Translational control during hypoxia: consequences for gene expression and hypoxia tolerance

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and hypoxia tolerance

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Translational control during hypoxia:
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and hypoxia tolerance

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Universiteit Maastricht,
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volgens het besluit van het College van Decanen,
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List of abbreviations

AMP	adenosine monophosphate
AMPK	amp activated protein kinase
ARNT	aryl hydrocarbon receptor nuclear translocator
ATF	activating transcription factor
ATP	adenosine triphosphate
BiP	immunoglobulin binding protein
BP	binding protein
CAIX	carbonic anhydrase IX
CHOP	C/EBP homologous protein
CITED	CREB-binding protein/p300-interacting transactivator with ED- rich tail
DDIT	dna damage inducible transcript
DNA	deoxyribonucleic acid
eIF	eukaryotic initiation factor
E.R.	Endoplasmic reticulum
GADD	growth arrest
GTP	guanidine triphosphate
HIF	hypoxia inducible factor
HK	hexokinase
HRE	hypoxia responsive element
HSP	heat shock protein
IRE	inositol requiring protein
ISR	integrated stress response
kDa	kilo Dalton
MEF	mouse embryonic fibroblast
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDK	phosphatidylinositol dependent kinase
PHD	prolyl hydroxylase domain
PI3K	phosphatidylinositol 3 kinase
PJS	Peutz-Jeghers syndrome
PKR	protein kinase RNA
PTEN	phosphatase and tensin homologue
RNA	ribonucleic acid
S51A	serine 51 to alanine mutation
SD	standard deviation
SEM	standard error of the mean
TSC	tuberous sclerosis complex
Q-PCR	quantitative PCR
UPR	unfolded protein response
VEGF	vascular endothelial growth factor
VHL	Von-Hippel Lindau
W.T.	wild-type

CHAPTER 1

General introduction

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Chapter 1

1. Cancer and hypoxia

Cancer remains a highly prevalent disease that has a tremendous cost to afflicted individuals, their families, and to society in general. In fact, 7.6 million people died world wide in 2007 alone according to the American Cancer Society¹. The term “cancer” refers to a collection of related diseases in which normal cells in the body acquire a series of genetic changes that enable them to proliferate in an uncontrolled manner. This results in the formation of a tumor mass or neoplasm at the site from which the cancer cell arose. The cancer cells often acquire further genetic changes that enable them to invade nearby tissues and to spread via the bloodstream or lymphatic system to other tissues in the body, a process known as metastasis.

The goal of current cancer treatment is to offer affected patients an improved quality of life by achieving cure of the disease, increasing their lifespan, or by diminishing symptomatic side effects. The standard treatment options available for cancer include surgery, chemotherapy and radiotherapy. For certain tumor types additional therapies such as hormone therapy (breast, prostate) or immunotherapy (melanoma) can be given. Advances in cancer research have certainly led to better prognosis for many cancer types especially in those cases where early diagnosis of the disease can be achieved. Improved methods for administering standard therapies, alone or in combination have also improved cancer therapy. For example, new technologies allow for much more conformed radiation dose delivery, allowing for reduced normal tissue toxicity and increased dose to the tumor. Nonetheless, available therapy remains ineffective in a large fraction of patients for reasons that are not clear. One of the potential explanations for the large degree of variability in patient response to treatment is the tremendous heterogeneity of the tumor microenvironment. The microenvironment of human tumors is unlike that of any other normal tissue, characterized by heterogeneities in nutrient supply, pH, and oxygenation. These unique features develop as a consequence of alterations in the metabolic and

proliferative status of the tumor cells together with a highly irregular vascular supply. In addition to the genetic and epigenetic changes that occur in cancer, this microenvironment is recognized to be an important contributor to altered gene expression and cellular function in cancer. In particular, both laboratory and clinical evidence strongly associate deficiencies in oxygenation (hypoxia) with tumor development, growth, metastasis and poor response to therapy. Hypoxia is known to directly influence the efficacy of cancer treatment (radiotherapy and chemotherapy) and also alters the behavior of tumor cells through a number of oxygen sensitive signaling pathways. This realization has stimulated efforts to understand the mechanisms through which hypoxia influences cell and cancer biology, and to develop new approaches to therapy by targeting these biological responses.

1.1 Hypoxia, an intrinsic resistance to therapy

The potential contribution of hypoxia to treatment resistance has been recognized for a long time. For radiotherapy, the lack of oxygen alters the chemical reactions which take place in the DNA upon irradiation such that only about one third as many lethal DNA lesions are produced in hypoxic cells compared with aerobic cells². For systemically delivered therapies, the hypoxic areas in tumors have also been thought to provide foci of resistance³⁻⁵. This is due in part to the unusual, chaotic, and insufficient organization of blood vessels within tumors which impairs the ability to deliver both oxygen and treatment to isolated regions of tumors^{4, 6, 7}. The poorly vascularized regions also limit cell proliferation which can provide resistance towards many chemotherapeutic agents targeted at proliferating cells. The intrinsic resistance to therapy caused by hypoxia has been demonstrated in many different model systems, and research programs have been designed to tackle this resistance. The importance of hypoxia has been demonstrated clinically where it predicts for worse outcome in the treatment of cancer of the head and neck, uterine cervix,

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and soft tissue sarcomas⁸⁻¹⁰. Clinical efforts towards overcoming hypoxia have focused on either eliminating it, for example by breathing high oxygen gas mixtures, or by replacing oxygen with drugs that can sensitize cells to radiation in a similar way to oxygen. These clinical studies have only modestly improved outcome and have been recently reviewed^{11, 12}.

1.2 Hypoxia, a molecular resistance to therapy

As mentioned, several clinical studies have demonstrated an association between the degree of hypoxia within a tumor and the overall patient prognosis. A series of measurements of pO_2 values in primary and metastatic tumors of the cervix, breast, head-and-neck and rectum^{8, 13, 14} have shown that patients with poorer tumor oxygenation exhibit a significantly lower probability of 5-year survival than patients with better-oxygenated tumors. Remarkably, the poorer prognosis for the more hypoxic tumors is valid irrespective of the modality of treatment (radiotherapy or radical surgery). These findings are supported by more recent studies of head-and-neck tumors and soft tissue sarcomas, and strongly suggest that hypoxia fundamentally alters tumor physiology to a more aggressive phenotype¹⁵.

This appears at first glance to be paradoxical, since hypoxia is a condition unfavorable to cell growth and in many situations can stimulate cell death. The resolution of this paradox lies in our understanding of the biological consequences of hypoxia for the tumor as a whole. In the past decade we have become aware of many important biological changes that occur when cells lack oxygen and the potential contribution of these pathways to the malignant phenotype of tumors. As we will see, the biological response to hypoxia is capable of promoting metastasis, angiogenesis, and selection of cells with diminished apoptotic potential. Consequently, the heterogeneity in oxygenation within a tumor can provide an overall positive advantage to tumor growth. This may explain the persistence of hypoxia, expected to arise very early in tumor

development, in most patients who present themselves in the clinic despite the fact that these tumors may be highly angiogenic. Of course, in order for hypoxia to produce relevant biological consequences, tumor cells must first be able to adapt and survive extended periods of time without oxygen.

We have hypothesized that the genetic and metabolic changes in cancer cells which drive the development of this "hypoxia tolerance" may be important contributors to the carcinogenic process itself^{11, 16}. The degree of hypoxia tolerance in a particular tumor may thus be an important determinant of the level of hypoxia which can be sustained within that tumor. We can thus view hypoxia tolerance as a property which provides a 'molecular resistance' towards effective therapy. This realization, along with our understanding of the key cellular pathways activated by hypoxia, leads to the possibility of targeting treatment against this molecular resistance. Several new studies have recently reported on this exciting possibility.

2. Mechanisms of adaptation to hypoxia

How does a tumor cell adapt to and survive in a nutrient-deficient and low-oxygen environment? Three key distinct oxygen sensing molecular pathways have emerged over the past several years which contribute to hypoxia tolerance in cancer (figure 1). The first is the inhibition or loss of pathways which stimulate cell death in response to hypoxia. This is best exemplified by the p53 pathway, which when functional can result in the rapid death of hypoxic, oncogenically transformed cells. Second is the activation of a conserved transcriptional program mediated in large part by the hypoxia-inducible-factors, HIFs (HIF1, HIF2, HIF3). This is a family of oxygen-sensitive transcription factors that activate more than 60 putative genes (for a review see¹⁷). The HIFs influence tumor response by stimulating angiogenesis, glycolysis, invasion and metastasis. Third is an adaptive response mechanism that involves the regulation of mRNA

translation. How these translational control pathways function and what their consequences are in terms of gene expression and hypoxia tolerance and is subject of this thesis.

Figure 1. Pathways to hypoxia tolerance. (A) Hypoxia causes the phosphorylation of p53 on Ser15 through ATR, resulting in apoptosis. During reoxygenation p53 phosphorylation is mediated by ATM, leading to apoptosis. This mechanism can provide the basis for selecting cells that have lost their apoptotic sensitivity. (B) Hypoxia and the activation of growth signaling pathways stabilize HIF-1 α , thereby activating the transcription of several HRE-target genes leading to increased glycolysis, angiogenesis and metastasis. (C) Hypoxia inhibits translation, thereby promoting energy homeostasis and increased cell survival during hypoxia.

2.1 Inhibition of mRNA translation

Exposure to hypoxia results in rapid and sustained inhibition of mRNA translation. The rate of protein synthesis is reduced by 60-70% within 1 hour of hypoxic exposure, and rapidly returns to state levels following reintroduction of oxygen¹⁸. Quantification of mRNA translation through analysis of polysomal RNA indicates that maximal inhibition occurs approximately 2hr after extreme hypoxia followed by a small recovery by 4 hr. However, mRNA translation remains significantly repressed for up to 24 hrs of hypoxia. This phenomenon has been observed in a large panel of cell lines (unpublished data) and appears to be a general response to hypoxia. The reduction in protein synthesis occurs through a HIF-independent, oxygen-sensing pathway which results in direct inhibition of

the initiation step of mRNA translation¹⁶. The involvement of a unique oxygen sensor in this pathway provides an explanation for HIF-independent hypoxia-induced biological changes.

mRNA translation is a highly regulated process that is sensitive to a variety of cellular stresses. The inhibition of translation is envisaged to affect tumor cells in two important ways. First, since protein synthesis is extremely energy costly, the inhibition of mRNA translation may act as a cellular survival mechanism by conserving energy demand during this period in which energy production is inefficient. This is exacerbated by the fact that deregulation of mRNA translation appears to be common in tumor cells. It has been known for many years that hypoxia causes a pronounced depression in the rate of oxygen consumption and energy turnover^{2, 19}. Studies performed in cell monolayers and in spheroid models of hypoxia have shown that the main ATP-demand pathways in hypoxic or anoxic cells are the Na⁺/K⁺ ATPase pump, protein synthesis and degradation, and gluconeogenesis^{4, 19-21}. Hochachka *et al* have calculated that under severe hypoxia, the ATP demand for protein synthesis drops to about 7% of that of normoxic cells²⁰. This drop correlates with a substantial and rapid drop in the rate of protein synthesis, which occurs initially at the levels of translation and later extends at the level of transcription as well. Secondly, the control of mRNA translation is increasingly being viewed as a means of controlling individual protein expression. Mechanisms which regulate global mRNA translation affect individual mRNAs to widely varying extents. The control of translation thus can be viewed as a means of rapidly changing the cellular proteome. The inhibition of overall mRNA translation paradoxically induces the expression of certain proteins that are essential for the adaptation to these conditions. Consequently, hypoxic control of mRNA translation undoubtedly influences individual gene expression and contribute to the hypoxic cell phenotype.

3. Regulation of mRNA translation

In recent years the importance of regulating mRNA translation for gene expression and cell phenotype has been convincingly demonstrated. This process is highly regulated and exquisitely sensitive to various forms of cellular stress as well as to growth factors and other cell signaling processes. It was known 25 years ago that cell proliferation and growth were proportional to the rate of protein synthesis^{22, 23}. Control of translation has been proven critical in regulating numerous other physiological processes in the cell including: growth stimulation²⁴, cell cycle progression²⁵, differentiation²⁶, ER stress²⁷, and oncogenic signaling in cancer²⁸.

The process of mRNA translation can be divided into initiation, elongation and termination phases with the initiation phase being the most complex part, underlined by the requirement of over 25 essential factors^{29, 30} (see figure 2). The initiation step in which ribosomal subunits are loaded onto the mRNA is also the primary control point for regulating mRNA translation efficiency. In eukaryotes, most translation is 'cap-dependent' requiring the assembly of an active eukaryotic initiation factor 4F (eIF4F) at the m⁷GpppN cap structure present at the 5' terminus of the mRNA (reviewed in³¹. eIF4F consists of the m⁷GpppN cap binding protein, eIF4E, a scaffolding protein eIF4G and an ATP-dependent helicase eIF4A. Formation of eIF4F serves as a major control point for regulating the efficiency of mRNA translation by the cell. Assembly of eIF4F at the cap facilitates recruitment of the 43S pre-initiation complex. This pre-initiation complex is composed of the small 40S ribosomal subunit, the initiation factor eIF3, and the ternary complex which consists of eIF2/GTP and the methionine-loaded initiator tRNA, Met-tRNA. Formation of the ternary complex constitutes the second major control point for the cell in regulating the initiation step of translation. A scan through the 5' UTR of the mRNA by this complex places it at the AUG initiation codon.

Here, the eIF5 protein stimulates hydrolysis of the eIF2-bound GTP into GDP, which promotes the release of the initiation factors and recruitment of the large 60S ribosomal subunit. Formation of this complete 80S ribosome represents the beginning of the elongation phase of translation³².

Figure 2. Control of the initiation step of mRNA translation. Initiation of translation begins following assembly of eIF4F (containing the cap-binding protein eIF4E, the scaffold protein eIF4G, and the RNA helicase eIF4A) at the m7GpppN 'cap' structure of the mRNA. Interaction with eIF3 facilitates recruitment of the small subunit of the ribosome together with the ternary complex containing eIF2/Met-tRNA/GTP. This complex then scans through the 5'UTR until it reaches the AUG start site. Here GTP is hydrolyzed releasing eIF2 and allowing recruitment of the large subunit of the ribosome. Control of initiation occurs principally at two steps. The first is through inhibition of eIF4F as a result of competitive binding of the 4E-BPs to eIF4E in their underphosphorylated form. The second is through phosphorylation of eIF2 α . Phosphorylation results in binding and inactivation of the GDP/GTP exchange factor eIF2B.

3.1 Control of translation through eIF4F

As mentioned above, the two major control points for stimulating or inhibiting translation are formation of the eIF4F and ternary complexes. Under normal circumstances protein levels of eIF4E are considered to be rate-limiting for assembly of eIF4F and translation initiation. Consequently, regulation of eIF4E availability is arguably the most common mechanism for influencing eIF4F formation. eIF4F assembly is repressed by a family of eIF4E binding proteins (4E-BP1, 4E-BP2 and 4E-BP3)^{33, 34}. These small proteins compete with eIF4G for an overlapping binding site within eIF4E. The 4E-BPs are themselves regulated by multiple sequential phosphorylation events on at least 5 known sites. Phosphorylation is controlled in large part through the mammalian target of rapamycin (mTOR) which integrates signals from various pathways (discussed below). Upon hyperphosphorylation, the 4E-BPs lose their affinity for eIF4E, resulting in increased eIF4E:eIF4G binding and stimulation of translation. eIF4G is also subject to direct regulation. During heat-shock, the HSP27 protein binds and sequesters eIF4G in granules to prevent its participation in translation³⁵. Furthermore, proteolytic cleavage of eIF4G is reported during apoptosis³⁶ and virus infection³⁷. The cleavage of eIF4G creates a fragment which lacks the eIF4E-binding motif and is therefore no longer compatible with cap-dependent translation.

3.2 mTOR

mTOR has emerged as an important central regulator of mRNA translation³⁸. mTOR is a large protein kinase containing sequence similarity to the catalytic domain of the lipid phosphatidylinositol 3-kinase (PI3K). mTOR stimulates mRNA translation by phosphorylation and inactivation of the eIF4E repressor 4E-BP1, and through phosphorylation and activation of S6 kinase (S6K1). mTOR directly phosphorylates at least two sites in 4E-BP1, Thr37 and Thr46, and may be involved in the phosphorylation of several others. S6K1 and its homolog S6K2 are also

both direct targets of mTOR. S6K promotes the translation of 5'TOP (terminal oligopyrimidine tract) mRNA's which contain a polypyrimidine stretch of nucleotides adjacent to the 5' cap³⁹⁻⁴¹. These mRNAs encode exclusively for components of the translational machinery, including all ribosomal proteins. The stimulation of translation via S6K is indirect and is mediated by other downstream proteins of S6K including the ribosomal protein S6 and the ribosomal recruitment protein eIF4B⁴².

3.3 Raptor/MLST8

The upstream regulators of mTOR include important proteins that are sensors of nutrients, energy status, and growth factor signaling. Nutrient sensing by mTOR remains poorly understood, but appears to be mediated by two mTOR binding proteins, Raptor and mLST8⁴³. In the absence of nutrients (amino acids) a tight interaction between these 3 proteins is thought to prevent interaction of mTOR with its substrates. In the presence of nutrients a conformational change may partially disrupt this interaction and promote substrate and phosphorylation by mTOR^{43, 44}.

3.4 PI3K/AKT signaling

Regulation of mTOR and consequently mRNA translation through growth factor signaling pathways is better understood. Insulin and other growth factors lead to phosphorylation of 4E-BP1 and S6K in an mTOR dependent manner. These growth factors signal through the PI3K pathway as evidenced by the fact that inhibition of PI3K blocks 4E-BP1 and S6K phosphorylation in a mTOR sensitive manner⁴⁵⁻⁴⁸. Signaling from PI3K to mTOR also involves activation of the AKT kinase via the phosphatidylinositol-dependent kinases PDK1 and PDK2 (see figure 3). AKT activation is negatively regulated by the lipid phosphatase PTEN. AKT appears to be an important mediator of mTOR activity since its activation has results in increased phosphorylation of 4E-BP1 and S6K. Furthermore, a dominant negative mutant of AKT blocks insulin mediated phosphorylation of 4E-BP1⁴⁹.

3.5 TSC1/2

Recent work has also shed light on the direct mechanism whereby growth factor signaling through the PI3K/AKT pathway activates mTOR. This mechanism is mediated by the tuberous sclerosis complex (TSC1/TSC2) which is a critical negative regulator of mTOR. Phosphorylation and activation of AKT leads to the inactivation of TSC2, and thus activation of mTOR. It was originally proposed that AKT directly phosphorylates TSC2, however the precise mechanism may be more complicated⁵⁰. Mutations in TSC1 or TSC2 cause deregulation of mTOR activity and an increase in cell and organ size in *Drosophila* in a manner consistent with mutations in PTEN⁵¹⁻⁵³. Mammalian cells deficient for TSC1 or TSC2 fail to down-regulate mTOR function in response to growth factor deprivation and demonstrate constitutively high levels of phosphorylated S6K1⁵⁴⁻⁵⁸.

As mentioned earlier, the process of mRNA translation is extremely energy costly and it is thus reasonable to assume that mTOR activity may also be sensitive to low cellular energy status. Indeed, activation of mTOR by insulin is inhibited when ATP is reduced by incubation in low glucose media⁵⁹. Recent studies have shown that TSC1/TSC2 also regulates mTOR function in response to changes in energy. AMP-activated protein kinase (AMPK) is a critical mediator of energy metabolism that becomes active when AMP levels are high⁶⁰. AMPK directly phosphorylates and activates TSC2⁶¹ leading to mTOR inhibition and a reduction in mRNA translation. Correspondingly, cells deficient for TSC1/TSC2 are unable to downregulate mTOR during ATP deprivation⁶¹. Recent work has shown that activation of AMPK and phosphorylation of TSC2 also requires the LKB1 tumor suppressor protein^{62, 63}. Under low energy conditions LKB1 phosphorylates and activates AMPK leading to mTOR inhibition. LKB1 and AMPK thus promote maintenance of cellular energy homeostasis through mTOR-dependent inhibition of mRNA translation.

Figure 3. Control of mRNA translation through mTOR. Activation of PI3K upon growth factor receptor signaling leads to an AKT-dependent inhibitory phosphorylation of the TSC2 protein. The TSC1/TSC2 complex is a negative regulator of mTOR, and thus inhibition of TSC2 activates mTOR. Low energy levels inhibit mTOR signaling following an activating phosphorylation of TSC2 by AMPK. Phosphorylation of S6K and 4E-BP1 by mTOR promotes synthesis of the translational machinery and stimulates cap-dependent translation respectively. Hypoxia inhibits mTOR and cap-dependent translation by induction of REDD1 in a TSC2-dependent manner. Hypoxia may additionally inhibit cap-dependent translation through alternative mechanisms.

3.6 Deregulated eIF4F in cancer

Considerable evidence supports the concept that changes in mRNA translation contribute to the development of cancer^{28, 64, 65}. For example, several genes that participate in signaling pathways upstream of mTOR are frequently altered in cancer. This includes the commonly disrupted PI3K/AKT pathway. Mutations in the tumor suppressor gene PTEN^{66, 67} result in deregulation of AKT and subsequent activation of mTOR and

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mRNA translation. Similarly, receptors such as HER2/Neu which signal to this pathway are also frequent targets of amplification or mutation in cancer⁶⁸. Mutations in either TSC1 or TSC2 results in formation of benign tumors called hamartomas which consist of multiple cell types⁶⁹. Mutations in the LKB1 tumor suppressor gene, which regulates TSC1/TSC2 in response to reductions in energy levels, causes Peutz-Jeghers syndrome (PJS)⁷⁰. This disease is characterized by the development of hamartomas and various neoplasms, which results as a direct consequence of mTOR deregulation⁷¹.

Downstream of mTOR several studies linking eIF4E to cancer provide more direct evidence for the role of mRNA translation in transformation and tumor progression. Activation of mTOR stimulates translation by increasing eIF4E availability. eIF4E is frequently over-expressed in human cancer and is can directly transform immortalized 3T3 fibroblasts^{65, 72}. Recently, two genetic studies in mice have demonstrated a clear role for eIF4E in tumorigenesis^{73, 74}. Transgenic mice overexpressing eIF4E showed significantly increased tumorigenesis⁷³. Modest overexpression of eIF4E (2.5-fold) also considerably accelerated lymphomagenesis in the Eμ-Myc transgenic B-cell lymphoma model^{73, 74}. In this system eIF4E accelerated tumor development through the inhibition of apoptosis⁷⁴.

3.7 Control of translation through eIF2

The second major control point for regulation of translation involves formation of the eIF2/GTP/Met-tRNA ternary complex. Upon the start of protein synthesis, the eIF5 protein stimulates hydrolysis of the eIF2-bound GTP into GDP, which results in the release of the initiation factors from the ribosome. Thus, in order to load another ribosome onto the RNA and initiate an additional round of protein synthesis, the exchange of eIF2/GDP for GTP is required. This reaction is catalyzed by the initiation factor eIF2B. However, this exchange reaction is inhibited when the eIF2 α subunit becomes phosphorylated at ser51. Phosphorylation of eIF2 α does not prevent the binding of eIF2B, but it does prevent the exchange of GDP

for GTP. Consequently, eIF2B remains bound to the phosphorylated and inactive eIF2 complex. Because the eIF2B subunit exists in significantly lower amounts than eIF2 α (about 20-30% of eIF2 α), a less than complete phosphorylation of eIF2 α molecules is sufficient to inhibit the exchange activity of eIF2B. Although phosphorylation of eIF2 α results in a general inhibition of mRNA translation, it also leads to increased translation of specific mRNAs as will be discussed later.

At least 4 distinct kinases have been shown to phosphorylate eIF2 α at ser51 and to repress translation in response to stress (reviewed in^{75, 76}). These are: (i) PKR, an interferon inducible, double-stranded RNA-activated protein kinase important during virus infection⁷⁷; (ii) HRI, a heme regulated inhibitor of translation found predominantly in erythroid cells⁷⁸; (iii) GCN2, a kinase originally identified in yeast which is activated upon nutrient (amino acid) deprivation⁷⁹; and (iv) PERK, an endoplasmic reticulum resident kinase which participates in a conserved pathway known as the unfolded protein response (UPR)⁸⁰. During the UPR, PERK is activated by autophosphorylation resulting in phosphorylation of eIF2 α and inhibition of translation. All of the kinases which phosphorylate eIF2 α are considered to be "stress-responsive" and are thought to play key roles in the adaptation of cells to adverse environments. PERK resides in the endoplasmic reticulum (ER) membrane with its kinase domain in the cytoplasmic side and is activated by ER stress⁸⁰. PERK knockout MEFs are unable to phosphorylate eIF2 α in response to ER stress⁸¹ and correspondingly display increased sensitivity to agents that cause ER stress⁸¹. Bertolotti et al demonstrated that the luminal domain of PERK is bound to BiP/GRP78 and that perturbation of protein folding leads to a dissociation of these two proteins and activation of PERK⁸². Therefore, a model has emerged in which BiP acts as a repressor of PERK activation under normal conditions. Upon an increase in protein misfolding, relief of this repression leads to PERK activation, eIF2 α phosphorylation and inhibition of translation.

4. Mechanistic basis and importance of translational control during hypoxia

During hypoxia mRNA translation is rapidly inhibited as evidenced by a massive reduction in ribosome associated or 'polysomal' RNA (see figure 4). Why is translational regulation employed under conditions of low oxygen availability? If one views the useful properties of translational control for the cell in general as stated by Mathews et al.⁸³ (e.g., directness and rapidity, reversibility, fine control, etc.) it becomes evident that from a teleological perspective that several of these properties would be beneficial under conditions of hypoxia. Transcriptional control is exerted at a much earlier step in the process of gene expression compared to translational control, and thus a change in protein levels requires much longer if regulated at the level of transcription, especially if the mRNA of the particular protein is abundant and/ or has a long half-life. Conversely, if the cell becomes re-oxygenated, translational control is a very efficient and rapid mechanism for quickly elevating protein synthesis levels to meet cellular demands. In tumors, fluctuations in oxygenation from near normal to near anoxia can occur with a periodicity of 15 to 20 minutes, clearly too short a time for transcriptional adaptation⁸⁴. The mechanisms which inhibit translation during hypoxia are multi-factorial and involve both of the primary control points used to regulate the initiation step of this process. Clues as to the mechanistic basis and importance of this inhibition have also recently become available.

4.1 Control of translation during hypoxia through eIF2 α

We previously reported that exposure of human diploid fibroblasts, minimally transformed cells, and various tumor cell lines to hypoxia leads to phosphorylation of eIF2 α ^{16, 85} and inhibition of protein synthesis. Importantly, this response is independent of HIF-1 α accumulation as both HIF-1 $\alpha^{+/+}$ and HIF-1 $\alpha^{-/-}$ MEFs similarly phosphorylate eIF2 α in response to hypoxia¹⁶. Phosphorylation of eIF2 α occurs within 1 hour of hypoxic

Figure 4. Reduction in polysomal RNA during hypoxic exposure. mRNA translation can be assessed by examining the association of ribosomes with mRNA. DU145 human prostate cancer cells were exposed to aerobic conditions or 4 hrs of hypoxia. RNA was then separated on the basis of size through a sucrose gradient. The first peak represents free 40S ribosomal subunits, the second free 60S subunits, and the third complete 80S ribosomes. Each subsequent peak represents 'polysomal' mRNA with 2, 3, 4, 5, etc ribosomes attached to the mRNA. Hypoxia results in an inhibition of translation as evidenced by a dramatic reduction in polysomal RNA and a shift to mRNAs that are less efficiently translated (fewer ribosomes). Transcripts that remain actively translated during hypoxia can be identified by microarray analysis of the remaining polysomal RNA.

exposure and before any significant changes in transcription or cellular energy status. Furthermore, the phosphorylation which occurs during hypoxia is completely reversible upon reoxygenation. As outlined above, the phosphorylation of eIF2 α prevents the exchange of GDP for GTP by eIF2B and thus prevents formation of an active ternary complex which can participate in translation initiation. Expression of a transdominant, non-phosphorylatable mutant allele of eIF2 α attenuates the repression of protein synthesis under hypoxia. We also demonstrated that the endoplasmic reticulum resident eIF2 α kinase, PERK, becomes hyperphosphorylated upon hypoxic stress, and overexpression of wt-PERK increases the levels of hypoxia-induced phosphorylation of eIF2 α . Cells

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stably expressing a dominant-negative PERK allele and mouse embryonic fibroblasts (MEFs) with a homozygous deletion of PERK exhibit attenuated phosphorylation of eIF2 α and reduced inhibition of protein synthesis in response to hypoxia. MEFs lacking PERK expression show not only attenuated inhibition of translation but also reduced survival after prolonged exposure to hypoxia¹⁶. Similar results were obtained using human tumor cells expressing a non-phosphorylatable eIF2 α construct. These results indicate that adaptation of cells to hypoxic stress requires activation of PERK and phosphorylation of eIF2 α and suggest that the mechanism of hypoxia-induced translational attenuation may be linked to ER stress¹⁶.

Phosphorylation of eIF2 α and inhibition of protein synthesis has also been demonstrated to occur in response to ischemia-reperfusion injury in neuronal tissue⁸⁶⁻⁸⁹. However, in these cases eIF2 α phosphorylation is observed only upon reoxygenation. While apparently similar to hypoxia, ischemia has distinct properties because of the more acute nature of the stress, the vulnerability of the neurons to the reperfusion and the fact that ischemia-reperfusion has a strong component of free-radical damage, which is not present in all modes of chronic hypoxia in tumor cells. Indeed, in our preliminary studies, not only did we observe eIF2 α phosphorylation in the absence of any reoxygenation, but also reoxygenation induced a rapid dephosphorylation of eIF2 α . Therefore, the regulation of eIF2 α phosphorylation by hypoxia in tumor cells appears to be governed by distinct pathways not present or utilized in ischemia-reperfusion. To our knowledge, this is the first demonstration of eIF2 α phosphorylation in response to hypoxia in non-neuronal cells.

4.2 Hypoxia and the UPR

The global decrease in mRNA translation is reminiscent of the heat-shock response when cells are exposed to temperatures of over 43°C⁹⁰⁻⁹³. Another striking similarity between the two responses is that they are paradoxically characterized by a robust upregulation of a few proteins

whose main function is to protect the cell. In fact, some of the stress-response proteins that are upregulated in the heat shock response, like heat shock protein 70 (HSP70) and glucose-regulated protein/binding protein (GRP78/BiP) are also upregulated under hypoxia⁹⁴⁻⁹⁹. Importantly, inhibition of BiP expression results in increased cell death in cells exposed to hypoxic stress¹⁰⁰. A likely explanation for the similarity between hypoxia and this heat-shock response is the induction of an evolutionarily conserved pathway known as the unfolded protein response (UPR).

Inhibition of mRNA translation through the phosphorylation of eIF2 α at Ser51 is an evolutionarily conserved response to a multitude of cellular stresses and has been termed the Integrated Stress Response (ISR)¹⁰¹. Other stresses which can activate the ISR include amino acid withdrawal and a variety of agents that activate endoplasmic reticulum (ER) stress. The lumen of the ER is an active site of protein folding in which all secretory and membrane-bound proteins are post- or co-translationally translocated into. The ER maintains a unique oxidizing environment necessary for the proper folding of these proteins, and is also important for posttranslational modifications such as glycosylation¹⁰². ER stresses, such as perturbations in calcium storage, redox status and inhibition of protein glycosylation, compromise the ER's capacity to correctly fold proteins, thereby leading to an accumulation of unfolded proteins within the ER¹⁰³. This imbalance between the ER's folding capacity and its protein load induces the UPR¹⁰⁴. The UPR constitutes three unique signaling pathways, one of which is characterized by activation of the ISR through PERK and subsequent phosphorylation of eIF2 α ¹⁰⁴(see figure 3). The activation of the UPR not only reduces ER protein load by inhibiting the translation of new proteins through the ISR, but also induces the expression of UPR target genes responsible for restoring ER homeostasis.

In mammalian cells, the UPR is mediated by three ER transmembrane proteins: PERK, IRE1 and ATF6. Each of these proteins has a luminal domain that is negatively regulated by the ER chaperone BiP/GRP78⁸². In unstressed cells, BiP binds the luminal domains of all three proteins,

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maintaining them in their inactive states. Upon accumulation of unfolded proteins, BiP dissociates from PERK, IRE1 and ATF6 to promote protein folding. Consequently, each of these three ER proteins activates a distinct pathway of the UPR, inducing a global translational attenuation as well as the transcriptional activation of UPR target genes. Such genes encode proteins involved in protein folding, glycosylation, calcium homeostasis and protein degradation¹⁰².

Several lines of evidence demonstrate that hypoxia is also able to activate the UPR and that cancer cells utilize the UPR as a survival mechanism under conditions of low oxygen. First of all, as described above, hypoxia activates the ISR through activation of PERK and phosphorylation of eIF2 α . This leads to attenuation of mRNA translation and a reduction in the protein content of the ER. This inhibition also paradoxically leads to increased expression of several proteins including the transcription factor ATF4. ATF4 plays an important role by transcriptionally regulating several other genes including CHOP (GADD153) and GADD34, and may be particularly important in preventing oxidative stress¹⁰¹. CHOP is a transcription factor implicated in activating apoptosis, whereas GADD34 is part of a negative feedback loop that promotes the dephosphorylation of eIF2 α and recovery from the ER stress response¹⁰⁵. Blais et.al. showed that ATF4 is similarly induced during hypoxia in a PERK and mRNA translation dependent manner⁸⁵. Both of the ATF4 target genes GADD34 and CHOP are also induced by hypoxia. Interestingly, primary human breast tumors also demonstrate higher ATF4 and CHOP expression as compared to normal tissues. The expression of ATF4 and CHOP has also been reported to occur in tumors in areas adjacent to necrosis and which are thought to be hypoxic¹⁰⁶. New data also demonstrates that activation of the PERK-eIF2 α pathway during hypoxia is necessary for efficient tumor growth *in vivo*. Transformed MEFs lacking PERK are unable to activate this pathway and produce much smaller tumors than their wild-type counterparts. The tumors grow slowly because the cells are unable to survive hypoxia within the tumor

microenvironment (Bi, M. *et al.* submitted). More importantly, human tumor cells expressing a dominant-negative PERK allele also show inhibited tumor growth *in vivo*, with hypoxic areas of the tumor co-localizing with areas of apoptosis (Bi, M. *et al.* submitted). Thus the UPR, activated under hypoxic conditions in tumors, may represent an important survival mechanism that contributes to hypoxia tolerance and cancer growth. There is also some evidence that hypoxia activates the other two arms of the UPR pathway mediated by ATF6 and IRE1. Upon activation of the UPR, ATF6 is known to be cleaved into a smaller fragment which is released from the ER. This cleaved form translocates to the nucleus where it functions as an active transcription factor. Hypoxia also induces ATF6 cleavage and nuclear localization in HeLa cells (unpublished data). Also, there is recent evidence that the UPR pathway controlled by IRE1 is activated and important during hypoxia. Activated IRE1 contains a nuclease activity that removes 26 bases from the XBP1 transcript. This frameshift allows the synthesis of a much more potent transcription factor compared with the unspliced form of the protein. XBP1 activates the transcription of several genes involved in the UPR that contain an ER stress response element (ERSRE) within their promoters. This includes genes such as BiP and protein disulfide isomerase (PDI) both of which are also activated by hypoxia. A recent report shows that MEFs derived from XBP1 knockout mice have increased levels of apoptosis and reduced clonogenic survival in response to hypoxia¹⁰⁷. Loss of XBP1 also inhibited tumor growth due to their reduced ability to survive in a hypoxic microenvironment. These studies thus implicate the IRE1/XBP1 arm of the UPR as an important factor for hypoxic stress and tumor growth. These data suggest that all three arms of the UPR are activated during hypoxia in a time- and oxygen concentration-dependent manner. Disruption of either of the pathways controlled by PERK or IRE1 sensitizes cells to hypoxia and impairs tumor growth.

