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Molecular radiobiology

Deregulation of cap-dependent mRNA translation increases tumour radiosensitivity through reduction of the hypoxic fraction

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ABSTRACT

Background and purpose: Tumour hypoxia is an important limiting factor in the successful treatment of cancer. Adaptation to hypoxia includes inhibition of mTOR, causing scavenging of eukaryotic initiation factor 4E (eIF4E), the rate-limiting factor for cap-dependent translation. The aim of this study was to determine the effect of preventing mTOR-dependent translation inhibition on hypoxic cell survival and tumour sensitivity towards irradiation.

Material and methods: The effect of eIF4E-overexpression on cell proliferation, hypoxia-tolerance, and radiation sensitivity was assessed using isogenic, inducible U373 and HCT116 cells.

Results: We found that eIF4E-overexpression significantly enhanced proliferation of cells under normal conditions, but not during hypoxia, caused by increased cell death during hypoxia. Furthermore, eIF4E-overexpression stimulated overall rates of tumour growth, but resulted in selective loss of hypoxic cells in established tumours and increased levels of necrosis. This markedly increased overall tumour sensitivity to irradiation.

Conclusions: Our results demonstrate that hypoxia induced inhibition of translational control through regulation of eIF4E is an important mediator of hypoxia tolerance and radioresistance of tumours. These data also demonstrate that deregulation of metabolic pathways such as mTOR can influence the proliferation and survival of tumour cells experiencing metabolic stress in opposite ways of nutrient replete cells.

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Solid tumour microenvironments are characterized by extreme heterogeneities in oxygenation. The percentage of viable hypoxic cells within individual tumours with otherwise similar clinical features varies tremendously among patients [1]. This is clinically important because high levels of tumour hypoxia correlate with poor prognosis and a more aggressive phenotype [1,2]. Hypoxia modifying approaches are therefore desired [3] and used to improve recurrence after radiotherapy [4].

The mechanisms responsible for promoting hypoxia tolerance in solid tumours are only partially understood. These include activation of the HIF transcription factors (HIF1, HIF2, HIF3), which induce a large number of genes involved in glycolysis, angiogenesis, pH

regulation and cell motility [5,6]. At least two other pathways have been implicated in promoting adaptation to low oxygenation [7]. This includes activation of the unfolded protein response (UPR), an evolutionarily conserved pathway that responds to endoplasmic reticulum (ER) stress, consisting of three parallel pathways mediated by, respectively, PERK, IRE1 and ATF6 signalling. These pathways mediate changes in transcription and translation that promote adaptation to ER stress through multiple mechanisms [7]. For example, PERK signalling is essential in the survival of hypoxic cells by preserving the cells' capacity to maintain high rates of autophagy [8]. Abrogation of PERK-mediated signalling results in loss of autophagic capacity and sensitizes cells to hypoxia induced cell death. Additionally, UPR-deficiency leads to decreased carbonic anhydrase 9(CA9)-expression, an endogenous marker for hypoxia [9] and leads to reduced survival in acidic microenvironments [10] and promotes expression of metastases related genes [11]. Finally, hypoxia results in the inhibition of mTOR (mammalian target of rapamycin) [7,12], a central regulator of cellular metabolism. mTOR integrates and transmits signals from a diverse array of

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signalling and nutrient sensing pathways to regulate cell growth and survival. Inhibition of mTOR under hypoxia prevents formation of the eIF4F translation initiation complex and thus suppresses protein synthesis. Under nutrient replete conditions, translation of eukaryotic mRNAs commences with the assembly of eIF4F at the m7GpppN cap structure of the mRNA. The limiting factor in the eIF4F complex is eIF4E, levels of which are 10- to 30-fold lower than other initiation factors [13]. Regulation of eIF4E is achieved through changes in the phosphorylation of eIF4E binding proteins (4E-BPs). Under nutrient replete conditions, the 4E-BPs are hyperphosphorylated by mTOR and do not bind eIF4E [14] reflecting high rates of translation initiation. During starvation or hypoxia mTOR activity is reduced causing hypophosphorylation of the 4E-BPs which increases their affinity for eIF4E and thereby cap-dependent translation is inhibited [15]. eIF4E is often overexpressed in human tumours promoting malignancy, recurrence of the tumour after treatment and predicting poor outcome for patients, an observation made in several tumour types [16]. Conversely, overexpression of eIF4E is capable of transforming cells *in vitro* and facilitates proliferation and tumour growth [17]. Previously, we have shown that constitutive loss of 4E-BP1 expression, and thus loss of eIF4E regulation, sensitized cells to hypoxia, reduced the viable hypoxic fraction within tumours and subsequently sensitized tumours to irradiation [18].

