrated the importance of HMHA1 in cancer cell invasion under hypoxic conditions.

3.5. HMHA1 promotes cancer cell invasion by enhancing MMP activity

In order to decipher the molecular mechanism by which HMHA1 regulated invasion activities, we next sought to examine how HMHA1 affected various biological processes pertaining to cancer cell invasion from a gain-of function approach.

To begin with, stable overexpression (OE) and control cell lines were established by infecting HeLa cells with lentivirus incorporating the HMHA1 coding sequence and lentivirus without any sequences inserted, respectively. Under normoxia, whilst the control cells showed only background level of expression, both mRNA and protein levels of HMHA1 in the OE cells were considerably higher in comparison, showing that the forced stable expression of HMHA1 was successful (Figure 5.1).

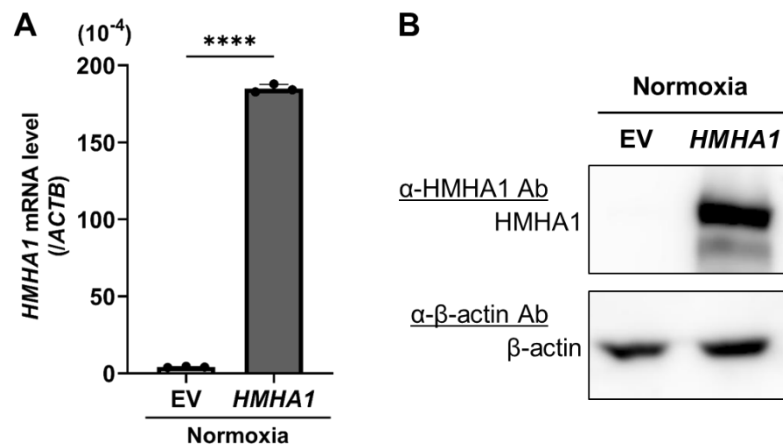


Figure 5.1 Confirmation of HMHA1 stable overexpression. (A-B) HeLa cells transduced with the HMHA1 coding sequence (*HMHA1*) or the corresponding empty vector control (EV) were cultured under normoxia. qPCR and Western blotting were performed to assess HMHA1 mRNA (A) and protein (B) expression levels, respectively. Results are shown as mean $\pm$ s.d. ($n = 3$); **** $P < 0.0001$ (Student's t -test).

Moreover, in transwell invasion assay, the OE cells had significantly more invading cells than the control cells under normoxic conditions. This confirmed that the artificial overexpression of HMHA1 could also instigate functional changes, and that HMHA1 overexpression could augment invasion activity even under normoxic conditions (Figure 5.2).

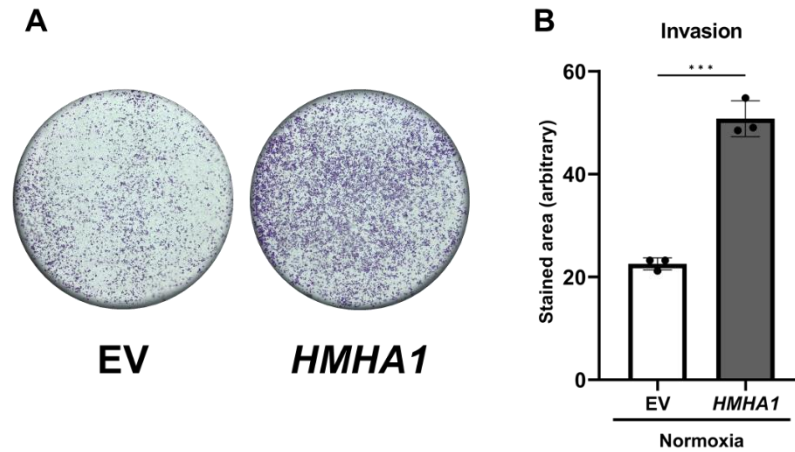


Figure 5.2 Overexpression of HMHA1 augments invasion under normoxia. (A) HeLa cells stably overexpressing HMHA1 and the control cells (EV) were subjected to the transwell invasion assay under normoxic (20% O₂) conditions. (B) Number of invading cells in (A) was quantified as the stained area. Results are shown as mean $\pm$ s.d. ($n = 3$); *** $P < 0.001$ (Student's t -test).

According to previous literature [30,31], HMHA1 possesses a GTPase-activating protein (GAP) domain and regulates the activities of small GTPases RHOA and RAC1, both of which are important for regulating actin cytoskeleton dynamics and cell morphologies. We therefore sought to study the effect of HMHA1 expression on actin filament structure. Using green fluorescein-conjugated Phalloidin, a chemical compound that binds to filamentous actin (F-actin) fibres, the structures of actin filaments in HMHA1 OE cells and control cells were examined. Intriguingly, whilst control cells displayed organised actin filaments and remarkable stress fibres, such structures were observed at much lower frequency in the OE cells and actin filaments also appeared relatively distorted (Figure 5.3A). Indeed, when assessing the cells based on the presence of stress fibres, significantly larger proportion of cells in the control group had prominent stress fibres when compared to the OE cells (Figure 5.3B). These data show that HMHA1 can regulate actin filament organisation and alter cytoskeleton structure, which may in turn render the OE cells deformability and invasiveness.

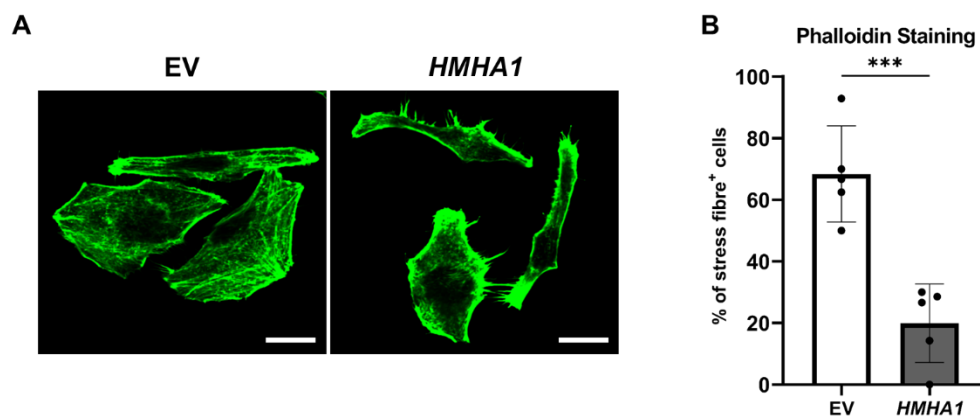


Figure 5.3 Overexpression of HMHA1 augments invasion under normoxia. (A-B) HeLa cells stably overexpressing HMHA1 and the control cells (EV) were cultured under normoxic (20% O₂) conditions and stained with phalloidin to visualise actin filaments (A), the percentage of cells with prominent stress fibres in each group was quantified (B). Results are shown as mean ± s.d. ($n = 5$); *** $P < 0.001$ (Student's t -test).

Since invasion can also be facilitated by cancer cells actively degrading the ECM, we next explored the possibility that HMHA1 might have potentiated ECM decomposition to enhance invasiveness. Generally, ECM degradation in tumour tissues is exacerbated by the secretion of proteolytic enzymes, which can cleave the ECM components, from cancer cells. The matrix metalloproteinases (MMPs), MMP-2 and MMP-9, are two major enzymes that can promote the degradation of collagen fibres in the ECM. Therefore, qPCR was first performed to examine whether HMHA1 overexpression upregulates the mRNA levels of these two MMPs. Unexpectedly, the mRNA levels of neither MMP2 nor MMP9 showed significant changes

when comparing the HMHA1-overexpressing cells and control cells, demonstrating that HMHA1 does not regulate MMP2 and MMP9 mRNA expression (Figure 5.4).

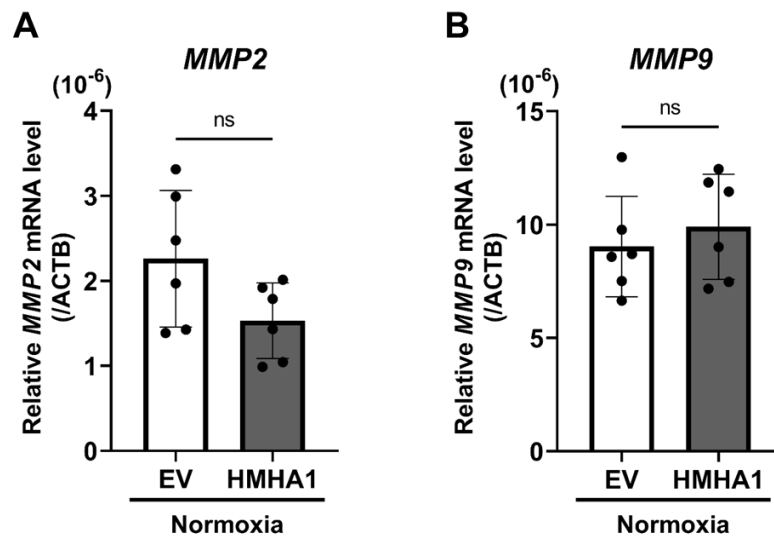


Figure 5.4 Overexpression of HMHA1 does not affect MMP mRNA levels. (A-B) HeLa cells stably overexpressing HMHA1 and the control cells (EV) cultured under normoxic (20% O₂) conditions were subjected to qPCR analysis to assess the mRNA expression levels of MMP2 (A) and MMP9 (B). Results are shown as mean $\pm$ s.d. ($n = 6$); ns: not significant (Student's t -test).

