Biochemical and Biophysical Research Communications 496 (2018) 1322–1330

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

The melatonin-MT1 receptor axis modulates tumor growth in *PTEN*-mutated gliomas

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ARTICLE INFO

Article history:
Received 26 January 2018
Accepted 2 February 2018

Keywords:
Glioma
MT1
Melatonin
PTEN

ABSTRACT

More than 40% of glioma patients have tumors that harbor *PTEN* (phosphatase and tensin homologue deleted on chromosome ten) mutations; this disease is associated with poor therapeutic resistance and outcome. Such mutations are linked to increased cell survival and growth, decreased apoptosis, and drug resistance; thus, new therapeutic strategies focusing on inhibiting glioma tumorigenesis and progression are urgently needed. Melatonin, an indolamine produced and secreted predominantly by the pineal gland, mediates a variety of physiological functions and possesses antioxidant and antitumor properties. Here, we analyzed the relationship between PTEN and the inhibitory effect of melatonin in primary human glioma cells and cultured glioma cell lines. The results showed that melatonin can inhibit glioma cell growth both in culture and *in vivo*. This inhibition was associated with PTEN levels, which significantly correlated with the expression level of MT1 in patients. In fact, c-fos-mediated MT1 was shown to be a key modulator of the effect of melatonin on gliomas that harbor wild type *PTEN*. Taken together, these data suggest that melatonin-MT1 receptor complexes represent a potential target for the treatment of glioma.

1. Introduction

Glioma is the most common malignancy of the central nervous system, and has a high recurrence rate and poor prognosis [1–3]. This is because of the rapid and uncontrolled proliferation and high invasiveness of glioma cells [4–6]. Therefore, new therapeutic strategies focusing on inhibiting glioma tumorigenesis and progression are urgently needed.

Melatonin is an endogenously produced hormone secreted by the pineal gland and the retina [7,8]. Within the body, the highest concentration of melatonin is in the brain [9,10]. As reported, melatonin plays an important role in regulating various physiological processes such as circadian rhythms, body temperature, seasonal reproduction, and inflammatory responses [11,12]. Most importantly, the potential benefits of melatonin have been evaluated based on their inhibitory effects on many cancer types such as breast, colon, and gastric [13–15]. However, the inhibitory effect of melatonin on glioma cell proliferation is somewhat controversial. In 2006, Martín et al. reported that melatonin suppresses glioma cell proliferation both *in vitro* and *in vivo*, and this was related to its inhibitory effect on key intracellular effectors such as PKC and NF-kB [16]. However, other studies indicated that melatonin did not induce glioma cell death and affect viability [17,18]. These differences in results could be attributed to different cellular genetic backgrounds, which might affect melatonin inhibitory responses in glioma patients.

