ARTICLE

# Lysine-40 succinylation of TAGLN2 induces glioma angiogenesis and tumor growth through regulating TMSB4X

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Protein lysine succinylation ($K_{succ}$) represents an important regulatory mechanism of tumor development. In this work, the difference of protein $K_{succ}$ between HCMEC/D3 co-cultured with U87 (glioma endothelia cells, GEC) and without U87 (normal endothelia cells, NEC) was investigated using TMT labeling and affinity enrichment followed by high-resolution LC-MS/MS analysis. Interestingly, TAGLN2 was highly succinylated at K40 in GEC (15.36 folds vs. NEC). Compared to the Vector group, $TAGLN2^{WT}$ and a succinylation-mimetic $TAGLN2^{K40E}$ greatly promoted the angiogenesis of glioma in vitro and in vivo. Furthermore, the adhesion and metastasis of U87 co-cultured with GEC in the $TAGLN2^{WT}$ or $TAGLN2^{K40E}$ group were also significantly promoted. This was consistent with the increased expression of VE-cadherin and actin cytoskeleton remodeling induced by TAGLN2 K40succ in GEC. In addition, high K40succ of TAGLN2 was associated with poor prognosis in patients with glioma. Overexpression of $TAGLN2^{K40E}$ also markedly promoted the proliferation and migration of glioma cells, further analysis of in vivo xenograft tumors showed that there was a significant decrease in tumor size and angiogenesis in the $TAGLN2^{K40R}$ group. Notably, the co-localization of TMSB4X and TAGLN2 mainly in the nucleus and cytoplasm of glioma cells was detected by immunofluorescence staining. We identified TMSB4X as a potential target of TAGLN2, which was proved to interact with $TAGLN2^{WT}$ rather than $TAGLN2^{K40A}$. And the inhibition of TMSB4X could markedly attenuate the proliferation and migration of glioma cells induced by TAGLN2 K40succ. The results revealed $K40_{succ}$ of TAGLN2 could be a novelty diagnosis and therapeutic target for gliomas.

*Cancer Gene Therapy* (2023) 30:172–181; https://doi.org/10.1038/s41417-022-00534-6

## INTRODUCTION

Glioblastoma (GBM), the WHO grade IV glioma, is the most common and lethal type of primary brain tumor associated with high rates of morbidity, relapse, and mortality. Currently, some relevant epigenetic biomarkers have been used for the classification of glioma and therapeutic basements, such as IDH and O-6-methylguanine-DNA methyltransferase promoter methylation. However, novel specific biomarkers for the prognosis and treatment of glioma should be further investigated. GBM, a highly vascularized tumor, is characterized by a high proliferation of endothelial cells and blood vessels [1, 2]. And the high degree of vascularization in the GBM can provide oxygen and nutrients to tumor cells and then fuels tumor proliferation and invasion [3]. Therefore, it is important to elucidate the molecular mechanisms that regulate the angiogenesis and proliferation of GBM for diagnosis, treatment, and prognosis of glioma.

Post-translational protein modification (PTM) is a dynamic and reversible protein chemical modification after translation and one of the most efficient biological mechanisms to regulate cell physiology [4], which can change the physicochemical properties of protein and influence the conformation of protein space [5]. Zhao et al demonstrated that succinylation on a lysine residue can induce the charge status to transform from +1 to −1 under physiological pH conditions, which in turn facilitates adjustment of the structure and function of substrate proteins [6]. As a result, lysine succinylation induces more significant changes in protein structure and function than lysine methylation and acetylation. Du et al. demonstrated that succinylated lysine could be desuccinylated by sirtuin-5 (SIRT5) localized mostly or exclusively to the mitochondrial matrix, a NAD-dependent protein lysine desuccinylase and demalonylase [7]. And desuccinylation or mutation of the SOD1 succinylation site can affect the elimination of reactive oxygen species, inhibiting the growth of lung tumor cells [8]. Thus, protein succinylation might be related to the pathological process of tumors. However, the effect of protein succinylation on glioma angiogenesis remains unclear. Therefore, differential identification of proteome and post-translational modification in normal endothelia cells (NEC) and glioma endothelia cells (GEC) would unquestionably provide new targets for drug research in glioma treatment. In the present study, we performed a quantitative analysis of lysine succinylome in GEC and NEC. Notably, the succinylation level of Transgelin-2 (TAGLN2) K40 ($K_{succ}$DVGRPQPGR) in GECs was significantly increased.

TAGLN2, an actin-binding protein, regulates cytoskeletal rearrangement by binding to actin [9]. Studies have demonstrated that the expression of TAGLN2 has great difference in various

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Received: 15 April 2022 Revised: 12 August 2022 Accepted: 6 September 2022
Published online: 21 September 2022

tumors, including gliomas. And a high expression of TAGLN2 is often associated with the grade of glioma and poor prognosis [10]. The study demonstrated that K40 of TAGLN2 is located in the CH domain that is involved in promoting G-actin polymerization [11]. And morphogenesis of the vascular system utilizes processes like cell adhesion, motility, and proliferation, which are closely related to the dynamics of actin filaments during angiogenesis [12]. Thus, we hypothesized that TAGLN2 $K40_{succ}$ could influence the angiogenesis of GECs by inducing the remodeling of actin fibers and then investigated the underlying effect and mechanism of TAGLN2 $K40_{succ}$ on glioma biological behavior.

## RESULTS

### Upregulation of lysine-40 succinylation in the TAGLN2 protein is confirmed

There are no significant differences in the expression level of TAGLN2 protein between GEC and NEC (Fig. 1A, B). High-resolution MS/MS spectra of TAGLN2 K40 succinylation peptide (**Ksucc**DVGRPQPGR) from GEC extract using anti-succinyl lysine pan antibody (Fig. 1C). Mass spectrometry analysis showed that TAGLN2 was succinylated on K40 in GECs, where the level of TAGLN2 succinylation was 15.36 times higher than that in NECs (Fig. 1D). The level of TAGLN2 $K40_{succ}$ was verified to be higher in GECs than in NECs by Western blots (Fig. 1E). The level of TAGLN2 $K40_{succ}$ was significantly higher in glioma tissue than in normal tissue, as indicated by Western blots and IHC (Fig. 1F, G).

### $K40_{succ}$ of TAGLN2 in GECs induced the remodeling of the actin cytoskeleton

The N-terminal CH domain of TAGLN2 is an important structural basis for promoting G-actin polymerization and stabilizing F-actin [9] and the 40th lysine is located in this domain. Thus, TAGLN2 $K40_{succ}$ may influence the stability of the actin skeleton. To elucidate the effect of TAGLN2 $K40_{succ}$ on the actin cytoskeleton, $TAGLN2^{WT}$, $TAGLN2^{K40E}$ (mimicking succinylation modification) or $TAGLN2^{K40R}$ (mimicking deletion) was stably expressed in HCMEC/D3 cells (Fig. 2A). Notably, compared with Vector and $TAGLN2^{K40R}$ group, the fluorescent signal of F-actin is mainly concentrated on the cell membrane, and many stress fibers are formed in $TAGLN2^{WT}$ and $TAGLN2^{K40E}$ group (Fig. 2B). These results suggest that TAGLN2 $K40_{succ}$ may affect the biological behavior of GECs by inducing rearrangement of the intracellular actin skeleton.

### $K40_{succ}$ of TAGLN2 in GECs promoted glioma angiogenesis in vitro and in vivo

And we explored the regulatory role of TAGLN2 $K40_{succ}$ on the angiogenesis of GECs. The results showed that the proliferation and migration of GECs in the $TAGLN2^{WT}$ and $TAGLN2^{K40E}$ group was significantly increased compared to the Vector group and the $TAGLN2^{K40R}$ group (Fig. 2C–F). At the same time, the 3D angiogenesis assays and tube formation assays showed that the length of the sprouting structure formed, the number of tube connections, the total tube length, and total branch length in the $TAGLN2^{WT}$ and $TAGLN2^{K40E}$ group were also significantly higher than in Vector group and $TAGLN2^{K40R}$ group (Fig. 2G–L). These results suggest that TAGLN2 $K40_{succ}$ in GECs can promote the angiogenesis of GECs in vitro. In addition, we mixed ECs and U87 cells in a ratio of 1:3 and injected them subcutaneously into the right flank of nude mice. And the expression of CD31 in the $TAGLN2^{K40R}$ group was significantly reduced (Fig. S1A, B). The results indicate that the desuccinylation of TAGLN2 K40 could inhibit the angiogenesis of glioma in vivo.

