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Hypoxic Glioma Cell-Secreted Exosomal miR-301a Activates Wnt/β-catenin Signaling and Promotes Radiation Resistance by Targeting TCEAL7

Xiao Yue,[1] Fengming Lan,[2] and Tingyi Xia[3]

[1]Department of Neorosurgery, The Affiliated Hospital of Xiangnan University (Clinical College), ChenZhou, Hunan, P.R. China; [2]Department of Radiation Oncology, National Cancer Center/Cancer Hospital & Shenzhen Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Shenzhen 518116, P.R. China; [3]Department of Radiation Oncology, PLA Airforce General Hospital, Beijing 100142, P.R. China

Recent evidence suggests that microRNAs (miRNAs) can be released to the extracellular microenvironment and mediate cell-cell communication through exosomes. The aim of this study was to identify exosomal miR-301a (exo-miR-301a) involved in glioblastoma (GBM) radioresistance and reveal the possible mechanisms. The exo-miR-301a specifically secreted by hypoxic GBM cells could transfer to corresponding normoxia-cultured cells and promote radiation resistance. Hypoxic exo-miR-301a directly targeted TCEAL7 genes, which were identified as a tumor suppressor in GBM malignancy and actively repressed its' expression in normoxic glioma cells. Our studies indicated that TCEAL7 negatively regulated the Wnt/β-catenin pathway by blocking β-catenin translocation from cytoplasm to nucleus. Interestingly, we clarified that the Wnt/β-catenin signaling was activated by miR-301a and TCEAL7 mediated the important procession. The exo-miR-301a was involved in the resistance to radiotherapy, and the effects would be reversed by miR-301a inhibition or TCEAL7 overexpression to regulate the Wnt/β-catenin axis. Here we show that exo-miR-301a, which is characteristically expressed and secreted by hypoxic glioma cells, is a potent regulator of Wnt/β-catenin and then depresses radiation sensitivity through targeting anti-oncogene TCEAL7. The newly identified exo-miR-301a/TCEAL7-signaling axis could present a novel target for cellular resistance to cancer therapeutic radiation in GBM patients.

INTRODUCTION

Glioblastoma (GBM) is the most common and malignant brain tumor of astroglial origin. GBM is associated with a dismal prognosis for the infiltration into the normal brain parenchyma.[1] Together with surgery, radiotherapy has always been the cornerstone of treatment for GBM; however, tumor recurrence is nearly universal.[2,3] Therefore, it's imperative to modulate the radiation resistance to GBM and develop novel strategies in treatment.

Exosomes are nanovesicles with a diameter ranging from 30 to 140 nm released from many cell types, including cancer cells. Exosomes secreted to the extracellular microenvironment are involved in the cell-cell neighboring communication and distant cells, favoring secretion of growth factors, cytokines, and angiopoietic factors. Active ingredients in exosomes can induct tumor proliferation, metastasis, therapeutic efficacy, and immune responses.[4] They can be isolated from blood or other bodily fluids to reveal cancerous progression occurring in the body.[5] In addition to being a reservoir of valuable biomarkers for cancer diagnosis and prognosis, exosomes also constitute vectors for therapeutic approaches in various tumors, which are responsible for stromal activation, induction of the angiogenic switch, vascular permeability enhancement, and immune escape.[6]

Exosomes are formed in the endosomal network and comprise different proteins and nucleic acids that vary according to cell types and mechanisms of biogenesis. One of the most distinct characteristics of exosomes is that they carry significant amounts of nucleic acids, including mitochondrial DNA, mRNA, long noncoding RNAs, small nuclear RNAs, and microRNAs (miRNAs). Accumulating evidence has demonstrated that the biogenesis of miRNAs is tightly controlled, and dysregulation of miRNAs is linked to various cancers' initiation and progression.[7,8] Furthermore, exosomes delivering miRNAs interact with various cells of the microenvironment to confer tumor-advantageous changes that influence tumor progression.

Nearly all human solid tumors are exposed to hypoxia, which plays a central role in tumor progression and resistance to therapy by fostering a variety of changes in cancer and stromal cell biology.[9] As is known, tumor hypoxia is a major contributing factor to the failures of anticancer therapies.[10] Tumor hypoxia is a therapeutic concern since it can reduce the effectiveness of conventional therapies, such as radiotherapy. In this study, we revealed that exosomal miR-301a (exo-miR-301a) released by hypoxic GBM cells can interfere with the sensitivity to radiotherapy by regulating the Wnt/β-catenin-signaling pathway.

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Correspondence: Fengming Lan, Department of Radiation Oncology, National Cancer Center/Cancer Hospital & Shenzhen Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Baohe Road 113, Longgang District, Shenzhen 518116, P.R. China.
E-mail: dreamlfm1986@163.com

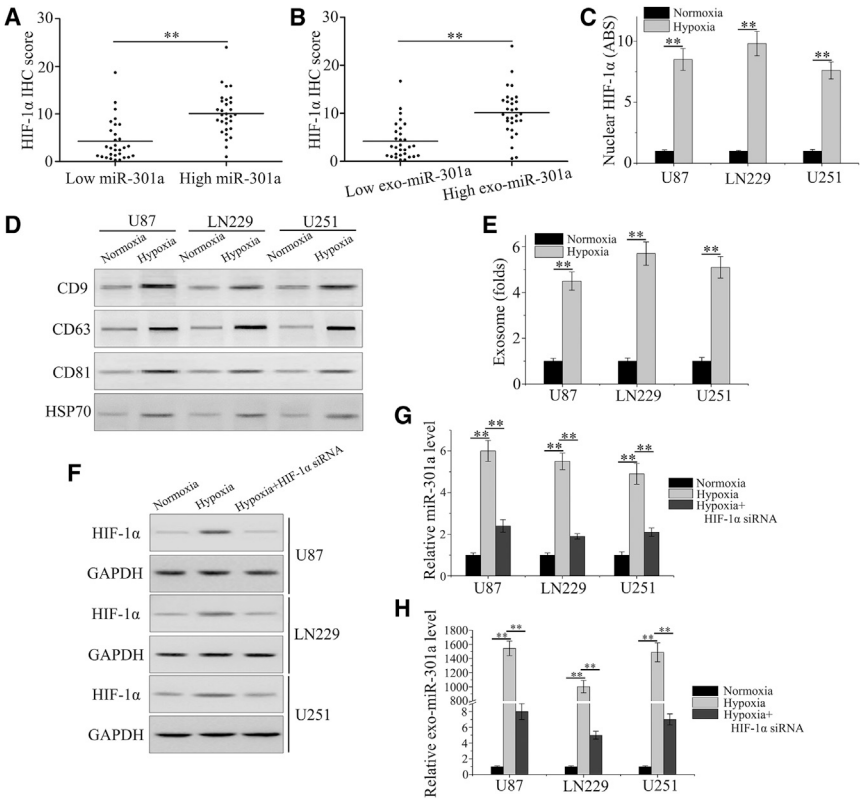

Figure 1. miR-301a Is Specifically Expressed and Secreted by Hypoxic GBM Cells and Transferred to Normoxic Glioma Cells via Exosome Secretion

High miR-301a (A) and serum exo-miR-301a (B) are strongly associated with HIF-1α protein. (C) ELISA assay for HIF-1α performed in GBM cells. (D) Western blot detected the markers of exosomes. (E) Quantification of exosomes by nanoparticle-tracking analysis. The expressions of HIF-1α (F), miR-301a (G), and exo-miR-301a (H) were detected using western blot and qPCR.