Considering that MMP activity can be regulated at multiple levels (e.g. post-translationally), we sought to directly investigate whether HMHA1 induction affects MMP activities by conducting gelatin zymography. In this assay, MMP activity is assessed by the decomposition of gelatin, the mono-chain form of collagen molecule, after applying the cell culture medium to electrophoresis in gelatin-containing polyacrylamide gel.

Strikingly, bands at the positions corresponding to the active forms of MMP-2 and MMP-9 were noticeably more intense for culture medium from HMHA1-overexpressing cells when compared to that from wild-type control cells (Figure 5.5A-C). This shows that HMHA1 enhanced MMP-2 and MMP-9 activities and thereby promoted ECM degradation. In parallel, the culture medium samples were also subjected to electrophoresis in polyacrylamide gel without gelatin so as to assess the amount of total protein in the medium loaded to the gel. Coomassie Blue staining of the gel showed generally similar intensity of smear in the HMHA1-overexpressing and control samples, meaning that the amount of loaded protein was similar between the two groups (Figure 5.5D). Thus, the change in MMP activity was not due to variation in protein loading. These data together demonstrate that HMHA1 enhances the gelatinolytic activities mediated by secreted MMP-2 and MMP-9, *via* post-transcriptional regulatory mechanism, to facilitate the decomposition of ECM, which potentially can promote cancer cell invasion.

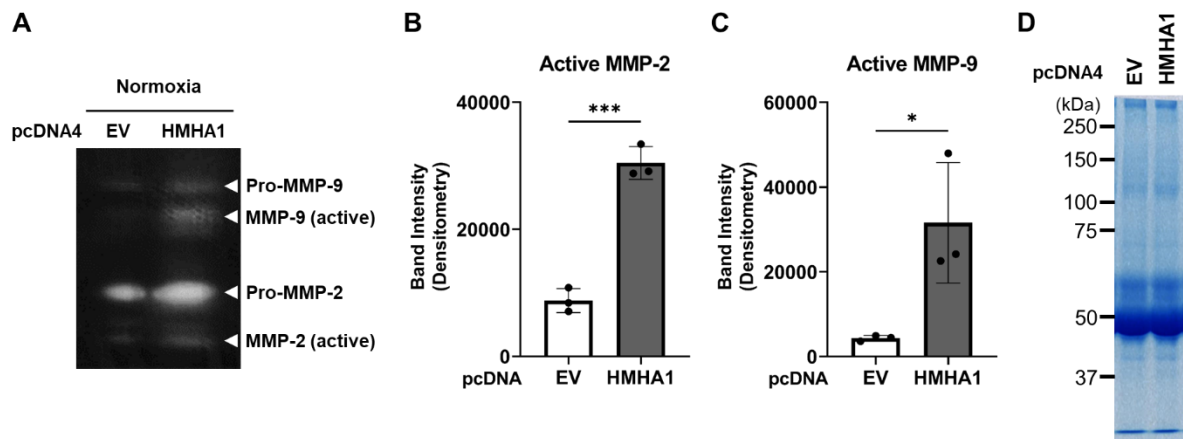


Figure 5.5 Overexpression of HMHA1 enhances MMP activity. (A) HT1080 cells transfected with the pcDNA4B/HMHA1 vector or the corresponding empty vector (EV) were cultured under normoxic (20% O₂) conditions for 48 hours. The culture medium samples were subjected to gelatin zymography to determine the enzymatic activities of excreted MMP-2 and MMP-9 proteins. (B-C) Band intensity in (A) was quantified by densitometric analysis for active MMP-2 (B) and active MMP-9 (C). (D) Total protein amount in the culture medium sample was assessed by electrophoresis in gelatin-free polyacrylamide gel and Coomassie Blue staining. Results are shown as mean $\pm$ s.d. ($n = 3$); $*P < 0.05$, $***P < 0.001$ (Student's *t*-test).

To further investigate the role of MMP in HMHA1-mediated invasion, transwell invasion assay was next performed whilst using a pharmacological inhibitor that specifically blocks MMP-2 and MMP-9 activities. HeLa OE cells and control cells were starved as aforementioned, and then re-seeded to the top chamber of the invasion transwell device either in the presence or absence of MMP-2/-9 inhibitor (MMPi). In the DMSO-treated control group, the OE cells had significantly more invading cells than the control cells, again showing that HMHA1 overexpression can promote cancer cell invasion. Importantly, when treated with MMPi, whereas the invasion of control cells remained unaffected, the invasion of OE cells was substantially suppressed (Figure 5.6A,B). The number of invading MMPi-treated OE cells was only slightly higher than that of the corresponding control cells, indicating that the inhibition of MMP activities in OE cells almost completely reversed the enhancement in invasion induced by HMHA1 overexpression (Figure 5.6B).

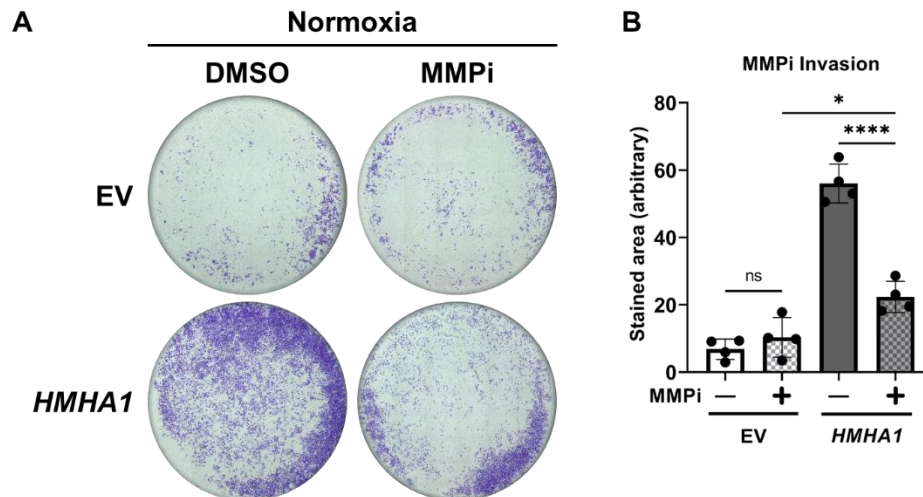


Figure 5.6 MMP inhibition reverses HMHA1-induced invasion. (A) HeLa cells stably overexpressing HMHA1 and the control cells (EV) were subjected to the transwell invasion assay under normoxic (20% O₂) conditions in the presence of DMSO or MMP-2/-9 inhibitor (MMPi). (B) Number of invading cells in (A) was quantified as the stained area. Results are shown as mean $\pm$ s.d. ($n = 4$); ns: not significant, * $P < 0.05$, **** $P < 0.0001$ (Student's t -test).

To examine whether MMPi treatment affected cell viability, OE cells and control cells were incubated with or without MMPi, and cell number was counted at 0 h and 24 h after treatment. As expected, regardless of the presence or absence of MMPi, there were no differences in cell number change across all groups, showing that MMPi treatment did not influence cell survival and proliferation (Figure 5.7). Thus, these results together demonstrated that HMHA1 indeed promotes cancer cell invasion by upregulating MMP activities.

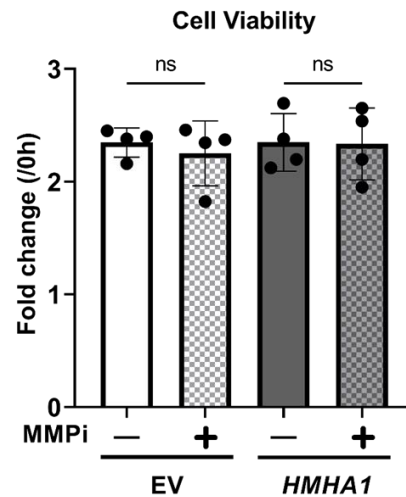


Figure 5.7 MMP inhibition does not affect cell viability. HeLa cells stably overexpressing HMHA1 and the control cells (EV) were cultured with or without MMP-2/-9 inhibitor (MMPi) for 24 hours. Cell count was performed before and after treatment, and viability is determined by the change in cell number. Results are shown as mean $\pm$ s.d. ($n = 4$); *ns*: not significant (Student's *t*-test).

All together, these results collectively unveiled the potential molecular mechanisms of HMHA1-induced invasion, not only by impeding stress fibre formation, which might contribute to cellular deformability, but also by enhancing MMP activities to aid ECM degradation.

3.6. HMHA1 expression is associated with hypoxia gene signature in cancer patients and links with poor prognosis

To evaluate the clinical relevance of hypoxia-induced HMHA1 expression, cancer patient data was extracted from The Cancer Genome Atlas (TCGA) database. According to their expression level of a previously established 24-gene hypoxia signature [29], patients with the same type of cancer were ranked and segregated. Patients with high expression of the hypoxia-inducible genes were deemed to have more hypoxic tumours, and were thus assigned to the “Hypoxia” group. Conversely, patients with low expression of the hypoxia-inducible genes were deemed to have less hypoxic or normoxic tumours, and were assigned to the “Normoxia” group (Figure 6.1).

