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MicroRNA-*124* expression counteracts pro-survival stress responses in glioblastoma

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Abstract

Glioblastomas are aggressive adult brain tumors, characterized by inadequately organized vasculature and consequent nutrient and oxygen (O_2)-depleted areas. Adaptation to low nutrients and hypoxia supports glioblastoma cell survival, progression, and therapeutic resistance. However, specific mechanisms promoting cellular survival under nutrient and O_2 deprivation remain incompletely understood. Here, we show that *miR-124* expression is negatively correlated with a hypoxic gene signature in glioblastoma patient samples, suggesting that low *miR-124* levels contribute to pro-survival adaptive pathways in this disease. Since *miR-124* expression is repressed in various cancers (including glioblastoma), we quantified *miR-124* abundance in normoxic and hypoxic regions in glioblastoma patient tissue, and investigated whether ectopic *miR-124* expression compromises cell survival, during tumor ischemia. Our results indicate that *miR-124* levels are further diminished in hypoxic/ischemic regions within individual glioblastoma patient samples, compared to regions replete in O_2 and nutrients. Importantly, we also show that increased *miR-124* expression affects the ability of tumor cells to survive under O_2 and/or nutrient deprivation. Moreover, *miR-124* re-expression increases cell death *in vivo*, and enhances the survival of mice bearing intracranial xenograft tumors. *miR-124* exerts this phenotype in part by directly regulating *TEAD1*, *MAPK14*/p38α and *SERP1*, factors involved in cell proliferation and survival under stress. Simultaneous suppression of these *miR-124* targets results in similar levels

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of cell death as caused by *miR-124* restoration. Importantly, we further demonstrate that *SERP1* re-introduction reverses the hypoxic cell death elicited by *miR-124*, indicating the importance of SERP1 in promoting tumor cell survival. In support of our experimental data, we observed a significant correlation between high *SERP1* levels and poor patient outcome in glioblastoma patients. Collectively, among the many pro-tumorigeneic properties of *miR-124* repression in glioblastoma, we delineated a novel role in promoting tumor cell survival under stressful microenvironments, thereby supporting tumor progression.

Keywords

miR-124; glioblastoma; hypoxia; ischemia; nutrient deprivation

Introduction

Glioblastoma Multiforme (GBM) is the most aggressive adult brain tumor. At only 12 months, median GBM patient survival is one of the lowest for all cancers (1). The standard of care for GBM treatment is surgery, followed by radiation and chemotherapy (2). However, glioblastomas rapidly become resistant to these therapies, thereby making them ineffective at significantly improving prognosis. GBM tumors are considered Grade IV (the most aggressive gliomas) and occur either *de novo* or as a progression from lower grade lesions (3). In both instances, the key feature of GBM, as compared with lower grade gliomas, is the presence of severely hypoxic/ischemic regions (4). Low oxygen tension (hypoxia) is defined as less than 2% O_2 and occurs in most solid tumors due to rapid proliferation, or aberrant angiogenesis, resulting in poor perfusion. The presence of hypoxic/ischemic areas is detrimental to GBM patients, as it positively correlates with recurrence and negatively correlates with patient survival (5, 6). Therefore, identifying factors mediating cellular adaptation to nutrient deprivation and hypoxia is crucial for improving therapeutic approaches to GBM.

Recent studies, such as the Cancer Genome Atlas (TCGA), have elucidated genetic aberrations associated with glioblastomas. In addition to deregulated oncoproteins and tumor suppressors (such as EGFR, PDGFR, PI3K, PTEN, NF1, etc.) (7, 8), numerous micro-RNAs (miRNAs) are differentially expressed in GBMs relative to adjacent non-neoplastic tissue (9, 10). miRNAs are ~22 nucleotide small RNAs that function as post-transcriptional negative regulators of ~30% of all mammalian genes (11, 12). While the inhibition of any single miRNA target is relatively modest, each miRNA impacts the expression of numerous genes. Thus, by targeting genes involved in multiple pathways, a single miRNA can significantly influence systems involved in cell cycle progression, differentiation, and cell death, as well as broad responses to stress (13).

Previous studies have measured miRNA levels in glioblastoma and compared them to adjacent non-neoplastic tissues, or to lower-grade gliomas. In particular, *miR-124* levels were shown to be significantly reduced in glioblastomas as compared to both adjacent non-neoplastic tissues (10) and lower-grade tumors (14, 15). *miR-124* is a brain-enriched miRNA critical for regulating neuronal differentiation (16-20). As *miR-124* levels are differentially

expressed in distinct brain cell types, low *miR-124* levels in glioblastoma may be a result of the cellular heterogeneity between glioma and adjacent tissue (21, 22). Alternatively, it is possible that *miR-124* functions as a tumor suppressor in GBM. This has been suggested in the context of other tumors (9, 23, 24) and the abundance of known *miR-124* targets negatively correlates with *miR-124* levels in brain tumor patient samples (15, 21), leaving open the possibility that glioblastoma cells expressing low *miR-124* levels exhibit a selective growth or survival advantage.

Hypoxic glioblastoma cells are often found in perinecrotic areas, where surviving cells experience low levels of O_2 in addition to diminished nutrient and growth factor availability (25). Such cells must therefore adapt to steep O_2 and nutrient gradients, and these adaptive responses are partly mediated by the Hypoxia Inducible Factors (HIFs) (26). Recent studies have shown that miRNAs also play a key role in modulating cellular survival or death under limiting O_2 and nutrient availability. For example, *miR-210* is elevated in hypoxic regions and promotes survival under low O_2 (27). Additionally, we have shown that restoring *miR-218* levels in glioblastoma opposes a Receptor-Tyrosine Kinase/HIF signaling pathway necessary for glioblastoma progression, particularly in the Mesenchymal subtype (28).

Here, we show that *miR-124* levels inversely correlate with a hypoxic signature in TCGA patient samples. Moreover, *miR-124* levels are diminished in pseudopalisading regions within individual glioblastoma patient tissues, when compared to relatively well-perfused regions. We demonstrate that increased *miR-124* expression in glioblastoma cells experiencing nutrient and O_2 deprivation promotes cell death, suggesting that *miR-124* targets factors important for glioblastoma survival under stressful microenvironments. Moreover, *miR-124* expression leads to increased cell death *in vivo*, in a doxycycline-induced tumor xenograft model that faithfully recapitulates hypoxic/ischemic regions in GBM. We identify three factors, TEAD1, p38α (MAPK14) and SERP1, as direct *miR-124* targets, and show that they are overexpressed in hypoxic/ischemic conditions. Combined inhibition of these targets recapitulates the increased cell death observed under low nutrient/O_2 stress, upon *miR-124* expression. In addition, SERP1 re-expression reverses the *miR-124* cell death phenotype in glioblastoma cells grown under hypoxia. Finally, we show that *miR-124* restoration confers increased mouse overall survival in an intracranial orthotopic xenograft model. These data suggest further investigation of *miR-124* and its targets, in particular SERP1, is strongly warranted.

Results

miR-124 levels inversely correlate with hypoxia signatures in glioblastoma patients and are further decreased in pseudopalisading necrotic regions within patient tissues

While several studies have shown that *miR-124* levels are low in glioblastomas as compared to adjacent non-neoplastic tissues, little is known about how *miR-124* levels correlate with low O_2 microenvironments. We took advantage of the large TCGA glioblastoma dataset (7), and compared *miR-124* levels to a “hypoxia” signature identified in the GBM patients’ expression profile. We defined a Hypoxia Inducible Factor (HIF) metagene by averaging the expression of known HIF-regulated genes (15) from each TCGA patient into a single expression value. A high HIF metagene score positively correlates with high levels of

hypoxia in each patient sample. Interestingly, *miR-124* levels negatively correlated with the HIF metagene, suggesting that GBM tissues experiencing highly hypoxic microenvironments have even lower *miR-124* levels (Figure 1a, S1). Furthermore, we stratified TCGA patients based on *miR-124* levels (high vs. low) and observed that patients expressing the lowest levels of *miR-124* exhibited a higher correlation with the HIF metagene. To validate our analysis, we also queried the levels of *miR-210*, a known miRNA stimulated by hypoxia, which positively correlated with the HIF metagene (Figure 1b).