### TAGLN2 $K40_{succ}$ destroyed the integrity of GEC in vitro

As shown in Fig. 3, compared with the Vector and the $TAGLN2^{K40R}$ group, the influx of FITC-dextran (40/100 kDa) was increased in the $TAGLN2^{WT}$ and $TAGLN2^{K40E}$ group, which indicates that the barrier between endothelial cells is impaired (Fig. 3A). VE-cadherin is fundamental to maintaining the integrity of the intercellular barrier between endothelial cells [13] and the expression of VE-cadherin was significantly down-regulated in $TAGLN2^{WT}$ and $TAGLN2^{K40E}$ group (Fig. 3B, C), suggesting that TAGLN2 $K40_{succ}$ could contribute to the disruption of the integrity by regulating the expression of VE-cadherin. U87 cells adhesion assays and transendothelial migration assays results showed that U87 cells in $TAGLN2^{WT}$ and $TAGLN2^{K40E}$ group adhered and migrated across GECs were significantly more than Vector and $TAGLN2^{K40R}$ group (Fig. 3D–G). These results further indicate that TAGLN2 $K40_{succ}$ induces disruption of the GEC barrier.

### $K40_{succ}$ of TAGLN2 promoted the glioma biological behavior and angiogenesis in vitro

To explore the biological function of TAGLN2 K40succ in glioma progression, $TAGLN2^{WT}$, $TAGLN2^{K40E}$ (mimicking succinylation modification) or $TAGLN2^{K40R}$ (mimicking deletion) were stably expressed in U87 and U251 cells with inhibition of endogenous TAGLN2 (Fig. S2A–F). Compared with the Vector and $TAGLN2^{K40R}$ groups, the cell proliferation rate was significantly enhanced in the $TAGLN2^{WT}$ and $TAGLN2^{K40E}$ groups (Fig. 4A, B). The wound healing assays revealed that the migration ability of the $TAGLN2^{WT}$ and $TAGLN2^{K40E}$ groups were significantly higher than those of the Vector and $TAGLN2^{K40R}$ groups in U87 and U251 cells (Fig. 4C, D). Next, HCMEC/D3 cells were co-cultured with U87 cells with different treatments. Interestingly, the number of tube connections, the total tube length, and total branch length of the GECs in the $TAGLN2^{WT}$ and $TAGLN2^{K40E}$ groups were also significantly higher than that in the Vector group and $TAGLN2^{K40R}$ group (Fig. 4E, F). These results have shown that TAGLN2 $K40_{succ}$ in U87 could promote the angiogenesis of GECs in vitro.

### Desuccinylation of TAGLN2 can inhibit glioma growth and angiogenesis in vivo

In vivo xenograft tumor analysis showed that there was a significant difference in tumor size and weight between the $TAGLN2^{WT}$ group and the $TAGLN2^{K40R}$ group (Fig. 5A–D). And compared to the $TAGLN2^{WT}$ group, the number of vascular and Ki67-positive cells in the $TAGLN2^{K40R}$ group was also greatly decreased (Fig. 5E–H), suggesting that desuccinylation of TAGLN2 in glioma cells inhibited glioma growth and angiogenesis.

### K40succ of TAGLN2 in U87 promoted the proliferation and migration via interacting with TMSB4X

GST-pull down with LC-MS/MS assay have shown that five proteins interacting with $TAGLN2^{WT}$ rather than $TAGLN2^{K40A}$ were regarded as important factors involved in glioma development (Table S2). The differential expression of the genes was analyzed in GBMs and low-grade gliomas (LGGs) as well as normal brain tissues from the GEPIA dataset (http://gepia2.cancer-pku.cn/#index). ARF4, TMSB4X, and SHOX2 were significantly upregulated in GBMs relative to normal brain (Fig. 6A). Moreover, the survival data from CGGA support the correlation between higher ARF4, TMSB4X, and SHOX2 expression and worse prognosis (Fig. 6B). Our previous study illustrated that K40succ of TAGLN2 modulate the remodeling of the actin cytoskeleton in U87 (Fig S3A). Given that TMSB4X, as a G-actin-binding protein preventing actin polymerization plays a role in cell motility by regulating actin cytoskeleton [14], we subsequently focused on the interaction between TAGLN2 and TMSB4X. The study found that TAGLN2 colocalized with TMSB4X in the nucleus and cytoplasm of U87 cells (Fig. 6C). Furthermore, the results showed that the expression of TMSB4X in $TAGLN2^{WT}$ and $TAGLN2^{K40E}$ group is higher than Vector and $TAGLN2^{K40R}$ group (Fig. 6D, E and Fig. S4A). Notably, the depletion of TMSB4X expression significantly attenuated cell proliferation and migration mediated by TAGLN2 K40succ (Fig S4B and Fig. 6F–I). F-actin staining assay demonstrated that the depletion