RESULTS

exo-miR-301a Is Specifically Expressed and Secreted by Hypoxic GBM Cells

Based on our previous observations, miR-301a was significantly upregulated in glioma tissues, and serum exo-miR-301a could serve as a potential diagnostic and prognostic biomarker for human glioma. Here we sought to detect the probable function of exo-miR-301a in hypoxic human GBM.[11,12] HIF-1α expression was performed as a hypoxic biomarker to compare with the expression of miR-301a in glioma patients. Higher miR-301a expression was observed in patients with high HIF-1α levels (Figure 1A), and the percent of serum exo-miR-301a (low versus high) was distributed according to the HIF-1α immunohistochemistry (IHC) score (Figure 1B). ELISA results displayed that GBM cells responded to hypoxia by increasing the levels of HIF-1α in the nucleus (Figure 1C).

Exosomal markers, containing CD9, CD63, CD81, and HSP70, were examined from GBM cells' exposure to normoxic or hypoxic conditions. As shown in Figure 1D, the levels of all exosomal markers were higher in hypoxic cells when compared with normoxic cells. Hypoxic conditions also significantly increased the number of vesicles present in exosomes, as detected by nanoparticle-tracking analysis (Figure 1E).

To explore the association between the increased expression of miR-301a and HIF-1α signaling, we observed the levels of miR-301a and exo-miR-301a in different statuses of HIF-1α. HIF-1α was induced by hypoxia and knocked down when transected with HIF-1α small interfering RNA (siRNA) (Figure 1F). Remarkably, hypoxia significantly increased the expressions of miR-301a and exo-miR-301a; meanwhile, downregulation of HIF-1α significantly decreased the expressions of miR-301a and exo-miR-301a (Figures 1G and 1H). Taken together, we demonstrated that exo-miR-301a is specifically secreted by hypoxic GBM cells and related to HIF-1α status.

exo-miR-301a from Hypoxic Glioma Cells Directly Targets TCEAL7 in Normoxic Glioma Cells

To explore the underlying mechanism of miR-301a in hypoxic glioma, the target genes of miR-301a were predicted by using the web-based software program Targetscan. TCEAL7 acts as a tumor suppressor gene and is decreased in various cancers.[13] To further explore the exact role of miR-301a in TCEAL7 regulation, we then detected the relationship of exo-miR-301a and TCEAL7 protein in glioma patients. The level of TCEAL7 was inversely correlated with the expression levels of miR-301a (Figure 2A). To confirm the direct binding between miR-301a and TCEAL7, the luciferase reporter assay was designed to conduct by the luciferase reporter plasmid containing wild-type (WT) and mutation (MT) TCEAL7 3′ UTRs with the miR-301a-binding site. TCEAL7 3′ UTR contains two highly conserved target sites of miR-301a, and the first target site is proven to be the binding site (Figure 2B). As demonstrated in Figure 2C, the luciferase activity in Luc-TCEAL7-UTR-transfected cells was significantly disrupted by miR-301a as compared with the TCEAL7 3′ UTR mutant group, both in normoxia and hypoxia.

Consistent with the results from the reporter assay, transfection with As-miR-301a resulted in a significant decrease in TCEAL7 expression under both normoxia and hypoxia conditions (Figure 2D). Furthermore, we examined the expression of TCEAL7 in normal cultured GBM cells that were treated with exosomes derived from normoxic or hypoxic cells. Hypoxic exosome treatment reduced luciferase activity when compared to the control and normoxic exosome groups (Figure 2E). Moreover, the protein levels of TCEAL7 were dramatically changed with hypoxic