Figure 5. Hypoxia and the UPR. Evidence suggests that hypoxia activates a conserved ER stress response program known as the UPR. This response is mediated by three ER transmembrane proteins PERK, IRE1, and ATF6. The activation of PERK and subsequent phosphorylation of eIF2 α is the best characterized arm of this response during hypoxia. Phosphorylation of eIF2 α leads to a general inhibition of overall mRNA translation, but stimulates the translation of a specific subset of proteins including ATF4. IRE1 activation removes a 26-nucleotide region of the XBP1 mRNA resulting in the formation of a potent transcription factor. Finally, ATF6 is cleaved, processed in the golgi, and translocates to the nucleus where it induces gene expression.

An unresolved issue which will be the focus for future research regards the mechanism by which the UPR is activated during hypoxia. The unique oxidizing environment of the ER provides some clues.

4.3 Control of translation during hypoxia through eIF4F

Our group has evidence that the observed inhibition of translation during hypoxia cannot be fully accounted for by the phosphorylation of eIF2 α . Phosphorylation of eIF2 α reaches a maximum during hypoxia and then declines even though translation rates as assessed by polysomal mRNA remain low. Furthermore, we have recent evidence that cells derived from a mouse in which a non-phosphorylatable allele of eIF2 α was knocked-in (S51A)¹⁰⁸ still display a substantial (albeit reduced) inhibition of translation. Another candidate mechanism that could account for this discrepancy is disruption of the cap-binding protein complex eIF4F. We have preliminary data suggesting that hypoxia also disrupts the eIF4F complex through multiple mechanisms including dephosphorylation of the 4E-BPs (unpublished data). This 'second' pathway of inhibiting translation occurs only after longer periods of exposure to hypoxia (4–16 h) and provides a possible mechanism for the sustained down-regulation of protein synthesis that needs further investigation.

5. Hypothesis and outline of this thesis

As outlined in the previous paragraphs, hypoxia has a profound effect on protein synthesis through inhibition of mRNA translation. Our hypothesis was that hypoxia influences translational control through two independent pathways that are each important contributors to gene expression and hypoxic cell phenotype. In **chapter 2** we investigated the first part of this hypothesis by characterizing the role and requirement of upstream signaling pathways that regulate translation initiation during hypoxia. In this study, we also demonstrated the importance of these regulatory pathways on the activation of several hypoxia regulated genes.

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The second part of our hypothesis relates to the effects of translational control by hypoxia on gene expression. We set out to identify and characterize specific genes that produce mRNAs which have an ability to remain translated under conditions in which overall protein synthesis is repressed. We reviewed the current understanding of the mechanisms leading to selective mRNA translation and gene expression during hypoxia in **chapter 3**. In **chapter 4** we assessed changes in the hypoxic gene expression using a proteomic approach combined with genome-wide analysis of well translated transcripts. The relative contributions of translational control to gene expression were further evaluated in **chapter 5**. In **chapter 6** and **chapter 7** we describe the novel regulation of two important hypoxia regulated genes, CITED2 and carbonic anhydrase 9 (CA9) which are controlled through hypoxia dependent changes in mRNA translation.

The last part of our hypothesis relates to the influence of translational control on the hypoxic cell phenotype. We tested this aspect of our hypothesis by initiating studies to determine if the translational control pathways are good candidates for the development of novel hypoxia sensitizing drugs. For this we generated an isogenic cell model in which we can modulate the translational control pathways in an inducible manner. The effect of targeting these pathways on hypoxia tolerance are described in **chapter 8**.

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CHAPTER 2

Gene expression during acute and prolonged hypoxia is regulated by distinct mechanisms of translational control

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Abstract

Hypoxia has recently been shown to activate the endoplasmic reticulum kinase PERK, leading to phosphorylation of eIF2 α and inhibition of mRNA translation initiation. Using a quantitative assay, we show that this inhibition exhibits a biphasic response mediated through two distinct pathways. The first occurs rapidly, reaching a maximum at 1–2 h and is due to phosphorylation of eIF2 α . Continued hypoxic exposure activates a second, eIF2 α independent pathway that maintains repression of translation. This phase is characterized by disruption of eIF4F and sequestration of eIF4E by its inhibitor 4E-BP1 and transporter 4E-T. Quantitative RT-PCR analysis of polysomal RNA indicates that the translation efficiency of individual genes varies widely during hypoxia. Furthermore, the translation efficiency of individual genes is dynamic, changing dramatically during hypoxic exposure due to the initial phosphorylation and subsequent dephosphorylation of eIF2 α . Together, our data indicate that acute and prolonged hypoxia regulates mRNA translation through distinct mechanisms, each with important contributions to hypoxic gene expression.

Introduction

The presence of hypoxic and anoxic areas in human tumors is well documented, and is prognostic for poor outcome (reviewed in^{1, 2}. The clinical importance of tumor hypoxia results from its ability to protect cells against both radiation and chemotherapy and from the fact that it can provide a selection pressure for apoptotically resistant cells³. Furthermore, the cellular response to hypoxia causes important changes in gene expression that affect cell behavior and influence patient prognosis. There has been particular focus on changes mediated through the family of hypoxia-inducible transcription factors (HIFs). HIF-1 and HIF-2 promote transcription of more than 60 putative downstream genes (for a review see⁴) that affect hypoxia tolerance, energy homeostasis, angiogenesis and tumor growth. Although the transcriptional response to hypoxia is clearly very important⁵⁻⁷, tumor cells also experience short, transient exposures to hypoxia and/or anoxia that occur over time frames too fast for an effective transcriptional response. Transient changes in oxygenation occur owing to the abnormal vasculature found in most tumors, characterized by immature, leaky and improperly formed vessels. Perfusion of these vessels can change dynamically in time, leading to rapid but transient episodes of severe hypoxia in the tumor cells dependent upon them^{8, 9}. Consequently, post-transcriptional responses are presumably important for adaptation to cycling oxygenation in tumors.

Control of mRNA translation during hypoxia is emerging as an important cellular response to hypoxia¹⁰⁻¹². As protein synthesis is energy costly, inhibition of mRNA translation may represent an active response to prevent loss of energy homeostasis during hypoxia. Indeed, it has been shown that overall mRNA translation is severely but reversibly inhibited during hypoxia^{10, 13, 14} with kinetics that precede ATP depletion¹⁵. Furthermore, regulation of mRNA translation can have a significant and rapid impact on individual gene expression. This is because the sensitivity of individual genes to changes in overall translation varies widely and in a

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manner that reflects the molecular mechanisms responsible for controlling translation^{16, 17}. Regulation of gene expression through control of mRNA translation is important during various pathologies including cancer¹⁸. The mechanisms responsible for inhibiting translation during hypoxia are not yet fully understood.

We have previously investigated the involvement of the endoplasmic reticulum (ER) kinase PERK in the hypoxia induced downregulation of protein synthesis¹⁰. PERK is activated as part of the evolutionarily conserved unfolded protein response (UPR) (reviewed in¹⁹. It phosphorylates eIF2 α , a subunit of eIF2, which in its GTP-bound form recruits the aminoacylated tRNA to the 40S ribosomal subunit. The exchange of GDP for GTP is mediated by the guanine nucleotide exchange factor eIF2B. Ser51-phosphorylated eIF2 α inhibits eIF2B, resulting in inhibition of translation initiation. eIF2 α phosphorylation results in a set of molecular events collectively termed the integrated stress response. These include the inhibition of global mRNA translation in conjunction with induced expression of the transcription factor ATF4 and its downstream target genes²⁰. We showed that hypoxia rapidly activated PERK, which led to reversible phosphorylation of eIF2 α ¹⁰. Hypoxia-induced inhibition of protein synthesis was severely attenuated in cells without functional PERK. After prolonged periods of hypoxia, PERK-deficient cells did show partial inhibition, suggesting that protein synthesis is regulated through additional mechanisms.

Another candidate mechanism for inhibiting translation during hypoxia is disruption of the cap-binding protein complex eIF4F, which consists of eIF4E, eIF4A and eIF4G (for recent reviews see^{21, 22}. eIF4E participates in a protein bridge between the mRNA and the ribosome by its simultaneous interaction with the mRNA 5' cap structure and the large scaffolding protein eIF4G, which in turn interacts with eIF3 that is bound to the 40S ribosomal subunit. eIF4E is regulated through a set of binding proteins (4E-BPs) that bind reversibly to eIF4E in their hypophosphorylated form, and this obstructs the interaction between eIF4E and eIF4G. The 4EBP1

protein becomes hyperphosphorylated in response to a number of stimuli, such as insulin, hormones, growth factors, mitogens and cytokines, as a result of activation of the PI3-kinase/Akt/FRAPmTOR pathway²³.

It remains unclear to what degree the lack of eIF4F assembly contributes to inhibition of translation during tumor hypoxia. Several studies have investigated the combined consequences of ischemia/reperfusion on eIF4F-related proteins in rat brains (reviewed in^{24, 25}. Proteolysis of eIF4G was reported during ischemia and reperfusion *in vivo*^{26, 27}, but not in neuronal cells cultured *in vitro* (NGF differentiated PC12 cells)²⁸. The reports addressing the expression and phosphorylation status of eIF4E during ischemia are conflicting, but 4E-BP1 dephosphorylation has been demonstrated both *in vivo* and *in vitro*^{27, 28}. The acuteness and complexity of ischemia/reperfusion stress and the high sensitivity of neurons to deprivation and reconstitution of both oxygen and nutrients are distinct properties of this model system and thus difficult to extrapolate to tumor hypoxia. In rat hepatocytes, 4E-BP1 becomes dephosphorylated and associates with eIF4E rapidly (15–60 min) upon mild hypoxia, but this could not explain the observed downregulation of protein synthesis²⁹. More recently, it was reported that hypoxia could influence 4E-BP1 phosphorylation by affecting the activity of mTOR³⁰. Serum-starved and hypoxic human embryonic kidney cells failed to activate mTOR, phosphorylate 4E-BP1 and dissociate 4E-BP1 from eIF4E in response to insulin treatment. Nonetheless, it remains unknown whether hypoxia alone is sufficient to disrupt the eIF4F complex and to what extent this influences overall translation during hypoxia. Here we show that hypoxia induces a biphasic inhibition of mRNA translation characterized by transient phosphorylation of eIF2 α and subsequent dissociation of eIF4F. These two mechanisms operate independently of each other and both have important consequences for gene expression during hypoxia.

Materials and methods

Cell culture

Exponentially growing cervical carcinoma HeLa cells (American Type Culture Collection CCL-2), lung adenocarcinoma A549 cells, normal human fibroblasts (AG1522) or MEFs that were WT or had a homozygous knock-in mutation for eIF2 α (S51A) (Scheuner et al, 2001) were grown on glass dishes or chamber slides in DMEM media supplemented with 10% fetal calf serum. The MEF media also contained MEM nonessential amino acids and 55 mM 2-mercaptoethanol (all Sigma-Aldrich). For preparation of extracts and viability assessments, see Supplementary data.

Hypoxic conditions

Cells were transferred to a hypoxic culture chamber (MACS VA500 microaerophilic workstation, Don Whitley Scientific). The composition of the atmosphere in the chamber consisted of 5% H₂, 5%CO₂, 0.0% O₂ and residual N₂.

m7GTP resin precipitation

A 1mg portion of HeLa extract was incubated with 25 ml of m7GTP sepharose resin (Amersham Biosciences) for 3 h at 4°C. The resin was washed, boiled in Laemmli buffer and the polypeptides were resolved by SDS-PAGE.

Western blotting

Cell extracts were boiled in Laemmli buffer and polypeptides were resolved by SDS-PAGE and transferred onto 0.2 mm nitrocellulose membranes (Amersham Corp.). For primary antibodies, see Supplementary data. Detection of peroxidase-coupled secondary antibodies was performed with Enhanced Chemiluminescence (Amersham Corp.).

Immunofluorescence

Cells were fixed with 4% paraformaldehyde and permeabilized in 4% paraformaldehyde and 0.1% Triton X-100. For antibodies, see Supplementary data. Cells were mounted in the ProLong™ Antifade Kit (Molecular Probes) and analyzed with a Zeiss inverted LSM 410 laser scan confocal microscope.

Polysomal fractionation and analysis

Polysomal fractionation and analysis were performed as described previously (Koritzinsky et al, 2005); see Supplementary data.

RNA isolation and reverse transcription

RNA isolation and reverse transcription were performed as described previously (Koritzinsky et al, 2005); see Supplementary data.

Quantitative PCR analysis

Real-time PCR was performed in either ABI 7700 or ABI 7500 (Applied Biosystems). For primers and probes, see Supplementary data. Unfractionated samples were normalized by 18S rRNA signal. Samples from polysome fractions were normalized by 18S rRNA measured by PCR divided by 18S rRNA measured by spectrometry during fractionation, corrected for loading. This facilitated correction for any differences in RNA isolation or reverse transcriptase efficiency between samples. The abundance of every gene was calculated relative to a master reference using standard curves.

Supplementary data

Supplementary data are available at The EMBO Journal Online.

Results

Kinetics of translation inhibition

To determine the effects of hypoxia on mRNA translation initiation in HeLa cells, we examined the association of ribosomes with mRNA at various time points. In this assay, the number of ribosomes found within the 'polysomal' fraction of mRNA (mRNA containing two or more ribosomes) is a reflection of de novo protein synthesis. This technique is advantageous to other methods such as 35S incorporation, which requires prior amino-acid starvation, a procedure that can itself influence translation initiation³¹. Figure 1A shows that at all time points examined, hypoxia causes a large decrease in polysomal mRNA and a corresponding increase in free ribosomes and ribosomal subunits. The reduction in translation is not influenced by cell death, as cell viability remains above 90% following 16 h of hypoxia (data not shown). Furthermore, the inhibition of translation is completely reversible upon reoxygenation (data not shown).

To assess quantitatively overall mRNA translation from the polysome profiles, we calculated the percentage of rRNA participating in polysomes and defined this as the overall translation efficiency. This value is reduced from 62 to 24% after 1 h of hypoxia, and then recovers somewhat stabilizing at 30% (Figure 1B). The drop in translation reproducibly exhibited this biphasic response with maximum inhibition after 1–2 h, followed by a small recovery. The magnitude of inhibition is comparable to that observed following complete disruption of the cellular redox environment with 1mM dithiothreitol (DTT) (17%) (data not shown).

Analysis of the polysome profiles in Figure 1A shows that hypoxia also causes a change in the distribution of the polysomal mRNA, with proportionally less signal in the higher molecular weight fractions. This indicates that the average number of ribosomes per mRNA transcript is also decreased during hypoxia, reflecting a reduction in translation initiation efficiency even for those transcripts that remain translated. From

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Figure 1. Hypoxia inhibits mRNA translation. HeLa cells were exposed to 0.0% O₂ for 0–16 h and cell lysates were separated on a sucrose gradient. (A) The optical density (OD) at 254nm is shown as a function of gradient depth for each time point. Actively translated mRNA is associated with high-molecular-weight polysomes deep in the gradient. (B) Translation efficiency in HeLa cells as a function of time in 0.0% O₂. As a measure of overall translation efficiency, the relative amount of rRNA participating in polysomes was estimated. This fraction is proportional to the integrated area under the curve containing polysomes, as marked in (A). (C) The average number of ribosomes per mRNA in the polysomes as a function of time in 0.0% O₂. This was calculated by differential integration of the profiles in (A).

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the polysome profiles, we calculated the average number of ribosomes per translated transcript (i.e. mRNAs containing two or more ribosomes) at different time points during hypoxia (Figure 1C). The kinetics of this parameter follow in large part that of the overall translation.

eIF2 α regulates translation during acute hypoxia

The eIF2 α kinase PERK is at least partly responsible for protein synthesis inhibition during acute hypoxia, as measured by radioactive labeling of newly synthesized proteins¹⁰. Thus, we hypothesized that the rapid inhibition and subsequent partial recovery in translation is due to changes in eIF2 α phosphorylation. Indeed, we found that the phosphorylation of eIF2 α is greatest after 1–2 h and then decreases by 8 h of hypoxia in several cell lines (Figure 2A). ATF4 protein levels also increase and then decrease during hypoxia in a manner that mirrors eIF2 α phosphorylation. The dynamics of eIF2 α phosphorylation and ATF4 protein induction thus correlate with the initial inhibition of translation and its subsequent recovery. To assess the requirement of eIF2 α phosphorylation for translation inhibition during hypoxia we examined the response of mouse embryo fibroblasts (MEFs) derived from eIF2 α knock-in mice containing an S51A mutation³². As expected, these cells were defective in phosphorylation of eIF2 α during hypoxia (Figure 2B). The translation efficiency in wild-type (WT) MEFs is similar to that in HeLa cells, with a rapid drop during acute hypoxia followed by a partial recovery (Figure 2C). In contrast, S51A MEFs display a substantial defect in their ability to inhibit translation during the initial phase.

Figure 2. Inhibition of translation during acute hypoxia is dependent on eIF2 α . HeLa cells, A549 cells, human normal fibroblasts (NF) and WT or S51A MEFs were exposed to 0.0% O₂ for 0–16 h, 1mM DTT or serum starvation (SS) for 1 h. Cell lysates were separated by SDS–PAGE. Immunoblots for (A) HeLa, A549 and NF or (B) MEFs were performed using antibodies against total or phosphorylated eIF2 α , ATF4 and β -actin. In (A), optical densitometry for phosphorylated eIF2 α or ATF4 normalized by total eIF2 α is also shown. Total eIF2 α expression has previously been shown to be constant during hypoxia¹⁰. (C) Cell lysates were separated on a sucrose gradient, and OD at 254nm was recorded. Translation efficiency as a function of time in 0.0% O₂ in WT and S51A MEFs was estimated as in Figure 1. (D) Average number of ribosomes per mRNA in the polysomes in WT and S51A MEFs as a function of time in 0.0% O₂ was calculated as in Figure 1.

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Nonetheless, after 16 h of hypoxia, both cell lines show a similar loss in translation efficiency. These data indicate that eIF2 α phosphorylation is indeed necessary for inhibition of translation during acute hypoxia, but not at later times. When the polysome profiles are analyzed in terms of the average number of ribosomes per translated transcript, S51A MEFs exhibit an even stronger defect in their response during acute hypoxia. Despite a small but detectable drop in translation efficiency during the first 4 h of hypoxia

(Figure 2C), S51A MEFs show no decrease in the average number of ribosomes per translated transcript (Figure 2D). The same result was found in cells treated with DTT, a known activator of PERK that causes eIF2 α phosphorylation. In contrast, WT MEFs show a strong reduction in average ribosomes per transcript during both acute hypoxia and DTT treatment. Interestingly, after 8 h of hypoxia, the average number of ribosomes per translated transcript increases again toward normal levels in WT cells and is equivalent to that in S51A MEFs by 16 h. These data provide further evidence that the inhibition of translation that occurs after acute and prolonged hypoxia is mechanistically distinct.

Disruption of the eIF4F complex during hypoxia

The assembly of the cap-binding complex eIF4F is a common control point for translation initiation and was thus a likely candidate for maintaining low rates of translation during prolonged hypoxia. We examined the levels of eIF4E and proteins that associate with it as an active complex (eIF4GI) or as an inactive complex (4E-BP1). Figure 3A shows that the levels of eIF4E do not change during hypoxia. In contrast, 4EBP1 (Figure 3B) shows both a small induction at 8 h and a strong dephosphorylation after 16 h of hypoxia. This protein runs as different migrating bands representing different phosphorylation levels. The fastest migrating band is substantially increased after 16 h of hypoxia, and represents the

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hypophosphorylated 4E-BP1, which is known to have a higher affinity for eIF4E. A small decrease in the abundance of the scaffold protein eIF4GI (Figure 3C) was observed after 8 h, consistent with a decrease in its rate of synthesis measured in a microarray study using polysomal RNA (unpublished data). Overexposure of the blots indicated no reproducible changes in the cleavage of eIF4G. The influence of hypoxia on 4E-BP1 phosphorylation appears to be largely independent of eIF2 α phosphorylation, as it is not differentially affected in the WT and S51A MEFs (unpublished data). However, until the relative contributions of various upstream signaling pathways to 4E-BP phosphorylation under hypoxia are better understood, it is premature to conclude that no connection between eIF2 α and eIF4F exists.

Figure 3. Expression of eIF4E, 4E-BP1 and eIF4GI during hypoxia. HeLa cells were exposed to 0.0% O₂ for 0–16 h and cell lysates separated by SDS-PAGE. Immunoblots were performed using antibodies against actin, (A) eIF4E, (B) 4E-BP1 and (C) eIF4GI. The phosphorylation forms of 4E-BP1 have different electrophoretic mobilities and are represented by several bands on the immunoblot. Full-length eIF4GI run at about 220 kDa; the blot is overexposed to detect cleavage products.

Figure 4. eIF4F is disrupted during prolonged hypoxia. HeLa cells were exposed to 0.0% O₂ for 0–16 h and cell lysates probed for the presence of various eIF4E complexes. Lysates were incubated with an m7-cap analogue ('Cap') or uncapped resin as a negative control. Immunoblots were performed with antibodies against actin, (A) eIF4GI, eIF4E, 4E-BP1 and (B) eIF4GII. 'Cap': proteins bound to the capped resin; 'Resin': proteins bound to the uncapped resin; 'C-FT': unbound fraction after incubation with capped resin; 'R-FT': unbound fraction after incubation with uncapped resin.

To more strictly assess the influence of hypoxia on eIF4F, we investigated the association of eIF4E with eIF4GI and eIF4GII as well as with its inhibitor 4E-BP1 in HeLa cells. During aerobic conditions where translation is efficient, eIF4E is associated with large amounts of both eIF4GI and eIF4GII, and only a small amount of 4E-BP1 (cap lanes in Figure 4A and B). Cap-associated eIF4G migrated somewhat slower than the overall pool of eIF4G, suggesting a possible modification of this phospho-protein when bound to the cap. In contrast, after 4 or 16 h of hypoxia, there is a dramatic loss in binding to both eIF4GI and eIF4GII, indicating dissociation of the eIF4F complex. At 16 h, this dissociation correlates with

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a large increase in binding between eIF4E and 4E-BP1, consistent with the increase in the hypophosphorylated levels of 4E-BP1 at this time. It also correlated with decreased phosphorylation of eIF4E (Supplementary Figure S1) at 16 h, but the physiological significance of this remains unclear. However, although dissociation of eIF4G and eIF4E is complete after 4 h of hypoxia, a corresponding change in eIF4E phosphorylation or eIF4E/4E-BP1 association is not seen at this time point. This suggests that a mechanism distinct from 4E-BP1 dephosphorylation may also inhibit eIF4F during hypoxia.

Translocation of eIF4E by 4E-T

A potential cause of eIF4F disruption that has not been well characterized is the translocation of eIF4E to the nucleus or to cytoplasmic bodies of mRNA processing (P-bodies). A 5–20% fraction of eIF4E is known to localize to the cell nucleus³³. The shuttling protein 4E-T is the only known regulator of eIF4E localization and is capable of binding and transporting it to the cell nucleus³⁴. eIF4E also colocalizes with 4E-T in P-bodies, where mRNA is degraded or stored³⁵. Hypoxia caused a redistribution of both eIF4E and 4E-T from predominantly cytoplasmic staining under aerobic conditions to substantial nuclear staining during hypoxia (Figure 5A–C). This redistribution occurred progressively over time in hypoxic conditions, correlating with the gradual dephosphorylation of 4E-T (Figure 5D). In addition, hypoxic cells exhibit significant eIF4E and 4E-T staining in the perinuclear area, which may be associated with the nuclear envelope or the ER. Interestingly, hypoxia also increased the number of 4E-T speckles, which have been described as P-bodies³⁶.

Gene-specific regulation of translation

As translation efficiency is highly gene specific, we anticipated that individual genes would show different patterns of translation efficiency during acute and prolonged hypoxia. To investigate this, we fractionated polysomal mRNA and subsequently measured the mRNA abundance of

individual genes by quantitative RT-PCR (Figure 6A). We first confirmed that concomitant with an increase in polysome association, the non/subpolysomal abundance decreased (Supplementary Figure S2).

Figure 5. 4E-T and eIF4E relocate during hypoxia. HeLa cells were treated with 0.0% O₂ for 0–16 h. Cells were stained with DAPI and (A) a polyclonal antibody against eIF4E, (B) a polyclonal antibody against 4E-T or (C) a monoclonal antibody against eIF4E and a polyclonal antibody against 4E-T. Cells were visualized by confocal microscopy and individual pictures merged to determine colocalization. (D) Cell lysates were separated by SDS-PAGE and immunoblots performed using antibodies against 4E-T. The ratio of the individual bands was quantified with optical densitometry. A crossreacting band is indicated (*).

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Subsequently, we quantified both the transcript recruitment and distribution within the polysomes (expressed as the relative fraction of translated transcripts and the average number of ribosomes per translated transcript, respectively).

We first measured the translational profile of the housekeeping gene β -actin (Figure 6B). In aerobic cells, it is efficiently translated with a majority of the mRNA in polysome fractions 5 and 6. After 1 h of hypoxia, there is a marked reduction in translation, as evidenced by a shift toward the lower polysome fractions, which recovers considerably by 16 h. The drop in translation efficiency at 1 h is due to reductions in the relative fraction of translated mRNA and in the average number of ribosomes per translated transcript (Figure 6B). At later time points, only the average number of ribosomes per transcript remained low. The kinetic changes in translation efficiency for β -actin are similar to those observed for overall translation efficiency.

Many proteins are induced at the transcriptional level by hypoxia and we suspected that these genes might be preferentially translated during hypoxia. We investigated the translation of the HIF-1 target gene carbonic anhydrase IX (CAIX), which is important for tumor cell growth and survival during hypoxia³⁷. Figure 6C shows an ~500-fold transcriptional induction of CAIX during hypoxia. Polysome analysis indicates that, similar to β -actin, CAIX is initially efficiently translated but severely inhibited after 1 h of hypoxia. A significant restoration of the polysome distribution occurs after 16 h and thus ensures protein mRNA. As for β -actin, the initial inhibition of CAIX translation is due to a drop in the recruitment of the mRNA into polysomes and in the number synthesis at this time where there is also significantly more cellular of ribosomes per transcript. However, during prolonged hypoxia, the mRNA recruitment recovers and lower translation efficiency is attributed only to a small reduction in the average number of ribosomes per transcript.

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Figure 6. Gene-specific regulation of translation during hypoxia. HeLa cells were exposed to 0.0% O₂ for 0–16 h and cell lysates were separated on sucrose gradients. (A) Fractions were collected as indicated, RNA was isolated and reverse transcribed. Thereafter, the total mRNA abundance of (B) β -actin, (C) CAIX, (D) ATF4, (E) CHOP and (F) GADD34 was determined using real-time quantitative PCR. The left panel shows total mRNA levels from unfractionated samples, normalized by 18S rRNA signal. The following three panels use black, gray and white bars to represent the gene abundance in polysome fractions following 0, 1 or 16 h hypoxia, respectively. The last two graphs show components of translation efficiency. This includes the relative fraction of transcripts in polysomes (i.e. corrected for total mRNA abundance) and the average number of ribosomes per mRNA. Graphs show the average from two independent experiments, and the histograms show the results from one representative experiment.

ATF4 is a central transcription factor mediating the UPR following ER stress. Both thapsigargin (which causes ER stress) and 16 h of hypoxia result in eIF2 α phosphorylation and translational induction of ATF4 in a PERK-dependent manner^{13, 17, 38}. Under normal conditions, the translation efficiency of this gene is low, with most of the mRNA found in fractions 1–3 (Figure 6D). In direct contrast to β -actin and CAIX, its translation is substantially increased during acute hypoxia, due to increased recruitment into the polysomes and an increase in the average number of ribosomes per transcript. In agreement with Blais et al³⁸, we also observed a further increase in ATF4 translation efficiency during prolonged hypoxia.

An important transcriptional target of ATF4 is the C/EBP transcription factor CHOP³⁹, which induces cell cycle arrest and apoptosis during ER stress. Figure 6E shows that CHOP is regulated both transcriptionally and translationally by hypoxia. Translation is only moderately inhibited during acute hypoxia, as shown by a drop in the average number of ribosomes per transcript. However, this reduction is much smaller than average overall reduction (Figure 1C) and the reductions observed for both β -actin and CAIX. After 16 h of hypoxia, translation of CHOP is stimulated, as indicated by a recovery in the number of ribosomes per transcript and a marked increase in the fraction of translated mRNA. Recovery from ER stress requires the GADD34 gene, which is induced in a PERK-dependent⁴⁰

and CHOP-dependent⁴¹ manner. GADD34 stimulates the activity of PP1c to dephosphorylate eIF2 α . We found that, like CHOP, GADD34 is regulated both transcriptionally and translationally during hypoxia. Interestingly, its translation efficiency is highest after 4 h of hypoxia, which coincides with the start of recovery from eIF2 α phosphorylation and overall translation inhibition (Figure 6F). In contrast, GADD34 mRNA is unable to completely bypass the translation inhibition after 16 h.

Gene-specific regulation of translation dependence upon eIF2 α

The gene-specific changes in translation noted above likely reflect the underlying eIF2 α and eIF4F-dependent mechanisms of translation control during hypoxia. We thus analyzed gene-specific translation in WT and S51A MEFs to establish the dependence of individual genes on eIF2 α regulation (Figure 7). In contrast to WT MEFs, S51A MEFs show no loss in translation efficiency of β -actin or CAIX during acute hypoxia (Figure 7A and B). The loss in translation efficiency for these genes in the WT cells is similar to that observed in HeLa cells and is due primarily to a reduction in the average number of ribosomes per transcript. For acute hypoxia, the translation efficiency during prolonged hypoxia is similar for these two genes in both cell lines. For ATF4 (Supplementary Figure S2), CHOP (data not shown) and GADD34 (Figure 7C), acute both these genes, S51A MEFs show virtually no reduction in this parameter during the acute phase of hypoxia. However, in contrast to hypoxia causes a stimulation of translation in WT MEFs that is similar to HeLa cells. However, the translational induction is entirely absent in S51A MEFs. The increase in translation efficiency for GADD34 in WT MEFs during acute hypoxia results mainly from an increase in the average number of ribosomes per transcript (Figure 7C). Cells that are defective in eIF2 α phosphorylation show impaired regulation of this parameter. Thus, for all genes examined,

the observed changes in translation efficiency during acute hypoxia are dependent on eIF2 α phosphorylation.

Discussion

Rapid and persistent downregulation of protein synthesis is thought to be a means of energy preservation and to protect against the lethal effects of hypoxia^{10, 42}. Here we show that the inhibition of global mRNA translation during hypoxia exhibits a biphasic response (Figure 8). The initial rapid inhibition (i.e. 15 min–4 h) is primarily dependent on eIF2 α phosphorylation, whereas inhibition during prolonged hypoxia is independent of eIF2 α . Phosphorylation of eIF2 α under conditions of anoxia is extremely rapid, occurring almost as quickly as we can establish hypoxia in our system (15–30 min). We have previously shown that eIF2 α is also phosphorylated under more moderate hypoxic conditions, although to a smaller degree and after longer times¹⁰. We speculate that this rapid anoxic response may be especially important during the acute exposures to hypoxia/anoxia that frequently occur in tumors due to the transient opening and closing of blood vessels. This rapid response may explain the importance of eIF2 α and ATF4 in the tolerance of cells to oxidative stress, which also occurs during hypoxia/ reoxygenation cycles²⁰. This hypothesis is supported by a recent study by Bi et al, who showed that activation of the PERK–eIF2 α pathway during hypoxia contributes to overall tumor growth¹³. Human tumor cells expressing a dominant-negative PERK allele as well as MEFs lacking PERK or expressing the S51A eIF2 α produce smaller tumors with increased cell death in hypoxic areas than their WT counterparts¹³. Thus, although activation of eIF2 α phosphorylation in response to hypoxia is transient, this response appears critical for long-term cell survival within hypoxic regions of tumors.

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Figure 7. Dependence on eIF2 α -P for gene-specific translational regulation. WT and S51A MEFs were exposed to 0.0% O₂ for 0–16 h and cell lysates were separated on sucrose gradients. RNA was isolated from polysome fractions and reverse transcribed. Thereafter, the abundance of (A) β -actin, (B) CAIX and (C) GADD34 was determined using real-time quantitative PCR. The first three panels use black, solid gray, hatched grey and white bars to represent the gene abundance in polysome fractions following 0, 1, 4 or 16 h hypoxia, respectively. The following graph depicts one component of translation efficiency, that is, the average number of ribosomes per mRNA.

Inhibition of translation during prolonged anoxia is associated with disruption of the mRNA cap-binding complex eIF4F and sequestration of eIF4E by both dephosphorylated 4E-BP1 and 4E-T. To our knowledge, this report is the first to show a physiological stress-induced regulation of the localization of eIF4E and its transporter 4E-T. Accumulation of eIF4E in the cell nucleus or P-bodies renders it unavailable for cytoplasmic translation. Relocalization of eIF4E may have additional roles apart from reducing cap-dependent protein translation, including effects on mRNA processing, transport and degradation. Moderate hypoxia (1%) has also been shown to affect the eIF4F complex through inhibition of mTOR³⁰. However, the kinetics and relative contribution of the eIF4F pathway on inhibition of global and gene-specific translation during more moderate hypoxia remain to be determined (see below).

