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Translational control is a major contributor to hypoxia induced gene expression

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ABSTRACT

Background and purpose: Hypoxia is a common feature of solid tumors that is associated with an aggressive phenotype, resistance to therapy and poor prognosis. Major contributors to these adverse effects are the transcriptional program activated by the HIF family of transcription factors as well as the translational response mediated by PERK-dependent phosphorylation of eIF2α and inhibition of mTORC1 activity. In this study we determined the relative contribution of both transcriptional and translational responses to changes in hypoxia induced gene expression.

Material and methods: Total and efficiently translated (polysomal) mRNA was isolated from DU145 prostate carcinoma cells that were exposed for up to 24 h of hypoxia (<0.02% O_2). Changes in transcription and translation were assessed using affymetrix microarray technology.

Results: Our data reveal an unexpectedly large contribution of translation control on both induced and repressed gene expression at all hypoxic time points, particularly during acute hypoxia (2–4 h). Gene ontology analysis revealed that gene classes like transcription and signal transduction are stimulated by translational control whereas expression of genes involved in cell growth and protein metabolism are repressed during hypoxic conditions by translational control.

Conclusions: Our data indicate that translation influences gene expression during hypoxia on a scale comparable to that of transcription.

The microenvironment of solid tumors is characterized by poor and heterogeneous oxygenation [1]. Clinical and experimental evidence associates deficiencies in oxygenation (hypoxia) with tumor development, growth, metastasis, and poor response to therapy [2–6]. Efforts have been taken to increase the efficacy of radiotherapy by modifying the hypoxic fraction [7]. Activation of multiple cellular adaptive pathways is essential to tolerate hypoxic stress and likely to form the basis for these adverse effects on tumor biology [8]. Insight into the regulation of these pathways is essential for the development of more effective anticancer therapies.

The best understood adaptive response to hypoxia is mediated by a family of hypoxia inducible transcription factors that regulate transcription of more than 60 genes involved in angiogenesis, glycolysis, pH regulation, invasion, and metastasis [9]. More recently, two other oxygen-sensitive pathways have been described that mediate changes in gene expression and affect important phenotypic tumor characteristics [8]. The first is regulated through activation of the unfolded protein response (UPR), a program of transcriptional and translational changes that takes place due to endoplasmic reticulum (ER) stress. Hypoxic exposure causes immediate inhibition of mRNA translation through transient phosphorylation of eukaryotic initiation factor 2α (eIF2α) at serine 51 by the ER kinase PERK in a diverse panel of cell lines from normal and neoplastic origin [10,11]. Deficiencies in PERK activation and eIF2α phosphorylation have important consequences for survival during hypoxic conditions [11,12] and overall tumor growth in xenograft models [13]. Recent studies have shown that a functional UPR is key for both maintaining autophagic flux [14–16] and pH homeostasis within the tumor microenviroment [17,18]. In addition activation of the UPR has also been linked to the expression of the metastasis associated gene LAMP3 [14,19]. The second recently described oxygen-sensitive pathway mediates inhibition of mTORC1 and results in hypophosphorylation of 4E binding protein 1 (4E-BP1) [12,20–23] and decreased protein synthesis due to repression of cap-dependent mRNA translation initiation [12,20–22]. Interestingly, preventing hypoxic regulation of this pathway by knockdown of 4E-BP1 altered mRNA translation of a small subset of genes and greatly decreased hypoxia tolerance [23,24].

Due to these various oxygen sensitive signaling pathways, gene expression changes in response to hypoxia occurrence through

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both transcriptional and translational changes [8,9,25–29]. However, while there have been many attempts to define the hypoxic transcriptome, the global contribution of translational control is unclear. To address this issue we compared the hypoxic transcriptome and translatome over a period of 2–24 h. Our data indicate that translational control is a highly dynamic process that contributes significantly 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% H_2, 5% CO_2 and residual N_2. 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

It was performed as described previously [27].