While the TCGA contains a large repository of patient samples, it does not compare normoxic and hypoxic regions of glioblastoma within the same tumor. To determine whether *miR-124* levels were changing in hypoxic/ischemic domains within an individual glioblastoma patient sample, we performed laser-capture microdissection, collected regions of pseudopalisading necrosis, and compared them to non-necrotic, better-perfused areas. (Figure 1c, Supplemental Figure 1b). We compared *miR-124* and *GLUT1* levels between the perfused ("normoxic") and hypoxic regions of each individual patient, and showed that, while *miR-124* levels are already low in glioblastoma tissue, they are further diminished in domains experiencing hypoxia/ischemia (Figure 1d, left panel). Conversely, *GLUT1*, a hypoxically induced HIF target gene, was elevated in the hypoxic regions (Figure 1d, right panel). Taken together, these findings suggest the possibility that *miR-124* levels may be further decreased in hypoxic/ischemic domains, potentially to confer a survival advantage within a stressful microenvironment.

miR-124 expression increases cell death in glioblastoma cells grown under limiting nutrients and oxygen

To determine whether *miR-124* levels affect glioblastoma cells during stress, we increased *miR-124* expression in U87MG and LN18 glioblastoma cells and incubated them for 48 hours under nutrient deprivation (no glucose, no serum) or hypoxia (0.5% O_2). Interestingly, we found that *miR-124* re-expression resulted in a significant increase in cell death in glioblastoma cells cultured under nutrient or O_2 deprivation, as measured by Annexin/PI staining and flow cytometry (Figure 2a, c). Consequently, a slight increase in cleaved PARP accumulation was observed (Figure 2b). As shown previously, no significant change in cell viability was observed in glioblastoma cells grown under replete O_2 and nutrients upon *miR-124* expression (Figure 2a), suggesting that cell death is due to *miR-124* negatively regulating factors responsible for pro-survival, adaptive responses specifically during stress. We confirmed these results by modulating serum and O_2 levels in U373-MG cells and imaging *miR-124*-dependent cell death (Figure S2a). Of note, *miR-124* enhanced cell death was also observed when cells were treated with tunicamycin, a small molecule that induces ER stress (Figures 2d-e, S2b). Based on these results, we hypothesized that *miR-124* may be targeting factors that are active under stress conditions, and subsequently their pro-survival effects.

TEAD1, MAPK14/p38α, and *SERP1* are *miR-124* direct targets

To identify potential *miR-124* targets promoting survival during stress, we queried various miRNA target prediction bioinformatic databases, including TargetScan™, miRBase™ and DIANA-LAB, as well as previously published microarrays of genes modulated by *miR-124*

overexpression (16, 20), and identified three putative *miR-124* targets that may play a role in pro-survival stress responses: *MAPK14*/p38α, *TEAD1* and *SERP1*.

TEAD1 (TEA Domain 1) is a transcription factor regulated by the Hippo pathway, which controls the expression of many pro-proliferation genes (29). Interestingly, a recent study has shown that TEAD1 overexpression leads to an anti-apoptotic phenotype in HeLa cells (30). p38α (also known as MAPK14, MAP kinase 14) is a kinase typically activated as a response to upstream stress signaling. Although p38α is thought to be tumor suppressive in some settings (31), it may have a pro-tumorigenic role in GBM: an activated p38-MAPK pathway signature correlates with poor survival in glioblastoma (14), and increased glioblastoma invasion (32). Interestingly, a recent study demonstrated that *miR-124* can decrease MAPK14 levels, to counteract p38α signaling in neurons (33). TEAD1 and p38α are upregulated in several cancers, including colon, prostate, lung and pancreatic cancer (30, 34), suggesting a pro-tumorigenic role for these proteins. SERP1 (stress-associated endoplasmic reticulum protein, also known as RAMP4) is a 7 kD peptide located within the sec61 ER translocon protein complex. While very little is known about SERP1 biological functions, it has been proposed that this peptide is critical for the folding of newly synthesized proteins under stress conditions: SERP1 was originally identified as a peptide exhibiting increased expression during hypoxia and hypoglycemia (35), and *Serp1*$^{-/-}$ mice are more susceptible to ER stress (36).

According to TargetScan™, *MAPK14* harbors a putative conserved *miR-124* binding site in its 3' Untranslated Region (UTR), while *TEAD1* and *SERP1* have two putative conserved binding sites each (Figure 3a). We ectopically increased Non-Targeting (NT) and *miR-124* levels in U87MG and LN18 cell lines, and assessed target mRNA levels by quantitative RT-PCR (Figure 3b) and protein levels in U87MG, U373 and LN18 cell lines by immunoblot (Figure 3c). In all cases, *miR-124* expression led to decreased levels of each of the three mRNA and protein targets, respectively. We then transduced lentiviruses expressing either a Non-Targeting or a pre-*miR-124* vector into human glioblastoma "stem like cells" grown as tumor spheres and again showed that *miR-124* overexpression leads to decreased levels of target mRNA (Figure 3d). To investigate whether *MAPK14, TEAD1* and *SERP1* are direct *miR-124* targets, we employed a 3' UTR luciferase assay comparing luciferase levels in cells transfected with plasmids expressing wild type 3' UTRs or those harboring mutations in the *miR-124* seed binding sites. We determined that all three targets are directly regulated by *miR-124*, as luciferase levels decreased upon *miR-124* expression in cells expressing the wild type 3'UTR regions, but not in cells expressing 3'UTR regions with mutated *miR-124* seed sequences (Figures 3e-g). (Note: only one *SERP1* seed sequence binding [S2] site appears to be a bona fide *miR-124* binding site [Figure 3g]).

TEAD1, MAPK14 and *SERP1* promote glioblastoma progression

To assess whether *MAPK14, TEAD1*, and *SERP1* play a role in glioblastoma tumorigenesis, we obtained RNA from formalin-fixed, paraffin embedded (FFPE) glioblastoma patient samples, procured from the University of Pennsylvania Department of Pathology. A comparison of average *miR-124* levels between glioblastoma RNA versus normal brain tissue RNA showed that *miR-124* levels are greatly diminished in GBM, relative to normal

brain, as previously shown (10) (Figure 4a). Of note, *MAPK14*, *TEAD1* and *SERP1* mRNA levels exhibited the opposite result: mRNA levels of these targets were higher in GBM samples compared to normal brain tissue (Figures 4b-d, S3a-d). These data suggest an important role for all three genes in glioblastoma. In order to verify whether they promote glioblastoma cell survival, we employed short hairpin RNAs (shRNAs) to inhibit *TEAD1*, *MAPK14*, and *SERP1* individually (Figure 4e; Figure 4f shows resulting mRNA levels) and as part of a combined triple depletion (Figure 4g; Figure 4h depicts corresponding protein levels). Decreased expression of each *miR-124* target partially enhanced cell death under nutrient or O_2 deprivation (Figure 4e); in contrast, combined loss of *TEAD1, MAPK14* and *SERP1* fully recapitulated the increased apoptosis (Figure 4g) observed in *miR-124* expressing cells (see Figure 2).