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https://doi.org/10.1016/j.bbrc.2018.02.010
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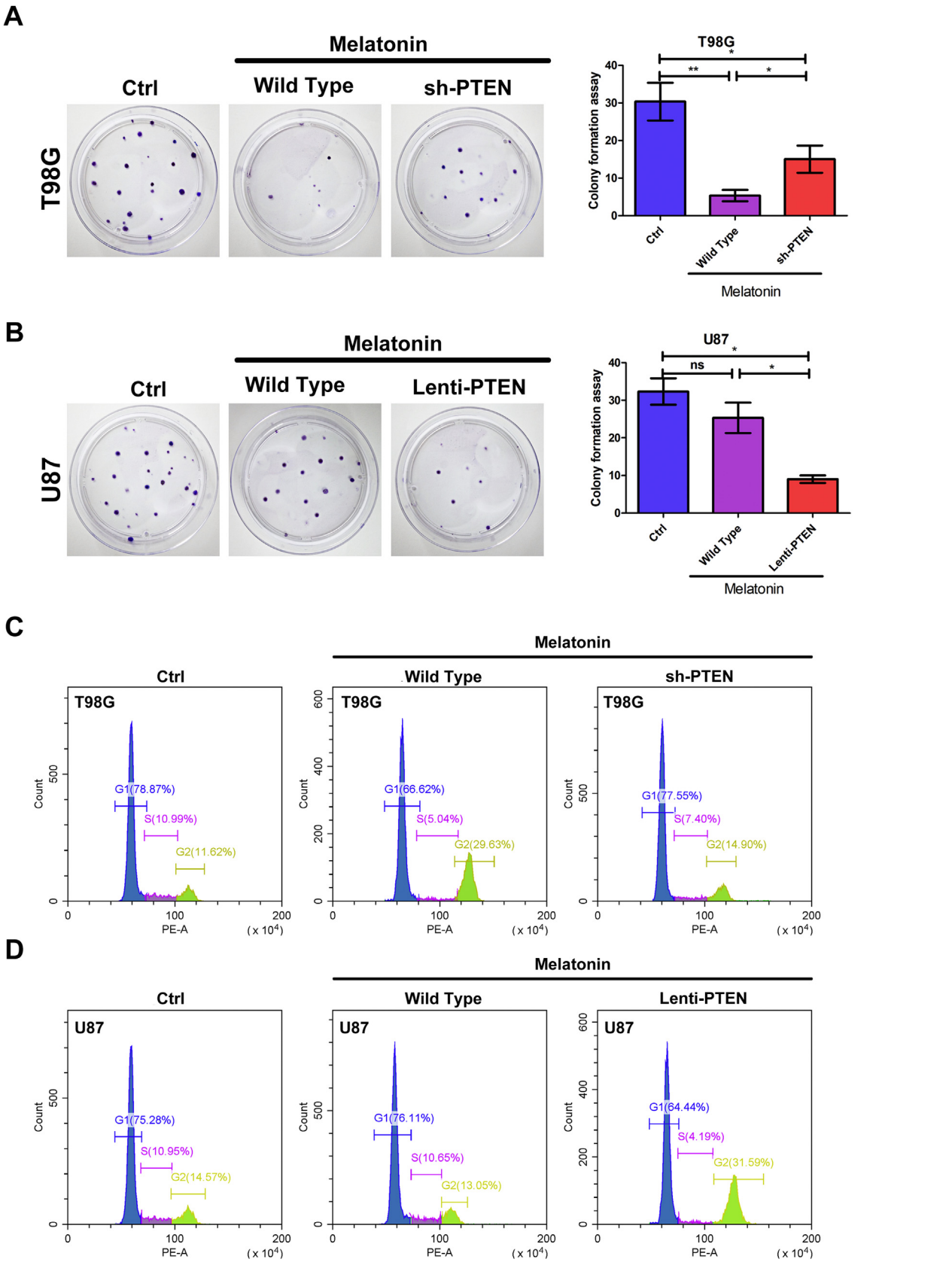

Fig. 1. Melatonin inhibited cell growth and induced G2/M arrest in PTEN-expressing glioma cells. (A) Clonal expansion analysis of PTEN^{WT} and sh-PTEN T98G cells with or without 48-hour melatonin (1 mM) treatment. (B) Clonal expansion analysis of PTEN-deficient and Lenti-PTEN U87 cells with or without 48-hour melatonin (1 mM) treatment. Data for each system are from at least three independent experiments. Data are shown as mean ± s.e.m. *$P < 0.05$; **$P < 0.01$; ns, not significant. (C) Cell cycle analysis of PTEN^{WT} and sh-PTEN T98G cells with or without 48-hour melatonin (1 mM) treatment. (D) Cell cycle analysis of PTEN-deficient and Lenti-PTEN U87 cells with or without 48-hour melatonin (1 mM) treatment.

Phosphatase and tensin homolog (PTEN) is a potent tumor suppressor for which loss-of-function mutations are often encountered in human cancers [19–21]. *PTEN* mutations are observed in 41–63% of glioma and are among the most frequent genetic alterations linked to glioblastoma multiforme [22,23]. Loss of PTEN function has been mechanistically linked to metastasis, in addition to lack of radio- and chemo-therapy response in brain and breast cancer patients, indicating that PTEN is a key regulator of tumor sensitivity to multiple therapeutic approaches [24–26]. Interestingly, several studies have reported that melatonin can affect the phosphorylation of PTEN, and associated signal transduction pathways [27,28]. Thus, the level and function of PTEN might be an important factor that modulates the inhibitory effect of melatonin on glioma; however, this is yet unclear.