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 phospho(serine 51)-eIF2α (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

Three independent biological experiments were performed to assess gene expression during anoxia. Equal amounts of RNA from each experiment were pooled and affymetrix arrays were performed as described previously [27]. 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 translatome (heavy polysomal mRNA). The change in translation efficiency (ΔT.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. Gene ontology was performed using the software program EASE [30]. Genes with an EASE score lower than 0.1 were considered to be significantly enriched.

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). Consistent with previous results, exposure to hypoxia caused a rapid decrease in polysomal mRNA and a corresponding increase in free ribosomal subunits (Fig. 1A). The fraction of ribosomes involved in mRNA translation changes from ~75% to ~45% within 2 h of exposure to hypoxia and remained low for at least 24 h. This repression is even more pronounced (from ~50% to 25%) in the fraction of highly efficiently translated mRNAs (associated with five or more ribosomes) (Fig. 1B). This inhibition of translation correlated with changes in both the UPR and mTOR signaling pathways. Hypoxia caused transient phosphorylation of eIF2α in DU145 cells similar to that reported for other cell types, (Fig. 1C) [10,11]. The transient nature in eIF2α phosphorylation is the result of a negative feedback loop involving upregulation of GADD34 [31]. Longer hypoxic exposure caused inhibition of the mTORC1 kinase and resulted in dephosphorylation of its target 4E-BP1 similar to that in other cell lines [10,20,32]. In DU145 cells 4E-BP1 is gradually dephosphorylated over 16 h of hypoxia, from the hyper-phosphorylated form (γ) into its fast-migrating hypophosphorylated active form (α), which prevents eIF4F assembly by sequestering eIF4E (Fig. 1D).

Next we performed gene profiling experiments to determine how translational control affects hypoxia induced gene expression genome-wide. For this we analyzed total mRNA (transcriptional changes) or mRNA isolated from the heavy polysomal fraction, defined as containing 5 or more ribosomes (referred to as 'translational' changes). Translational changes are influenced by both changes in transcription and translation efficiency (i.e. the rate at which a specific mRNA is translated into protein) and are closely related to de novo protein production. Transcriptional and translational changes were assessed after exposure to hypoxia for 2, 4, 8, 12, 16 and 24 h. Fig. 2 shows dotplots for both transcriptional and translational (polysomal) mRNA expression for all hypoxic time points versus the aerobic control and illustrates the increased expression changes that occur as a function of time. As expected, transcriptional changes (total mRNA) are limited at early time points, with very few changes within 2 h of hypoxia. In contrast, the changes in translation appear to occur much earlier, as evidenced by a large number of changes in the polysomal mRNA already within 2 h.

To identify a robust set of hypoxia regulated genes at both the transcriptional and translational levels we filtered the data shown in Fig. 2 for genes demonstrating more than 3-fold change in expression at two consecutive time points. The number of genes that fulfill these criteria is indicated in Fig. 2 in each plot. At the transcriptional level, only 53 induced genes are identified after 2 h, and this rises steadily with exposure time affecting 350 genes after 16 h. A consistently smaller number of transcriptionally repressed genes are identified with these filter criteria, where we identified only 21 genes at 2 h and as many as 239 at 12 h. Interestingly, a much larger number of differentially expressed genes are identified at the translational level, particularly for the repressed set of genes. Within 2 h 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 h and then increased again dramatically reaching as many as 689 at 24 h. Consequently, translational control appears to exert a very strong influence on repressed genes during both acute (2 h) and chronic (16–24 h) hypoxic exposure.