Analysis of mRNA distribution within polysomes at different times also revealed interesting mechanistic differences during acute and prolonged hypoxia. Acute hypoxia caused a substantial drop in the average number of ribosomes per translated transcript. This presumably results from a reduction in the rate at which ribosomal subunits can be loaded onto mRNA, as each subunit requires a new nonphosphorylated eIF2 α molecule. Inhibition of translation during prolonged hypoxia via eIF4F did not show this effect. Instead, translation was suppressed mainly by reducing the fraction of mRNA found within the polysomes. These results are consistent with a model in which the eIF4F cap-binding complex remains bound to the mRNA allowing sequential rounds of initiation by

available eIF2 α complexes. When translation is inhibited via eIF4F, many transcripts will lack this cap binding complex and hence will not be competent to initiate translation. However, those transcripts that do contain eIF4F will be translated normally (Figure 8). A similar phenomenon has been observed for a subpopulation of mRNAs that contains 5' terminal oligopyrimidine tracts (5'TOPs), which alternate between translationally repressed and active states in response to various stimuli (reviewed in⁴³). It is thus conceivable that the overall reduction in mRNA translation observed during prolonged hypoxia affects a subset of genes, such as those with 5'TOPs, to a greater degree than others.

Figure 8 Model of the effects of hypoxia on overall mRNA translation. Acute hypoxia causes transient eIF2 α phosphorylation due to PERK activation as a part of the UPR. This results in inhibition of the rate of translation initiation. Following prolonged hypoxic conditions, activation of 4E-BP and 4E-T causes disruption of eIF4F, which inhibits the recruitment of mRNA to polysomes. Both molecular mechanisms affect specific mRNAs to varying degrees, resulting in differential gene expression.

An important issue that arises from our study is the nature of the oxygen-sensing pathways upstream of eIF2 α and eIF4F. Substantial evidence suggests that the oxygen sensors are largely independent of the HIF oxygen-sensing pathway^{10, 44}. In the case of eIF2 α , its phosphorylation occurs in an HIF-independent manner. Instead, it requires PERK activation¹⁰ and is associated with activation of the UPR in response to ER stress^{13, 45}. The upstream signaling that leads to eIF4F disruption is less clear, with perhaps both HIF-dependent and independent components. Hypoxia has been shown to prevent insulin stimulation of mTOR and

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phosphorylation of its substrate 4E-BP1 during conditions of moderate hypoxia and serum starvation³⁰. Similarly, Brugarolas et al showed that induction of REDD1 during hypoxia resulted in activation of the mTOR inhibitory complex TSC1/TSC2⁴⁶. As we also observe a decrease in the phosphorylation of 4E-BP1 after prolonged hypoxia, the eIF4F-dependent changes in translation reported here may also be due in part to inhibition of mTOR via REDD1 and TSC1/2. However, it is unlikely that this accounts entirely for eIF4F disruption and translation inhibition during hypoxia. REDD1 is an HIF-dependent gene and both mTOR inhibition and translation inhibition during hypoxia occur in HIF1 α -knockout cells^{10, 30}. Furthermore, our data indicate that eIF4F disruption occurs before substantial binding of eIF4E to 4E-BP1. Here we have identified redistribution of eIF4E into the cell nucleus via 4E-T as an additional mechanism for eIF4F disruption during hypoxia. Further work will be needed to establish to what degree inhibition of translation is due to suppression of mTOR/4E-BP1 phosphorylation and 4E-T activation, as well as to the requirements of HIF in both of these pathways.

The fact that both eIF2 α and eIF4F independently affect translation during hypoxia has important implications for the regulation of gene expression. mRNAs preferentially translated during acute hypoxia must be less dependent on eIF2 α availability, whereas mRNAs that are actively translated during prolonged hypoxia must be less dependent on eIF4F. The translation of ATF4, which contains two upstream open reading frames (uORFs) in its 5'UTR, is perhaps the best example of a mammalian gene that displays this type of preferential translation¹⁷. When eIF2 α availability is high, translation begins at the 5' most uORF and re-initiation occurs efficiently at the subsequent uORF, preventing translation from the correct start codon of ATF4. When eIF2 α is phosphorylated, there is a higher probability of skipping the second uORF and re-initiating at the bona fide start codon⁴⁷. Here, we found that in addition to ATF4, the downstream genes CHOP and GADD34 are also translationally induced during acute hypoxia. The S51A MEFs, which are unable to phosphorylate

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eIF2 α , are defective in this translational regulation. This result is consistent with a report showing that ER stress-induced expression of GADD34 protein can be prevented by keeping eIF2 α dephosphorylated⁴⁰. The bypass of translation inhibition may thus facilitate the ability of GADD34 to dephosphorylate eIF2 α and promote recovery from ER stress.

Our results predict that during prolonged hypoxia, gene transcripts with lower dependency on eIF4F should be preferentially translated. The translation of ATF4 and CHOP was in fact stimulated in HeLa cells after 16 h of hypoxia when the eIF4F complex was disrupted. Preferential translation under conditions of limiting cap-binding complex activity can occur through a higher than average affinity for eIF4F⁴⁸. Another group of mRNAs that can be translated independently of the eIF4F complex are those that contain an internal ribosomal entry site (IRES) in their 5'UTR^{22, 49}. The presence of an IRES enables translation initiation under conditions where eIF4F-dependent translation is inhibited. Importantly, both mouse HIF-1 α and VEGF have been shown to contain functional IRESs within their 5'UTR, although their biological importance is not yet firmly established^{50, 51}. This provides a mechanism to ensure their translation during prolonged hypoxia where eIF4F is disrupted. The mechanism responsible for the selective translation of ATF4 and CHOP during prolonged hypoxia remains to be identified.

In conclusion, we have shown that mRNA translation is inhibited through multiple independent pathways with differing activation kinetics during hypoxia. These distinct modes of translational control influence the translation of individual genes to varying degrees and consequently can influence hypoxia-regulated protein expression in complex ways. An important finding is that inhibition of translation via eIF2 α is transient, leading to dynamic changes in the translation efficiency of genes over the first 8 h of hypoxia. Our selected analysis of gene translation during hypoxia suggests that many genes may be differentially regulated by hypoxia. A complete survey of the genome for differentially translated genes during various exposures to hypoxia and their dependence of eIF2 α

is possible and will undoubtedly identify novel and important hypoxia-regulated proteins¹¹.

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CHAPTER 3

Translational control of gene expression during hypoxia

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Abstract

Poor oxygenation is a unique and prevalent feature of solid tumors associated with poor patient prognosis. In part, this is caused by a series of adaptive cellular responses that together have a large impact on gene expression and cell phenotype. HIF plays a key role in this response by activating a transcriptional program that stimulates genes involved in angiogenesis, cell metabolism, cell survival and cell invasion. Recently, hypoxia has also been shown to suppress protein synthesis through the regulation of the initiation step of mRNA translation. This appears to be a common feature of the cell in response to hypoxia and is mediated by two distinct pathways. The first occurs rapidly, is transient, and is associated with activation of the unfolded protein response (UPR) that occurs in response to endoplasmic reticulum (ER) stress. Translation inhibition during this initial phase is due to phosphorylation of eukaryotic initiation factor 2 α (eIF2 α) in a PERK dependent manner. Although this effect is transient, overall levels of translation remain low during hypoxia due to inhibition of a second eukaryotic initiation complex, eIF4F. This second mechanism is multi-factorial, but due at least in part to inhibition of the mTOR kinase. Although each of these pathways leads to a general inhibition in translation, the consequence at the individual gene level is highly variable. This is due to sequences in the 5' and 3' untranslated regions (UTRs) of mRNA that confer their ability to maintain, or even increase, translation efficiency in spite of the overall inhibition. Consequently, regulation of mRNA translation appears to be an important mediator of gene expression during hypoxia.

Introduction

It is well documented that the majority of solid tumors contain regions of poor oxygenation¹. The oxygen concentration in these areas varies from complete anoxia to ~4% O₂, while the range in normal tissues is ~3-9% O₂. There are several mechanisms that contribute to the formation of tumor hypoxia. As tumor cells proliferate, the oxygen demand on individual blood vessels increases until a point where the oxygen diffusion from the vessel becomes a limiting factor. This is referred to as 'chronic' hypoxia and can result in complete anoxia at distances of ~150-200 μm from the vessel wall. In addition, tumor cells also experience transient periods of low oxygen, often called 'acute' hypoxia due to transient changes in blood flow. This leads to areas of cycling oxygenation characterized by moderate to severe hypoxia dependent on the relative reduction in blood flow². Therefore, large proportions of tumor cells are subject to hypoxic exposure and must be capable of adapting to this stress.

Clinical studies with oxygen electrodes and hypoxia markers have demonstrated not only that tumors are poorly oxygenated, but also that the level of hypoxia correlates with poor prognosis. This is true for patients treated by radiotherapy and chemotherapy, as might be expected due to the protection hypoxia offers against these treatments, and importantly also for patients treated by surgery alone³⁻⁵. High levels of hypoxia in primary tumors is also associated with an increased prevalence of metastasis^{5, 6}. Together these data support the idea that hypoxia contributes to a more malignant tumor phenotype⁷.

Understanding the various cellular responses to hypoxia that are responsible for its negative impact on tumor biology is important in order to improve and/or develop effective anticancer therapies. Cellular responses to hypoxia can affect overall tumor growth and behavior in two distinct ways. First, they can contribute to 'hypoxic tolerance', the survival of the cell itself to the hypoxic stress. In minimally transformed cells, hypoxia leads to rapid cell death through the induction of p53 and

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apoptosis. Consequently, hypoxia provides an environment for the selective outgrowth of clones with reduced apoptotic potential.⁸ Second, assuming that the cells can survive, various hypoxia induced cellular responses alter gene expression and thus change the behavior of the cell.

The best described response to hypoxia is mediated by the hypoxia inducible factor (HIF) family of transcription factors (reviewed in ref.⁹). These transcription factors are activated primarily via posttranscriptional mechanisms during hypoxia and drive the expression of a large number of genes whose products are involved in angiogenesis, erythropoiesis, metabolic transition to anaerobic glycolysis, and cell motility and invasion. Consequently, HIF both promotes hypoxic cell tolerance through its ability to stimulate glycolysis and maintain ATP levels under conditions where oxidative phosphorylation is inhibited, and alters cell function in ways that can promote malignancy (e.g. stimulation of metastasis and angiogenesis).

Regulation of mRNA translation

In recent years, the regulation of mRNA translation has emerged as a second important mediator of the cellular response to hypoxia. Hypoxia causes a rapid and sustained inhibition in protein synthesis^{10, 11}. This is primarily due to a HIF-independent oxygen sensing pathway that directly inhibits the initiation step of mRNA translation^{12, 13}. In a manner analogous to HIF, the inhibition of translation during hypoxia is hypothesized to be important through its ability to both promote cell survival and to alter gene expression and cell phenotype. Protein synthesis is a highly energy demanding process, and thus its inhibition during hypoxia promotes energy homeostasis and may sustain survival during this period of inefficient ATP production. Indeed, cells that are compromised in their ability to inhibit translation during hypoxia show increased apoptosis and reduced clonogenic survival to this stress¹⁴. However, suppression of mRNA translation can also induce important changes in protein expression. A general inhibition in protein synthesis

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may alter the relative levels of expressed proteins (proteome) due to differences in protein half-life. Furthermore, proteome changes are expected due to the fact that not all mRNA transcripts are affected to the same degree by the pathways that regulate translation during hypoxia. In some cases, the inhibition of overall mRNA translation can paradoxically induce the expression of certain proteins that are normally translationally repressed. Finally, because translation efficiency can be rapidly modulated, this mechanism allows for much faster protein changes than are possible through changes in transcription. In this review we focus our discussion on recent data describing both the mechanisms that control mRNA translation during hypoxia, and how they impact both overall and specific gene expression.

Translation initiation

Translation of eukaryotic mRNA transcripts generally begins with the assembly of the eukaryotic initiation factor 4F (eIF4F) at the m⁷GpppN cap structure of the mRNA¹⁵. eIF4F consists of three subunits; the cap binding protein eIF4E, a scaffolding protein eIF4G and an ATP-dependent helicase eIF4A. Binding of eIF4F to a transcript facilitates the recruitment of the 43S pre-initiation complex, which is necessary to initiate translation. This complex is formed by the small 40S ribosomal subunit, the initiation factor eIF3 and the ternary complex which consists of eIF2-GTP and the methionine-loaded initiator tRNA (Met-tRNA). The 43S pre-initiation complex scans through the 5'UTR of the mRNA until it recognizes the AUG initiation codon. There it recruits the 60S ribosomal subunit to assemble the complete 80S ribosome and starts the elongation phase of translation. Both the binding of the eIF4F complex to the mRNA as well as the formation and recruitment of the ternary complex form important regulatory control points during the translation initiation process in response to many forms of cells stress¹⁶. Furthermore, both of these control points are affected during hypoxic exposure.

Translational control during hypoxia via eIF2

Formation of the ternary complex requires exchange of eIF2-GDP for GTP and is carried out by the eIF2B exchange factor. This reaction is strongly inhibited by phosphorylation of the eIF2 α subunit at serine 51, and because eIF2B exists in smaller amounts than eIF2 α (about 20-30%), small increases in eIF2 α phosphorylation can dramatically suppress translation initiation. We and others have shown that both severe (<0.05%) and more moderate (~1%) hypoxia cause phosphorylation of eIF2 α ^{12, 13}. In the case of severe hypoxia, this phosphorylation is extremely rapid, occurring within 30 min of exposure. This effect is fully reversible upon return to oxygenation, and thus not due to depletion of ATP or other nutrients in the media. Under severe hypoxia, eIF2 α phosphorylation levels are highest after short exposures and show a partial recovery after 4-8 hours, consistent with the activation of a negative feedback loop (see below). In contrast, phosphorylation of eIF2 α during moderate hypoxia (0.2% and 1% oxygen) requires significantly longer hypoxic exposures (>6 hours). Phosphorylation of eIF2 α during hypoxia requires activation of the ER resident kinase PERK, which is known to be involved in the unfolded protein response (UPR).

Figure 1. Polysome analysis of cells grown under aerobic conditions (solid line) or after exposure to 1 hour of hypoxia (dashed line). mRNA and its associated proteins was separated through sedimentation in sucrose gradients. The absorbance as a function of depth is indicated. The first 2 peaks represent free 40S, 60S subunits and the 3rd peak the 80S ribosome. Subsequent peaks represent mRNAs that have 2, or more ribosomes attached and are actively engaged in protein synthesis.

Translational control of gene expression during hypoxia

We have examined the translational responses of several cell lines as a function of time during severe (<0.05%) hypoxia. This has been carried out primarily by analyzing the levels of ribosome association with mRNA (polysome profiles). Figure 1 shows a typical polysome profile, in which it is possible to estimate both the percentage of ribosomes involved in translation (overall translation) as well as the average number of ribosomes per transcript (ribosome density). We have shown that hypoxia causes a rapid, eIF2 α dependent loss in polysomal mRNA¹³. Overall translation was estimated to drop from over 60% under normal conditions to ~20% within 1 hour of hypoxia. Furthermore, the polysomes that remained were shifted to lower molecular weight reflecting a significant drop in ribosome density. This is consistent with a drop in ternary complex availability and a slowing in the recruitment of ribosome complexes to the mRNA. Similar to the phosphorylation of eIF2 α , there appeared to be a partial recovery in overall translation after 4-8 hours, and a very large recovery in ribosome density. Cells derived from knock-in mice containing a non-phosphorylatable allele of eIF2 α (S51A) were unable to effectively inhibit overall translation during short hypoxic exposures, and showed no changes in ribosome density at any time during hypoxia. However, these cells displayed similar levels of translational suppression as the wild type cells after 16 hours of hypoxia. Together these data indicate that severe hypoxia elicits a biphasic inhibition of translation. The first phase is due to transient eIF2 α phosphorylation, which blocks ternary complex formation and slows the recruitment of ribosomes onto mRNA transcripts. The second phase occurs more slowly in an eIF2 α independent manner and is capable of reducing overall translation without reducing ribosome density. This second phase of translation inhibition correlates with disruption of the cap binding complex eIF4F (see figure 2).

Translational control during hypoxia via eIF4F

The availability of the cap-binding protein eIF4E is rate-limiting under normal conditions. The necessity for strict regulation of this protein is

underlined by the fact that eIF4E is often over expressed in tumors and that it plays a clear role during tumorigenesis^{17, 18}. This regulation is achieved by a family of eIF4E binding proteins (4E-BPs), which sequester eIF4E in their hypophosphorylated form.¹⁹ Phosphorylation of the 4E-BPs is largely controlled by the mammalian target of rapamycin (mTOR) which integrates signals from several upstream signaling pathways. Hyperphosphorylation of the 4E-BPs lowers the affinity for eIF4E, resulting in an increased interaction between eIF4E and eIF4G and stimulation of translation.

Figure 2. Control of translation initiation during hypoxia is mediated through two distinct mechanisms; (i) eIF4F disruption and (ii) inactivation of the ternary complex. The first is through loss of interaction between eIF4E and eIF4G as a result of increased binding of eIF4E with 4E-BP1 or 4E-T. This affects the fraction of mRNA that is involved in translation not the ribosome density on that mRNA. The second mechanism is through PERK dependent phosphorylation of eIF2α. Here the ribosome loading onto the mRNA is impaired due to inactive ternary complex, whereas the fraction of mRNA involved in translation remains the same.

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A number of publications have suggested that hypoxia can disturb formation of the eIF4F complex through a reduction in mTOR activity and a corresponding decrease in the phosphorylation of 4E-BP1. Moderate hypoxia (1.5%) in combination with serum deprivation effectively inhibited the activation of mTOR and subsequent hypophosphorylation of its substrates 4E-BP1 and S6K²⁰. However, it is less clear to what extent this effect mediates the eIF2 α independent changes in overall translation described above. We found that hypophosphorylation of 4E-BP1 and increased association with eIF4E occurred only after prolonged hypoxia (16 h), whereas decreased association between eIF4E and eIF4G (indicative of eIF4F disruption) was already reduced after 4 hrs of hypoxia. Similarly, the inhibition of mTOR during moderate hypoxia (0.5-1%) did not cause any immediate changes in overall translation, despite measurable differences in 4E-BP phosphorylation²¹. In this study, decreases in protein synthesis under moderate hypoxic conditions did not occur for more than 24 hours. It also appears that growth factor signaling can modulate the ability of mTOR to inhibit translation, since serum deprived cells demonstrate ~30% inhibition in protein synthesis after 16 hours of moderate hypoxia. Similarly, Kaper reported that severe hypoxia is also less effective at inhibiting protein synthesis in cells that express mutations in upstream regulators of mTOR²². Also consistent with these data, Connolly et al. have shown that inhibition of protein synthesis during hypoxia might strongly depend upon cell transformation.²³ They showed that hypoxia caused a 4E-BP1 dependent decrease in protein synthesis in untransformed cells, but no decrease in cancer cells. We found that the disruption of eIF4F at 4 hours may also be influenced by the redistribution of eIF4E and the eIF4E translocator (4E-T) into either the nucleus or to recently discovered cytoplasmic bodies of mRNA processing (P-bodies)^{24, 25}. Translocation of eIF4E into either of these cellular locations makes it unavailable to participate in translation initiation. These data suggest hypoxia induced

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eIF4F disruption may be influenced at least in part by an mTOR/4E-BP1 independent mechanism.

Although changes in mTOR activity may not dramatically influence levels of overall translation during moderate or severe hypoxia, it may still influence the translation of specific gene transcripts (see below). Changes in the regulation of mTOR occur frequently in cancer, and thus its regulation by hypoxia may have important implications for the hypoxic phenotype. The regulation of mTOR activity during hypoxia occurs through at least two independent mechanisms. Although mTOR activity is strongly influenced by growth factor signaling through the PI3K/AKT pathway, this pathway does not seem to be responsible for mTOR inhibition during hypoxia^{20, 26, 27}. However, a downstream effector of the PI3K/AKT pathway, the tuberous sclerosis complex (TSC), was shown to be essential. This complex functions to inhibit mTOR, and cells lacking TSC1 or TSC2 are unable to downregulate mTOR signaling during hypoxia²⁷. Brugarolas et al. showed convincingly that activation of TSC2 and inhibition of mTOR during hypoxia occurred due to the induction of a protein called REDD1²⁷. Transcription of REDD1 is induced in a HIF-1 dependent manner suggesting a role for oxygen sensing in the activation of this pathway.²⁸

The second pathway that contributes to inhibition of mTOR during hypoxia also sits upstream of TSC2. Under energy deprived conditions AMPK is activated by LKB1 which results in phosphorylation of TSC2 and subsequent inhibition of mTOR^{29, 30}. This pathway is thus considered as an energy sensor that can slow protein synthesis under conditions of low ATP. A recent report by Liu et al. showed that AMPK becomes progressively activated during 20 hours of exposure to moderate hypoxia in serum replete conditions²¹. Interestingly, under serum deprived conditions AMPK activation and mTOR inhibition occurred more rapidly (within 30 min), although no corresponding effect on overall translation was observed at this time. Unlike REDD1, the activation of AMPK under these conditions

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can explain why inhibition of mTOR occurs independently of HIF activation^{20, 21}.

Translational mechanisms for selective gene expression during hypoxia

Upstream open reading frames and ATF4

Although hypoxia causes a global inhibition in translation there are clearly many proteins that affect cell survival and cell behavior that are induced under these conditions. How can such proteins bypass the general inhibition in protein synthesis? The answer to this question lies largely within the 5' and 3' untranslated regions (UTR) of their transcripts. Translation efficiency is influenced by both positive and negative regulatory elements within the UTRs, the importance of which is demonstrated by their conservation throughout evolution³¹. Translation is suppressed by the presence of long 5'UTRs with stem loops and other secondary structures that impede scanning of the 43S preinitiation complex for the start codon. Translation can also be repressed by the presence of upstream AUG (uAUG) codons and their associated upstream open reading frames (uORFs), and by RNA binding proteins present in either the 5' or 3' UTRs³²⁻³⁴. Translation initiation occurs primarily at the first AUG encountered by the pre-initiation complex, and a uORF can thereby effectively block initiation from the correct ORF. However, several examples have demonstrated that uORFs can also confer selective translation of a specific transcript under certain circumstances. The best mammalian example of this is ATF4 which codes for a transcription factor activated during the UPR following phosphorylation of eIF2 α ^{35, 36}. ATF4 contains two conserved uORFs within its 5'UTR that together ensure that ATF4 protein is made only under conditions where eIF2 α is phosphorylated. Following translation of the first uORF, the ribosome, which normally would dissociate from the mRNA, stays intact and is able to re-initiate at either the second uORF or the ATF-4 ORF. Before this can

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occur the ribosome needs to acquire a new ternary complex containing eIF2-GTP and Met-tRNA. Under normal circumstances where ternary complex availability is high, re-initiation begins at the second uORF. Re-initiation following translation of the second uORF does not occur and thus expression of ATF4 is prevented. However, under conditions where eIF2 α is phosphorylated, ternary complex availability is low, and there is a higher chance that the ribosome will not have acquired a ternary complex by the time it reaches the second uORF. A scanning ribosome that picks up a ternary complex after passing the second uAUG can then initiate at the ATF4 ORF.

Blais and colleagues were the first to demonstrate that hypoxia can stimulate the translation of ATF4 in a PERK dependent manner³⁷. Furthermore, they showed that the 5'UTR of ATF4 could confer selective translation of a reporter plasmid. ATF4 has recently been demonstrated to play a critical role in protecting cells against hypoxia and facilitating tumor growth¹⁴. This is likely due to its ability to activate downstream genes that are important mediators of reactive oxygen stress³⁸. By analyzing the distribution of mRNA within polysomes, we also found that ATF4 translation was rapidly stimulated during hypoxia. Furthermore, two of its downstream targets, CHOP and GADD34 which also contain uORFs are also selectively translated in a manner that is dependent on eIF2 α phosphorylation^{13, 14}.

At the moment it is unclear how many other genes might be subject to selective translation through a similar process during hypoxia. It has been estimated that 10-30% of genes contain one or more uORFs, and 15-53% of mRNAs contain upstream start codons^{39, 40}. Furthermore, the ability of uORFs to control translation can be regulated by mechanisms other than phosphorylation of eIF2 α . Translation of the Her2 oncogene is also repressed under normal conditions due to an uORF⁴¹. Mehta et al. recently showed that translation of Her2 is derepressed by so called 'translational deregulation elements' (TDE) in its 3'UTR⁴². These TDEs facilitate binding of HuR and HnRNPC1/C2, proteins that are also

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required for efficient Her2 translation. This example highlights the fact that uAUGs and uORFs may affect translation through many different mechanisms, some of which are yet to be described.

Internal ribosome entry sites

Other elements in the UTRs can act as positive regulators of translation, including internal ribosome entry sites (IRESs). An IRES consists of an mRNA secondary structure that facilitates direct ribosome binding independent of formation of eIF4F at the cap. This mechanism was originally discovered in viruses as a means to promote translation of viral mRNAs in infected cells that shut-down host protein synthesis through blockade of eIF4F⁴³. The list of mammalian mRNAs reported to contain IRES activity is growing, and includes a number of hypoxia regulated genes including HIF-1 α (see below), VEGF and BIP⁴⁴⁻⁴⁸. Thus, IRES dependent translation might contribute to selective gene expression during hypoxia when eIF4F integrity is disrupted. The ability of an IRES to support translation may also be regulated by various initiation factors and other associated proteins⁴⁹. Consequently IRES function of specific mRNAs can be induced under specific circumstances⁴⁹. An interesting example is the IRES-dependent translation of cat-1, which can be stimulated through phosphorylation of eIF2 α ⁵⁰.

5' Terminal oligopyrimidine tract (TOP) sequences

As described earlier mTOR signaling results in phosphorylation of its two well known substrates S6K and 4E-BP1. S6K phosphorylation promotes translation of a subset of mRNAs that contain terminal oligopyrimidine tract (5'TOP) sequences in their 5'UTR. This was originally thought to be due to its ability to phosphorylate the ribosomal protein S6, although this recently was shown not to be the case⁵¹. However, 5'TOP sequences are found in many components of the translational machinery, including all ribosomal proteins, and their translation is greatly stimulated by mTOR⁵².

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Interestingly the 5'UTR of HIF-1 α has also been reported to contain these 5'TOP sequences.

Translational control of HIF-1 α

Oncogene and growth factor mediated translation of HIF-1 α

HIF-1 α is the oxygen sensitive subunit of HIF that together with the stable HIF-1 β subunit regulates the expression of more than 60 putative genes⁹. Its selective degradation under aerobic conditions occurs due to modification of two proline residues in its oxygen dependent degradation (ODD) domain, by one of a family of prolyl hydroxylase enzymes (PHD1-3). The hydroxylated form of HIF-1 α is recognized by VHL, which targets it for ubiquitination and degradation. HIF-1 α levels are under tight control, evidenced by the fact that the PHD enzymes are themselves targets of HIF, leading to the establishment of a negative feedback loop. The importance of HIF expression in cancer is highlighted by the fact that defects in the proteins that control its degradation, for example through VHL loss or PHD deregulation, can lead to cancer⁵³⁻⁵⁵. Although degradation is the primary means of HIF-1 α regulation, like many genes regulated at the protein level, its expression also depends on its rate of synthesis. HIF-1 α expression is increased in response to growth factor signaling through the PI3K/AKT pathway^{56, 57}. Increased expression of HIF-1 α has been demonstrated for Ras- transformed cells, PTEN defective glioblastoma cells and after over expression of heregulin, the HER2/neu ligand^{56, 58-60}. Several lines of evidence suggest that these increases in HIF-1 α occur through increased translation (see figure 3). Perhaps the strongest evidence comes from the observation that both HIF-1 α and its downstream target VEGF, are overexpressed in hamartomas derived from Peutz-Jeghers syndrome and tuberous sclerosis complex that arise from mutations in LKB1 and TSC1/2 respectively²⁷. Mutations in either of these genes lead to deregulation of mTOR and increased rates of translation. Increased HIF-1 α levels are also seen in cells derived from TSC2 knockout

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mice, and this effect is completely blocked by the mTOR inhibitor rapamycin²⁷. The increased translation of HIF-1 α therefore provides a possible link between mTOR induced cell growth and the need for increased metabolism and angiogenesis as suggested previously⁶¹. It is not yet clear how HIF-1 α synthesis is selectively stimulated in response to mTOR activation, although it is unlikely that this is due to its reported IRES. An IRES allows translation to occur in a cap-independent manner, whereas mTOR signaling stimulates cap-dependent translation by phosphorylating the eIF4E inhibitor 4E-BP1. A recent study by Thomas et al. suggested that the reduction in HIF-1 α translation upon mTOR inhibition with rapamycin is strongly dependent on its 5'UTR sequence⁶². Cells engineered to express a HIF-1 α construct lacking the 5'UTR were resistant to rapamycin induced growth inhibition. This preferential translation was hypothesized to occur due to its 5' TOP sequences.

Figure 3. Current understanding of HIF-1 α mRNA translation. Under aerobic conditions HIF-1 α translation can be stimulated through mTOR dependent growth factor signaling. This appears to be mediated by 5'TOP motives within the 5'UTR of HIF1. During hypoxic exposure mTOR signaling and therefore cap-dependent translation is inhibited. It is thought that hypoxia can promote IRES-dependent translation of HIF1 which eliminates the need for eIF4F.

Translation of HIF-1 α during hypoxia

The fact that HIF-1 α translation is sensitive to mTOR inhibition suggests that hypoxia, which inhibits mTOR, may paradoxically also inhibit HIF-1 α synthesis. In contrast to this expectation HIF-1 α mRNA is preferentially

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translated during hypoxia and thus able to 'escape' the effects of hypoxia on mTOR as well as the overall reduction in mRNA translation^{45, 63}. Lang et al. originally showed that this effect was due IRES activity in the 5'UTR of murine HIF-1 α ⁴⁵. However, there remains a great deal of controversy over the authenticity of the HIF-1 α IRES as well as several other mammalian mRNAs reported to have IRES activity^{64, 65}. Furthermore, the group that originally identified the murine HIF-1 α IRES recently reported that its apparent activity is largely caused by cryptic promoter activity that is stimulated by the SV40 enhancer element in their reporter plasmid⁶⁵. Using RNA transfection experiments the authors showed that the HIF-1 α 5'UTR does support a small amount of IRES translation but this activity is nowhere near to that reported previously. However, it has also been suggested that efficient IRES activity in HIF-1 α may require association of other cellular proteins with specific sequences in the HIF-1 α 5'UTR. The polypyrimidine tract binding (PTB) protein was shown to stimulate translation of HIF-1 α by binding to its 5' TOP sequences and promoting IRES activity⁶⁶. Clearly, several questions remain regarding the mechanism of selective HIF-1 α mRNA translation.

The selected examples above demonstrate that our understanding of the effect of translational regulation during hypoxia on gene expression is still very limited. The ability of translation to regulate individual gene expression on a genome wide scale has been investigated in only a few circumstances^{43, 67-70}. In these studies changes in the levels of efficiently translated (polysomal) mRNA were evaluated using microarray technology and compared to transcriptional (total mRNA) changes. Although limited, these studies have shown that subtle changes in overall translation can influence gene expression in important ways. For example, it was shown that AKT activation causes large changes in the levels of genes found in polysomes at time points that precede changes in mRNA transcription and without significant changes in overall translation⁷⁰.

Several similar studies have begun to assess the contribution of translational regulation during hypoxia on a genome wide scale. Using

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microarray analysis Blais et al. identified several genes that displayed an increased translation efficiency during hypoxia, including ATF4 as described above³⁷. This study was performed using polysome mRNA from HeLa cells isolated after 16 hours of exposure to severe hypoxia and thus translation inhibition at this time point is expected to be dominated by inhibition of eIF4F. We performed a similar study after only 4 hours of hypoxia in which we expected to observe changes mediated by both eIF2 α and eIF4F. We were able to show that of the top 50 genes with the largest induction during hypoxia, 20% displayed a selective ability to be translated⁷¹. This included several genes dependent on HIF-1 or involved in the UPR. A genome-wide analysis of translation in Arabidopsis during hypoxia also revealed distinct clusters of genes that show a preferential ability to maintain translation⁷². These genes were involved in abiotic and biotic stress responses and were characterized by having a low GC content in their 5'UTRs. Analysis of polysomal RNA in various cell lines under different levels and durations of hypoxia will also play an important role elucidating 5' and 3' UTR translational control elements that mediate preferential translation of various genes. Studies in genetic models which lack the ability to control translation through either eIF2 α or eIF4F will also help to clarify the relative importance of these two pathways on gene expression during hypoxia.

Concluding remarks

Translational control through both UPR dependent and independent mechanisms has emerged as an important regulatory process in the response to hypoxia that affects both hypoxic cell tolerance and the hypoxic phenotype. Changes in mRNA translation affect the key hypoxia regulated transcription factors ATF4 and HIF, and thus indirectly influence the expression of many other genes. Although the pathways that direct translational control during hypoxia have become partly elucidated, several questions remain for future investigation. One challenge will be to

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elucidate the exact mechanisms through which genes like HIF-1 α are preferentially translated during hypoxia. Understanding of this process might be important in the design of more effective anti-tumor treatments. Interestingly, targeting HIF-1 α translation is already an active area of research. In a screen for compounds able to block HIF activity, Rapisarda et al. identified captothecin, a topo1 inhibitor⁷³. They subsequently showed that its ability to inhibit HIF-1 α occurred through suppression of its translation. Similarly, the anti-cancer agents taxol and 2ME2, both which disrupt microtubules, have also been suggested to block HIF-1 α through a translational mechanism⁷⁴. It is also important to keep in mind that inhibition of growth factor signaling, and thus PI3K/AKT-mTOR signaling, can also suppress HIF activation by inhibiting its translation. This effect was recently demonstrated with tyrosine kinase inhibitors to EGFR⁷⁵.

A second important area that has not been adequately addressed is the degree to which control of mRNA translation during hypoxia may be altered in cancer. Control of mRNA translation via disruption of eIF4F is indeed expected to vary considerably amongst different tumors since the upstream pathways that control the assembly of this complex are frequently disrupted in cancer. eIF4F has emerged as an important target of PI3K signaling during tumor development. It will thus be important to assess the differences in regulation of translation and gene expression during hypoxia in cells with specific defects in these upstream signaling pathways. Furthermore, although eIF4F assembly under hypoxia is disrupted, the importance of this effect on tumor growth or hypoxia tolerance has yet to be addressed.

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CHAPTER 4

The hypoxic proteome is influenced by gene-specific changes in mRNA translation

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Koritzinsky M, Seigneuric R, Magagnin MG, van den Beucken T, Lambin P, Wouters BG. The hypoxic proteome is influenced by gene-specific changes in mRNA translation.

