Thyroid Hormone Receptor β1 Suppresses Atypical Meningioma Proliferation in Vitro

shen zhang (zhangshen1919@163.com)
First Affiliated Hospital of Dalian Medical University https://orcid.org/0000-0002-7343-0445
Feihui Zhou
Kunming Medical University First Affilliated Hospital
Jingchun Pei
Kunming Medical University First Affilliated Hospital
Xinkui Zhang
Kunming Medical University First Affilliated Hospital
Chang Liu
Kunming Medical University First Affilliated Hospital
Zhigao Li
Kunming Medical University First Affilliated Hospital
Linjie Mu
Kunming Medical University First Affilliated Hospital
Congqian Wang
Kunming Medical University First Affilliated Hospital
Yue Liu
xiangyangshi diyi renmin yiyuan: Xiangyang No 1 People's Hospital Affiliated to Hubei University of Medicine
Zhiwei Tang
Kunming Medical University First Affilliated Hospital https://orcid.org/0000-0001-9314-4465

Research Article

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Abstract

Objective

To study the role of TRβ1 in atypical meningioma.

Methods

Forty-four meningioma specimens were collected, including 38 cases of WHO grade I and 6 cases of WHO grade II. The Ki67 index and TRβ1 mRNA level were detected in 44 cases. TRβ1 protein expression was evaluated in the meningiomas by IHC and Western blot analysis. Primary meningioma cells were cultured and identified with EMA and vimentin antibodies. Cell proliferation was evaluated using MTT, EdU and SRB assays. siRNA was used to silence TRβ1 expression. Western blotting assays were employed to detect p-ERK expression.

Results

There was a negative correlation between TRβ1 mRNA and the Ki67 index in meningioma tissues. The expression of TRβ1 protein in atypical meningiomas was lower than that in benign meningiomas ($p < 0.05$). T3 suppressed meningioma cell proliferation and cell survival in a dose-dependent manner. Moreover, we found that T3 significantly inhibited p-ERK protein expression ($p < 0.05$). Furthermore, TRβ1 knockdown almost completely rescued the T3-induced suppression of meningioma cell proliferation and partially rescued the T3-induced ERK activation.

Conclusion

Our study is the first to report that TRβ1 suppresses atypical meningioma cell proliferation by attenuating ERK phosphorylation.

Introduction

Meningiomas are common tumors of the central nervous system that originate from the cap cells of arachnoid granules. The incidence is approximately 8.03/100,000, which is approximately 1/3 of all intracranial tumors[1]. According to the 2016 WHO classification, there are three grades of meningiomas: benign meningioma (grade I), atypical meningioma (grade II) and interstitial meningioma (grade III) [2]. Benign meningioma accounts for approximately 80% of all meningiomas, atypical meningioma accounts for approximately 18.5% of all meningiomas, and interstitial meningioma accounts for approximately 1.5% of all meningiomas [3]. The survival and recurrence rates of meningiomas are closely related to the pathological grade of the tumor. The overall five-year survival rate is 92% for benign meningiomas, 78%

for atypical meningiomas, and 47% for interstitial meningiomas[4]. The recurrence rate is 7–20% for benign meningiomas, 29–40% for atypical meningiomas, and 50%-94% for interstitial meningiomas [5, 6].

Because of its relatively high incidence and poor prognosis, it is urgent to find new diagnosis and treatment strategies for atypical meningiomas. Therefore, it is particularly important to explore the molecular pathogenesis of atypical meningioma and provide a theoretical basis for clinical treatment.

Thyroid hormone receptors (TRs), including TRα1, TRα2, TRβ1 and TRβ2, are encoded by the THRA or THRB gene. TRs are widely distributed in human tissues and play important roles during development and internal environment homeostasis maintenance [7]. The distribution of TR isoforms is different in various tissues. For example, TRα1 is predominantly expressed in skeletal muscle and brown fat, TRα2 predominates in the brain, TRβ1 predominates in the brain, liver and kidney, and TRβ2 predominates in the central nervous system and developing retina [8] [9] [10] [11].