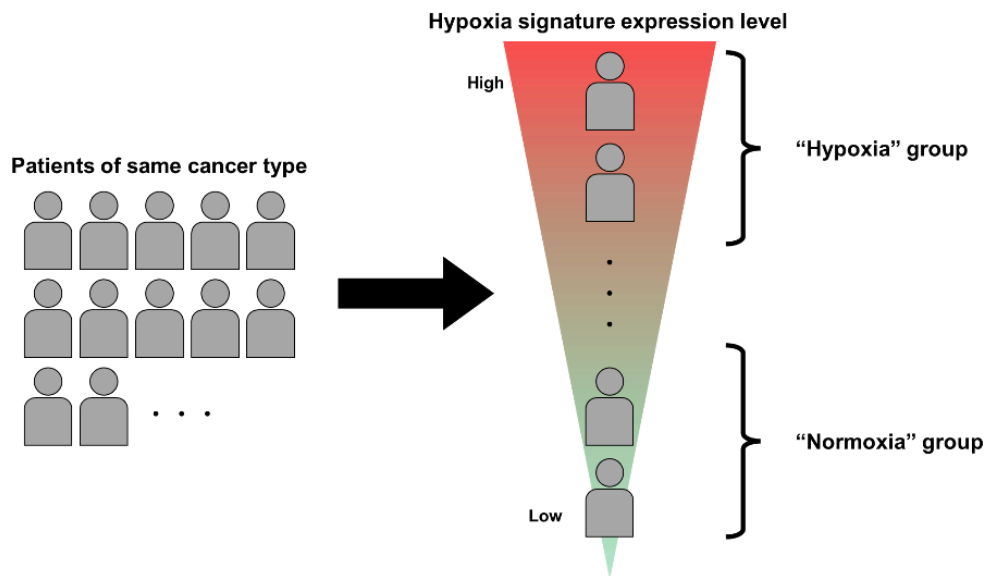


Figure 6.1 Schematic diagram of patient segregation. Cancer patient data was obtained from TCGA database. For each type of cancer analysed, patients were ranked according to their expressions of hypoxia signature genes, and those fulfilling the set criteria were put into the “Hypoxia” or “Normoxia” group correspondingly.

Next, HMHA1 expression levels of the patients in the two groups were analysed. Concordant to the *in vitro* and *in vivo* findings, it was found that patients in the “Hypoxia” group had significantly higher HMHA1 expression than the patients in the “Normoxia” group (Figure 6.2). And, such trend could be consistently observed across various cancer types, including ovarian cancer, gastric cancer, bladder cancer, low-grade glioma, and glioblastoma, showing that HMHA1 expression is associated with tumour hypoxia in cancer patients.

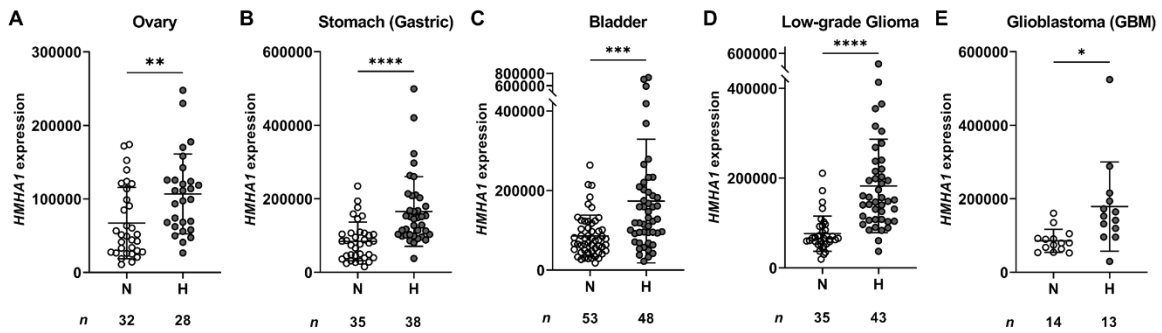


Figure 6.2 HMHA1 expression is associated with the expression of hypoxia signature in cancer patients. (A-E) The HMHA1 expression levels of patients in the “Normoxia” group (N) and “Hypoxia” group (H) were retrieved from the dataset of ovarian cancer (A), gastric cancer (B), bladder cancer (C), low-grade glioma (D) and glioblastoma (GBM; E). Results are shown as mean $\pm$ s.d. Number of patients (*n*) in each group is shown below the graph; **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001 (Student’s *t*-test).

Furthermore, the overall survival of patients in the “Hypoxia” and “Normoxia” groups were analysed by the Kaplan-Meier method. Results show that patients in the “Hypoxia” group, who also have higher HMHA1 expression, had significantly poorer overall survival outcomes when compared to the “Normoxia” group patients (Figure 6.3).

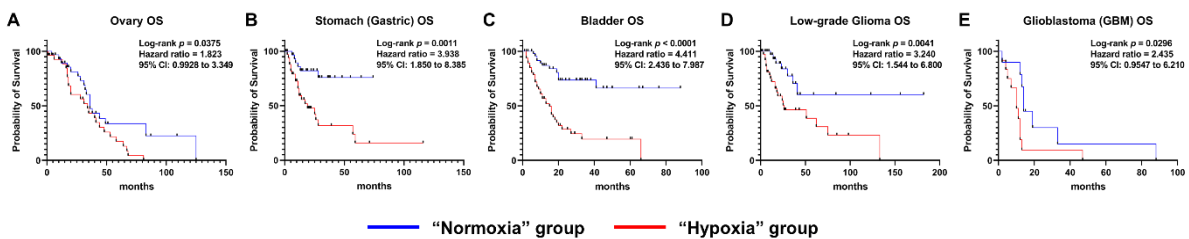


Figure 6.3 Patients in “Hypoxia” group have worse survival outcome than patients in “Normoxia” group. (A-E) Overall survival analyses were performed using the Kaplan-Meier method. Survival data of patients in the “Normoxia” group and “Hypoxia” group were retrieved from the dataset of ovarian cancer (A), gastric cancer (B), bladder cancer (C), low-grade glioma (D) and glioblastoma (GBM; E). *P*-values, hazard ratios, and 95% confidence intervals are as indicated (Log-rank test).

Taken together, these results provided additional evidence supporting that HMHA1 is a hypoxia-responsive gene, and indicated that high HMHA1 expression levels may predict poor prognosis in patients

Chapter 4

**Further induction of HMHA1 expression
after irradiation and reoxygenation
treatment promotes the invasion activity
of “ex-hypoxic” cancer cells**

4.1. Post-irradiation reoxygenation treatment further enhances HMHA1 expression in “ex-hypoxic” cancer cells in a HIF-dependent manner

It has previously been reported that, after radiation therapy, severely hypoxic tumour cells locating distant from blood vessels undergo reoxygenation, acquire HIF activity, and subsequently translocate to the proximal regions of blood vessels *in vivo* [24]. So, we next sought to investigate whether HMHA1 is also involved in this process. To simulate the *in vivo* conditions, HeLa cells were incubated under hypoxic (< 0.1%) conditions for 24 hours before being subjected to irradiation and subsequent reoxygenation treatment. Samples were collected immediately (0 h) after reoxygenation (“Reoxygenation –”) and 6 hours after reoxygenation (“Reoxygenation +”) for qPCR analysis (Figure 7.1A).

At 0 h, both irradiated and unirradiated hypoxia samples showed higher HMHA1 expression than the normoxia samples, meaning that hypoxia treatment induced HMHA1 and the expression was maintained even after irradiation and reoxygenation treatment (Figure 7.1B,C). Of note, the expression level of HMHA1 was comparable between the irradiated and unirradiated hypoxia samples at 0 h, indicating that irradiation and reoxygenation treatment did not immediately change HMHA1 expression, and that the two groups had the same basal levels of hypoxia-induced HMHA1 immediately after reoxygenation treatment (“Reoxygenation –”). After 6 hours of reoxygenation, the unirradiated cells showed a drop in HMHA1 mRNA level, likely because re-exposure to oxygen caused the rapid degradation of HIF α -subunits and the transcription of HMHA1 gene discontinued in the absence of hypoxia stimuli (Figure 7.1B). Contrarily, HMHA1 mRNA levels of the irradiated cells was further increased at 6 h, in spite of the re-exposure to oxygen (Figure 7.1C). Moreover, such increase in HMHA1 mRNA level was only observed in the cells incubated under hypoxic conditions before irradiation-reoxygenation treatment, but not in the control cells incubated under normoxic conditions (Figure 7.1C). These data demonstrate that neither irradiation treatment, nor reoxygenation of hypoxic cells alone could enhance HMHA1 expression; but irradiation under hypoxic conditions and the subsequent reoxygenation, which is the conditions observed in the distal regions of blood vessels in tumour tissues after radiation therapy, conjointly triggered the further induction of HMHA1 in the “ex-hypoxic” cells.