To address this issue, we analyzed the relationship between PTEN and the inhibitory effect of melatonin, using human glioma primary cells and cell lines. We also identified a key modulator of this interaction with respect to the malignant progression of gliomas.

2. Materials and methods

2.1. Human glioma specimens and primary glioma cell culture

Human glioma tissue specimens were collected from 50 patients (ages 25–71) who underwent curative resection for glioma between 2013 and 2015 at Cancer Hospital of Hefei Institutes of Physical Science, Chinese Academy of Sciences (CAS), with Institutional Review Board approval. Within hours of surgical removal, tumors were broken down into single cells by trypsinization and transferred to Neurobasal medium (Thermo Scientific, Waltham, MA, USA) containing B27 supplements (Invitrogen, Grand Island, NY, USA) and 1% 100 × penicillin/streptomycin (Hyclone, Logan, UT, USA) for culturing. Trypan blue staining, followed by fluorescence-activated cell sorting (Beckman Coulter, Indianapolis, IN, USA) analysis were performed to evaluate cellular viability. All glioma cases were histologically confirmed by trained pathologists. No patients had received chemotherapy or radiotherapy prior to surgery, and informed consent was obtained from all patients.

2.2. Glioma cell line culture and reagents

Human glioma cell lines U87 ($PTEN^{-/-}$) and T98G ($PTEN^{WT}$) were obtained from the American Type Culture Collection and cultured in DMEM/F12. All culture media were supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 2 mM L-glutamine, and all cells were grown at 37 °C in a humidified incubator with 5% CO_2 atmosphere.