We hypothesized that eIF4E overexpression and thus reversing the inhibitory effect on translation mediated by reduced mTOR functioning during hypoxia, would render the cells less capable to adapt to hypoxia. Furthermore, that this would lead to increased hypoxic cell killing, a reduction in tumour hypoxia and increased sensitivity of tumours to irradiation. To test this hypothesis, we engineered isogenic cells that inducibly overexpress eIF4E upon tetra- or doxycycline exposure. These isogenic models allowed us to mimic the therapeutic situation of targeting mTOR-regulation during hypoxia and compare the temporal regulation of eIF4E before and after irradiation.

Materials and methods

Cells and equipment

Our studies included U373-MG (glioblastoma-astrocytoma) and HCT116 (colorectal adenocarcinoma) cells that were maintained as described by the American Type Culture Collection and as described previously [19]. Cells were engineered to induce eIF4E after exposure to tetracycline (invitrogen) or doxycycline (Sigma) by using a flip-in T-rex system according to the manufacturers' instructions (invitrogen). For hypoxia exposure cells were transferred to a hypoxic culture chamber (MACS VA500 microaerophilic workstation, Don Whitley Scientific, Shipley, UK). In the clonogenic survival experiments, tetracycline was removed from the culture medium during the colony formation period. The EcoRI restriction site within the hygromycin resistance gene of pCDNA5/FRT/TO was removed by PCR amplification using the primers se: CTTCCTTCTCGCCACGTTTC; as: CCGCGCTGGAGGAT-CATCCA; mut: TTGACATTGGGGAGTTTAGC and reintroduced into the flanking NGOMIV restriction site, creating pCDNA5(+)/FRT/TO. eIF4E was cloned into the shuttle vector, pCDNA5(+) between EcoRI and XhoI.

Western blotting

Western blotting was performed as described previously [20]. Protein transfers were probed with antibodies directed against eIF4E (Cell Signalling Technologies) or β -actin (Sigma). Bound antibodies were visualized using HRP-linked secondary antibodies (Cell Signalling Technologies) and ECL luminescence (Pierce).

Tumour model

For *in vivo* experiments, U373 or HCT116 cells were subcutaneously injected in NMRI-*nu* (*nu/nu*) female mice (28–32 g). When and if applicable, mice received doxycycline added to their drinking water (2 g/L in 5% sucrose). Tumours were irradiated (15 Gy (U373) or 10 Gy (HCT116), 16 MeV) with a tumour specific dose. For immunohistochemical analysis, mice were killed after 7 days of doxycycline treatment. To visualize viable hypoxic cells, animals were injected with pimonidazole (60 mg/kg I.P. (hypoxyprobe-1, Chemicon, Temecula, CA, USA) 60 min prior to killing. All animal experiments were in agreement with national guidelines, approved by the Animal Ethics Committee of the University of Maastricht, the Netherlands.

Immunohistochemistry and image processing

Frozen, acetone fixed sections were stained using anti-pimonidazole (Chemicon, Chandlers ford, UK), 9F1 (rat monoclonal antibody to mouse endothelium, Department of Pathology, Radboud University Nijmegen Medical Center, The Netherlands). For quantitative analysis, the slides were scanned by a computerized digital image processing system using a high-resolution intensified solid-state camera on a fluorescence microscope (Zeiss Axioskop) with a computer-controlled motorized stepping stage. Tumour necrosis was evaluated morphologically using hematoxylin and eosin (H&E) staining.

Statistics

Data were analysed with Graphpad Prism, using a repeated measures ANOVA with a bonferroni *post hoc* test. Survival curves were generated using the Kaplan–Meier method. As end-point, reaching 4 times the initial or irradiated volume was chosen. Tumour growth was followed up to 90 days after irradiation. Data were considered statistically significant when $p < 0.05$.