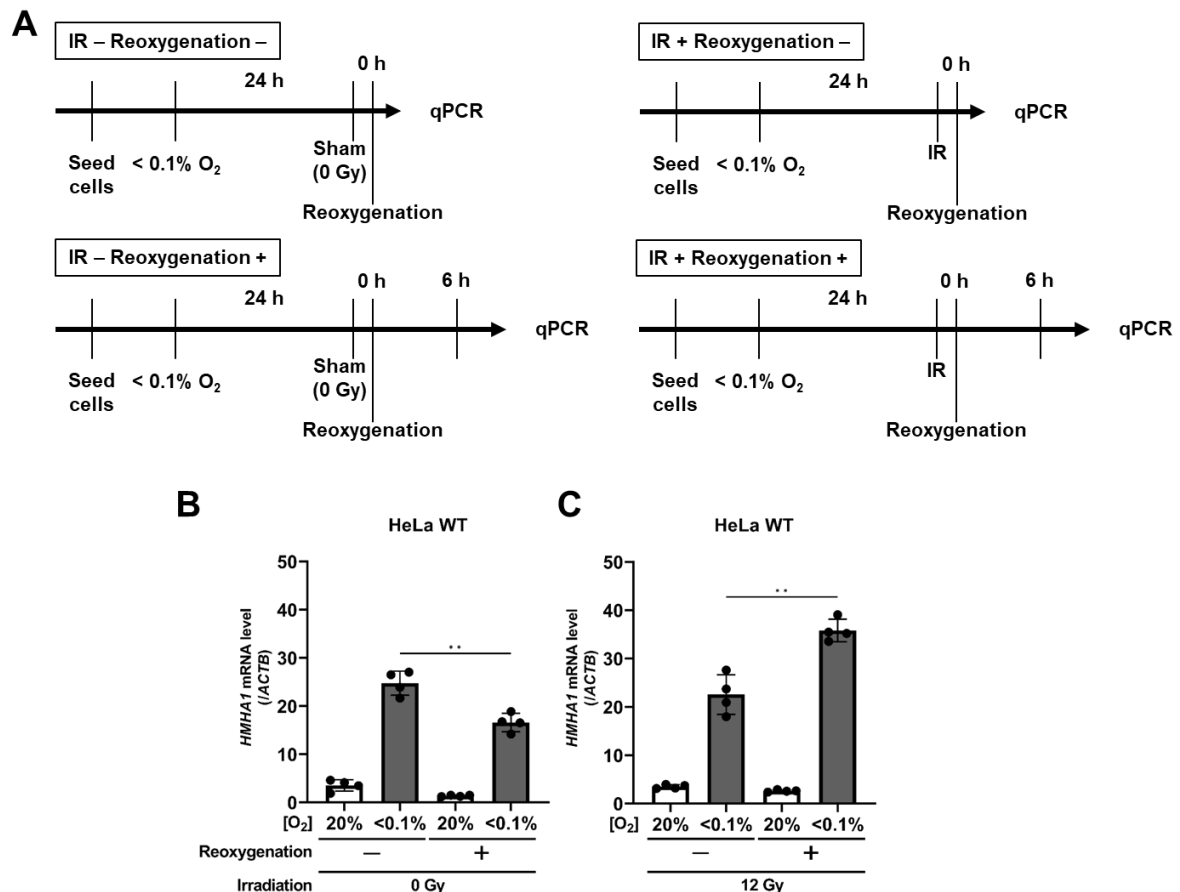


Figure 7.1 Post-irradiation reoxygenation further increases HMHA1 expression. (A) Schedule of irradiation (IR) and reoxygenation treatment. (B-C) HeLa cells cultured under normoxic (20% O₂) or hypoxic (< 0.1% O₂) conditions were subjected to 0 Gy γ -ray sham treatment (B) for negative control or to 12 Gy of γ -ray irradiation (C). Cells cultured under hypoxic conditions were reoxygenated for 6 hours after irradiation treatment. qPCR was performed to assess HMHA1 mRNA expression levels.

Next, to examine whether the further induction of HMHA1 after irradiation and reoxygenation treatment was regulated by HIF, HIF-1 β knockout HeLa cells were subjected to the same treatment and HMHA1 expression was analysed by qPCR. In line with previous data, HMHA1 mRNA expression remained at basal levels for both normoxic and hypoxic cells at 0 h, regardless of irradiated or not (Figure 7.2A,B). This recapitulated the abrogation of hypoxia-induced HMHA1 expression upon the knockout of HIF-1 β . At 6 h after reoxygenation treatment, unlike the wild-type parental cells, the irradiated HIF-1 β knockout HeLa cells showed no changes in HMHA1 mRNA levels when compared to 0 h (Figure 7.2B), demonstrating that the further increase in HMHA1 expression induced by post-irradiation reoxygenation treatment is indeed dependent on HIF.

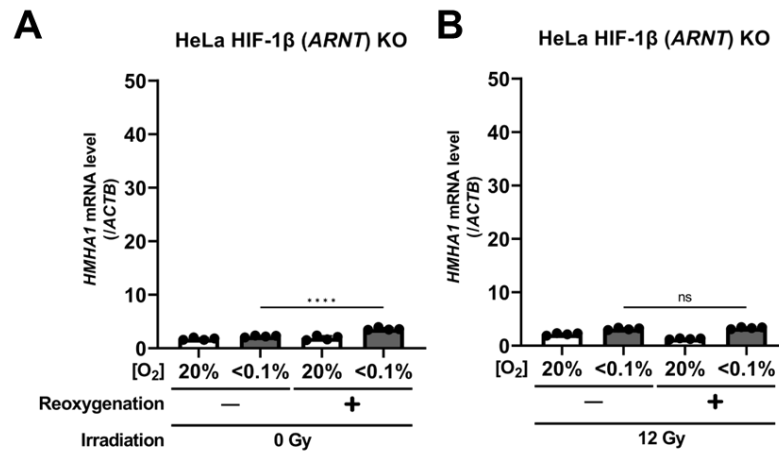


Figure 7.2 Knockout of HIF-1 β abrogates the induction of HMHA1 expression after irradiation and reoxygenation treatment. (A-B) HIF-1 β knockout (KO) HeLa cells were cultured under normoxic (20% O₂) or hypoxic (< 0.1% O₂) conditions and then subjected to 0 Gy γ -ray mock treatment (A) for negative control or to 12 Gy of γ -ray irradiation (B). Cells cultured under hypoxic conditions were reoxygenated for 6 hours after irradiation treatment. qPCR was performed to assess HMHA1 mRNA expression levels.

In order to clarify how post-irradiation reoxygenation provoked HIF activity to induce HMHA1 expression, the involvement of reactive oxygen species (ROS) was examined. This is because ROS is known to be generated both after irradiation as well as upon post-hypoxia reoxygenation, and to be able to activate the HIF α -subunit.

To inhibit ROS production, we employed an antioxidant, NAC (N-acetyl-L-cysteine). HeLa cells were pre-incubated and maintained in NAC-containing culture medium from 1 hour before irradiation. As expected, in the PBS-treated control group, HMHA1 expression was further induced after irradiation and reoxygenation treatment when compared to the unirradiated control (Figure 7.3). Conversely, when pre-treated with NAC, although HMHA1

expression was significantly higher than the normoxia control group, the level remained the same between the irradiated and unirradiated cells, meaning that NAC treatment abolished the further induction of HMHA1 after irradiation and reoxygenation (Figure 7.3). This shows that post-irradiation reoxygenation treatment increases HMHA1 expression in a ROS-dependent manner.

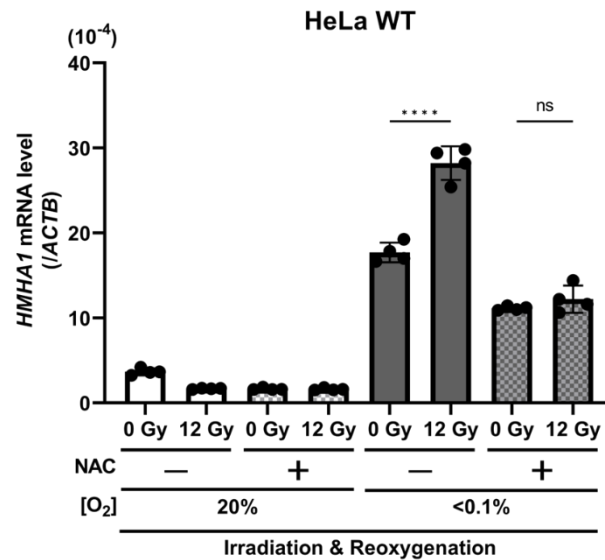


Figure 7.3 HMHA1 induction after irradiation and reoxygenation is dependent on ROS. HeLa cells were cultured under normoxic (20% O₂) or hypoxic (< 0.1% O₂) conditions and then incubated with or without NAC from 1 hour before 0 Gy γ -ray mock treatment for negative control or 12 Gy of γ -ray irradiation. Cells cultured under hypoxic conditions were reoxygenated for 6 hours after irradiation treatment. qPCR was performed to assess HMHA1 mRNA expression levels.

Taken together, these results provided supporting evidence to the model of severely hypoxic cells undergoing irradiation and reoxygenation treatment activate the ROS-HIF pathway to further enhance HMHA1 expression.

4.2. Post-irradiation reoxygenation HMHA1 induction promotes the invasion activity of irradiated “ex-hypoxic” cancer cells

To investigate whether the post-irradiation reoxygenation-mediated induction of HMHA1 could effectuate changes in invasion activities, HMHA1 knockout HeLa clones were established by the lentiviral transduction CRISPR/Cas9 system, and the knockout clones were used in the transwell invasion assay in combination with irradiation and reoxygenation treatment. First, successful knockout of HMHA1 was confirmed by genome sequencing (Figure 8.1A,B) in addition to Western blotting, wherein both clones showed no expression of HMHA1 protein after hypoxia treatment (Figure 8.1C).