Abstract

Background and purpose: Hypoxia causes a rapid reduction in mRNA translation efficiency. This inhibition does not affect all mRNA species to the same extent and can therefore contribute significantly to hypoxia-induced differential protein expression. Our aim in this study was to characterize changes in gene expression during acute hypoxia and evaluate the contribution of regulation via mRNA translation on these changes. For each gene, the contribution of changes in mRNA abundance versus mRNA translation was determined.

Materials and Methods: DU145 prostate carcinoma cells were exposed to 4 hours of hypoxia (<0.02% O₂). Efficiently translated mRNAs were isolated by sedimentation through a sucrose gradient. Affymetrix microarray technology was used to evaluate both the transcriptional and translational contribution to gene expression. Results were validated by quantitative PCR.

Results: 120 genes were more than 4-fold upregulated by hypoxia in the efficiently translated fraction of mRNA, in comparison to only 76 genes at the level of transcription. Of the 50 genes demonstrating the largest changes in translation, 11 were found to be more than 2-fold over represented in the translated fraction in comparison to their overall transcriptional level. The gene with the highest translational contribution to its induction was CITED-2, which is a negative regulator of HIF-1 transcriptional activity.

Conclusions: Gene-specific regulation of mRNA translation contributes significantly to differential gene expression during hypoxia.

Introduction

The presence of hypoxic (poorly oxygenated) areas in human solid tumors is well documented¹⁻⁹. Hypoxia causes radiation resistance and limits radiotherapy efficacy^{2-4, 10, 11}. Tumor hypoxia has also been associated with a lower susceptibility to apoptosis^{12, 13}, increased angiogenesis¹⁴⁻¹⁷ as well as higher metastatic and invasive potential^{2, 10, 18-20}. The cellular response to hypoxia which underlies its negative impact on tumor biology is believed to be due in large part to dynamic changes in gene expression.

A vast number of hypoxia-induced changes in gene expression have been described. There has been particular focus on changes mediated through the hypoxia inducible transcription factor HIF-1. HIF-1 promotes transcription of its target genes through binding a conserved hypoxia responsive element (HRE) gene promoter together with its co activator CBP/p300. More than 60 putative downstream target genes have been identified (for a review see²¹), including genes involved in glycolysis (e.g. PGK-1)²², angiogenesis (e.g. VEGF)^{23, 24}, pH regulation (CA9) and survival (e.g. EPO)^{25, 26}. HIF-1 is activated during hypoxia through protein stabilization of its subunit HIF-1 α , which is targeted for ubiquitin mediated degradation by pVHL (von-Hippel Lindau) during normoxia²⁷.

Although the transcriptional response to hypoxia is clearly very important, cells in normal and cancerous tissue often experience short transient exposures to hypoxia which do not allow sufficient time for a productive transcriptional response²⁸⁻³⁰. However, a biological response to acute hypoxia is presumably required to maintain energy homeostasis and provide adaptation to the quickly changing redox microenvironment. A potentially important point for regulating gene expression that is able to respond rapidly to changes in the microenvironment is the process of mRNA translation (protein synthesis). Indeed, it has been shown that overall mRNA translation is severely but reversibly inhibited during hypoxic conditions^{13, 31, 32}, and this inhibition can be detected after only a few minutes of oxygen deprivation. The hypoxia-induced inhibition in mRNA translation is at least in part mediated through PERK-dependent

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phosphorylation of the translation initiation factor eIF2 α ³¹. PERK is known to be activated in response to endoplasmic reticulum (ER) stress suggesting a possible mechanism through which oxygen activates this pathway.

Protein synthesis is the highest cellular consumer of ATP^{33, 34}, and thus its inhibition during hypoxia probably serves to maintain energy homeostasis. However, it is important to realize that the inhibition of global mRNA translation potentially affects different mRNA species (different genes) to highly varying degrees. This variation is based on the fact that mRNAs from different genes have different dependencies and/or affinities for the cellular translation machinery^{35, 36}. Thus while some mRNAs may be completely dependent on the translation factors that are inhibited during hypoxia, others can remain translated through alternative mechanisms and thereby bypass the overall translation block. For example, the transcription factor ATF-4 is translationally induced by ER stress in spite of PERK-dependent eIF2 α phosphorylation³⁵, and ATF-4 in turn causes transcriptional activation of a conserved gene expression program called the unfolded protein response (UPR) (reviewed in³⁷). Hence, the inhibition of overall translation is a potentially powerful cellular tool for rapidly changing gene expression during hypoxia.

In this study, our aim was to evaluate the translational contribution to differential gene expression during acute hypoxia. Utilizing Affymetrix microarray technology we here show that there are significant changes in gene-specific mRNA translation efficiency during hypoxia.

Materials and Methods

Cell culture and hypoxic conditions

Exponentially growing cervical (HeLa) and prostate (DU145) carcinoma cells were seeded on glass dishes in DMEM or McCoy media with 10% foetal calf serum (FCS) (Sigma-Aldrich) and transferred to a hypoxic culture chamber (MACS VA500 microaerophilic workstation, Don Whitley

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Scientific, Shipley, UK). The atmosphere in the chamber consisted of 5% H₂, 5% CO₂, the desired concentration of O₂ and residual N₂. An anoxic atmosphere was ensured by the inclusion of a catalyst in the hypoxic chamber that catalysed the conversion of H₂ with any O₂ to H₂O.

2D gel electrophoresis

Cells were washed twice with ice-cold 10mM Tris/250 mM sucrose buffer pH 7.0 and lysed in a 7M urea/2 M thiourea based lysisbuffer. The samples were applied to 24 cm pH 3-10 NL IPG strips (Amersham Biosciences). Following isoelectric focusing, the strips were placed on the Protean Plus Dodeca Cell (Bio-Rad Laboratories) to perform a second dimension separation using 12% SDS-PAGE gels. The gels were stained with SYPRO Ruby dye (Bio-Rad Laboratories) and imaged on a Molecular Imager FX (Bio-Rad Laboratories).

Polysomal fractionation and analysis

Cells were treated with 0.1 mg/ml cycloheximide (CHX) for 3min at 37°C, washed twice with ice cold PBS/CHX and harvested by scraping in lysis buffer (1% Triton X-100, 0.3M NaCl, 15mM MgCl₂, 15mM Tris (pH 7.4), 0.1mg/ml CHX, 100 units RNase-In (Ambion)) at 4 °C. Nuclei were removed, 200ug/ml heparin was added and residual debris was removed by centrifugation. The lysate was layered on a 10ml continuous sucrose gradient (20-50% sucrose in 15mM MgCl₂, 15mM Tris (pH 7.4), 0.3M NaCl). After 90min of centrifugation at 39.000 rpm in an SW41-Ti rotor at 4°C, the absorbance at 254nm was measured continuously as a function of gradient depth in a BioRad Laboratories UV monitor. To assess overall translation efficiency, we integrated the area under the curve that represented mRNA attached to 2 or more ribosomes, and compared this to the total area under the curve.

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RNA isolation

Samples were collected in 0.55M final concentration of guanidinium-HCl, subjected to 4 precipitation steps (ethanol, sodium acetate and lithium chloride), one phenol-chloroform extraction and one column extraction (RNeasy Mini Kit, Qiagen) with extensive washing in between. RNA quantity and quality was checked using the ND-1000 spectrometer (NanoDrop Technologies) and RNA Nano LabChip kit on the 2100 Bioanalyzer (Agilent) respectively.

Affymetrix gene array measurements

20ng RNA was pooled from 3 independent experiments, representing total and highly efficiently translated RNA from aerobic and hypoxic cells. The RNA was processed according to the manufacturer's standard protocol (Affymetrix) for 2-cycle amplification. The biotin-labelled samples were hybridized to HG U133 Plus 2.0 chips. GCOS software (Affymetrix) was used for the absolute call (present, marginal, absent) of 54.675 probe sets. Intensities were filtered with a threshold of 200. Two lists of genes were generated by sorted according to (Polysomal Hypoxia)/(Polysomal Normoxia) intensity ratios or (Total Hypoxia)/(Total Normoxia) intensity ratios respectively.

Quantitative PCR analysis

The RNA was reverse transcribed using random decamers (Ambion) and MML reverse transcriptase (Sigma-Aldrich). Real-time PCR was performed in ABI 7500 (Applied Biosystems). The abundance of the following genes were detected with SYBR® Green I (Applied Biosystems) using the indicated forward and reverse primers: β -Actin: F-accatggatgatgatcgcc and R-gccttgacatgccgg; ATF-4: F-tggccaagcacttcaaacct and R-gttgttgaggaggactgaccaa; DDIT3: F-ggagcatcagtcctccactt and R-tgtgggattgagggtcacatc; VEGF: F-gactccggcggaagcat and R-tccgggctcggtgattta. The following genes were measured using TaqMan® Gene Expression Assays (Applied Biosystems): 18SrRNA: Hs99999901_s1

and EGLN3: Hs00222966_m1. The abundance of every gene was calculated using standard curves, and the relative gene abundances were normalized by 18S signal.

Results

We have reported that hypoxia results in a rapid inhibition of mRNA translation. In order to investigate the potential contribution of this inhibition on protein expression we analyzed the cellular proteome after short (1 hr) and long (24 hrs) exposures to hypoxia. The proteome from aerobic or hypoxic HeLa cells was separated in two dimensions on the basis of charge and mass and proteins visualized by staining with SYPRO-Ruby. The early time point (1 hr) was chosen to minimize the contribution of changes in transcription on protein expression. Thus, most of the changes in protein expression are due to post-transcriptional changes including the inhibition of mRNA translation which is maximal at this time. We compared the results at 1 hr with the proteomic changes in chronically (24 hours) hypoxic cells, where differential gene expression was assumed to be affected by both transcriptional and post-transcriptional changes. This allowed us to assess the relative importance of post-transcriptional regulation during hypoxia. This experiment was repeated in triplicate thus producing 3 separate 2D-gels for each condition (representative gels from each experimental condition are shown in figure 1A). Proteins were matched across all gels to identify those that consistently showed changes during each of the experiments. The levels of these matched proteins during hypoxia and normoxia are plotted in figure 1B. Table 1 also lists the number of protein spots that showed strong consistent expression under one condition, but were not present in the other. These spots are therefore likely to represent real induced or repressed proteins, and are not a result of technical artifacts. The substantial change in the hypoxic proteome visible within 1 hour suggests that post-transcriptional events are important regulators of protein expression under these conditions.

Figure 1. Hypoxia-induced changes in the proteome. HeLa cells were exposed to hypoxia for 0, 1 or 24 hours. Whole cell extracts were separated by 2D gel electrophoresis. (A): The resolved proteins were stained with SYPRO Ruby dye and imaged on the Molecular Imager FX (Bio-Rad). (B): The gels were analysed using PDQuest software (Bio-Rad). The relative abundance of each matched protein during anoxia vs. hypoxia was plotted with the diagonal lines representing 2-fold changes.

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Table 1. Hypoxia-induced changes in the proteome.

Hours of hypoxia	Detected proteins	Matched proteins	>2-fold change	Present only in normoxia	Present only in hypoxia
0	1440				
1	982	685	70	30	10
24	1102	882	77	20	25

Regulation of mRNA translation efficiency, which is known to occur during hypoxia, is a possible mechanism that can influence the rapid changes in protein expression shown above. To assess overall mRNA translation efficiency in DU145 cells during hypoxic conditions, we separated cell lysates on sucrose gradients and then measured RNA levels as a function of gradient depth. The resulting profiles are shown in figure 2A. The two shallowest peaks (readily detected in the aerobic sample) represent the 40S and 60S ribosomal subunits respectively. The subsequent peaks represent mRNAs with increasing numbers of attached ribosomes. Efficiently translated mRNA is associated with many ribosomes (polysomes) simultaneously and therefore sediments deep in the gradient. Figure 2A demonstrates that hypoxia inhibits overall translation in DU145 cells since there is a clear reduction in the high molecular weight polysomal RNA. The percentage of ribosomes participating in translation can be determined by integrating the polysomal area and dividing by the total integrated area. The average results from three independent experiments are presented in figure 2B. This figure shows that the fraction of ribosomes participating in translation dropped from 74% in aerobic cells to 45% after 4 hours of hypoxia. We also calculated the fraction participating in highly efficient translation (figure 2A), which we defined as mRNA with 5 or more ribosomes. This fraction dropped from 48% to 20% after 4hrs of hypoxia.

Figure 2. Hypoxia inhibits mRNA translation. DU145 cells were exposed to hypoxia for 0 or 4 hours. Whole cell extracts were separated by sedimentation through sucrose gradients. (A): Optical density (O.D.) at 254nm as a function of depth in the gradient. The area representing RNA that participates in complexes containing 2 or more ribosomes is marked “polysomes”. RNA was isolated for microarray and PCR analysis from the fraction indicated “highly efficient translation”. (B): Quantification of translation efficiency as the percentage of rRNA participating in complexes of two or more ribosomes. This equals the integration of the area under the “polysomes” part of the curve divided by the total area under the curve. The fraction participating in “highly efficient translation” is also indicated.

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In order to investigate the potential contribution of changes in mRNA translation on hypoxia induced gene expression, we utilized microarray technology. Changes in mRNA translation were assessed by isolating RNA from the highly efficiently translated polysomal fractions as depicted in figure 2A. Transcriptional changes were assessed by isolating total mRNA from the same samples. Since this technique allowed us to evaluate both transcriptional and translational contributions to differential gene expression, we chose to measure these entities after 4 hours of hypoxia. At this time point both transcriptional and translational control of gene expression are expected to be active, and with the microarray technique we could directly compare these events. In addition to providing more information, this also allowed us to validate our results through literature comparison, since many hypoxia-induced transcriptional changes are well described. Equivalent RNA amounts from three independent experiments from cultures growing under normoxia or following 4 hours of hypoxia were pooled and then assessed using Affymetrix microarray chips containing 54,675 probe sets (including 38,500 well-characterized human genes).

Figure 3 shows the scatter plots of gene intensities (log base 2) for hypoxic and aerobic samples. For all genes that demonstrate a less than 2-fold change during hypoxia, the plotted points fall within the two indicated parallel lines on these plots. Figure 3A summarizes the transcriptional (total RNA) changes during hypoxia. Of the 22,332 gene probe sets that could be evaluated, 654 (3%) were induced more than 2-fold during hypoxia with 76 of these increasing more than 4-fold. Changes in the highly translated RNA fraction are summarized in Figure 3B. In this highly translated fraction we found that 1082 genes (~5%) increased more than 2-fold during hypoxia with 120 of these increasing more than 4-fold. The amount of an individual gene in the highly translated mRNA fraction reflects both the total mRNA available for translation, as well as the gene's translation efficiency. However, since both the total mRNA and the highly translated fraction of mRNA are known, it is possible to

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calculate the relative translation efficiency for each gene on the array. This is simply equal to the signal in the highly translated fraction divided by the signal from the total cell. We calculated the translation efficiency for each gene under both aerobic and hypoxic conditions to determine those genes exhibiting the largest increases in translation efficiency. Figure 3C shows that the translation efficiency of 508 genes increased more than 2-fold with 41 of these increasing more than 3-fold and 5 more than 4-fold. These results demonstrate that although overall translation is inhibited during hypoxia, a significant number of genes are induced through specific increases in their translation efficiency.

Table 2 shows a ranked list of the 50 genes showing the largest increase in mRNA translation during hypoxia (derived from figure 3B). Table 2 also lists the increase in total mRNA abundance and hence the component that can be attributed to changes in mRNA production and/or stability. The change in mRNA translation efficiency during hypoxia can be deduced by comparing these values. Of the 50 most highly induced genes, 11 demonstrated a significant contribution from changes in translation efficiency (more than 2-fold increase in translation efficiency). For each of these genes, their rank within the top 50 was much higher than their rank evaluated by the increase in total mRNA levels. As shown in Table 2, a majority of the 50 genes showing the largest changes in translation have previously been shown to be induced by hypoxia either at the mRNA or protein level. This result helps to validate the results presented here. To further validate our microarray data, we performed quantitative real-time PCR (qPCR) on several of the genes shown to be transcriptionally upregulated. The results from these validation experiments are shown in figure 4. As a negative control, we measured the abundance of β -actin, which was not induced by hypoxia on the microarray. As expected, β -actin was also not induced when measured by qPCR. We also measured the mRNA abundance for ATF-4, EGLN3, VEGF and DDIT3. The results from the qPCR were consistent with the data from the microarray. In general, the induction factors were somewhat higher when measured by qPCR.

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This might be related to the fact that the gene abundance using this method is normalized by the abundance of 18S rRNA, which may fall slightly during hypoxia. Nonetheless, the qPCR results verified the data obtained by microarray analysis.

Figure 3. Microarray signal distributions. Total and polysomal RNA isolated from DU145 cells after exposure to hypoxia for 0 or 4 hours was hybridized to Affymetrix microarray chips. (A) and (B) Logarithm of raw (not normalized) intensities of probe sets under hypoxia vs. anoxia for total mRNA. Diagonal lines represent the threshold for 2-fold expression changes. (A) Total, i.e. unfractionated mRNA in aerobic versus hypoxic samples. (B) mRNA with high translation efficiency in aerobic versus hypoxic samples. (C) Representation of the number of genes with hypoxia induced translation efficiency, calculated by dividing the gene abundance from the “highly efficient translation” by that obtained from the unfractionated sample.

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Table2. Genes most highly induced by hypoxia measured by microarray.

Probe set	Gene symbol	Poly Rank	Total Rank	Ratio Poly	Ratio Total	Ratio Poly:Total	Hypoxia Induced	Pathway	References
202934_at	HK2	1	2	30.1	24.6	1.2	yes	H ¹	[26, 69]
221009_s_at	ANGPTL4	2	6	21.3	15.2	1.4	yes	H	[3, 39]
202769_at	CCNG2	3	1	16.7	29.4	0.6	yes	H	[40, 71]
209357_at	CITED2	4	42	16.0	5.4	3.0	yes	H	[5, 60]
217028_at	CXCR4	5	13	15.0	10.2	1.5	yes	H	[61, 69]
235857_at	KCTD11	6	7	14.6	14.3	1.0			
218507_at	HIG2	7	30	13.9	6.7	2.1	yes	H	[14, 69]
204284_at	PPP1R3C	8	10	12.1	12.1	1.0	yes	H	[40, 60]
229830_at	PDGFA	9	20	11.7	8.2	1.4	yes		[19]
203438_at	STC2	10	9	11.5	12.9	0.9	yes	H, U ²	[25, 40]
227337_at	Lrp2bp	11	14	10.8	10.1	1.1			
242517_at	GPR54	12	5	10.2	17.2	0.6			
223333_s_at	ANGPTL4	13	8	10.2	13.2	0.8	yes	H	[3, 39]
202887_s_at	DDIT4	14	12	10.1	11.5	0.9	yes	H	[9, 40]
202364_at	MXI1	15	47	9.6	5.2	1.8	yes	H	[69]
209383_at	DDIT3	16	4	9.3	20.3	0.5	yes	H, U	[12, 42]
228499_at	PFKFB4	17	3	9.2	22.2	0.4	yes	H	[48, 60]
206424_at	CYP26A1	18	32	9.0	6.5	1.4			
209201_x_at	CXCR4	19	21	8.6	8.1	1.1	yes	H	[61, 69]
218274_s_at	FLJ10415	20	75	8.4	4.0	2.1			
212366_at	ZNF292	21	52	8.3	4.7	1.8			
221567_at	NOL3	22	22	7.8	8.1	1.0			
218149_s_at	ZNF395	23	62	7.8	4.3	1.8			
201170_s_at	BHLHB2	24	26	7.5	7.2	1.0	yes	H	[71, 72]
201313_at	ENO2	25	67	7.5	4.1	1.8	yes		[60]
208763_s_at	DSIPI	26	29	7.5	6.7	1.1			
203574_at	NFIL3	27	24	7.5	7.7	1.0	yes		[40]
201466_s_at	JUN	28	36	7.5	6.0	1.2	yes	H, U	[36, 60, 64]
205463_s_at	PDGFA	29	59	7.3	4.4	1.7	yes		[19]
202843_at	DNAJB9	30	16	7.3	9.0	0.8		U	[35]

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Continued Table2. Genes most highly induced by hypoxia measured by microarray.

Probe set	Gene symbol	Poly Rank	Total Rank	Ratio Poly	Ratio Total	Ratio Poly:Total	Hypoxia Induced	Pathway	References
226863_at		31	123	7.3	3.4	2.2			
231798_at	NOG	32	23	7.0	7.8	0.9			
1554452_a_at	HIG2	33	19	6.8	8.3	0.8	yes	H	[14, 69]
203439_s_at	STC2	34	18	6.8	8.6	0.8	yes	H, U	[15, 40]
215446_s_at	LOX	35	114	6.8	3.4	2.0	yes	H	[60, 69]
212368_at	ZNF292	36	37	6.7	5.8	1.2			
202973_x_at	FAM13A1	37	68	6.7	4.1	1.6			
200632_s_at	NDRG1	38	17	6.4	8.9	0.7	yes	H	[24, 60]
241348_at	FLJ10997	39	130	6.3	3.3	1.9			
1554462_a_at	DNAJB9	40	35	6.2	6.1	1.0		U	[35]
210512_s_at	VEGF	41	39	6.1	5.6	1.1	yes	H, U	[24, 26, 43, 68]
204285_s_at	PMAIP1	42	159	6.1	3.0	2.0	yes	H	[31]
209339_at	SIAH2	43	459	6.0	2.2	2.7	yes		[50]
211527_x_at	VEGF	44	270	5.9	2.5	2.3	yes	H, U	[24, 26, 43, 68]
224610_at	RNU22	45	286	5.9	2.5	2.4			
202672_s_at	ATF3	46	11	5.6	12.0	0.5		U	[28]
224797_at	ARRDC3	47	56	5.5	4.6	1.2			
224741_x_at	GAS5	48	277	5.5	2.5	2.2	yes		[29]
219232_s_at	EGLN3	49	433	5.4	2.2	2.4	yes	H	[2, 44]
204286_s_at	PMAIP1	50	174	5.4	2.9	1.9	yes	H	[31]

¹ H=HIF pathway

² U= unfolded protein response (UPR)

Discussion

There is substantial evidence that tumor oxygenation varies dynamically in time at frequencies that would be difficult to compensate for by changes in transcription. Thus, it is not clear to what degree the cellular proteome, and ultimately cell behavior, changes during acute exposures to hypoxia. To address this question we assessed the effect of both acute (1 hour) and chronic (24 hours) hypoxia on the overall pool of expressed proteins. Both 1 hour and 24 hours of hypoxia clearly caused significant changes in the proteome, as demonstrated by a large number of proteins showing a greater than 2-fold change in expression. As listed in Table 1, significantly fewer protein spots were detected during hypoxia compared to normoxia, consistent with hypoxia-induced inhibition of mRNA translation (and transcription at later time points). Of the proteins that were detected in all conditions, approximately 10% changed their expression 2-fold or more during hypoxia. Of these, 59% had increased expression during 1 hour hypoxia, versus 34% after 24 hours. The results shown in figure 1 and table 1 demonstrate that significant changes in the proteome take place very early in the hypoxic exposure, at a time where transcriptional changes are thought to contribute to a negligible extent.

Figure 4. Microarray validation by quantitative PCR. DU145 cells were exposed to hypoxia for 0 or 4 hours. Gene-specific mRNA abundance was measured for β -actin, ATF-4, EGLN3, VEGF and DDIT3 using quantitative real-time PCR. Gene abundance was normalized by 18S rRNA signal and the induction factors were calculated using the abundance measured in three independent experiments. The standard error of the mean is shown.

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These data provide evidence that a non-transcriptional mechanism is important for the changes in protein expression that occur during hypoxia. Although we argue that gene-specific mRNA translation can contribute significantly to the observed changes, other mechanisms may also be important contributors, including both mRNA and protein stabilization.

A strong suppression of mRNA translation was observed after 4 hours of hypoxia. However, the global decrease in translation efficiency assayed in this way represents the average reduction in translational efficiency for all genes. The change in translational efficiency for any particular gene under these conditions may differ significantly from this global average. This variability is thus a potentially important contribution to changes in protein expression during hypoxia. In order to evaluate this contribution we used microarrays to characterize both transcriptional and translational changes of individual genes. After 4 hours of hypoxia we found a substantially higher number of genes that showed significant increases in translation in comparison to the number showing only transcriptional changes. This is due to the fact that increased translation arises from increases in both mRNA abundance and in gene-specific translation efficiency. Importantly, these translated genes accurately reflect *de novo* protein production and thus are likely contributors to biological behavior. The gene found to show the largest increase in translation during hypoxia was the HIF-1 target gene hexokinase 2 (HK-2), which is involved in glucose metabolism³⁸. The protein production of HK-2 increased by 30-fold during 4 hours of hypoxia versus normoxia, while the mRNA abundance in the cells increased by 25-fold. The interpretation of this result is that HK-2 is both transcriptionally and translationally induced, with the transcriptional component being dominant.

A majority of the genes showing the highest increases in translation have previously been shown to be targets of the HIF transcription factors (table 2). The criteria for this annotation were non stringent and genes that contain putative HREs in their promoter, or that are induced by either overexpression of HIF or loss of pVHL have been included together with

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bona fide HIF targets. It is clear from the table that the potential influence of HIF through hypoxia-induced transcription is significant. However, the HIF target genes displayed a large spread in translation efficiency during hypoxia, ranging from 0.4 for PFKFB4 to 3.0 for CITED-2. CITED-2 was highly induced during hypoxia (number 4 on the list) with a 16-fold increase in translation and only a 5-fold increase in total mRNA. This gene product binds p300 and thereby inhibits the HIF-1-p300 interaction required for HIF-1-dependent gene transcription^{39, 40}. Hypoxia has previously been shown to induce both mRNA and protein levels of CITED-2⁴⁰, and the results presented here indicate that gene-specific up regulation of mRNA translation contributes significantly to the increased protein levels. Interestingly, the CITED-2 promoter also contains three HREs⁴⁰, indicating that it is a part of a negative feedback loop controlling expression of HIF-1 target genes. Apparently, CITED-2 mRNA is in addition able to bypass the overall repression of mRNA translation to ensure its robust expression during hypoxia.

Other genes that have a high translational component of induction include hypoxia-inducible protein 2 (HIG-2), MAX interactor 1 (MXI-1), zinc finger protein (ZNF) 292 and 395 and enolase 2 (ENO-2). As exemplified by HK-2 and CITED-2, several of the genes shown in table 2 are induced both at the transcriptional level and through increased mRNA translation efficiency. It is conceptually not surprising that transcriptionally activated mRNA species possess the ability to be preferentially translated. Without this ability, the transcriptional induction would clearly be inefficient and result in uneconomical use of the cells limited energy sources.

The mechanistic basis for preferential translation is not well understood, although elements in the regulatory (i.e. untranslated) parts of the mRNA seem to be important. ATF-4 is an example of a gene that, paradoxically, is more efficiently translated during ER stress when global translation is inhibited through PERK-dependent eIF2 α phosphorylation³⁵. PERK is also activated during hypoxia and necessary for at least a

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component of the hypoxia-induced inhibition in translation³¹. ATF-4 is thought to be translationally induced during eIF2 α phosphorylation because its upstream open reading frames (uORFs) in the 5' untranslated region (5'UTR) of the mRNA prevent proper ribosome initiation under normal conditions³⁵. Phosphorylation of eIF2 α makes initiation less efficient which increases the probability that ribosomes will read through the false uORFs and start translating at the *bona fide* start site. In our microarray, ATF-4 was indeed translationally induced during hypoxia, consistent with results from a previous microarray experiment performed after a longer hypoxia exposure in HeLa cells⁴¹. Here, we obtained an induction of 3.5 in the polysomes versus 2.3 in the totals, resulting in a poly/total ratio of 1.52. It is also noteworthy that 8 of the 50 top induced genes listed in table 2 have been previously implicated in the UPR which requires ATF4 activation.

Gene-specific translation during hypoxia has also been reported for mouse HIF-1 α and human VEGF. These genes have been reported to contain functional internal ribosomal entry sites (IRES) in their 5'UTR⁴²⁻⁴⁵. The presence of an IRES makes it possible for ribosomes to bind the mRNA independently of the 5' cap-structure, and therefore bypasses the requirement for certain translation factors. However, although it has been shown that the IRES-dependent translation is relatively less affected by the overall hypoxia-induced translation inhibition, it is not clear to which extent it contributes to overall protein expression and phenotype. HIF-1 α did not appear as a translationally regulated gene on our list, perhaps indicating differences between mouse and human 5'UTRs in this gene. In contrast, VEGF was hypoxia-induced on our array and showed a poly/total ratio of 1.1 and 2.3 for two different probe sets. Many other genes had a higher contribution from changes in mRNA translation efficiency to their induction than ATF-4 and VEGF, and the molecular mechanisms responsible for this are yet to be identified.

An intriguing group of genes are those that have a large transcriptional induction, but not a corresponding increase in translation. (e.g. cyclin G2,

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GPR54, DDIT3, PFKFB4, ATF3). It is possible that this reflects different induction kinetics and that these genes will be efficiently translated after longer exposures to hypoxia. Our previous results have indicated that at least two distinct molecular pathways contribute to the inhibition of overall mRNA translation during acute and chronic hypoxia^{31, 46}. It is likely that a switch of molecular mechanisms can facilitate different groups of genes to be efficiently translated at different times. Another possibility is that the transcriptionally induced but translationally inhibited mRNAs produce proteins that are important in coping with the stress represented by reoxygenation. It may be important for the cells to have mRNA available to be translated as soon as oxygen is reintroduced and overall translation inhibition is reversed. It will thus be of interest to extend these studies of translation efficiency to cells exposed to hypoxia plus reoxygenation. Furthermore, our current results are limited to a single time point and a single oxygen concentration. It will be of interest to further evaluate the contribution of mRNA translation at a range of oxygen concentrations typical of those seen in tumors (i.e. 0.02%-5% O₂), and at different time points. The substantial change in the proteome observed as early as 1 hour after hypoxia suggests that translation control may also be important in these short exposures that may arise *in vivo* through transient changes in oxygenation.

In conclusion, we identified a large number of genes that demonstrate a selective ability to be translated during hypoxia exposure. Gene-specific regulation of translation therefore plays an important role in contributing to the hypoxic proteome.

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CHAPTER 5

Regulation of mRNA translation is a major contributor to hypoxia regulated gene expression

Submitted for publication.

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Abstract

Hypoxia is a common feature of solid tumors associated with an aggressive phenotype, resistance to therapy and poor prognosis. Many of these adverse effects are mediated by hypoxia induced changes in protein expression. At the transcriptional level this occurs in large part through stabilisation and activation of the HIF family of transcription factors. In addition, hypoxia has recently been shown to regulate the initiation step of mRNA translation through PERK dependent phosphorylation of eIF2 α and through inhibition of the mRNA 5' cap binding complex eIF4F. Although many studies have investigated transcriptional changes during hypoxia, the contribution of regulation at the translational level is less clear. We used gene profiling to characterize global changes in both total and efficiently translated (polysomal) mRNA in DU145 prostate carcinoma cells subjected to hypoxia for different periods of time. Our data indicate that changes in translation efficiency contribute significantly to both hypoxia induced and repressed genes at all time points examined. The contribution is particularly important for the initial response to hypoxia (2-4 hrs) accounting for the majority of identified genes. The influence of transcription becomes more important after prolonged hypoxia, reaching a maximum after 8 hours. Interestingly, the influence of translation rises again after 16 hours particularly for the set of hypoxia repressed genes. The biphasic contribution of translational regulation on differential gene expression during hypoxia is consistent with the early and transient phosphorylation of eIF2 α followed by a more delayed inhibition of eIF4F. Together, our data indicate that translation influences gene expression during hypoxia on a scale comparable to that of transcription.

Introduction

The microenvironment of solid tumors is characterized by poor and heterogeneous oxygenation¹. Clinical and experimental evidence associates deficiencies in oxygenation (hypoxia) with tumor development, growth, metastasis and poor response to therapy²⁻⁴. Activation of multiple cellular adaptive pathways is essential to tolerate hypoxic stress and likely forms the basis for these adverse effects on tumor biology^{5, 6}. Insight into the regulation of these pathways is essential for the development of more effective anticancer therapies.

A large part of the adaptation to hypoxia has been attributed to gene regulation by the HIF family of transcription factors. This is not surprising as HIF triggers a transcriptional program that involves over 60 putative genes coding for proteins involved in angiogenesis, metabolism and metastasis⁷. HIF-1 is a heterodimeric transcription factor that consists of HIF-1 α and HIF-1 β . Both subunits are ubiquitously expressed, but HIF-1 α is rapidly degraded by the 26S proteasome under aerobic conditions in a pVHL (von Hippel-Lindau protein) dependent manner⁸. When oxygen levels become limited HIF-1 α protein is stabilized, binds to HIF-1 β and mediates transcription of target genes like vascular endothelial growth factor (VEGF) and carbonic anhydrase 9 (CA9) by binding to the hypoxia responsive element (HRE) in their promoters⁷.

More recently, two other oxygen-sensitive pathways have been described that mediate important cellular processes during hypoxia⁹⁻¹². The first is through activation of the unfolded protein response (UPR), a program of transcriptional and translational changes occurring as a consequence of endoplasmic reticulum stress. This causes rapid inhibition of mRNA translation through transient phosphorylation of eukaryotic initiation factor 2 α (eIF2 α) at serine 51 by the ER kinase PERK^{9, 12}. Although phosphorylation of eIF2 α is transient, translational repression is maintained by a second pathway that deregulates the 5'cap binding complex eIF4F. This complex consists of the cap-binding protein eIF4E, the scaffolding protein eIF4G and the RNA helicase eIF4A. Exposure to

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hypoxia for periods longer than 4 hours causes disruption of the eIF4F complex through the sequestration of eIF4E by 4E binding proteins (4E-BPs)^{9-11, 13} and relocalization of eIF4E into the nucleus and/ or p-bodies by the 4E-T transporter protein⁹.

Paradoxically, the overall down regulation of protein synthesis occurs in concert with the initiation of a transcriptional program that is required for adaptation to hypoxia. This raises the question as whether hypoxia induced proteins demonstrate a selective ability to be synthesized under these conditions. It is known that individual mRNA transcripts are translated into protein with widely differing efficiencies and have different reliance on individual initiation factors. Furthermore, we and others have previously shown that specific mRNA transcripts remain associated with polysomes during hypoxia, indicative of de novo protein synthesis^{9, 14}. This provides tumor cells with a potential mechanism to respond quickly to changes in their microenvironment. We hypothesize that this might be especially important under circumstances where tumor cells experience short, transient periods of hypoxia due to perfusion changes in the tumor vessels^{15, 16}.