Results

We and others have shown previously that adaptation to hypoxia occurs in part through inhibition of eIF4E activity and thus initiation of mRNA translation. To directly investigate the role of eIF4E activity on cell growth and hypoxia tolerance, we developed isogenic U373 and HCT116 [21] cells that overexpress eIF4E in a tetracycline/doxycycline inducible manner. Exposure to tetracycline or doxycycline induced the basal expression of eIF4E in both lines (Fig. 1A). eIF4E has been described to transform cells *in vitro* and facilitate proliferation [17]. As expected eIF4E overexpressing U373 cells displayed increased proliferation under normal conditions compared to empty vector control cells (pCDNA5(+)) (Fig. 1B, left panel, $p < 0.01$). As shown previously [8], control cells exposed to moderate hypoxia (0.2%) show a reduction in proliferation (Fig. 1B, right panel). Surprisingly the growth advantage of eIF4E overexpressing U373 cells seen under ambient oxygen was abrogated in moderate hypoxia. To explore whether this decrease was due to increased cell death, we assessed hypoxia induced changes in clonogenic survival. Exposure to either moderate or severe hypoxic (<0.02%) conditions resulted in a significant reduction in colony formation in cells induced to overexpress eIF4E compared to empty vector controls (Fig. 1C, $p < 0.05$ for both). Although delayed, the sensitization effect of eIF4E-overexpression was also observed in HCT116 cells (Supplementary Fig. 1). The observed delay may be a consequence of higher endogenous eIF4E in HCT116 compared to U373 (data not shown), and, therefore, a lower proportional overexpression and less severe disruption of the balance between eIF4E and its inhibitors.

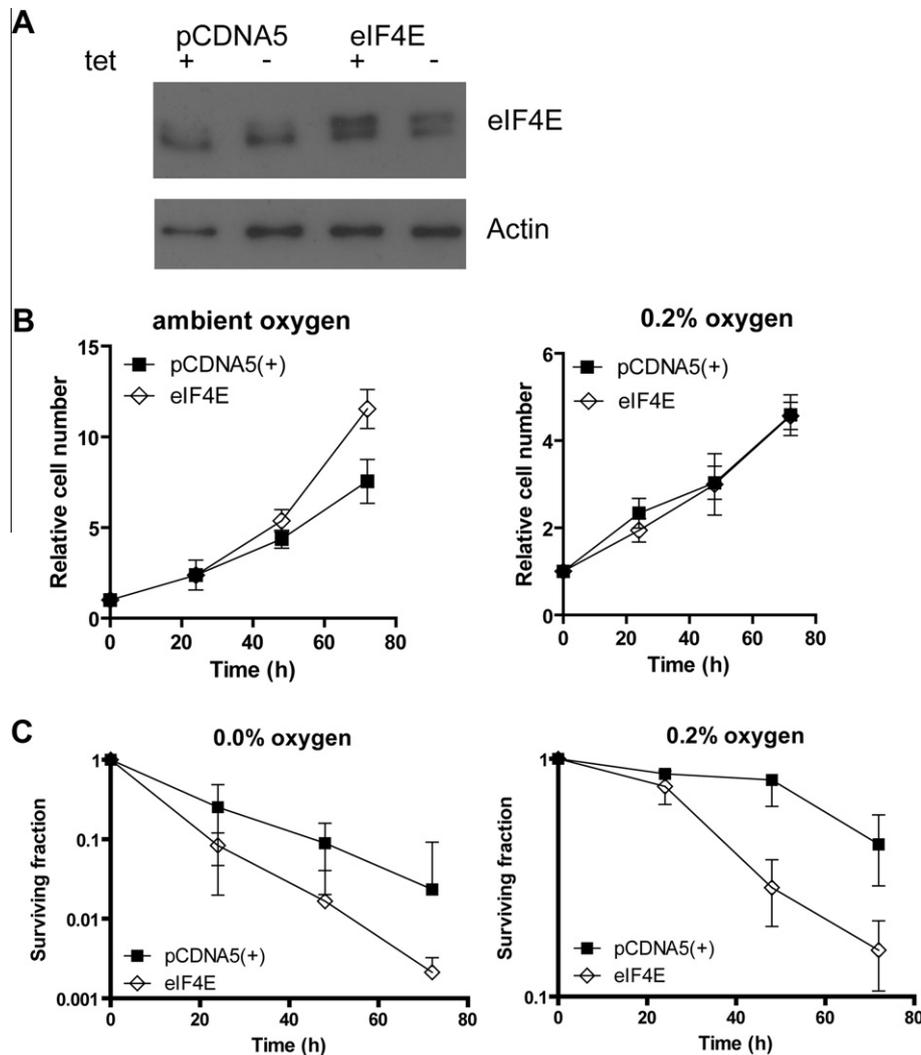


Fig. 1. eIF4E over expression sensitizes cells to hypoxia. (A) Isogenic flip-in T-rax U373 cells with tetra-/doxycycline inducible empty vector (pCDNA5(+)) or eIF4E expression were cultured in the presence or in the absence of 1 μ g/ml tetracycline for 24 h. eIF4E induction was assessed by immunoblotting. (B) After induction (24 h) cellular proliferation was determined under ambient (21%, left panel) oxygen or hypoxia (0.2%, right panel) in the presence of 1 μ g/ml tetracycline for 3 days (mean \pm SEM, $n = 3$). (C) Clonogenic survival of cells exposed to <0.02% oxygen (left panel) or 0% oxygen (mean \pm SEM, $n = 3$).