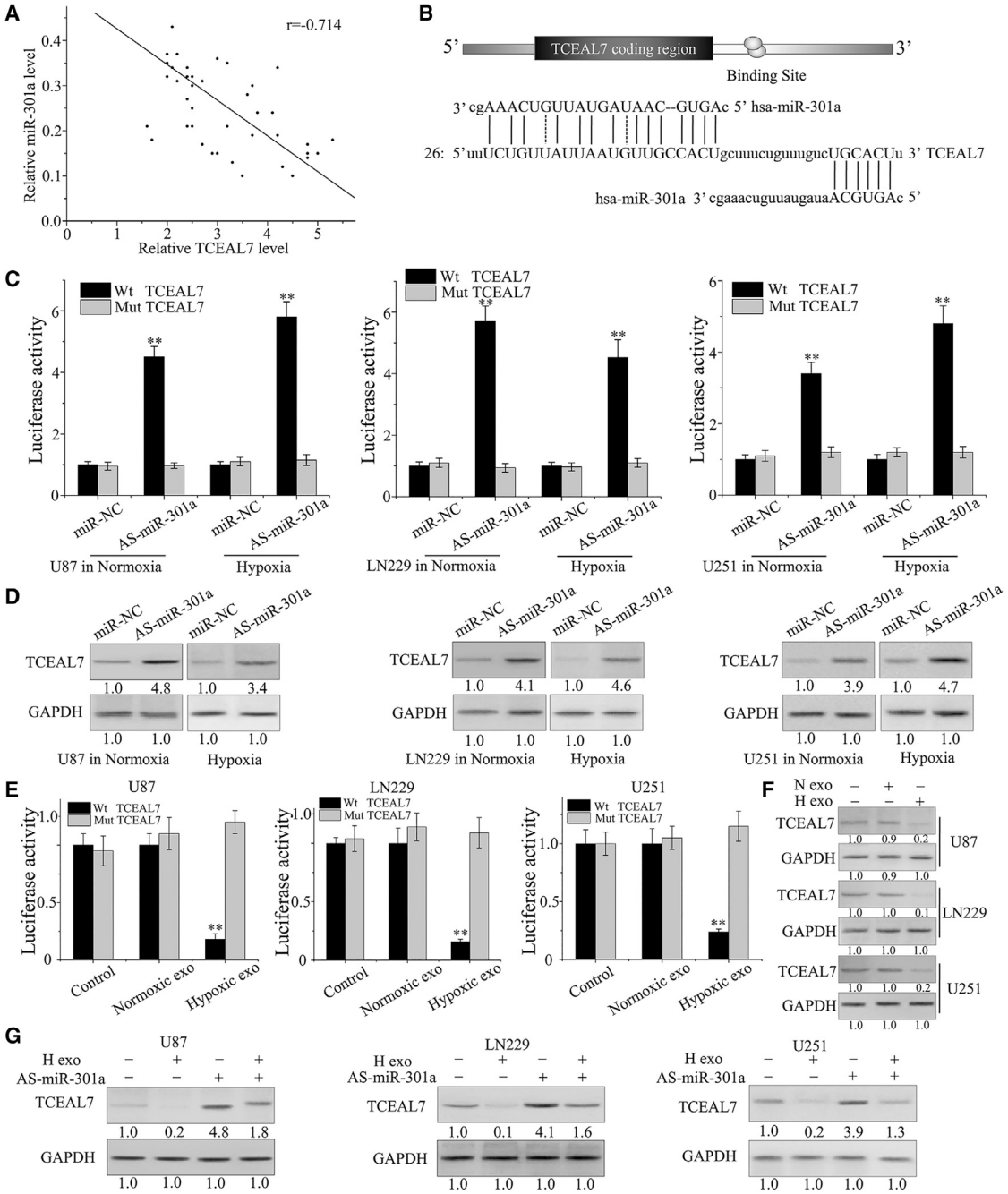

Figure 2. Exosomal miR-301a Derived from Hypoxic Glioma Cells Targets TCEAL7

(A) The inverse correlation of miR-301a with TCEAL7. (B) Schematic representation of the putative binding sites in TCEAL7. (C) mRNA 3′ UTR for miR-301a. pGL3-WT-TCEAL7-3′ UTR-Luc and pGL3-MUT-TCEAL7-3′ UTR-Luc reporters were transfected with As-miR-301a or NC. (D) Western blot analysis was performed to evaluate the expression of TCEAL7 in normoxic and hypoxic glioma cells, which were transfected with As-miRNA-301a. (E) Luciferase reporter co-transfected with exosomes. (F) Hypoxic-derived exosomes decreased the expression of TCEAL7. (G) As-miR-301a prevents augmentation of hypoxic exosomes on TCEAL7 protein downregulation. **$p < 0.01$.

exosome treatment compared to those in the control and normoxic groups (Figure 2F). The effect of hypoxia-derived exosomes on TCEAL7 downregulation was prevented by transfecting As-miR-301a (Figure 2G). Collectively, these findings indicate that hypoxic exo-miR-301a directly targeted TCEAL7 and actively repressed the expression in glioma cells.

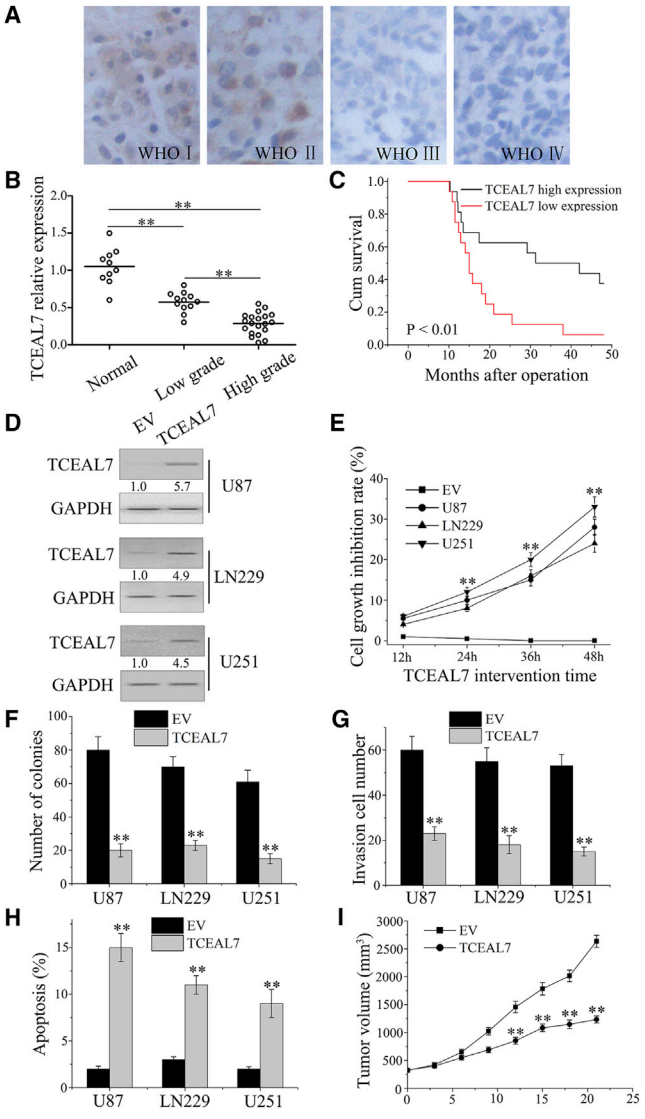

Figure 3. TCEAL7 Is Frequently Downregulated in GBM Malignancy

(A) Immunohistochemical analysis showed the expression of TCEAL7. (B) TCEAL7 expression in glioma and normal brain tissues as detected by qPCR. (C) Kaplan-Meier survival curves analyzed glioma patients who had high or low expression of TCEAL7. (D) Western blot was performed to evaluate the expression levels of TCEAL7. (E) TCEAL7 remarkably inhibited the cell viability of glioma cells. (F) Fewer colonies were observed in TCEAL7 vector than control cells. (G) TCEAL7 vector significantly reduced invasive capacity. (H) TCEAL7 vector induced apoptosis detected by fluorescence-activated cell sorting (FACS) analysis. (I) Inhibition of the growth of U87 xenografts treated with TCEAL7 vector.

Table 1. Association of TCEAL7 Expression in Human Glioma with Different Clinicopathological Features

Clinicopathological Feature	Number of Cases	TCEAL7 Expression		p Value
		High, n (%)	Low, n (%)	
WHO Grade				
I	6	6 (100.0)	0 (0.0)	<0.01
II	6	5 (83.3)	1 (16.7)	
III	8	4 (50.0)	4 (50.0)	
IV	12	1 (8.3)	11 (91.7)	
Age (Years)[a]				
≤44	14	8 (57.1)	6 (42.9)	NS
>44	18	8 (44.4)	10 (55.6)	
Gender				
Male	17	7 (41.2)	10 (58.8)	NS
Female	15	9 (60.0)	6 (40.0)	
KPS				
≤80	22	7 (31.8)	15 (68.2)	<0.01
>80	10	9 (90.0)	1 (10.0)	

[a]Median age was 44 years.

TCEAL7 Is Frequently Downregulated in GBM Malignancy

In our previous study, immunohistochemical staining analysis revealed that protein levels of β-catenin were overexpressed in high-grade glioma.[12] The protein level of TCEAL7 was detected in similar tissues and downregulated along with an increased state of malignancy (Figure 3A). TCEAL7 was inversely correlated with β-catenin in glioma tissues, so we speculated that TCEAL7 might be a negative regulator of Wnt/β-catenin-signaling activity. Furthermore, qPCR was performed to analyze TCEAL7 expression in glioma and normal brain specimens. As shown in Figure 3B, the expression of TCEAL7 in glioma tissues was lower than in normal tissue counterparts, and it decreased with ascending pathological grade.