Finally, we treated U87-MG cells with scrambled or *pre-miR-124* lentivirus and subjected them to low O_2 or serum-free/low O_2 conditions for 24 hours. Upon stress, particularly the serum-free/low O_2 conditions, a marked increase in all three targets was observed (6-8 fold) (Figure 4i). *miR-124* treatment counteracted this increase, bringing target levels closer to baseline. At the same time, ectopic *miR-124* expression under serum-free/low O_2 conditions significantly increased *NOXA* levels, a marker of apoptotic cell death (Figure 4j).

miR-124 expression affects glioblastoma cell proliferation and survival *in vivo*

In order to establish a role for *miR-124* re-expression/target gene downregulation *in vivo*, we transduced U87-MG cells with control or pre-*miR-124* – GFP, and assessed growth both *in vitro* (cell counts) and *in vivo* (subcutaneous xenografts). Additionally, we performed the same experiments comparing non-targeting shRNA vs. *TEAD1*, *SERP1*, or *MAPK14* shRNAs. As previously established (10), *miR-124* re-expression leads to decreased cell proliferation *in vitro* (Figure S4a) and subcutaneous tumor growth *in vivo* (Figure 5a). *TEAD1* and *MAPK14*/p38α inhibition also resulted in decreased *in vitro* proliferation when U87-MG cells were grown under replete media (Figures S4b, c). *SERP1* knockdown did not result in significant differences in *in vitro* proliferation under replete media, but resulted in decreased proliferation when cells were grown under 0.5% O_2. (Figure S4d). *TEAD1* and *SERP1* inhibition led to decreased tumor growth *in vivo* (Figure 5b, c; p38α modulation has been previously shown to lead to decreased tumor growth in glioblastoma growth *in vivo* [37], thus not shown here).

Since increased *miR-124* expression led to a significant difference in tumor size (Figure 5a), it was difficult to determine whether increased cell death occurred in areas experiencing limited nutrient and O_2 availability, since smaller tumors exhibit fewer hypoxic/ischemic regions. In order to mitigate this scenario, we devised a doxycycline-inducible system where *miR-124* expression is regulated by doxycycline administration. Briefly, we created stable U87-MG cell lines expressing either a doxycycline-inducible control or *miR-124*, along with GFP, to assess the robustness of the system. As shown in the schematic in Figure 5d, subcutaneous tumors were allowed to grow for 20 days in nude mice, so that they achieved a size sufficient for the natural generation of O_2/nutrient gradients. At day 20, doxycycline was administered, thereby activating expression of *miR-124*-GFP, or the control-GFP construct. The doxycycline-inducible system was validated *in vitro* as described in Figure

S5a-b. Surprisingly, no statistically significant difference in tumor size was observed after doxycycline treatment. (Figure 5e). Subcutaneous tumor architecture was similar in both control and *miR-124* expressing tumors, and both showed regions of hypoxia as demonstrated by HIF-1α staining (Figure 5f). However, when we stained for GFP (the proxy for expression of either the control or the *miR-124* construct), a significant loss of GFP-expressing cells was detected in the tumor tissues expressing the *miR-124* construct (Figures 5g, h), even though both constructs expressed GFP in a doxycycline-dependent manner *in vitro* (Figure S5a). We then quantified cell death using the terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) assay, and observed increased apoptosis in tissues expressing *miR-124*, compared to those expressing the control construct (Figure 5i, j). While there are many possible explanations for these observations, increased TUNEL staining in *miR-124* expressing tumors, coupled with decreased numbers of GFP-expressing cells, suggest that *miR-124* expressing cells may be selected against in the seven days of doxycycline treatment, due to their inability to survive O_2 and nutrient deprivation.

SERP1 is an important pro-survival target in glioblastoma

Since SERP1 has been shown to be elevated during brain ischemia (35), we focused on dissecting the role for SERP1 in survival under stress. We decreased *SERP1* levels via an shRNA-mediated lentivirus in U87-MG cells and subjected them to low O_2 or serum-free/low O_2 conditions for 24 hours. Just as we observed in Figure 4i, *SERP1* transcripts are elevated under stress (Figure 6a). Importantly, we demonstrate that shRNA treatment recapitulates the *miR-124* phenotype by eliciting increased *NOXA* levels (Figure 6b). We therefore determined if re-expression of the *SERP1* Open Reading Frame (lacking the 3'UTR) reverses *miR-124*-mediated cell death. Increased *miR-124* expression led to enhanced cell death under hypoxia, while SERP1 re-expression almost completely abrogated *miR-124*-mediated cell death under hypoxia (Figure 6c). This underscores a previously unappreciated role for SERP1 in glioblastoma. As observed with our FFPE patient samples in Figure 4d, TCGA patient data also suggest that *SERP1* mRNA levels are elevated in glioblastoma samples, as compared to normal brain tissue (p - value < 0.0001) (Figure 6d). Additionally, a query into the REMBRANDT database (https://caintegrator.nci.nih.gov/rembrandt/) showed that patients with increased *SERP1* mRNA exhibit lower overall survival, compared to patients with low or intermediate levels (p - value = 0.006) (Figure 6e). Taken together, these data suggest that SERP1 may be an attractive target for further evaluation in glioblastoma survival.

miR-124 expression increases overall survival in an orthotopic intracranial mouse model

While several studies have investigated the role for *miR-124* in glioblastoma (10, 38), the impact of *miR-124* expression on glioblastoma cell survival *in vivo* is unclear. To test whether *miR-124* expression led to a change in tumor cell survival, we performed an intracranial orthotopic experiment in Nu/Nu mice expressing either control or pre-*miR-124* construct, as well as GFP. *miR-124* levels were increased only ~50 fold (data not shown), similar to the difference in *miR-124* levels between GBM and adjacent non-neoplastic tissue measured in patients (10, 39). GFP levels were similar between control and *miR-124* constructs (Figure S5c). Mice were sacrificed upon showing neurological symptoms associated with tumor burden. We observed that *miR-124* re-expression conferred mice with

a higher survival rate (p - value < 0.0001) (Figure 7a, 7b shows brain sections upon sacrifice), suggesting the importance of silencing *miR-124* during GBM tumor progression.

Discussion

Glioblastomas are difficult to treat, primarily due to their resistance to standard of care therapy and eventual tumor recurrence. Both resistance to therapy and recurrence are closely associated with the frequent occurrence of hypoxic/ischemic regions in grade IV gliomas. The ability of glioblastoma cells to survive under nutrient and O_2 deprivation ensures the opportunity for glioblastoma recurrence. Many factors are over - and underexpressed to promote cellular survival, and a majority have yet to be elucidated. Several micro-RNAs have been demonstrated to promote survival under stress. Here, we show that *miR-124* loss in glioblastoma allows for survival during nutrient and O_2 deprivation, as *miR-124* re-expression in glioblastoma cells increased cell death under these conditions.