The contrasting changes in cell proliferation and survival observed in aerobic and hypoxic cells following induction of eIF4E prompted us to assess this effect *in vivo*. Utilization of inducible cell lines also allowed us to compare the effects of constitutive eIF4E overexpression with that of transient overexpression in established tumours. Consistent with the increased proliferation observed in aerobic cells *in vitro*, overexpression of eIF4E prior to and following implantation significantly increased the onset of palpable tumours (above 60 mm³) (pCDNA5(+) +dox vs eIF4E +dox and eIF4E –dox vs eIF4E +dox, $p < 0.05$) (Fig. 2A and B). Consistent with this observation, the growth of eIF4E expressing tumours (doxycycline treated) once established was also significantly increased compared to the uninduced controls or the empty vector controls with or without doxycycline (Fig. 2C, pCDNA5(+) +dox vs eIF4E +dox and eIF4E –dox vs eIF4E +dox, $p < 0.05$). These results are in line with previous reports indicating that overexpression of eIF4E is sufficient to transform cells in culture and increase the rate of tumour growth in breast, head and neck, colon, cervical and bladder cancers [16].

To distinguish potential differences between the role of eIF4E in tumour establishment/initiation and tumour proliferation, we examined the consequences of eIF4E overexpression after tumours

had established. Similar to that observed during constitutive expression, induction of eIF4E through administration of doxycycline in established tumours also resulted in a significant acceleration in tumour growth (Figs. 2D and 4C (–dox vs +dox, $p < 0.05$), suggesting that the effect seen on tumour establishment is due to increased proliferation of the cells rather than initial changes in the tumour microenvironment. To confirm overexpression of eIF4E after addition of dox to the drinking water of mice, tumours were harvested after seven days doxycycline and assessed for eIF4E by immunoblotting. As expected eIF4E was overexpressed compared to the empty vector control (Fig. 2E).

In contrast to the growth promoting effects observed under normoxic conditions, cells overexpressing 4E were sensitized to hypoxia-induced cell death *in vitro* (Fig. 1C). Thus, we examined changes in the fraction of viable hypoxic cells in tumours following induction of eIF4E. The fraction of hypoxic cells was determined in established tumours treated with or without doxycycline to overexpress eIF4E for seven days. Remarkably these data indicate that this transient increase in eIF4E resulted in a 25% reduction in hypoxic fraction of the viable (non-necrotic) tumour area (Fig. 3C, $p < 0.05$). Furthermore, eIF4E overexpression was associated with an approximate 1.5-fold increase in tumour necrosis (Fig. 3B and