TRβ1 plays important roles in tumor suppression in a variety of tumors. Aberrant methylation of the TRβ1 gene was reported in breast cancer tissues, suggesting an inhibitory role in the development of breast cancer [13]. Park et al. have also shown that T3-bound TRβ1 can downregulate the Src signaling pathway and inhibit tumor cell growth[14]. TRβ1 activation by T3 could also inhibit tumor cell proliferation by downregulating the JAK-STAT-cyclin D signaling pathway [15]. It has been reported that there is a relationship between thyroid disease and meningiomas [16]. However, it is unclear whether TRβ1 plays a role in the proliferation of atypical meningiomas.

Materials And Methods

Ethics statement

This study was approved by the ethics committee of the First Affiliated Hospital of Kunming Medical University.

Materials

Forty-four meningioma specimens were collected, including 38 cases of WHO grade I and 6 cases of WHO grade II, at the First Affiliated Hospital of Kunming Medical University. These specimens were collected from a total of 10 males and 34 females who had an average age of 49.75 ± 13.9 years. All samples were divided into three parts. One part was formalin fixed and paraffin embedded for IHC staining and Ki67 index detection. The second part was frozen at -80°C for PCR and Western blot assays. The third part was acutely dissected and isolated for primary meningioma cell culture.

Primary meningioma cell culture. Fresh meningioma tissues were minced into small pieces of approximately 1 mm^3 and digested with 0.25% trypsin at 37°C for 18 minutes. The primary meningioma cells were collected and cultured in Dulbecco's modified Eagle medium (DMEM, Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, BI, Israel) and 1% penicillin/streptomycin (Gibco,

Carlsbad, CA, USA) [17]. The primary cells were passaged when the cells reached 90% confluency. Early passage (less than five passages) primary cells were used for all designed experiments.

Immunohistochemistry (IHC). Fresh tissues were collected and fixed in 4% formaldehyde and embedded in paraffin. Tissue sections (5 μm) were deparaffinized, and antigens were retrieved by boiling the slides in 0.1 M citrate acid buffer for 5 min. Immunostaining for detecting TRβ1 (Clone J51, Santa Cruz Biotechnology, Inc., USA) was performed according to previously described protocols[18]. Quantification of the immunostaining signal was performed based on the intensity and percentage of immunopositive cells.

Quantitative real-time PCR. Total mRNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed using Iscript™ cDNA reverse transcription reagent (BIO-RAD) to obtain cDNA for quantitative PCR analysis. The mRNA levels of the target genes were quantified using RT Real-Time™ SYBR Green PCR master mix (Thermo Fisher Scientific, USA) and detected with an ABI-7900 system (Applied Biosystems). The sequences of the primers were as follows: human TRβ1: F-5'-TACAGCCTGGGACAAACC-3', R-5'-GGCGACGACTGTTCATTT-3'; human GAPDH: F-5'-GGAGCGAGATCCCTCCAAAAT-3', R-5'-GGCTGTTGTCATACTTCTCAT GG-3'.

Cell proliferation assay. T3 was dissolved in 1 N NaOH solution and formulated into 1 ng/ml, 5 ng/ml, 10 ng/ml, and 20 ng/ml. The same concentration of NaOH was used as the negative control. Cells were seeded in a 96-well plate at 4,000 cells/well in 100 μl of complete medium and cultured at 37°C. Twenty-four hours after plating, the culture medium was switched to complete medium supplemented with T3 or the NaOH control at the indicated concentrations (1 ng/ml, 5 ng/ml, 10 ng/ml, and 20 ng/ml). The MTT (5 mg/ml) reagent was added 48 hours after T3 intervention, and the OD value (490 nm wavelength) was measured with a spectrophotometric plate reader (Bio-Tek, USA). Cells were plated in 48-well plates at $0.2–12\times10^5$ cells/well. Twenty-four hours later, the cells were treated with either T3 (20 ng/ml) or the control at the indicated dosage for another 4 days. The cells were then fixed with 10% trichloroacetic acid (TCA) for 60 minutes at room temperature followed by washing with deionized water 5 times. The cells were stained with 0.4% (W/V) SRB for 15 minutes at RT followed by washing with 1% acetic acid 5 times. Finally, 10 mM Tris base was added to dissolve the dye, and the optical densities were measured at 530 nm with a spectrophotometric plate reader (Bio-Tek, USA). To determine the cell proliferation rate, cells were seeded on cell culture slides at 1×10^5 cells/well. Twenty-four hours after plating, the cells were treated with T3 (20 ng/ml) and EdU (1 ng/ml). Twenty-four hours later, EdU incorporation was measured using a Click-iT EdU Alexa Fluor 488 Imaging Kit (Invitrogen) following the manufacturer's instructions. All experiments were performed in triplicate and repeated twice.