miRNAs function by targeting and modestly inhibiting a vast array of mRNAs and proteins. Given that miRNAs can inhibit up to 30% of the mammalian genome (13), differentially expressed miRNAs are a powerful discovery tool in cellular processes impacting development, physiology and disease. While a single miRNA inhibits any given mRNA or protein only ~10-20%, global pathway changes can be significant. Additionally, modulating miRNA levels often reveals new targets or pathways that have been under-investigated or not well understood. Previous studies have identified an anti-proliferative role for *miR-124* in glioblastoma (10), but no consistent evidence has linked *miR-124* to cell death under limiting O_2 and nutrient conditions in the context of glioblastoma. During the preparation of this manuscript, a recently published study showed that *miR-124* levels are downregulated in pulmonary artery smooth muscle cells (PASMC) subjected to hypoxia, implying that *miR-124* expression could be utilized in the future to target effectors of pulmonary arterial hypertension (40). In this paper, we show that *miR-124* levels (while decreased in glioblastoma compared to normal brain tissue) are further diminished in pseudopalisading necrotic/hypoxic regions as compared to better-perfused tissue. Based on TCGA data, the more patient samples express a hypoxic signature, the lower *miR-124* levels become. While the observation that *miR-124* levels anti-correlate with hypoxic regions and hypoxic signatures in patients (Figure 1) does not necessarily imply that *miR-124* has been selectively inhibited to confer survival, endogenous *miR-124* expression has a significant effect in inhibiting cell survival in hypoxic/ischemic glioblastoma tissue. We demonstrate for the first time how *miR-124* controls cell death through inhibition of various pro-survival targets elevated under low nutrients, growth factors and O_2, making *miR-124* an attractive target for further investigation in glioblastoma. Our current model suggests that, due to lack of *miR-124* expression, tumor cells acquire the ability to uncontrollably proliferate, as these cells express high levels of pro-proliferation factors including CDK-6 (10), and TEAD1 (Figure 3). During growth and expansion, glioblastoma tissues invariably experience gradients of nutrient and O_2 availability, and in many cases, regions where nutrients and O_2 are significantly depleted. Our study suggests that lack of *miR-124* allows cells in these regions to upregulate pro-survival factors (such as SERP1, a novel *miR-124* target). Taken together, lack of *miR-124* expression allows glioblastoma cells to proliferate, but also survive under stress conditions (Figure 7c, model). While at the present time miRNA-based

therapeutics await further development, investigating miRNA targets should lead to insights into the biological processes of glioblastoma, as well as potential new therapeutic modalities.

One interesting novel *miR-124* target described here is SERP1. No previous connection has been made between SERP1 and glioblastoma progression, and very little is known about the biological roles for SERP1 in the endoplasmic reticulum. So far, SERP1 appears to have a role in modulating responses to ER stress (36) and protecting proteins from degradation while promoting consequent glycosylation (35). Additionally, it was shown that SERP1 levels are elevated in astrocytes during rat brain ischemia (35). Since *miR-124* re-expression led to increased cell death under tumor ischemic and ER stress conditions, we hypothesized that *miR-124* counteracts pro-survival stress responses in glioblastoma, in part by targeting SERP1. Here, we identify SERP1 as an important factor in glioblastoma patient survival. Additionally, we show that SERP1 overexpression rescues the cell death phenotype conferred by *miR-124*, suggesting that SERP1 may provide a pro-survival (hence, pro-tumorigenic) role *in vivo*.

Liu et al. have recently shown that *miR-124* levels decrease in the sub-ventricular zone when mice are subjected to cerebral ischemia, in a model of stroke (41). The authors suggest that decreased *miR-124* allows for increased proliferation in the ischemic region during stroke-induced neurogenesis. We demonstrate that, in addition to allowing for cellular proliferation, the decrease in *miR-124* may also be permissive for cellular survival during stroke, potentially by allowing for SERP1 upregulation under low nutrients and O_2, as described by Yamaguchi and colleagues (35). Thus, more research regarding the connection between *miR-124*, SERP1, and hypoxia/ischemia is warranted, in the settings of stroke and glioblastoma tumorigenesis.

In summary, we have shown glioblastoma cells undergo apoptosis under hypoxic/ischemic conditions, upon *miR-124* reintroduction. This increase in cell death may be partially mediated by direct *miR-124* inhibition of TEAD1, *MAPK14*/p38α and SERP1, in addition to numerous other factors. Further research on *miR-124* targets involved in survival under nutrient and O_2 deprivation may lead to novel glioblastoma therapeutics.

Materials and Methods

Cell Culture conditions, reagents, and lentiviral transduction

U87MG, U373, LN18 and HEK 293T cells were obtained from ATCC (Manassas, VA), and cultured in DMEM containing 10% FBS, glutamine, non-essential amino-acids, Penicillin/Streptomyicin antibiotics and HEPES buffer, and passaged on average every three days. Cells cultured in the nutrient deprived media were grown in glucose-free DMEM containing glutamine, non-essential amino-acids, antibiotics and HEPES buffer, without the addition of FBS. Cells were grown under hypoxic incubation in a Ruskinn invivo$_2$ 400 workstation. Patient-derived glioblastoma tumor spheres were a gift from Dr. Jeremy Rich (Cleveland Clinic, Cleveland, OH). These cells were maintained in Neurobasal medium, supplemented 1:50 with B27 (Invitrogen, Carlsbad, CA), Epidermal Growth Factor (Sigma) at 20 ng/mL, and basic Fibroblast Growth Factor at 20 ng/mL. Non-targeting and miR-124 mimics were

obtained from Dharmacon (Lafayette, CO). microRNA mimic transfection was performed using HiPerFect transfection reagent (Qiagen). 100 mM miRNA mimics were used for all experiments. Tunicamycin (Sigma) was diluted in DMSO was administered at 1 μg/mL for 24 hours. All viruses were packaged using the third generation lentivector system (Invitrogen, USA) and expressed in HEK 293T cells. Supernatant containing virus was collected at 24 hr and 48 hr timepoints and concentrated using 10-kDa Amicon Ultra-15 centrifugal filter units (Millipore). Overexpression of SERP1 Open Reading Frame was achieved by cloning into the pCDH-CMV-MCS-EF1-Puro vector (Systems Biosciences) via EcoRI and NotI restriction sites.

Immunoblots

Lysates were collected using a whole cell elution buffer previously described. 20 or 40 μg protein was loaded in 12% or 15% SDS-PAGE gels, transferred to nitrocellulose and blotted with various antibodies for TEAD1 (Novus), p38α (Cell Signaling), SERP1 (Genetex), β-tubulin, PARP, cleaved PARP, (Cell Signaling). Primary antibodies were diluted 1:1000 in 5% non-fat milk TBST.

Cell death assays

Cell death was assessed using Annexin-PI staining on Flow Cytometry, using the FITC Annexin V Apoptosis Detection Kit I from BD Pharmingen (San Jose, CA). For nutrient deprivation/hypoxia experiments, cells were kept under stress conditions for 48 hours before cell death was assayed. For tunicamycin experiments, cells were kept under stress conditions for 24 hours.

In vivo xenograft assays

All experiments were performed in accordance with NIH guidelines and approved by the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC). Sub-cutaneous xenografts were performed on Nu/Nu mice (Charles River) as previously described. 1,000,000 U87-MG cells expressing the empty pCDH-EF1-copGFP lentivector or the pCDH-EF1-copGFP-pre-*miR-124* lentivector were injected on flanks of nude mice and the tumors were subsequently measured by caliper over the period of a month. Additionally, sub-cutaneous xenografts were performed with U87-MG cells expressing: Scr shRNA, TEAD1 shRNA, SERP1 shRNA, doxycycline-inducible miR-124 and doxycycline inducible control. Tumor volume was measured using the calculation: $(X \bullet Y^2)\pi/6$, where X is the longest caliper measurement, Y is the shortest, and $\pi=3.14$. For the doxycycline-inducible experiments, mice were fed 2 mg/mL doxycycline and 5% glucose in their drinking water for 7 days.

Orthotopic xenograft injections were performed by stereotactically injecting 500,000 U87-MG glioblastoma cells in 5 uL PBS, 5mm in the right cerebral hemisphere in 8-week-old Nu/Nu mice. As in the sub-cutaneous xenograft experiment, the U87-MG cells were expressing either the empty pCDH-EF1-copGFP, or the pCDH-EF1-copGFP-pre-*miR-124* lentivector. Prior to injection, the mouse was prepared as previously described. The animals were sacrificed at the first sign of neurological symptoms.

Immunohistochemistry (IHC) and Immunofluorescence (IF) assays

IHC and IF were performed on 5 μm sections according to standard protocols. Antibody concentrations are the following: HIF1α: 1:100 (Abcam), GFP: 1:100 (Cell Signaling). Images were taken using a Leica 500 microscope (Leica) at 10X magnification. Paraffin embedded sections were stained for TUNEL using an ApopTag Plus Fluorescein In situ Apoptosis Detection Kit (Chemicon, CA, USA) according to the manufacturer's instructions. Images were taken at 20X magnification using an Olympus IX81 microscope.

