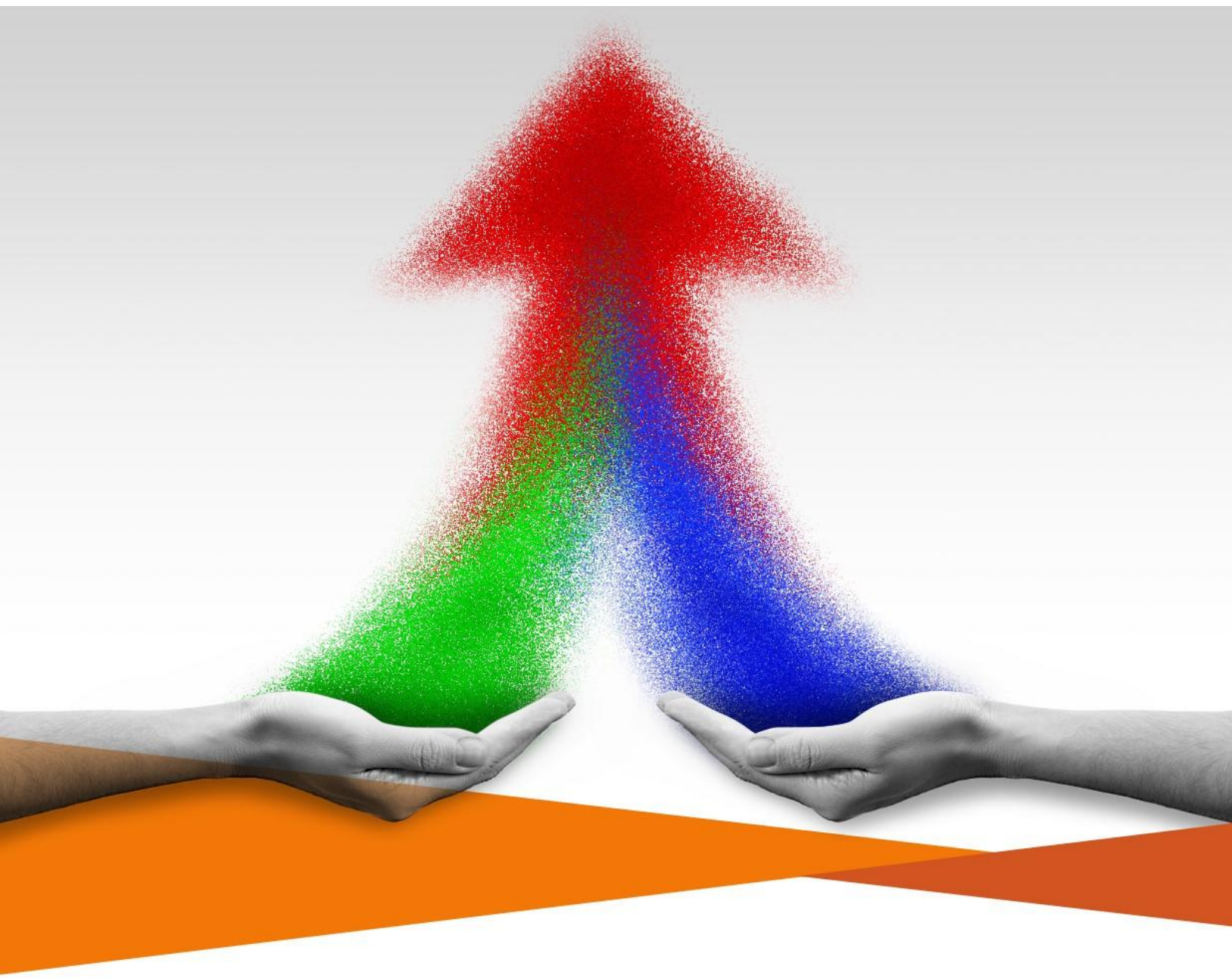

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Short Report

FAT1 modulates EMT and stemness genes expression in hypoxic glioblastoma

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Glioblastoma (GBM) is characterized by the presence of hypoxia, stemness and local invasiveness. We have earlier demonstrated that FAT1 promotes invasiveness, inflammation and upregulates HIF-1α expression and its signaling in hypoxic GBM. Here, we have identified the role of FAT1 in regulating EMT (epithelial-mesenchymal transition) and stemness characteristics in GBM. The expression of FAT1, EMT (Snail/LOX/Vimentin/N-cad), stemness (SOX2/OCT4/Nestin/REST) and hypoxia markers (HIF-1α/VEGF/PGK1/CA9) was upregulated in ≥39% of GBM tumors ($n = 31$) with significant positive correlation ($p \leq 0.05$) of the expression of FAT1 with LOX/Vimentin/SOX2/HIF-1α/PGK1/VEGF/CA9. Furthermore, positive correlation ($p \leq 0.01$) of FAT1 with Vimentin/N-cad/SOX2/REST/HIF-1α has been observed in TCGA GBM-dataset ($n = 430$). Analysis of cells (U87MG/A172) exposed to severe hypoxia (0.2%O_2) revealed elevated mRNA expression of FAT1, EMT (Snail/LOX/Vimentin/N-cad), stemness (SOX2/OCT4/Nestin/REST) and hypoxia markers (HIF-1α/PGK1/VEGF/CA9) as compared to their normoxic (20%O_2) counterparts. FAT1 knockdown in U87MG/A172 maintained in severe hypoxia and in normoxic primary glioma cultures led to significant reduction of EMT/stemness markers as compared to controls. HIF-1α knockdown in U87MG cells markedly reduced the expression of all the EMT/stemness markers studied except for Nestin and SOX2 which were more under the influence of FAT1. This indicates FAT1 has a novel regulatory effect on EMT/stemness markers both via or independent of HIF-1α. The functional relevance of our study was corroborated by significant reduction in the number of soft-agar colonies formed in hypoxic-siFAT1 treated U87MG cells. Hence, our study for the first time reveals FAT1 as a novel regulator of EMT/stemness in hypoxic GBM and suggests FAT1 as a potential therapeutic candidate.