Preferential translation is mediated by particular RNA elements within the 5' and 3' untranslated regions (UTRs) and include internal ribosome entry sites (IRESs) and upstream open reading frames (uORFs)¹⁷. An IRES is a secondary RNA structure that allows direct binding of the ribosomes to the mRNA transcript¹⁸. This mode of translation appears to be important during hypoxia as it has been reported for HIF-1 α , VEGF and BiP¹⁹⁻²¹. Probably the best example for regulation of gene expression by uORFs is ATF-4. This transcription factor is translationally induced by ER stress in spite of PERK-dependent eIF2 α phosphorylation. Translation of ATF-4 is prevented under normal conditions by the presence of uORFs in its 5'UTR. When eIF2 α becomes phosphorylated initiation is less efficient, allowing ribosomes to read through the false uORFs and engage initiation at the proper start codon²². Changes in translation of specific genes under hypoxic conditions can also be regulated by a small class of non-coding

Microarray analysis of translated mRNA during hypoxia

RNAs, called microRNAs (miRNAs)²³⁻²⁵. Although most currently known miRNAs repress gene specific mRNA translation, there is one report suggesting that they may also stimulate translation of specific transcripts as well²⁶.

Several recent studies have investigated the effect of translational control during hypoxia on a genome-wide scale^{14, 27-29}. By microarray analysis Blais et al. identified several genes, including ATF4, that displayed increased translation efficiency following exposure to 16 hours of hypoxia²⁸. Similar experiments have also been conducted after 4 hours of exposure to severe hypoxia^{14, 29}, and 20 hours of more moderate hypoxia (1.0% O₂)²⁷. Although these studies focused on a single time point after hypoxic exposure, they clearly demonstrate that preferential translation does contribute to hypoxia induced gene expression. Nevertheless, it remains unclear to what extent changes in translation efficiency contribute to global changes (up or down) in gene expression. This contribution is expected to vary significantly during hypoxic exposure, given the fact that the pathways mediating translational control operate with different kinetics and influence translation through different mechanisms⁹. To address this issue we assessed the hypoxic translome over a period of 2-24 hours of hypoxic exposure and compared it with changes in transcription over a similar period. Our results indicate that translational control is a dynamic but significant contributor to changes in gene expression on a level comparable to that of transcription.

Materials and methods

Tissue culture and hypoxic conditions

Exponentially growing prostate carcinoma cells (DU145) were seeded on glass dishes in DMEM media with 10% fetal calf serum (FCS) (Sigma-Aldrich) and transferred to a hypoxic culture chamber (MACS VA500 microaerophilic workstation, Don Whitley Scientific, Shipley, UK). The atmosphere in the chamber consisted of 5% H₂, 5% CO₂ and residual N₂.

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An anoxic atmosphere was ensured by the inclusion of a catalyst in the hypoxic chamber that catalyzed the conversion of H_2 with any O_2 to H_2O .

Polysomal fractionation and analysis

Cells were treated with 0.1 mg/ml cycloheximide (CHX) for 3 min at 37 °C, washed twice with ice cold PBS/CHX and harvested by scraping in lysis buffer (1% Triton X-100, 0.3 M NaCl, 15 mM $MgCl_2$, 15 mM Tris (pH 7.4), 0.1 mg/ml CHX, 100 units RNase-In (Ambion)) at 4 °C. Nuclei were removed, 200 µg/ml heparin was added and residual debris was removed by centrifugation. The lysate was layered on a 10 ml continuous sucrose gradient (20–50% sucrose in 15 mM $MgCl_2$, 15 mM Tris (pH 7.4), 0.3 M NaCl). After 90 min of centrifugation at 39,000 rpm in an SW41-Ti rotor at 4 °C, the absorbance at 254 nm was measured continuously as a function of gradient depth in a BioRad Laboratories UV monitor. To assess overall translation efficiency, we integrated the area under the curve that represented mRNA attached to more than one or more than five ribosomes, and compared this to the total area under the curve. Samples were collected in 0.55 M final concentration of guanidinium-HCl, subjected to four precipitation steps (ethanol, sodium acetate and lithium chloride), one phenol-chloroform extraction and one column extraction (RNeasy Mini Kit, Qiagen) with extensive washing in between. RNA quantity and quality was checked using the ND-1000 spectrometer (NanoDrop Technologies) and RNA Nano LabChip kit on the 2100 Bioanalyzer (Agilent), respectively.

Western blotting

Cells were washed twice with cold PBS and scraped in RIPA lysis buffer. After centrifugation at 16,000 rpm supernatant was boiled in Laemmli buffer for 10 min and proteins were resolved by SDS-PAGE. After electrophoresis proteins were transferred onto nitrocellulose membranes and subsequently blocked overnight at 4°C in PBS containing 0.1% Tween-20 (PBS-T) supplemented with 5% milk powder. Membranes were probed for 1 h with antibodies directed against phosphor (serine 51)-

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eIF2 α (Research Genetics), eIF4E, eIF2 α , 4EBP1 (all Cell Signaling Technologies) or β -actin (Sigma). Bound antibodies were visualized using HRP-linked secondary antibodies (anti-rabbit (Cell Signaling Technologies) and anti-mouse (Sigma)) and ECL luminescence (Pierce).

Affymetrix gene array measurements

Twenty nanograms of RNA was pooled from three independent experiments, representing total or highly efficiently translated RNA from either aerobic or hypoxic cells. The RNA was processed according to the manufacturer's standard protocol (Affymetrix) for 2-cycle amplification. The biotin-labelled samples were hybridized to HG U133 Plus 2.0 chips. GCOS software (Affymetrix) was used for the absolute call (present, marginal, absent) of 54,675 probe sets. To identify the groups of genes regulated by transcription or translation, we filtered for genes demonstrating more than 3-fold change at two consecutive time points. This resulted in four datasets, representing induced and repressed genes in the hypoxic transcriptome (total mRNA) and translome (heavy polysomal mRNA). The change in translation efficiency ($\Delta T.E.$) was calculated as the ratio of polysomal signal to total signal for hypoxic exposure divided by the ratio of polysomal signal to total signal during normoxic exposure.

Results

To evaluate the overall changes in mRNA translation that occur in the human prostate carcinoma cell line DU145 during hypoxia, we assessed the amount and distribution of ribosome associated mRNA (polysomes). To this end mRNA transcripts isolated from cells exposed to hypoxia for different periods of time were fractionated through sucrose gradients. This technique enables separation of transcripts based on the number of associated ribosomes and thus on their translation efficiency. Typical absorption profiles of fractionated RNA as a function of gradient depth are

shown in figure 1A. The two first peaks represent the 40S and 60S ribosomal subunits respectively, whereas the subsequent peaks represent mRNAs with increasing number of attached ribosomes. Consistent with previous results, exposure to hypoxia caused a rapid decrease in polysomal mRNA and a corresponding increase in free ribosomal subunits. Overall translation efficiency can be estimated by dividing the integrated area under the absorption profile representing the translated fraction by the total integrated area⁹. Figure 1B shows the estimated fraction of ribosomes participating in translation as a function of hypoxic exposure from three independent experiments. The fraction of ribosomes involved in mRNA translation changes from ~75% to ~45% within 2 hours of exposure to hypoxia and remains low for at least 24 hours. This repression is even more pronounced (from ~50% to 25%) in the fraction of highly efficiently translated mRNAs (associated with five or more ribosomes).

The observed overall inhibition of mRNA translation during hypoxia is caused by at least two independent mechanisms as described previously^{9, 12}. The first involves activation of an evolutionarily conserved pathway known as the unfolded protein response (UPR). This causes the immediate translational repression through phosphorylation of eIF2 α by the ER-resident kinase PERK¹². We therefore examined the phosphorylation status of eIF2 α as a function of time in the DU145 cell line. Similar to what has been reported in other cell types, hypoxia causes a transient increase in eIF2 α phosphorylation that peaks around 4 hours after exposure to hypoxia (Figure 2A) followed by a gradual loss of phosphorylation^{9, 12}. Loss of eIF2 α phosphorylation during persistent ER stress is known to occur as a result of the activation of a negative feedback loop involving upregulation of GADD34³⁰.

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Figure 1. Overall inhibition of mRNA translation during hypoxia. DU145 cells were exposed to 0% O₂ for 0-24 h and subsequently cell lysates were separated through sucrose gradients. (A) The optical density profiles at 254 nm as function of gradient depth are shown after hypoxic exposure for 0 (bold), 4 (dotted) and 16 (thin) hrs. (B) Translation efficiency in DU145 as function of exposure time. This was calculated by dividing the integrated area under the curve for the total polysomal (solid line) or high translated polysomal area (dashed line) by the total integrated area.

Longer exposure to hypoxic conditions activates a second mechanism that antagonizes the activity of mammalian target of rapamycin (mTOR) kinase on its target 4E-BP1^{9, 11, 13}. This results in hypo-phosphorylation of 4E-BP1, sequestering of eIF4E and disruption of the 5'cap binding complex eIF4F. 4E-BP1 appears as three different phosphorylation forms with distinct migration patterns (α, β, γ) upon Western blotting³¹. Figure 2B shows that under aerobic conditions 4E-BP1 is mainly present in the slow migrating hyperphosphorylated inactive form (γ) and that 4E-BP1 is gradually dephosphorylated over 16 hrs of hypoxia, ending up mostly in

its fast-migrating hypo-phosphorylated active form (α). This form of 4E-BP1 prevents eIF4F assembly by sequestering eIF4E. Protein levels of eIF4E remain unchanged during hypoxia. The data presented in Figure 2 illustrates that both phosphorylation of eIF2 α as well as eIF4F disruption by 4E-BP1 contribute to translational repression during hypoxia in DU145 cells.

Figure 2. Hypoxia inhibits translation through two molecular pathways. DU145 cells were exposed to 0, 1, 4, 8 and 16 hrs of 0% O₂ and subjected to Western blot analysis. (A) Phosphorylated eIF2 α with total eIF2 α as a loading control. Treatment with 1 mM DTT for 1 h was used as a positive control for eIF2 α phosphorylation. (B) 4E-BP1 with eIF4E and actin as loading controls. The SDS page resolves 3 4E-BP1 species (γ , β , α) representing hyper-, intermediate- and hypo-phosphorylated 4E-BP1 respectively.

Next we performed a series of gene profiling experiments to determine how regulation of mRNA translation affects hypoxia induced gene expression at the individual gene level. This was done by analyzing mRNA isolated from whole cells (transcriptional changes) or from the heavy polysomal fraction, defined as containing 5 or more ribosomes (referred to as 'translational' changes). Translational changes are influenced both by changes in transcription and by changes in translation efficiency (i.e. the rate at which a specific mRNA is converted into protein) and are closely related to de novo protein production. Transcriptional and translational

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Figure 3. Gene profiling of efficiently translated and total mRNA. Efficiently translated mRNA and total mRNA were isolated from DU145 cells as a function of hypoxic exposure. Equal amounts of mRNA were pooled from 3 independent experiments and hybridized to Affymetrix microarrays. Logarithm of raw intensities for each probe set under hypoxia versus normoxia is shown for total mRNA and efficiently translated mRNA. The number of genes showing more than 3-fold change in expression at two consecutive time points is indicated in each plot. The diagonal upper and lower lines represent threshold for 3-fold change in expression.

changes were assessed in DU145 cells after exposure to hypoxia for 2, 4, 8, 12, 16 and 24 hours. In figure 3A the total and polysomal mRNA levels are shown separately as dotplots for all hypoxic time points versus their respective aerobic control. The data from each array are normalized by the total array intensity such that the total signal for all arrays is equal. Consequently the translation plots do not reflect the average overall inhibition in mRNA translation that occurs during hypoxia as quantified in figure 1B. Instead, these dotplots identify the probes that show significant deviations (up regulated and down regulated) from the overall average inhibition. Visual examination of the dotplots in figure 3A suggests that, as expected, changes in transcription between hypoxic and aerobic cells increase as a function of time. This is evident by the fact that total mRNA levels are most similar between hypoxic and aerobic cells at 2 hrs and then diverge for longer exposures. In contrast, by comparing dotplots of poly and total mRNA at 2 hrs, it appears that changes in translation occur earlier than changes in transcription.

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In order to identify a robust set of hypoxia regulated genes at both the transcriptional and translational levels we filtered the data shown in figure 3 for genes demonstrating more than 3-fold changes in expression at two consecutive time points. The number of genes that fulfill these criteria are indicated in figure 3A in each plot. At the transcriptional level, only 53 induced genes are identified after 2 hrs, and this rises steadily with exposure time affecting 350 genes after 16 hrs. The small drop at 24 hrs to 238 genes likely arises from the conditions of our filter since it lacks an additional later consecutive time point to compare with. A consistently smaller number of transcriptionally repressed genes are identified with these filter criteria, where we identified only 21 genes at 2 hrs and as many as 239 at 12 hours. Interestingly, a much larger number of differentially expressed genes are identified at the translational level, particularly for the repressed set of genes. Within 2 hrs we identified 95 up regulated and 318 down regulated genes respectively. After longer exposures the number of genes induced at the translational level was more similar to that for transcription. However, the translationally repressed genes showed a greater dependence on exposure time. The number of genes dropped from 318 to only 124 at 8 hrs and then increased again dramatically reaching as many as 689 at 24 hrs. Consequently, translational control appears to exert a very strong influence on repressed genes during both acute (2 hrs) and chronic (16-24 hrs) hypoxic exposure.

Although it is clear from figure 3 that hypoxia regulates different numbers of genes at the transcriptional and translational levels, this figure does not directly address the concordance between the identified gene sets. Differences found between the hypoxia regulated changes in transcription and translation are mediated by potential changes in translation efficiency. In order to provide better insight on this aspect of gene regulation during hypoxia, we analyzed the contributions of transcription and translation efficiency in more detail.

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Figure 4. Analysis of hypoxia induced genes. (A) The number of genes showing more than 3-fold increase in expression at two consecutive time points at the total mRNA level (black), the polysomal level (grey) or both (white) were determined and plotted as a function of hypoxic exposure. (B) The changes in T.E. (log2 values) were determined as the ratio of polysomal signal to total signal for each gene at the hypoxic time point divided by the same ratio for that gene under aerobic conditions. Threshold levels for no change in TE (solid line) and 2-fold changes (broken lines) are indicated. (C) The percentage of genes induced in polysomal expression showing preferential translation as a function of hypoxic exposure $\Delta T.E. > 2$ ($\blacktriangle$), $\Delta T.E. > 3$ ($\bullet$), $\Delta T.E. > 4$ ($\blacklozenge$).

The results of this analysis for induced and repressed genes are shown in figures 4 and 5 respectively.

Figure 4A shows the number of genes that were identified only at the transcriptional or translational level as well as those identified in both. Interestingly, the number of genes identified as both transcriptionally and translationally induced is relatively small, with only 27 genes meeting these criteria at 2 hrs. This group of co-regulated genes increases over time to a maximum number of 153 genes after 16 hrs of hypoxia. The percentage of translationally induced genes exhibiting transcriptional co-regulation increases from 28% at 2 hrs to 74% at 24hrs of exposure to hypoxia. This is perhaps not surprising as increases in translation arise through both increased transcription and increased translation efficiency of the mRNA.

Despite this overlap, it appears from figure 4A that a transcriptional change is frequently not accompanied by a corresponding change at the level of translation and vice versa. This suggests that changes in the translation efficiency of individual genes is contributing significantly to the hypoxia regulated genes found from polysomal mRNA analysis. One potential concern with this analysis is that the lack in overlap between transcriptional and translational identified genes might be caused by the arbitrary threshold values we set to identify the hypoxia regulated genes. To assess this issue more directly we calculated the change in translation efficiency ($\Delta T.E.$) for each gene in the set of translationally regulated genes. The translational efficiency is evaluated by comparing the level of each gene in the polysome to that in the whole cell. As calculated (see methods) $\Delta T.E.$ represents the fold change in this number relative to all other genes on the array (i.e. it does not reflect the overall average drop in translation depicted in figure 1). Figure 4B shows the calculated $\Delta T.E.$ values, in ascending order, for all translationally regulated genes. At all time points, the majority of genes demonstrate a higher $\Delta T.E.$ than the average gene on the array (average is 0 in this dataset) indicating that they are preferentially translated. Both the percentage of genes and the

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degree of increase in Δ T.E. are highest at early time points (2-4 hrs). In figure 4C, we have plotted the percentage of genes that demonstrate a 2-, 3- or 4-fold increase in translational efficiency. Nearly 80 percent of the induced genes in the polysome double their T.E. after a 2 hr exposure to hypoxia compared to aerobic conditions and 50% have an increase of 4-fold or more. The contribution of changes in translation efficiency varies with hypoxic exposure time and is maximal at early times when the UPR is activated. The percentage of genes showing this preferential regulation drops to a minimum after 8 hours (less than 20% showing a 2-fold increase), and then increases again with a kinetic pattern that is consistent with the disruption of eIF4F. At these later time points it is clear that most of the increases in translation are driven by transcriptional changes.

The same type of analysis was performed on the identified sets of down regulated genes. Figure 5A shows the number of genes repressed at the level of transcription, translation or both. It is evident from these data that the changes in translation play an even larger role for repression than for induction. After 2 hours of hypoxia, the expression of over 318 genes is more than 3-fold repressed in the polysomal fraction, this in sharp contrast to the 21 genes repressed at the transcriptional level. Furthermore there is a very limited number of genes identified as transcriptionally and translationally regulated during early hypoxic time points (2-4 hrs). Although a slight recovery in translational repression is observed after 8 hours (124 genes affected), this increases again at late time points affecting a maximum number of 601 genes after 24 hrs of hypoxia. Transcriptional repression is not observed for the majority of translationally repressed genes, with the exception of the 8 hr time point where 71% of translationally repressed genes are also transcriptionally repressed.

Since transcription did not appear to account for the majority of translationally repressed genes, we also calculated Δ T.E. values for all

Figure 5. Analysis of hypoxia repressed genes. (A) The number of genes showing more than 3-fold decrease in expression at two consecutive time points at the total mRNA level (black), the polysomal level (grey) or both (white) were determined and plotted as a function of hypoxic exposure. (B) The changes in T.E. (log2 values) were determined as the ratio of polysomal signal to total signal for each gene at the hypoxic time point divided by the same ratio for that gene under aerobic conditions. Threshold levels for no change in TE (solid line) and 2-fold changes (broken lines) are indicated. (C) The percentage of genes reduced in polysomal expression showing preferential translation as a function of hypoxic exposure $\Delta T.E. < 0.5$ ($\blacktriangle$), $\Delta T.E. < 0.33$ ($\bullet$), $\Delta T.E. < 0.25$ ($\blacklozenge$).

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translationally repressed genes (shown in ascending order in figure 5B). Most of the genes exhibit a lower $\Delta T.E.$ than the average gene on the array indicating selective repression. The percentage of genes demonstrating a 2-, 3-, or 4-fold decrease in translation efficiency is shown in figure 5C. After 2 hours of hypoxia, nearly 100% of the translationally repressed genes demonstrate a $\Delta T.E.$ below 0.5 and 70% even below 0.25. Similar to the translationally enhanced genes, preferential regulation of the repressed genes reaches a minimum after 8 hours of hypoxic exposure. These kinetics correlate well with transient phosphorylation of eIF2 α at early time points and the disruption of eIF4F at prolonged hypoxia (figure 2). Interestingly, the number of genes that demonstrate large reductions in translation efficiency rises sharply at 16 and 24 hrs consistent with the reduction in cap-dependent translation at these times.

To determine whether translational control influences important biological processes mediated during hypoxia we performed gene ontology (GO) analysis. Enrichment of particular GO terms was determined for both the translationally induced genes (>2-fold induced and $\Delta T.E.>2$) and translationally repressed genes (>2-fold repressed and $\Delta T.E.<0.5$) at early (2-8 hrs) and late (12-24 hrs) hypoxic time points using the software program EASE³². Genes with an EASE score lower than 0.1 were considered to be significantly enriched. Biological themes including transcription, signal transduction and protein transport are selectively affected by translational control during acute hypoxia (table 1). Prolonged exposure to hypoxia mainly influences metabolic processes, although transcription and signal transduction are affected too, albeit to a lesser degree compared to acute hypoxia (table 2). Known hypoxia regulated processes like glycolysis and cell death are also over repressed in the translationally induced genes (data not shown). However these genes do not show an increased change in translation efficiency and are thus not predominantly regulated by translational control. The translationally repressed genes that show significantly reduced mRNA translation rates

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include the themes transport, steroid metabolism, cell growth and protein metabolism during acute hypoxia (table 3) and protein metabolism, RNA metabolism and mitotic cell cycle during prolonged hypoxia (table 4). Many of the down regulated genes, especially after longer exposures encode proteins that are involved in mRNA translation, including ribosomal proteins L19, S12, S18B and L4 as well as translation initiation factors such as eIF5 and eIF2B.

Table1. Identification of over represented processes in subset of translationally induced genes with $\Delta T.E. > 2.0$ during acute hypoxia.

Gene Category	EASE score	Gene symbols
<i>Nucleobase, nucleoside, nucleotide and nucleic acid metabolism</i>	1.27E-02	BHLHB2, MXI1, PBX2, CTNNBIP1, AK3, ZBTB1, NFAT5, CITED2, TEAD3, ZNF292, JMJD2B, MFHAS1, HIPK2, C6orf111, SPTBN1, TLE3, PBF
Regulation of DNA-dependent transcription	3.94E-03	BHLHB2, MXI1, PBX2, CTNNBIP1, ZBTB1, NFAT5, CITED2, TEAD3, ZNF292, JMJD2B, MFHAS1, HIPK2, TLE3, PBF
<i>Signal transduction</i>	4.45E-02	RRBP1, KIF13B, CTNNBIP1, IGF1R, STC1, NFAT5, MFHAS1, SNX27, FLJ11220, MKNK2, FLJ22570, TLE3, ASB3
<i>Protein transport</i>	4.49E-02	RRBP1, TPR, MXI1, KIF13B, SNX27
Intracellular protein transport	3.76E-02	RRBP1, TPR, MXI1, KIF13B, SNX27
Protein targeting	1.32E-02	RRBP1, TPR, MXI1, KIF13B
<i>Phosphorylation</i>	9.98E-02	IGF1R, STC1, HIPK2, MKNK2
Protein amino acid phosphorylation	7.73E-02	IGF1R, STC1, TAO1, HIPK2, MKNK2, STC1

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Table2. Identification of over represented processes in subset of translationally induced genes with $\Delta T.E. > 2.0$ during prolonged hypoxia.

Gene Category	EASE score	Gene symbols
Metabolism	3.08E-02	RRBP1, RYBP, EGFR, MXI1, WASPIP, KIF13B, IGF1R, ENO3, STC1, ZBTB1, GAD1, DUSP6, TEAD3, PRB1, RUNX1, ZNF292, JMJD2B, TTBK2, SFPQ, NTRK3, LZTS1, FBXL3A, STAT2, PYGB, PBF, KIAA1238, LOC51057, PURG, PTP9Q22, C6orf130
Protein metabolism	5.13E-02	RRBP, EGFR, MXI1, WASPIP, KIF13B, IGF1R, STC1, GAD1, DUSP6, TTBK2, NTRK3, FBXL3A, KIAA1238, PTP9Q22
Protein modification	6.35E-03	EGFR, IGF1R, STC1, GAD1, DUSP6, TTBK2, NTRK3, FBXL3A, KIAA1238, PTP9Q22
Energy derivation by oxidation of organic compounds	7.10E-02	ENO3, GAD1, PYGB
Phosphate metabolism	3.13E-02	EGFR, IGF1R, STC1, DUSP6, TTBK2, NTRK3, PTP9Q22
Protein amino acid phosphorylation	9.62E-02	EGFR, IGF1R, STC1, TTBK2, NTRK3
Glutamine family amino acid catabolism	4.45E-02	GAD1, LOC51057
Signal transduction		
Transmembrane receptor protein tyrosine kinase signaling pathway	5.69E-02	EGFR, IGF1R, NTRK3
Transcription		
DNA dependent regulation of transcription	9.95E-02	RYBP, MXI1, ZBTB1, TEAD3, RUNX1, ZNF292, JMJD2B, LZTS1, STAT2, PBF, PURG

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Table3. Identification of over represented processes in subset of translationally repressed genes with $\Delta T.E. < 0.5$ during acute hypoxia.

Gene Category	EASE score	Gene symbols
Transport		
Vesicle-mediated transport	5.26E-03	SNX17, LDLR, SEC23A, SNX4, HPCAL1, EXOC7, PICALM, SEC15L1
Protein transport	7.42E-03	M11S1, HSPD1, SNX17, SEC23A, RNP24, SNX4, EXOC7, TIMM22, SEC15L1
Intracellular protein transport	5.31E-03	M11S1, HSPD1, SNX17, SEC23A, SNX4, TIMM22, SEC15L1
Steroid metabolism		
Sterol metabolism	9.04E-03	LDLR, MVK, OPRS1, APOL2
	7.54E-04	LDLR, MVK, HSA9761, OPRS1, APOL2
Cholesterol metabolism	6.17E-03	LDLR, MVK, APOL2
Cell growth and/ or maintenance		
	8.72E-03	M11S1, HSPD1, TM4SF8, SNX17, SLC29A1, LDLR, CDC2, SLC7A6, RFC3, SEC23A, RNP24, CXCL1, SNX4, HPCAL1, IL18, TNFSF7, PSEN1, FSTL1, RFP, SKP2, HSA9761, ORC5L, CDK2, CCNE2, EXOC7, DAZAP2, FTSJ1, PICALM, APOL2, TIMM22, ARPM1, DKFZP434K0427, CTL1, SEC15L1, CLASP1
Protein metabolism		
	2.63E-03	RPL11, DNAJB1, M11S1, HSPD1, SNX17, PPIF, PRKAG1, LDLR, CDC2, SLC7A6, DNAJB4, MVK, STK19, IL18, CSNK1A1, EIF4EL3, BAG2, HSPA8, MGAT2, MAP3K7, CDK2, PICALM, BMP2K, DKFZp761P1010, MRPL42, EIF3S5, EFG2, USP15, BMP2K
Protein folding	1.30E-03	DNAJB1, HSPD1, PPIF, DNAJB4, BAG2, HSPA8

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Table4. Identification of over represented processes in subset of translationally repressed genes with $\Delta T.E. < 0.5$ during prolonged hypoxia.

Gene Category	EASE score	Gene symbols
Protein metabolism	2.56E-04	DNAJB1, M11S1, HSPD1, EIF5A, ARIH2, PSMC3, PPIF, ETF1, EIF2B1, CCT2, LDLR, PLK, UBE2S, CDC20, PPP2R1B, CDC2, CAPN7, MRPL19, MAP4K5, UK114, DNAJB4, NUPL2, PPID, MRPS12, MRPS12, FLJ10613, EIF2S3, IL18, CDC2L1, PTK2, STK6, EIF5, MALT1, HSPA8, MRPS18B, UFD1L, C2F, BAG2, AURKB, BUB1, CDKN3, PARD3, FKBP1A, HSPA8, CDC2, MGAT2, TGM2, HSPCA, DKFZP564M182, ZRF1, HSPCB, ADAM10, PICALM, TOPK, HSP70-4, FLJ10634, UCHL5, KPNA3, MGC2744, SDPR, HSPC150, USP47, MRPL4, MRPL4, GC14421, CTSC, KIAA1970, CCT5, DNAJA2, NEDL2, EFG1, RARSL, FLJ12525, MtfMT
Protein folding	9.70E-08	DNAJB1, HSPD1, PPIF, CCT2, DNAJB4, PPID, HSPA8, BAG2, FKBP1A, HSPA8, HSPCA, ZRF1, HSPCB, FLJ10634, CCT5, DNAJA2
Mitotic cell cycle	9.03E-05	HMGB1, PLK, CDC20, PPP2R1B, CDC2, CCNA2, RFC3, NOLC1, CHC1, CDC2L1, STK6, BUB1, CDKN3, CDC2, ORC5L, MCM4, PRIM2A, TOPK, CCNB1, DNAJA2
Mitosis	1.65E-04	PLK, CDC20, CDC2, CCNA2, NOLC1, CHC1, CDC2L1, STK6, BUB1, CDC2, TOPK, CCNB1
RNA metabolism	7.45E-07	HSPA1A, HNRPF, DKC1, SFPQ, SFRS1, CCT2, PPP2R1B, SFRS3, RNU3IP2, NOLC1, SFRS6, HNRPH3, CDC2L1, C2F, HSA9761, SR140, SFRS5, POP1, HNRPH1, RRP41, MGC2744, THOC4, WDR4, KIAA1970, RARSL, CPSF2
RNA processing	7.87E-07	HNRPF, DKC1, SFPQ, SFRS1, CCT2, PPP2R1B, SFRS3, RNU3IP2, NOLC1, SFRS6, HNRPH3, CDC2L1, C2F, HSA9761, SR140, SFRS5, POP1, HNRPH1, RRP41, MGC2744, THOC4, WDR4, KIAA1970, RARSL, CPSF2
rRNA processing	6.26E-04	DKC1, RNU3IP2, NOLC1, C2F, HSA9761, RRP41

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Discussion

It is becoming increasingly clear that translational control mechanisms play key roles in regulating gene expression that are relevant in cancer. This includes regulation of genes in response to a wide range of cellular stresses such as oncogene activation³³, radiation³⁴ and hypoxia^{14, 27, 28}. These studies have clearly shown that a substantial number of genes can be preferentially translated during stressful conditions, allowing for rapid changes in protein expression. This effect may be particularly important during hypoxia, which has emerged as a strong regulator of the initiation step of mRNA translation by virtue of its ability to regulate both eIF4E and eIF2 α . What remains unclear however, is the extent to which translational mechanisms contribute to differential gene expression compared with that of transcription. To address this point we have studied the effect of hypoxia on gene expression in detail as a function of time and determined the relative contributions of transcriptional and translational responses.

Here we show that mRNA translation is rapidly repressed in DU145 human prostate carcinoma cells during hypoxia. This inhibition is similar to what has been reported for other cell lines^{9, 12}. We furthermore demonstrate that two previously identified mechanisms involved in translational repression during hypoxia, activation of the UPR and inhibition of mTOR are also activated in DU145 cells⁹⁻¹³ albeit with different kinetics. Rapid but transient phosphorylation of eIF2 α is followed by the much slower dephosphorylation of 4E-BP1 causing the disruption of the eIF4F complex.

An important aspect of the large decrease in overall rates of translation is its contribution to maintenance of energy homeostasis since protein synthesis is one of the most energy consuming processes within the cell³⁵. A second aspect is that this allows the cell to 'reprogram' the translation state of available mRNA transcripts. All available initiation factors can selectively assemble onto different mRNA species, ensuring the translation of important transcripts needed for the adaptation to hypoxic stress. Our group has previously shown for a small number of genes that regulation of

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eIF2 α and eIF4F has dramatically different effects on gene specific translation⁹. To obtain a broader overview of this effect we assessed transcriptional and translational changes at different time points during hypoxia on a genome-wide scale. Our results indicate that translational control plays a key role in the up regulation of genes during acute hypoxia, whereas transcriptional control is dominant after prolonged hypoxic exposure. After 2 hours of exposure to hypoxia only 28% of the translational induced genes show signs of transcriptional co-regulation, whereas this increases over time to 74% after 24 hours. Interestingly, translational control appears to be the main mechanism for down regulation of gene expression during hypoxia. Our data demonstrate that more than 90% of the down regulated genes during acute hypoxia are repressed as a consequence of translational control.

Another interesting result of this study is that selective translation (stimulation or repression) is highly dynamic over time. Almost 80% of the translational induced genes demonstrate a 2-fold increased translation efficiency during acute hypoxia. This percentage of genes reaches a minimum after 8 hours and then goes up and stabilizes at 44%. A similar effect is observed for the repressed genes where after 2 hours of hypoxia nearly all of the translationally regulated genes exhibit over 2-fold reduction in translation efficiency. This translational repression is somewhat suppressed after 8 hrs but becomes dominant again after prolonged hypoxic exposure. This kinetic picture of translational control extends earlier results from our lab from a single time point¹⁴ and presumably are reflective of the difference in regulation of eIF2 α and eIF4F at these different time points. Preferentially translated mRNAs during acute hypoxia are less dependent on eIF2 α availability while efficiently translated mRNAs after longer periods of hypoxia are less dependent on eIF4F. We cannot segregate the effects of these pathways in the current study, apart from their known kinetics of induction. As a result we are unable to clearly designate the ability for preferential translation of certain genes to either of these particular pathways. This

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would require similar profiling of transcriptional and translational changes during hypoxia in cell lines defective in either pathway and these experiments are underway.

Transcripts that are less dependent on eIF2 α availability have been shown to harbor uORFs within their 5'UTR. Genes, like ATF-4 are translationally silenced under normal conditions by these RNA elements. Initiation of translation at uORFs occurs efficiently under normal conditions and can prevent translation of the actual ATF-4 ORF. However under conditions of ER stress eIF2 α is phosphorylated and the translation efficiency of these decoy uORFS is impaired. Subsequently, this allows the ribosomes to scan along the mRNA, bypass the decoy uORFs and initiate translation at the proper ATF-4 start codon²². Separate mechanisms confer translational specificity to manuscripts under conditions that affect the translation initiation complex eIF4F. Some mRNAs can utilize secondary structures within the 5'UTR that act as internal ribosome entry sites to facilitate direct recruitment of the ribosomes to the transcript. Such transcripts are thus less dependent on initiation at the 5'cap structure on the mRNA and thus also independent of the 5'cap binding complex eIF4F. Since prolonged exposure to hypoxia induces disruption of eIF4F, this seems an ideal mechanism to maintain translation of important mRNA species. Several reports have shown that translation of HIF-1 α , VEGF and BiP is mediated through this mechanism during hypoxia¹⁹⁻²¹, although this remains controversial for HIF-1 α ^{36, 37}. Changes in gene expression that arise through regulation of translation are also expected to show a strong dependency on oxygen concentration. Several publications have shown that during more moderate hypoxia (0.2-1.5% O₂) phosphorylation of eIF2 α is substantially delayed^{10, 13, 27}. It is very likely that this will affect gene expression differently than during anoxic conditions. Therefore it will be of interest to extend this study to other oxygen concentrations (0.2-5% O₂). This has recently been done using PC-3 human prostate cancer cells after a single exposure to 1.0% O₂ for 20 hours²⁷. In this study, Thomas and Johannes identified 104 induced

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genes and 56 repressed genes showing more than 2-fold change in the polysomal fraction. Of these, only 33 and 31 genes of the induced and repressed genes respectively, demonstrated changes in translation efficiency. This number of translationally regulated genes is significantly smaller than that we observed in this current study and suggests that the severe hypoxia has a much stronger effect on translational control than moderate hypoxia. Nevertheless, to study this effect in more detail it will be important to utilize the same cell line throughout these studies as there might be differences in the dependence of different cell lines on the pathways that regulate translational control during hypoxia.