TRβ1 knockdown with siRNA. Meningioma cells were plated in six-well plates (1 × 106 cells/well) and transfected with siRNA targeting TRβ1 (GCGCTATGACCCAGA A AGT) or control siRNA (final concentration 20 nM; RiboBio, China) using Lipofectamine 2000 (Life Technologies) according to the manufacturer's protocol. Seven hours after transfection, the cell culture medium was replaced with fresh

medium. The efficiency of siRNA-mediated gene silencing was detected 72 h after transfection by Western blotting analysis.

Western blot analysis. Frozen meningioma tissues or cells were collected and lysed in cold RIPA buffer supplemented with protease inhibitor cocktail (Sigma). Western blot analysis was performed as described previously [19]. Briefly, 30 μg of each protein sample was subjected to SDS–PAGE and blotted onto polyvinylidene fluoride (PVDF) membranes. After incubation with specific primary antibodies at 4°C overnight, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature for 1 hour.

Statistical analysis. Data were analyzed using the Statistical Package for the Social Sciences (SPSS) version 19.0 (SPSS, Inc. Chicago, IL, USA). Two-tailed Student's t-test was used for comparisons between two groups. P values < 0.05 were considered to be significant.

Results

TRβ1 expression was lower in human atypical meningioma.

Ki67 has been widely studied for its relationship with tumorigenesis, tumor development, clinical prognostic indicators, and tumor recurrence. Studies have also shown that Ki67 is correlated with the degree of malignancy of meningioma. The higher the expression of Ki67 is, the more malignant the tumor[20]. By comparing the Ki67 index and TRβ1 mRNA expression, we found a negative correlation among the 44 meningioma tissues, as shown in Fig. 1a. High mRNA expression of TRβ1 was correlated with a lower Ki67 index, and vice versa. IHC staining also revealed that there were fewer TRβ1-positive cells in atypical meningiomas than in benign meningiomas (37.25 ± 5.56% vs. 90.75 ± 18.73%, $p < 0.01$, Fig. 1b). We also analyzed TRβ1 protein expression in the two types of meningioma tissues by Western blotting. Compared with that in benign meningiomas, the expression of TRβ1 protein in atypical meningioma was lower (1.32 ± 0.56 > 0.32 ± 0.15 *P < 0.05 Fig. 1c-d).

T3 Inhibited Atypical Meningioma Cell Proliferation In Vitro

To determine the role of TRβ1 in atypical meningiomas, we established a primary meningioma cell culture system. The cells were identified with immunostaining. The ratio of EMA-positive cells was 99%, and the ratio of Vim-positive cells was 99% (Fig. 2a).

Primary atypical meningioma cells were treated with T3, and cell proliferation was measured using the MTT assay. T3 treatment effectively downregulated the expression in a concentration-dependent manner (*P < 0.05, **P < 0.01, ***P < 0.001, Fig. 2b). Subsequently, to further elucidate the role of T3 in meningioma cell proliferation, we performed EdU analysis. The results showed that the ratio of EdU-positive cells was 27.45 ± 4.61% in the T3 group and 48.59 ± 2.22% in the control group. These findings indicate that T3 inhibited the proliferation of atypical meningioma cells (Fig. 2c-d). We also examined the

cell growth curve by SRB assay. The cell numbers were measured 1 day, 2 days, 3 days and 4 days after T3 (20 ng/ml) treatment. We found that the proliferation of atypical meningioma cells was significantly inhibited in the T3 group compared with the control group (Fig. 3).