Introduction

Glioblastoma is the most aggressive of all brain tumors[1,2] and its resistance to therapy is attributed to the presence of glioma stem cells (GSCs).[1,3,4] The hallmark of GBM is the presence of hypoxic regions around the necrotic core of the tumor with aberrant vascular supply.[3] Hypoxia drives the sustenance and expansion of GSCs in the tumor microenvironment.[3,5] Hypoxia also enhances the invasiveness of tumor cells via EMT, which exacerbates GBM aggressiveness.[6–9] Hence, there is a need to identify newer target(s) regulating multiple factors like stemness and EMT which contribute to tumor aggressiveness and poor patient survival.

FAT1 gene, an ortholog of *Drosophila* tumor suppressor gene *fat*,[10] has been implicated in human cancers.[11–17] It has been documented to have both oncogenic and tumor suppressor roles in different tumors.[11–15,17,18] In GBM, we have earlier reported a crucial role of FAT1 as an oncogene required for the maintenance of pro-inflammatory microenvironment[15] and regulation of HIF-1α via Akt/mTOR pathway.[14] In many cancers including glioma, FAT1 is already known to increase migration/invasion of tumor cells.[14,15,18] However, the role of FAT1 in the maintenance of stemness and EMT process during glioma pathogenesis is unknown so far. Here, we report a novel role of FAT1 in augmenting EMT and stemness characteristics of hypoxic GBM tumors.

Key words: FAT1, hypoxia, glioblastoma, EMT, stemness
Abbreviations: EMT: epithelial-mesenchymal transition; GBM: glioblastoma; HIF-1α: hypoxia inducible factor-1α
Additional Supporting Information may be found in the online version of this article.
Grant sponsor: All India Institute of Medical Sciences, intramural grant; **Grant number:** A-500; **Grant sponsor:** Defence Research and Development Organisation; **Grant number:** DLS/81/4822/LSRB-167/ID/2008
DOI: 10.1002/ijc.31092
History: Received 5 May 2017; Accepted 25 Sep 2017; Online 9 Oct 2017
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What's new?

Glioblastoma multiforme (GBM), the most aggressive form of glioma, is characterized by the presence of hypoxia, cancer stem cells, and high invasiveness. In many cancers including glioma, FAT1 is already known to increase migration/invasion of tumor cells, but its role in the maintenance of stemness and epithelial-mesenchymal transition (EMT) process during glioma pathogenesis remains unclear. This study demonstrates a novel role of FAT1 in the expression regulation of EMT/stemness markers and clonogenic capacity of glioma cells under hypoxia. FAT1 thus emerges as one of the key factors for maintaining EMT/stemness properties in hypoxic GBM and as a potential therapeutic target.

Results

FAT1 expression positively correlates with the expression of hypoxia, EMT and stemness markers in GBM tumors: mRNA expression of EMT (Snail/LOX/Vimentin/N-cad/E-cad) and stemness (SOX2/OCT4/Nestin) markers was analyzed in a cohort of GBM samples ($n = 31$) previously analyzed for FAT1 and hypoxia markers (PGK1/VEGF/CA9/HIF-1α).[15,19] FAT1 was upregulated (≥1.5-fold of normal brain RNA) in 45% of the tumors while hypoxia markers were upregulated in ≥55% of the tumors [PGK1 (81%), VEGF (77%), CA9 (74%) and HIF-1α (55%)] (Table S1). On analyzing the same GBM tumors for EMT and stemness markers, we found increased expression (≥1.5-fold) of EMT markers [LOX (61%), Vimentin (VIM) (55%), Snail (42%) and N-cad (26%)] in ≥26% of the tumors and stemness markers [SOX2 (84%), Nestin (81%) and OCT4 (39%)] in ≥39% of the tumors (Table S1). E-cadherin expression was undetectable in 98% of the studied samples (Table S1).

We have previously reported significant positive correlation ($p < 0.05$) between the expression of FAT1 and hypoxia markers (HIF-1α/VEGF/PGK1/CA9) in GBM ($n = 35$).[14] On analyzing Spearman's correlation of the expression of FAT1 with EMT/stemness/hypoxia markers in 31 GBM samples, FAT1 expression was observed to positively correlate with EMT markers [LOX ($p < 0.01$) and Vimentin (approaching significance of $p = 0.062$)], stemness marker [SOX2 ($p < 0.05$)] and hypoxia markers [HIF-1α ($p < 0.05$), VEGF ($p < 0.01$), PGK1 ($p < 0.01$) and CA9 ($p < 0.01$)] (Fig. 1*a*).

Further in-group comparison of the differential expression of EMT/stemness/hypoxia markers was done across tumors having high and low expression of FAT1. GBM tumors ($n = 31$) were arranged in decreasing order of FAT1 expression and grouped by tertiles to eliminate the intermediate group having heterogenous population of GBM with high and low FAT1 expression (Table S2). A heat map was generated which revealed upregulation of Vimentin ($p = 0.01$), SOX2 ($p = 0.04$), HIF-1α ($p = 0.02$), VEGF ($p = 0.001$) and PGK1 ($p = 0.02$) in the high FAT1 tertile as compared to the low FAT1 tertile (Fig. 1*b*). This confirmed the positive correlation of FAT1 expression with EMT and stemness markers in the GBM tumors analyzed.