Translational control also appears to play an important role during tumorigenesis^{33, 38-40}. Mutations in genes that code for proteins involved in signaling to mTOR are frequently observed in cancer. This includes mutations in membrane kinases receptors like HER2/neu⁴¹ or in tumor suppressor genes like TSC1, TSC2^{42, 43} and LKB1⁴⁴. In addition several studies have clearly linked the mTOR regulated gene eIF4E directly to cancer development. Over expression of eIF4E causes transformation of immortalized 3T3 fibroblasts^{38, 45} and human mammary epithelial cells⁴⁶. Knock down of eIF4E reduces cell proliferation and reverts the transformed phenotype⁴⁷⁻⁴⁹. Furthermore, transgenic mice over expressing eIF4E showed a significant increase in tumor development^{50, 51}. The effect of eIF4E on tumorigenesis has been attributed to its ability to decrease apoptosis in at least some tumor models⁵¹. Recently 2 reports performed polysomal gene profiling following overexpression of eIF4E^{39, 52}. Interestingly, Mamane et al. observed enhanced translation of transcripts encoding anti-apoptotic proteins³⁹. Our study extends these results by demonstrating that translational control and its effects on gene expression are also highly dynamic and strongly influenced by hypoxic stress in tumors.

We performed gene ontology analysis on the selectively repressed and induced translated genes during acute and prolonged hypoxia in order to provide more insight into the mechanisms by which translational control

affect hypoxia tolerance. Hypoxia down regulates translation rates of genes involved in protein metabolism, including translation initiation factors, heat shock proteins and ribosomal proteins. These results seem consistent with mTOR inhibition that occurs during hypoxia as mTOR mediates increased mRNA translation of these genes⁵³. In addition, synthesis rates of proteins involved in cell growth and cell cycle are decreased and may contribute to the well known ability of hypoxia to decrease cell proliferation. Our data suggest that hypoxic cells utilize translation control to shut down these pro-proliferative processes. This might lead to a more persistent repression of mRNA translation compared to the rapid and reversible inhibition we observe during the first 24 hours of hypoxia. By decreasing the energy demand on the cell this could serve as an important survival mechanism to tolerate long term hypoxic stress.

The genes that we identified as being preferential translated during hypoxia encode for proteins involved in metabolism, signal transduction and transcription. Although these processes are clearly important in tumor cells, it remains to be seen how important these individual proteins are for hypoxia tolerance. Nevertheless the finding that many of the translationally enhanced genes encode for proteins involved in regulating transcription is interesting. This includes direct effects of translation on mRNA transcripts encoding transcription factors like MXI1 and CITED2 as well as transcriptional co regulators. These factors can antagonize c-myc and HIF transcriptional activity respectively and might therefore play a role during tumor development and the hypoxic response. We previously identified and characterized CITED2 as a preferentially regulated gene during hypoxia^{14, 54}.

In summary, we have shown that translational control contributes significantly to hypoxia regulated gene expression on a level that is comparable to transcriptional regulation. The magnitude of this effect suggests that translational control may be an interesting therapeutic target to pursue for hypoxia directed therapies.

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CHAPTER 6

Regulation of Cited2 expression
provides a functional link between
translational and transcriptional
responses during hypoxia

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Abstract

Background and purpose: Protein synthesis rates are greatly reduced under hypoxic conditions as a consequence of an overall inhibition of mRNA translation. Certain specific mRNA species have the ability to escape this general translational repression. At the cellular level this results in differential protein expression during hypoxic conditions. The objective of this study was to characterize the translational regulation of the postulated HIF-1 α antagonist Cited2.

Materials and methods: DU145 prostate carcinoma cells and mouse embryonic fibroblasts with a homozygous knock-in mutation for eIF2 α (S51A) or wild-type eIF2 α were exposed to severe hypoxia after which both total mRNA and efficiently translated mRNA were isolated. Quantitative RT-PCR was used to measure and compare changes in transcription (total mRNA) with changes in translation (efficiently translated mRNA fraction).

Results: We show using HIF-1 α null MEF cells that transcriptional induction of Cited2 during hypoxia is dependent on HIF-1 α . Although global mRNA translation is inhibited during hypoxia Cited2 mRNA remains efficiently translated. An evolutionary conserved upstream open reading frame (uORF) in the 5'UTR of Cited2 did not stimulate translation in an eIF2 α dependent manner during hypoxia.

Conclusions: Selective translation Cited2 by an eIF2 α independent mechanism establishes a link between translation and HIF-1 dependent transcription during hypoxia.

Introduction

Tumor cells are frequently exposed to conditions of stress including nutrient starvation, oxidative stress, and hypoxia. Cellular adaptation to these conditions facilitates both cell survival and overall tumor growth. For tumor hypoxia, these adaptive mechanisms also appear to contribute to increased malignancy and poor prognosis by promoting a more aggressive tumor phenotype¹⁻⁴. The best understood adaptive response to hypoxia occurs through activation of hypoxia inducible transcription factor 1 (HIF-1). This transcription factor plays a pivotal role in the cellular response to hypoxia by inducing transcription of more than 60 down stream genes⁵. HIF-1 is a heterodimer consisting of α and β subunits that are both ubiquitously expressed. However, under aerobic conditions HIF-1 α has an extremely short half-life due to its efficient ubiquitination by the von Hippel-Lindau tumor suppressor protein (pVHL) and subsequent degradation⁶. The ability of pVHL to recognize HIF-1 α is dependent on hydroxylation of proline residues 402 and 564 in HIF-1 α which is carried out by a family of oxygen-sensitive prolyl hydroxylases (PHD1-3). Under hypoxic conditions hydroxylation cannot occur, resulting in HIF-1 α stabilization and formation of a functional transcription factor with HIF-1 α . This complex mediates transcription by binding to hypoxia responsive elements (HREs) within HIF responsive genes. In addition to regulation by protein stability, hypoxia also influences the transcriptional activity of HIF-1. Efficient transcriptional activity requires interaction of the carboxy-terminal transactivation domain (CTAD) in HIF-1 α with the transcriptional co-activator CBP/p300^{7, 8}. Under aerobic conditions, this interaction is suppressed by hydroxylation of asparagine residue 803 in the CTAD by another oxygen sensitive enzyme called FIH^{9, 10}.

A second important mechanism for regulating gene expression during hypoxia is through control of mRNA translation¹¹. Translational control provides a rapid and reversible manner of regulating gene expression, in contrast to transcriptional effects that can take hours to occur. Therefore, regulating gene expression by translation might be especially important in

tumor cells that experience transient periods of hypoxia that are too short to elicit a significant transcriptional response. Such transient fluctuations in oxygen supply are well documented^{12, 13} ranging from severe hypoxia (0%) to ~4% O₂ and are the consequence of the irregular and immature tumor vasculature. Regulation of mRNA translation has emerged as an important contributor to differential gene expression not only during hypoxia^{14, 15} but also after irradiation¹⁶ or oncogenic signaling¹⁷. Work from our group and others has shown that global initiation of mRNA translation is rapidly inhibited upon exposure to hypoxia^{18, 19}. This inhibition is mediated by at least two independent pathways that display different activation kinetics. A rapid, but transient, response is elicited through activation of the endoplasmic reticulum kinase PERK and subsequent phosphorylation of eukaryotic initiation factor 2 α (eIF2 α)²⁰. This pathway dictates the early response to hypoxia. During chronic hypoxia, data suggest that disruption of eukaryotic initiation factor 4F (eIF4F) complex and sequestration of eIF4E by both its inhibitor 4E-BP1, and its transporter 4E-T maintain inhibition of translation. Although both of these pathways lead to global inhibition in translation, individual mRNA transcripts can be affected to highly variable degrees¹⁸, ultimately resulting in hypoxic specific changes in protein expression.

In order to identify translationally controlled genes we previously performed a gene profiling study on the subset of efficiently translated mRNAs in DU145 prostate carcinoma cells after exposure to hypoxia¹⁵. We identified the transcriptional activator Cited2 as one of the genes whose translation was significantly increased during hypoxia. Cited2 has also been shown to be regulated at the transcriptional level during hypoxia²¹. Although it has not been definitively demonstrated, this induction is likely due to HIF-1, since Cited2 has been reported to contain 3 putative HREs that HIF-1 can bind to²¹. Interestingly, Cited2 can compete with HIF-1 for binding the coactivator CBP/p300 through its CH1 domain²². Therefore, by sequestration of CBP/p300, Cited2 is able to act as a negative feedback loop that limits the transcriptional activity of HIF-1 and thus HIF-1

responsive gene activation²³. Here we have investigated in detail the transcriptional and translational regulation of Cited2 to explore a possible link between hypoxic control of mRNA translation and HIF activity during hypoxia.

Materials and Methods

Tissue culture conditions

Exponentially growing prostate carcinoma cells (DU145), mouse embryonic fibroblasts (MEFs) wild type, HIF-1 α null or that had a homozygous knock-in mutation for eIF2 α (S51A) were grown on glass dishes in McCoy and DMEM media respectively supplemented with 10% foetal calf serum (FCS) (Sigma-Aldrich). For hypoxic exposure cells were transferred into a MACS VA500 microaerophilic workstation (Don Whitley Scientific, Shipley, UK). The atmosphere in the chamber consisted of 5% H₂, 5% CO₂, 0% O₂, and 90% N₂.

Isolation of polysomal RNA

Polysomal fractionation was performed as described previously¹⁸. Briefly, after treatment with cycloheximide (CHX) for 3 min at 37°C cells were washed twice with ice-cold PBS/CHX, scraped and lysed in 1% Triton X-100 and 100 units RNase-In (Ambion) in Buffer A (0.3 M NaCl, 15 mM MgCl₂, 15 mM Tris (pH 7.4), 0.1 mg/ml CHX) at 4°C. Nuclei were removed, 200 mg/ml heparin was added and residual debris was removed by centrifugation. The lysate was layered on a 10 ml continuous sucrose gradient (20–50% sucrose and 0.1mg/ml heparin in Buffer A). After 90 min of centrifugation at 39,000 rpm in an SW41-Ti rotor at 4 °C, the absorbance at 254 nm was measured continuously as a function of gradient depth in a BioRad Laboratories UV monitor and fractions were collected in 0.55 M final concentration of guanidinium-HCl. RNA isolation was performed as previously described¹⁸.

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Quantitative Real-time PCR

Real-time PCR was performed in ABI 7500 (Applied Biosystems). RNA samples were reverse transcribed using MML reverse transcriptase (Sigma-Aldrich) and random decamers (Ambion). The abundance of the following genes were detected with Taqman[®] Gene Expression Arrays (Applied Biosystems); human cited2 Hs00366696-m1, mouse actin: Mm00607939_s1, mouse ATF4: mm00515324_m1, mouse CA9: mm00519870_m1, mouse cited2 Mm00516121_m1 and 18SrRNA: Hs99999901_s1. Following genes were measured with SYBR[®] Green I (Applied Biosystems) using the indicated forward and reverse primers: human β -Actin: F-accatggatgatgatcgcc and R-gccttgccacatgccgg; human ATF-4: F-tggccaagcacttcaaacct and R-gttgttgaggaggactgaccaa. Total mRNA samples were normalized by 18S rRNA. Samples from polysomal fractions were normalized by 18S rRNA measured by PCR divided by 18S rRNA measured by spectrometry during fractionation. This facilitates correction for any differences in RNA isolation or reverse transcription efficiency between samples. Error bars represent the standard error of the mean from 3 independent experiments.

Results

As discussed above, Cited2 has been shown to be transcriptionally regulated during hypoxia. Since it contains a putative HRE, we first investigated whether transcriptional induction was strictly dependent on HIF-1. To this end, we measured transcriptional induction of Cited2 in HIF-1 α WT and HIF-1 α null MEFs. As can be seen in Fig.1, transient upregulation of Cited2 is limited to HIF-1 α proficient MEF cells implying that Cited2 is indeed regulated by HIF-1 during hypoxia. Several other known HIF target genes including VEGF and CA9 were also exclusively induced in WT HIF-1 α MEFs (data not shown). These data suggest that HIF-1 may indeed limit its own transcriptional activity via upregulation of Cited2.

Regulation of Cited2 expression during hypoxia

In order to study the effect of hypoxia on Cited2 mRNA translation we isolated efficiently translated polysomal mRNA (associated with five or more ribosomes) from DU145 cells using sucrose gradients. Two

Figure 1. HIF-1 dependent induction of Cited2 mRNA upon hypoxia. Cited2 mRNA levels were determined using quantitative real-time PCR in wild type (black bars) and HIF null (white bars) MEF cells. Representative result of 2 independent experiments is shown.

representative polysome profiles from aerobic and hypoxic (2h) conditions are shown in fig. 2A. The lowest molecular weight peaks represent the 40S and 60S ribosomal subunits respectively. The following peaks represent mRNAs with increasing numbers of associated ribosomes. From these profiles the percentage of ribosomes that are participating in translation can be obtained by dividing the integrated area of the translated fraction by the total integrated area. This value gives an estimate of the amount of ongoing *de novo* protein synthesis. Under aerobic conditions approximately 54% of the ribosomes reside in the highly translated fraction, whereas this drops to only 22% after 2 hours of hypoxia. Translation in these cells remains inhibited for up to 24 hours (data not shown).

To assess the translation efficiency of Cited2 we measured Cited2 transcript levels in both the total mRNA fraction as well as in the highly translated polysomal fraction. Fig. 2B shows that total cellular levels of Cited2 mRNA rise rapidly (>3 fold within 2 hours) and continuously over

Figure 2. Cited2 mRNA transcripts are efficiently translated during hypoxia. DU145 cells were exposed to 0% hypoxia for 0-16 hrs and cell lysates were separated on sucrose gradients. (A) Typical optical density profile at 254 nm is shown as a function of gradient depth for aerobic exposure (left panel) and after 2 hrs of hypoxia (right panel). Total mRNA levels (white bars) and transcript abundance of efficiently translated mRNAs in polysomal fraction (black bars) were determined by quantitative RT-PCR for Cited2 (B) and β -actin (C) in DU145 cells.

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m -----GCCTGGCGGTCTTGCGGAGCGGCTAGGGCAGCGGAGGAAA 40
r -----TTGTTGGCAGCTGCCTGGCGGTCTGAGGAGCGGCTAGGGCAGCGGAGGAA 52
h ACAGCTCATTGTTGGCAGCTGCCGGGCGGTCTGCGGAGCTGTGAGGGCAACGGAGGGGA 60
      *** ***** ** ***** * ***** ***** *
m AG-AAAAGGGAACGGCTCGGAATTTGCTCCAGCGGCTGCTGCAAGACCTCGGCGCCAACC 99
r AG-AAAAGGGAACGGCTCGGAATTTGCTCCAGCGGCTGCTGCAAGACCTCGGCGCCAACC 111
h AATAAAAGGGAACGGCTCCGAATCTGCCCCAGCGGCCGCTGCGAGACCTCGGCGCCGACA 12
  * ***** ***** ***** ** ***** ***** ***** **
m TCGCACCAGCGA-GCGCCTCACAGCCCATCGGCTGTCCCTCTATGTGCTGCTGAGCCGGT 158
r TCGCACCAGCGA-GCGCCCCACAGCCCTTCGGCTGCCCCCTCTATGTGCTGCTGAGCCGGT 170
h TCGCGACAGCGAAGCGCTTTGCACGCCAGGAAGGTCCCTCTATGTGCTGCTGAGCCGGT 180
      **** * **** ***** ** ** ***** *****
m CCTGGACTCGACGAGCCCCGCTTCGGTGTTCGAGCAGAAATCGCAAAGACGGAAGGACT 218
r CCTGGACCCGACGAGCCCCGCTTCGGTGTTCGAGCCGAAATCGCAAAGACGGAAGGACT 230
h CCTGGACGCGACGAGCCCCGCTTCGGTGTTCGAGCAGAAATCGCAAAAACGGAAGGACT 240
      ***** ***** ***** ***** ***** ***** *****
      M A D H M M A M N H G R F P D G T N
m GGAAATGGCAGACCATATGATGGCCATGAACCACGGGCGCTTCCCCGACGGCACCAACGG 278
r GGAAATGGCAGACCATATGATGGCCATGAACCACGGTTCGCTTCCCCGACGGCACCAACGG 290
h GGAAATGGCAGACCATATGATGGCCATGAACCACGGGCGCTTCCCCGACGGCACCAATGG 300
      ***** ***** ***** ***** ***** *****

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Figure 3. Evolutionary conserved uORF in 5'UTR of Cited2. Sequence alignment of human, mouse and rat 5' untranslated regions of Cited2. GenBank™ accession number respectively NM_006079, NM_010828 and NM_053698. Identical nucleotides are marked by an asterisk, the conserved uORF is marked by a box and ATG start codon for the Cited2 ORF is indicated in bold.

time for up to 16 hours of hypoxia. Interestingly, despite the overall mRNA found within the well-translated fraction over time is similar to the fold-increase in transcription. This demonstrates that Cited2 remains efficiently translated during hypoxia under conditions where overall mRNA suppression in mRNA translation (Fig. 2A), the fold-increase in Cited2 translation is greatly reduced. In comparison, the amount of mRNA that remains efficiently translated for the housekeeping gene β -actin decreases to less than 40% after 2 hours of hypoxia. At this time point, no significant change in total cellular β -actin transcripts is observed. Thus, as expected, β -actin translation falls during hypoxia to levels that are similar to the overall drop in cellular translation.

Selective translation of specific transcripts under certain circumstances is often determined by sequences within the 5' or 3' untranslated regions

(UTRs) of those transcripts. We reasoned that if such a sequence was present and important for regulation of Cited2 it should be evolutionarily conserved. Interestingly sequence alignment of the 5'UTR from several different species revealed a high sequence similarity (Fig. 3). Furthermore, all species contained a conserved upstream open reading frame (uORF) within its 5'UTR. The presence of uORFs has previously been implicated in the translational induction of other genes, including the hypoxia responsive gene activating transcription factor 4 (ATF4). Consistent with these previous findings, we found that hypoxia stimulated the translation of ATF4 at 2 and 4 hours of exposure even though total mRNA abundance remained unchanged (Fig. 4).

A

Figure 4. Translational control of ATF-4 regulation. (A) ATF-4 mRNA abundance in DU145 cells was measured as function of time during hypoxia. (B) Presence of efficiently translated ATF-4 transcripts in polysomal fraction. Results from 3 independent experiments are shown with SEM.

B

Although both ATF4 and Cited2 contain a uORF, the fact that the increase in translation of ATF4 is transient and occurs without a corresponding transcriptional induction suggests that their translational control may be

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distinct. To test this further, we assessed translational changes in ATF4 and Cited2 in cells that have been shown to be defective in hypoxic regulation of ATF4²⁴. Since ATF4 induction is strictly dependent on eIF2 α phosphorylation we used MEF cells with a homozygous S51A knock-in mutation in eIF2 α . This mutant eIF2 α allele cannot be phosphorylated upon hypoxia and thus the S51A cells are unable to inhibit overall mRNA translation during acute hypoxia¹⁸. We measured both total cellular and well-translated transcript levels after 4 hours of hypoxia. In addition, we measured transcript levels following 1 hour of reoxygenation because at this time both eIF2 α phosphorylation and overall mRNA translation return to normal levels. Both Cited2 and ATF4 showed a small increase ($\sim$ 2 fold) in total cellular mRNA after 4 hours of hypoxia and this did not differ between the WT and S51A cells (Fig.5A). In the WT cells, translation of

Figure 5. Translation control of Cited2 is independent of eIF2 α phosphorylation. (A) Total mRNA levels in WT MEF and S51A MEF cells after acute hypoxia or hypoxia plus reoxygenation. (B) Accumulation of ATF4 and Cited2 mRNA transcripts in highly translated fraction relative to the housekeeping gene β -actin. Results from 3 independent experiments are shown with SEM.

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ATF4 was greatly increased after 4 hours of hypoxia. Compared with β -actin, which behaves similarly to overall changes in translation, the amount of well-translated ATF4 increased by ~ 25 fold (Fig.5B). This increase was dependent on eIF2 α phosphorylation as it did not occur in the S51A cells and it returned to near normal levels after 1 hour of reoxygenation. In contrast, the amount of well-translated Cited2 mRNA showed a comparatively much smaller increase after 4 hours. Furthermore, translation of Cited2 mRNA was not impaired in S51A MEFs and may even be slightly increased, although this is not significant ($p > 0.15$). These data suggest that despite the presence of a conserved uORF, translational control of Cited2 during hypoxia is independent of eIF2 α phosphorylation.

Discussion

The oxygen-sensing pathways present in tumor cells are important determinants of hypoxia tolerance, gene expression and cell phenotype. Several important pathways that influence protein expression during hypoxia have emerged, including a transcriptional response driven in large part by HIF-1. In addition, hypoxia causes a rapid and sustained inhibition in global translation that can also lead to selective gene expression due to the fact that mRNA transcripts from specific genes show differences in their ability to be translated during hypoxia¹⁸. This may account for the ability of cells to rapidly alter protein expression, as evidenced by detectable changes in the proteome within 1 hour of exposure to hypoxia¹⁵. To date, the transcriptional and translational pathways appear to be largely distinct with little known influence on each other.

We recently identified Cited2 as part of the subset of genes whose translation is induced during hypoxia¹⁵. Cited2 has been postulated to act as a negative feedback loop for HIF-1 transactivation by competing for the transcriptional co-activator CBP/p300^{21, 23}. This feedback loop may be generated by HIF-1 itself, since both hypoxia and deferoxamine induce Cited2 at the mRNA level²¹ and because the Cited2 promoter contains 3

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consensus HREs (5'RCGTG) that bind the HIF-1 heterodimer. However, definitive evidence that HIF-1 is required for induction has not, to our knowledge been demonstrated. Our data presented in figure 1 provide strong evidence that Cited2 is indeed a bona fide HIF-1 α target since the transcriptional induction is completely abolished in HIF-1 α null cells.

Since Cited2 acts as an inhibitor of HIF-1 transcriptional activity, its additional regulation by translation provides a potential link between translational changes during hypoxia and HIF-1 mediated transcriptional changes. We therefore explored in detail the transcriptional and translational control of Cited2 during acute severe hypoxia. Our results show that Cited2 is induced to similar levels in both transcription and translation and this occurs within 2 hours and continues to rise for up to 16 hours. (Fig.2). The ability for preferential translation during hypoxia is illustrated by the fact that the increase in translation closely follows the transcriptional induction. This while overall levels of mRNA translation are greatly decreased like we showed for β -actin which represents the average gene. It will be interesting to determine whether Cited2 is also preferentially translated during moderate hypoxia. Especially since our lab recently found that acute moderate hypoxia also causes inhibition of mRNA translation mediated by the same mechanism as during severe hypoxia²⁵.

There still is limited knowledge regarding the mechanisms responsible for selective translation of different genes during cellular stress, including hypoxia. The translation efficiency of individual genes is thought to be regulated largely by the 5' and 3' untranslated parts of the mRNA. Sequence alignment of the 5'UTR of Cited2 from different species revealed an evolutionary conserved uORF. Under normal growth conditions an uORF acts as a repressor of protein synthesis by engaging the ribosome at its start site and thereby preventing translation of the correct downstream ORF. However, somewhat paradoxically, translation of uORF containing transcripts can be stimulated under conditions where eIF2 α is phosphorylated and overall mRNA translation is inhibited. Phosphorylation

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of eIF2 α reduces the rate at which initiation of translation can occur, and this is thought to allow time for the ribosome to scan through the 'decoy' uORF and reach the correct downstream ORF before beginning protein synthesis. This is the case for ATF4, whose transcript is more efficiently translated during hypoxia when eIF2 α is phosphorylated^{20, 26}. However, we found that although Cited2 contains a conserved uORF, its preferential translation during hypoxia was not similarly dependent on phosphorylation of eIF2 α .

It remains unclear if the conserved uORF in Cited2 contributes to its selective translation during hypoxia, and there are other candidate elements in the 5'UTR that might be important. It has been reported that a number of hypoxia regulated genes including HIF-1 α , VEGF and BiP contain an internal ribosome binding site (IRES) within their 5' UTR²⁷⁻²⁹. An IRES consists of a secondary mRNA structure that facilitates direct ribosome binding to the mRNA independent of the eIF4F complex at the 5'cap structure. Since disruption of the eIF4F complex also occurs during hypoxia¹⁸, it is tempting to speculate that the Cited2 5'UTR may harbor an IRES that stimulates its translation during hypoxia. This possibility warrants further investigation.

Regardless of the exact mechanism, our data show that hypoxia induced changes in translation efficiency effect the expression of Cited2, and thus the transcriptional potency of HIF-1. This provides the first evidence of a link between the rapid activation of translation inhibition by hypoxia and the transcriptional response driven by HIF-1. Interestingly, a link from HIF-1 back to translation has also been reported. HIF-1 is responsible for induction of the REDD1 gene, which functions as an inhibitor of mTOR signaling³⁰. Inhibition of mTOR has been reported to occur during hypoxia and may be responsible in part for decreased translation due to its ability to regulate cap-dependent translation³¹. Together with the data presented here, these results indicate that cross-talk between transcriptional and translational changes during hypoxia may be more ubiquitous than previously recognized.

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CHAPTER 7

Hypoxia-induced carbonic anhydrase 9 expression is dependent on the unfolded protein response

Submitted for publication.

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Abstract

Adaptation to tumor hypoxia is mediated in large part by changes in protein expression. These are driven by multiple pathways including activation of the HIF-1 transcription factor and the endoplasmic reticulum kinase PERK, a component of the unfolded protein response (UPR). Through gene expression profiling we discovered that induction of the HIF-1 target gene CA9 was defective in mouse embryo fibroblasts derived from mice harboring an eIF2 α S51A knock-in mutation. This finding was confirmed in isogenic human U373 glioblastoma and HCT116 colon carcinoma cells with an engineered defect in eIF2 α phosphorylation. Impaired CA9 expression was not due to changes in HIF activity or CA9 mRNA stability. Using chromatin immunoprecipitation we show instead that the PERK/eIF2 α translationally regulated gene ATF4 binds directly to the CA9 promoter and is associated with loss of the transcriptional repressive histone 3 lysine 27 tri-methylation mark. Transient knock-down or overexpression of ATF4 confirmed its role in CA9 induction during hypoxia. Our data indicate that regulation of CA9 is achieved through independent activation of both the HIF-1 and UPR hypoxia response pathways.

Introduction

Mammalian cells have evolved a number of sophisticated mechanisms to allow adaptation to changes in the supply of essential metabolic factors. These include mechanisms for sensing the presence of nutrients, energy, and oxygen. Many of these mechanisms are exploited by tumor cells to facilitate survival to the unique conditions found within the microenvironment of solid human tumors¹. In particular, adaptation to hypoxia is considered to be an important factor that contributes to the poor prognosis of patients with high levels of tumor hypoxia². The hypoxia inducible factor (HIF) plays an essential role in this adaptation by transcriptionally regulating the expression of a large number of genes involved in angiogenesis, anaerobic glycolysis, invasion and metastasis³.

HIF-1 is a heterodimeric transcription factor consisting of α and β subunits that are both ubiquitously expressed. Under aerobic conditions HIF-1 α is ubiquitinated by the von Hippel-Lindau tumor suppressor protein (pVHL) causing its rapid degradation within the 26S proteasome⁴. This process relies on hydroxylation of proline residues 402 and 564 by a family of oxygen-sensitive prolyl hydroxylases (PHD1-3). Under hypoxic conditions hydroxylation cannot occur, resulting in HIF-1 α stabilization and formation of a functional transcription factor with HIF-1 β . This complex drives transcription of HIF responsive genes by binding to hypoxia responsive elements (HREs) in their promoters.

One of the best characterized targets of HIF-1 is carbonic anhydrase (CA) 9. The CA9 promoter contains a single HRE element that is essential for its transcriptional increase during hypoxia⁵. CA9 is frequently over expressed in human tumors and is associated with poor prognosis^{6, 7}. CA9 belongs to a family of zinc metalloenzymes involved in regulating pH by catalyzing the reversible conversion of carbon dioxide to bicarbonate and H⁺. CA9 is found in the plasma membrane with its active site on the extracellular domain. Consequently, it has been shown to contribute to the acidification of the extracellular tumor environment during hypoxia⁸. At the same time, the bicarbonate produced by this reaction can be used

by bicarbonate transporters for import to cytoplasm and neutralization of intracellular pH. Low extracellular pH in tumors is associated with increased invasion and metastasis^{9, 10} and can also negatively affect cancer treatment by modulating uptake of anticancer drugs¹¹. The potential importance of CA9 as a marker of hypoxia and regulator of tumor pH has made it an attractive diagnostic and therapeutic target¹²⁻¹⁴.

In addition to regulation by HIF through the HRE element, five potentially important regions within the CA9 promoter have been identified by DNase I footprinting (PR1-PR5)¹⁵. PR1 plays an important role in CA9 induction during culture at high cell density via binding of SP1/3 transcription factors¹⁶. In vitro cell culture at high cell density leads to a small decrease in the pericellular oxygen concentration due to high rates of cellular oxygen. Under these conditions, transcriptional activation of CA9 requires cooperation of SP1 with minimal HIF-1 activity and operates through the PI3K pathway¹⁶. The MAPK/ERK pathway has also been implicated in regulation of CA9 expression in response to both hypoxia and culture at high cell density^{17, 18}. Simultaneous inhibition of PI3K and MAPK pathways is sufficient to prevent CAIX expression during both conditions¹⁷.

Changes in protein expression during hypoxia also arise through HIF independent mechanisms. Several recent reports indicate that hypoxia activates an adaptive program called the unfolded protein response (UPR)¹⁹⁻²¹. Activation of the UPR during hypoxia results in an immediate but reversible reduction in overall protein synthesis²². This is mediated through phosphorylation of the eukaryotic translation initiation factor eIF2 α by the ER resident kinase PERK. Mouse embryonic fibroblasts (MEFs) deficient in PERK or expressing an allele of eIF2 α mutated at the PERK phosphorylation site (S51A) are unable to inhibit translation during hypoxia and demonstrate increased hypoxia sensitivity and profoundly decreased tumor growth compared to their WT controls²⁰. Although activation of the UPR causes a global decrease in mRNA translation, the degree of inhibition is highly gene specific²²⁻²⁴. The sensitivity of any

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particular mRNA to inhibition of translation is influenced by sequences in its 5' and 3' untranslated regions (UTRs). Paradoxically, the translation of some mRNA's, including the transcription factor ATF-4, are stimulated under conditions where eIF2 α is phosphorylated^{22, 23}.

In this study we investigated the effect of UPR dependent signaling during hypoxia on the regulation of CA9. We found that CA9 expression is regulated through a PERK/eIF2 α /ATF4 dependent pathway. We show that ATF4 directly binds to the CA9 promoter and is required for transcriptional induction of CA9 during hypoxia. Our data indicate that regulation of CA9 is achieved through independent activation of both the HIF-1 and UPR hypoxia response.

Materials and Methods

Cell culture

Exponentially growing U373 (human glioblastoma-astrocytoma) and HCT116 (human colon carcinoma) cells were grown in Mem alpha and DMEM respectively, supplemented with 10% foetal calf serum (FCS) (Sigma-Aldrich). Mouse embryonic fibroblasts (MEFs) wild type or with a homozygous knock-in mutation for eIF2 α (S51A)²⁵ were grown in DMEM plus 10% FCS and supplemented with 55 μ M β -mercaptoethanol and non-essential amino acids. For hypoxic exposure cells were transferred into a MACS VA500 microaerophilic workstation (Don Whitley Scientific, Shipley, UK). The atmosphere in the chamber consisted of 5% H₂, 5% CO₂, 0% O₂, and 90% N₂.

RNA extraction and quantitative RT-PCR

RNA was isolated using SV Total RNA Isolation System (Promega) according manufacturers' instructions. RNA samples were reverse transcribed using iScript kit as described by manufacturer (Biorad). Real-time PCR was performed in ABI 7500 (Applied Biosystems). The abundance of human and mouse transcripts were determined using

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Taqman Gene Expression Arrays (Applied Biosystems) or with primers in combination with SYBR Green I (Applied Biosystems) as described in supplementary material and methods. Total mRNA samples were normalized by 18S rRNA. For determination CA9 mRNA half life cells were treated with 5 µg/ml actinomycinD (Sigma) for the indicated times.

Western blot analysis

Cells were washed twice with cold PBS and scraped in 50 mM Tris HCl pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS supplemented with protease inhibitors (Roche). After centrifugation at 10,000 g supernatants were boiled in Laemmli buffer for 10 min and proteins were resolved by SDS-PAGE. Proteins were subsequently transferred onto nitrocellulose membranes and blocked overnight at 4°C in PBS containing 0.1% Tween-20 (PBS-T) supplemented with 5% skim milk powder. Membranes were probed for 1 h with antibodies directed against CAIX (M75²⁶), HIF-1α (Transduction Laboratories) or β-actin (Sigma). Bound antibodies were visualized using HRP-linked secondary antibodies (anti-rabbit (Cell Signaling Technologies) and anti-mouse (Sigma)) and ECL luminescence (Pierce).

Transfection of reporter constructs and siRNA

U373 and MEF cells were transiently cotransfected with CA9 promotor constructs and pcDNALacZ using Lipofectamine (Invitrogen) as described by the manufacturer. Transfected cells were sub cultured 16 h post transfection, exposed to hypoxia and finally harvested 48 hrs after transfection. Luciferase and β-galactosidase activity was measured using commercial luciferase kit (Promega) and galacto light system kit (Applied Biosystems) in a LB9507 luminometer (Berthold). For transient knock down U373 cells were transfected with 100nM siRNA duplexes using Oligofectamine according to the manufacturers' instructions. siRNA duplex targeting ATF4 122372, CHOP 146321, ATF6 115889 and HIF1α 42840 was obtained from Ambion and scramble control siRNA was from

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Dharmacon. Transfected cells were sub cultured 24 h post transfection and harvested 72 hrs after transfection. The ATF4 open reading frame was amplified from pCMV_XL5 (Origen) using following primers ATF4_Fw 5'-gtatgcggaggatccccgcaacatgaccgaaatgagc-3' and ATF4_Rv 5'-tccgcatacgaattcctcctgactatcctcaactagg-3' and cloned into pcDNA5 (Invitrogen) using *Bam*HI and *Eco*RI restriction sites. Transfection of U373 cells with pcDNA5-ATF4 or empty vector control was done as for the reporter constructs described above.