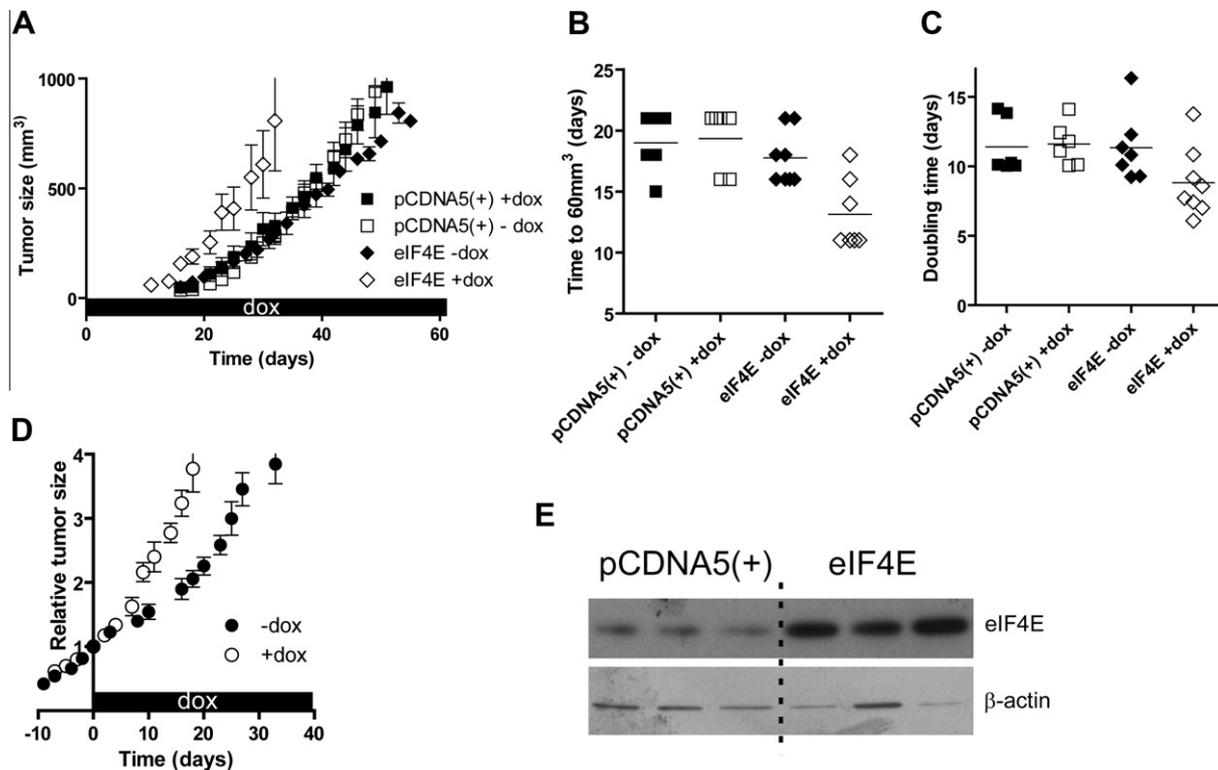


Fig. 2. eIF4E overexpression increases tumour growth. (A) U373 isogenic cells harbouring doxycycline-inducible genes (empty vector or eIF4E) were injected subcutaneously in nude mice. When indicated (+dox), the transgene was induced 24 h before implantation and the mice received doxycycline (2 g/L) for the whole duration of the experiment (mean \pm SEM, pCDNA5(+)+dox, $n = 6$ -dox $n = 6$, eIF4E -dox $n = 7$, eIF4E +dox $n = 8$). (B) Graphical representation of palpability ($>60 \text{ mm}^3$) of the tumours. (C) Doubling time of the individual tumours. (D) U373 isogenic cells harbouring doxycycline-inducible eIF4E were injected subcutaneously in nude mice. When tumours were established and reached a size of 150 mm^3 , mice were fed doxycycline (2 g/L) to induce the eIF4E. Tumour size was assessed as a function of time (mean \pm SEM, -dox $n = 7$, +dox $n = 8$). (E) After 7 days tumours were harvested and assessed for eIF4E induction by immunoblotting (each lane represents an individual tumour).

D, $p < 0.05$). In line with the *in vitro* results, these data are suggesting increased hypoxic cell death *in vivo*. Comparable results on reduction of the hypoxic tumour fraction were obtained in HCT116 xenografts (Suppl Fig. 2).

Tumour hypoxia is an important limiting factor in radioresponsiveness of tumours. To determine if the reduction of hypoxia in tumours with overexpressed eIF4E as assessed by pimonidazole is sufficient to change the response to radiotherapy, tumour bearing mice received doxycycline in their drinking water for 7 days after reaching a tumour size of 150 mm^3 . After 4 days of doxycycline exposure the xenografts were irradiated with a tumour specific dose of 15 Gy on U373 tumours and 10 Gy on the relatively less radioresistant HCT116 tumours and regrowth of the tumours was followed over time. The growth delay of tumours to this large single dose is primarily determined by the fraction of viable hypoxic cells in the tumour as these cells require up to 3-fold more radiation to achieve a comparable degree of cell killing in comparison to well-oxygenated cells. The given dose resulted in a significant tumour growth delay in comparison to unirradiated tumours (Fig. 2D) for both controls and eIF4E-overexpressing tumours (Fig. 4A). Strikingly, of the 8 irradiated eIF4E tumours 4 failed to regrow during the follow up of 3 months after irradiation, compared to regrowth of all the control tumours during the identical period. As a measure for radioresponsiveness, we calculated the time that was required to reach 4 times the irradiated volume and plotted as survival analysis, thereby visualizing the effect of irradiation on tumour regrowth and the sensitizing effect of eIF4E overexpression (-dox 15 Gy vs dox 7 days 15 Gy $p < 0.01$). The experiment was terminated after 90 days regrowth. The sensitizing effect of eIF4E-overexpression was also observed, although less dramatic, in HCT116 (Supplementary Fig. 3).

Although we observed a large difference in radiation response of the eIF4E-overexpressing tumours, consistent with the observed

reduction in hypoxic fraction, the increased radioresponsiveness could also be a result in changes in intrinsic radiosensitivity of the cells. Therefore, we measured radiosensitivity of control and eIF4E-overexpressing cells under ambient oxygen or anoxic conditions. No differences in radiosensitivity were observed (Fig. 4D). These data indicate that the observed effect of pre-irradiation overexpressed eIF4E on radiosensitivity of the tumour is due to changes in tumour hypoxia and the decreased viable tumour mass, rather than eIF4E-overexpression as a radiosensitizer.