The 32 patients with glioma were divided into high and low TCEAL7 expression groups using the median expression value as the cutoff point. Statistically significant correlations were observed among the low TCEAL7 expression, advanced pathological grade, and ≤80 Karnofsky performance status (KPS), and no significant association was found with other clinicopathological parameters (Table 1). The Kaplan-Meier analysis revealed the survival rate of patients with high TCEAL7 expression was significantly greater than for those with low TCEAL7 expression ($p < 0.01$; Figure 3C). We found a significant association between TCEAL7 levels and overall survival (OS) in glioma patients by Cox regression analyses (Table 2).

The level of TCEAL7 protein was significantly increased in the TCEAL7 vector group compared with the empty vector (EV) (Figure 3D). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) results revealed that TCEAL7 vector-treated cells showed lower viability than the negative control (NC) group cells (Figure 3E). Clonogenic assays showed that the cells transfected with TCEAL7 vector formed significantly fewer colonies on soft agar than the control group (Figure 3F). As shown in Figure 3G, the results suggested that upregulated TCEAL7 expression could significantly reduce the GBM cell-invasive capacity. Annexin V/propidium iodide (PI) double staining was conducted, and the

Table 2. Univariate and Multivariate Analyses of Prognostic Parameters in Patients with Glioma

	Univariate Analysis		Multivariate Analysis	
	p Value	Hazard Ratio, 95% CI	p Value	Hazard Ratio, 95% CI
Age[a] (≤44, >44 years)	0.72	4.5 (1.8–6.7)		
Gender (F, M)	0.85	3.2 (1.1–5.8)		
KPS (≤80, >80)	<0.01	6.5 (2.8–14.3)	<0.01	4.1 (1.5–7.3)
WHO grade (I–II, III–IV)	<0.01	7.4 (4.7–17.6)	<0.01	6.5 (3.4–13.8)
TCEAL7 expression (high, low)	<0.01	7.9 (2.3–13.0)	<0.01	4.8 (2.0–9.1)

CI, confidence interval; F, female; M, male.
[a]Median age was 44 years.

percentage of apoptotic cells significantly increased in the TCEAL7 vector (Figure 3H).

Next, we established subcutaneous tumor-bearing U87 cell lines, and, as observed in Figure 3I, there was no significant difference in tumor size between the two groups during the first 9 days; however, starting from day 10, the TCEAL7 vector group exhibited a marked decrease in the size of xenograft tumors compared with the control groups.

TCEAL7 Negatively Regulates the Wnt/β-catenin Transcriptional Pathway by Binding to β-catenin without Suppressing the Stability of β-catenin

To examine the interaction between the TCEAL7 and Wnt/β-catenin signaling, we first investigated the effect of overexpression of TCEAL7 on β-catenin/T cell factor (TCF) transcriptional activity, which was conducted by TOP and FOP FLASH in H4 cells. The TOP FLASH activity, but not the FOP FLASH activity, was markedly enhanced by the addition of β-catenin. Nevertheless, overexpression of TCEAL7 suppressed the β-catenin-stimulated activation of the TOP FLASH reporter in a dose-dependent manner (Figure 4A). Similarly, elevated expression of TCEAL7 significantly inhibited Wnt/β-catenin activity, as detected by TOP/FOP reporter constructs in high-grade glioma cells U87 and LN229 (Figure 4B).

We then examined whether TCEAL7 regulates the expression of Wnt-targeted genes; expression levels of Wnt-targeted genes were significantly decreased in the TCEAL7 overexpression cells (Figure 4C). Remarkably, overexpression of TCEAL7 did not inhibit β-catenin protein levels (Figure 4D). To further examine the effect of TCEAL7 overexpression on the stability of β-catenin, we performed the cycloheximide-chase experiment. Importantly, the results showed that β-catenin was not significantly changed in cells overexpressing TCEAL7 compared to control cells (Figure 4E). These above results demonstrate that the overexpression of TCEAL7 suppresses β-catenin-stimulated TCF transcriptional activity without suppressing the stability of β-catenin.

To gain insight into the mechanism by which TCEAL7 suppresses the apparent TCF activity, we examined whether TCEAL7 physically interacts with β-catenin. Recombinant TCEAL7 was bacterially expressed, purified, and subjected to pull-down assays with glutathione S-transferase (GST) or GST-β-catenin. The purified TCEAL7 was co-precipitated with GST-β-catenin, but not with GST alone (Figure 4F). Moreover, FLAG-TCEAL7 and hemagglutinin (HA)-β-catenin were expressed in H4 cells and subjected to immunoprecipitation with an anti-FLAG antibody. HA-β-catenin was co-precipitated with FLAG-TCEAL7 (Figure 4G). Endogenous β-catenin was co-precipitated with endogenous TCEAL7 and formed a complex in U87 and LN229 cells (Figure 4H).

Finally, we examined nuclear translocation of β-catenin when we increased the expression of TCEAL7 in glioma cells. As immunofluorescence demonstrated in Figure 4I, overexpression of TCEAL7 blocked β-catenin translocation from cytoplasm to nucleus compared with empty control vector. All in all, these results suggest that TCEAL7 as a necessary tumor suppressor can inhibit nuclear translocation of β-catenin and obstruct the transcriptional activity of Wnt/β-catenin signaling.

exo-miR-301a Derived from Hypoxic Glioma Cells Transfers to Normoxic Glioma Cells and Increases the Activity of Wnt/β-catenin Signaling by Targeting TCEAL7

Based on the above results, we conclude that the increase in miR-301a reflects the exosome-mediated miRNA transfer, but not an induction of miR-301a's endogenous expression in the recipient cells. Initially, GW4869, a drug that could block exosome secretion, was used to investigate the involvement of the exo-miR-301a in hypoxic glioma cells. GW4869 treatment significantly reduced the number of vesicles present in exosomes and increase in intracellular levels of miR-301a (Figure S1).

To confirm that hypoxic glioma cell-secreted miR-301a can be transferred to normoxic cells via exosomes, we measured the miR-301a levels in secreted exosomes derived from different glioma cells treated with normoxic or hypoxic conditions. An increase in the cellular level of mature miR-301a was observed in exosomes that originated from recipient hypoxic cells (Figure 5A), but no pri- or pre-miR-301a changes were observed for exosome uptake (Figure 5B). Moreover, the levels of miR-301a in exosome-treated cells were not significantly affected by an RNA polymerase II inhibitor (Figure 5C).

