International Journal of Biomedical Engineering and Clinical Science
2025, Vol. 11, No. 2, pp. 11-27
https://doi.org/10.11648/j.ijbecs.20251102.11

Research Article

Inhibition of TRF1 Can Accelerate Aging and Induce Autophagy Through the P53-SIRT6 Pathway in Glioblastoma Multiforme

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Abstract

Glioblastoma (GBM) is a kind of intractable brain tumor. The effect of surgical treatment, radiotherapy and chemotherapy is not ideal. TRF1 is one of the important components of shelterin complex, which plays an important role in human telomere protection. Previous studies have reported that inhibition of TRF1 expression can inhibit the growth and proliferation of GBM without causing serious physiological dysfunction. However, the specific mechanism of inhibition of GBM growth and proliferation caused by decreased TRF1 expression has not been fully elucidated. To further elucidate this mechanism, we knockdown TRF1 by siRNA. We detected the levels of cell senescence, autophagy through biological experiments. It has been found that the knockdown of TRF1 can cause significant increase in the aging, autophagy of GBM. In addition, SIRT-6 is a NAD^+- dependent deacetylase. Previous studies have reported that SIRT-6 can maintain the stability of telomere function. Moreover, telomere dysfunction can cause the decrease of SIRT-6 expression. Therefore, we want to study the effect of SIRT-6 expression level on TRF1 knockdown induced aging, autophagy in GBM. The experimental results showed that the knockdown of TRF1 caused the decrease of SIRT-6 expression level, and the increase of SIRT-6 expression level could inhibit the aging, autophagy caused by TRF1 knockdown. This study provides a new direction for the treatment of GBM.

Keywords

Glioblastoma, TRF1, SIRT-6, Autophagy, Senescence, Telomere Damage

1. Introduction

Glioblastoma (GBM) is a common malignant neuroepithelial tumor of the central nervous system in adults, accounting for about 30% of the total brain and central nervous system tumor [1]. GBM is highly invasive and can metastasize in a large area. It is mixed with healthy brain tissue, which makes it almost impossible to remove the tumor by surgery without

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Received: 17 April 2025; **Accepted:** 28 April 2025; **Published:** 29 May 2025

serious consequences. In addition, GBM tumor has some stem cell-like cells (also called glioma stem cell-like cells), which can reproduce the original tumor after injection into immunodeficient mice, thus causing resistance to radiotherapy and chemotherapy [2]. Based on the above points, GBM through general radiotherapy and chemotherapy treatment effect is very unsatisfactory, the average survival time of patients is still only 12-15 months [3]. The clinical symptoms caused by GBM, such as headache and convulsions, will significantly reduce the quality of life and cause serious social risk. Therefore, looking for innovative and effective strategies and methods to treat GBM has become a top priority.

Mammalian telomere is a heterochromatin structure located at the end of linear chromosome, which is composed of TTAGGG repeats bound by a series of related proteins called shelterin [4]. Shelterin complexes include TRF1, TRF2, TIN2, RAP1, TPP1 and POT1. The main function of telomeres is to stabilize the end structure of chromosomes and prevent the end connection between chromosomes. Telomere is closely related to the aging of human body. The loss of telomere DNA can induce telomere DNA damage reaction, and then induce cell aging through p53 pathway. In the process of cell replication, DNA polymerase can't completely replicate the ends of linear molecules, so telomere shortens after each round of replication, which is mainly offset by telomerase [5]. Telomerase is a kind of reverse transcriptase (TERT). It uses RNA component (TERC) as template to prolong telomere by adding telomere repeat sequence at the end of chromosome, so as to prevent telomere erosion. The activity of telomerase in normal human tissues is inhibited, and it can only be detected in dividing cells (such as hematopoietic cells, stem cells, germ cells and tumor cells).

For cancer, maintaining telomere length above the minimum is the key to maintain unlimited cell proliferation. Therefore, telomere is regarded as an important anti-cancer target [6]. At present, most researches on cancer telomeres focus on telomerase inhibition as a treatment to prevent telomere extension of cancer cells [7]. The most advanced anti-telomerase drug in the world is GRN163L. However, telomerase inhibition as a cancer treatment strategy shows obvious defects and deficiencies. First of all, in cancer, the anti-tumor effect of telomerase inhibitors can only be achieved when telomeres reach a very short length, and this effect will be lost in the absence of p53 [8]. Unfortunately, many tumors have mutations in the p53 tumor suppressor gene. In addition, inhibition of telomerase activity may activate the alternative telomere lengthening mechanism based on recombination [9]. Therefore, telomerase as a target of tumor therapy is not ideal in clinical practice.

It is urgent to find a tumor therapy that does not rely on telomerase to target telomeres. TRF1 is directly combined with TTAGGG telomere repeat sequence, which is very important for telomere protection [10]. Loss of TRF1 in vivo can cause DNA damage at telomeres. Telomere dysfunction can fragment DNA and release it into cytoplasm. Cytoplasmic DNA activates cGAS-STING pathway, which leads to autophagy [11]. On the other hand, it is well known that telomere damage can cause replication aging of cells [12]. Previous studies have reported that inhibition of TRF1 in mouse lung cancer cells can inhibit tumor growth and proliferation independent of telomere length, and more importantly, inhibition of TRF1 will not affect the survival or tissue function of mice [13]. So we boldly speculate that TRF1 can be an important target for the treatment of glioblastoma.

Sirtuin (SIRT) is a kind of protein with NAD^+-dependent deacetylation or ADP ribosyltransferase activity, which is involved in the regulation of energy metabolism, stress response, cell survival and longevity [14, 15]. Up to now, seven SIRT proteins have been found in mammals, they have different tissue, subcellular localization and biological functions. SIRT-1 and SIRT-2 are expressed in nucleus and cytoplasm, SIRT-3, SIRT-4 and SIRT-5 are mitochondria, while SIRT-6 and SIRT-7 are located in nucleus [14]. SIRT1 plays a role in chromatin regulation and genome stabilization. SIRT2 was found to be related to mitotic structure and ensured normal cell division. SIRT3 regulates mitochondrial fatty acid oxidation. SIRT4 inhibits pyruvate dehydrogenase complex. SIRT6 is a nuclear ADP ribosyltransferase [16]. There is evidence that SIRT6 deacetylates H3K9Ac at telomere to prevent telomere dysfunction, and telomere damage can inhibit the expression of SIRT6 through p53 pathway [17, 18]. Therefore, we speculate that TRF1 knockdown in GBM can activate autophagy and p53-dependent cell senescence, and this process can be regulated by SIRT-6.

2. Materials and Methods

2.1. Bioinformatics Analysis

The expression level of TRF1 mRNA in GBM and corresponding normal tissues was analyzed by GEPIA database (http://www.proteinatlas.org/). TRF1 mRNA expression in different grades of gliomas was downloaded from TCGA and CCGA databases. Cbioportal database (http://www.cbioportal.org/) was used to collect the information of TRF1 mRNA expression and survival time of GBM patients, and then the statistical analysis was carried out by Graphpad Prism 7 software, and the survival analysis chart was drawn. ATLAS database was used to analyze the distribution of SIRT-6 in cells and the expression level of SIRT-6 in various tissues of normal people. At last, we downloaded the relevant KEGG pathway of SIRT-6 and the interaction network between TRF1 and SIRT-6 through String database (https://string-db.org/), and make diagrams through Graphpad Prism 7 and Cytoscape software respectively.

2.2. RNAi

The transfection reagent used in this experiment was Lipofectamine 3000 (Invitrogen) [19]. In the six well plate,

when the cell density of U87 reached 40% - 50%, the cells were transfected with 20 nmol / L TRF1-siRNA (GenePharma) according to the manufacturer's protocol. The sequence of TRF1 siRNA is shown in Table 1.

2.3. Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted by trziol method, and the concentration of RNA was determined by UV spectrophotometer (NanoDrop). The volume of 1 ug RNA was calculated from the measured concentration. The cDNA was synthesized by reverse transcription kit (Promega). All reverse transcription products were analyzed by the Roche LightCycler System with SYBR green incorporation (Roche). The final Cq value was analyzed by the $2^{-\Delta\Delta Ct}$ method, and then the relative mRNA expression of the target gene was calculated. The primer sequences used in this study are shown in Table 2.