In addition, TCGA GBM data ($n = 430$) were analyzed and found to have significant upregulation of FAT1, EMT markers, stemness markers and hypoxia markers as compared to normal brain ($n = 10$). Spearman's correlation and heat map analysis of GBM grouped in tertiles [GBM tertile arranged with FAT1 expression in decreasing order: high FAT1 ($n = 143$), medium FAT1 ($n = 144$) and low FAT1 ($n = 143$)] showed significant positive correlation of FAT1 with EMT markers [Vimentin ($p = 0.01$) and N-Cad ($p = 0.0001$)], stemness markers [SOX2 ($p = 0.01$) and REST ($p = 0.0001$)] and Hypoxia markers [HIF-1α ($p = 0.0001$) and VEGF ($p = 0.0002$)] (Figs. S1*a* and S1*b*). While overall, for most of the markers our in-house GBM set was in concordance with TCGA dataset, for some like PGK1, Snail and OCT4, there was a discrepancy. This could be a reflection of the heterogenous nature of the two datasets. Overall, analysis of GBM tumors suggests positive correlations between the expression of FAT1, EMT (LOX and Vimentin) and stemness (SOX2) genes.

FAT1 knockdown decreases expression of EMT and stemness markers in GBM cell lines exposed to severe hypoxia: The functional association between FAT1 and EMT/stemness markers was studied in GBM cell lines (U87MG and A172) exposed to severe hypoxia (0.2%O_2), which simulates *in vivo* GBM hypoxic microenvironment. FAT1 was found to be upregulated by ≥6-fold in hypoxia-siControl as compared to normoxia-siControl cells (Fig. 2*a*). Similarly, EMT markers (Snail/Vimentin/LOX/N-cad) and stemness markers (SOX2/OCT4/Nestin/REST) were significantly increased by ≥2-fold in hypoxia-siControl cells as compared to normoxia-siControl cells (Figs. 2*b* and 2*c*). To analyze whether FAT1 has any regulatory effect on the expression of EMT/stemness markers, siRNA-mediated FAT1 knockdown was done in U87MG/A172 under severe hypoxia. FAT1 mRNA levels were attenuated by ≥90% in hypoxic-siFAT1 cells as compared to their respective hypoxic-siControls cells (Fig. 2*a*). The expression of EMT markers (Snail/Vimentin/LOX/N-cad) as well as stemness markers (SOX2/OCT4/Nestin/REST) was found to be significantly downregulated ($p < 0.05$) in hypoxic-siFAT1 cells as compared to the hypoxic-siControl cells (Figs. 2*b* and 2*c*). The maintenance of the hypoxia was confirmed by the increased expression of markers CA9, VEGF and PGK1 (≥5-fold) in hypoxia-siControl as compared to normoxia-siControl of U87MG and A172 cells (Figs. S2*a* and S2*b*).

Our lab has previously demonstrated FAT1 to positively regulate the function and activity of HIF-1α in hypoxic GBM

a

Gene		HIF1α	VEGF	PGK1	CA9	LOX	VIM	SNAIL	NCAD	SOX2	NESTIN	OCT4
FAT1	Correlation Coefficient	0.327	0.671	0.528	0.385	0.431	0.283	0.118	0.102	0.396	0.192	0.213
	Sig. (1-tailed)	**0.036** (*)	**0.000** (*)	**0.001** (*)	**0.016** (*)	**0.008** (*)	0.062	0.263	0.292	**0.014** (*)	0.150	0.125

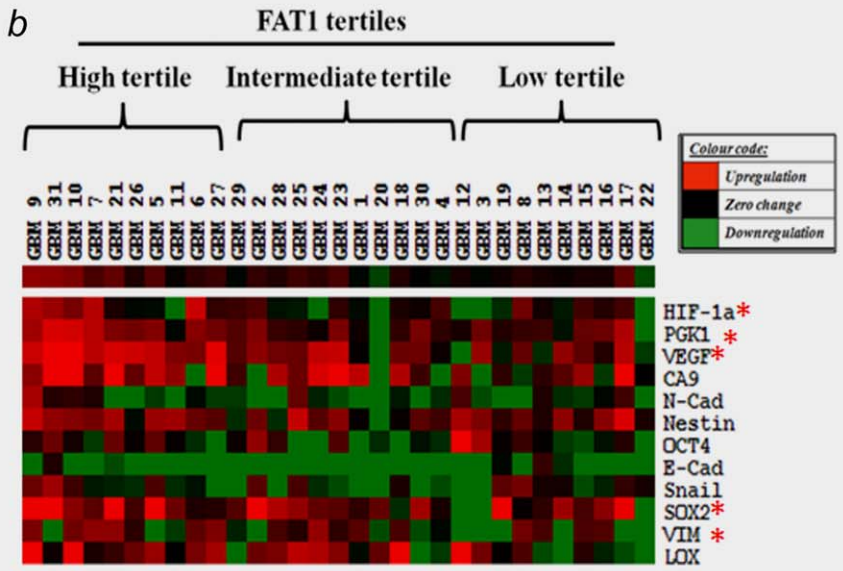

Figure 1. (*a*) Spearman's rank correlation between the expression of FAT1, EMT and stemness markers in 31 GBM tumors. A positive correlation of FAT1 with EMT markers [LOX ($p = 0.008$) and Vimentin (VIM) ($p = 0.062$)], stemness marker [SOX2 ($p = 0.014$)] and hypoxia markers (HIF-1α ($p < 0.01$), VEGF ($p < 0.01$), PGK1 $p < 0.01$) and CA9 ($p = 0.0001$), has been observed. (*b*) Heat map showing clustering of hypoxia markers, EMT markers and Stem cell markers in 31 tumors arranged in decreasing order of FAT1 generated by Cluster 3.0 and Java TreeView. Heat map represents greater upregulation of Vimentin ($p = 0.01$), SOX2 ($p = 0.04$) and HIF-1α ($p = 0.02$), VEGF ($p = 0.001$), PGK1 ($p = 0.02$), in the high FAT1 GBM tertile as compared to the low FAT1 GBM tertile with a difference across the tertiles. Genes with significant ($p < 0.05$) are marked with star (*). [Color figure can be viewed at wileyonlinelibrary.com]