We investigated the Wnt/β-catenin transcriptional activity to clarify the involvement of TCEAL7 in exo-miR-301a-mediated Wnt/β-catenin signaling. Figures 5D and 5E show that the expression and transcriptional activity of Wnt/β-catenin signaling was markedly increased by miR-301a mimic transfection and hypoxic glioma cell-derived exosome exposure. In contrast, high levels of TCEAL7 decreased Wnt/β-catenin activity that was induced by miR-301a mimic or exposed to hypoxic exosomes. Similarly, the inhibition of TCEAL7 in hypoxic cells abolished the effects of

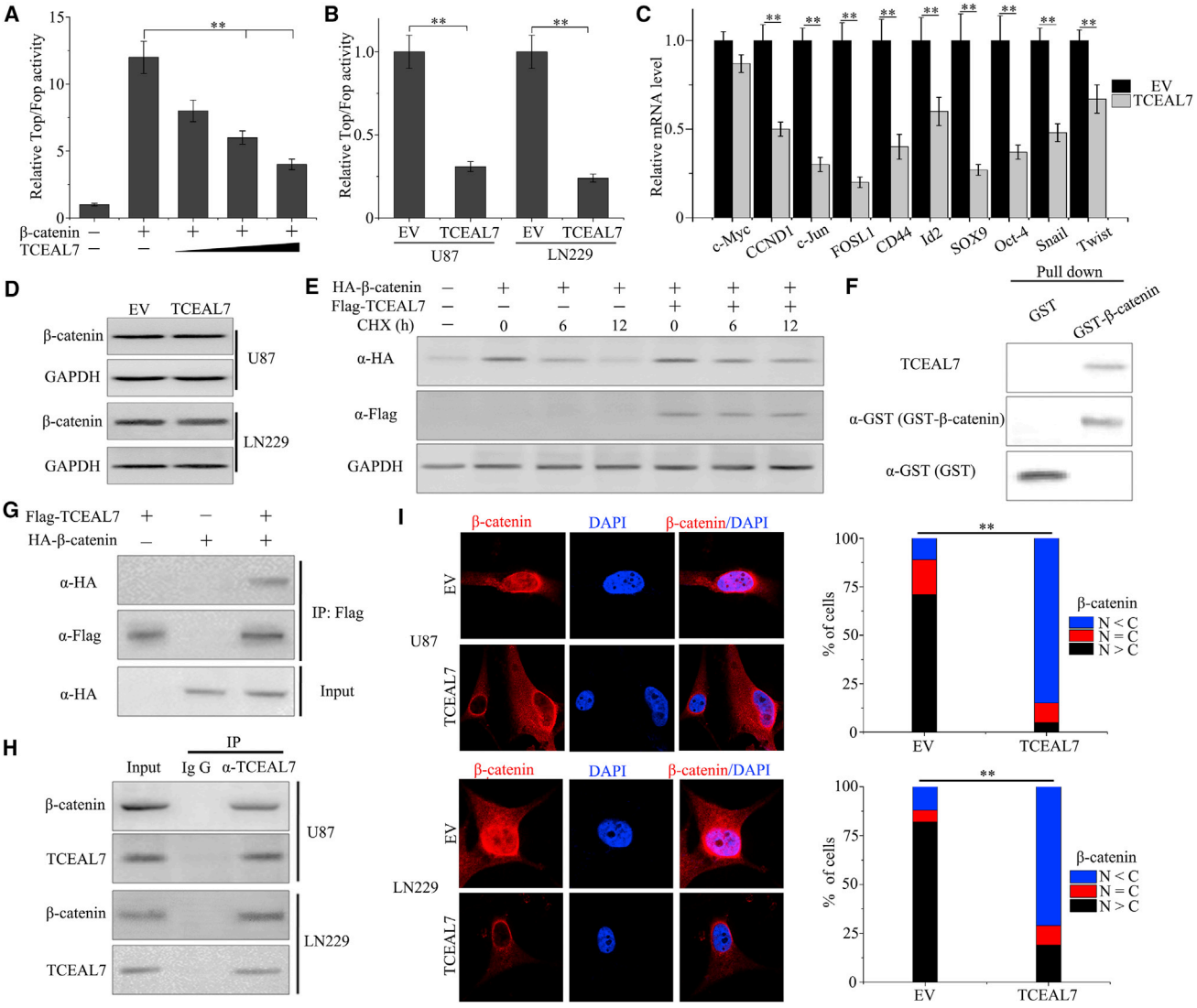

Figure 4. TCEAL7 Negatively Regulates the Wnt/β-catenin Pathway by Binding to β-catenin without Suppressing the Stability of β-catenin

(A and B) TOP/FOP assay revealed the Wnt/β-catenin transcriptional activity when treated with TCEAL7 vector. (C) Knockdown of TCEAL7 promotes the expression of Wnt target genes. (D) Western blot was performed to evaluate the protein levels of β-catenin. (E) The effect of TCEAL7 overexpression on the stability of β-catenin. Cells were treated with cycloheximide (CHX) (20 mg/mL) for the indicated hours. (F) Interaction between purified TCEAL7 and β-catenin proteins. (G) The interaction between ectopically expressed β-catenin and TCEAL7 was examined using immunoprecipitation assay. (H) The interaction between endogenous β-catenin and TCEAL7 was examined using immunoprecipitation assay. (I) Immunofluorescence detected β-catenin translocation from cytoplasm to nucleus.

As-miR-301a in TOP-FOP examination. Furthermore, we established primary GBM culture cells and verified that exo-miR-301a increased the activity of Wnt/β-catenin signaling by targeting TCEAL7 (Figure S2). The expressions of β-catenin in nucleus were increased when exposed to exosomes isolated from hypoxic cells. Furthermore, As-miR-301a and TCEAL7 vector rescued the effects of hypoxic cell-derived exosomes on Wnt/β-catenin activity (Figure 5F). Therefore, hypoxic exo-miR-301a can transfer to normoxic glioma cells and activate Wnt/β-catenin signaling by targeting TCEAL7.

miR-301a Is Involved in the Radiosensitization of Hypoxic Exosome-Co-cultured Glioma Cells

We certified that the activity of Wnt/β-catenin signaling transcription can lead to the radioresistance of H4 glioma cells (Figure S3), so we hypothesized that miR-301a would disturb the radiosensitivity of glioma cells by interfering with Wnt/β-catenin signaling. As shown in Figure S4, As-miR-301a enhances radiosensitization; nevertheless, overexpression of β-catenin or downregulated TCEAL7 reverses the effect of As-miR-301a *in vitro* and *in vivo*.