Trβ1 Regulated Atypical Meningioma Proliferation Via Erk

Since T3 inhibited atypical meningioma cell proliferation, it is of interest to determine whether the effect of T3 is dependent on TRβ1. Therefore, we knocked down TRβ1 expression with siRNA in the cells to detect whether the role of T3 was blocked.

TRβ1 expression was significantly silenced with siRNA (silencing 47.64%, Fig. 4b,4d). Then, we used the MTT assay to measure cell proliferation. The data showed that T3 significantly decreased the cell number in the NC group (0.35 ± 0.008 vs. 0.41 ± 0.005, $^*P < 0.05$), and siRNA treatment rescued the effect of T3 on decreasing cell proliferation (0.42 ± 0.003 vs. 0.43 ± 0.007 Fig. 4a). These data indicate that T3 inhibits the proliferation of atypical meningioma cells, at least partially, through TRβ1.

We further explored the possible mechanism of TRβ1. TRβ1 is able to activate several signaling pathways, including the ERK pathway. Our data showed that T3 downregulated ERK phosphorylation (p-ERK) compared with the control group (0.52 ± 0.099 vs. 0.64 ± 0.099, $^*P < 0.05$, Fig. 4c). However, when TRβ1 was silenced, the effect became less obvious (0.83 ± 0.35 vs. 0.76 ± 0.28, Fig. 4c). These results indicate that TRβ1 might inhibit the proliferation of atypical meningioma cells by inhibiting ERK activation.

Conclusion

Atypical meningioma is a type of refractory meningioma with a high recurrence rate and invasive activity. Treatments, such as surgery, radiotherapy and chemotherapy, are available in clinical practice. However, the overall prognosis of patients with atypical meningiomas is still poor, and the recurrence rate is high. It is particularly important to further explore the pathogenesis of atypical meningiomas to provide targets for clinical diagnosis and treatment.

Studies have also shown that Ki67 is correlated with the degree of malignancy of meningiomas, and high expression of Ki67 indicates more malignancy. In our current study, we found a negative correlation between the expression of TRβ1 mRNA and the Ki67 index in meningioma tumor tissues. Our results may suggest that TRβ1 is associated with the pathological grades of meningioma. We performed IHC staining and Western blot assays and further confirmed that TRβ1 was more highly expressed in benign meningiomas than in atypical meningiomas. In fact, the expression of TRβ1 was almost absent in atypical meningiomas, suggesting that TRβ1 may play roles in restricting meningioma progression and/or metastasis. Recent studies have reported that TRβ inactivation or loss of expression can promote the occurrence and development of multiple tumors. In gastric cancer, hypermethylation of the TRβ gene promoter region is positively correlated with clinical and pathological features, such as infiltration, lymph

node metastasis, and distant metastasis of tumors [23]. In a mouse model in which the TRβ gene is deleted, the incidence of spontaneous follicular thyroid cancer and distant metastasis were increased [24]. Ectopic overexpression of TRβ in human thyroid cancer cells (FTC-133 and FTC-236) significantly inhibited proliferation and metastasis in vitro and in vivo. In a xenograft model, ectopic overexpression of TRβ could significantly downregulate the expression level of vascular endothelial growth factor and inhibit tumor growth by inhibiting neovascularization [25].

TRβ1 activity is dependent on ligand binding, such as T3. To further verify whether TRβ1 plays a role in meningiomas, we examined the effects of T3 on the proliferation of meningioma cells by MTT, SRB and EdU analysis. The results suggested that T3 suppressed cell proliferation in a concentration-dependent manner and significantly inhibited cell proliferation at a concentration of 20 ng/ml. To further clarify the role of TRβ1 in mediating such effects, we knocked down TRβ1 expression by siRNA and detected cell proliferation by MTT assay. We found that the inhibitory effect of T3 on cell proliferation was weakened. These results suggest that the proliferation inhibition effect of T3 is mediated via the TRβ1 pathway.