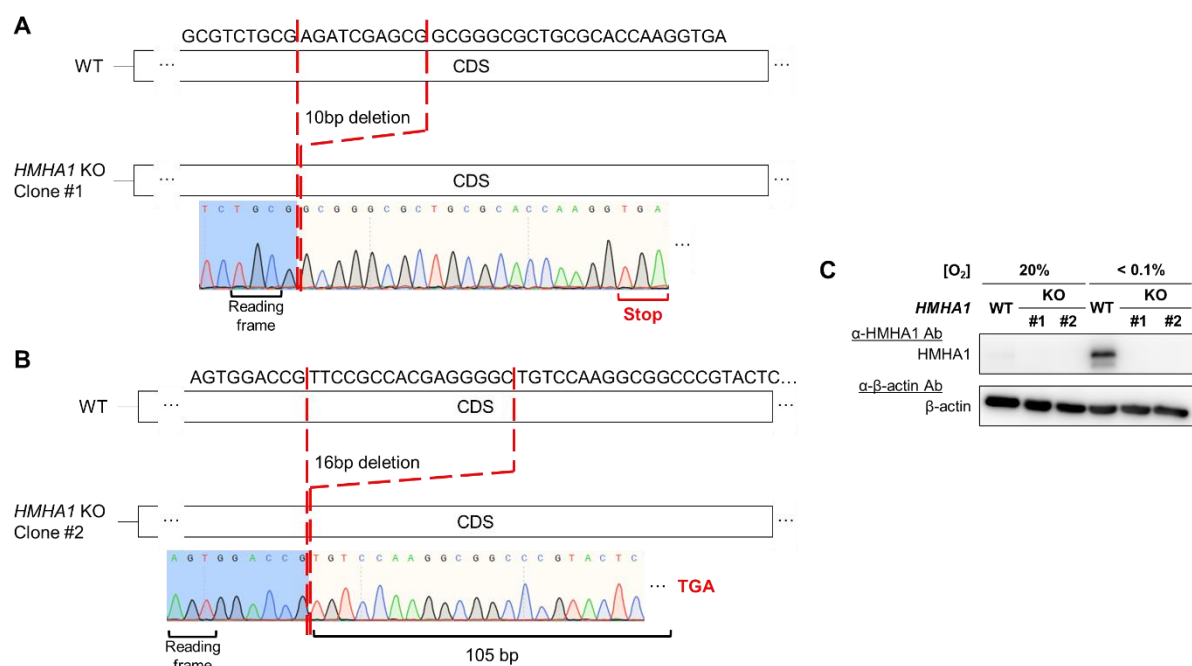


Figure 8.1 Confirmation of HMHA1 knockout (KO) in HeLa cells. (A-B) Genome sequencing of the *HMHA1* gene locus was performed to verify knockout in Clone #1 (A) and Clone #2 (B). (C) HMHA1 knockout HeLa clones (#1 and #2) and control cells (WT) were incubated under normoxic (20% O₂) or hypoxic (< 0.1% O₂) conditions. Western blotting was performed to assess HMHA1 protein expression levels.

For wild-type cells, hypoxic incubation followed by irradiation and reoxygenation significantly enhanced cellular invasion when compared to the unirradiated reoxygenated control group (Figure 8.2A, left). This suggests that the previously reported post-radiotherapy translocation of surviving tumour cells could be successfully recapitulated in this *in vitro* experimental setting, and shows that irradiation indeed promotes the invasion of surviving reoxygenated cells.

Next, using HMHA1 knockout HeLa cells, it was found that the number of invading cells drastically reduced even under unirradiated conditions when compared to the wild-type control cells (Figure 8.2A, upper row). Such low level of invasion activities again supports that HMHA1 is required for the stimulatory effect of hypoxia on invasion. Moreover, when comparing the irradiated and unirradiated group, unlike wild-type control cells, the HMHA1 knockout cells showed no differences in the number of invading cells (Figure 8.2A,B). This shows that knocking out HMHA1 negated the increase in invasion induced by irradiation and reoxygenation treatment to hypoxic cells.

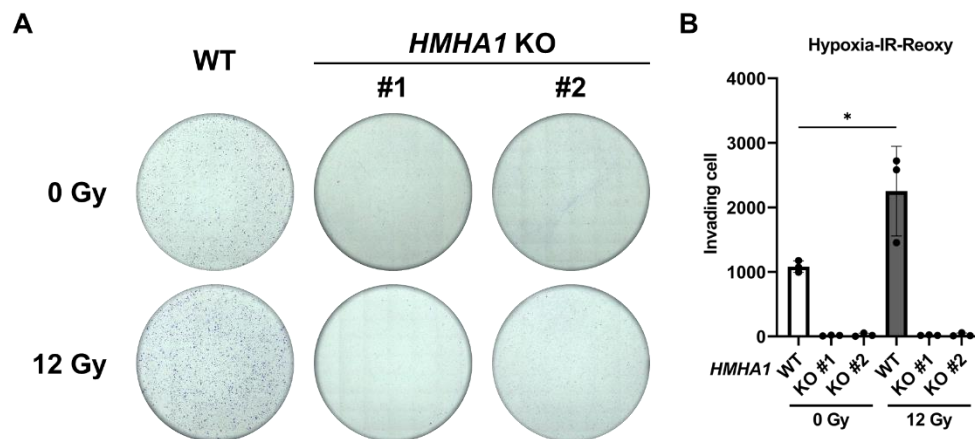


Figure 8.2 Knockout of HMHA1 abrogates the invasion activity of reoxygenated cells after irradiation treatment. (A) HMHA1 knockout HeLa clones (#1 and #2) and wild-type control cells were incubated under hypoxic (< 0.1% O₂) conditions, irradiated and then reoxygenated. Transwell invasion assay was subsequently performed to assess invasion activities. (B) Number of invading cells in (A) was counted using ImageJ.

In order to assess whether HMHA1 expression status would affect the survival of cells after irradiation and reoxygenation treatment, the changes in cell number of wild-type control cells and HMHA1 knockout cells was compared. Cells were subjected to the same protocol of treatment as in the transwell invasion assay, and cell viability was reflected as the cell number counted immediately (0 h) and at 24 h after reoxygenation. As expected, there were no differences in cell number amongst the wild-type control cells and the HMHA1 knockout cells (Figure 8.3). Thus, the changes in invasion activity observed in transwell invasion assay were not due to changes in cell viability or proliferation after irradiation and reoxygenation treatment.

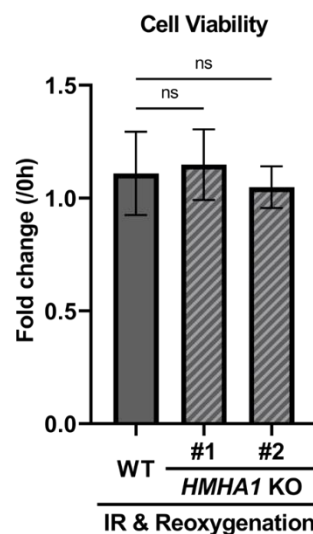


Figure 8.3 HMHA1 status does not affect cell viability after irradiation and reoxygenation treatment. HMHA1 knockout HeLa clones (#1 and #2) and wild-type control cells were incubated under hypoxic ($< 0.1\% \text{ O}_2$) conditions, irradiated and then reoxygenated for 24 hours. Cell count was performed before and after reoxygenation, and viability is determined by the change in cell number.

Collectively, these results demonstrated that HMHA1 is required for the post-irradiation invasion activity of the reoxygenated, “ex-hypoxic” cells.

Chapter 5

Discussion

The goal of this study is to elucidate the molecular mechanism underlying the hypoxia-inducible factor (HIF)-dependent invasion of hypoxic cells surviving radiotherapy. Given the complexity of cellular responses occurring after exposure to hypoxia stimuli and radiotherapy, I started with the identification of a hypoxia-responsive gene that can potentially regulate cancer cell invasiveness. Based on existing reports and previous analyses our group performed, the *HMHA1* gene was considered a potential candidate. In this study, I confirmed the induction of HMHA1 expression under hypoxic conditions and clarified the regulatory mechanism behind in relation to HIF. I also demonstrated the role of HMHA1 in promoting cancer cell invasion under hypoxia and attempted to resolve the molecular mechanism in detail. Furthermore, I successfully recapitulated the post-radiotherapy invasion of hypoxic cancer cells in *in vitro* settings, with which I unveiled the involvement of the ROS-HIF-HMHA1 axis (Figure 9.1).

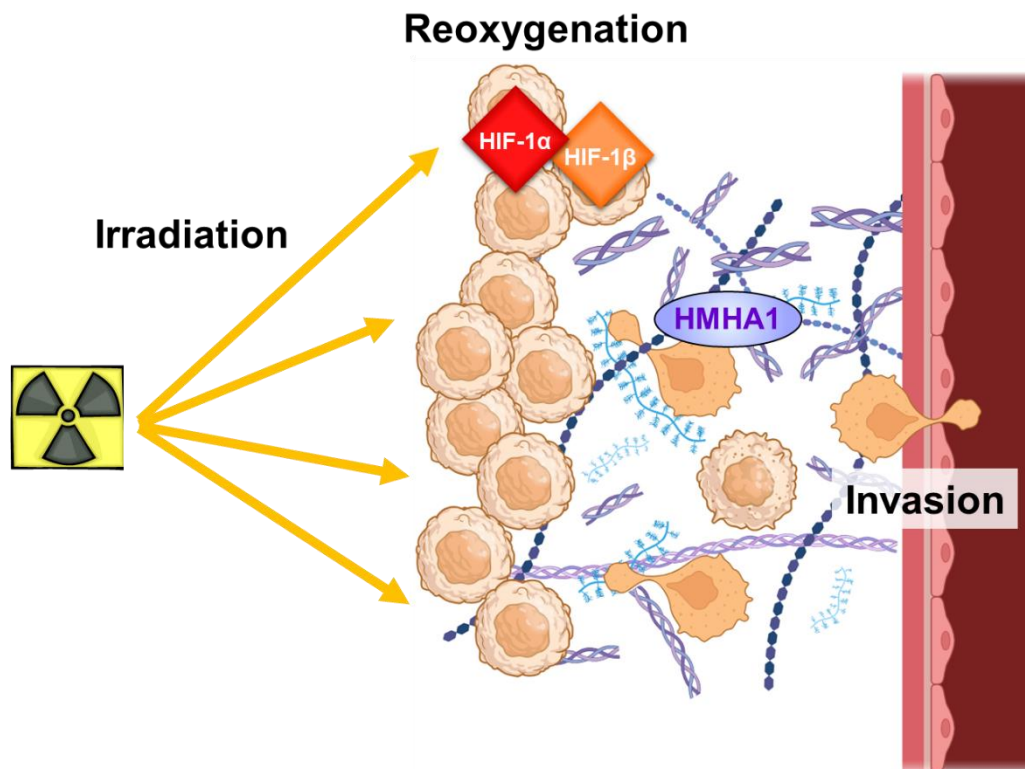


Figure 9.1 Graphical summary showing the putative molecular mechanism underlying the post-radiotherapy invasion of “ex-hypoxic” cancer cells.