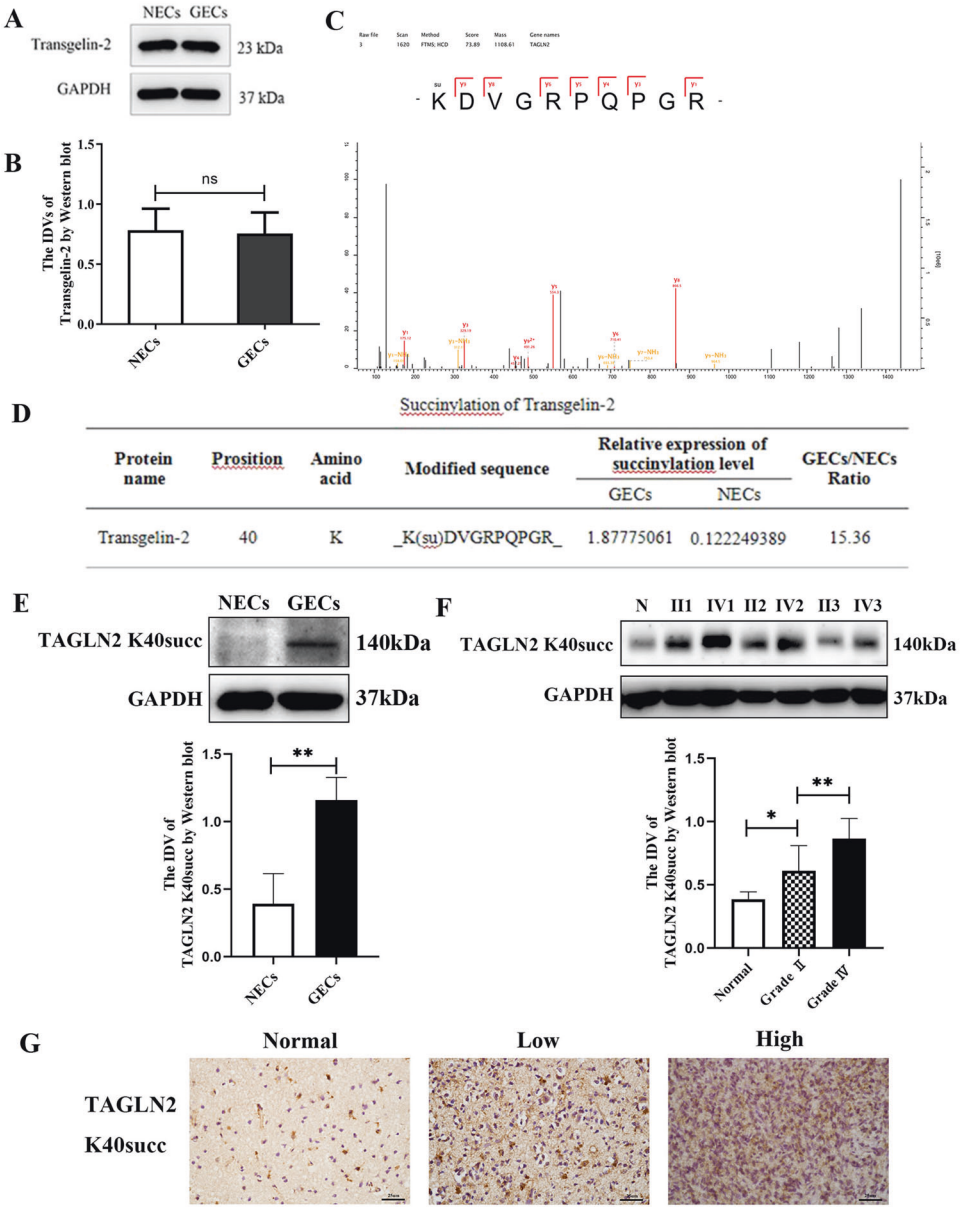


**Fig. 1 Upregulation of lysine succinylation on TAGLN2 protein is confirmed. A** The expression of TAGLN2 was measured by western blots in GECs and NECs. **B** The relative gray value of TAGLN2 normalized to GAPDH. **C**, **D** Succinylation of TAGLN2 in GECs, mass spectrometric verification of TAGLN2 succinylation at K40 (KsuccDVGRPQPGR). **E** The expression of TAGLN2 K40succ was measured by western blots in GECs and NECs. The relative gray value of TAGLN2 K40succ normalized to GAPDH. **F** The expression of TAGLN2 K40succ was measured by western blots in different grade gliomas and normal brain specimens. The relative gray value of TAGLN2 K40succ normalized to GAPDH. **G** Representative images of IHC staining for TAGLN2 K40succ in different grade gliomas and normal brain specimens (scale bar = 25 μm). Data were shown as the mean ± SD, $*p < 0.05$, $**p < 0.01$.

of TMSB4X decreased the expression of F-actin in $TAGLN2^{WT}$ and $TAGLN2^{K40E}$ groups (Fig. S4C). Collectively, these results support the possibility that K40succ of TAGLN2 in U87 promotes proliferation and migration via interacting with TMSB4X.

## DISCUSSION

Post-translational modifications (PTM) are crucial for protein structure and function. In recent years, studies have found that succinylation modification can result in a group with a larger structure and thus significantly change the structure and function of proteins [15]. And studies have confirmed that protein succinylation induced the alteration of the activity and structure of enzymes and participated in function regulation of tumor metabolism and signal transduction [16]. Aberrant vascularization is a typical feature of glioblastoma. The interactions between tumor cells and the brain microenvironment, principally brain vascular endothelium, are the critical determinants in their tumor progression and migration [17]. More importantly, crosstalk among global succinylome was studied in HCMEC/D3 co-cultured without or with U87, which may considerably provide new biology targets for drug research in glioma treatment.

TAGLN2 is an actin-binding protein (ABP), which could be combined with F-actin or G-actin to block the binding of other ABPs and actin, thereby regulating the dynamic changes of the cytoskeleton [18, 19]. The expression of TAGLN2 have a significant difference in various tumors including gliomas [10], and is associated with tumor development [20]. In addition, TAGLN2

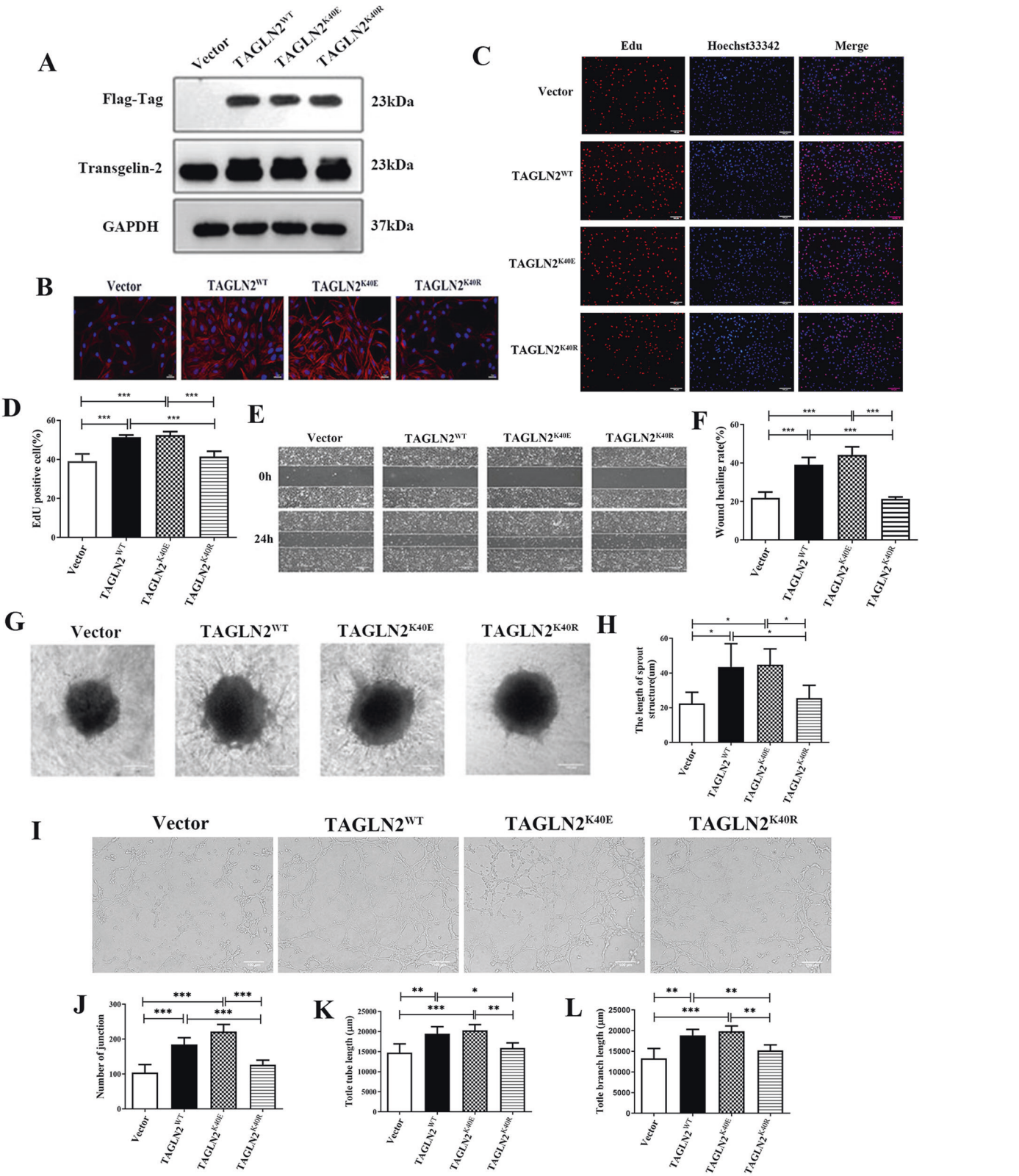


**Fig. 2 $K40_{succ}$ of TAGLN2 in GECs promoted glioma angiogenesis in vitro. A** The expression of Flag-Tag and TAGLN2 were measured by western blots. **B** The distribution of F-actin in GECs detected by fluorescent probes (scale bar = 25 μm). **C** EdU assay was used to detect the proliferation ability of cells in GECs (scale bar = 100 μm). **D** The relative proportion of EdU staining cells. **E** Wound healing was used to detect the migration ability between different groups (scale bar = 200 μm). **F** Quantification of wound healing rate. **G** The 3D angiogenesis experiment was used to detect the sprouting ability of each group of GECs (scale bar = 100 μm). **H** Quantification of the length of sprouting structures. **I** The tube formation assay was used to detect the lumen forming ability of the GECs (scale bar = 100 μm). **J**–**L** Quantification of the tube connection numbers, total length, and branch length. Data were shown as mean ± SD, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