In previous studies, TRβ1 was found to act as a tumor suppressor by affecting different signaling pathways, including the Src signaling pathway, PI3K-AKT signaling pathway, Wnt/β-Catenin signaling pathway, and STAT signaling pathway. It has been reported that TRβ can reduce the phosphorylation of AKT, attenuate the PI3K-AKT-mammalian target of rapamycin (mTOR)-ribosomal protein S6 kinase (p70S6K) signaling pathway, and inhibit tumor growth in vivo [26]. When activated by T3, TRβ1 attenuates the Src-FAK-ERK signaling pathway and inhibits tumor proliferation and invasiveness [24]. In liver cancer cells, the activation of the TR/DKK4/Wnt/β-Catenin signaling pathway promotes the degradation of β-Catenin and downregulates the expression of CD44, CyclinD1 and c-Jun. Thus, it inhibits the growth and invasion of liver cancer cells[27]. In addition, TRβ can also inhibit JAK and STAT phosphorylation, attenuate the JAK-STAT-cyclin D signaling pathway, inhibit tumor cell proliferation, and inhibit tumor growth [15].

Sharma et al. analyzed the proteome of human meningioma tissue by mass spectrometry and found that 2367 proteins were differentially expressed in meningiomas of different grades. Functional analysis of these differentially expressed proteins suggested that integrin signaling was included. The Wnt signaling pathway, Ras signaling pathway, FGF signaling pathway, EGF growth signaling pathway, and apoptotic signaling pathway are involved [28]. The receptor tyrosine kinases that have also been shown to play roles in the development of meningiomas include epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), and Erb-B2 receptor tyrosine. Kinase 2 (ERBB2), insulin-like growth factor 1 receptor (IGF1R) and vascular endothelial growth factor receptor (VEGFR) are widely expressed in meningioma tumor cells. The Ras/Raf/MEK signaling pathway is downstream of the receptor tyrosine kinases, and the PI3K/Akt/mTOR signaling pathway may be involved in the development of meningiomas. In addition to an elevated expression of ERK, pS6RP and Akt, Hilton et al. demonstrated that phosphorylated JNK and MEK are also increased in most meningiomas of different grades[29–32].

Considering the important roles of PI3k/AKT and ERK in meningioma development, we detected the expression of activated AKT and ERK in meningioma cells. Our study showed that T3 treatment attenuated p-ERK levels in atypical meningioma cells, and TRβ1 depletion almost completely rescued this effect. In contrast, there was no obvious difference in PI3k/AKT activation (data not shown).

In conclusion, our study reported that TRβ1 inhibits atypical meningioma cell proliferation. However, whether this role of TRβ1 acts through the ERK signaling pathway in atypical meningiomas requires further verification. Future functional experiments using atypical meningioma cell models and xenograft mouse models are needed. Although the detailed mechanism remains to be delineated, this preliminary study provides further knowledge of the biological functions of TRβ1 in atypical meningiomas and suggests that TRβ1 could be a potential target for atypical meningioma therapy.

Declarations

Acknowledgments

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Conflict of interest

The authors declare no conflicts of interest.

Informed consent

Informed consent was obtained from all individual participants included in this study.