2.4. Western Blot Analysis

Total protein of U87 cells was extracted by RIPA reagent. The protein concentration was determined by BCA method (Thermo Fisher Scientific). According to the obtained concentration, the loading volume of each sample was calculated (the mass of samples used in this study was designed as 50ug). Prepare electrophoresis solution and protein membrane transfer solution according to the instructions. The protein transfer solution was stored in a refrigerator at 4 ℃, while the electrophoresis solution was stored at normal temperature. Prepare 10% SDS-PAGE separation gel, prepare 5% concentrated gel after solidification, and insert it into a toothed comb. Add the specified volume of protein into the lane, then place the electrophoresis protein gel on PVDF membrane (Millipore), and transfer it to the membrane through membrane transfer solution. Then the PVDF membrane was sealed with 5% milk for 2 hours. The PVDF membrane containing the target protein was cut off and incubated in the designated antibody diluent for 12-16 hours. Wash the membrane three times with TBST detergent for 10 minutes each time. The membrane was incubated in secondary anti rabbit or anti mouse antibody at room temperature for 2 hours, and then washed with TBST. Target protein and internal reference protein were detected with the ECL Blotting Detection Reagents (Thermo Fisher Scientific). The antibodies used in this experiment are as follows: GAPDH (Abcam), TRF1 (Abcam), Beclin-1 (Abcam), LC3 (Abcam), p16 (Abcam), SIRT-6 (Abcam), p53 (Abcam).

2.5. Trypan Blue Staining

TRF1-siRNA (GenePharma) was transfected into U87 cells, and NMN solution was added 24 hours after transfection. After 48 hours of transfection, the medium in the six well plate was discarded. Trypsin was added to digest the cells for 90 seconds, and then the trypsin was discarded. Add 900ul of complete medium and 100ul of 0.4% trypan blue dye to the six well plate, shake well and let stand for 3 minutes. Finally, 10ul trypan blue cell suspension (Solarbio) was dropped on the special cell counting plate and counted under the ordinary light microscope (MARIENFELD).

2.6. Cell Counting Kit-8 (CCK-8)

U87 cell suspensions of TRF1-siRNA and TRF1-siRNA+NMN were inoculated in 96-well plates, with about 100ul per well. The culture plate was pre-cultured in an incubator for 4 hours. Add 10μl CCK-8 solution (Beyotime Institute of Biotechnology) to each well, and then put the culture plate into an incubator to incubite for 1-4 hours. The absorbance value (OD) at 450nm was determined by microplate reader. The cells were cultured for 24 hours, 48 hours and 72 hours, and the above steps were repeated.

2.7. Wound Healing

Draw three parallel lines across the hole on the bottom of the six-well plate, and spread the cells to the plate. 24 hours later, TRF1-siRNA was transfected into cells. After 48 hours of transfection, the 200ul pipette tip was used to scratch in the direction perpendicular to the drawn parallel line. Discard the old medium and add 1 ml PBS for washing. Add 2ml serum-free medium, observe and take pictures with fluorescence microscope. The scratch width was measured again 24 hours after the first scratch, and the percentage of healing and recovery of scratch width between cells in each well was compared.

2.8. β-galactosidase Assays (Visible Spectrophotometry)

TRF1 siRNA was transfected into U87 cells, and NMN dilution was added. The experiment was started 48 hours after transfection. According to the specific operation steps in the manual, β-galactosidase Assay Kit (Solarbio) was used to extract β-GAL enzyme from cells. The absorbance value of the extracted β-GAL enzyme was measured at 400nm by microplate reader. Establish the corresponding standard curve, and calculate the β-GAL enzyme activity of the sample.

2.9. Cell Clone Formation Experiment

U87 cells were treated with TRF1-siRNA and NMN. The cells were slowly washed twice with deionized water, then 4% tissue cell fixative (Biosharp) was added and the cell culture dish was allowed to stand for 20 minutes. The cells were washed with deionized water for three times, then 0.5% crystal violet dye solution (Sangon Biotech) was added to completely infiltrate the cells, and the cells were allowed to stand at room temperature for 30 minutes. Deionized water was added and the cell culture dish was slowly washed three times on a shaker for 2 minutes each time. Finally, put the cell culture dish upside down on the toilet paper, drain the water, take photos of the results and analyze the results with

image J.

2.10. Statistical Analysis

Each biological experiment was repeated at least three times. Data are presented as means ±standard deviation (SD). Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software). The experimental results were analyzed by one-way ANOVA and Student's t-tests. P values < 0.05 were considered statistically significant.

Table 1. Sequence of TRF1-siRNA.

	Sence	Antisence
TRF1-siRNA1	GGUGAUCCAAAUUCUCAUATT	UAUGAGAAUUUGGAUCACCTT
TRF1-siRNA2	GGUACAGUAUCCUUAUUGATT	UCAAUAAGGAUACUGUACCTT
Negative control (NC)	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT

Table 2. Primer Sequence of qRT-PCR.

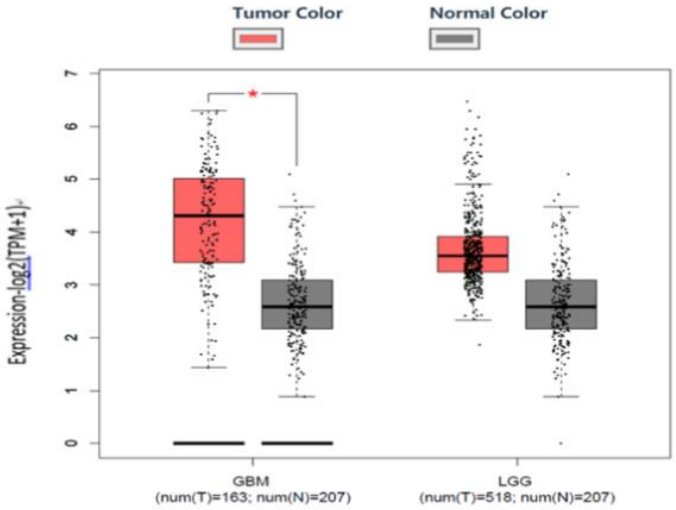
	Sence	Antisence
TRF1	AACAGCGCAGAGGCTATTATTC	CCAAGGGTGTAATTCGTTCATCA
p16	GGAGGCCGATCCAGGTCAT	CACCAGCGTGTCCAGGAAG
SIRT-6	TCCCCGACTTCAGGGGTC	GTTCTGGCTGACCAGGAAGC
p53	AAGTCTAGAGCCACCGTCCA	CAGTCTGGCTGCCAATCCA
ATG-5	ATGATAATGGCAGATGACAAGG	TCAGTCACTCGGTGCAGG
Beclin-1	AGGTTGAGAAAGGCGAGACA	AATTGTGAGGACACCCAAGC
IL-1α	GGTTGAGTTTAAGCCAATCCA	TGCTGACCTAGGCTTGATGA
IL-6	CAGGAGCCCAGCTATGAACT	GAAGGCAGCAGGCAACAC
GAPDH	GTCTCCTCTGACTTCAACAGCG	ACCACCCTGTTGCTGTAGCCAA

3. Results

3.1. The Expression Level of TRF1 in GBM and Its Knock Down Efficiency

Glioblastoma (GBM) is a kind of brain tumor with high malignancy. Although surgery combined with radiotherapy and chemotherapy is used, the curative effect is still not ideal [20, 21]. Therefore, new treatment strategies and methods are needed to deal with GBM. Previous studies have shown that TRF1 inhibition can target telomeres independently of telomere length, thus inhibiting the growth and proliferation of lung cancer [13]. TRF1 is also overexpressed in several types of cancer, such as renal cell carcinoma and gastrointestinal cancer [22]. Therefore, we speculate that TRF1 is an important therapeutic target for GBM. We analyzed the expression level of TRF1 in glioblastoma by GEPIA database. As shown in Figure 1A, the expression level of TRF1 mRNA in GBM patients was higher than that in the corresponding normal population, although the results were not statistically significant. We analyzed the expression level of TRF1 in different grades of gliomas through CCGA and TCGA databases and found that the higher the expression level of TRF1 mRNA with the increase of glioma grade (Figure 1B and 1C). Then, we used ATLAS database to know the relationship between TRF1 mRNA expression and prognosis of GBM patients. From Figure 1D, we can clearly see that the survival prognosis of GBM patients with low TRF1 expression is significantly better than that of GBM patients with high TRF1 expression. The above results make us more convinced that TRF1 is of great significance in treating GBM. TRF1-siRNA was designed and synthesized by GenePharma. TRF1-siRNA was transfected into U87 cells by Lipofectamine 3000. Then, in order to verify the effectiveness of siRNA, we verified the knockdown efficiency of siRNA by qRT-PCR and Western blot experiments from two aspects of mRNA and protein

expression. As shown in Figure 1E, 1F and 1G, compared with the control group, the mRNA and protein levels of TRF1 in the experimental group were significantly decreased with statistical significance. These results indicate that the TRF1 siRNA sequence is correct and can be used in this study.