Laser Capture Microdissection

FFPE slides were lightly stained with Hematoxylin and Eosin and immediately subjected to laser capture under RNase-Free conditions. For pseudopalisading regions, the internal necrotic core was initially cut and discarded, before the pseudopalisading region was collected for testing. Microdissection was performed using a Leica microscope and the Leica LMD software. Upon collection, RNA was extracted using the Qiagen miRNeasy FFPE kit.

Patient samples

LCM tissue: At the Hospital of the University of Pennsylvania, we searched the departmental surgical pathology database for cases with the diagnosis of "Glioblastoma, WHO grade IV" over a two year period (2011-2013). Only primary glioblastomas that had not received prior radiation or chemotherapy were included. Subsequently, the cases were screened by a neuropathologist (P.P.G) for adequate areas of both pseudopalisading necrosis and solid tumor, and whether it was possible to obtain ten 10 um unstained tissue sections from the archived paraffin embedded formalin fixed tissue blocks.

mRNA extraction from whole patient sample: the formalin-fixed, paraffin-embedded (FFPE) patient samples were obtained from the University of Pennsylvania Department of Pathology and Laboratory Medicine (Philadelphia, PA). GBM blocks were screened by a neuropathologist (S.V) contained > 95% tumor cells. Controls (temporal lobectomy tissue obtained from intractable epilepsy patients) showed histopathologic evidence of mild to focally moderate gliosis, but no lesions. GBM patients age range: 24 to 89 years, (Median: 63 years). Control patients age range: 22 to 61 years, (Median: 38 years). RNA was obtained from FFPE samples using the RecoverAll™ Total Nucleic Acid Isolation kit (Ambion, CA, USA).

Bioinformatic analyses

mRNA expression data were downloaded from the TCGA Data Portal (https://tcga-data.nci.nih.gov/tcga/). Data generated on the Affymetrix microarray platform HT_HG-U133A for 385 tumor and 10 normal samples was subjected to GCRMA normalization (GCRMA background correction, quantile normalization, log2 transformation and Median polish probeset summarization) was used to determine mRNA expression. To obtain expression, previously normalized TCGA Level2 data from 426 tumor samples and 10 normal samples run on Agilent's miRNA microarray was utilized. Data were analyzed using the Partek software (Partek Inc., St. Louis, MO, USA). To determine the association between *miR-124* and expression of the HIF signature, we utilized the Gene Set Analysis

(GSA) implementation (version 1.03) of Gene Set Enrichment Analysis (GSEA). We defined the HIF signature by using previously - established HIF regulated genes (15). To create a single value that represents the expression of the HIF signature (HIF metagene), the average expression of all the genes in the HIF signature was calculated for each tumor sample (using TCGA GBM data). This HIF metagene was then used as a continuous variable for various comparisons between groups. Analysis was performed using the R language and environment for statistical computing.

Statistics

All statistical analysis was performed using the GraphPad Prism™software (CA, USA). All data are represented as mean ± S.E.M. To establish whether a difference between two values is statistically significant, we performed Student's t-test, where $p < 0.05$ defines statistical significance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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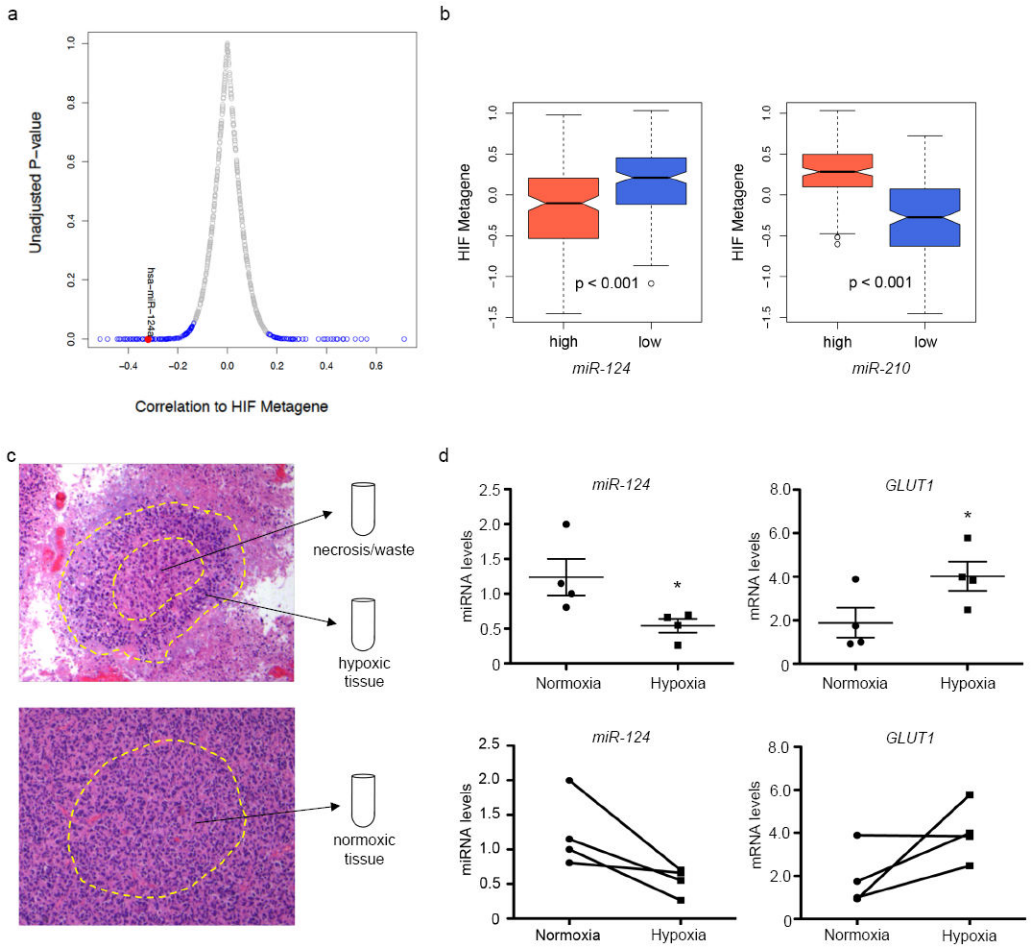

Figure 1. *miR-124* levels inversely correlate with hypoxia signatures in glioblastoma patients and are further decreased in pseudopalisading necrotic regions within patient tissues

(a) Hypoxia Inducible Factor (HIF) metagene correlation to miRNA levels. MiRNAs that are positively and negatively correlated with the metagene are indicated blue = p - value < 0.05. Red dot = *miR-124*. **(b)** HIF metagene in TCGA GBM patient samples divided into high and low *miR-124 / miR-210* levels. **(c)** Schematic of Laser-Capture Microdissection. Top: region of pseudopalisading necrosis. The inner necrotic region is discarded, while the pseudopalisade is collected as “hypoxic tissue”. Bottom: Non-necrotic region, collected as the perfused (“normoxic”) tissue counterpart. **(d)** *miR-124* and *GLUT1* mRNA levels in perfused or “normoxic” and hypoxic regions in patient samples. Top: averages; bottom: paired representation (each pair is from an individual patient). * p - value = 0.047 (*miR-124* changes); p - value = 0.035 (*GLUT1* changes). n = 4 patients.