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Figures

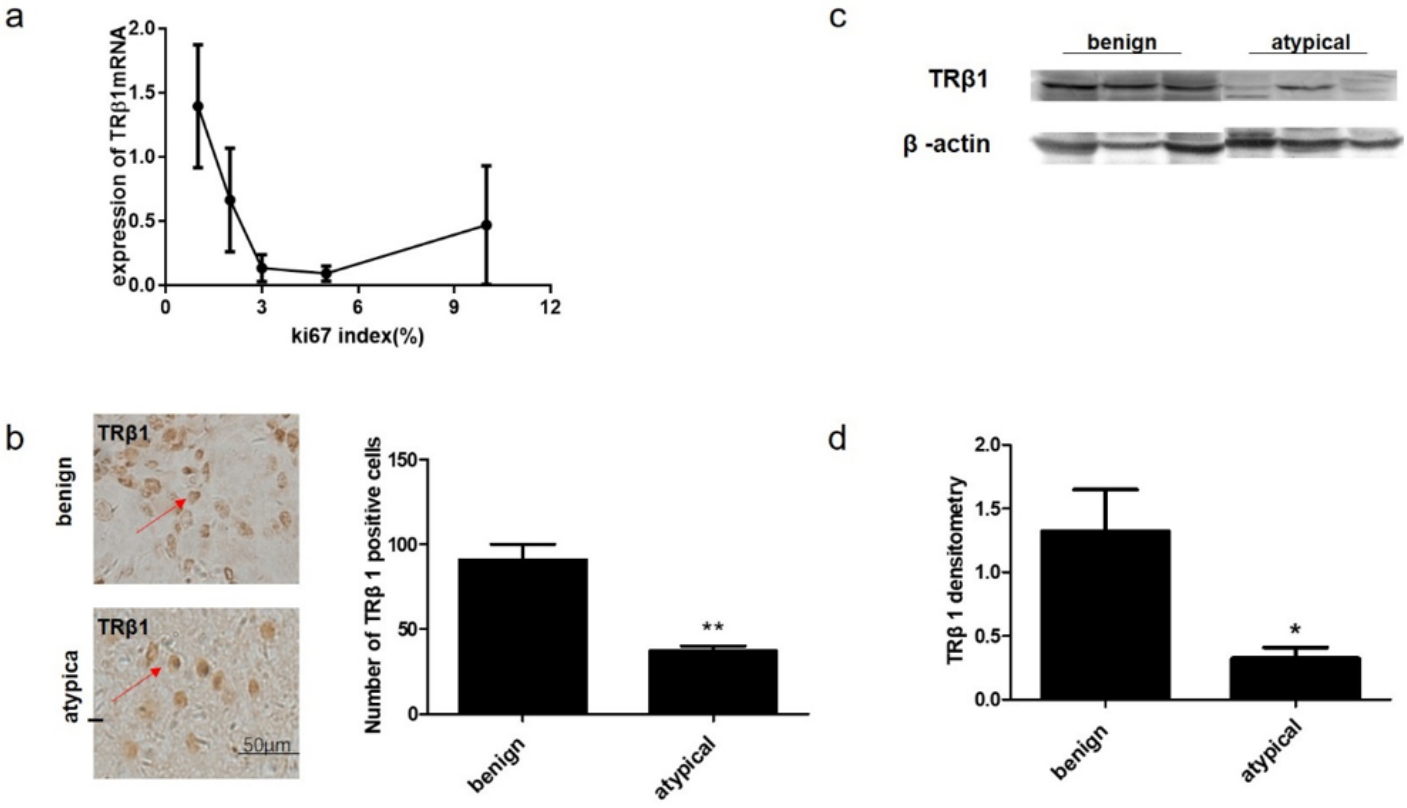

Figure 1

TRβ1 expression is negative correlated meningioma malignancy. a: The expression of TRβ1 mRNA in meningiomas is negative correlated with the ki67 index. b: The ratio of TRβ1 positive cells in atypical meningioma tissues was less than that in benign meningioma tissues（**P<0.01）. c: Representative

images of the TRβ1 protein in both atypical and benign meningiomas. d: Statistical comparison of TRβ1 protein expression in both atypical and benign meningiomas（*P<0.05）

Figure 2

T3 inhibited Atypicalatypical meningioma cell proliferation in vitro. a: Identification of primary meningioma cells with EMA and Vimentin staining. b: MTT assay showed that T3 suppressed atypical meningioma cell proliferation in a concentration-dependent manner (*P<0.05, **P<0.01, ***P<0.001). c: Representative images of EdU-positive cells treated with（right） or without T3（left）. d: Statistical comparison showed that T3 inhibited the proliferation of atypical meningioma cells (**P<0.01).