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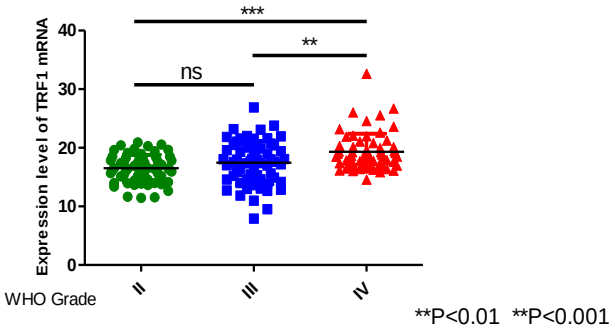

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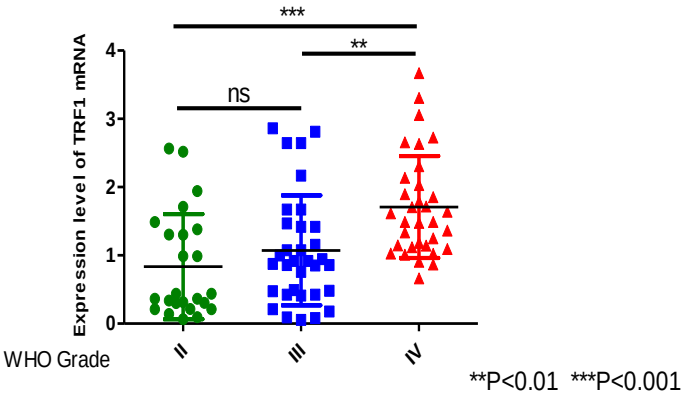

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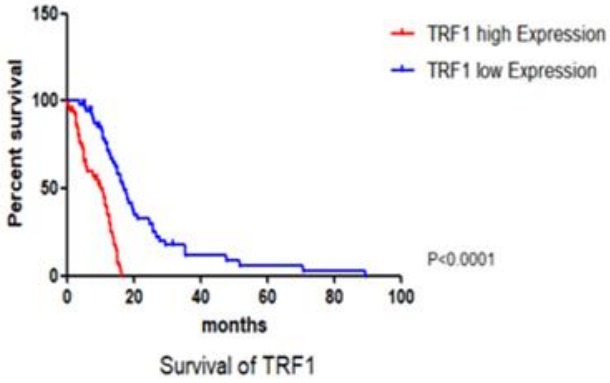

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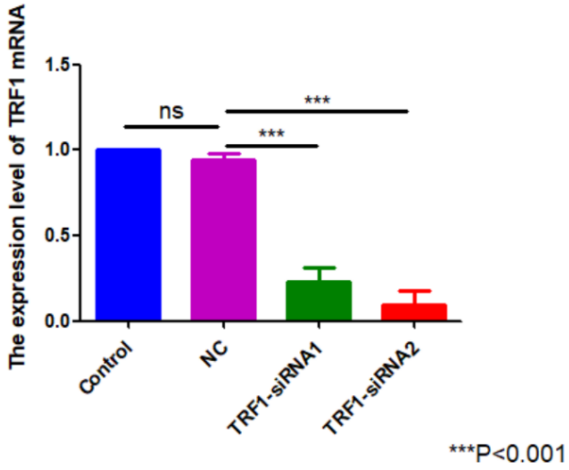

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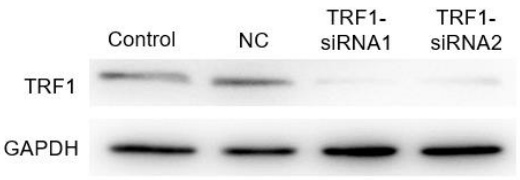

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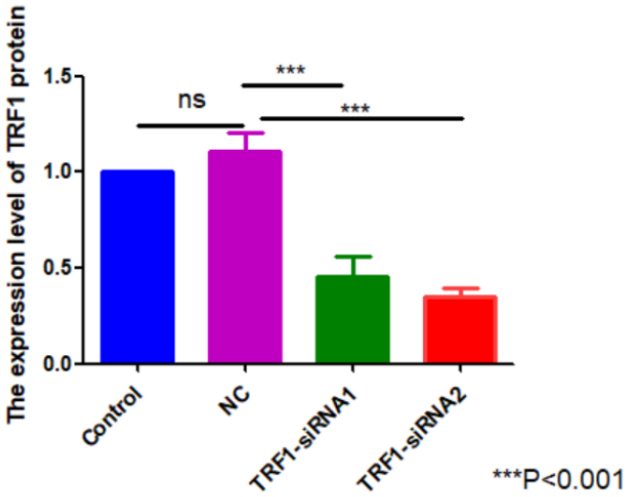
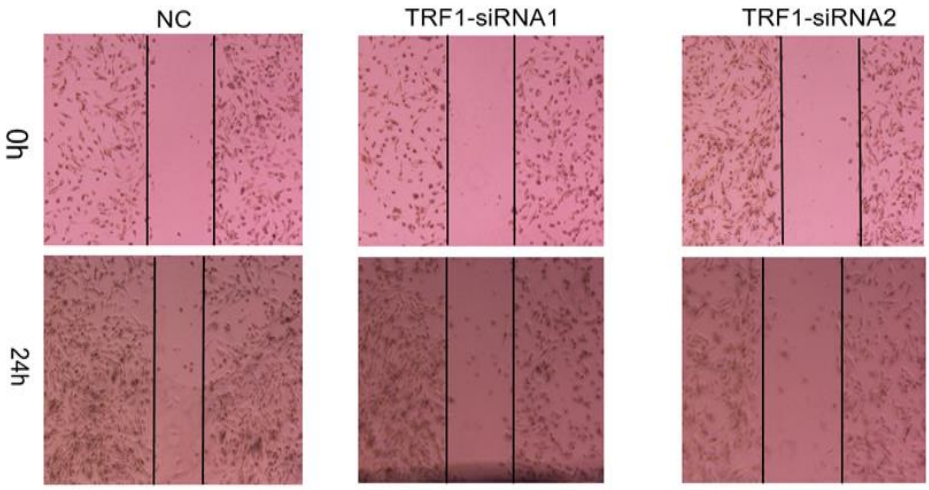


G

Figure 1. *The expression level of TRF1 in glioma and the knockdown efficiency of TRF1. (A) The expression of TRF1 mRNA in glioma was analyzed by GEPIA database. (B) The expression levels of TRF1 mRNA in different grades of gliomas were analyzed by CCGA database. (C) The expression levels of TRF1 mRNA in different grades of gliomas were analyzed by TCGA database. (D) ATLAS database speculates the relationship between TRF1 mRNA expression and prognosis of glioma patients. The subjects of the statistics are all IDH wild-type glioma patients. (E) The knockdown efficiency of TRF1 in U87 cells was detected by qRT-PCR. GAPDH was used as internal control. (F) The knockdown efficiency of TRF1 was detected by Western blot. (G) Quantitative analysis of (D).* $^{***}P<0.001$.

3.2. Knockdown of TRF1 Can Inhibit the Growth and Proliferation of GBM

It has been reported that TRF1 is overexpressed in adult stem cells and pluripotent stem cells, which are important for maintaining tissue homeostasis [27]. And TRF1 is overexpressed in many tumor tissues. Therefore, we want to explore the effect of TRF1 knockdown on the growth and proliferation of glioblastoma. We transfected U87 cells with TRF1-siRNA, and then detected the growth and proliferation of U87 cells by scratch test, CCK-8 cell proliferation test and trypan blue staining test. The experimental results are shown in Figure 2A-2D. The growth and proliferation status of cells in the control group is significantly better than that in the siRNA transfected experimental group. This suggests that TRF1 may be an important therapeutic target for GBM.