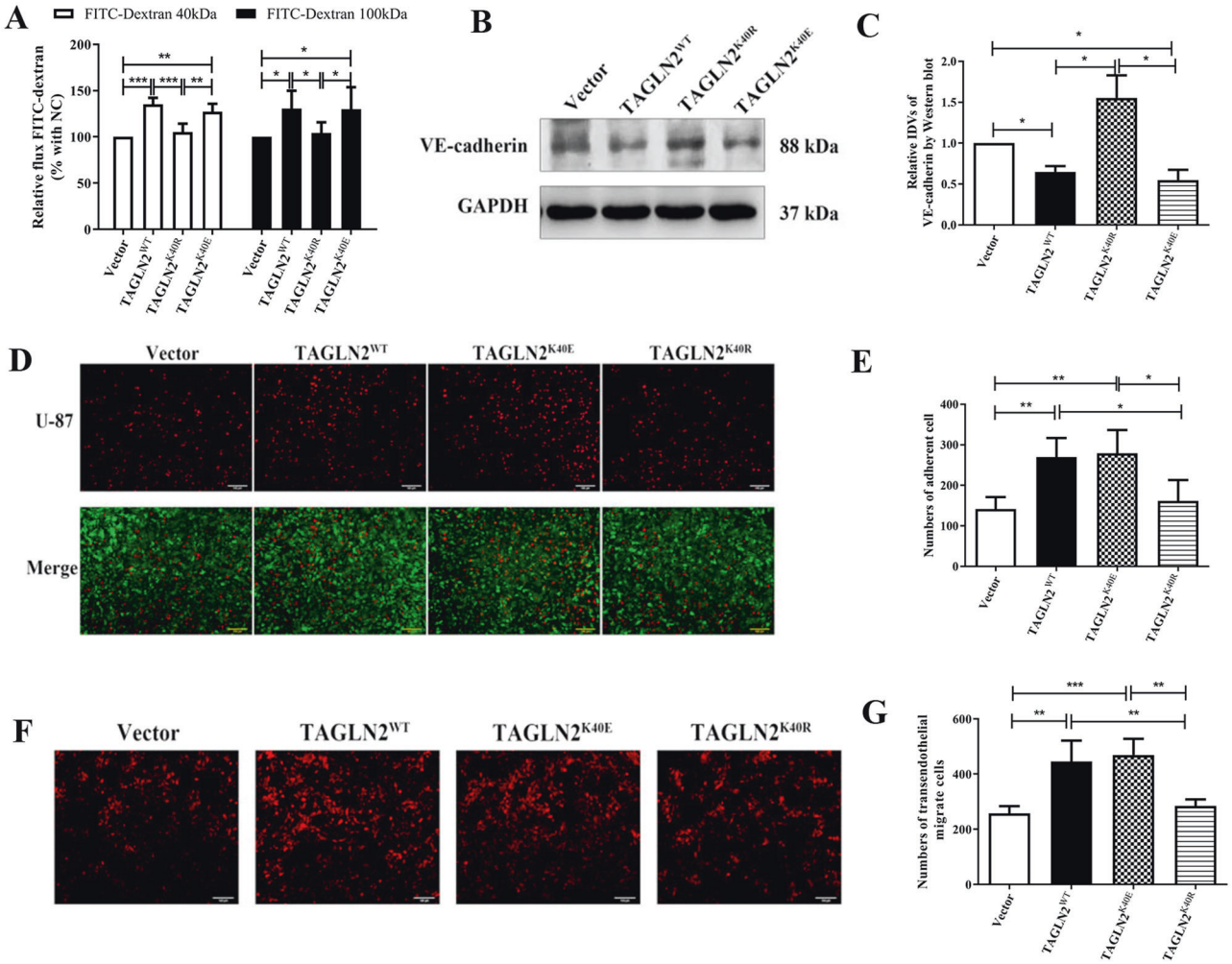


**Fig. 3 $K40_{succ}$ of TAGLN2 destroyed the integrity of GECs in vitro. A** 40 kDa/100 kDa-FITC-dextran permeability experiment. **B** The expression of VE-cadherin was measured by western blots in GECs. **C** The relative gray value of VE-cadherin normalized to GAPDH. **D** The adhesion of U87 cells (scale bar = 100 μm). **E** Quantification of the number of adherent cells. **F** The transendothelial migration of U87 cells (scale bar = 100 μm). **G** Quantification of the number of transendothelial migration cells. Data were shown as the mean ± SD, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

can regulate the skeleton of endothelial cells and promote the migration and tube formation ability of HUVECs [21]. And the researchers observed that the expression of TAGLN2 was significantly increased in the microvascular endothelial cells of lung cancer tissues [22], which means that TAGLN2 could play an important role in regulation of tumor angiogenesis. Notably, our result demonstrated that lysine at position 40 of TAGLN2 was highly succinylated in GECs, which was approximately 15.36 times that of NECs. And the increased succinylation of TAGLN2 K40 promoted the proliferation, migration and tube forming ability of GECs. Moreover, the blood vessel formation of xenograft tumors in the K40R group was less than that in the WT group, which indicated that the desuccinylation of TAGLN2 K40 could inhibit the angiogenesis of GECs in vivo. The above-mentioned results suggested that TAGLN2 succinylation contributed to glioma angiogenesis.

Endothelial cell proliferation and migration are important steps in angiogenesis, and the remodeling of the actin cytoskeleton can strictly regulate this process [12]. And actin fibers are the main cytoskeletal components of endothelial cells, which can be continuously remodeled to form filopodia, lamellipodia and stress fibers to generate force to drive the movement of cells [23, 24]. TAGLN2 can stabilize and bind to F-actin [9] and K40 of TAGLN2 is located in the CH domain, which is necessary to promote the polymerization of G-actin [25]. Interestingly, our results showed that the K40succ of TAGLN2 facilitates the formation of GECs stress fibers and a concentration of actin in the cell periphery, which is beneficial to maintain the directional migration of cells [24]. The results indicated that TAGLN2 K40succ could promote the remodeling of the actin cytoskeleton in GECs, thereby causing the cells to contract and ultimately resulting in the increase of their exercise ability.

Previous studies revealed that a high level of TAGLN2 expression was related to hepatocellular carcinoma and breast and pancreatic cancer progression [26]. It was also reported that TAGLN2 might exert a vital role in promoting the development of human glioma [10]. In this study, we further demonstrated that the level of TAGLN2 K40succ was higher in human glioma tissues than in normal tissues. TAGLN2 K40succ promoted the proliferation and migration of glioma cells. In addition, TAGLN2 K40succ in U87 also promoted the angiogenesis of GECs. To explore the specific molecular mechanism of TAGLN2 K40succ on the glioma biological behavior, the differential proteins that interact with $TAGLN2^{WT}$ or $TAGLN2^{K40A}$ were screened. Among the identified proteins ARF4 [27], TMSB4X [28], NEK10, SHOX2 [29], and ANOS1 [30] were involved in the regulation of human cancers progression. After CGGA and GEPIA datasets analysis, the ARF4, TMSB4X and SHOX2 were significantly upregulated in glioma and associated with patient prognosis, suggesting that they might