cells.[14] HIF-1α is known to modulate EMT and stemness genes in GBM.[3] To confirm whether the regulatory effect of FAT1 on the expression of EMT and stemness genes is via HIF-1α or independent of it, we did siRNA mediated individual/dual knockdown of FAT1 and HIF-1α in U87MG cells under severe hypoxia. On HIF-1α knockdown (Fig. S3), similar to FAT1 knockdown, the expression of EMT markers (Snail/N-Cad/Vimentin/LOX) and stemness markers (OCT4 and REST) was observed to be significant downregulated ($p < 0.05$) in U87MG under hypoxia. On dual knockdown of FAT1 + HIF-1α, the downregulation ($p < 0.05$) of these markers were similar to that observed with single gene knockdown of only FAT1 and only HIF-1α (Figs. 2*d* and 2*e*). Nestin and SOX2 expression was significantly downregulated in hypoxia-siFAT1 treated cells (Fig. 2*e*). Interestingly, Nestin was not reduced and SOX2 was only 26% reduced in hypoxia-siHIF-1α cells (Fig. 2*e*). Expression of both Nestin and SOX2 was significantly lesser in hypoxia-siFAT treated cells than in hypoxia-siHIF-1α treated cells *p*-values <0.001 and *p*-values <0.05 for Nestin and SOX2, respectively. The significantly reduced expression of SOX2 observed in hypoxia-siFAT1 treated cells as compared to hypoxia-siHIF-1α treated cells was consistent, reflecting the role of FAT1 beyond the effect of HIF-1α thus suggesting a novel role of FAT1 in regulating Nestin and SOX2 expression in GBM under hypoxia.

To study the effect of exogenous FAT1 overexpression on EMT/stemness genes, we tried to clone and express full-length FAT1-cDNA but was unsuccessful because of its large size (>13Kb) and multiple-cadherin repeats. In literature, truncated-FAT1 constructs with only 2 cadherin-repeats have been used.[16] We overexpressed this truncated-FAT1 (kind gift by Prof. Chen[16]) in U87MG, but there was no significant effect on the expression of EMT/stemness markers (data not shown). This could be because the truncated-FAT1 protein did not fully replicate the properties of the full-length endogenous-FAT1 with 34 cadherin-repeats. Overexpression of endogenous FAT1, induced by severe-hypoxia, led to increased expression of EMT/stemness markers reflecting the regulatory effect of FAT1 on EMT/stemness genes expression.

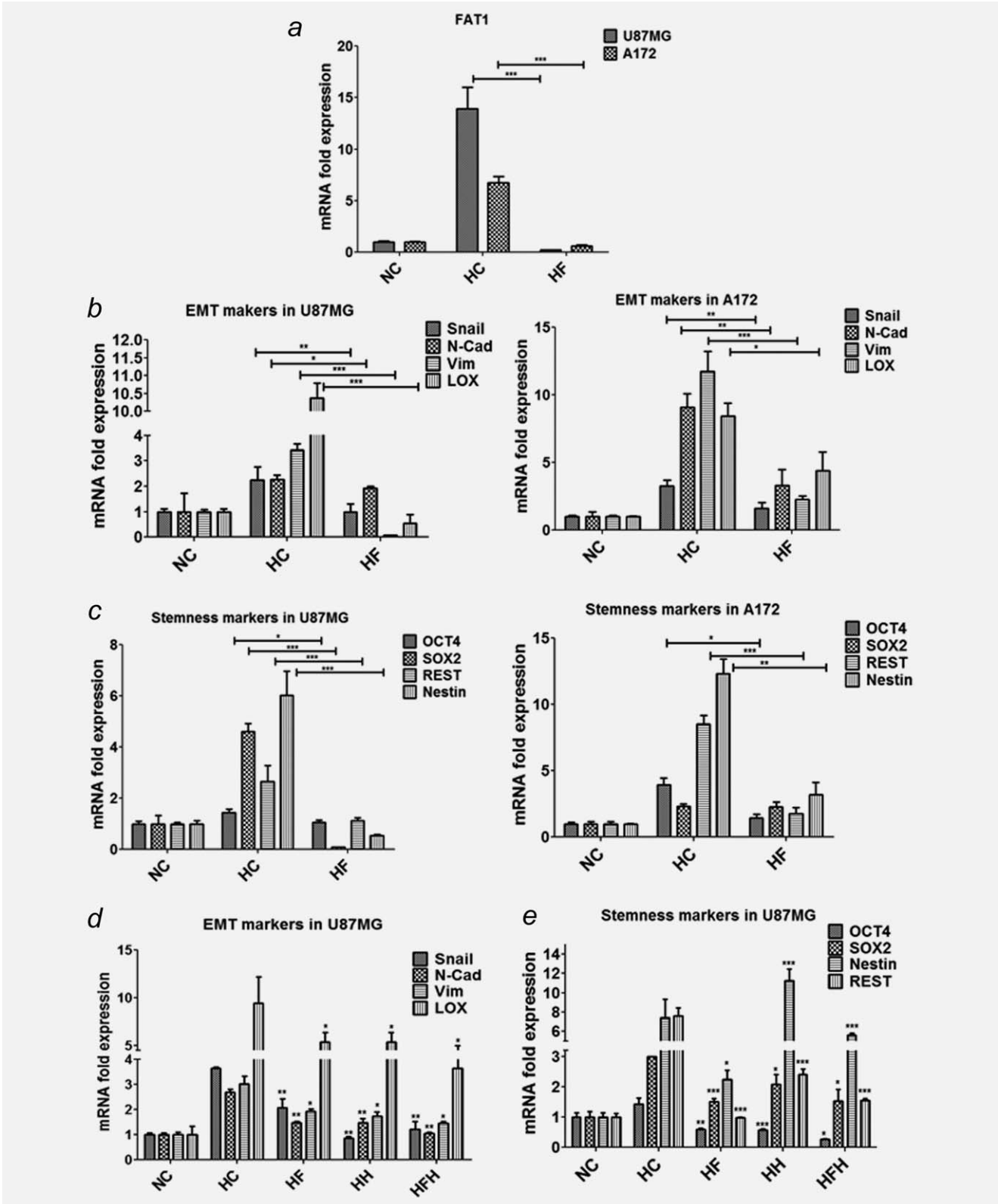
a
FAT1
U87MG
A172
mRNA fold expression
NC
HC
HF
b
EMT makers in U87MG
Snail
N-Cad
Vim
LOX
EMT makers in A172
c
Stemness markers in U87MG
OCT4
SOX2
REST
Nestin
Stemness markers in A172
d
EMT markers in U87MG
HH
HFH
e
Stemness markers in U87MG