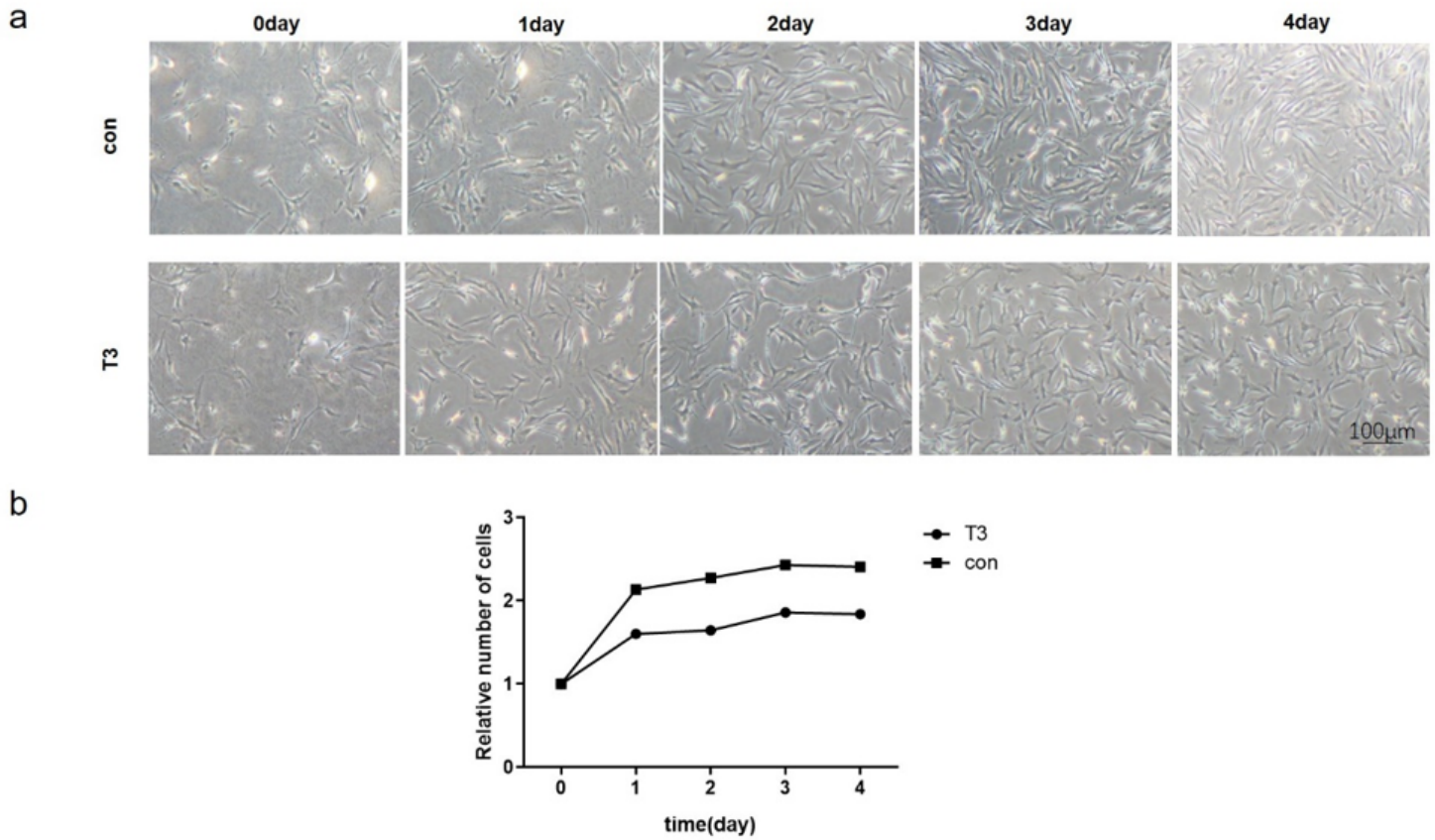

Figure 3

T3 inhibits the proliferation rate of atypical meningioma. a: Representative images of primary atypical meningioma cells treated with or without T3. b: Statistical comparison of relative number of cells showed that T3 treatment decreased the cells growth (Average cell proliferation rate :159±35%vs205±60%)