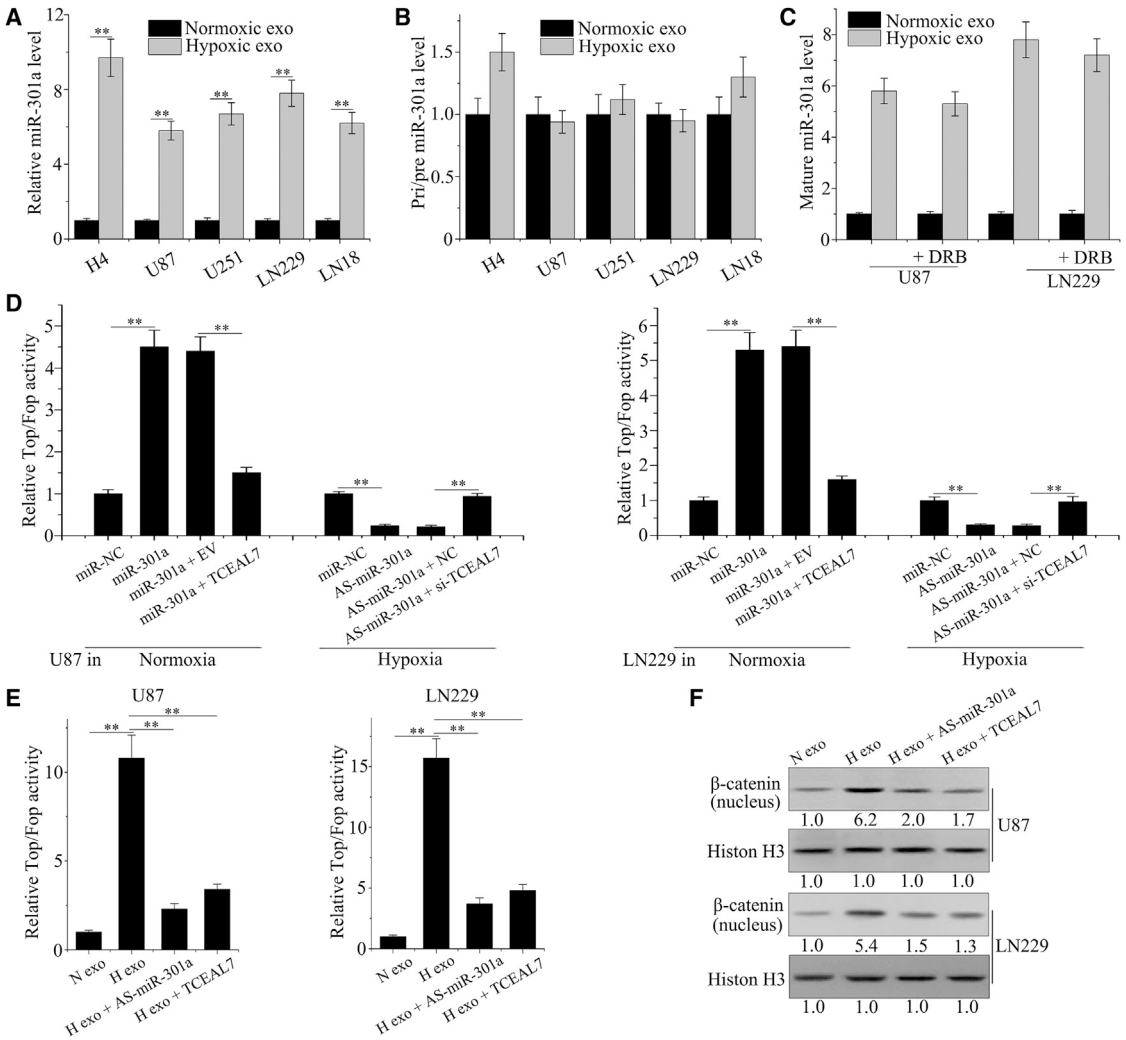

Figure 5. Exosomal miR-301a Derived from Hypoxic Glioma Cells Increases Wnt/β-catenin Signaling by Targeting TCEAL7

(A) RNA was extracted from normoxic cells incubated with hypoxic exosomes and analyzed for miR-301a level. (B) RNA extracted from normoxic cells incubated with hypoxic exosomes of different origins for 24 h (or PBS as control) was analyzed for the level of pri-miR-301a or pre-miR-301a. (C) Hypoxic-secreted exosomes were fed to normoxic cells in the presence or absence of 5,6-dichlorobenzimidazole 1-β-D-ribofuranoside (DRB) (20 μM). After 24 h, RNA extracted from the recipient cells was analyzed for miR-301a level. (D) Glioma cells were treated with miR-301a mimics, TCEAL7 vector, As-miR-301a, or si-TCEAL7, and TOP/FOP assays detected the activity of Wnt/β-catenin signaling. (E) Hypoxic glioma cells were treated with As-miR-301a or TCEAL7 siRNA, and TOP/FOP assays detected the activity of Wnt/β-catenin signaling. (F) Western blot analysis was performed to evaluate the protein levels of β-catenin in the nucleus and cytoplasm.

To explore if hypoxic exo-miR-301a levels mediate the radiosensitization of glioma cells, we detected the survival capability of cells after irradiation. MTT and clonogenic assays were performed to examine the cell survival. The survival curves for cells were detected after exposure to irradiation over a dose range of 0–8 Gy. The results showed that hypoxic exosome groups presented radiation resistances and no significant decrease in irradiation-induced survival compared with normoxic cells. Furthermore, the downregulation of miR-301a or increasing TCEAL7 markedly increased cellular sensitivity to radiation ($p < 0.01$) (Figure 6A). In addition, hypoxic exosome groups couldn't be further suppressed in cell viabilities compared with normoxic exosome groups; however, the conditions were reversed when transfected with As-miR-301a or TCEAL7 vector (Figure 6B).

We further investigated the effect of miR-301a on cell apoptosis after irradiation. As shown in Figure 6C, the percentages of apoptotic cells induced by 4- or 8-Gy irradiation in the normoxic exosome-cultured groups were significantly greater than those in the hypoxic exosome group, and the apoptotic abilities were restored when accompanied with As-miR-301a or TCEAL7 vector.