Figure 2.

FAT1 knockdown decreases the expression of EMT and stemness markers in primary GBM culture: Freshly resected glioma grade-II (PC1) and grade-IV (PC2) tumors were processed and cultured in primary culture medium in normoxia. These cells did not survive under hypoxia. Expression of FAT1 under normoxia in PC1 was 4.6-fold ±0.73 and in PC2 was 6.3-fold ±1.05 as compared to normoxic U87MG cells but was comparable to hypoxic U87MG cells (5.72-fold ± 0.53) (Fig. 3*a*). FAT1 knockdown in PC1and PC2 led to significant reduction in the expression of EMT markers as compared to their siControl cells (Figs. 3*b* and 3*c*). Among stemness markers, Nestin and SOX2 were significantly down-regulated in siFAT1 transfected PC2 (grade-IV) only (Fig. 3*d*).

FAT1 knockdown decreases anchorage-independent growth of U87MG cells under hypoxia: Anchorage-independent growth is the capacity of transformed cells to proliferate independent of a solid surface, representing stemness properties of a cancer cell. We evaluated the effect of FAT1 knockdown on U87MG clonogenicity under hypoxia. The number of colonies was more in hypoxic-siControl cells ($n = 32.8 \pm 5.29$; size = 21.32 ± 4.29 μm) than in normoxic-siControl cells ($n = 15.6 \pm 0.89$; size = 39.29 ± 13.23 μm) (Fig. 3*e*). Significant reduction in the number of colonies in hypoxic-siFAT1 cells ($n = 15.75 \pm 2.48$; size = 21.07 ± 4.92 μm) as compared to the hypoxic-siControl cells ($n = 32.8 \pm 5.29$; size = 21.32 ± 4.29 μm) after 4 weeks of culture (Fig. 3*e*). Decreased colony forming capacity of U87MG cells under hypoxia on FAT1 knockdown reflects the importance of FAT1 in maintenance of its stemness properties.

Material and Methods

Patients and tumor samples

Thirty-one surgically resected GBM samples were collected from Department of Neurosurgery/Neuropathology, AIIMS, New Delhi, after obtaining ethical clearance (IESC/T-106/04.03.2011) and written consent from the patients.[16,19] Control normal human-brain RNA was purchased from Stratagene/Clontech (La Jolla/Mountain View, CA). TCGA-data Level-3 (http://www.cbioportal.org) was analyzed for expression of FAT1/EMT/stemness/hypoxia markers in 430 GBM samples with respect to 10 normal-brains.

In vitro cell culture, glioma primary culture and siRNA transfection

Glioma cell lines U87MG/A172 were procured from ATCC, Manassas, VA. Methodology on cell culture and siRNA-transfection is explained in supplementary methodology-M1 (*in vitro* culture) and M2 (primary culture of resected-glioma).

Soft agar colony formation assay

Assay was done in hypoxic U87MG cells transfected with siFAT1. Detail in supplementary methodology-M3.

q-PCR

Total-RNA was isolated from GBM tumors and cell lines, followed by cDNA preparation and q-PCR analysis as described previously.[16,19] Primers detail in Table S3.

Gene clustering and GBM tertile analysis

Semi-supervised gene clustering of EMT/stemness markers performed and Heat-map analysis done using Cluster 3.0/Java TreeView as described previously.[19]

Statistical analysis

Spearman's rank correlation (non-parametric; one-tailed) was calculated using SPSS-11.5, GraphPad. Statistical significance determined at the level of $p \leq 0.05$.