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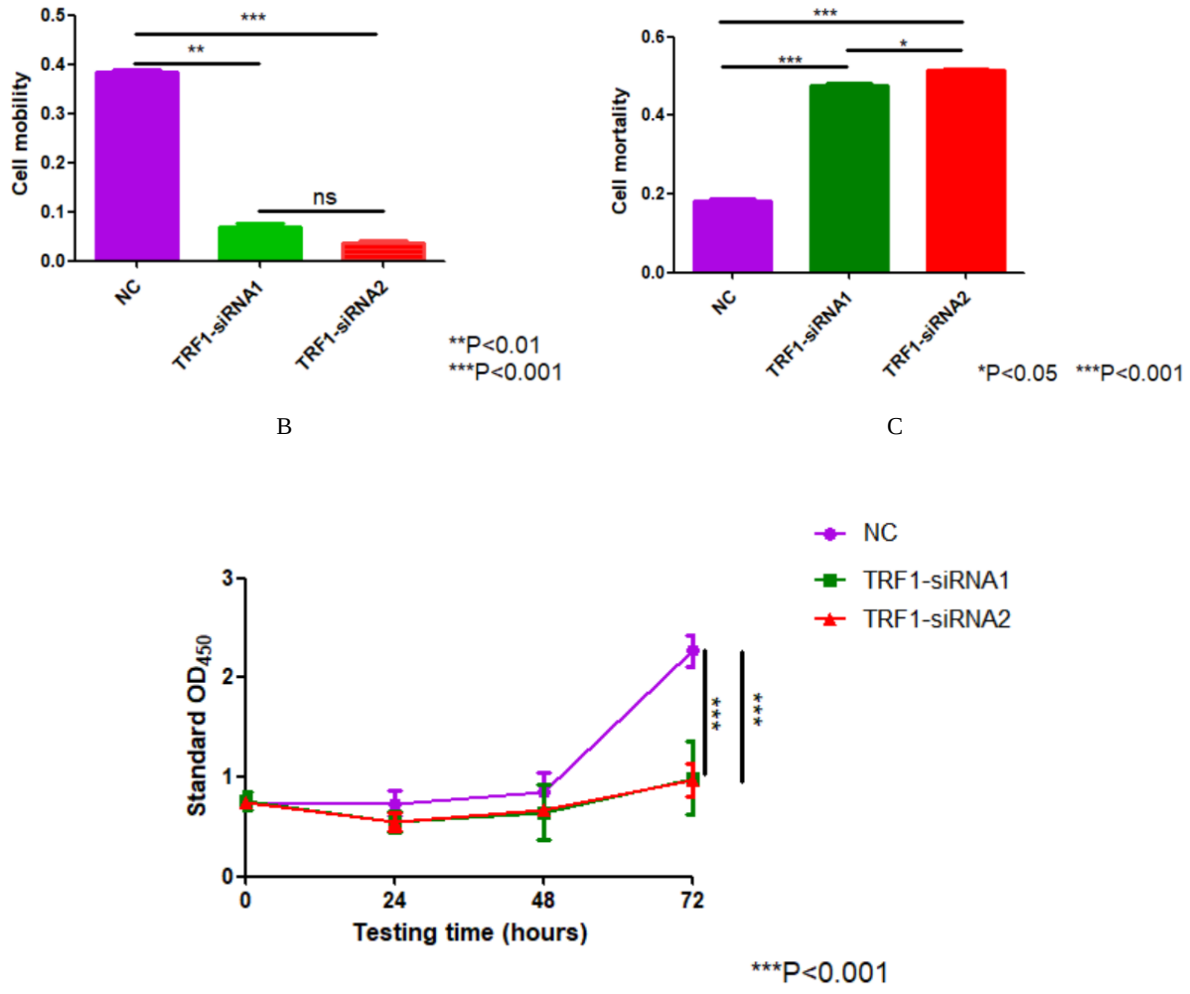


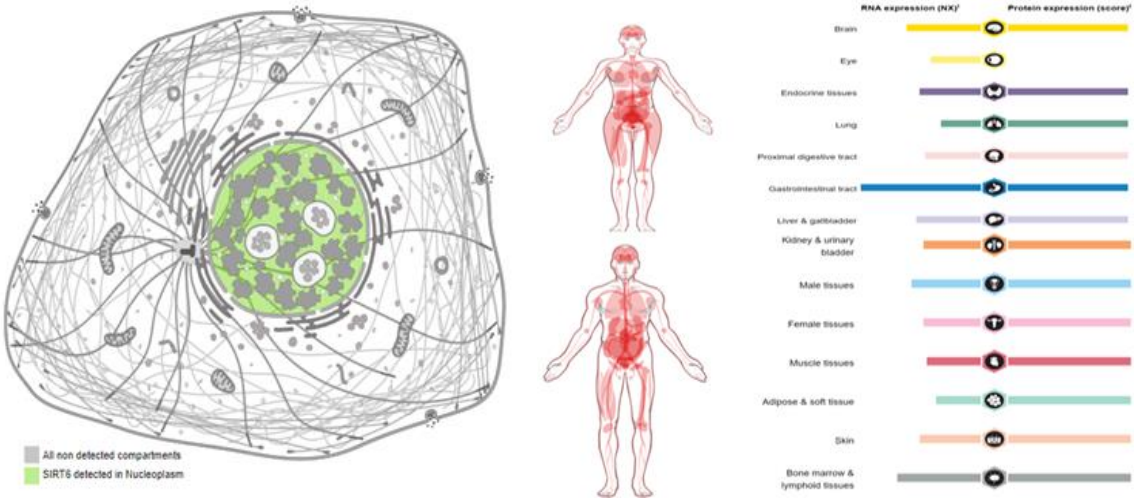
Figure 2. *Effect of knocking down TRF1 on the growth and proliferation of U87 cells. (A) Scratch test was used to detect the effect of TRF1 knockdown on U87 cell migration. (B) Quantitative analysis of (A). (C) Trypan blue assay was used to detect the effect of TRF1 knockdown on the survival status of U87 cells. (D) CCK-8 assay was used to detect the effect of TRF1 knockdown on the proliferation of U87 cells.* $^{*}P<0.05$ $^{**}P<0.01$ $^{***}P<0.001$.

3.3. The Relationship Among TRF1, p53 and SIRT-6

Inhibition of TRF1 can cause telomere damage, and then activate p53 pathway, leading to cell aging or apoptosis [23]. It has been proved that telomere damage can reduce the expression of SIRT-6 through p53 pathway, and then accelerate telomere damage [24]. SIRT-6 is a NAD^+- dependent histone deacetylase, mainly located in the nucleus. SIRT-6 can maintain telomere stability. When SIRT-6 is absent, it can cause genomic instability, chromosome end-to-end fusion and premature cell senescence [25]. Therefore, we estimate the relationship among TRF1, p53 and SIRT-6 as follows: the telomere damage of U87 cells caused by TRF1 knockdown can inhibit the expression of SIRT-6 through p53 pathway, while increasing the expression of SIRT-6 can inhibit p53 signaling pathway by inhibiting telomere dysfunction. NMN is the precursor of NAD^+, which can activate SIRT-6 by increasing the expression level of NAD^+ [24, 26].

First, we used ATLAS database to learn the distribution of SIRT-6 in glioma cells. Results as shown in figure 3A, SIRT-6 was mainly located in the nucleus of glioma cells. We used ATLAS database to analyze the expression level of SIRT-6 in human tissues. As shown in figures 3B and 3C, SIRT-6 is widely expressed in various human tissues and organs. We used String database to speculate the relationship between TRF1, p53 and SIRT-6. Next, we downloaded the KEGG pathway of SIRT-6 interaction gene through the string database. As shown in Figure 3D, SIRT-6 interaction gene is mainly enriched in alcoholism related pathway, systemic lupus erythematosus related pathway and necroptosis related pathway. Figure 3E shows that there may be a direct biological relationship among TRF1, p53 and SIRT-6. Then, we conducted a series of biological experiments to confirm this conclusion. We used siRNA to knock down TRF1 in U87 cells, and then detected the expression levels of SIRT-6 and p53 by qRT-PCR and Western blot analysis. From figures 3F

to 3H, we can see that knockdown of TRF1 can reduce the expression level of SIRT-6 and increase the expression level of p53. In order to further confirm the correctness of the conclusion, we carried out SIRT-6 rescue experiment. As shown in Figures 3I to 3J, the increase in SIRT-6 expression inhibited the expression of p53 in TRF1 knockdown U87 cells. Therefore, we believe that TRF1 knockdown can affect the expression of SIRT-6 through p53 pathway in glioma.