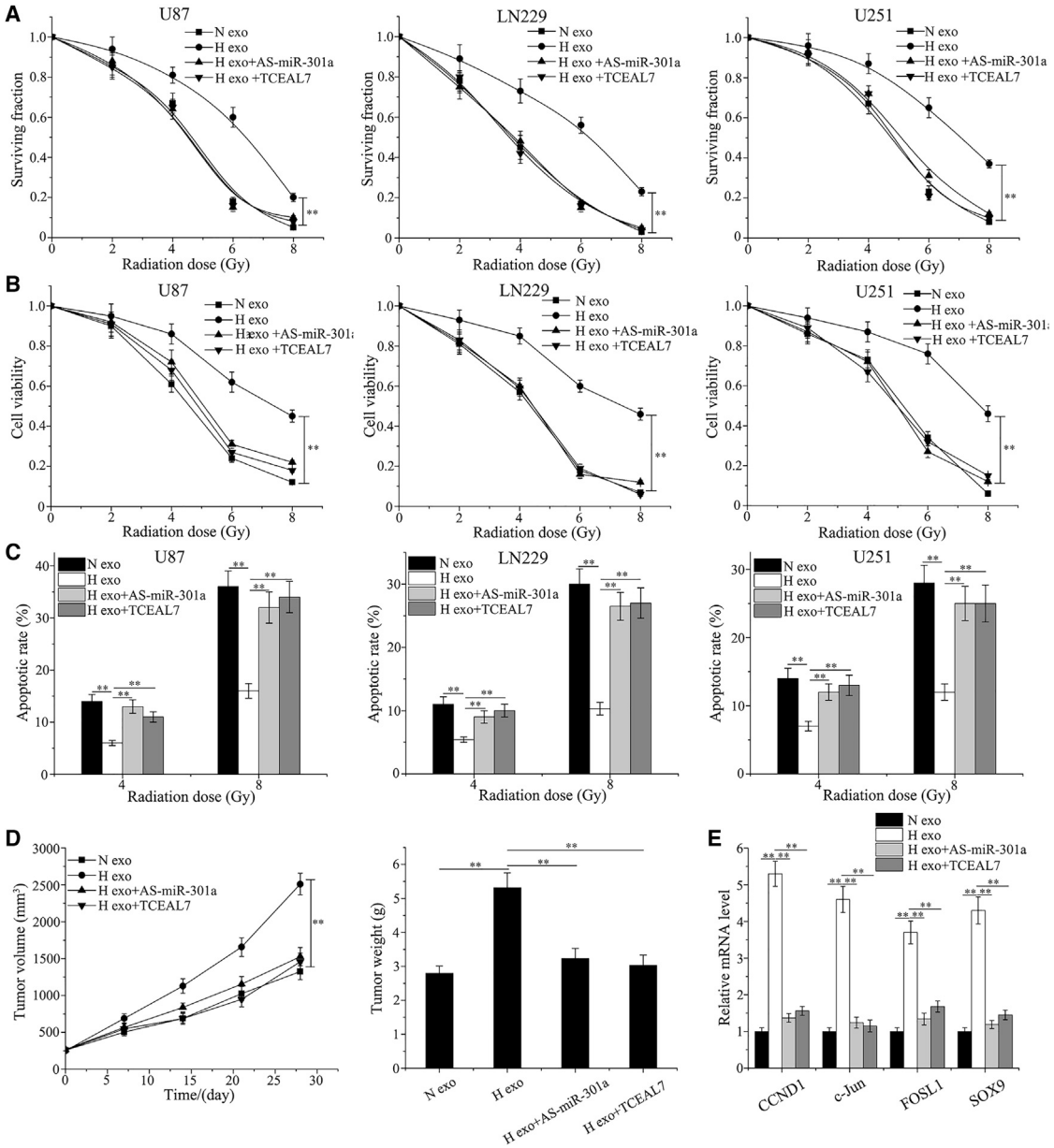

Figure 6. miR-301a Is Involved in the Radiosensitization of Hypoxic Exosome-Co-cultured Glioma Cells

(A) Clonogenic assays of cells treated with 0, 2, 4, 6, and 8 Gy radiation. (B) Cell viability is expressed as a percentage relative to scramble cells. (C) Flow cytometry analysis of apoptosis using annexin V and propidium iodide in cells treated with 4 or 8 Gy. (D) Tumor volume of each group was detected. Solid tumors were weighed after observation in different groups. (E) The expressions of Wnt/β-catenin signaling downstream genes were detected by qPCR. **$p < 0.01$.

To facilitate *in vitro* experiments, subcutaneous tumors were established by using the U87 glioma cell line to further assess the role of miR-301a in the radiation resistance of hypoxic glioma. In the xenograft growth assay, radiation alone could not significantly reduce tumor growth in the hypoxic exosome group as compared with the control group, and the effect of tumor inhibition was enhanced by As-miR-301a or TCEAL7 vector, as observed by *in vitro* detection analyses (Figure 6D). Solid tumors were weighed after observation, and significant differences existed in As-miR-301a and TCEAL7 vector groups compared with hypoxic exosome groups ($p < 0.01$).

Then qPCR was performed to examine the level of mRNAs that were transcriptional regulated by the Wnt/β-catenin pathway. Wnt/β-catenin downstream genes were activated in the hypoxic exosome condition; however, As-miR-301a or TCEAL7 could significantly inhibit the overexpression, which was similar to the results

obtained from the *in vitro* study (Figure 6E). Taken together, these data suggested that hypoxic exosomes could promote the radioresistance of glioma cells and the effects would be reversed by As-miR-301a or TCEAL7 to regulate the Wnt/β-catenin axis.

DISCUSSION

It is well established that the tumor microenvironment supports tumor growth and limits the effectiveness of clinical therapy. Hypoxia, which occurs as a consequence of inadequate or irregular blood supply, is a common feature of highly aggressive and rapidly growing tumors. Under hypoxic stress, cancer cells contribute to therapeutic resistance, heterogeneity, and progression by activating various cellular pathways.[14,15] Tissue hypoxia has a strong impact on tumor cell biology, and it has been regarded as a central factor for tumor aggressiveness and metastasis through a number of adaptive responses. In particular, the hypoxic tumor microenvironment, as a therapeutic concern in this regard, may be important in preventing or reverting malignant conversion and enhancing the effectiveness of conventional therapies as well as radiotherapy.[16] Thus, understanding how tumors respond to hypoxia will allow for the design of innovative combined cancer therapies that can overcome the radiotherapy barriers. In this study, we discussed how exo-miR-301a-driven transient compositional GBM heterogeneity is involved in hypoxic stress and the molecular mechanisms induced by tumor cell hypoxia, with a special emphasis on radiotherapeutic resistance.

Recent independent studies have confirmed that exosomes containing miRNAs play important roles in cancer biology, with both diagnostic and therapeutic implications. The presence of circulating exosomal miRNAs in the serum of cancer patients has raised the possibility that they may serve as significant diagnostic markers. miRNA can be horizontally transferred into the extracellular environment through exosomes to modify the microenvironment. Extracellular miRNA is emerging as an identified group of messengers and effectors in intercellular communication.[17] As reported, the presence of exosomal miRNAs in the blood of cancer patients has raised the possibility that they may serve as a necessary diagnostic marker.

Although the role of exosomal miRNAs in cancer progression and metastasis is still poorly understood, emerging evidence demonstrates that cancer-secreted exosomal miRNAs are functioning as mediators of tumor progression and metastasis.[18] Serum exosomal miR-4772-3p is a predictor of recurrence in stage II and III colon cancer.[19] Exosomal miR-24-3p impedes T cell function by targeting FGF11 and serves as a potential prognostic biomarker for nasopharyngeal carcinoma.[20] miR-21 and miR-1246 are enriched in human breast cancer exosomes and significantly elevated in the plasma of patients with breast cancer.[21] Exosome-mediated delivery of miR-9 induces cancer-associated fibroblast-like properties in human breast fibroblasts.[22] Breast cancer-secreted exosomal miR-939 downregulates VE-cadherin and destroys the barrier function of endothelial monolayers.[23] Mesenchymal stem cell-derived exosomes containing miR-222/223 stimulate cycling quiescence and early breast cancer dormancy in bone marrow.[24] These above findings suggest that the horizontal transfer of exosomal miRNAs from cancer cells to neighboring cells or distant non-malignant cells can modify the microenvironmental niche for their own advantage. Here we demonstrate that exo-miR-301a, which is specifically expressed and secreted by hypoxic GBM cells, promotes radiation resistance by targeting TCEAL7 and then activating the Wnt/β-catenin pathway.

TCEAL7 is a member of the transcription elongation factor A (SII)-like gene family and relatively poorly reported. TCEAL7 was first cloned as a proapoptotic nuclear protein and shown to function as a tumor suppressor gene, which is downregulated in ovarian cancer.[25] Furthermore, TCEAL7 has been observed to have decreased expression in a variety of human tumors. TCEAL7, as a cell death-regulatory protein, has been associated with a reduced risk of invasive serous ovarian cancers.[25] Additional studies showed that TCEAL7 bound to E-boxes of gene promoters and, thereby, negatively modulated Myc activity.[26] Another mechanism has been clarified: TCEAL7 in the negative regulation of nuclear factor κB (NF-κB) signaling at the basal level by modulating the transcriptional activity of NF-κB on its target gene promoters in ovarian cancer cells.[13] At this point, we have illuminated that TCEAL7 is frequently downregulated in GBM malignancy and functions as a tumor suppressor.