Figure 2. Decrease in EMT and stemness genes on FAT1 knockdown under hypoxia. qPCR was done to measure the mRNA expression of EMT/stemness genes, 18S rRNA as internal control. (*a*) U87MG/A172 cells transfected with FAT1-specific siRNA (siFAT1) and control-siRNA (siControl) were exposed to severe hypoxia ($0.2\%O_2$) 24 hr post siRNA transfection. Expression was checked 72 hr post siRNA transfection. As compared to normoxia-siControl cells (NC) significant ($p < 0.001$) increased expression of FAT1 observed in hypoxia-siControl cells (HC) in both U87MG and A172 cells. The knockdown efficiency of FAT1-specific siRNA was >98% ($p < 0.001$) in hypoxia-siFAT1 cells (HF). (*b*) Expression of EMT markers (Snail/N-Cad/Vimentin/LOX) in U87MG/A172 was significantly increased in hypoxia-siControl cells (HC) as compared to normoxic-siControl cells (NC). In hypoxia siFAT1 cells (HF), decreased expression of EMT markers [Snail-($p < 0.01$), N-cad-($p < 0.01$), Vimentin-($p < 0.001$) and LOX-($p < 0.05$)] was found as compare to hypoxia-siControl cells (HC). (*c*) Expression of stemness markers (OCT4/SOX2/REST/Nestin) in U87MG and A172 was significantly increased in hypoxic-siControl (HC) as compared to normoxic-siControl cells (NC). In hypoxia-siFAT1 cells (HF), decreased expression of stemness markers OCT4-($p < 0.05$), REST-($p < 0.001$) and Nestin-($p < 0.01$) was found in both U87MG and A172 as compared to hypoxia siControl cells (HC). Decreased expression of SOX2-($p < 0.001$) was observed in hypoxia-siFAT1 (HF) treated U87MG cells only. (*d* and *e*) U87MG cells were transfected with either siFAT1/siHIF-1α or simultaneously with siFAT1 + siHIF-1α and exposed to severe hypoxia ($0.2\%O_2$) 24 hr post siRNA transfection. Expression analysis was done 72 hr post siRNA transfection. Similar to hypoxic-siFAT1 cells (HF), expression of EMT markers (Snail/Ncad/Vimentin/LOX) and stemness markers (OCT4 and REST) was significantly decreased in hypoxia-siHIF-1α (HH) and hypoxia-siFAT1 + siHIF-1α (HFH) treated cells as compared to hypoxia-siControl cells (HC). Nestin expression was significantly decreased in hypoxia-siFAT1 (HF) and hypoxia-siFAT1 + siHIF-1α cells (HFH) but not in hypoxia-siHIF-1α (HH) cells as compared to hypoxia-siControl cells (HC). SOX2 expression was decreased in hypoxia-siFAT1 (HF) and hypoxia-siFAT1 + siHIF-1α (HFH) as compared to hypoxia-siControl cells (HC). Expression of SOX2 was decreased only 26% in hypoxia-siHIF-1α cells (HH) as compared to hypoxia-siControl cells (HC). Each bar is expressed as mean ± SD. Each experiment was done in triplicate and repeated twice. Bar graph represents fold mRNA expression of genes. Asterisk sign (*) signify *p*-values (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) calculated by *t*-test.

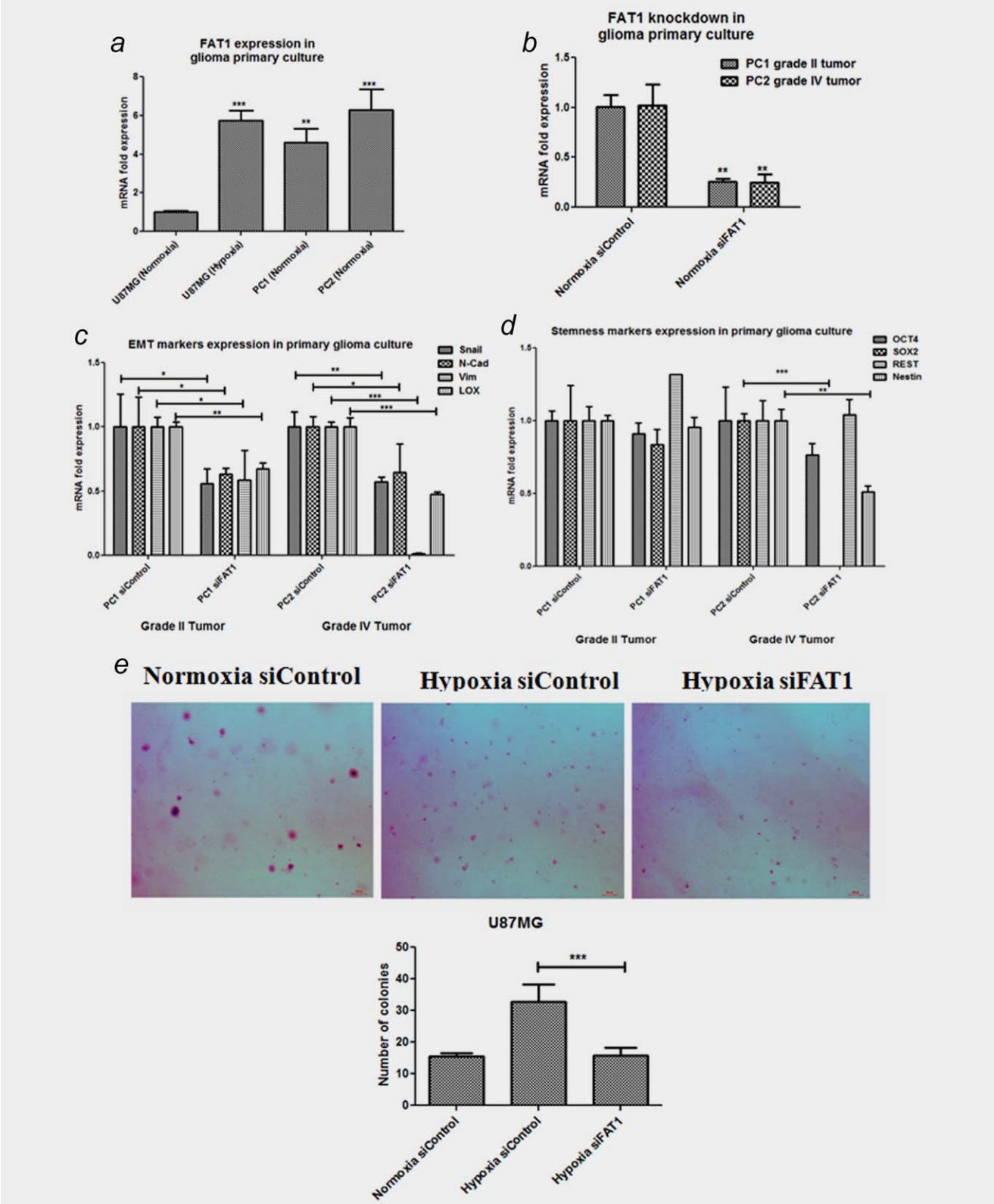
a
FAT1 expression in glioma primary culture
mRNA fold expression
U87MG (Normoxia)
U87MG (Hypoxia)
PC1 (Normoxia)
PC2 (Normoxia)
b
FAT1 knockdown in glioma primary culture
PC1 grade II tumor
PC2 grade IV tumor
mRNA fold expression
Normoxia siControl
Normoxia siFAT1
c
EMT markers expression in primary glioma culture
Snail
N-Cad
Vim
LOX
mRNA fold expression
PC1 siControl
PC1 siFAT1
PC2 siControl
PC2 siFAT1
Grade II Tumor
Grade IV Tumor
d
Stemness markers expression in primary glioma culture
OCT4
SOX2
REST
Nestin
mRNA fold expression
PC1 siControl
PC1 siFAT1
PC2 siControl
PC2 siFAT1
Grade II Tumor
Grade IV Tumor
e
Normoxia siControl
Hypoxia siControl
Hypoxia siFAT1
U87MG
Number of colonies
Normoxia siControl
Hypoxia siControl
Hypoxia siFAT1