Chromatin immunoprecipitation assays

ChIPs were performed and analyzed essentially as described previously²⁷, see supplementary materials and methods.

Tumor xenograft growth

Animal experiments were performed using adult NMRI (nu/ nu) female mice (28- 32 g) from the animal facility of the Catholic University of Leuven in Belgium. The animal facilities and experiments were in accordance with local institutional guidelines for animal welfare and were approved by the Animal Ethics Committee of the university. Three million U373 pcDNA3, U373 HIF-1 α and U373 S51A cells were resuspended in 100 μ l growth medium and injected sub-cutaneously in to the lateral flank of recipient mice. Tumors were excised after they reached approximately 100-300 mm³ and fixed in 1-4 % formaldehyde and embedded in paraffin.

Immunohistochemical analysis of CAIX

5 μ m sections were deparaffinized in xylene, rehydrated in a graded alcohol series and finally with water. Endogenous peroxidase was quenched using 0.3 % hydrogen peroxide in absolute methanol. Epitope retrieval was done by microwave treatment in the presence of 10 mM sodium citrate (pH 6.0). Sections were incubated with rabbit polyclonal anti CAIX antibody (1:1000 dilution, Novus) for 60 min at RT. A secondary biotinylated polyclonal goat anti rabbit antibody (1:500 dilution,

DakoCytomation) was applied, followed by incubation with the vectastain ABC kit (1:200 dilution, Vector Laboratories). Staining was visualized using DAB chromogen (Dako). Sections were rinsed and counterstained with hematoxylin (Klinipath).

Results

Hypoxic induction of CA9 requires eIF2 α phosphorylation

In an ongoing screen to characterize changes in gene expression during hypoxia mediated by the UPR we compared transcriptional profiles of wild-type and eIF2 α S51A mutant MEFs. We identified CA9 as one of the top differentially regulated genes between the WT and S51A cells which are unable to phosphorylate eIF2 α ²⁵ (data not shown). Quantitative RT-PCR analysis confirmed that CA9 mRNA levels during hypoxia are approximately 5 to 8-fold lower in the S51A MEFs compared with their WT controls (Figure 1A+B). The reduction in CA9 expression in the S51A mutants is observed during culture under both severe (<0.02% O₂) and more moderate (0.2% O₂) hypoxia.

We also assessed hypoxia induced CA9 expression in a series of human cell lines engineered to have defects in eIF2 α phosphorylation. We created isogenic stable cell lines expressing different constructs at a single genomic site that effectively prevent eIF2 α phosphorylation (Rouschop and Wouters, in preparation) (data not shown). These included expression of a dominant negative eIF2 α (S51A)²⁸ that is insensitive to PERK signaling, a C-terminal fragment of GADD34 that promotes dephosphorylation of eIF2 α , and a shRNA against PERK (shPERK). Figure 1C shows that expression of each of these three constructs in U373 glioblastoma cells effectively prevents induction of CA9 during hypoxia.

Figure 1. CA9 expression is impaired in cells defective in eIF2 α phosphorylation during hypoxic conditions. WT and S51A MEFs were exposed to severe (0.0% O₂) (A) or moderate (0.2% O₂) (B) hypoxia and CA9 mRNA levels were assessed using Q-PCR after 0, 8 and 24 hrs. (C) CAIX levels measured in isogenic U373 cells expressing dominant negative eIF2 α (S51A), GADD34 or shPERK after 0, 8 and 24 hrs of severe hypoxia. (D) U373 pcDNA5 and S51A were seeded at different densities and analyzed with Q-PCR for CA9 mRNA expression. (E) CA9 protein expression was assessed by western blotting in isogenic U373 cell lines after exposure to hypoxia.

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Similar results were observed in HCT116 cells carrying the same constructs (data not shown). The requirement for eIF2 α phosphorylation seems specific to hypoxia since upregulation of CA9 during culture at high cell density under aerobic conditions is similar in wild type and S51A expressing cells (Figure 1D). Importantly, the reduction in CA9 mRNA levels in the eIF2 α phosphorylation deficient cells also results in reduced CA9 protein expression during hypoxia (Figure 1E).

The effects of eIF2 α phosphorylation on CA9 are independent of HIF

Since the transcription of CA9 is strongly regulated by the HIF-1 transcription factor, we investigated the possibility that HIF-1 signaling was aberrant in cells with defective eIF2 α phosphorylation. However, no differences in the levels of HIF-1 α (Figure 2A) or HIF-1 β (Figure 2B) mRNA were observed between S51A MEF and their WT controls. HIF activity as assessed using a HRE luciferase reporter plasmid was also similar in the two cell lines (Figure 2C). We additionally assessed HIF-1 α protein levels in the human isogenic U373 cell lines after exposure to hypoxia and found similar levels in the three eIF2 α defective cell lines as in the empty vector control (Figure 2D). Finally, we assessed the transcriptional induction of two additional endogenous HIF dependent genes, GLUT-1 and VEGF. In contrast to CA9, the expression of both VEGF (Figure 2E+F) and GLUT-1 (Figure 2G+H) was unaffected in the cell lines with defective eIF2 α phosphorylation. Together these data suggest that HIF-1 activity is not affected by eIF2 α phosphorylation.

Figure 2. HIF-1 expression and activity does not require UPR signaling. Analysis of HIF-1 α (A) and HIF-1 β (B) mRNA levels in WT and S51A MEF cells. (C) HIF activity was measured in WT and S51A MEF cells using HRE-luciferase constructs normalized to co-transfected β -galactosidase. (D) Western blot analysis of HIF-1 α levels in isogenic U373 cell lines. Induction of HIF-1 α target genes VEGF (E) and GLUT-1 (G) in U373 cells after 0, 8 and 24 hrs of hypoxia and VEGF (F) and GLUT-1 (H) in WT and S51A MEF cells measured by Q-PCR.

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eIF2 α phosphorylation is required but not sufficient for CA9 induction

These data suggested that activation of PERK and phosphorylation of eIF2 α contributes to CA9 transcription during hypoxia. We therefore investigated whether other activators of PERK and the UPR might also regulate CA9. To this end we induced ER stress in U373 cells by treatment with either with DTT or tunicamycin. Although both drugs clearly elicited a robust UPR response, as demonstrated by induction of the UPR responsive gene CHOP (Figure 3), neither affected CA9 mRNA levels.

CA9 is regulated at the transcriptional level by ATF4

In order to explain the reduced CA9 mRNA levels in UPR impaired cells we first examined whether mRNA stability was dependent upon eIF2 α phosphorylation. To this end we determined CA9 mRNA half life after blocking transcription with actinomycin D for various lengths of time. However, the results presented in Figure 4A (MEFs) and B (U373) indicate that CA9 mRNA half life not altered in the eIF2 α phosphorylation deficient cells. These data suggest that differences in CA9 expression are likely due to changes in transcription. Therefore we investigated CA9 promoter activity using luciferase reporter constructs. Promoter region -174 to +37 (pGL3-PR5) contains the 5 previously identified regulatory promoter elements (PR1-PR5) and a hypoxia responsive element (HRE) that influence transcriptional activation of CA9^{5, 15}. The minimal promoter region necessary for hypoxia induced expression of CA9 is -50 to +37 (pGL3-PR1). Essential for this are the SP1 transcription factor in PR1 and the HRE immediately downstream of PR1. In our experiments both promoter sequences (-174 to +37) and (-50 to +37) showed basal levels of activity in UPR impaired cells and their controls under normoxic conditions (Figure 5A). However exposure to hypoxia resulted in a significant induction of promoter activity in all cell lines examined. Furthermore, mutations in either the SP1 binding site or the HRE element completely abolished hypoxia induceability. The fact that we did not

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observe any differences between UPR impaired cells and control suggests either that elements outside the (-174 to +37) promoter region are responsible for the UPR dependent effect on CA9 expression or that the UPR does not influence transcription of these non-chromatin containing plasmids.

Figure 3. ER stress does not induce CA9 expression during normoxia. U373 cells were stimulated with 1 μ g/ml tunicamycin or 1 mM DTT for 8 or 24 hrs. Expression levels for CA9, CHOP and actin were determined using Q-PCR. Bars represent average expression of at least two independent experiments plus standard deviation.

ATF4 binds the CA9 promoter

Since our reporter constructs did not cover the complete promoter we attempted to identify transcription factors that are able to bind the CA9 promoter using chromatin immunoprecipitation (ChIP). Using this approach we tested three transcription factors that are known to be activated by the UPR, ATF4, CHOP and ATF6. ATF4 is induced at the translational level in response to PERK dependent phosphorylation of eIF2 α and has also been shown to occur during hypoxia^{23, 29}. CHOP is a transcriptional target of both ATF4 and ATF6 and is also preferentially translated during hypoxia^{22, 29}. Interestingly, CHOP has previously been implicated in the regulation of CA VI³⁰. ATF6 on the other hand is also

Figure 4. Stability of CA9 mRNA is not dependent on eIF2 α phosphorylation. (A) CA9 mRNA levels were induced in WT and S51A MEF cells with 100 μ M cobalt chloride for 16 hours after which CA9 mRNA levels were assessed using Q-PCR at different time points after addition of actinomycin D. (B) CA9 mRNA stability in U373 pcDNA5 and U373 S51A cells was determined after exposure to 16 hours of hypoxia.

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induced by the UPR, but has no known dependency upon PERK or eIF2 α phosphorylation. U373 and HCT116 cells were exposed to aerobic or hypoxic conditions (4 h) after which ChIPs were performed using antibodies against ATF4, CHOP, ATF6 and the HA epitope tag. We observed enrichment of the CA9 promoter after performing the ChIP procedure for ATF4 in U373 (Figure 5B) and HCT116 (Figure 5C). In contrast, there was no enrichment using CHOP or ATF6. ATF-4 binding could be explained by the presence of 2 putative amino acid response elements (AAREs) in the CA9 promoter region that closely resemble the ATF-4 DNA binding sequence in the CHOP promoter (ATTGCATCA)³¹ (Figure 5D). Furthermore we show that we could efficiently immuno precipitate the CA9 promoter using an antibody directed against HIF-1 α (Figure 5B). As additional controls for the ChIP procedures we determined the enrichment of known interacting promoter regions over that of an unrelated promoter region of ATG12. The ATF4 and ATF6 ChIPs effectively pulled down the CHOP promoter (up to 28-fold and 6-fold enrichment respectively) and CHOP pulled down the GADD34 promoter (~5-fold enriched) (Figure 5E). We also performed ChIP analysis using an antibody directed against the histone H3 methylation mark on lysine 27 (H3K27me). The presence of H3K27me correlates with repression of gene expression³². With U373 cells cultured under aerobic conditions we obtained significant enrichment of the CA9 promoter using an antibody against the H3K27me mark compared to an unrelated control antibody (Figure 5F). Strikingly this H3K27me mark at the CA9 promoter disappears rapidly upon exposure to hypoxia. Similar results were obtained using HCT116 cells (data not shown). These data suggest that ATF4 mediates endogenous transcriptional activation of CA9 by direct binding to its promoter and that activation occurs following a change in histone methylation.

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Figure 5. ATF4 binds to the CA9 promoter region. (A) WT CA9 promoter construct or mutant versions with PR2-5 deleted, sp1 binding site or HRE inactivated were transiently cotransfected with pcDNA3-LacZ into WT MEF or S51A MEF cells. Luciferase activity was measured relative to β -galactosidase expression after 24 hrs of hypoxia after which fold induction was calculated for each construct. Bars represent the average of 4 independent experiments plus standard deviation. ChIP analysis was performed on U373 (B) and HCT116 (C) cells after exposure to normoxia or hypoxia using indicated antibodies. Q-PCR was used to determine fold enrichment of the CA9 promoter region over that obtained with negative control antibody HA. (D) Localization of putative ATF4 binding sites in CA9 promoter region. (E) Positive controls for ChIP analysis. Fold enrichment of CHOP and GADD34 promoter regions for ATF4/ ATF6 and CHOP respectively over unrelated promoter region of ATG12 gene. (F) ChIP analysis using U373 cells with antibodies against H3K27me during hypoxia, fold enrichment over negative control anti-HA Ab.

To investigate the functional importance of ATF4 in regulating CA9 expression during hypoxia we knocked down ATF4 using siRNA. In addition we knocked down the ATF4 regulated transcription factor CHOP as well as HIF-1 α and ATF6 as positive and negative controls respectively. We achieved 50-90% knock-down of our target genes compared to scrambled (SCR) siRNA control in U373 (Figure 6A) and HCT116 cells (supplementary figure 3A). U373 cells depleted from HIF-1 α or ATF4 exhibited a strong reduction in CA9 mRNA compared with the SCR controls after 16 hrs of hypoxia (Figure 6B). Depletion of the other UPR related transcription factors CHOP and ATF6 did not affect CA9 induction during hypoxia.

To determine if cellular levels of ATF4 were directly influencing endogenous CA9 expression we also over-expressed ATF4 in U373 cells and exposed them to hypoxic conditions. Again basal mRNA levels of CA9 were unaffected under aerobic conditions (data not shown), however after 16 hours of hypoxia CA9 mRNA levels were approximately 2-fold higher compared to cells bearing an empty vector control (Figure 6C). Taken together, our data support a model in which hypoxic induction of CA9 is dependent on the PERK controlled arm of the UPR involving direct binding of ATF4 to the CA9 promoter.

Figure 6. ATF4 is required for CA9 mRNA expression during hypoxia. U373 cells were transiently transfected with siRNA duplexes directed against HIF1 α , ATF4, CHOP and ATF6. Twenty four hrs post transfection cells were exposed to 16 hrs of hypoxia. (A) The knockdown efficiency was determined for gene specific siRNAs using Q-PCR. Expression was normalized to mRNA levels in cells transfected with scrambled control. (B) Relative expression of CA9 mRNA post transfection with siRNA after culturing under normoxia or 16 hours of hypoxia. (C) Exogenous ATF4 was over expressed in U373 cells and exposed to 0, 8 and 16 hours of hypoxia. Relative levels of CA9 mRNA were determined using Q-PCR compared to empty vector control under aerobic conditions.

The UPR mediates expression of CA9 in vivo

Finally to investigate the role of the UPR on induction of CA9 expression *in vivo*, xenograft tumors were grown from isogenic U373 cells bearing empty vector, eIF2 α S51A or shRNA against HIF-1 α as a positive control. Immunohistochemical staining using an antibody against CA9 revealed an

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overall decreased staining intensity in the HIF-1 α knock-down tumor sections compared to the empty vector controls (Figure 7A+B). Tumors derived from the U373 cells with defective eIF2 α phosphorylation also showed a reduction in CA9 levels that was comparable to the HIF-1 α knock-down tumors (Figure 7C).

Figure 7. Overall CA9 expression is reduced in section of U373 S51A xenograft tumors. Immunohistochemical analysis of CA9 expression in xenograft tumors derived from U373 cells bearing empty vector, eIF2 α S51A or shRNA against HIF-1 α . Sections were stained with anti-CA9 polyclonal antibody (A) U373 pcDNA, (B) U373 HIF-1 α knock-down, (C) U373 S51A and (D) negative control. Staining is predominantly membranous and focused to perinecrotic regions in all tumors. *n*, regions of necrosis.

Discussion

It is well established that hypoxic conditions induce CA9 transcription through the transcription factor HIF, which binds to the HRE element in the CA9 promoter⁵. Here we showed that in addition to HIF, PERK signaling to eIF2 α is necessary for CA9 induction during hypoxia. This was demonstrated using several different genetic models in both human and mouse cells. The requirement for UPR signaling during hypoxia does not extend to CA9 induced by high cell density under aerobic conditions³³, which has been shown to involve the PI3K pathway¹⁶ and MAPK signaling¹⁷. These results support the existence of two distinct mechanisms for CA9 transcriptional regulation, a HIF dependent mechanism activated under low oxygen levels and a PI3K/ MAPK dependent pathway at high cell density. Here we provide evidence that the former mechanism relies on simultaneous PERK/eIF2 α signaling.

The dependence of CA9 expression on eIF2 α phosphorylation is directly mediated by ATF4. Knockdown or overexpression of ATF4 decreased or increased CA9 expression during hypoxia respectively. Furthermore, we showed that ATF4 directly binds to the CA9 promoter. Interestingly published microarray data from several studies has shown that CA6 is also responsive to UPR activation^{30, 34, 35}. In contrast to CA9 this effect is dependent on the ATF4 responsive transcription factor CHOP and not directly through ATF4^{30, 35}. Nonetheless these data suggest an important and broader role for PERK/eIF2 α /ATF4 in regulating pH levels through up regulation of carbonic anhydrases.

The function of ATF4 on the CA9 promoter and its potential co-operation with HIF1 is not fully understood. Induction of the UPR under non-hypoxic conditions using DTT or tunicamycin was unable to induce CA9. Interestingly we found that the CA9 promoter contains trimethylation marks on lysine residue 27 of histone H3 under aerobic conditions. These marks are generally correlated to a transcriptional repressive state³². We found that this repressive mark was lost after exposure to hypoxia for periods as short as 1 hour. These data suggest

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that hypoxia influences CA9 expression in part through an epigenetic mechanism. This result is interesting in relation to a recent report suggesting that hypoxia may affect global changes in histone methylation³⁶. To what extent this is dependent on ATF4 expression remains unclear.

ATF4 may also influence CA9 through its known interaction with the transcriptional co-activator p300/CBP³⁷ or p300/CBP-associated factor (PCAF)³⁸. Both p300/CBP and PCAF contain histone acetyltransferase (HAT) activity and function as co-activators in the transcriptional regulation of many genes. Our data indicate that recruitment of HIF during hypoxia is essential but not sufficient for complete transcriptional activation of CA9. Since p300/CBP is required for HIF mediated transcription it is attractive to suggest a model in which ATF4 and HIF co-operate through their ability to recruit p300/CBP and/or PCAF. Such cooperation would be meaningful in certain hypoxic range, which simultaneously activates both pathways.

Adaptation to hypoxic conditions through activation of the UPR is an important survival mechanism for tumor cells. The Ire1/Xbp1 and PERK/eIF2 α arms of the UPR have been shown to play a critical role in determining hypoxia sensitivity^{20, 39}. PERK knockout MEFs have decreased hypoxia tolerance and tumor growth in xenograft studies due to their inability to activate eIF2 α phosphorylation²⁰. It is tempting to speculate that this growth defect could be due in part to decreased CA9 expression. It has previously been shown that knock-down of CA9 in MDA468 and MDA231 breast carcinoma cell lines using siRNA resulted in a significant reduction in clonogenic survival after hypoxic exposure compared to control cells¹⁴. However whether decreased CA9 expression is responsible for the decrease growth rate observed with the eIF2 α deficient tumors needs further investigation. PERK/eIF2 α phosphorylation and upregulation of ATF4 is also involved in other important processes including angiogenesis³⁴, and maintenance of energy homeostasis⁴⁰. Thus, as is the case for HIF, the contribution of the UPR to hypoxia tolerance is likely to

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be multi-factorial. Our data also contribute to a growing overlap in the contribution of UPR and HIF dependent responses to the hypoxic tumor phenotype. pH regulation, angiogenesis, and energy homeostasis are also all strongly regulated by HIF³. In this regard it is important to note that the oxygen dependencies of HIF activation and the UPR are not the same. Whereas HIF is activated at relatively moderate hypoxic conditions (1-2%), full activation of the UPR requires more severe hypoxia^{19, 41}. Consequently the UPR and HIF pathways may allow cells to adjust the strength of these responses as a function of oxygen concentration.

CA9 has become widely adopted as a surrogate marker of tumor hypoxia used to classify patients that likely respond poorly to therapy^{42, 43}. Although CA9 expression correlates well with exogenous marker of hypoxia pimonidazole its correlation with HIF-1 α is much weaker^{44, 45}. CA9 expression is often detected in the perinecrotic regions of solid tumors where HIF1 α levels are reduced⁴⁵. Several possible explanations have been suggested for this poor co-localization⁴⁵ including the large difference in protein half-life between HIF-1 α and CA9. Due to its long half-life CA9 expression may be present in areas that have been reperfused with oxygen and no longer express HIF-1 α . Our results provide an additional mechanism to explain high CA9 expression in perinecrotic tumor areas. Tumor cells in these regions are likely to exhibit activation of PERK due to the extremely hypoxic nature of these regions. Therefore simultaneous activation of the UPR and HIF, might drive higher CA9 expression in these areas. The requirement of UPR signaling may also explain why some HIF-1 positive tumors fail to express CA9.

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CHAPTER 8

Targeting translational control pathways; an in vitro study

In preparation

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Abstract

Hypoxia is a common feature of solid tumors and is associated with an aggressive phenotype, resistance to therapy and poor prognosis. These adverse effects are mediated by changes in protein expression that occur at both the transcriptional and translational level through several oxygen-sensitive pathways. Here we report the development of an isogenic tumor model in which three of these pathways can be conditionally regulated. Inhibition of the PERK/ eIF2 α pathway of the unfolded protein response (UPR) was achieved by overexpression of eIF2 α S51A, GADD34 or shRNA against PERK. Likewise HIF signaling was targeted through shRNA against HIF-1 α and mTOR signaling by shRNA against TSC1 or overexpression of eIF4E. All cell lines were validated using Q-PCR or Western blot analysis. Using these cell lines we demonstrated that HIF signaling is important for cell growth under hypoxic conditions as HIF-1 α knockdown cells exhibit 3-fold reduced proliferation rates. Conversely, all pathways independently contribute to hypoxia tolerance since their disruption significantly sensitized cells to hypoxia induced cell death. Finally we showed that expression of this isogenic inducible system can be used efficiently in established xenograft tumors by administration of doxycycline to the drinking water of tumor bearing mice. This will allow us in the future to determine the relative importance of targeting these pathways *in vivo* in pre-established tumors in a manner that closely mimics the therapeutic situation.

Introduction

The majority of solid human tumors contain areas that are poorly oxygenated¹. It has been demonstrated that tumor hypoxia causes treatment resistance through induction of angiogenesis, metastasis and selection for cells with reduced apoptotic potential²⁻⁴. Together these studies indicate that hypoxia promotes a more malignant tumor phenotype which is of high clinical relevance⁵. The negative impact on patient prognosis is caused in part by the activation of several oxygen sensitive signaling pathways. These hypoxia response pathways have therefore received widespread attention and are being evaluated as potential targets for the treatment of human tumors⁶. The best understood of these pathways involves the stabilization and activation of the HIF family of transcription factors. Their activation during hypoxia mediates the induction of more than 60 genes affecting important pathways like glycolysis and angiogenesis².

Work from our lab and others has demonstrated that global mRNA translation is inhibited during hypoxia⁷⁻⁹. This inhibition contributes to maintaining cellular energy homeostasis, but is not secondary to energy depletion as it occurs very rapidly. Moreover, it has a major affect on hypoxia induced gene expression by modulating gene specific translation and thus on the hypoxic cellular phenotype^{7, 10, 11}. Hypoxia causes an overall inhibition of mRNA translation through at least two different signal transduction pathways. The first response is mediated rapidly during acute hypoxia through PERK-dependent phosphorylation of eukaryotic initiation factor alpha (eIF2 α). This prevents the formation of a functional ternary complex and thus translation initiation. Data suggest that this type of translation inhibition is transient, with significant recovery during continued exposure to hypoxia^{7, 12}. However, translation rates remain inhibited due to the activation of a second response mediated via disruption of the eIF4F complex which is essential for cap-dependent translation. Hypoxia prevents eIF4E:eIF4G interaction and thus functional

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eIF4F through increased eIF4E:4E-T and eIF4E:4E-BP1 complex formation^{7, 12, 13}.

It has been shown that the cells capacity to mediate translational control through eIF2 α is important for their survival in response to hypoxia, and for promoting tumor growth or response to treatment^{9, 14}. This has generated attention for translational control pathways in addition to HIF as potential targets for tackling hypoxia in cancer treatment. However, the majority of these results have used mouse embryo fibroblasts from knockout or knockin mice whose applicability to epithelial human cancers is questionable. It also remains unclear as to the relative importance of these pathways in contributing to tumor growth, metastasis, or response to therapy. This is further complicated by the tumor microenvironment which harbors areas of acute, chronic or cycling levels of hypoxia, causing a range of different oxygen tensions^{15, 16} that differentially affect these various oxygen sensitive pathways.

In this study, we have developed and tested a new set of tumor cell lines to investigate the relative importance of multiple adaptive mechanisms during hypoxia. We describe a novel inducible isogenic cell model in which different hypoxia responsive pathways can be either activated or inactivated by administration of tetracycline or doxycycline. We propose that this model system can provide valuable information about the importance of these hypoxia response pathways in established tumors and their potential for targeting in combination with other therapies.

Material and Methods

Tissue culture conditions

Exponentially growing human colon cancer cell line HCT116 and human glioblastoma cell line U373 were grown in DMEM and MEM alpha medium respectively supplemented with 10% foetal calf serum (FCS) (Sigma-Aldrich). For hypoxic exposure cells were transferred to a hypoxic culture chamber (MACS VA500 microaerophilic workstation, Don Whitley

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Scientific, Shipley, UK). The atmosphere in the chamber consisted of 5% H₂, 5% CO₂, the desired concentration of O₂ and residual N₂. An anoxic atmosphere was ensured by the inclusion of a catalyst in the hypoxic chamber that catalysed the conversion of H₂ with any O₂ to H₂O.

Generation isogenic Flp-in expressing cells

Stable isogenic U373 and HCT116 cell lines expressing a single Flp-in recombination site were generated using the Flp-inTM T-Rex system (Invitrogen) according to the manufacturers' instructions. Briefly, the Flp-in recombination plasmid pFRT/LacZeo was transfected into U373 and HCT116 using lipofectamine as described by the manufacturer (Invitrogen). Stable β -galactosidase positive clones were obtained after selection zeocin rich media. Next the tet-repressor was introduced into these clones by transfection with pcDNA6/TR and selection for blastocidin resistance. Clones were selected that only contained 1 Flp-in recombination site. Proliferation under aerobic and hypoxic conditions of the final clones as well as their hypoxia sensitivity was found to be exactly as the parental cell line.

Western blotting

Cells were lysed directly in hot (95 °C) sample buffer (125mM Tris-HCl, 5% glycerol, 0.04% sodium dodecyl sulphate and 1M β -mercaptoethanol), resolved by SDS-PAGE and blotted onto polyvinylidene fluoride (PVDF) membranes. Blots were probed with antibodies directed against phospho(serine 51)-eIF2 α (Research Genetics), PERK (Santa Cruz), eIF4E and TSC1 (Cell signaling). Bound antibodies were visualized using HRP-linked secondary antibodies (anti-rabbit (Cell Signaling Technologies) and anti-mouse (Sigma)) and ECL luminescence (Pierce). Exposure to 1mM DTT for 1 hour was used as a positive control for eIF2 α phosphorylation. This strongly reducing agent causes protein misfolding in the endoplasmic reticulum which activates the eIF2 α kinase PERK.

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Growth and survival

Cell number was counted before (N_0) and after (N) hypoxic exposure. Doubling time T after growth time t was calculated from the formula $N=N_02^{(t/T)}$. For clonogenic survival, single cells were plated and exposed to hypoxic conditions for the indicated time period. Colonies containing more than 50 cells were scored as a surviving cell. Surviving fraction was calculated as the ratio between surviving cells and cells plated, corrected for plating efficiency (PE), i.e. survival in untreated controls.

Tumor xenograft growth

Animal experiments were performed using adult NMRI (nu/ nu) female mice (28- 32 g) from the animal facility of the Catholic University of Leuven in Belgium. The animal facilities and experiments were in accordance with local institutional guidelines for animal welfare and were approved by the Animal Ethics Committee of the university. Three million U373 and U373-eGFP cells were resuspended in 100 μ l growth medium and injected sub-cutaneously in to the lateral flank of recipient mice. Tumors were grown until they reached 300 mm³ after which the mice were left untreated or received 2g/ L doxycycline in their drinking water. Tumors were excised after 4 days and fixed in 1-4 % formaldehyde. GFP expression was assessed using the Bonsai imaging apparatus (Siemens Germany).

Results

Development of an isogenic inducible cell model

The objective of this study was to compare the relative importance of several hypoxia responsive pathways on cellular phenotype during this stress. For this purpose we created a new tumor cell model based on the commercial Flp-inTM T-Rex system (Invitrogen). In this system, creation of an acceptor cell line results in the ability to easily introduce a number of different transgenes or shRNAs into a single genomic site through a flp mediated recombination event. The cell lines created from this acceptor

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cell line are truly isogenic containing only a single integration at a common genomic site. In addition, the system we engineered is under the control of the tetracycline responsive promoter and can thus expression of the inserted gene or shRNA is dependent on the addition of tetracycline.

We successfully generated U373 and HCT116 cell lines that contain a Flp-in recombination site at a single location in the chromosome (see materials and methods). We introduced an ORF coding for enhanced green fluorescent protein (eGFP) in these two cell lines to validate inducibility and expression levels. Figure 1A shows that eGFP expression is readily visible after addition of 1 $\mu\text{g/ml}$ tetracycline for 24 h. The inducibility was assessed in more detail using FACS analysis. Within 4 hrs after addition of tetracycline eGFP levels were more than 50-fold elevated and reach maximum levels after 24 hrs (Figure 1B). The expression was furthermore not compromised under hypoxic conditions. Similar data were observed for HCT116 cells expressing eGFP (data not shown).

Figure 1. Validation of isogenic model system using eGFP. A. U373 cells stably expressing eGFP from an inducible integrated construct were left untreated (top panel) or exposed to 1 $\mu\text{g/ml}$ tetracycline for 24 hrs (bottom panel). B. Quantitative FACS analysis of GFP expression after addition of tetracycline under aerobic (filled boxes) and hypoxic (open circles) conditions.

Modulation of hypoxia responsive pathways

The low basal and high inducibility observed following introduction of eGFP indicated that the acceptor lines would be suitable for use in developing cell lines targeting hypoxia responsive pathways. We used multiple approaches to disable oxygen sensing through each of the three main hypoxia response pathways that affect transcription (HIF) and translation (PERK/eIF2 α and eIF4F) (see figure 2A).

Figure 2. Interference with hypoxia activated response pathways. (A) Deactivation of these pathways is achieved either through expression of shRNA, dominant negative (DN) or wild type versions of the indicated target molecules. (B) Western blot analysis of generated cell lines during hypoxia. Expressed constructs listed on the left and protein detected in WB on the right. (C) Validation of HIF-1 α knockdown cells was done using Q-PCR.

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For this purpose we targeted the PERK/eIF2 α pathway at 3 different levels, overexpressing GADD34, dominant negative eIF2 α S51A, or short hairpin interfering RNA (shRNA) against PERK (shPERK). GADD34 expression leads to the activation of a phosphatase that dephosphorylates eIF2 α and thus prevents hypoxic inactivation of this protein. The HIF response was disrupted by shRNA against HIF-1 α . Signaling through eIF4F during hypoxia was disrupted by targeting either mTOR activation (using a shRNA against TSC1) or by overexpression of eIF4E. For validation of the different constructs cells were cultured for 48 hours in the presence of tetracycline followed by 4 hours of hypoxic (Figure 2 B+C) or aerobic (data not shown) conditions. Subsequently, the expression levels of the inserted transgenes or their targets were evaluated. shRNA was effective at silencing TSC1, PERK and HIF1 α . Probing the phosphorylation of eIF2 α indicated that overexpression of GADD34 was effective. Expression of the eIF2 α S51A allele has no major effect on endogenous eIF2 α phosphorylation levels (Figure 2C). However it significantly rescues the translational repression during hypoxia compared to control cells as determined by polysomal RNA fractionation (data not shown).

Figure 4. Effect of disrupted hypoxia response pathways on proliferation. HCT116 cells defective in indicated pathways were grown under 0.2% oxygen. Subsequently fold increase in doubling time (T) was calculated.

Figure 5. Relative importance of hypoxia response pathways on hypoxia sensitivity. HCT116 cells defective in indicated pathways were seeded at low concentrations and exposed to anoxic conditions ($>0.02\%$ O_2) for the indicated period of time. Clonogenic survival was determined after ~ 14 days.

Next we investigated whether interference with any of these mechanisms might lead to differences in proliferation rates under aerobic or hypoxic conditions. For this purpose, HCT116 cells were treated with tetracycline for 48 hrs and subsequently grown for different periods under

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either aerobic or hypoxic conditions. As expected, we observed that interference with any of these pathways had no influence on cell growth under optimal aerobic conditions (Figure 4). Interestingly this is also true for culture during hypoxia for most of the tested cell lines except for the HIF knock down cells. These cells exhibit a ~3-fold reduction in proliferation rate upon hypoxia.

The ability to activate the PERK/ eIF2 α pathway in response to hypoxia has previously been shown to be vital for cells to tolerate hypoxic stress^{9, 14}. However the relative importance of PERK/ eIF2 α activation compared to the other two pathways is currently unknown. Therefore we investigated this by determining clonogenic cell survival after exposure to severe and moderate hypoxic conditions. We were able to confirm the importance of PERK/ eIF2 α activation during hypoxia for cell survival. Preventing eIF2 α phosphorylation significantly sensitized HCT116 cells to severe hypoxia (Figure 5A-C). This was observed with all constructs that prevent eIF2 α phosphorylation, although overexpression of eIF2 α S51A was most potent in sensitizing cells to hypoxia induced cell death. Knockdown of HIF-1 α revealed a similar effect on clonogenic survival as the PERK/eIF2 α pathway (Figure 5D). For both pathways this effect was apparent after 24 hours of hypoxia. In contrast, interference with mTOR signaling on hypoxia tolerance achieved through overexpression of eIF4E or knockdown of TSC1 became evident after 72 hours of exposure to hypoxia (Figure 5E, F). Exposure to more moderate hypoxia (0.2% O₂) was found to delay induction of cell death by about 24 hours, but did not alter the relative sensitivity of any individual cell line (data not shown).

Finally we investigated whether we could use this system to inducibly regulate expression of a transgene within a pre-established tumor. To obtain proof of principle we implanted U373-eGFP cells sub-cutaneously into the flank of nude mice. Expression of GFP was induced when the tumor size reached 300 mm³ by addition of doxycycline to the drinking water of the mice. After 4 days fluorescence images were taken *ex vivo*. Significant induction of eGFP was visible in doxycycline treated U373-eGFP

tumors (Figure 6), although some 'leaky' GFP expression was observed in untreated tumors. Nevertheless these data demonstrate that expression of desired transgenes can be induced in established xenograft tumors.

Figure 6. Validation of inducible system *in vivo*. U373-eGFP cells were implanted sub-cutaneously in nude mice. Tumors were grown until they reached 300 mm³ after which the mice were left untreated or received doxycycline in their drinking water. Tumors were excised after 4 days and eGFP expression (top panel) was assessed *ex vivo*. Image of total tumor is shown in bottom panel.