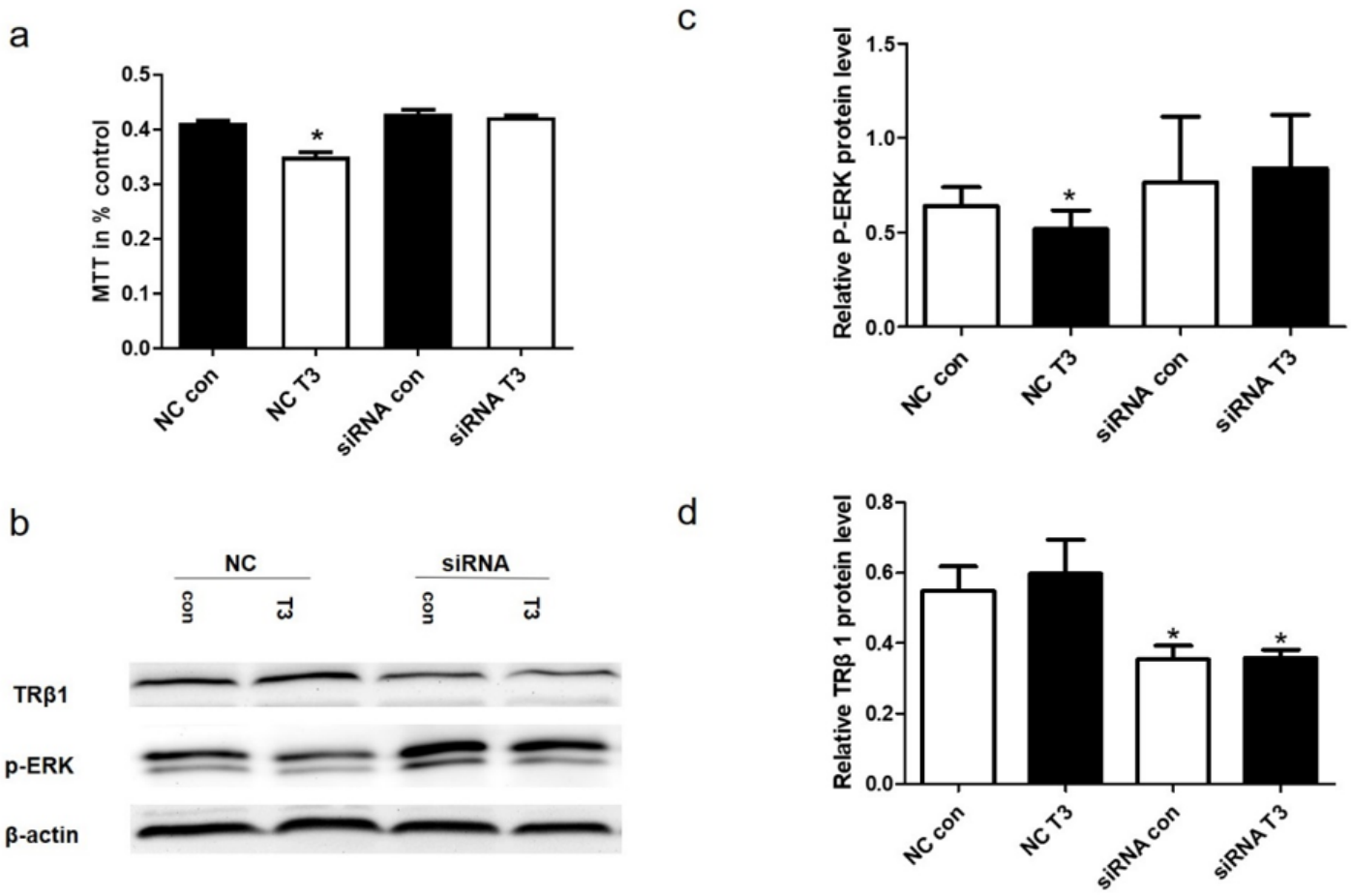

Figure 4

TRβ1 inhibits expression of p-ERK a: After knocked down TRβ1, the inhibitory effect of T3 on atypical meningioma cells is reduced (*P<0.05). b: Representative images of TRβ1 and p-ERK expression treated with or without T3 after siRNA treatment. c: Statistical comparison showed that TRβ1 depletion blocked T3-induced downregulation of p-ERK (*p<0.05). d: Statistical comparison showed that siRNA decreased the TRβ1 protein expression significantly (*p<0.05).