5.1. Hypoxic induction of HMHA1 expression is dependent on HIF-1 and HIF-2

In this study, it is shown that the induction of HMHA1 expression under hypoxic condition is dependent on both HIF-1 and HIF-2. Using a combination of knockout and siRNA-mediated knockdown, the expression of HIF-1 α and HIF-2 α could be simultaneously suppressed. Indeed, in both qPCR and Western blotting, I confirmed that the dual suppression of HIF-1 and HIF-2 abrogated the hypoxic induction of HMHA1 expression. However, a shortcoming of this design of experiment is that the efficacy of siRNA for knocking down the target gene would greatly influence the result. Herein, whilst the siRNAs targeting HIF1A effectively abolished HIF-1 α expression (Figure 2.10A), that for HIF2A was relatively less efficacious (Figure 2.9B, Lane 8 and 11). This could have led to ambiguity if the results were not as unequivocal. Ideally, HIF-1 α /HIF-2 α double knockout cell lines should be established in order to obtain more definitive results.

Next, in order to examine the recruitment of HIF to the *HMHA1* gene locus, I performed ChIP-qPCR using an anti-HIF-1 α antibody and narrowed down the region where HIF-1 potentially binds to. In this particular experiment setting, because the primer sets for qPCR were designed to amplify consecutive neighbouring regions, chromatin DNA must be adequately (but not overly) sheared. Otherwise, distal regions would also be amplified, leading to false positive results. To corroborate the current results and better identify the HIF binding site, similar experiment using anti-HIF-1 β antibody could be conducted. Moreover, it is not known whether HIF-1 α and HIF-2 α are recruited to the same binding site to regulate HMHA1 expression. Comparing the three sets of data from using antibodies against HIF-1 α , HIF-2 α and HIF-1 β , respectively, may provide more details. Notably, in this study, the exact HRE in the *HMHA1* locus that HIF binds to was not identified. In the HMHA1 intron 1 locus, there exists multiple sites containing the HRE consensus sequence (5'-RCGTG-3'), and some are less than 20 base pairs apart, which technically cannot be distinguished by ChIP-qPCR. Thus, in order to identify the responsible HRE(s), mutants with each consensus sequence respectively modified, either in the genome or in the corresponding plasmids for reporter assay, should be generated.

HIF-1 and HIF-2 have been reported to regulate an overlapping, but not identical, set of downstream genes. Functionally, HIF-1 and HIF-2 have also been shown to play differential roles by regulating their own unique set of target genes [35-38]. Moreover, it has been suggested that HIF-1 triggers acute responses to hypoxic stress, whereas prolonged hypoxia causes a shift from HIF-1 to HIF-2 [39,40]. This allows the coordinated temporal regulation of

hypoxia-driven biological responses [38]. In this study, I demonstrated that HMHA1 expression under hypoxic conditions is completely abolished upon HIF-1 β knockout, whereas HIF-1 α and HIF-2 α knockout respectively resulted in partial HMHA1 suppression to different extents. This indicates that HIF is likely the dominant regulator of HMHA1 expression, but HIF-1 α and HIF-2 α contributes differently to the hypoxic induction of HMHA1 expression. Thus, HMHA1 may actually be a putative model gene for studying the mechanism of the transition from HIF-1 to HIF-2, as well as how HIF-1 and HIF-2 complement each other in regulating their common target genes.

5.2. HMHA1 promotes the disassembly of stress fibres

I demonstrated that HMHA1 overexpression subdues stress fibre formation. HMHA1 has been reported to function as a GTPase-activating protein (GAP), which inactivates small GTPases by hydrolysing GTP into GDP, for RHO and RAC [30,31]. In general, activated RHO (GTP-bound RHO) promotes the bundling of actin filament into stress fibres, whereas activated RAC (GTP-bound RAC) triggers rapid actin polymerisation and branching to facilitate membrane ruffling and lamellipodia formation [41-43]. And the mutual antagonism between RHO and RAC has been widely reported [44,45]. Based on the phalloidin staining results, HMHA1 may inactivate RHO under hypoxia and thereby suppresses stress fibre formation. On the other hand, HMHA1-overexpressing cells did appear to have more ruffling at the periphery of the plasma membrane (Figure 4.3A). This may indicate that HMHA1 suppresses RHO and activates RAC signalling under hypoxia. However, the links between RHO/RAC status, the reduced formation of stress fibre, and the enhanced cancer cell invasion upon HMHA1 overexpression still remain unclear.

In a previous *in vivo* study, HMHA1 has been found to regulate T cell migration into lymph nodes by maintaining cell deformability [32]. Intriguingly, the suppression of actin filament bundling and stress fibre formation can potentially lower cell stiffness and thus allow for cell deformation [46]. If so, this may act as a mechanism by which hypoxic cancer cells acquire invasiveness *via* the HIF-HMHA1 pathway. To test this hypothesis, experiments can be performed to assess the regulation of cell stiffness/deformability by HMHA1 under hypoxic conditions in the future. This would help clarify the contribution of HMHA1, as a small GTPase regulator, to cancer cell invasion.

5.3. HMHA1 augments cancer cell invasiveness by regulating MMP activities

HMHA1 is crucial for cancer cell invasion under hypoxic conditions, and mechanistically, HMHA1 enhances invasion by upregulating MMP activities. We showed that HMHA1 overexpression does not change the mRNA expression levels of MMP2 and MMP9 (Figure 4.4). This seems reasonable as the HMHA1 protein has not been reported to possess any DNA-binding domains, nor has it been shown to function as a transcription factor/cofactor. So, the enhanced total MMP activities is unlikely due to increases in the expression levels of MMP-2 and MMP-9. Instead, the regulation by HMHA1 occurs at post-transcriptional level. However, the detailed molecular mechanism is not explored in this study.

Importantly, apart from the major F-BAR and GAP domains, the HMHA1 protein also contains a C1 domain, which commonly binds to phospholipid, and a C-terminal PDZ-binding region, which may be involved in signal transduction *via* protein-protein interaction. Thus, I sought to investigate the potential interacting partners of the HMHA1 protein. From an online protein interactome database [47], it was found that HMHA1 may interact with several proteins whose function is related to protein trafficking (Figure 9.2). Since MMP-2 and MMP-9 are secretory proteins functioning in the extracellular matrix, with the above information, one possible mechanism is that HMHA1 may regulate the transport of MMP-2 and MMP-9 proteins, thereby enhancing the secreted, extracellular MMP activities to promote invasion.

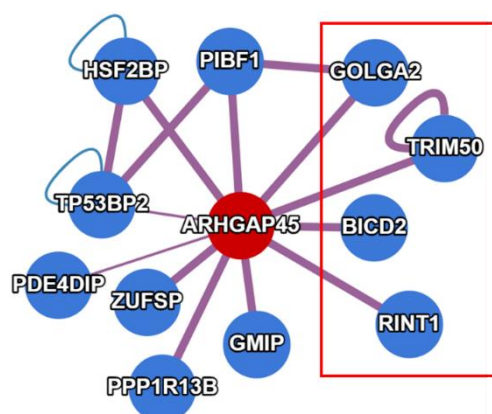


Figure 9.2 Putative interactome of HMHA1 (ARHGAP45). Proteins potentially interact with HMHA1 are shown. Those in the red box may have function related to protein trafficking. (Retrieved from <http://www.interactome-atlas.org/>)

5.4. The role of HMHA1 in metastasis

As mentioned in the Introduction section, metastasis involves a sophisticated cascade of biological events, and local invasion to surrounding normal tissue is the major process that cancer cells first exit the primary tumour site. In this study, I employed the transwell invasion assay to assess the invasiveness of cancer cells *in vitro*. Whilst this assay is considered the “orthodox method” to study invasion activities, it should be noted that other processes of the metastatic cascade are not appraised. To fully elucidate the role of HMHA1 in metastasis, the involvement of HMHA1 induction in other facets of metastasis should be tested as well. For instance, the expression of EMT markers can be assessed to determine whether HMHA1 regulates EMT. Moreover, functional assays like the scratch assay and transwell migration assay can be performed to investigate whether HMHA1 regulates cell mobility. In addition, a modified transwell invasion assay, by seeding endothelial cells prior to cancer cells into the device, can mimic the process of cancer cell traversing the blood vessel wall and can therefore be used to evaluate whether the intravasation or extravasation process is affected.

Furthermore, in order to demonstrate HMHA1 can ultimately promote metastasis, further *in vivo* experiments should be performed. Of note, a common design for modelling metastasis *in vivo* is to directly inject cancer cells into the circulatory system of mice (e.g. intravenously), but such design only simulates the biological processes after intravasation, which may not necessarily suit the case of HMHA1. A spontaneous tumour metastasis model from, such as a pulmonary metastasis after orthotopic breast cancer transplantation, can be employed in the future.