Melatonin (M5250, with a purity of ≥98% determined by high

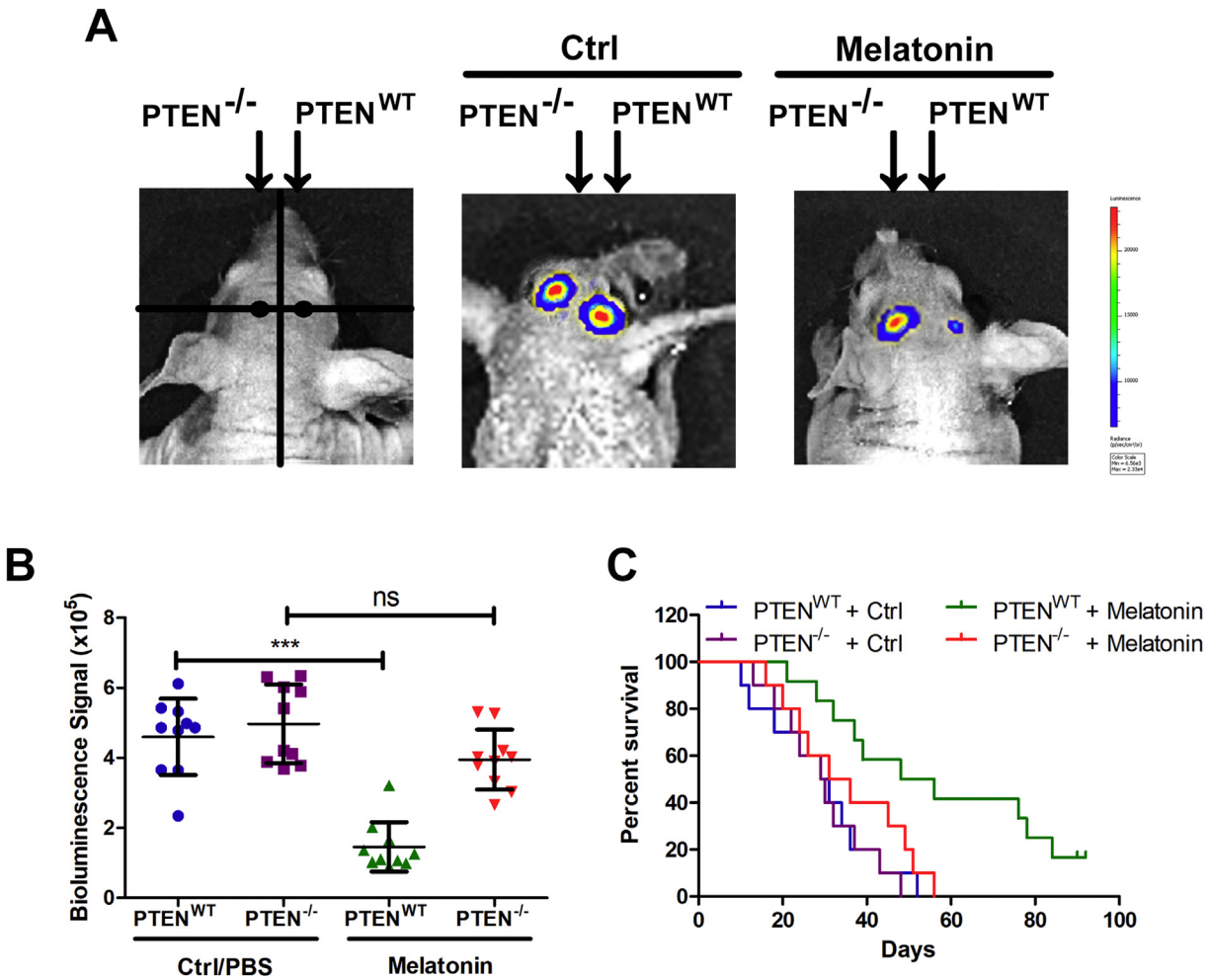

Fig. 2. Melatonin inhibited PTEN-expressing glioma growth in intracranial tumors mice. (A) Representative photon flux images from the brain of NOD/SCID mice implanted with luciferase-expressing $PTEN^{WT}$ (right) and $PTEN^{-/-}$ (left) T98G cells with or without melatonin. (B) Bioluminescence signal intensity in intracranial tumors was compared among the four experimental groups of $PTEN^{WT}$ (right) and $PTEN^{-/-}$ (left) T98G cells with or without melatonin. n = 10. Data are shown as mean ± s.e.m. ***$P < 0.001$; ns, not significant. (C) Kaplan–Meier survival curve showing the survival of all the animals injected with $PTEN^{WT}$ (right) and $PTEN^{-/-}$ (left) T98G with or without melatonin.

performance liquid chromatography; Sigma-Aldrich) was dissolved in ethanol (95%) to form a 0.1 g/mL stock solution and added to cells at 1 mM. Luzindole was also purchased from Sigma Chemical (St. Louis, MO) and added to cells at 50 μM. 2-iodo-melatonin was purchased from Tocris Bioscience (Ellisville, Missouri) and added to cells at 50 μM.

2.3. Cell growth inhibition assay (MTS assay)

Cells (1000/well in 96-well plates) were exposed to 1 mM melatonin. After 72 h, they were analyzed by the MTS viability assay according to the manufacturer's protocol (Cell Titer 96® AQueous One Solution Cell Proliferation Assay, Promega, Madison, WI, USA), and absorption was measured at 490 nm using a plate reader (Asys UVM340, Biochrom, Cambridge, UK). The IC_{50} value, indicating the concentration of melatonin resulting in 50% inhibition of cell growth, was calculated statistically by comparing to that of controls.

2.4. Xenograft tumor model

The methods were performed as previously described [29,30]. Before intracranial implant, T98Gs were transduced with lentivirus expressing luciferase. Then, 2 μL of a 2×10^6-cells/mL cell