Although it is clear from Fig. 2 that hypoxia regulates different numbers of genes at the transcriptional and translational levels, this analysis does not directly address the concordance between the identified gene sets. To assess the role of changes by translational control we compared changes in transcription and translation of all hypoxia induced (Fig. 3) and repressed (Fig. 4) genes in more detail. Fig. 3A shows the number of induced genes that were identified at the transcriptional or translational level as well as those identified at both levels. Interestingly, the number of genes identified as both transcriptionally and translationally induced is

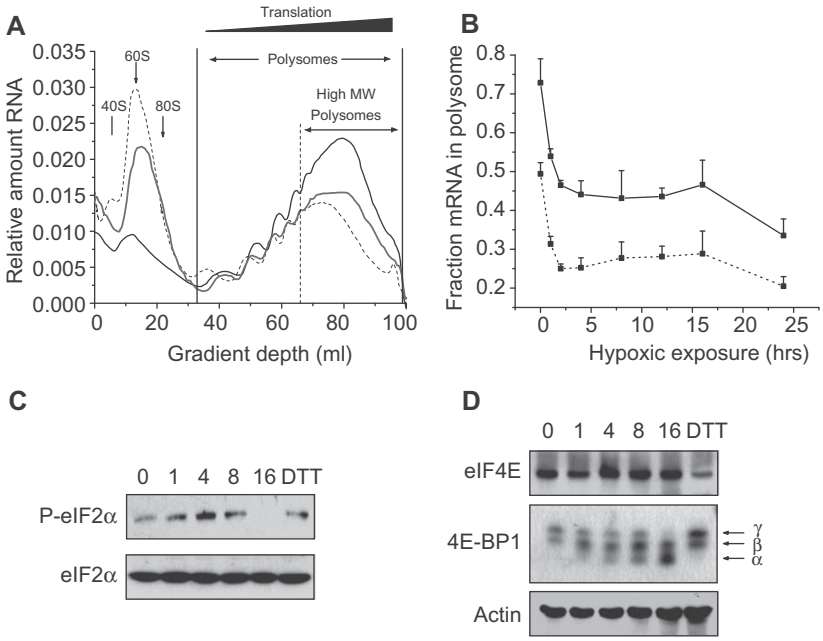

Fig. 1. Overall inhibition of mRNA translation during hypoxia. DU145 cells were exposed to 0% O_2 for 0–24 h and subsequently cell lysates were separated through sucrose gradients. (A) The optical density profiles at 254 nm as function of gradient depth are shown after hypoxic exposure for 0 (bold), 4 (dotted) and 16 (gray) h. (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. (C) Phosphorylated eIF2α with total eIF2α as a loading control. Treatment with 1 mM DTT for 1 h was used as a positive control for eIF2α phosphorylation. (D) 4E-BP1 with eIF4E and actin as loading controls. The SDS page resolves 3 4E-BP1 species (α, β, γ) representing hyper-, intermediate- and hypo-phosphorylated 4E-BP1, respectively.