5.5. HMHA1 and patient prognosis

To investigate the clinical relevance of the hypoxic induction of HMHA1 expression, patient data from TCGA database was analysed. By segregating patients based on their expressions of hypoxia signature genes, I showed the association between HMHA1 expression and tumour hypoxia. However, the prognostic value of HMHA1 was not thoroughly explored. Given the potential role of HMHA1 in promoting metastasis, further analysis can be done to examine whether high HMHA1 expression level predicts poorer disease-free, or more specifically, distant metastasis-free survival outcomes. In addition, more stringent criteria for data filtering/screening to focus on patients who received radiotherapy may better estimate the clinical relevance of the post-irradiation induction of HMHA1 upon tumour reoxygenation.

With such analyses, it is envisioned that HMHA1 can potentially be established as an independent prognostic factor.

5.6. Post-irradiation reoxygenation further increases HMHA1 expression

To investigate the response of hypoxic tumour cells surviving radiotherapy to post-irradiation tumour microenvironment changes, I attempted to replicate the *in vivo* condition by coupling hypoxic cell culture and irradiation treatment *in vitro*. As expected, qPCR analyses showed that subjecting hypoxic cells to irradiation and reoxygenation treatment further induces HMHA1 expression in ROS- and HIF-dependent manners. Nonetheless, such induction of HMHA1 expression in the “ex-hypoxic” cells was only confirmed at mRNA level in the present study. Ideally, Western blotting should also be performed to assess the changes in HMHA1 protein expression levels. Moreover, although it is evident that ionising radiation can stimulate ROS generation and thereby stabilises HIF- α subunit upon reoxygenation [48-50], I did not confirm the expression levels of HIF proteins herein. Additionally, assessing the activity of HIF, for instance by the 5HRE/Luciferase reporter system which expresses luciferase under the control of HIF [51], would have consolidated the results.

Intriguingly, whilst ionising radiation and hypoxia/reoxygenation are known to be able to induce ROS production independently, individual treatment alone did not enhance HMHA1 expression. Only the combinatory treatment of irradiation and reoxygenation showed a stimulatory effect. A plausible explanation is that the individual treatment alone could not produce sufficient amount of ROS that reach the threshold for the HIF-mediated induction of HMHA1 expression. This can be examined by performing further experiments to compare the ROS levels and HIF activities in each of the mentioned conditions.

5.7. HMHA1 induction and post-irradiation translocation of “ex-hypoxic” tumour cells

Our group previously reported that radiotherapy induces the translocation of hypoxic tumour cells in a xenograft tumour mouse model [24]. Based on the findings in this study, it is worthwhile to examine whether HMHA1 expression is upregulated in the translocating cells by performing immunohistochemical staining of the irradiated tumour sections. Also, having established the HMHA1 knockout HeLa cell lines, wild-type and HMHA1 knockout HeLa xenograft tumours can be generated for irradiation treatment, and the difference in post-radiotherapy invasion activity of the surviving reoxygenated cells can be examined. This will clarify whether HMHA1 is involved in this process and provide more details of the molecular mechanism underlying the post-radiotherapy distant metastasis and recurrence.

5.8. HMHA1 as a member of the ARHGAP family

HMHA1, also called ARHGAP45, belongs to the ARHGAP family. Whilst this study investigated the role of HMHA1 under hypoxia, the involvement of other members of the ARHGAP family in hypoxic responses, especially whether they have overlapping functions with HMHA1, can be further explored. Likewise, there are other reported roles of the ARHGAP family members in cancer progression, one of which is immune cell infiltration [52-55]. Given that HMHA1 is highly expressed in some immune cells under normal physiological conditions, it is worthwhile to investigate whether the hypoxic induction of HMHA1 exert any effects on the immune landscape within the hypoxic tumour microenvironment.

5.9. Conclusion and Future Perspectives

Hypoxia can be considered as one of the intrinsic natures of rapid-growing solid tumours, which can occur to different extent in different cancer patients. Tumour hypoxia is not epiphenomenal, but clinically significant. It is shown that hypoxic cancer cells acquire malignant properties and thus contribute to poor prognosis of patients. This study, in particular, aimed to elucidate how the master regulator of hypoxic responses, HIF, contributes to hypoxic cancer cell invasion as well as to post-radiotherapy metastasis and recurrence. By detailing the underlying molecular mechanisms, it is expected that the results would shed light on targeting HIF to improve therapeutic outcomes and on the identification of novel potential therapy targets.

Herein, I characterised HMHA1 as a HIF downstream gene, and demonstrated its role to promote cancer cell invasion under hypoxic conditions as well as in the dramatic changes in tumour microenvironments after irradiation and reoxygenation treatment. I also sought to investigate how HMHA1 augments invasiveness. However, as discussed above, there are remaining parts and details that I was not able to address. The lack of *in vivo* metastasis studies is perhaps the largest gap in this study. Moreover, although I have examined the mechanism of HMHA1 enhancing invasiveness from a gain-of-function approach, all the experiments were carried out under normoxic conditions. It remains to be confirmed whether HMHA1 functions in the same fashion under hypoxia and upon post-irradiation reoxygenation. And the clinical prognostic value of HMHA1 also awaits further analyses. Finally, whilst this study focused on the role of HMHA1 in cancer cell invasion, its other potential functions still remain to be explored. Future findings are expected to deepen the understanding of HMHA1 functioning as a hypoxia-responsive gene and to provide insights into its clinical significance.

References

1. Schito, L., & Semenza, G. L. (2016). Hypoxia-Inducible Factors: master regulators of cancer progression. *Trends in Cancer*, 2(12), 758–770.
2. Shi, R., Liao, C., & Zhang, Q. (2021). Hypoxia-Driven Effects in Cancer: characterization, mechanisms, and therapeutic implications. *Cells*, 10(3), 678.
3. Chen, Z., Han, F., Du, Y., Shi, H., & Zhou, W. (2023). Hypoxic microenvironment in cancer: molecular mechanisms and therapeutic interventions. *Signal Transduction and Targeted Therapy*, 8(1).
4. Bertout, J. A., Patel, S. A., & Simon, M. C. (2008). The impact of O₂ availability on human cancer. *Nature Reviews. Cancer*, 8(12), 967–975.
5. Wigerup, C., Pålman, S., & Bexell, D. (2016). Therapeutic targeting of hypoxia and hypoxia-inducible factors in cancer. *Pharmacology & Therapeutics*, 164, 152–169.
6. Vaupel, P., & Mayer, A. (2007). Hypoxia in cancer: significance and impact on clinical outcome. *Cancer and Metastasis Reviews*, 26(2), 225–239.
7. Semenza, G. L., Agani, F., Booth, G., Forsythe, J., Iyer, N., Jiang, B. H., et al. (1997). Structural and functional analysis of hypoxia-inducible factor 1. *Kidney International*, 51(2), 553–555.
8. Maynard, M. A., Qi, H., Chung, J., Lee, E. H., Kondo, Y., Hara, S., et al. (2003). Multiple Splice Variants of the Human HIF-3 α Locus Are Targets of the von Hippel-Lindau E3 Ubiquitin Ligase Complex. *Journal of Biological Chemistry*, 278(13), 11032–11040.
9. Hara, S., Hamada, J., Kobayashi, C., Kondo, Y., & Imura, N. (2001). Expression and characterization of Hypoxia-Inducible Factor (HIF)-3A in human kidney: Suppression of HIF-Mediated gene expression by HIF-3A. *Biochemical and Biophysical Research Communications*, 287(4), 808–813.
10. Maynard, M. A., Evans, A. J., Hosomi, T., Hara, S., Jewett, M. A. S., & Ohh, M. (2005). Human HIF-3 α 4 is a dominant-negative regulator of HIF-1 and is down-regulated in renal cell carcinoma. *FASEB Journal*, 19(11), 1396–1406.
11. Villareal, L. B., Falcon, D. M., Xie, L., & Xue, X. (2024). Hypoxia-inducible factor 3 α 1 increases epithelial-to-mesenchymal transition and iron uptake to drive colorectal cancer liver metastasis. *British Journal of Cancer*.
12. Lu, X., & Kang, Y. (2010). Hypoxia and Hypoxia-Inducible Factors: master regulators of metastasis. *Clinical Cancer Research*, 16(24), 5928–5935.
13. Wicks, E. E., & Semenza, G. L. (2022). Hypoxia-inducible factors: cancer progression and clinical translation. *The Journal of Clinical Investigation*, 132(11).
14. Wong, C. C., Zhang, H., Gilkes, D. M., Chen, J., Wei, H., Chaturvedi, P., et al. (2012). Inhibitors of hypoxia-inducible factor 1 block breast cancer metastatic niche formation and lung metastasis. *Journal of Molecular Medicine*, 90(7), 803–815.