In several types of cancer eIF4E is constitutively overexpressed and associated with a poorer prognosis and faster regrowth of the tumour after treatment [16]. As we observed the increased sensitivity of eIF4E tumours to irradiation (Fig. 4A) and also faster growth (Fig. 2A and D), we questioned what the consequences of eIF4E overexpression post-irradiation would be. At a size of 150 mm^3 inducible eIF4E tumours were irradiated with a single dose of 15 Gy. Half of the mice received doxycycline in their drinking water after irradiation till the end of the experiment and tumour regrowth was followed over time. As expected, the tumours overexpressing eIF4E regrew faster than the tumours that temporarily overexpressed eIF4E and even partly abolished the effect on tumour growth delay mediated by irradiation (Fig. 4B and C (-dox 15 Gy vs dox after irradiation (15 Gy) $p < 0.001$). Thus, the improvement in tumour response is limited to conditions where eIF4E is overexpressed prior to treatment and thus capable of selectively killing the therapeutically resistant hypoxic cells.

Discussion

We found that eIF4E-overexpression facilitates growth of normoxic cells, as also reflected in tumour growth, but sensitized cells to

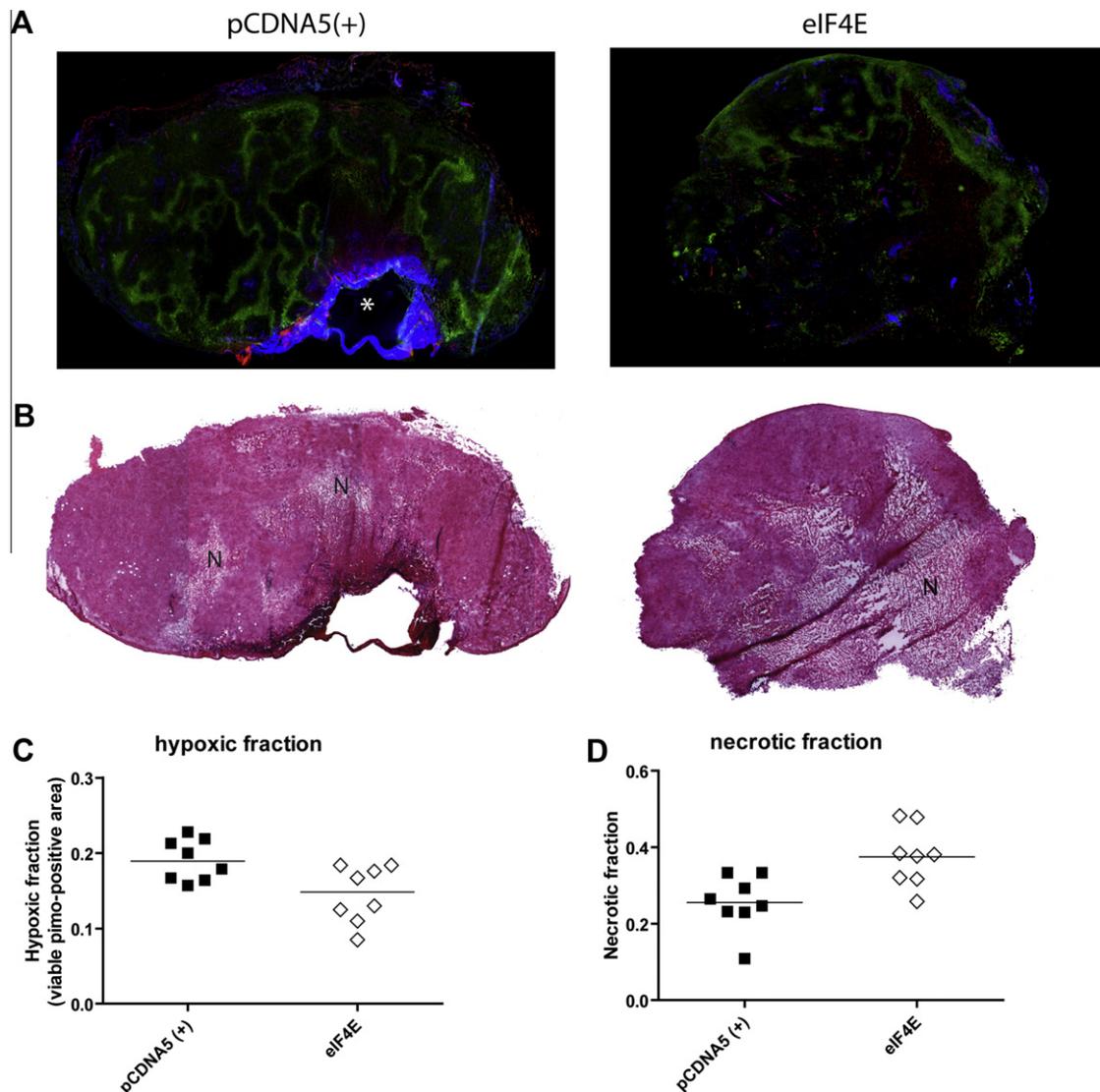


Fig. 3. eIF4E overexpression reduces the hypoxic fraction of tumours. After 7 days of doxycycline the tumours were harvested and analysed for changes in the microenvironment. (A) Pimonidazole immunohistochemistry revealed a decreased hypoxic fraction (pimonidazole positive viable fraction, Green; blood vessels, Red; Hoechst, Blue) in eIF4E overexpressing tumours. * indicates aspecific staining (B) Haematoxylin and eosin (H&E) staining revealed increased necrosis in the eIF4E overexpressing tumours. (C) Quantification of pimonidazole immunohistochemistry of the viable tumour tissue and (D) for necrotic fraction based on morphology.

hypoxia exposure. The reduced hypoxic fraction within tumours sensitized tumours to irradiation when targeted prior to irradiation. Targeting eIF4E after irradiation increased regrowth of the tumours. Using these unique conditional overexpression models, we were able to separate the effect on tumour growth, mainly caused by normoxic cells and the effect on tumour hypoxia.