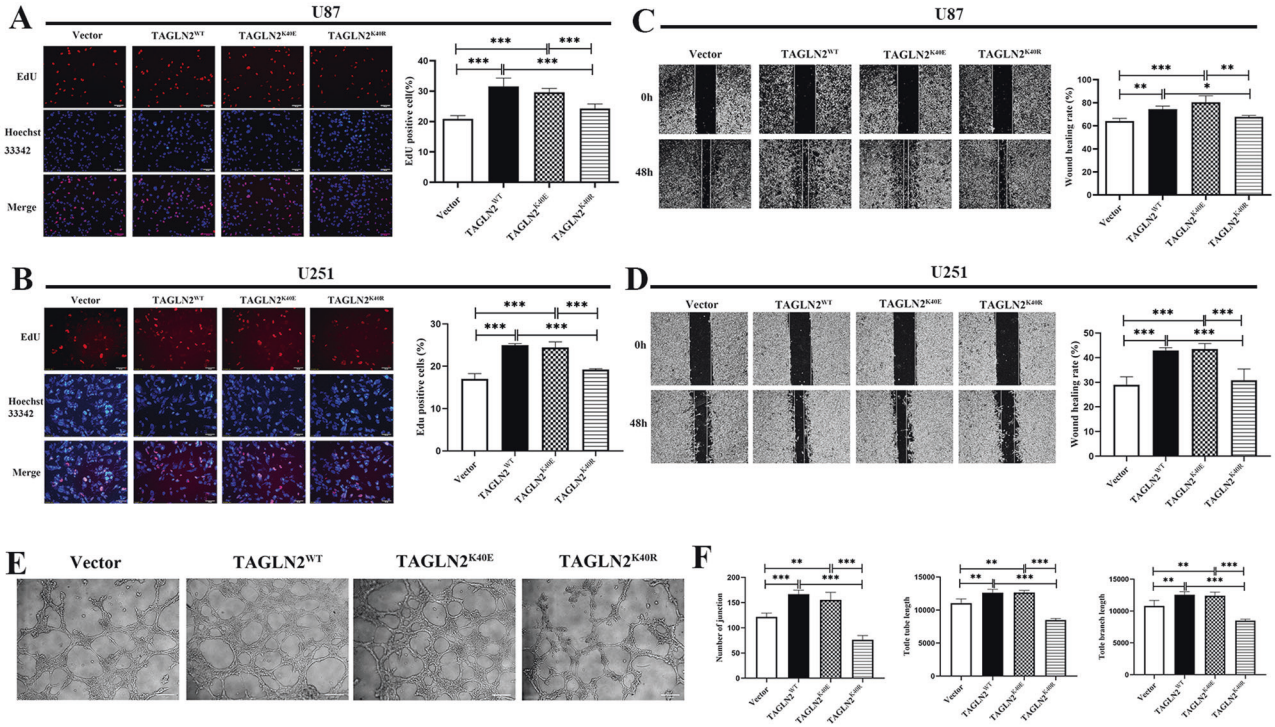


**Fig. 4 $K40_{succ}$ of TAGLN2 in U87 promoted the glioma biological behavior in vitro. A**, **B** EdU assay was used to detect the proliferation ability of U87 and U251 cells (scale bar = 50 μm). The relative proportion of EdU staining cells. **C**, **D** Cell migration ability of U87 and U251 cells was tested by wound healing assay (scale bar = 200 μm). Quantification of wound healing rate. **E** The tube formation assay was used to detect the lumen forming ability of the GECs (scale bar = 100 μm). **F** Quantification of the tube connection numbers, total length, and branch length. Data were shown as mean ± SD, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

be potential targets for further research. In addition, TAGLN2 is expressed not only in the cytoplasm but also in the nucleus. However, little is known about the function of TAGLN2 in the nucleus. The emerging roles of actin and actin-binding proteins in the nucleus have become a new frontier in cell biology.

TMSB4X could sequester monomeric actin that prevents G-actin from joining a filament [31]. It was reported that TMSB4X influenced neuronal motility via modulating the actin dynamics [32] and promoted the adhesion and thus motility of melanoma cells by regulating the number and morphology of focal adhesions. Additionally, a correlation between TMSB4X expression level and exhibition of EMT features was also demonstrated [28]. TMSB4X has also been implicated in the stemness and invasiveness of glioblastoma [33] and affects actin cytoskeletal rearrangement by controlling G-actin transport between cells and supporting Formin protein-mediated actin polymerization [34]. Interestingly, our results clarified that TAGLN2 K40succ increased the expression of TMSB4X in U87. And then the depletion of TMSB4X significantly attenuated TAGLN2 K40succ-dependent cell migration and proliferation, suggesting that TMSB4X plays a crucial role in regulating cytoskeletal rearrangement and participates in the process of TAGLN2 K40succ-regulating cell migration.

Currently, our study puts more emphasis on translational relevance, but there are still some limitations in this research. I. The effect of TAGLN2 K40succ on glioma in vivo intracranial models or primary culture models should be verified. II. The effect of exogenous blocking of the TAGLN2 K40succ via cell-penetrating peptide or Liposomes on glioma proliferation and angiogenesis should be further investigated. III. The level of TAGLN2 K40succ in other tumor cells or tissues should be also evaluated.

In conclusion, our results revealed that TAGLN2 K40succ could promote glioma angiogenesis and induce the rearrangement of the actin skeleton via interacting with TMSB4X and ultimately enhanced the proliferation and migration of glioma. Notably, K40-succinylated TAGLN2 is also associated with poor prognosis in patients with glioma. Therefore, targeting succinylated TAGLN2 provides a novel strategy for glioma therapy.

## MATERIALS AND METHODS

### Glioma samples

The tumor samples in this study were taken from eight patients with primary brain glioma who had not received chemotherapy or any other treatment before surgery and were surgically removed with the patient's informed consent (Table S1). The patients were from the General Hospital of Northern Theater Command (Shenyang, China). This study was approved by the Hospital Ethics Committee of the General Hospital of Northern Theater Command and carried out in accordance with the principles of the Declaration of Helsinki.

### Proteomic quantification of lysine succinylation

TMT labeling, HPLC fractionation, affinity enrichment, and mass spectrometry-based quantitative proteomics were performed to quantify the dynamic difference of lysine succinylome between the GEC and NEC in PTM Biolabs (Hangzhou, China). Intensive bioinformatic analysis was then carried out to annotate the quantifiable targets, including protein annotation, functional classification, functional enrichment, and functional enrichment-based cluster analysis.

### Cell culture

Immortalized human cerebral microvascular endothelial cell line (HCMEC/D3, a gift from Dr. Pierre-Olivier Couraud, Department of Neurology and Stroke Centre, Bichat University Hospital, Paris, France) was cultured in endothelial basal medium 2 (EBM-2, Lonza, Walkersville, MD, USA) on an established protocol [35]. Human glioblastoma cell lines, U87 and U251 cell lines were obtained from the Chinese Academy of Sciences Cell Bank and maintained in Dulbecco's modified Eagle medium (DMEM) and supplemented with 10% fetal bovine serum (FBS) (Gibco, life technologies, Grand Land, NY, US). The source of cell lines was recently authenticated by STR profiling and tested for mycoplasma contamination.