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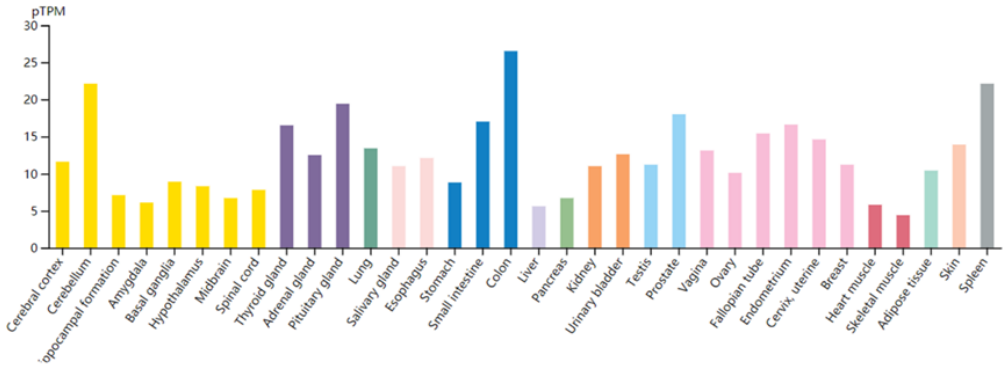

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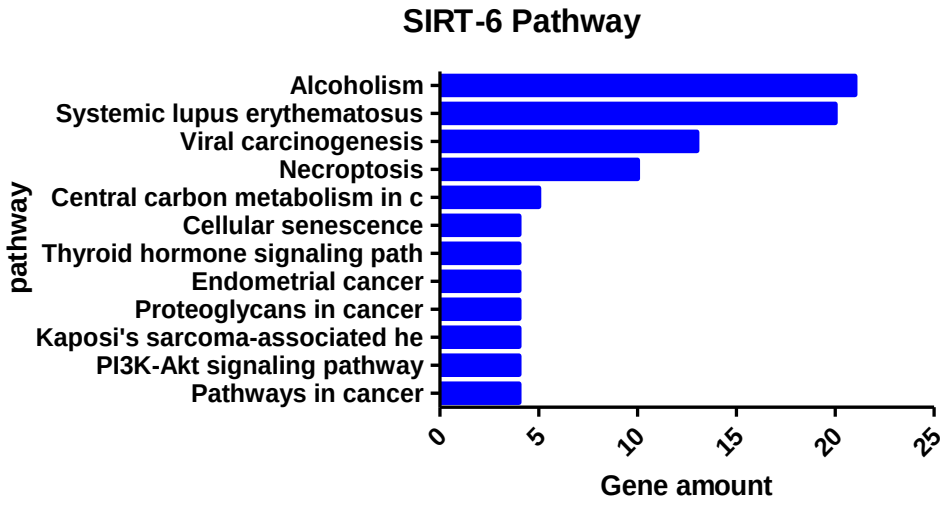

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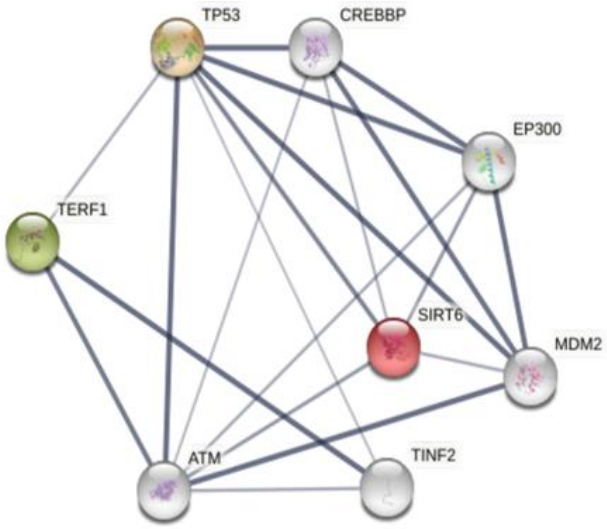
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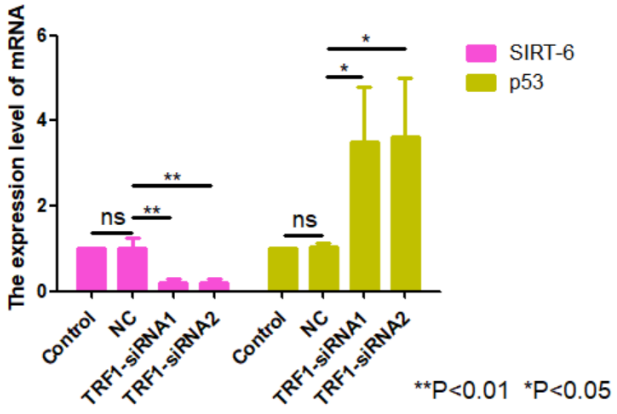
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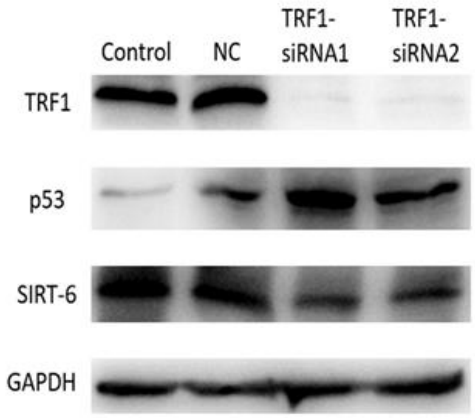
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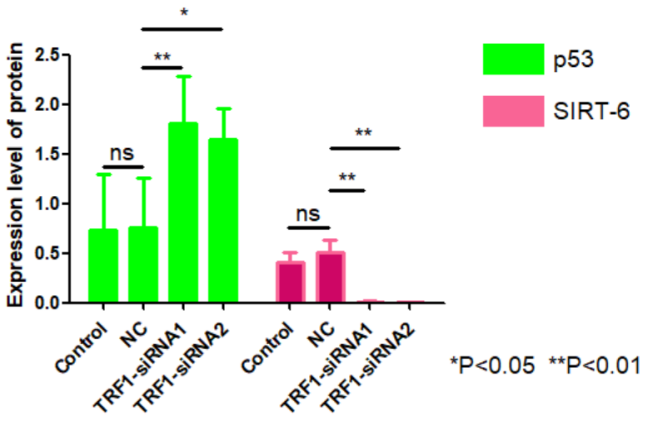
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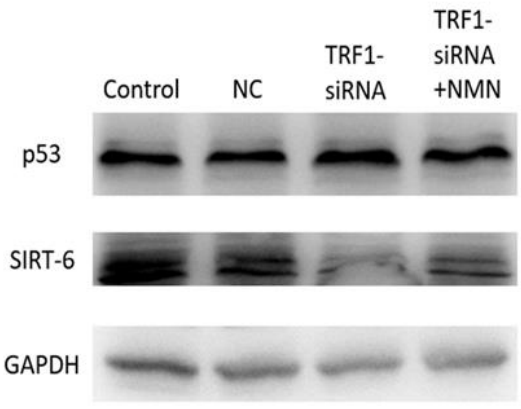
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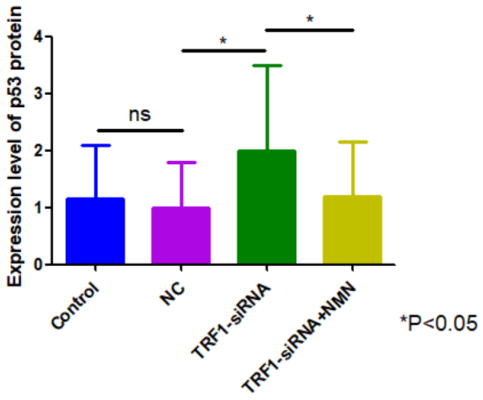

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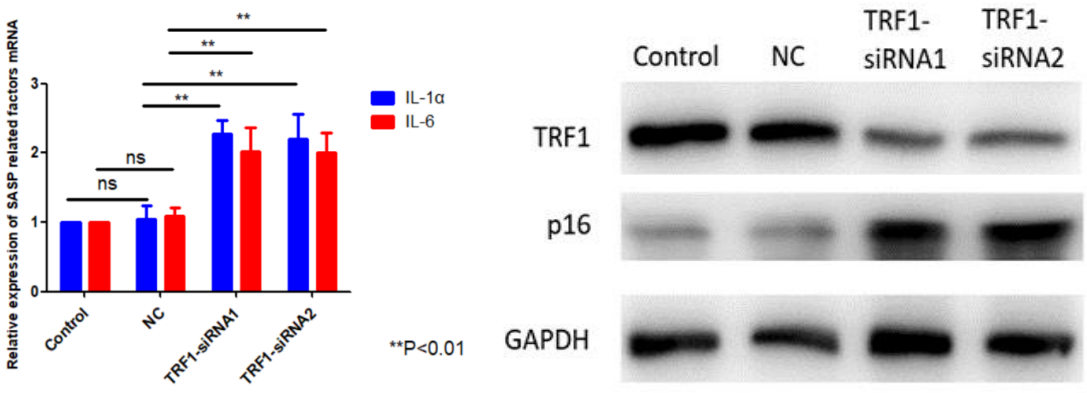
Figure 3. *The relationship between TRF1, p53 and SIRT-6. (A) Distribution of SIRT-6 in glioma cells analyzed by Atlas Database. (B-C) Protein and mRNA expression of SIRT-6 in human tissues. (D) The relationship among TRF1, p53 and sirt-6 was inferred by String database. (E-F) The mRNA and protein expression levels of sirt-6 and p53 in TRF1 knockdown cells were detected. (G) The quantitative analysis of (H) The expression of SIRT-6 and p53 protein in U87 cells added with NMN after TRF1 knockdown was detected by Western blot. The final concentration of NMN was 250uM. (I) The quantitative analysis of (I).* $^{*}P<0.05$ $^{**}P<0.01$.