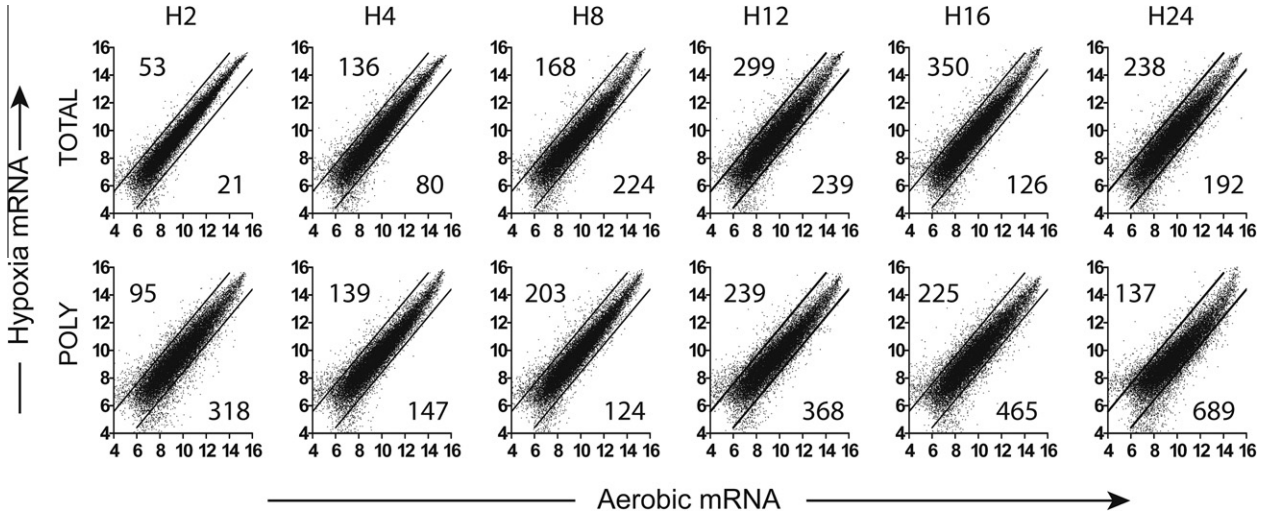

Fig. 2. Gene profiling of efficiently translated and total mRNA. Logarithm of raw intensities for each probe set under hypoxia versus normoxia is shown for total mRNA and efficiently translated mRNA. The number of genes showing more than 3-fold change in expression at two consecutive time points is indicated in each plot. The diagonal upper and lower lines represent threshold for 3-fold change in expression.

relatively small, with only 27 genes meeting these criteria at 2 h. This group of co-regulated genes increases over time to a maximum number of 153 genes after 16 h of hypoxia. The percentage of translationally induced genes exhibiting transcriptional co-regulation increases from 28% at 2 h to 74% at 24 h 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 Fig. 3A 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 are 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 (ΔT.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 ΔT.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 Fig. 1). Fig. 3B shows the

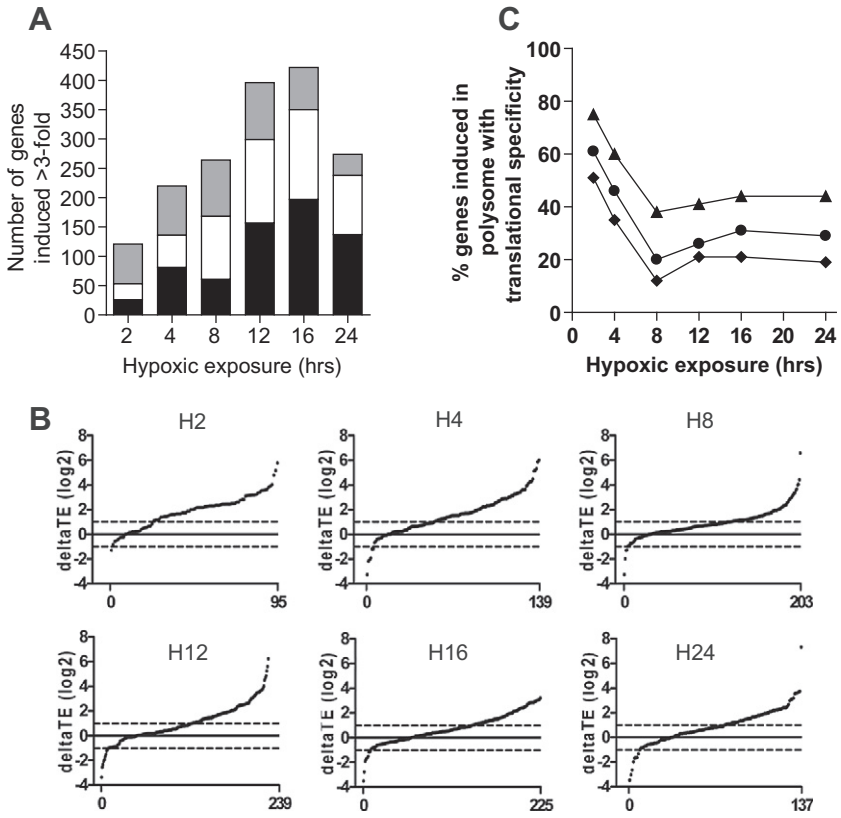

Fig. 3. Analysis of hypoxia induced genes. (A) The number of genes showing more than 3-fold increase in expression at two consecutive time points at the total mRNA level (black), the polysomal level (gray) or both (white) was determined and plotted as a function of hypoxic exposure. (B) The changes in T.E. (log 2 values) were determined as the ratio of polysomal signal to total signal for each gene at the hypoxic time point was divided by the same ratio for that gene under aerobic conditions. Threshold levels for no change in T.E. (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 ΔT.E. > 2 (▲), ΔT.E. > 3 (●), ΔT.E. > 4 (♦).