Figure 3.

Discussion

We have earlier observed FAT1 to have an oncogenic role in gliomas by upregulating migratory and invasive properties of GBM cells as well as by increasing expression of pro-inflammatory molecules like COX2, IL1β and IL6 through the transcription factor AP1.[15] We also showed that FAT1 upregulates HIF-1α expression and functions under hypoxic conditions, thus affecting their metabolic status and invasive properties.[14] Studies have demonstrated that the hypoxic microenvironment with increased HIF-1α promotes stemness and invasiveness, which favors tumor growth and therapeutic resistance.[3,19] Here, for the first time, we have demonstrated the role of FAT1 in regulating EMT and stemness properties in GBM under hypoxia, highlighting the importance of FAT1 signaling in aggressive GBM.

On analyzing our GBM tumor cohort and the TCGA GBM dataset, FAT1 expression was observed to have a significant positive correlation ($p \leq 0.05$) with the expression of EMT (LOX and Vimentin) and stemness (SOX2) markers. A significant positive correlation ($p < 0.05$) of FAT1 with the expression of hypoxia markers (HIF1-α/VEGF/PGK1/CA9) was observed in our GBM cohort analyzed, similar to our earlier finding.[14] Also, a significant positive correlation ($p < 0.05$) of FAT1 with the expression of HIF-1α and VEGF was observed in TCGA GBM dataset. These correlations of gene expression may reflect the functional interactions of genes in GBM especially under hypoxia. This functional correlation of FAT1 with hypoxia, EMT and stemness genes in GBM was further explored in *in vitro* GBM cells (U87MG and A172) exposed to severe hypoxia ($0.2\%O_2$), to mimic the *in vivo* tumor hypoxic microenvironment.

On exposure to severe hypoxia ($0.2\%O_2$), increased expression of FAT1 along with increased expression of hypoxia markers (HIF1-α/PGK1/VEGF/CA9), EMT markers (Snail/LOX/N-cad/Vimentin) and stemness markers (SOX2/OCT4/Nestin/REST) was observed in both U87MG and A172 cells. The regulatory effect of FAT1 on the expression of EMT/stemness markers was further determined by knocking down FAT1 expression using FAT1-specific siRNA, in GBM cells (U87MG/A172) maintained under severe hypoxia. FAT1 knockdown in glioma cells led to significant decrease in the expression of EMT markers (Snail/LOX/N-cad/Vimentin) and stemness markers (SOX2/OCT4/Nestin/REST). This *in vitro* observation of decreased EMT/stemness markers on FAT1 knockdown corroborated with the findings in the GBM samples, where positive correlation was observed between FAT1 and EMT/stemness genes expression under hypoxic tumor microenvironment, as reflected by increased expression of HIF-1α and other hypoxia markers. FAT1 is documented to have an oncogenic role in glioma by upregulating proinflammatory microenvironment and HIF-1α signaling under hypoxia and its role in promoting migration/invasion in various cancers is well established.[14,15,18,20] The observation of the role of FAT1 in regulating the expression of EMT/stemness is novel and it further strengthens the oncogenic role of FAT1 in GBM.

We have earlier reported the role of FAT1 in modulating HIF-1α expression and function in hypoxic-glioma cells,[14] where FAT1 knockdown under severe-hypoxia led to decreased expression and function of HIF-1α along with decreased migration/invasion of GBM cells.[14] HIF-1α is known to alter the expression of EMT and stemness genes in GBM.[3] HIF-1α knockdown led to reduced expression of all EMT markers (Snail/LOX/N-cad/Vimentin) and the stemness markers (OCT4 and REST) similar to that observed on FAT1 knockdown. However, the expression of Nestin was not reduced on HIF-1α knockdown but was significantly downregulated on FAT1 knockdown. Similarly, SOX2 expression reduction was