3.4. The Effect of TRF1 Knockdown on GBM Cell Senescence and Its Specific Mechanism

Telomere DNA damage can lead to cell aging, thus hindering the growth and proliferation of cells, which is of great benefit to the prevention and treatment of tumors. Aging cells can produce related biomarkers, such as SASP related factors, p16, β-galactosidase and so on [28, 29]. SIRT-6 is a kind of deacetylase located in heterochromatin, which provides protection against telomere and genomic DNA damage, thus preventing cell aging [30]. Previous studies have shown that telomere injury can downregulate the expression of SIRT-6 [18]. Therefore, we believe that TRF1 knockdown can induce cell senescence by reducing the expression of SIRT-6 in glioma cells. First of all, we need to confirm that TRF1 knockdown does cause cell senescence. We detected the important cell senescence markers (IL-1α, IL-6 and p16) by qRT-PCR and Western blot. From Figure 4A to 4D, we can see that the expression level of senescence markers in TRF1 knockdown cells was significantly higher than that in control cells. Through the above results, we proved that the knockdown of TRF1 can cause cell aging of glioma cells. Next, we want to confirm that knockdown of TRF1 can lead to cell aging by reducing the expression of SIRT-6. We observed whether the activator of SIRT-6 (NMN) could inhibit cell aging caused by TRF1 knockdown by β-GAL activity test (visible light spectrophotometry). From figure 4E and 4G, we can observe that the activity of β -galactosidase in the experimental group which knocks down TRF1 and adds NMN is obviously decreased compared with the experimental group which knocks down TRF1 only. This results indicate that SIRT-6 activator can inhibit the aging of glioma cells induced by knocking down TRF1. Finally, we confirmed by Western blot that SIRT-6 inhibited the aging of glioma cells induced by TRF1 knockdown. The experimental results are shown in 4E and 4I. we can clearly see that the increase of SIRT-6 expression level does inhibit the expression level of p16, a marker of cell aging. All in all, these results indicate that TRF1 knockdown can induce cell aging of glioma cells by reducing the expression level of SIRT-6.



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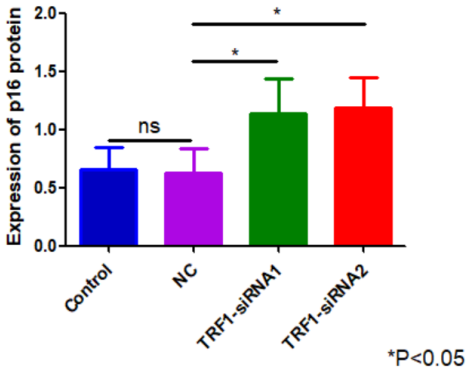

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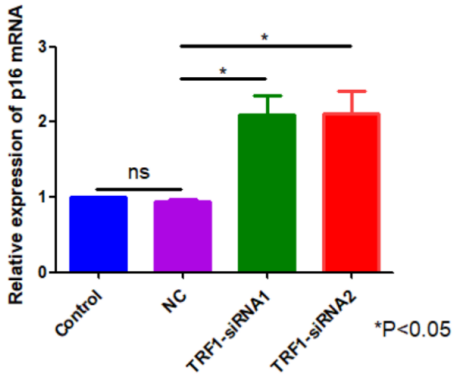

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	NC	TRF1-siRNA	TRF1-siRNA+NMN	200 nmol/ml	100 nmol/ml	50nmol/ml	25nmol/ml	12.5nmol/ml	6.25nmol/ml	0nmol/ml
Measuring tube	0.057	0.058	0.058	0.886	0.423	0.253	0.149	0.117	0.101	0.051
Control tube	0.128	0.281	0.129	0.059	0.058	0.058	0.059	0.058	0.06	0.059
	0.052	0.052	0.054	0.058	0.059	0.059	0.059	0.059	0.059	0.058

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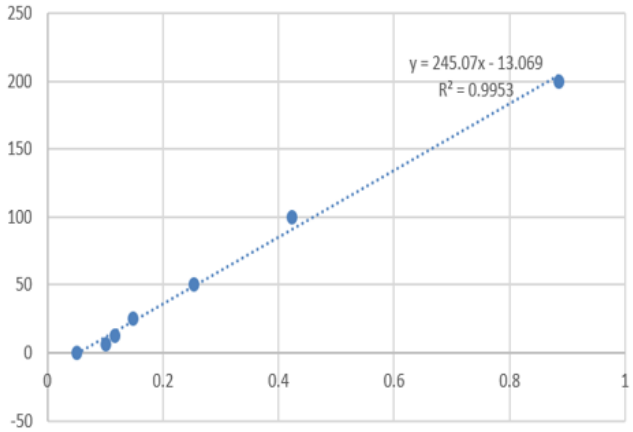

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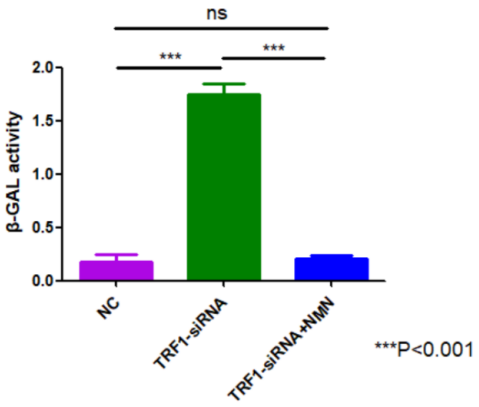

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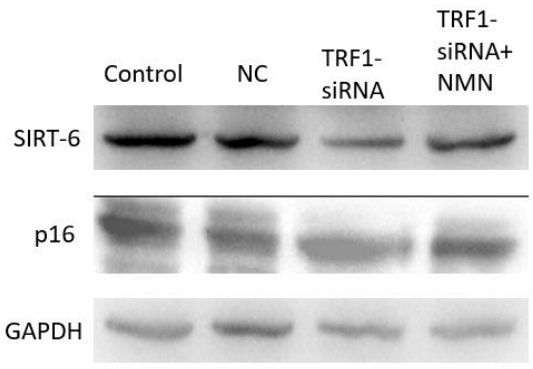

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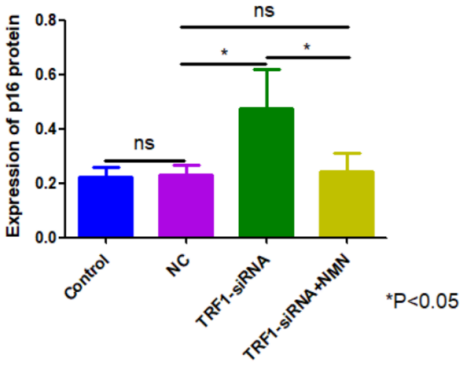