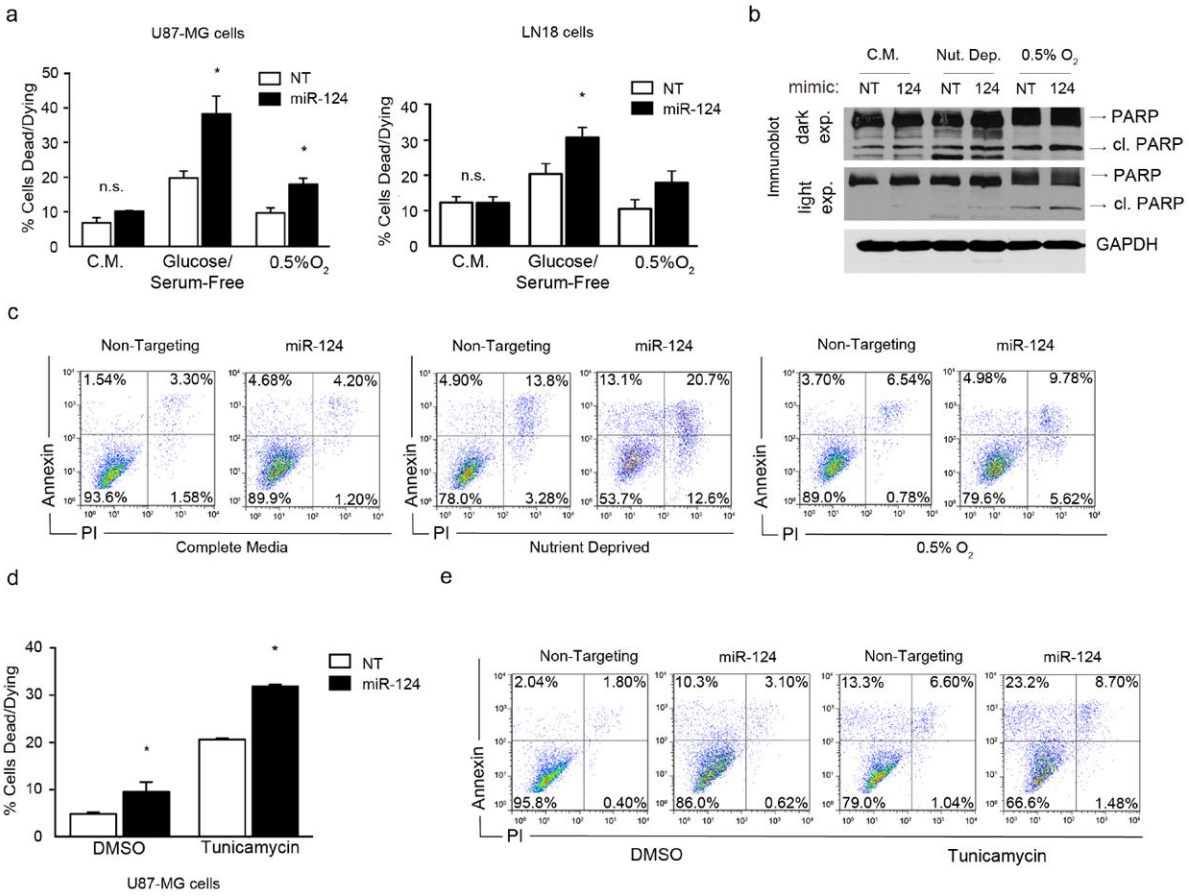

Figure 2. *miR-124* expression increases cell death in glioblastoma cells grown under limiting nutrients and oxygen

(a) U87-MG and LN18 cells were grown under the following conditions: Complete Media (C.M.), Glucose/Serum-Free, and Hypoxia (0.5% O_2). Cells were transfected with either a Non-Targeting (NT) or *miR-124* mimic (miR-124) for 48 hours, followed by Annexin/PI staining and flow cytometry for cell death analysis. **(b)** Immunoblots of cleaved PARP in cells treated as described in Figure 1a. **(c)** Representative Annexin – PI flow cytometry plots of U87MG cells corresponding to Figure1a. **(d)** U87-MG cells were treated with tunicamycin or DMSO control, and transfected with either a NT or *miR-124* mimic for 24 hours, followed by Annexin/PI staining and cell death analysis by flow cytometry. **(e)** Representative Annexin – PI flow cytometry plots from Figure 1d. * p - value < 0.05.

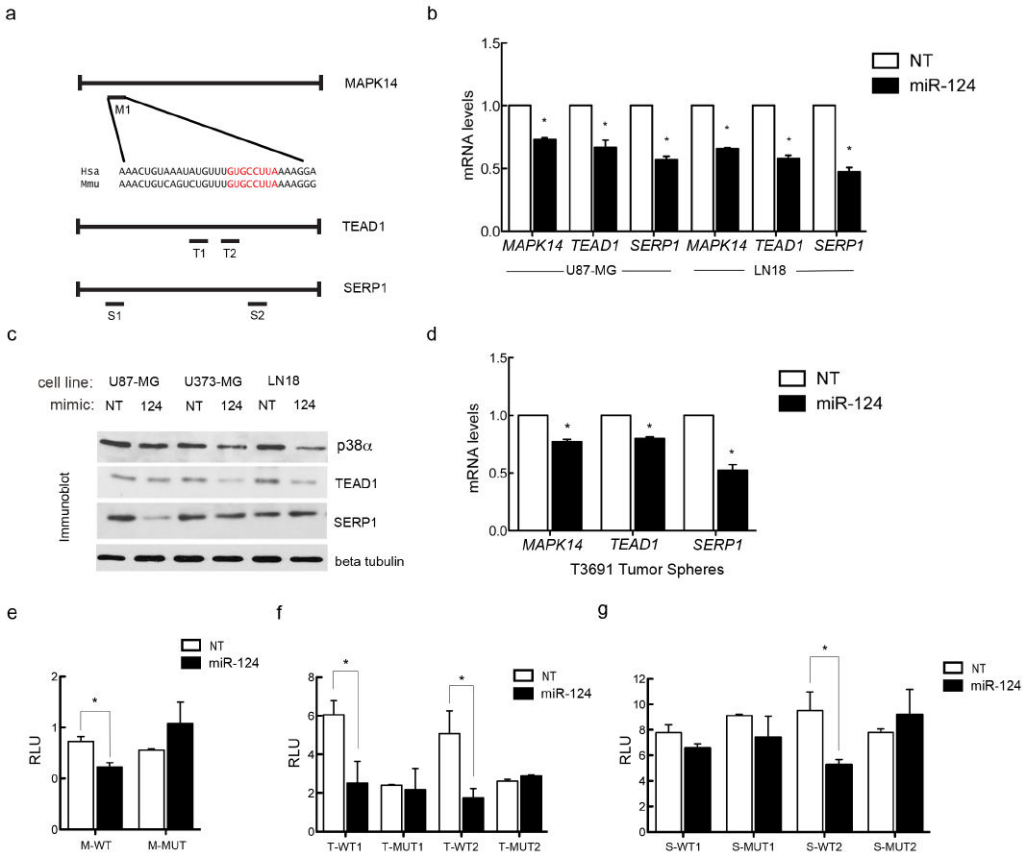

Figure 3. *TEAD1, MAPK14*/p38α, and *SERP1* are *miR-124* direct targets

(a) Schematic of *miR-124* seed sequence binding sites in the 3' untranslated regions of the three putative targets. **(b)** *MAPK14*/p38, *TEAD1* and *SERP1* mRNA levels in U87-MG and LN18 cells treated either with a Non-Targeting (NT) or a *miR-124* mimic after 12 hours of transfection. **(c)** p38, TEAD1 and SERP1 protein levels in U87-MG, U373-MG, and LN18 cells treated either with a Non-Targeting (NT) or *miR-124* mimic after 72 hours of transfection. **(d)** mRNA levels of glioblastoma stem like cells grown as tumor spheres transduced with either an empty control or pre-*miR-124* expressing virus. **(e-g)** T98G cells transfected with pMIR-REPORT with intact or mutated seed sequences were tested for luciferase activity in the presence of stable *miR-124* expression. Luciferase levels for **(e)** *MAPK14*, **(f)** *TEAD1* and **(g)** *SERP1* 3'UTR constructs.