Discussion

Tumor cells adapt to hypoxic conditions by activating molecular response pathways that include those regulating both mRNA translation (PERK/eIF2 α and mTOR/eIF4F) and transcription (HIF). Failure to execute this adaptive response program sensitizes tumor cells to hypoxia induced cell death. These pathways therefore provide interesting targets for the development of novel cancer therapies.

In this study we report the development of a novel tumor model designed to investigate the relative importance of different hypoxia response pathways. A major advantage of this system is that these pathways can be manipulated in established xenograft tumors and thus more closely mimic the clinical situation as compared to conventional xenograft models. This system allows us to assess the potential of

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targeting these different pathways as well as targeting different points in the oxygen signaling pathways. An important aspect in this regard is that the xenograft tumors are established with the pathway of interest intact and then are induced with doxycycline to express interfering RNAs or transgenes to inactivate the oxygen signaling pathway. This eliminates any potential disturbances (i.e. on tumor take or growth) before tumors have completely formed. Most studies have been complicated by directly injecting gene knockout cells or their wild type controls. This might also explain some of the controversial results obtained for HIF-1 α . Several groups have reported reduced tumor growth rates upon HIF-1 α loss whereas others describe the opposite effect¹⁷⁻¹⁹. The tumor microenvironment furthermore has a major impact on the outcome of HIF-1 α loss of function and potentially can affect other molecular targets in a similar manner²⁰.

Here we successfully engineered U373 and HCT116 tumor cell lines to accept transgenes at a single genomic location. We were able to interfere with PERK/eIF2 α , mTOR/eIF4F and HIF signaling by overexpression of either activating or inhibitory constructs (Figure 2). These three independent hypoxia responsive pathways each mediate different downstream processes and thus differentially influence hypoxic cell phenotype. Our data reveals that of these three pathways only HIF-1 α is important for maintaining proliferation rates *in vitro* under low oxygen tensions. This is in agreement with previously published data and might partly explain the reduced tumor growth rates of MEFs lacking HIF-1 α ^{17, 21}. In contrast to the effect on proliferation all three pathways proved to be important for maintaining survival after a hypoxic insult. Of all tested constructs, overexpression of dominant negative eIF2 α (S51A) was most potent in sensitizing cells to hypoxia induced cell death. From published experiments with PERK knockout cells it was already known that phosphorylation of eIF2 α is important for cell survival during hypoxia^{9, 14}. Our data support this and moreover suggest that there might be better ways to interfere with eIF2 α phosphorylation than targeting PERK.

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The importance of mTOR signaling to eIF4F through its ability to phosphorylate 4E-BP1 in determining hypoxia tolerance had not previously been investigated. However, clear evidence has been provided by several groups, including ours, that the mTOR pathway is inhibited upon exposure to hypoxia^{7, 12, 13}. Here we were able to show that interfering with the ability of hypoxia to suppress eIF4F via mTOR signaling indeed affects cell survival during hypoxia. However this only becomes evident after prolonged exposure periods (72 hr) in contrast to the relatively early effects we observe on cell survival when PERK/ eIF2 α or HIF signaling have been disrupted. This suggests that mTOR/ eIF4E might not be as important for cell survival during acute hypoxia as PERK/ eIF2 α and HIF signaling.

Interestingly, exposure to different levels of hypoxia did not alter the relative importance of a particular pathway in promoting hypoxia tolerance. This suggests that targeting PERK/eIF2 α or HIF signaling would be the most effective strategy at both extreme hypoxia (<0.02% O₂) and more moderate hypoxic (0.2% O₂) conditions. However, at this point we cannot rule out that one of the pathways might dominate cell survival at higher oxygen concentrations (1.0-2.0% O₂) or in the more relevant context of the tumor microenvironment. At these relatively higher oxygen tensions HIF-1 α is maximally activated and this therefore could shift the sensitivity more towards HIF targeting.

The complicated oxygenation status of solid tumors makes it virtually impossible to predict the best pathway from *in vitro* data. Tumor cells are exposed to acute, chronic and cycling levels of hypoxia as well as a range of different oxygen tensions^{15, 16, 22}. Since this complex situation cannot be replicated *in vitro* it will be crucial to determine the most effective pathway from *in vivo* animal experiments. Furthermore, the relative contribution of the different hypoxia response pathways to phenotypes associated with hypoxia such as metastasis, angiogenesis, metabolism and genomic instability are unknown. Here we demonstrate that our model system is suitable to address these issues. We provided proof of

principle that we can induce the expression of desired genes in established tumors using eGFP as a reporter. Animal experiments using xenograft tumors with defects in PERK/eIF2 α , mTOR/ eIF4E and HIF signaling are ongoing to resolve the relative importance of these pathways for hypoxia tolerance. Finally, these results will determine which of these pathways will be the best candidate to target in combination with other cancer therapies.

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CHAPTER 9

General discussion and summary

General discussion and summary

This thesis describes the consequences of regulating mRNA translation on gene expression during hypoxia. It has been known for more than a decade that hypoxia causes a profound drop in the rate of overall protein synthesis^{1, 2}. In 2002 and just prior to the start of this thesis it was found that this reduction in protein synthesis was due to a HIF independent, oxygen-sensitive pathway that results in direct inhibition of mRNA translation³. This initial publication also demonstrated that phosphorylation of eIF2 α plays an important role in the repression of overall mRNA translation. As described in the general introduction, eukaryotic translation is a highly regulated process which involves many eIFs and complicated signaling cascades. This fact and preliminary data from our lab led us to hypothesize that translational control is mediated through several distinct pathways during hypoxia.

In the study described in chapter 2 we were able to define the contribution of eIF2 α phosphorylation to translational control in hypoxic cells. Using MEF cells that are compromised in their ability to phosphorylate eIF2 α upon hypoxia, we showed that this pathway was responsible for the majority of the initial drop in protein synthesis, but that additional pathways contributed to translational repression especially during chronic exposure. As hypothesized, we found an additional hypoxia sensitive regulatory step in the cap-binding complex eIF4F. Our data shows that the formation of eIF4F is severely compromised during hypoxia by different mechanisms. First, prolonged exposure to hypoxic conditions leads to dephosphorylation of 4E-BP1. This hypophosphorylation results in an increased affinity for the cap-recognizing protein eIF4E. As a consequence, eIF4E is unable to participate in the formation of eIF4F and translation initiation is repressed.

Regulation of 4E-BP1 is controlled by the kinase mTOR which integrates signals from several upstream pathways. Arsham and colleagues have previously shown that 4E-BP1 hypophosphorylation is the result of mTOR inhibition during moderate hypoxia in combination with

serum deprivation⁴. In a recent paper Liu and colleagues demonstrate that moderate hypoxia causes repression of mTOR through activation of AMPK⁵. Strikingly, this only affects overall protein synthesis rates after hypoxic exposure periods of over 24 hours. From these studies it remains unclear what the relative contribution of mTOR inhibition to the overall inhibition of mRNA translation observed during hypoxia really is. This needs to be addressed in the following years. Likewise it will be interesting to establish the role of mTOR in hypoxia tolerance and hypoxia-induced gene expression.

The observation that disruption of eIF4F took place prior to complete dephosphorylation of 4E-BP1 led us to the discovery of a new interesting step in the regulation of eIF4F. We were able to show that eIF4F formation is hampered by translocation of eIF4E together with the shuttling factor 4E-T into the nucleus or to recently discovered cytoplasmic bodies of mRNA processing (P-bodies)^{6, 7}. Although the exact mechanism of how this takes place is unclear, our data suggests that dephosphorylation of 4E-T might be important. Thus, as hypothesized hypoxia leads to the activation of at least two distinct pathways that inhibit mRNA translation. A rapid, transient response through phosphorylation of eIF2 α and a prolonged effect through disruption of eIF4F.

In chapter 2 we also showed for a variety of genes that gene-specific translation is modulated by hypoxia. This data led us to our second hypothesis that translational control plays an important role in mediating rapid changes in gene expression upon hypoxia (chapter 4). To test this hypothesis we assessed the hypoxic phenotype after acute (1 h) and chronic (24 h) hypoxia using a proteomic approach. Remarkable changes in the expression of specific proteins were observed after only 1 hour of hypoxic exposure. Due to the short exposure time it is very unlikely that these changes are due to a transcriptional response. These data suggest that mechanisms other than transcription are responsible for the observed proteomic changes.

To investigate whether rapid changes in protein expression might be mediated through modulation of gene-specific mRNA translation we applied a technique that allowed us to assess translation efficiency on a genome-wide scale. For this purpose, both total mRNA as well as efficiently translated mRNAs from hypoxia exposed cells was hybridized to Affymetrix microarrays. Using a similar technique, Blais et al. had identified multiple preferentially translated genes during prolonged hypoxia (16h)⁸. As described above, at this time point translation inhibition is predominantly regulated through disruption of eIF4F. Since we were interested to determine genes affected by both eIF2 α and eIF4F regulation we performed a similar study after acute hypoxia (4 h) (chapter 4). We were able to show that of the top 50 genes with the largest induction during hypoxia, 20% displayed a selective ability to be translated⁹. This included several genes dependent on HIF-1 or involved in the UPR. These data showed that regulation of gene-specific mRNA translation contributes to the hypoxic proteome.

One important question that was raised by these data was what the relative contributions of transcriptional and translational control are on overall changes in gene expression during hypoxia. To address this issue we studied the effect of hypoxia on gene expression in more detail. Since the activation of these mechanisms during hypoxia are complex and operate with different kinetics (chapter 2), we extended our pilot study described above by assessing mRNA translation as a function of hypoxic exposure time (chapter 5). Our results reveal that translational regulation contributes predominantly to gene induction during acute hypoxia, whereas transcriptional control seems more important after prolonged hypoxic exposure. In contrast, translational control appears to be the main mechanism for down regulation of gene expression during hypoxia. These data suggest that translational control may be especially important during acute hypoxia. Translationally regulated genes reach a minimum after 8 hours of hypoxia, and then increase again with a kinetic pattern that is consistent with the disruption of eIF4F. Altogether our data

demonstrates that gene expression regulated through translational control during hypoxia is a highly dynamic process. This might be important for the adaptive response during acute hypoxia. Especially since oxygenation levels within the tumor can fluctuate dramatically over very short periods of time that are often too short to elicit effective transcriptional response¹⁰. Hypoxia down regulated mRNA translation rates of genes encoding for translation factors, ribosomal proteins as well as proteins involved in cell growth. We believe that this might lead to a more persistent inhibition of mRNA translation compared to the transient repression by eIF2 α phosphorylation and eIF4F disruption. Whether this might provide tumor cells with a survival mechanism to tolerate long term hypoxic stress needs to be further evaluated. On the other hand hypoxia stimulated mRNA translation of genes involved in signal transduction, metabolism and transcriptional regulation. The possibility that translational control regulates the transcriptional response during hypoxia is an interesting finding and will be worthwhile to explore in the future.

One of the genes that we characterized in more detail, Cited2, was identified as one of the top translationally regulated genes in our gene profiling studies (chapter 4 and 5). Cited2 is interesting because it had been proposed to act as negative regulator of HIF-1 transcriptional activity by antagonizing its interaction with coactivator CBP/p300^{15, 16}. In chapter 6 we provided the first clear evidence using HIF-1 α null cells that Cited2 is indeed a genuine HIF-1 α target and thus can act as postulated antagonist. We were able to validate the gene expression results and showed that the translational and transcriptional inductions mimic each other closely. This results in a net increase in Cited2 protein synthesis as the overall protein synthesis in the cell drops. To find an explanation for the preferential translation of Cited2 we analyzed the 5'UTR sequences from different species and discovered an evolutionary conserved uORF. As discussed earlier, this RNA element is essential for gene specific translation of ATF4 during hypoxia. We were able to show that Cited2 translation is not impaired in cells that have lost the ability to induce translation of uORF

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regulated genes like ATF4. Despite of this, our data shows that translational control regulates the expression of Cited2 during hypoxia and thus indirectly affects HIF-1 transcriptional activity. This study also indicates that cross-talk between transcriptional and translational changes during hypoxia may be more ubiquitous than previously recognized.

In chapter 7 we followed up on one of the leads we obtained from an ongoing screen in our laboratory, CA9. This screen involves characterization of UPR mediated changes in gene expression during hypoxia by transcriptional profiling of wild-type and eIF2 α S51A mutant MEFs. We identified CA9 as one of the top differentially regulated genes between the WT and S51A cells. Using different model systems we were able to show that expression of CA9 was dependent on the cells capacity to phosphorylate eIF2 α . We were able to show that PERK/eIF2 α translationally regulated gene ATF4 binds directly to the CA9 promoter and that this was associated with loss of the transcriptional repressive histone 3 lysine 27 (H3K27) tri-methylation mark. We furthermore confirmed the role of ATF4 in regulating CA9 during hypoxia by transient knock-down or over expression of ATF4. This study indicates that CA9 expression is mediated through independent activation of both the HIF-1 and UPR hypoxia response pathways. These data may have important implications for the use of CA9 as a diagnostic tumor marker.

In chapter 8 we described the development of a novel tumor model designed to investigate the relative importance of different hypoxia response pathways. The rational behind this new tumor model is that we can manipulate these hypoxia responsive pathways in established xenograft tumors. This model allows us to more accurately determine the importance of the various pathways compared to conventional xenograft models. Our *in vitro* data showed that PERK/eIF2 α , mTOR/eIF4F and HIF signaling independently contribute to hypoxia tolerance as their disruption significantly sensitized cells to hypoxia induced cell death. Finally, we demonstrated that this isogenic inducible system can be used to efficiently express transgenes in established xenograft tumors by administration of

doxycycline to the drinking water of tumor bearing mice. This enables us in the future to determine the relative importance of targeting these pathways *in vivo* in pre-established tumors in a manner that closely mimics the therapeutic situation.

Concluding remarks and future perspectives

Translational control has emerged as an important adaptive response to hypoxia by changing the hypoxic phenotype and increasing the cellular tolerance towards this condition. Modulation of mRNA translation affects essential hypoxia regulated transcription factors like HIF and ATF4. Consequently, this also causes changes in the expression of many downstream genes in an indirect manner. The discovery of the pathways that mediate translational control in response to hypoxia has provided insight into the link between translational control and hypoxia tolerance. However, as often new findings raise more research questions. One important question to resolve in the coming years is how specific mRNA species become preferentially translated during hypoxia. There is good evidence that suggests a pivotal role for the untranslated regions of mRNA transcripts in their ability to become translated. It will be interesting to identify RNA elements that stimulate mRNA translation on a genome-wide scale. For this it will be very useful to use genetic models in which particular translational control pathways have been disrupted. For example, the MEF S51A model is an excellent model to identify genes regulated by uORFs like ATF4, as its induction is abolished under hypoxia in S51A MEF cells compared to the wild type controls. However these kind of studies need further validation on an individual basis. This will be a difficult task, requiring a lot of effort. Even for a widely studied gene, like HIF-1 α which it has been proven difficult to unravel its translational regulation and it still remains partly unclear. In addition to IRES activity and uORFs it is has recently become clear that changes in translation of specific genes may also result from expression of a class of small non-coding RNAs called microRNAs (miRNAs)¹⁷. Interestingly, two recent

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studies have shown that hypoxia alters the expression pattern of known miRNAs, although the importance of these miRNAs and their targets on the hypoxic phenotype remain unknown^{18, 19}. An interesting hypothesis to investigate in the future is that regulation of miRNA expression during hypoxia contributes to changes in mRNA translation and that this is relevant for cell phenotype and tumor growth.

A second point of research will be to address to which degree translational control pathways are altered in cancer. This is important if we consider these pathways as new therapeutic targets. Especially for the eIF4F dependent response, since the upstream signaling pathways are often mutated in tumors. Furthermore, it will be interesting to sort out the relative importance of the various known hypoxia responsive pathways on cancer growth and treatment response in the context of a real tumor microenvironment. This will provide guidance in developing new types of therapies aimed at targeting tumor hypoxia and its cellular response. We made some progress on this by developing an inducible model system in which the different hypoxia response pathways can be inactivated. Efforts are currently underway to determine the relative importance of these pathways in xenograft tumor models. Although these issues remain to be addressed it certainly does not slow down the development of these novel therapeutic agents by the pharmaceutical industry. Finally the success of these new therapeutic drugs will be dependent upon their careful and logical introduction into the clinic. Since hypoxia is highly heterogeneous within the tumour it is crucial to consider targeting hypoxia only in combination with other traditional treatments.

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Algemene discussie en samenvatting

In dit proefschrift zijn de gevolgen van translationele regulatie op gen expressie tijdens hypoxie beschreven. Het is al meer dan tien jaar bekend dat hypoxie een grote daling in totale eiwit synthese veroorzaakt^{1,2}. In 2002, vlak voor de start van dit promotieonderzoek, werd bekend dat deze reductie in eiwit synthese door een HIF onafhankelijk, zuurstofgevoelig mechanisme wordt gereguleerd dat resulteert in een directe inhibitie van mRNA translatie³. Deze initiële publicatie demonstreerde ook dat fosforylatie van eIF2 α een belangrijke rol speelt in de remming van totale mRNA translatie. Zoals beschreven in de algemene introductie, is eukaryotische translatie een strict gereguleerd proces waarin vele eIFs en ingewikkelde signaal transductie routes een rol spelen. Dit feit en preliminaire data van ons lab hebben geleid tot de hypothese dat translationele regulatie wordt veroorzaakt door verscheidene mechanismen tijdens hypoxie.

In hoofdstuk 2 wordt een studie beschreven waarin de bijdrage van eIF2 α fosforylatie op translationele controle in hypoxische cellen wordt gedefinieerd. Door MEF cellen te gebruiken die niet in staat zijn om eIF2 α te fosforyleren tijdens hypoxie, konden we aantonen dat dit mechanisme grotendeels verantwoordelijk is voor de initiële daling in eiwit synthese. Er blijken echter additionele mechanismen actief die ook zorgen voor remming van mRNA translatie voornamelijk tijdens chronische blootstelling aan hypoxie. Zoals veronderstelt, hebben we een extra regulatie mechanisme gevonden in het cap-binding complex eIF4F. Onze data laat zien dat de formatie van eIF4F ernstig belemmerd wordt door verschillende mechanismen tijdens hypoxie. Ten eerste, een langdurige blootstelling aan hypoxische condities leidt tot defosforylatie van 4E-BP1. Deze hypofosforylatie resulteert in een verhoogde affiniteit voor het cap-herkende eiwit eIF4E. Het resultaat hiervan is dat eIF4E niet meer deel kan nemen in de formatie van eIF4F en de initiatie van translatie wordt geremd.

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De regulatie van 4E-BP1 wordt gecontroleerd door de kinase mTOR die signalen integreert van verschillende bovenliggende mechanismen. Arsham en collega's hebben al eerder laten zien dat 4E-BP1 hypofosforylatie het resultaat is van mTOR inhibitie tijdens matige hypoxie in combinatie met serum depletie⁴. In een recent onderzoeksartikel beschrijven Liu en collega's dat matige hypoxie onderdrukking van mTOR veroorzaakt door AMPK te activeren⁵. Opmerkelijk is dat dit alleen effect heeft op totale eiwit synthese na blootstelling aan hypoxische condities voor perioden langer dan 24 uur. Na deze studies blijft het onduidelijk wat de relatieve bijdrage van mTOR inhibitie op de totale remming van mRNA translatie waargenomen tijdens hypoxie werkelijk is. Verder onderzoek is nodig om dit te verduidelijken. Evenzo zal het interessant zijn om te onderzoeken hoe mTOR de gevoeligheid voor hypoxie en hypoxie-geïnduceerde gen expressie beïnvloed.

De observatie dat eIF4F desintegreerd voordat 4E-BP1 volledig gedefosforyleerd was leidde tot de ontdekking van een nieuwe interessante controle punt in de regulatie van eIF4F. We hebben kunnen aantonen dat eIF4F formatie wordt voorkomen door translocatie van eIF4E samen met de shutteling factor 4-ET naar de nucleus of naar de recent ontdekte cytoplasmatische bodies waar mRNA processing (P-bodies) plaatsvindt^{6,7}. Hoewel het exacte mechanisme nog onduidelijk is, wijst onze data erop dat defosforylatie van 4-ET belangrijk kan zijn. Derhalve, zoals veronderstelt leidt hypoxie tot de activatie van tenminste twee duidelijke mechanismen die mRNA translatie remmen. Een snelle, tijdelijke reactie door fosforylatie van eIF2 α en een langdurig effect door eIF4F desintegratie.

In hoofdstuk 2 hebben we voor verscheidene genen laten zien dat gen-specifieke translatie wordt gemoduleerd door hypoxie. Deze data heeft geleid tot onze tweede hypothese, dat translationele controle een belangrijke rol speelt in het veroorzaken van snelle veranderingen in gen expressie tijdens hypoxie (hoofdstuk 4). Om deze hypothese te testen hebben we bepaald wat het hypoxische fenotype is na acute (1 uur) en

chronische (24 uur) hypoxie. Opmerkelijke veranderingen in eiwit expressie werden al 1 uur na blootstelling aan hypoxie waargenomen. Vanwege de korte blootstellingstijd is het erg onwaarschijnlijk dat deze veranderingen tot stand komen door een transcriptionele inductie. Deze data duiden erop dat er andere mechanismen dan transcriptie verantwoordelijk zijn voor de waargenomen veranderingen in eiwit expressie.

Om te onderzoeken of snelle veranderingen in eiwit expressie veroorzaakt worden door veranderingen in gen-specifieke mRNA translatie, hebben we een techniek toegepast die ons in staat stelt om translatie efficiëntie op een genoomwijde schaal te bepalen. Voor dit doeleinde, werd zowel totaal mRNA als efficiënt getransleerd mRNA van cellen die blootgesteld waren aan hypoxie, gehybridiseerd op Affymetrix microarrays. Gebruik makend van dezelfde techniek, hebben Blais et al. meerdere specifiek getransleerde genen tijdens hypoxie (16 uur) geïdentificeerd⁸. Zoals hierboven beschreven, is op dit tijdpunt translatie inhibitie voornamelijk gereguleerd door desintegratie van eIF4F. Omdat we graag genen wilden identificeren die door zowel eIF2 α als eIF4F gereguleerd worden hebben we een vergelijkbare studie uitgevoerd na acute hypoxie (4 uur) (hoofdstuk 4). Hierin hebben we kunnen aantonen dat van de top 50 genen met de hoogste inductie tijdens hypoxie, 20% een selectief vermogen om getransleerd te kunnen worden lieten zien⁹. Hierbij inbegrepen waren meerdere genen die afhankelijk zijn van HIF-1 of die in rol spelen in de UPR. Deze data laten zien dat regulatie van gen-specifieke mRNA translatie bijdraagt aan het hypoxische fenotype.

De relatieve bijdrage van transcriptionele en translationele controle op de totale veranderingen in gen expressie tijdens hypoxie bleef onbekend. Om hier meer inzicht in te krijgen hebben we in meer detail gekeken naar de effecten van hypoxie op gen expressie. Aangezien de activatie van de mechanismen die mRNA translatie beïnvloeden tijdens hypoxie zeer complex is (hoofdstuk 2), hebben we ervoor gekozen om onze pilot studie, zoals hierboven beschreven uit te breiden door mRNA translatie te

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bepalen als functie van hypoxische blootstellingstijd (hoofdstuk 5). Onze resultaten tonen aan dat translationele regulatie voornamelijk een rol speelt bij gen expressie tijdens acute hypoxie, terwijl transcriptionele controle belangrijker is na langdurige blootstelling aan hypoxie. Verder lijkt translationele controle het belangrijkste mechanisme te zijn voor de onderdrukking van gen expressie tijdens hypoxie. Dit wijst erop dat translationele controle vooral belangrijk is tijdens acute hypoxie. Het aantal translationeel gereguleerde genen bereiken een minimum na 8 uur hypoxie en neemt dan weer toe met een kinetisch patroon dat gelijk is aan de desintegratie van eIF4F. Gezamenlijk toont onze data aan dat gen expressie gereguleerd door translationele controle tijdens hypoxie een zeer dynamisch proces is. Hierdoor kan het functioneren als een belangrijk aanpassings mechanisme voor tumor cellen, aangezien zuurstof concentraties in tumoren dramatisch kunnen veranderen over een heel korte tijd¹⁰. Onze data laat zien tijdens hypoxia mRNA translatie specifiek geremt wordt voor transcripten die coderen voor transcriptie factoren, ribosomale eiwitten en eiwitten betrokken bij cel groei. Dit zou mogelijk kunnen leiden tot een meer langdurigere inhibitie van mRNA translatie vergeleken met de tijdelijke remming veroorzaakt door verstoring van eIF4F en eIF2 α fosforylatie. Of dit tumor cellen in staat stelt om langdurige blootstelling aan hypoxische condities te overleven moet verder onderzocht worden. Aan de ander kant tonen onze resultaten aan dat hypoxia de mRNA translatie van genen stimuleerd die betrokken zijn bij signaal transductie, metabolisme en de regulatie van transcriptie. Dit suggereerd dat de transcriptionele respons tijdens hypoxia beïnvloed wordt door regulatie van mRNA translatie. Deze hypothese is zeer interessant voor verder onderzoek.

Een van de genen die we in meer detail bestudeerd hebben, Cited2, hebben we geïdentificeerd in onze microarray studies als een van de meest translationeel gereguleerde genen (hoofdstuk 4 en 5). Dit is zeer interessant aangezien Cited2 mogelijk de transcriptionele activiteit van HIF-1 negatief beïnvloed door de interactie met coactivator CBP/p300 te

verhinderen^{15, 16}. In hoofdstuk 6 tonen we eenduidig aan dat Cited2 mRNA expressie afhankelijk is van HIF-1 α en dat Cited2 dus als negatief respons mechanisme voor HIF activatie kan dienen. We hebben de microarray data succesvol gevalideerd en aangetoond dat Cited2 zowel transcriptioneel als translationeel in dezelfde mate geïnduceerd wordt. Dit resulteert in een netto toename van Cited2 eiwit synthese aangezien de totale eiwit synthese in de cel afneemt. Om deze preferentiele mRNA translatie te verklaren hebben wij de niet-coderende gedeeltes van het Cited2 transcript verder onderzocht. In het 5' gedeelte troffen we een evolutioneër geconserveerd uORF aan. Zoals eerder besproken stimuleert dit RNA element ondermeer translatie van de hypoxia gereguleerde transcriptie factor ATF4. Onze resultaten tonen echter aan dat het uORF in Cited2 niet de translatie bevordert zoals bij ATF4. Desondanks laat deze data zien dat de regulatie van mRNA translatie HIF-1 activiteit indirect beïnvloed via Cited2. Tevens blijkt hieruit dat transcriptionele en translationele veranderingen elkaar meer beïnvloeden dan voorheen gedacht.

In hoofdstuk 7 hebben wij het eiwit CA9 verder bestudeerd dat tijdens een nog voortdurende screening in ons laboratorium opgepikt is. Deze screening is gebaseerd op het bepalen van transcriptie profielen in WT en S51A MEF cellen. CA9 was een van de genen die een zeer groot expressie verschil vertoonde tussen de WT en S51A MEFs. In verschillende model systemen hebben wij laten zien dat CA9 expressie afhankelijk is van eIF2 α fosforylatie. We hebben kunnen aantonen dat de PERK/eIF2 α afhankelijke transcriptie factor ATF4 bind aan de promoter regio van CA9. De CA9 promoter toonde tevens een verlies van histon 3 lysine 27 (H3K27) trimethylatie aan, een histon modificatie die geassocieerd is met inhibitie van transcriptie. De betrokkenheid van ATF4 in de regulatie van CA9 hebben we verder gevalideerd door tijdelijke uitschakeling of overexpressie van ATF4. Onze studie toont aan dat CA9 expressie onafhankelijk gereguleerd wordt door HIF-1 α en de UPR. Deze resultaten

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zijn mogelijk van groot belang wanneer CA9 als een diagnostische tumor marker gebruikt zou worden.

In hoofdstuk 8 beschrijven we de ontwikkeling van een nieuw tumor model waarmee het belang van verschillende hypoxia respons mechanismen bepaald kan worden. In dit nieuwe tumor model kunnen deze mechanismen uitgeschakelt worden in reeds ontwikkelde xenograft tumoren. Dit stelt ons in staat om het belang van de verschillende hypoxia respons mechanismen beter te bepalen vergeleken met de conventionele xenograft modellen. Onze in vitro data toont aan dat PERK/eIF2 α , mTOR/eIF4F en HIF allen bijdragen aan de gevoeligheid voor hypoxische condities. Tot slot hebben we laten zien dat dit induceerbaar isogeen model gebruikt kan worden voor het tot expressie brengen van transgenen in reeds ontwikkelde xenograft tumoren. Hiermee kunnen we in de toekomst op een meer relevante manier bepalen welke van de verschillende hypoxia respons mechanismen geschikt is als aangrijpingspunt voor een mogelijk nieuwe therapie.

Conclusies en toekomst perspectieven

Regulatie van mRNA translatie manifesteert zich als een belangrijk mechanisme dat door tumor cellen gebruikt wordt om de gevoeligheid voor hypoxische condities te verminderen. Veranderingen in mRNA translatie hebben *rechstreeks invloed op belangrijke transcriptie factoren* zoals HIF en ATF4. Indirect beïnvloedt dit de expressie van een groot aantal genen die door deze transcriptie factoren gereguleerd worden. Het ontdekken van de mechanismen die mRNA translatie reguleren tijdens hypoxie heeft geleid tot nieuwe inzichten in de relatie tussen mRNA translatie en de *gevoeligheid voor hypoxische condities*. Natuurlijk leidt deze ontdekking tot nieuwe vraagstellingen. Zo zal het interessant zijn om te onderzoeken hoe specifieke mRNA transcripten actief getransleerd blijven terwijl translatie van het merendeel van mRNAs geremt is. Er zijn duidelijke aanwijzingen dat de niet-coderende gedeeltes van een mRNA

transcript hierin een rol spelen. Of er specifieke RNA elementen bestaan in de niet-coderende mRNA gedeeltes die mRNA translatie stimuleerd moet verder bepaald worden. Hiervoor zouden microarrays uitgevoerd kunnen worden met cel modellen waarin specifieke mRNA regulatie mechanismen uitgeschakeld zijn. Zo zou het MEF S51A model ideaal zijn om mRNA transcripten te identificeren die voor actieve translatie afhankelijk zijn van uORFs in hun niet-coderende mRNA. Translatie van deze mRNAs wordt namelijk niet geïnduceerd in de S51A MEFs vergeleken met de WT MEFs. Aangezien alle geïdentificeerde genen op individuele basis gevalideerd moeten worden zal dit veel tijd en inzet vergen. Zelfs voor veelvuldig bestudeerde genen zoals HIF-1 α is het moeilijk gebleken om het mechanisme dat translatie van HIF-1 α mRNA mogelijk maakt te bepalen en blijft het gedeeltelijk onbekend. Naast de reeds bekende IRES en uORF elementen is recent gebleken dat mRNA translatie ook beïnvloed kan worden door expressie van kleine, niet-coderende mRNA moleculen die microRNAs (miRNAs) worden genoemd¹⁷. Uit twee recente studies is gebleken dat de expressie van bekende miRNAs beïnvloed wordt door hypoxia^{18, 19}. Hoewel deze resultaten zeer interessant zijn blijft het momenteel onbekend of dit ook het hypoxische fenotype van de cel verandert. De gedachte dat veranderingen in miRNA expressie translatie van specifieke mRNA transcripten beïnvloed die van belang zijn voor het hypoxische fenotype en tumor ontwikkeling is een interessante hypothese om verder te bestuderen.

Een ander interessant onderwerp voor verder onderzoek is om te bepalen in hoe verre de mechanismen die mRNA translatie beïnvloeden veranderd zijn in tumoren. Informatie hierover is van groot belang als we nieuwe behandelwijzen willen baseren op deze mechanismen. In het bijzonder voor de eIF4F afhankelijke respons aangezien de bovenliggende signaal transductie routes vaak veranderd zijn in tumoren. Verder zal het belangrijk zijn om te achterhalen welk hypoxia respons mechanisme het meest interessant is om een nieuwe therapie op te richten. Belangrijk hierbij is dat dit bepaald wordt in de context van een echt tumor

micromilieu. Dit bevordert de ontwikkeling van een nieuw soort therapie die gericht is op de hypoxische cellen in een tumor. We hebben enige voortgang geboekt met de ontwikkeling van een induceerbaar xenograft tumor model systeem waarin de verschillende hypoxia respons mechanismen uitgeschakeld kunnen worden. Momenteel zijn er muizen studies aan de gang waarin de relevantie van deze mechanismen getest wordt. Ondanks dat er nog veel vragen onbeantwoord blijven is de ontwikkeling van nieuwe therapeutische middelen door de farmaceutische industrie in volle gang. Het succes van nieuwe therapeutische middelen zal afhangen van een rationele en zorgvuldige invoering in de kliniek. Vanwege de zeer heterogene aard van tumor hypoxia zal het aan te raden zijn om een dergelijke nieuwe therapie alleen te gebruiken in combinatie met traditionele behandelwijzen.

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Curriculum Vitae

Twan van den Beucken was born on March 17th, 1977 in Venlo, The Netherlands. In 1994 he started the study "Technical Microbiology" at the school of higher education in Venlo, where he in 1998 graduated "cum laude". In the period of 1998 to 2003 Twan worked for biotechnology companies Target Quest and Dyax as associate scientist and senior developmental scientist respectively. Twan started his PhD in July 2003 at the department of radiation oncology (Maastricht) within the University of Maastricht, to determine the effect of hypoxia on translational control, gene expression and cell survival in cancer. Twan is currently appointed as a post-doctoral fellow at the department of radiation oncology in Maastricht. As part of this appointment Twan is now working at the Princess Margaret Hospital/ Ontario cancer institute in Toronto, Ontario, Canada.

List of publications

Twan van den Beucken, Marianne Koritzinsky, Hanneke Niessen, Ludwig Dubois, Kim Savelkouls, Hilda Mujcic, Barry Jutten, Juraj Kopacek, Sylvia Pastorekova, Philippe Lambin, Willem Voncken, Kasper M.A. Rouschop and Bradly G. Wouters. Hypoxia-induced carbonic anhydrase 9 expression is dependent on the unfolded protein response. Submitted

Twan van den Beucken, Michael G. Magagnin, Barry Jutten, Renaud Seigneuric, Philippe Lambin, Marianne Koritzinsky and Bradly G. Wouters. Regulation of mRNA translation is a major contributor to hypoxia induced gene expression. Submitted

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