calculated ΔT.E. values, in ascending order, for all translationally regulated genes. At all time points, the majority of induced genes demonstrate a higher ΔT.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 degree of increase in ΔT.E. are highest at early time points (2–4 h). In Fig. 3C, we have plotted the percentage of genes that demonstrate a 2-, 3- or 4-fold increase in translational efficiency. Nearly 80% of the induced genes in the polysome double their T.E. after a 2 h exposure to hypoxia compared to aerobic conditions and 50% have an increase of 4-fold or more.

The same type of analysis was performed on the identified sets of down-regulated genes. Fig. 4A shows the number of identified repressed genes at the level of transcription, translation or both. These data show that changes in translation play an even larger role for repression than for induction. After 2 h of hypoxia, the expression of over 318 genes is more than 3-fold repressed in the polysomal fraction, and in sharp contrast to the 21 genes repressed at the transcriptional level. Although a slight recovery in translational repression is observed after 8 h (124 genes affected), this increases again at late time points affecting a maximum number of 601 genes after 24 h of hypoxia. Transcriptional repression is not observed for the majority of translationally repressed genes, with the exception of the 8hr time point (71%), where overall changes in translation were comparably smaller.

Since transcription did not appear to account for the majority of translationally repressed genes, we also calculated ΔT.E. values for all translationally repressed genes (shown in ascending order in Fig. 4B). Most exhibit a lower ΔT.E. than the average indicating selective translational repression. The percentage of genes demonstrating a 2-, 3-, or 4-fold decrease in translation efficiency is shown in Fig. 4C. After 2 h of hypoxia, nearly 100% of the translationally repressed genes demonstrate a ΔT.E. below 0.5, and 70% even below 0.25.

To determine whether translational control influences important or unique biological processes we performed gene ontology (GO) analysis. Enrichment of particular GO terms was determined for both the translationally induced genes (>2-fold induced and ΔT.E.>2) and translationally repressed genes (>2-fold repressed and ΔT.E.<0.5) at early (2–8 h) and late (12–24 h) hypoxic time points Transcription, signal transduction and protein transport are selectively affected by translational control during acute hypoxia whereas prolonged exposure mainly influences metabolic processes (Supplementary Table 1 and 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 from these categories 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 efficiency include the themes transport, steroid metabolism, cell growth and protein metabolism during acute hypoxia and protein metabolism, RNA metabolism and mitotic cell cycle during prolonged hypoxia (Supplementary Table 3 and 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.