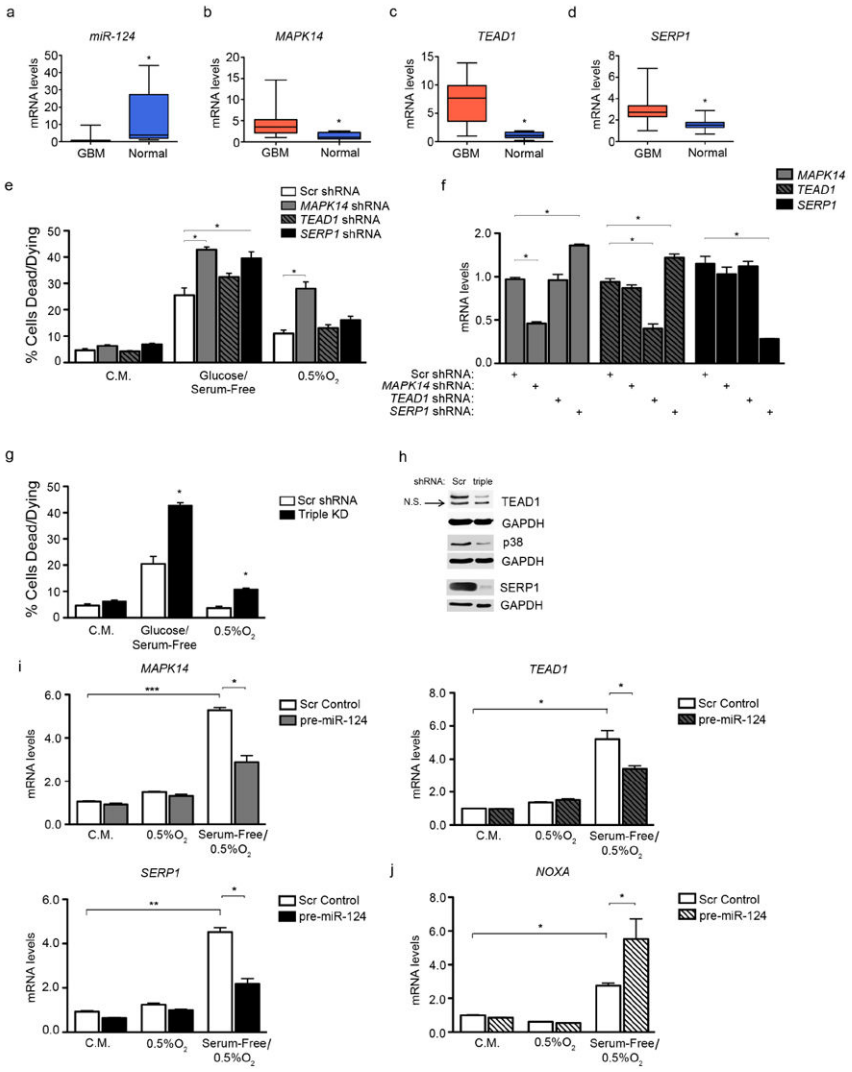

Figure 4. *TEAD1, MAPK14* and *SERP1* promote glioblastoma progression

(a) *miR-124*, **(b)** *MAPK14*, **(c)** *TEAD1*, and **(d)** *SERP1* levels in GBM (n=30) and normal brain samples (n=10). * p value < 0.05. **(e)** shRNA-mediated inhibition of individual targets partially recapitulates the cell death phenotype observed in Figure 2a. * p value < 0.05. **(f)** Transcript levels of all three targets, upon shRNA-mediated inhibition of any single target. **(g)** Cell death levels after shRNA-mediated depletion of all three targets combined (Triple KD). **(h)** Immunoblot showing target protein levels upon triple ablation. Scr = Scrambled control shRNA, triple = shRNAs for all three targets. **(i)** mRNA levels of *MAPK14* (top left), *TEAD1* (top right), and *SERP1* (bottom left) under the following conditions: Complete Media (C.M.), Hypoxia (0.5% O_2), and Serum Free + Hypoxia (0.5% O_2). U87-MG cells were transduced with scrambled control (Scr) or pre-*miR-124*-expressing lentivirus and subjected to these conditions for 24 hours. (j) *NOXA* levels from experiment in (**4i**) * p - value < 0.05; ** p - value < 0.001, *** p - value < 0.0001.

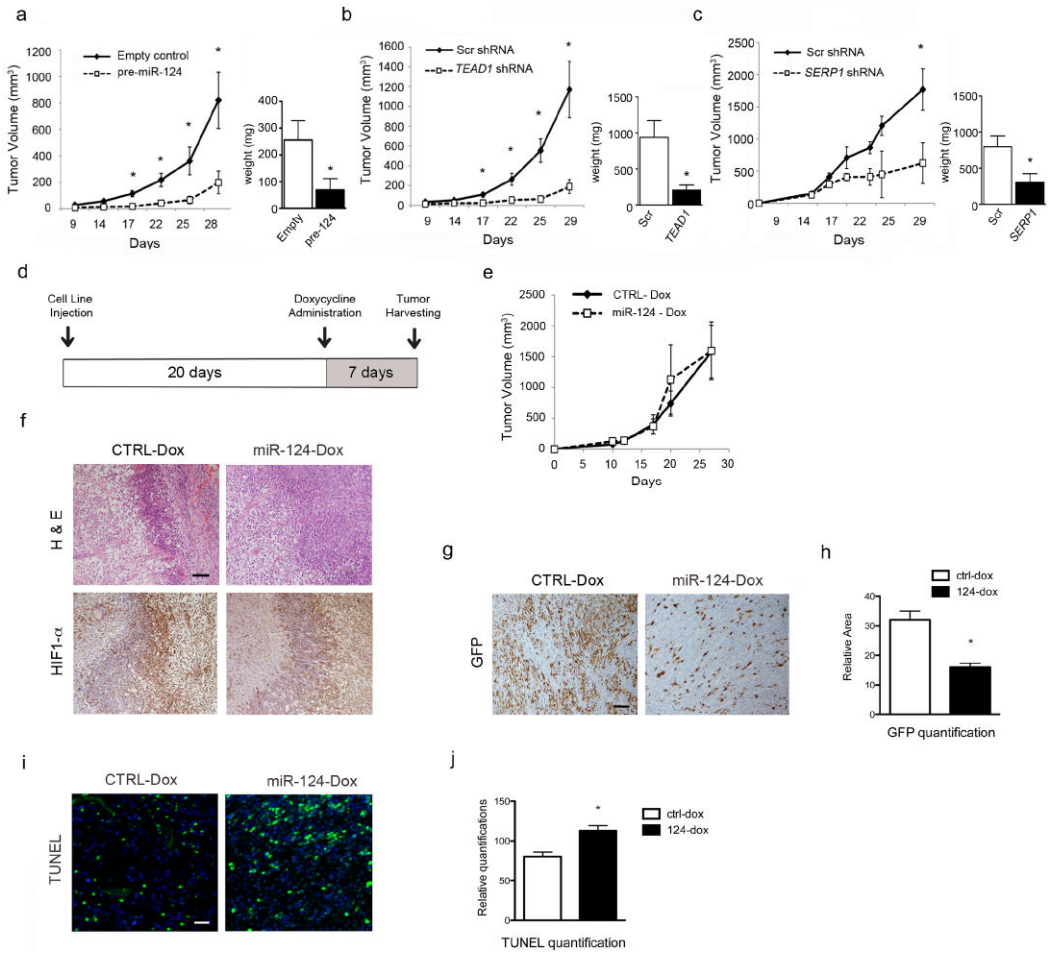

Figure 5. *miR-124* expression affects glioblastoma cell proliferation and survival *in vivo*

Growth curves and tumor weights of subcutaneous xenografts of U87-MG cells expressing *miR-124* (**a**, n=9), *TEAD1* shRNA (**b**, n=9), and *SERP1* shRNA (**c**, n=6). **(d)** Schematic of doxycycline-inducible subcutaneous xenograft assay. **(e)** Growth curve of doxycycline inducible subcutaneous xenografts after expressing control or *miR-124* for seven days. **(f)** H&E and HIF-1α IHC staining of doxycycline-inducible subcutaneous xenograft tumor sections. Scale bar is 200 μm. **(g, h)** GFP IHC staining and quantification, **(i, j)** TUNEL staining and quantification of doxycycline-inducible subcutaneous xenograft tumor sections. Scale bar is 100 μm. * p - value < 0.05.