Figure 3. (*a*) Increased expression of FAT1 in glioma primary culture cells: Glioma primary cultures (PC1; grade-II and PC2; grade-IV tumors) maintained in DMEM F-12 medium supplemented with 10%-FBS in normoxic-condition (Normoxia, $20\%O_2$). Expression of FAT1 was analyzed in PC1 (Normoxia, $20\%O_2$), PC2 (Normoxia, $20\%O_2$), U87MG cells (severe-hypoxia, $0.2\%O_2$) and compared to U87MG cells (Normoxia, $20\%O_2$). Increased expression of FAT1 was observed in hypoxic U87MG cells, PC1 (Normoxia) and PC2 (Normoxia) as compared to U87MG cells (Normoxia). (*b*) FAT1 knockdown in glioma primary culture: glioma primary cultured cells were transfected with siFAT1/siControl maintained in normoxia, $20\%O_2$. After 72 hr of siRNA treatment, total-RNA was isolated for gene expression analysis. The knockdown efficiency of FAT1-specific siRNA has been observed to be >78% ($p < 0.001$) in both the siFAT1-transfected PC1 and PC2 cells as compared to siControl-treated PC1 and PC2 cells. (*c*) Expression of EMT markers (Snail/N-Cad/Vimentin/LOX) was significantly decreased in siFAT1-transfected PC1 and PC2 cells as compared to siControl-transfected PC1 and PC2 cells. (*d*) Expression of stemness markers (OCT4/SOX2/REST/Nestin) was not altered significantly in siFAT1-transfected PC1 cells as compared to siControl-treated PC1 cells. Only SOX2 and Nestin was significantly decreased siFAT1-transfected PC2 cells as compared to siControl-transfected PC2 cells. Bar graph (mean ± SD) represents fold mRNA expression of genes. Asterisk sign (*) signify *p*-values (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) calculated by *t*-test. Abbreviations: PC1, primary culture-1; PC2, primary culture-2. (*e*) Soft agar colony formation assay after FAT1 knockdown under hypoxia: after 24 hr of siControl/siFAT1 transfection, U87MG cells were exposed to severe hypoxia ($0.2\%O_2$) for 48 hr. Treated cells were seeded in 0.3% agarose in DMEM supplement with 10% FBS for 4 weeks. We observed significant decreased number of colonies in hypoxia-siFAT1 treated cells ($n = 15.75 \pm 2.48$; $p < 0.001$) as compared to hypoxic-siControl cells ($n = 32.8 \pm 5.29$). The number of colonies was observed to be more in hypoxic-siControl cells ($n = 32.8 \pm 5.29$) than in normoxic-siControl cells ($n = 15.6 \pm 0.89$). The size of colonies in normoxia-siControl (size = 39.29 ± 13.23 μm) was bigger as compared to hypoxia-treated cells (hypoxic-siControl colony size = 21.32 ± 4.29 μm and hypoxic-siFAT1 colony size = 21.07 ± 4.92 μm) with no difference in the size of the colonies between hypoxic-siControl and hypoxic-siFAT1. Microscopic images were taken at 4× magnification (Scale bar, 100 μm). Bar graph represents number of soft agar colonies (mean ± SEM, $n = 5$). The experiment was performed in triplicates and repeated twice. Asterisk sign (***) signifies $p < 0.001$). *t*-test was applied to calculate *p*-values. [Color figure can be viewed at wileyonlinelibrary.com]

more significant on FAT1 knockdown than on HIF-1α knockdown. Our group has previously reported that SOX2 expression is mainly regulated by HIF-2α.[21] Expression of SOX2 and Nestin was also downregulated significantly on FAT1 knockdown in GBM primary culture cells. Further studies are required to confirm the pathway(s) through which FAT1 regulates Nestin and SOX2 expression in GBM under hypoxia. Overall, our study for the first time documents the novel role of FAT1 in regulating the expression of EMT/stemness genes both via or independent of HIF-1α pathway in hypoxic GBM.

Since, FAT1 has been observed to substantially influence the expression of EMT/stemness markers in GBM cells, further corroboration on the long-term survival effect of FAT1 on GBM cells was analyzed by clonogenicity of glioma cells after FAT1 knockdown by soft agar assay. This is a cell-based assay to study the effectiveness of specific agents on the survival and proliferation of cells. It represents most potential *in vitro* model mimicking *in vivo* 3 D cellular microenvironment of GBM tumors for studying stemness properties of GBM cells.[20] A recent report on the antagonistic effect of FAT1 on caspase 8 has shown FAT1 knockdown to reduce the clonogenic capacity of glioma cells.[22] In hypoxic-siControl cells, the number of colonies was more than normoxia-siControl U87MG cells corroborating previous findings.[23,24] However, we found a significant reduction in the number of agar colonies on FAT1 knockdown in U87MG cells under hypoxia as compared to hypoxic-siControl cells, thus providing functional evidence of FAT1 critically regulating the clonogenicity of GBM. In conclusion, our study for the first time identified FAT1 to be a crucial regulator of EMT and stemness gene expression and functionally essential for maintaining the clonogenic capacity of glioma cells. We hereby state that FAT1 is one of the key factors for maintaining EMT/stemness properties of hypoxic GBM and may be a target for therapeutic intervention in GBM subset having increased FAT1 expression.

Acknowledgements

This work was funded by grant from Defence Research and Development Organisation (DRDO) and intramural grant from All India Institute of Medical Sciences (AIIMS), New Delhi, India to K.C. S.S. acknowledges the JC Bose fellowship of DST (Department of Science and technology) India. Research fellowship from AIIMS to C.S. (Chitrangda Srivastava) and DST-SERB fellowship to K.I. We acknowledge Ms. Jyoti, Mr. Manoj Sharma and Lab attendants Mr. Pappu and Mr. Mukesh for their assistance in routine lab work and cell culture work. We acknowledge Prof. Timothy A. Chen from Memorial Sloan-Kettering Cancer Center, New York, NY 10065 for his kind gift of the FAT1 overexpressing construct.

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