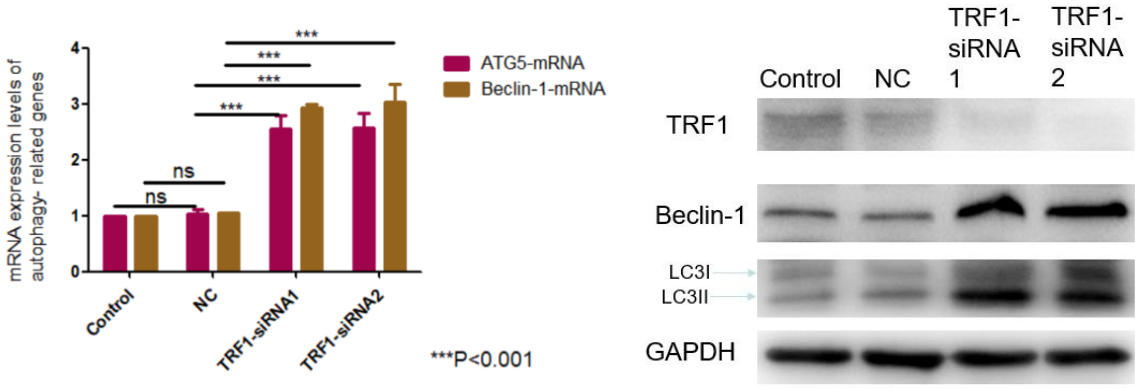
I

Figure 4. *Effect of knocking down TRF1 on senescence of U87 cells and its specific mechanism. (A) The mRNA expressions of IL-1α and IL-6 in TRF1 knockdown cells were detected by qRT-PCR. (B-D) Western blot and qRT-PCR were used to detect the expression of p16 protein and mRNA in the TRF1 knockdown cells. (E) β- Gal activity (UV spectrophotometry) was used to detect the aging status of TRF1 knockdown and NMN added after TRF1 knockdown. (F) Standard curve of β-GAL activity experiment. (G) Quantitative analysis of β-GAL activity. (H) Western blot was used to detect the p16 expression of TRF1 knockdown and NMN after TRF1 knockdown. (I) Quantitative analysis of (H).* $^{*}P<0.05$ $^{**}P<0.01$ $^{***}P<0.001$.

3.5. Autophagy Induced by TRF1 Knockdown in GBM Cells and Its Mechanism

Autophagy is a process of phagocytizing cytoplasmic proteins or organelles, coating them into vesicles, fusing with lysosomes to form autophagic lysosomes and degrading their contents [31]. Autophagy is a double-edged sword. On the one hand, autophagy is crucial for eukaryotic cells to maintain homeostasis; on the other hand, excessive autophagy can lead to cancer cell death [32, 33]. It has been reported that telomere damage can cause autophagy [11]. Therefore, we want to know whether the knockdown of TRF1 will cause autophagy in GBM cells. TRF1 in U87 cells was knocked down by siRNA, and the changes of protein and mRNA expression levels of autophagy markers (Beclin-1, LC3II/I, ATG5) were detected by Western blot and qRT-PCR respectively. Results as shown in Figure 5A to 5C, the expression level of autophagy markers in the experimental group treated with siRNA was significantly higher than that in the control group. These results suggest that TRF1 knockdown can induce autophagy in GBM cells.

Previous studies have shown that SIRT-6 can prevent telomere damage in human endothelial cells [15]. Moreover, SIRT inhibitors can induce autophagy in colorectal cancer cells [34]. Therefore, we want to observe whether the increase of SIRT-6 expression in GBM cells inhibits autophagy caused by TRF1 knockdown. NMN is the precursor of NAD^+, so it can increase the expression level of SIRT-6. By Western blot, we found that the expression of SIRT-6 was significantly increased in NMN group compared with siRNA group; Importantly, with the increase of SIRT-6 expression, the protein expression level of autophagy markers was significantly lower than that of siRNA group (Figure 5D, 5E). Therefore, we hypothesized that SIRT-6 can inhibit autophagy caused by TRF1 knockdown in GBM cells.



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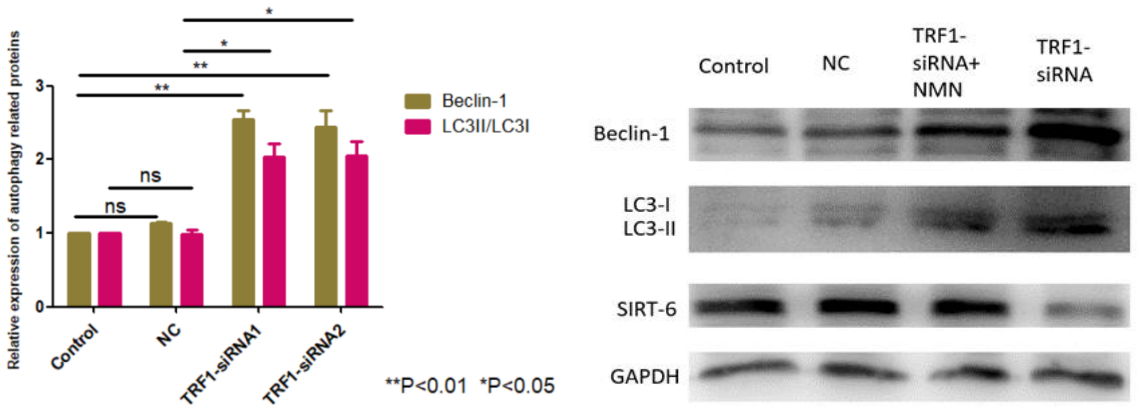

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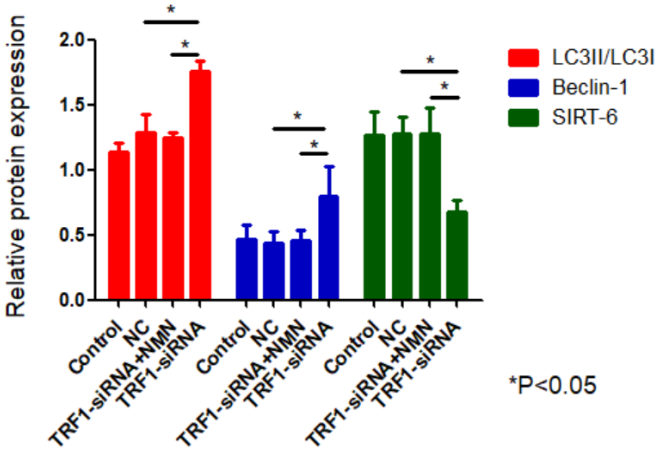

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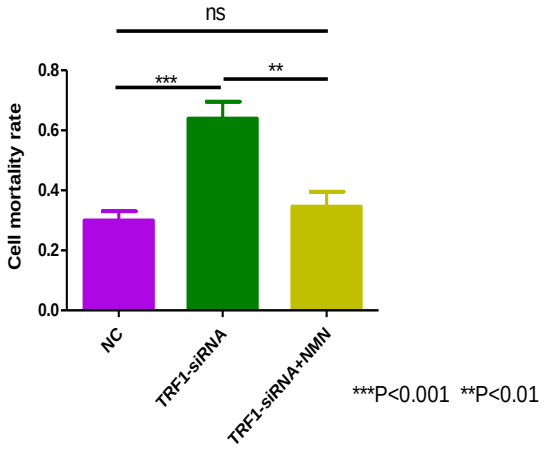
Figure 5. *Mechanism of autophagy induced by knocking down TRF1 in U87 cells. (A, B) The mRNA and protein expression levels of autophagy markers after TRF1 knockdown were detected by qRT-PCR and Western blot. (C) Quantitative analysis of (B) (D) TRF1 knockdown and the changes of autophagy markers expression caused by adding NMN were detected by Western blot. (E) Quantitative analysis of (D).* $^{*}P<0.05$ $^{**}P<0.01$ $^{***}P<0.001$.

3.6. SIRT-6 Can Attenuate the Inhibition of GBM Cell Growth and Proliferation Caused by TRF1 Knockdown

Previous studies have shown that SIRT-6 deficient mice have a phenotype related to aging [35]. In addition, some literatures have pointed out that SIRT-6 can stabilize telomere structure and prevent telomere damage caused by stress. Therefore, we speculate that SIRT-6 may be involved in the weakening of GBM cell growth inhibition caused by TRF1 knockdown. Firstly, we detected the cell mortality of the control group and the experimental group by trypan blue staining. Results as shown in Figure 6A, the cell mortality of TRF1 knockdown group was significantly higher than that of TRF1 knockdown and treated with NMN (SIRT-6 can be activated). Next, we further verified our view through CCK-8 experiment and cell clone formation experiment. The experimental results of CCK-8 are shown in Figure 6B, the growth state of cells in the experimental group with TRF1 knockdown and treated with NMN is significantly better than that in the experimental group with TRF1 knockdown. In the cell clone formation experiment shown in Figure 6C, we can clearly see that the number of cell clones in the experimental group with TRF1 knockdown and treated with NMN is significantly higher than that in the experimental group with TRF1 knockdown only. The above experimental results further suggested that SIRT-6 could reduce the inhibition of TRF1 knockdown on the growth and proliferation of GBM cells.