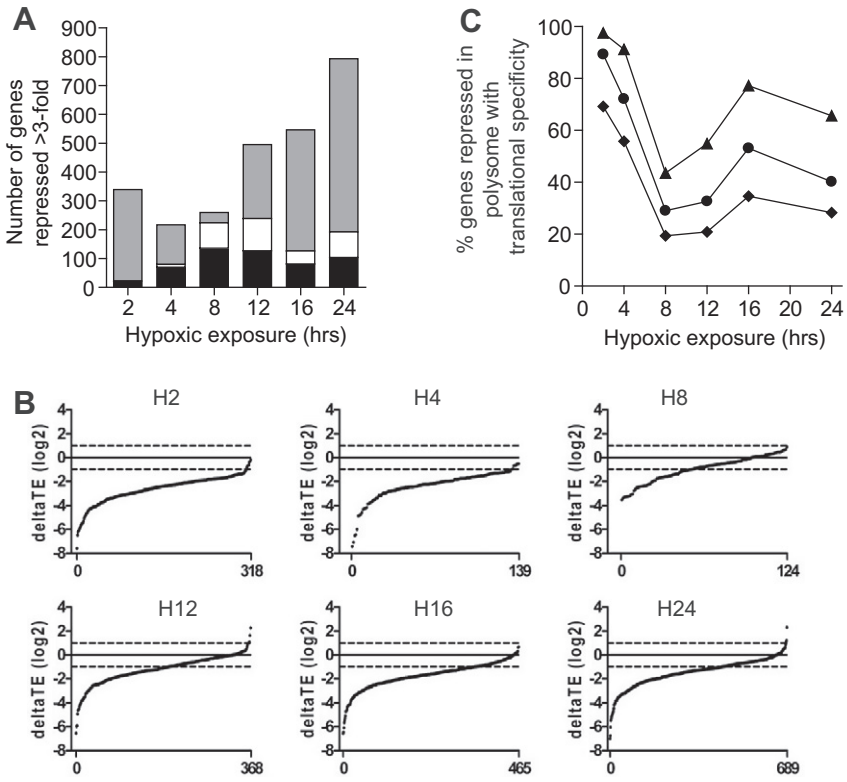

Fig. 4. Analysis of hypoxia repressed genes. (A) The number of genes showing more than 3-fold decrease in expression at two consecutive time points at the total mRNA level (black), the polysomal level (gray) or both (white) was determined and plotted as a function of hypoxic exposure. (B) The changes in T.E. (log 2 values) were determined as the ratio of polysomal signal to total signal for each gene at the hypoxic time point was divided by the same ratio for that gene under aerobic conditions. Threshold levels for no change in T.E. (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 ΔT.E. < 0.5 (▲), ΔT.E. < 0.33 (●), ΔT.E. < 0.25 (♦).

Discussion

It is becoming increasingly clear that translational control mechanisms play key roles in regulating gene expression under conditions that are relevant in cancer, including oncogene activation [33], radiation [34], and hypoxia [25–27]. Although these studies have undoubtedly shown that a substantial number of genes can be preferentially translated during stressful conditions, it remains unclear to what extent translational mechanisms contribute to differential gene expression. To address this point we compared the hypoxic transcriptome and translatome over a period of 2–24 h. 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 h of exposure to hypoxia only 28% of translationally induced genes show signs of transcriptional co-regulation, whereas this increases over time to 74% after 24 h. Interestingly, translational control appears to be the main mechanism for down-regulation of gene expression during hypoxia, over 90% of the down-regulated genes during acute hypoxia are repressed as a consequence of translational control.

A second key finding 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 h and then increases and stabilizes at ~44%. A similar effect is observed for the repressed genes where after 2 h of hypoxia nearly all of the translationally regulated genes exhibit over 2-fold reduction in translation efficiency. This translational repression is somewhat smaller after 8 h but becomes dominant again after prolonged hypoxic exposure. This kinetic picture of translational control extends earlier results from our lab from a single time point [27]. The percentage of translationally enhanced genes showing preferential regulation drops to a minimum after 8 h (less than 20% showing a 2-fold increase). Similarly, preferential regulation of the repressed genes is highest at early and late time points, reaching a minimum after 8 h of hypoxic exposure. These kinetics correlate well with transient phosphorylation of eIF2α at early time points and the disruption of eIF4F at prolonged hypoxia. Preferentially translated mRNAs during acute hypoxia are less dependent on eIF2α availability while efficiently translated mRNAs after longer periods of hypoxia are less dependent on eIF4F. It will be interesting to determine the individual contributions of both pathways to translational control in more detail. This would require similar profiling of transcriptional and translational changes during hypoxia in cell lines defective in either pathway and these experiments are underway.

We performed gene ontology analysis on the selectively repressed and induced translated genes during acute and prolonged hypoxia in order to provide insight into the mechanisms by which translational control may 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 genes [35]. 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 h 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 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 [27,36].

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.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.radonc.2011.05.058.

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