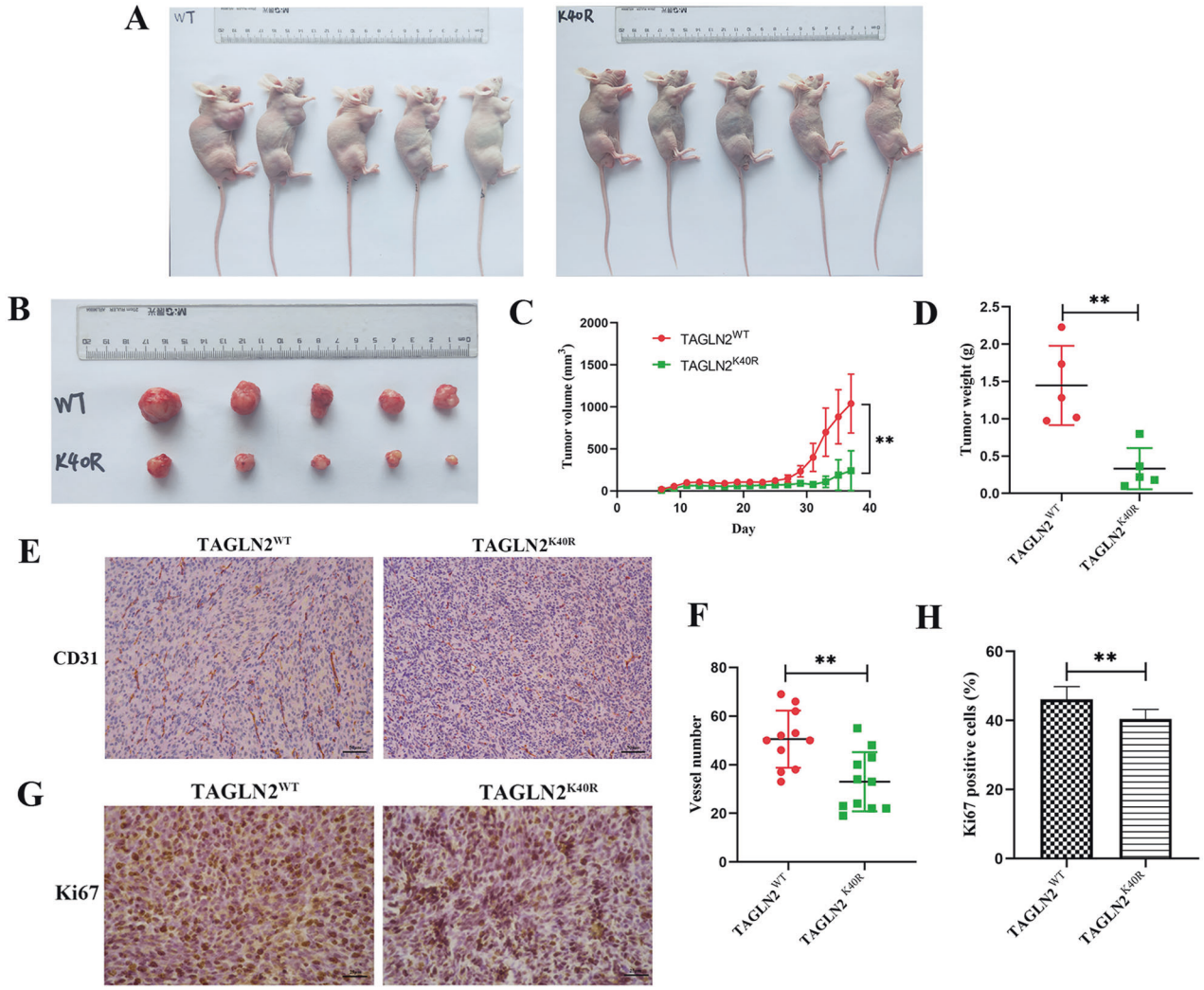


**Fig. 5 Desuccinylation of TAGLN2 in glioma cells can inhibit glioma growth and angiogenesis in vivo.** The respective image of mice (**A**) with implantation of U87 and tumor tissues (**B**) from different groups. Heterotopic xenograft tumor volume (**C**) and weight (**D**) were detected in nude mice. The expression and number analysis of CD31 + vessels (**E**, **F**) were measured by immunohistology (scale bar = 50 μm). Data were shown as the mean ± SD. The number analysis of Ki67-positive cells (**G**, **H**) was measured by immunohistology (scale bar = 25 μm). Data were shown as the mean ± SD, $^{**}p < 0.01$.

### Generation of stable cell lines

The stable cell lines were generated using a lentivirus system. Briefly, Full-length WT cDNA carrying Flag or cDNA with point mutation of the TAGLN2 gene (K40E/K40R) was synthesized from GenePharma (Shanghai, China), and cloned into indicated vectors LV5 (EF-1a/GFP&Puro) (GenePharma, Shanghai, China) and then transfected into cells using Lipofectamine 3000. Cells were seeded in a 24-well cell culture plate and infected with virus when the cell fusion rate reached 20%. After 24 h, fresh medium was replaced for further culture for 72 h, 1 μg/mL purinomycin (Solarbio, Beijing, China) was added to select stable transfected cell lines for 2 weeks.

### RNA interference analysis

Small interfering RNA (siRNA) against the coding region of TMSB4X and the 3′ UTR of TAGLN2 were synthesized (GenePharma; Shanghai, China). siRNAs were transfected with Lipofectamine 3000 reagent (Thermo Fisher Scientific; Waltham, MA, USA) according to the manufacturer's protocol. Knockdown efficiency was evaluated at 72 h after transfection by Western blots and ICC assays. Sequences of siRNA targeting TAGLN2 ($n = 1$): si-TAGLN2#1: 5′-GCUGUGGCUGCAGGAACUU-3′. Sequences of siRNA targeting TMSB4X ($n = 3$): si-TMSB4X#1: 5′-GUCUGACAAACCCGAUAUG-3′, si-TMSB4X#2: 5′-GCCUUCCAAAGAAACGAUU-3′, si-TMSB4X# 3: 5′-GCGAAUCGUAAUGAGGCGU-3′.

### HCMEC/D3 culture with or without U87

The six-well plate or 24-well plate is separated into upper and lower chambers by Transwell nested chambers (0.4 μm pore size; Corning, NY, USA), allowing only the exchange of metabolites or secreted substances between the upper and lower chambers. According to the purpose of the experiment, HCMEC/D3 and U87 cells were inoculated in the upper or lower compartment, respectively. The GECs could be obtained after the coculture of HCMEC/D3 and U87 cells for 6 days.

### Cell proliferation assay

Proliferation was assessed using the EdU Imaging Kits (Cy3) (APExBIO, America). Briefly, GECs were seeded at a density of $1 \times 10^4$ cells/mL and incubated 48 h at 37 °C. Dye incorporation was assayed as a measure of proliferation according to manufacturer's instructions.

### Western blots

Total proteins were extracted from GECs with RIPA buffer containing protease inhibitors (Beyotime Institute of Biotechnology, Jiangsu, CN) on ice, and the supernatant extracts were quantified for protein level using the BCA protein assay kit (Beyotime Institute of Biotechnology, Jiangsu, CN). And then subjected to SDS-PAGE based on an established protocol [36]. After being transferred onto polyvinylidene fluoride membranes,

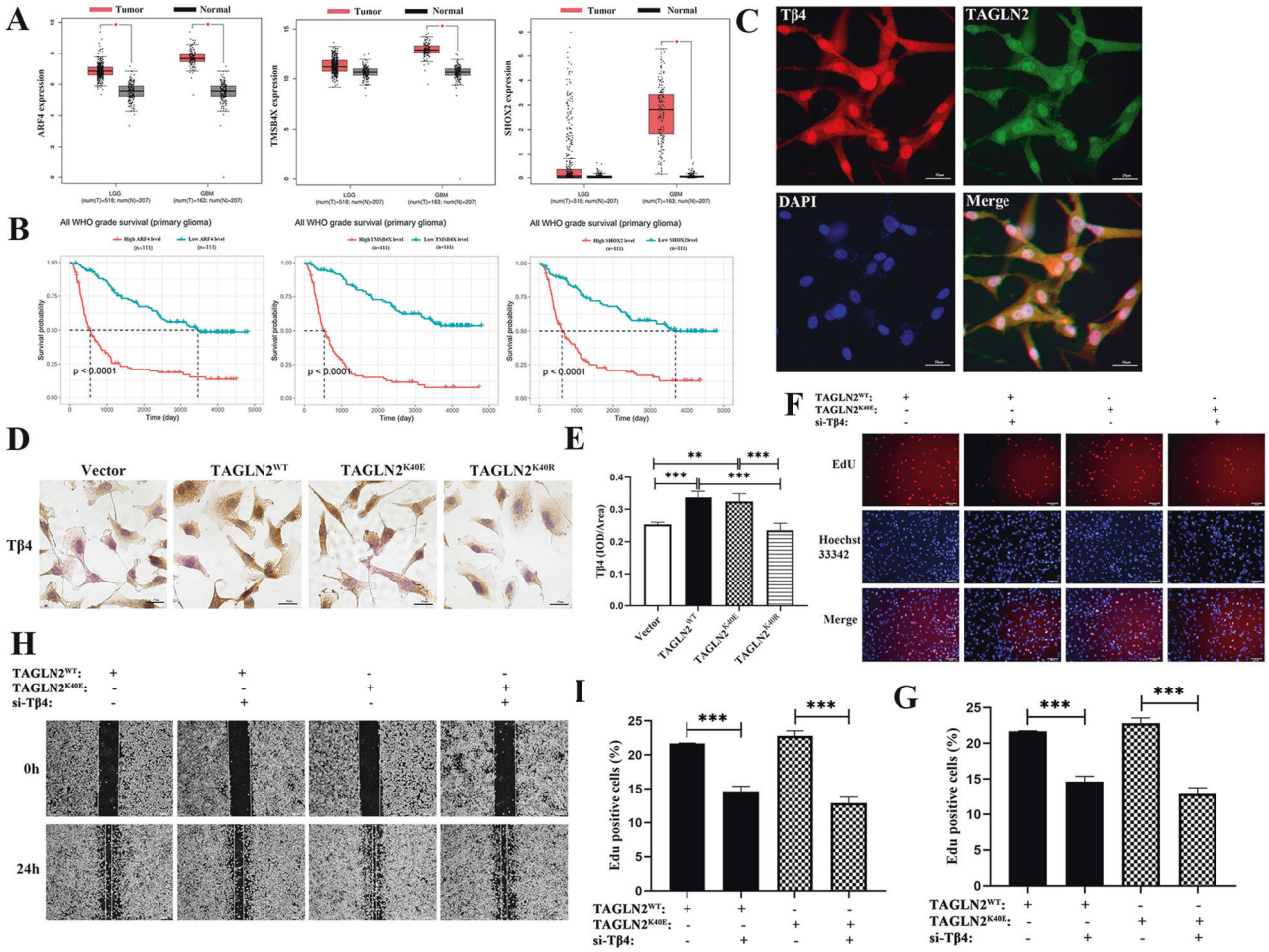


**Fig. 6 K40succ of TAGLN2 in U87 promoted the proliferation and migration via interacting with TMSB4X. A** Quantification of ARF4, TMSB4X, and SHOX2 mRNA expression levels in gliomas in GEPIA dataset. **B** The survival analysis according to ARF4, TMSB4X, and SHOX2 expression in glioma using CGGA datasets. **C** IF images of TAGLN2 (green) and TMSB4X (red) with DAPI staining (blue) in U87 cells (scale bar = 25 μm). **D** Expression of TMSB4X in U87 stable cells by ICC (scale bar = 25 μm). **E** Quantification of the expression of TMSB4X. **F** The effects of TMSB4X depletion on the proliferation of U87 cells with TAGLN2$^{WT}$ and TAGLN2$^{K40E}$ expression (scale bar = 50 μm). **G** The relative proportion of EdU-positive cells. **H** Wound healing assay was used to detect the migration ability of U87 cells (scale bar = 200 μm). **I** Quantification of wound healing rate. Data were shown as the mean ± SD, $^{**}p < 0.01$, $^{***}p < 0.001$.