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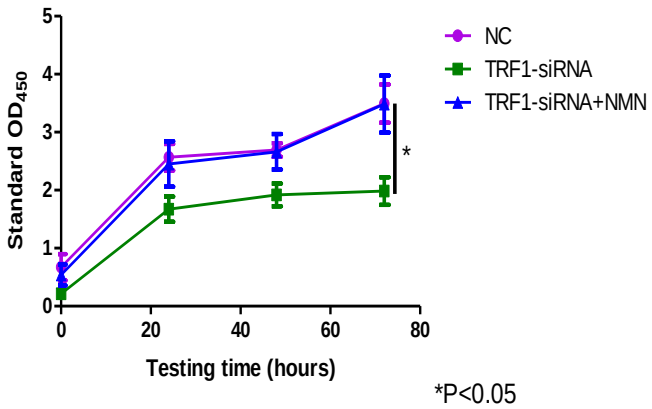

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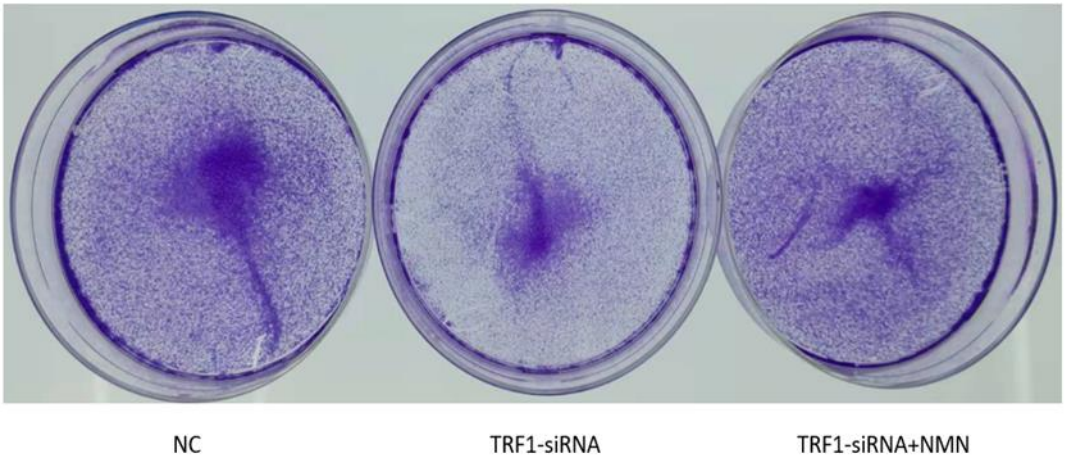

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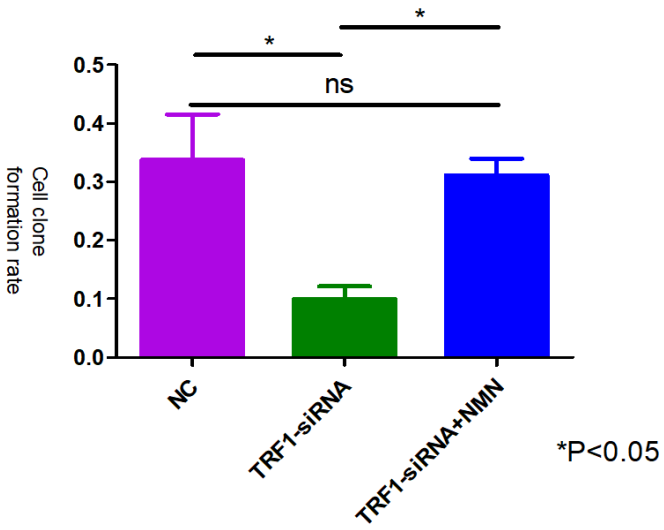

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Figure 6. *SIRT-6 can reduce the growth and proliferation restriction of U87 cells caused by TRF1 knockdown. (A) The death number of U87 cells in each experimental group was detected by trypan blue staining. (B) The ultraviolet absorbance at 450nm of U87 cells in each experimental group was detected by CCK-8 experiment. (C) The growth and proliferation of U87 cells in each experimental group were detected by cell clone formation experiment. (D) Quantitative analysis of (C).* $^{*}P<0.05$ $^{**}P<0.01$ $^{***}P<0.001$.

4. Discussion

Telomere is a special structure composed of short, repeated untranslated sequences (TTAGGG) and some binding proteins. Besides providing a buffer for untranslated DNA, telomere can also protect the ends of chromosomes from fusion and degeneration. It plays an important role in chromosome location, replication, protection and control of cell growth and longevity, and is closely related to apoptosis, cell transformation and immortalization. In tumors, cancer cells almost universally acquire telomere maintenance mechanism (TMM). Among them, 85% of tumors extend telomeres by telomerase, while the remaining 15% extend telomeres by ALT [36]. Telomeres are surrounded by six protective proteins, which are called shelterin proteins. TRF1 and TRF2 are homologous to each other, and they both directly bind to telomere DNA double strand [37]. As we all know, the deletion of telomere protective protein will cause damage to telomere DNA, which in turn will cause cell growth and proliferation restriction [38]. In addition, previous studies have shown that TRF1 is overexpressed in various tumors (such as renal cell carcinoma and gastrointestinal tumors). Most importantly, TRF1 is overexpressed in adult stem cells and pluripotent stem cells, which are crucial to maintain the steady state and pluripotency of tumor cells, respectively [39]. This provides a new idea for the clinical treatment of many kinds of tumors-that is, to induce telomere DNA damage of tumor cells to cause tumor cell death.

Glioma originated from neuroepithelium and is the most

common primary intracranial tumor. Glioblastoma is one of the most malignant gliomas, most of its tumor cells grow in the supratentorial cerebral hemisphere. Surgical resection is considered to be the best treatment for glioblastoma, but the effect of surgical treatment is poor because its tumor cells can invade the surrounding tissues. Therefore, new strategies and methods are needed for the clinical diagnosis and treatment of glioblastoma. It has been previously reported that in the mouse model of glioblastoma, inhibition of telomere protective protein TRF1 can cause telomere damage, thereby impairing the initiation and progression of glioblastoma. This indicates that TRF1 may be an important therapeutic target for glioblastoma. However, it is still unclear how inhibition of TRF1-induced telomere damage can cause the growth and proliferation restriction of glioblastoma cells. In this paper, the specific mechanism of how the decrease of TRF1 inhibits the growth of glioblastoma cells is described.

Autophagy is a conservative cellular pathway. In this process, dysfunctional or damaged organelles were removed by lysosomal degradation, and products were recovered to meet the metabolic needs of cells. Therefore, autophagy is very important to maintain the balance in vivo, and mediates the resistance to anti-cancer therapies such as radiotherapy, chemotherapy and some targeted therapies [40]. However, the role of autophagy in cancer is still controversial, and it may inhibit tumors during the development of cancer, so the specific role of autophagy seems to be highly dependent on the cellular environment [41]. Telomere DNA damage caused by the deletion of shelterin protein in telomeres causes autophagy through cGAS-STING pathway. In addition, telomere DNA damage can also induce cell senescence through p53 pathway. Therefore, we speculate that the knockdown of TRF1 in glioblastoma can induce cell aging and autophagy through telomere DNA damage.

SIRT-6 is an NAD^+-dependent deacetylase, which can prevent telomere dysfunction. As we all know, knocking down TRF1 will cause telomere dysfunction. Therefore, we speculate that SIRT-6 can be involved in the process of weakening telomere dysfunction and a series of adverse consequences caused by TRF1 knockdown in glioblastoma.

The experimental results of this study show that TRF1 knockdown can induce cell senescence and autophagy of GBM cells, and SIRT-6 can participate in the inhibition of cell senescence and autophagy caused by TRF1 knockdown.

Abbreviations

TRF1	Telomere Repeat Sequence Binding Factor 1
TRF2	Telomere Repeat Sequence Binding Factor 2
GBM	Glioblastomamultiforme
SIRT6	NAD-dependent Protein Deacetylase Sirtuin-6
SASP	Senescence-associated Secretory Phenotype
ATM	Ataxia Telangiectasia Mutated
IL-1α	Interleukin-1α
IL-6	Interleukin-6
LC3	Microtubule-associatedprotein1 Light Chain 3
ATG-5	Autophagy-relatedprotein-5

Author Contributions

Ziyang Nie: Data curation, Formal Analysis, Software, Writing – original draft, Writing – review & editing

Siqi Liu: Formal Analysis, Methodology, Software, Validation, Writing – original draft

Xinyu Huang: Formal Analysis, Methodology, Software, Validation, Writing – original draft

Xin Geng: Conceptualization, Data curation, Funding acquisition, Investigation, Supervision, Validation, Visualization, Writing – review & editing

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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