The protein eIF4E is rate limiting in translation and is tightly controlled by interacting proteins. During hypoxia or starvation, reduced mTOR activity causes dephosphorylation of the 4E-BPs. These will scavenge eIF4E and prevent it from participating in the eIF4F complex and thus prevent cap-dependent translation. As most cellular ATP is consumed during translation, it is tempting to speculate that translation is inhibited to preserve energy during periods where ATP-production is limited. In addition, the decrease in overall translation allows the cell to redirect these efforts towards specific translation of proteins required for overcoming or surviving hypoxia [22].

mTOR-signalling or downstream targets are often deregulated in human tumours. In particular, overexpression of eIF4E promotes malignancy, recurrence of the tumour and predicts poor outcome

for patients in several tumour types [16]. In line with the literature, we observed increased growth tumours upon induction of eIF4E and faster recurrence of the tumour when induced after irradiation (Fig. 4B). Given the widespread deregulation of signalling pathways that affect mTOR and eIF4E activity in cancer, there has been interest in developing therapeutical agents to target these pathways. Although these strategies have clear impact on tumour growth and cellular proliferation, the current and previous data [18,23,24] indicate that caution should be taken in clinical regimens targeting these pathways as these drugs may promote tumour hypoxia. For example, the use of rapamycin *in vitro* increases hypoxia tolerance, whereas preventing decreased mTOR-signalling during hypoxia by AMPK-inhibitors leads to increased hypoxic cell killing [24]. Furthermore, administration of rapamycin to tumour bearing mice increased tumour hypoxia, and although a clear effect on tumour growth was seen, failed to improve response to fractionated radiotherapy [23].

Consistent with its ability to inhibit eIF4E, 4E-BP1 has been reported to have a number of antiproliferative and tumour suppressor functions [25]. Paradoxically, eIF4E and 4E-BP1 overexpression