nonspecific binding was blocked with 5% nonfat milk. The blots were probed with the following primary antibodies: Flag-Tag antibody (T0003, Affinity, USA); TAGLN2 antibody (sc-373928, Santa Cruz, Dallas, TX, USA); CD31 antibody (ab9498, Abcam, Cambridge, UK); GAPDH (60004-1-Ig, Proteintech, Rosemont, USA); VEGFR2 (2415-1-AP, Proteintech, Rosemont, USA); VE-cadherin (66804-1-Ig, Protintech, Rosemont, USA). Secondary antibodies included goat anti-rabbit IgG H&L (HRP, ab6721, Abcam, Cambridge, UK), and goat anti-mouse IgG H&L (HRP, ab6789, Abcam, Cambridge, UK).

## Permeability assays

In the upper well of the Transwell (0.4 μm), the medium was replaced with 50 mM fluorescein isothiocyanate (FITC) labeled dextran (40 kDa/100 kDa) (Xian ruixi Biological Technology, CN). Samples were analyzed using a Florescence Multiwell Plate Reader (FlexStation 3; Molecular Devices, San Jose, CA, USA). The results were expressed as permeability coefficients using the slopes of the curves, which were obtained through the volume clearance plotted against time.

## Cell migration assay

GECs were starved overnight, trypsinized, and suspended at a final concentration of $1 \times 10^6$ cells/mL, 200 μL/well seeded in a six-well cell culture plate. After the cells are fused overnight, 200 μL sterile pipette was used to scratch the cell layers of each group. Then photographs were taken at 0 and 24 h, respectively. The intersection of the bottom line and the cell scratch line was considered as the observation point.

## Three-dimensional angiogenesis assay system

The GECs were trypsinized and suspended at a final concentration of $1 \times 10^5$ cells/mL, 10 μL suspension was dripped onto the cell culture plate cover and the appropriate culture medium was added to the culture plate, which was incubated for 24 h at 37 °C after closing the plate cover. The GECs spheres was removed with an appropriate amount of EBM-2 and added to a 96-well plate covered with rat tail tendon collagen type I (Xinyou Biotechnology. Hangzhou, CN) at 37 °C for 48 h and observed with the inverted microscope.

## F-actin staining assay

Cells were fixed with acetone for 10 min at room temperature, followed by incubation with goat serum for 30 min at room temperature, according to a previous study [37]. Cells were then stained with Actin-Tracker Red-555 for 1 h at room temperature. The cells were then counterstained with DAPI (Beyotime Institute of Biotechnology) for 15 min at room temperature and visualized with the microscope.

## Immunofluorescence

The cells were fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.1% Triton x-100 for 5 min. After blocking for 1 h in

5% bovine serum albumin at room temperature, the cells were incubated overnight at 4 °C with primary antibodies anti-TMSB4X (1:50, Cat# 19850-1-AP, Proteintech, Wuhan, China) and anti-TAGLN2 (1:50, sc-373928, Santa Cruz, Dallas, TX, USA). Subsequently, the Alexa Fluor™ 594 goat anti-rabbit IgG (H + L) and Alexa Fluor™ 488 goat anti-mouse IgG (H + L) (1:1000, Thermo Fisher Scientific; Waltham, MA, USA) secondary antibodies were added for 1 h at room temperature followed by counterstaining with DAPI.

### Immunocytochemistry (ICC) and immunohistochemistry (IHC)

The following procedures were performed according to the Ultra-Sensitive SP instructions (Maxim Institute of Biotechnology, Fuzhou, CN): After blocking for 10 min in nonspecific staining blocker, the tissues were incubated overnight at 4 °C with primary antibody anti-CD31 (1:1000, ab9498, Abcam, Cambridge, UK) or anti-TMSB4X (1:50, Cat# 19850-1-AP, Proteintech, Wuhan, China) or rabbit polyclonal antibodies against the human TAGLN2 K40-succinylated peptide (PDLGTD**Ksucc**DVGRPQPGR) were generated from three rabbits (ChinaPeptides Co., Ltd., Shanghai). The images were visualized by following standard protocols of the DAB rendering kit (Maxim Institute of Biotechnology, Fuzhou, CN) and then observed via microscope.

### Tumor xenograft experiments

Male BALB/c nude mice (6–8 weeks, 18–20 g) were purchased from Vital River Laboratory Animal Technology (Beijing, China) and were raised at the Laboratory Animal Center of Shenyang Pharmaceutical University. All animal experiments were performed in accordance with the Animal Care Committee of Shenyang Pharmaceutical University. In angiogenesis experiment in vivo, ECs and U87 cells were mixed in a ratio of 1:3, and $5 \times 10^5$ cells were injected into the right flank of BALB/c nude mice. For the glioma experiment in vivo, mice were randomly divided into two groups ($n = 5$): WT and K40R. Cells that stably expressed TAGLN2$^{WT}$ or TAGLN2$^{K40R}$ were mixed in Matrigel and subcutaneously injected into nude mice. Tumor volume was measured every 3 days and calculated using the formula: tumor volume ($mm^3$) = (length × width$^2$)/2. After 40 days, tumors were removed, weighed, and stored at −80 °C.

### GST-pull down

The fusion protein of GST-TAGLN2 and GST-TAGLN2$^{K40A}$ were expressed and purified using a prokaryotic expression system and GST affinity chromatography. The lysates of U87 cells were added to the GST affinity chromatography column. Then GST Elution-Buffer was used to elute the interacting proteins. The eluted proteins were subjected to LC-MS/MS analysis.

### Statistical analysis

All data are presented as the mean ± standard deviations (SD). Student's *t*-test was used for comparing two groups' data and the one-way ANOVA test was used for more than two groups' data comparisons using SPSS software. When ANOVA was significant, post hoc testing of differences between groups was performed using the least significant difference (LSD) test. Differences with a $p$ value of <0.05 were considered statistically significant.

## DATA AVAILABILITY

Quantitative analysis of global proteome in HCMEC/D3 and proteomic quantification of lysine succinylation in human project accession: PXD005001. All other data supporting the findings of this study and the experimental materials generated in this study are available from the corresponding authors upon request.