We have previously shown that miR-301a was activated by Wnt/β-catenin and then promoted the invasion of glioma cells by inhibiting the expression of SEPT7 *in vitro* and *in vivo*.[12] Moreover, miR-301a that was significantly upregulated in glioma and serum exo-miR-301a could serve as a potential diagnostic and prognostic biomarker for human glioma.[11] In this work, we further clarified that hypoxic glioma cell-secreted exo-miR-301a activates the Wnt/β-catenin pathway and promotes radiation resistance by targeting TCEAL7.

We cultured GBM cell lines in hypoxic condition with high exo-miR-301a expression to investigate the role in sensitivity to radiation. Hypoxia significantly increased the expression of miR-301a and exo-miR-301a; meanwhile, knockdown of HIF-1α significantly decreased the expression of miR-301a and exo-miR-301a. The exo-miR-301a, which is specifically secreted by hypoxic GBM cells, could transfer to normoxia-cultured glioma cells *in vitro*. To explore the probable mechanisms, TCEAL7 was inversely correlated with miR-301a expression in the glioma tissues, and it was identified as a direct target gene of miR-301a in glioma cell lines. It is important to note that our studies indicated that TCEAL7 negatively regulated the Wnt/β-catenin pathway by blocking β-catenin translocation from cytoplasm to nucleus. Interestingly, we clarified that Wnt/β-catenin signaling could be regulated by exo-miR-301a and the important mediating gene was TCEAL7. In addition to the established roles in hypoxic GBM cells, exo-miR-301a mediated the resistance to radiotherapy, and downregulated miR-301a expression could enhance the sensitivity to radiation in glioma cells.

In conclusion, the adverse role that hypoxia plays in treatment resistance and disease progression has led to the development of

hypoxia-targeted treatments. Therefore, discovery and validation of therapeutic targets and critical pathways derived from the hypoxic tumor microenvironment is of major importance. In this context, hypoxia-associated exo-miR-301a is identified as an attractive target for enhancing the radiosensitivity of GBM. Our work provides evidence that miR-301a can be transported through exosomes from among different cell types, facilitating cell-to-cell communication, which can lead to radiation resistance. These results suggest that miR-301a promotes radiation resistance by repressing anti-oncogene TCEAL7 expression and then activity of the Wnt/β-catenin-signaling pathway. The newly identified exo-miR-301a/TCEAL7-signaling axis could present a novel target for cellular resistance to cancer therapeutic radiation in GBM patients. Only by understanding the mechanisms through which hypoxia modulates the malignant phenotype of GBM to foster radiotherapy suppression can we rationally design future interventions that overcome hypoxia-induced resistance.

MATERIALS AND METHODS

Cell Culture

The normal oxygen cells were cultured in a humidified atmosphere containing 5% CO_2 at 37°C, while the hypoxic cells were maintained in 95% CO_2 conditions.

Patients and Clinical Samples

This study was approved by The Affiliated Hospital of Xiangnan University (Clinical College), the National Cancer Center/Cancer Hospital & Shenzhen Hospital, and the PLA Airforce General Hospital. All participants signed the appropriate informed consent to collect the blood samples prior to recruitment. The 32 patients identified as having glioma, including 6 pilocyticastrocytomas (World Health Organization [WHO] I), 6 diffuse astrocytomas (WHO II), 8 anaplasia astrocytomas (WHO III), and 12 primary GBMs (WHO IV), were recruited into the study from October 2011 to July 2013.

Isolation of Exosomes from Serum and Conditioned Media

The fractionation and purification of exosomes from blood serum and conditioned media (CMs) were performed as described previously.[11]

Luciferase Assays

TOP and FOP-FLASH (Millipore, Billerica, MA, USA) reporter constructs were performed to detect the transcriptional activity of Wnt/β-catenin, as described previously.[12,27]

ChIP-PCR

Glioma cells were incubated with either control or 0.4 mM palmitate for 12 h, and a chromatin immunoprecipitation (ChIP) assay was performed using the chromatin immunoprecipitation assay kit (Millipore), according to the manufacturer's protocol; the details have been described previously.[12,27]

Co-immunoprecipitation

FLAG-tagged proteins were immunoprecipitated from cell lysates by incubation with anti-FLAG M2 affinity-gel (Sigma) for 2 h at 41°C. After extensive washing with PBS, the precipitated proteins were eluted by incubation with binding buffer containing FLAG peptides for 1 h at 41°C. For immunoprecipitation of endogenous proteins, cell lysates were incubated with a rabbit anti-TCEAL7 antibody or a control rabbit immunoglobulin G (IgG).

Recombinant Proteins and GST Pull-Down Assay

To construct plasmids expressing GST-TCEAL7, a cDNA corresponding to human TCEAL7 was cloned into a vector. The resultant β-catenin protein was incubated with the beads, on which either GST or GST-β-catenin (Abcam) was immobilized, for 2 h at 41°C. After washing with PBS, the bound TCEAL7 protein was analyzed by immunoblotting. The 5 μg GST-TCEAL7 fusion protein and 500 μg cell lysates were incubated at 4°C overnight. Then 50 μL glutathione-Sepharose-4B beads were added to the samples and incubated at 4°C for 1 h to capture the GST fusion proteins. After washing with lysis buffer three times, the proteins were eluted in Laemmli buffer and analyzed by SDS-PAGE.

Establishment of Subcutaneous Tumor-Bearing U87 Cells in the Nude Mouse Model

All animal experimental procedures were performed according to the regulations and internal biosafety and bioethics guidelines. The 40 mice were randomized into 4 groups (normoxic exosome control, hypoxic exosome group, hypoxic exosome +As-miR-301a, and hypoxic exosome + TCEAL7 vector) when subcutaneous tumors reached a mass of approximately 200 mm^3 in size. Lipofectamine 2000 with synthetic reagents was injected subcutaneously once every 4 days. At the end of 28 days, all the mice were sacrificed and subcutaneous tumors were excised for further experiments.

Irradiation

Cell irradiation was performed at room temperature by using Faxitron Cabinet X-ray System (Faxitron, IL), with a dose rate of 0.36 Gy/min at various doses (0, 2, 4, 6, and 8 Gy). Subcutaneous tumors in the nude mice were irradiated 20 min for a total of 4 times (on days 0, 6, 12, 18, and 24; 8 Gy each time), and lead shields were used to avoid radiation injury.

Statistical Analysis

All of the statistical analyses were assessed by commercially available software SPSS version 13.0 (SPSS, Chicago, IL, USA). The ANOVA was performed to evaluate the statistical differences among groups. Statistical significance was determined as $p < 0.01$ (**).

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.ymthe.2019.07.011.

AUTHOR CONTRIBUTIONS

X.Y. and F.L. initiated and guided the study. F.L., T.X., and X.Y. performed the analyses, wrote the paper, and designed the figures.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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