15. Ma, Z., Wang, L. Z., Cheng, J., Lam, W. S. T., Ma, X., Xiang, X., et al. (2021). Targeting Hypoxia-Inducible Factor-1-Mediated metastasis for cancer therapy. *Antioxidants & Redox Signaling*, 34(18), 1484–1497.
16. Dellas, K., Bache, M., Pigorsch, S. U., Taubert, H., Kappler, M., Holzapfel, D., et al. (2008). Prognostic Impact of HIF-1 α Expression in Patients with Definitive Radiotherapy for Cervical Cancer. *Strahlentherapie Und Onkologie*, 184(3), 169–174.
17. Chen, L., Shi, Y., Yuan, J., Han, Y., Qin, R., Wu, Q., et al. (2014). HIF-1 Alpha Overexpression Correlates with Poor Overall Survival and Disease-Free Survival in Gastric Cancer Patients Post-Gastrectomy. *PloS One*, 9(3), e90678.
18. Chen, Y., Li, X., Wu, S., Xu, G., Zhou, Y., Gong, L., et al. (2014). Expression of HIF-1 α and CAIX in nasopharyngeal carcinoma and their correlation with patients' prognosis. *Medical Oncology*, 31(12).
19. Han, S., Huang, T., Hou, F., Yao, L., Wang, X., & Wu, X. (2019). The prognostic value of hypoxia-inducible factor-1 α in advanced cancer survivors: a meta-analysis with trial sequential analysis. *Therapeutic Advances in Medical Oncology*, 11.
20. Bouleftour, W., Rowinski, E., Louati, S., Sotton, S., Wozny, A., Moreno-Acosta, P., et al. (2021). A review of the role of hypoxia in radioresistance in cancer therapy. *Medical Science Monitor*, 27.
21. Suwa, T., Kobayashi, M., Nam, J., & Harada, H. (2021). Tumor microenvironment and radioresistance. *Experimental and Molecular Medicine*, 53(6), 1029–1035.
22. Zhu, Y., Zhao, T., Itasaka, S., Zeng, L., Yeom, C. J., Hirota, K., et al. (2012). Involvement of decreased hypoxia-inducible factor 1 activity and resultant G1–S cell cycle transition in radioresistance of perinecrotic tumor cells. *Oncogene*, 32(16), 2058–2068.
23. Harada, H., Itasaka, S., Kizaka-Kondoh, S., Shibuya, K., Morinibu, A., Shinomiya, K., & Hiraoka, M. (2009). The AKT/MTOR pathway assures the synthesis of HIF-1 α protein in a glucose- and reoxygenation-dependent manner in irradiated tumors. *Journal of Biological Chemistry*, 284(8), 5332–5342.
24. Harada, H., Inoue, M., Itasaka, S., Hirota, K., Morinibu, A., Shinomiya, K., et al. (2012). Cancer cells that survive radiation therapy acquire HIF-1 activity and translocate towards tumour blood vessels. *Nature Communications*, 3(1).
25. Trastour, C., Benizri, E., Ettore, F., Ramaoli, A., Chamorey, E., Pouysségur, J., & Berra, E. (2007). HIF-1 α and CA IX staining in invasive breast carcinomas: Prognosis and treatment outcome. *International Journal of Cancer*, 120(7), 1451–1458.
26. Dales, J., Garcia, S., Meunier-Carpentier, S., Andrac-Meyer, L., Haddad, O., Lavaut, M., et al. (2005). Overexpression of hypoxia-inducible factor HIF-1 α predicts early relapse in breast cancer: Retrospective study in a series of 745 patients. *International Journal of Cancer*, 116(5), 734–739.
27. Ishikawa, H., Sakurai, H., Hasegawa, M., Mitsuhashi, N., Takahashi, M., Masuda, N., et al. (2004). Expression of hypoxic-inducible factor 1 α predicts metastasis-free survival after radiation therapy alone in stage IIIB cervical squamous cell carcinoma. *International Journal of Radiation Oncology, Biology, Physics*, 60(2), 513–521.

28. Chow, C. C., Kobayashi, M., Kambe, G., & Harada, H. (2023). ZBTB2 is Recruited to a Specific Subset of HIF-1 Target Loci to Facilitate Full Gene Expression Under Hypoxia. *Journal of Molecular Biology*, 435(15).
29. Yang, L., Taylor, J., Eustace, A., Irlam, J. J., Denley, H., Hoskin, P. J., et al. (2017). A Gene Signature for Selecting Benefit from Hypoxia Modification of Radiotherapy for High-Risk Bladder Cancer Patients. *Clinical Cancer Research*, 23(16), 4761–4768.
30. de Kreuk, B. J., Schaefer, A., Anthony, E. C., Tol, S., Fernandez-Borja, M., Geerts, D., et al. (2013). The human minor histocompatibility antigen1 is a RHOGAP. *PloS One*, 8(9), e73962.
31. Amado-Azevedo, J., Reinhard, N., Van Bezu, J., Van Nieuw Amerongen, G., Van Hinsbergh, V., & Hordijk, P. (2018). The minor histocompatibility antigen 1 (HMHA1)/ArhGAP45 is a RacGAP and a novel regulator of endothelial integrity. *Vascular Pharmacology*, 101, 38–47.
32. He, L., Valignat, M., Zhang, L., Gelard, L., Zhang, F., Guen, V. L., et al. (2021). ARHGAP45 controls naïve T- and B-cell entry into lymph nodes and T-cell progenitor thymus seeding. *EMBO Reports*, 22(4).
33. Xu, P., Ma, J., Ma, J., Zhang, W., Guo, S., Jian, Z., et al. (2017). Multiple pro-tumorigenic functions of the human minor Histocompatibility Antigen-1 (HA-1) in melanoma progression. *Journal of Dermatological Science*, 88(2), 216–224.
34. McKeown, S. R. (2014). Defining normoxia, physoxia and hypoxia in tumours – implications for treatment response. *British Journal of Radiology*, 87(1035).
35. Hu, C., Wang, L., Chodosh, L. A., Keith, B., & Simon, M. C. (2003). Differential roles of Hypoxia-Inducible factor 1A (HIF-1A) and HIF-2A in hypoxic gene regulation. *Molecular and Cellular Biology*, 23(24), 9361–9374.
36. Downes, N. L., Laham-Karam, N., Kaikkonen, M. U., & Ylä-Herttuala, S. (2018). Differential but complementary HIF1A and HIF2A transcriptional regulation. *Molecular Therapy*, 26(7), 1735–1745.
37. Hoeflin, R., Harlander, S., Schäfer, S., Metzger, P., Kuo, F., Schönenberger, D., et al. (2020). HIF-1 α and HIF-2 α differently regulate tumour development and inflammation of clear cell renal cell carcinoma in mice. *Nature Communications*, 11(1).
38. Koh, M. Y., & Powis, G. (2012). Passing the baton: the HIF switch. *Trends in Biochemical Sciences*, 37(9), 364–372.
39. Bartoszewski, R., Moszyńska, A., Serocki, M., Cabaj, A., Polten, A., Ochocka, R., et al. (2019). Primary endothelial cell-specific regulation of hypoxia-inducible factor (HIF)-1 and HIF-2 and their target gene expression profiles during hypoxia. *FASEB Journal*, 33(7), 7929–7941.
40. Jaśkiewicz, M., Moszyńska, A., Króliczewski, J., Cabaj, A., Bartoszevska, S., Charzyńska, A., et al. (2022). The transition from HIF-1 to HIF-2 during prolonged hypoxia results from reactivation of PHDs and HIF1A mRNA instability. *Cellular & Molecular Biology Letters*, 27(1).
41. Tapon, N., & Hall, A. (1997). Rho, Rac and Cdc42 GTPases regulate the organization of the actin cytoskeleton. *Current Opinion in Cell Biology*, 9(1), 86–92.

42. Machesky, L. M., & Hall, A. (1997). Role of Actin polymerization and adhesion to extracellular matrix in RAC- and RHO-induced cytoskeletal reorganization. *The Journal of Cell Biology*, 138(4), 913–926.
43. Jaffe, A. B., & Hall, A. (2005). RHO GTPases: Biochemistry and Biology. *Annual Review of Cell and Developmental Biology*, 21(1), 247–269.
44. Burridge, K., & Wennerberg, K. (2004). Rho and Rac Take Center Stage. *Cell*, 116(2), 167–179.
45. Parri, M., & Chiarugi, P. (2010). Rac and Rho GTPases in cancer cell motility control. *Cell Communication and Signaling*, 8(1).
46. Alibert, C., Goud, B., & Manneville, J. (2017). Are cancer cells really softer than normal cells? *Biology of the Cell*, 109(5), 167–189.
47. Luck, K., Kim, D. K., Lambourne, L., Spirohn, K., Begg, B. E., Bian W., et al. (2020). A reference map of the human binary protein interactome. *Nature*, 580(7803), 402–408.
48. Moeller, B. J., Cao, Y., Li, C. Y., & Dewhirst, M. W. (2004). Radiation activates HIF-1 to regulate vascular radiosensitivity in tumors. *Cancer Cell*, 5(5), 429–441.
49. Sabharwal, S. S., & Schumacker, P. T. (2014). Mitochondrial ROS in cancer: initiators, amplifiers or an Achilles' heel? *Nature Reviews. Cancer*, 14(11), 709–721.
50. Wang, H., Jiang, H., Van De Gucht, M., & De Ridder, M. (2019). Hypoxic radioresistance: Can ROS be the key to overcome it? *Cancers*, 11(1), 112.
51. Shibata, T., Giaccia, A. J., & Brown, J. M. (2000). Development of a hypoxia-responsive vector for tumor-specific gene therapy. *Gene Therapy*, 7(6), 493–498.
52. Yang, C., Wu, S., Mou, Z., Zhou, Q., Zhang, Z., Chen, Y., et al. (2021). Transcriptomic analysis identified ARHGAP family as a novel biomarker associated with Tumor-Promoting immune infiltration and nanomechanical characteristics in bladder cancer. *Frontiers in Cell and Developmental Biology*, 9.
53. Fan, B., Ji, K., Bu, Z., Zhang, J., Yang, H., Li, J., & Wu, X. (2021). ARHGAP11A is a prognostic biomarker and correlated with immune infiltrates in gastric cancer. *Frontiers in Molecular Biosciences*, 8.
54. Fu, M., Pan, S., Cai, X., Hu, Y., Zhang, W., & Pan, Q. (2022). Analysis of ARHGAP4 expression with colorectal Cancer Clinical characteristics and Prognosis. *Frontiers in Oncology*, 12.
55. Yao, L., Li, Y., Li, S., Wang, M., Cao, H., Xu, L., & Xu, Y. (2023). ARHGAP39 is a prognostic biomarker involved in immune infiltration in breast cancer. *BMC Cancer*, 23(1).

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