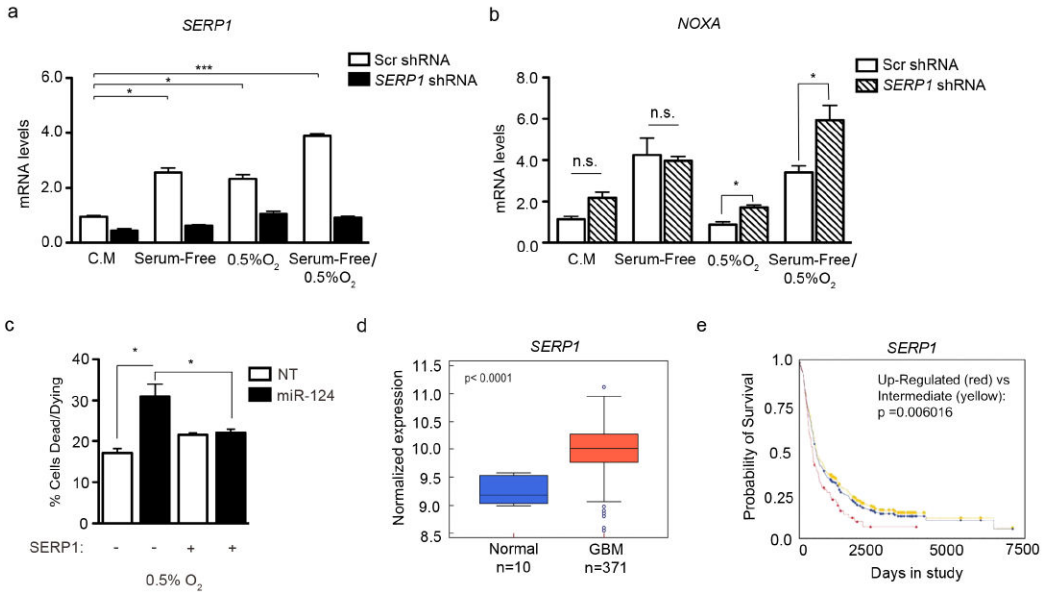

Figure 6. SERP1 is an important factor in glioblastoma

(a) *SERP1* and **(b)** *NOXA* mRNA levels in U87-MG cells grown under the following conditions: Complete Media (C.M.), Serum-Free, Hypoxia (0.5% O_2), and Serum Free + Hypoxia (0.5% O_2). Cells were transduced with scrambled (Scr) or *SERP1* shRNA and subjected to these conditions for 24 hours. * p - value < 0.05; ** p - value < 0.001, **(c)** *SERP1* Open Reading Frame re-expression leads to rescue of cell death in U87-MG expressing *miR-124* under hypoxia. **(d)** SERP1 levels in normal brain and GBM tissues from the TCGA dataset (p - value < 0.0001). **(e)** Kaplan – Meier survival curve of patients from the REMBRANDT database, stratified by SERP1 expression levels (p - value = 0.006).

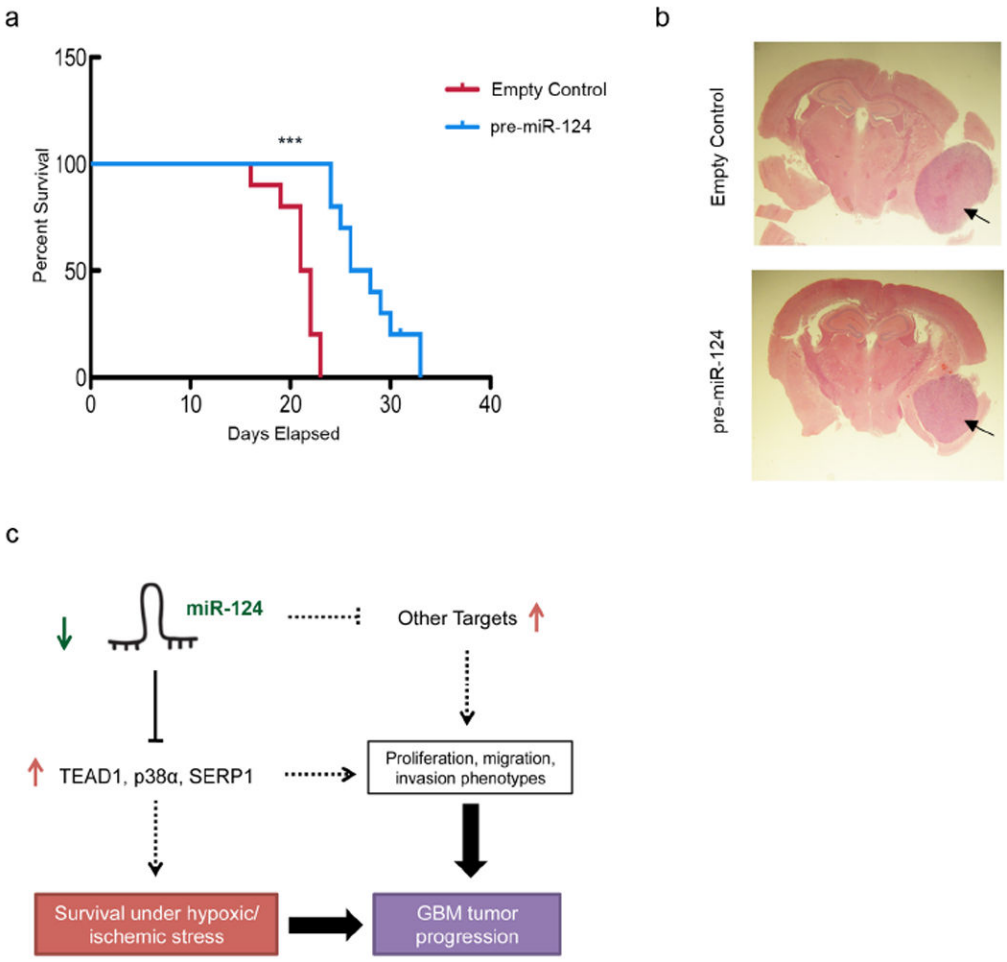

Figure 7. *miR-124* expression increases overall survival in an orthotopic intracranial mouse model

(a) U87-MG cells transduced with either an empty control or pre-*miR-124* expressing virus were injected into nude mice, subsequently surveyed for survival. Kaplan-Meier curve, *** p - value < 0.0001. **(b)** Representative H&E images of xenografts in the brain showing tumor lesions (arrows) at the time of sacrifice. **(c)** Model for *miR-124* role in glioblastoma. *miR-124* loss contributes to glioblastoma tumorigenesis by promoting the elevated expression of cell proliferation factors, as well as pro-survival factors, especially in regions experiencing nutrient and oxygen deprivation.