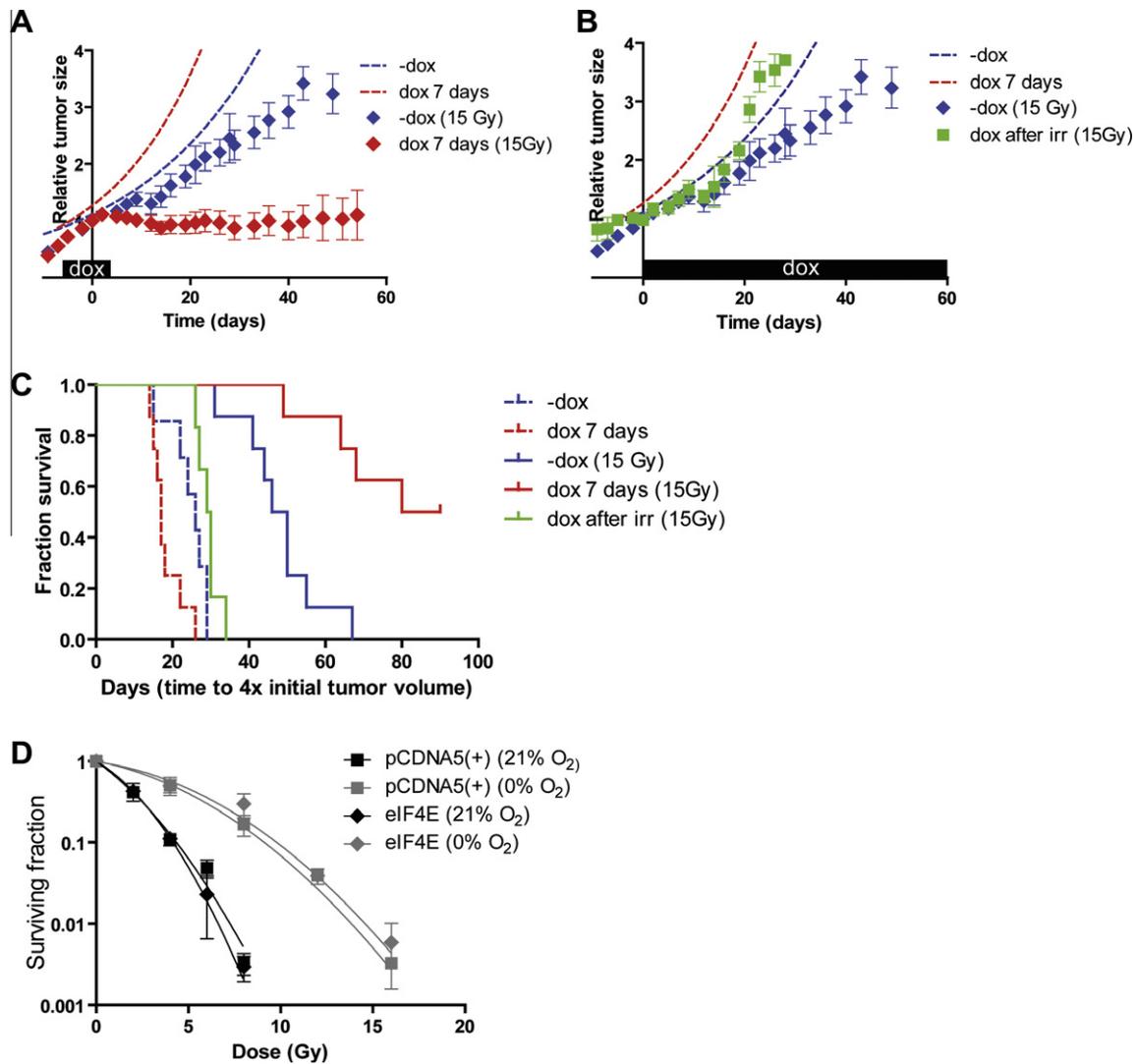


Fig. 4. eIF4E overexpression prior to irradiation sensitizes tumours to irradiation. U373 isogenic xenografts were established in nude mice and irradiated, when indicated (15 Gy) with 15 Gy at 150 mm³. (A) Doxycycline (2 g/L) was administered for 7 days (day -4 to 3, irradiation at $t = 0$) to induce the transgene and tumour size was assessed as function of time (mean \pm SEM, $n = 9$). Regular growth of the tumours without irradiation (-dox and +dox) is included for comparison of tumour growth delay. (B) Doxycycline (2 g/L) was administered after irradiation ($t = 0$) to induce the transgene and tumour size was assessed as a function of time (mean \pm SEM, -dox 15 Gy $n = 9$, +dox 15 Gy $n = 5$). Regular growth of the tumours (-dox and +dox) is included for comparison of tumour growth delay. (C) Kaplan Meier survival for tumours in 2D, 4A and 4B to reach four times the initial volume. (D) Clonogenic survival of empty vector and eIF4E overexpressing (24 h induction) cells after irradiation under normal and hypoxic (4 h, <0.02% O₂) conditions.

in tumour tissues is often simultaneously observed [26]. Our current results provide a potential explanation for this apparent paradox. Under nutrient rich, normoxic conditions when mTOR is active, overexpression of eIF4E facilitates growth and promotes malignancy. We speculate that co-overexpression of 4E-BP1 may provide a mechanism to inhibit translation despite eIF4E-overexpression under conditions of metabolic stress such as hypoxia. We showed that cells with an imbalance in eIF4E/4E-BP1 ratio are highly proliferative, but sensitized to hypoxia induced cell death. Thus, tumour hypoxia may provide an environment that would allow selection of high 4E-BP1-expressing cells in tumours. If true, then one would predict that tumours with overexpression of eIF4E and 4E-BP1 may be particularly hypoxia tolerant. It will be interesting to test this idea in the future.

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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.2011.05.047.

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