## REFERENCES

1. Jhaveri N, Chen TC, Hofman FM. Tumor vasculature and glioma stem cells: contributions to glioma progression. Cancer Lett. 2016;380:545–51.
2. Ahir BK, Engelhard HH, Lakka SS. Tumor development and angiogenesis in adult brain tumor: glioblastoma. Mol Neurobiol. 2020;57:2461–78.
3. Onishi M, Ichikawa T, Kurozumi K, Date I. Angiogenesis and invasion in glioma. Brain Tumor Pathol. 2011;28:13–24.
4. Alleyn M, Breitzig M, Lockey R, Kolliputi N. The dawn of succinylation: a post-translational modification. Am J Physiol Cell Physiol. 2018;314:C228–32.
5. Olsen JV, Mann M. Status of large-scale analysis of post-translational modifications by mass spectrometry. Mol Cell Proteom. 2013;12:3444–52.
6. Zhang Z, Tan M, Xie Z, Dai L, Chen Y, Zhao Y. Identification of lysine succinylation as a new post-translational modification. Nat Chem Biol. 2011;7:58–63.
7. Du J, Zhou Y, Su X, Yu JJ, Khan S, Jiang H, et al. Sirt5 is a NAD-dependent protein lysine demalonylase and desuccinylase. Science. 2011;334:806–9.
8. Lin ZF, Xu HB, Wang JY, Lin Q, Ruan Z, Liu FB, et al. SIRT5 desuccinylates and activates SOD1 to eliminate ROS. Biochem Biophys Res Commun. 2013;441:191–5.
9. Kim HR, Park JS, Karabulut H, Yasmin F, Jun CD. Transgelin-2: a double-edged sword in immunity and cancer metastasis. Front Cell Dev Biol. 2021;9:606149.
10. Han MZ, Xu R, Xu YY, Zhang X, Ni SL, Huang B, et al. TAGLN2 is a candidate prognostic biomarker promoting tumorigenesis in human gliomas. J Exp Clin Cancer Res. 2017;36:155.
11. Kim HR, Kwon MS, Lee S, Mun Y, Lee KS, Kim CH, et al. TAGLN2 polymerizes G-actin in a low ionic state but blocks Arp2/3-nucleated actin branching in physiological conditions. Sci Rep. 2018;8:5503.
12. Lamalice L, Le Boeuf F, Huot J. Endothelial cell migration during angiogenesis. Circ Res. 2007;100:782–94.
13. Dejana E. Endothelial cell-cell junctions: happy together. Nat Rev Mol Cell Biol. 2004;5:261–70.
14. Padmanabhan K, Grobe H, Cohen J, Soffer A, Mahly A, Adir O, et al. Thymosin beta4 is essential for adherens junction stability and epidermal planar cell polarity. Development. 2020;147:dev193425.
15. Yang Y, Gibson GE. Succinylation links metabolism to protein functions. Neurochem Res. 2019;44:2346–59.
16. Sreedhar A, Wiese EK, Hitosugi T. Enzymatic and metabolic regulation of lysine succinylation. Genes Dis 2020;7:166–71.
17. Zeng Z, Li Y, Pan Y, Lan X, Song F, Sun J, et al. Cancer-derived exosomal miR-25-3p promotes pre-metastatic niche formation by inducing vascular permeability and angiogenesis. Nat Commun. 2018;9:5395.
18. Merino F, Pospich S, Raunser S. Towards a structural understanding of the remodeling of the actin cytoskeleton. Semin Cell Dev Biol. 2020;102:51–64.
19. Na BR, Kim HR, Piragyte I, Oh HM, Kwon MS, Akber U, et al. TAGLN2 regulates T cell activation by stabilizing the actin cytoskeleton at the immunological synapse. J Cell Biol. 2015;209:143–62.
20. Dvorakova M, Nenutil R, Bouchal P. Transgelins, cytoskeletal proteins implicated in different aspects of cancer development. Expert Rev Proteom. 2014;11:149–65.
21. Xiao Y, Li Y, Han J, Pan Y, Tie L, Li X. Transgelin 2 participates in lovastatin-induced anti-angiogenic effects in endothelial cells through a phosphorylated myosin light chain-related mechanism. PLoS ONE. 2012;7:e46510.
22. Jin H, Cheng X, Pei Y, Fu J, Lyu Z, Peng H, et al. Identification and verification of transgelin-2 as a potential biomarker of tumor-derived lung-cancer endothelial cells by comparative proteomics. J Proteom. 2016;136:77–88.
23. Fonseca CG, Barbacena P, Franco CA. Endothelial cells on the move: dynamics in vascular morphogenesis and disease. Vasc Biol. 2020;2:H29–43.
24. Svitkina T. The actin cytoskeleton and actin-based motility. Cold Spring Harb Perspect Biol. 2018;10:a018267.
25. Yin LM, Ulloa L, Yang YQ. Transgelin-2: biochemical and clinical implications in cancer and asthma. Trends Biochem Sci. 2019;44:885–96.
26. Zeng C, Zhang H. Mechanisms of transgelin-2 in tumorigenesis. Disco Med. 2021;32:23–8.
27. Jang SY, Jang SW, Ko J. Regulation of ADP-ribosylation factor 4 expression by small leucine zipper protein and involvement in breast cancer cell migration. Cancer Lett. 2012;314:185–97.
28. Makowiecka A, Malek N, Mazurkiewicz E, Mrowczynska E, Nowak D, Mazur AJ. Thymosin beta4 regulates focal adhesion formation in human melanoma cells and affects their migration and invasion. Front Cell Dev Biol. 2019;7:304.
29. Teng Y, Loveless R, Benson EM, Sun L, Shull AY, Shay C. SHOX2 cooperates with STAT3 to promote breast cancer metastasis through the transcriptional activation of WASF3. J Exp Clin Cancer Res. 2021;40:274.
30. Murcia-Belmonte V, Esteban PF, Martinez-Hernandez J, Gruart A, Lujan R, Delgado-Garcia JM, et al. Anosmin-1 over-expression regulates oligodendrocyte precursor cell proliferation, migration and myelin sheath thickness. Brain Struct Funct. 2016;221:1365–85.
31. Xue B, Leyrat C, Grimes JM, Robinson RC. Structural basis of thymosin-beta4/profilin exchange leading to actin filament polymerization. Proc Natl Acad Sci USA. 2014;111:E4596–605.
32. Lee CW, Vitriol EA, Shim S, Wise AL, Velayutham RP, Zheng JQ. Dynamic localization of G-actin during membrane protrusion in neuronal motility. Curr Biol. 2013;23:1046–56.
33. Wirsching HG, Krishnan S, Florea AM, Frei K, Krayenbuhl N, Hasenbach K, et al. Thymosin beta 4 gene silencing decreases stemness and invasiveness in glioblastoma. Brain. 2014;137:433–48.
34. Vitriol EA, McMillen LM, Kapustina M, Gomez SM, Vavylonis D, Zheng JQ. Two functionally distinct sources of actin monomers supply the leading edge of lamellipodia. Cell Rep. 2015;11:433–45.

35. Zhang C, Zhang X, Wang J, Di F, Xue Y, Lin X, et al. Lnc00462717 regulates the permeability of the blood-brain tumor barrier through interaction with PTBP1 to inhibit the miR-186-5p/Occludin signaling pathway. FASEB J. 2020;34:9941–58.
36. Jia P, He J, Li Z, Wang J, Jia L, Hao R, et al. Profiling of blood-brain barrier disruption in mouse intracerebral hemorrhage models: collagenase injection vs. autologous arterial whole blood infusion. Front Cell Neurosci. 2021;15:699736.
37. Li Q, Lan X, Han X, Wang J. Expression of Tmem119/Sall1 and Ccr2/CD69 in FACS-sorted microglia- and monocyte/macrophage-enriched cell populations after intracerebral hemorrhage. Front Cell Neurosci. 2018;12:520.

## ACKNOWLEDGEMENTS

We thank PTM Biolabs (Hangzhou, China) for help and support in the Proteomic Quantification of Lysine Succinylation. This work was supported by the National Natural Science Foundation of China (No. 82072793 and No. 81773289); the Natural Science Foundation for Liaoning Province (No. 2021-MS-213).

## AUTHOR CONTRIBUTIONS

Y.G. conceived and designed the research. X.Z. and J.H. performed the experiments and acquired data; D.F., X.L., C.Z., J.B., H.H., H.Z., and J.W. helped acquire data; X.Z. and J.H. analyzed the data; X.Z. and J.H. wrote the manuscript; Y.G. edited the paper and obtained funding. All authors revised the paper and approved the final version.

## COMPETING INTERESTS

The authors declare no competing interests.

## ETHICS

Animal procedures were approved via the Institutional Animal Care and Use Committee of Shenyang Pharmaceutical University and were processed in accordance with the guideline of the Animal Care and Use Committee of the National Institutes of Health. The use of the clinical samples was approved by the Hospital Ethics Committee of General Hospital of Northern Theater Command and carried out in accordance with the principles of the Declaration of Helsinki.

## ADDITIONAL INFORMATION

**Supplementary information** The online version contains supplementary material available at https://doi.org/10.1038/s41417-022-00534-6.

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