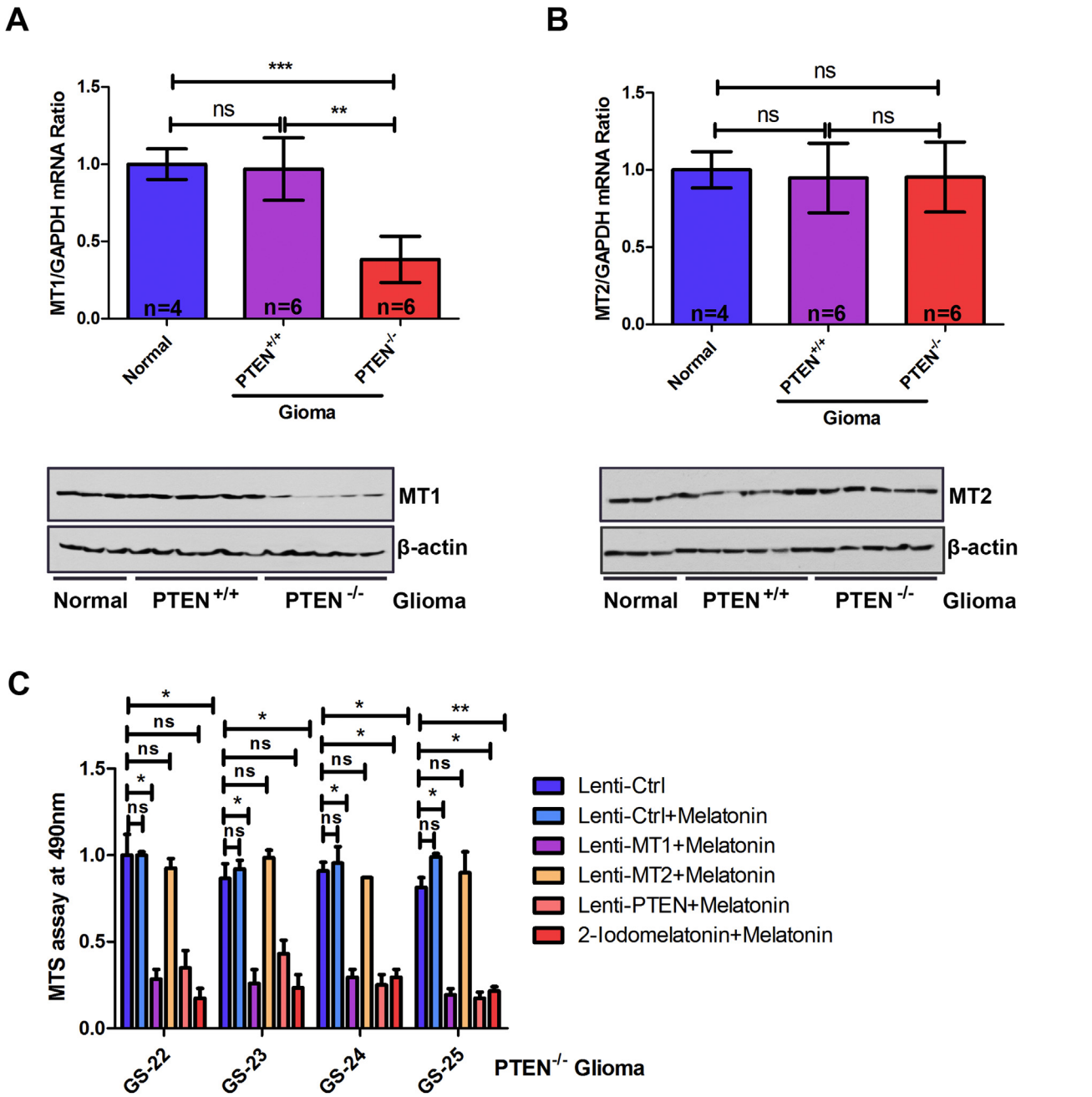

Fig. 3. MT1 but not MT2 receptor id depleted in PTEN-deficient gliomas. (A) The mRNA and protein expression of MT1 in normal brain tissues, PTEN-expressing gliomas and PTEN-deficient gliomas. (B) The mRNA and protein expression of MT2 in normal brain tissues, PTEN-expressing gliomas and PTEN-deficient gliomas. (C) The cellular viability of PTEN-deficient glioma cell transfected with Lenti-MT1, Lenti-MT2 or Lenti-PTEN with or without 48-hour melatonin (1 mM) or 2-Iodomelatonin (50μM) treatment. Data for each system are from at least three independent experiments. Data are shown as mean ± s.e.m. $^*P < 0.05$; $^{**}P < 0.01$; ns, not significant.

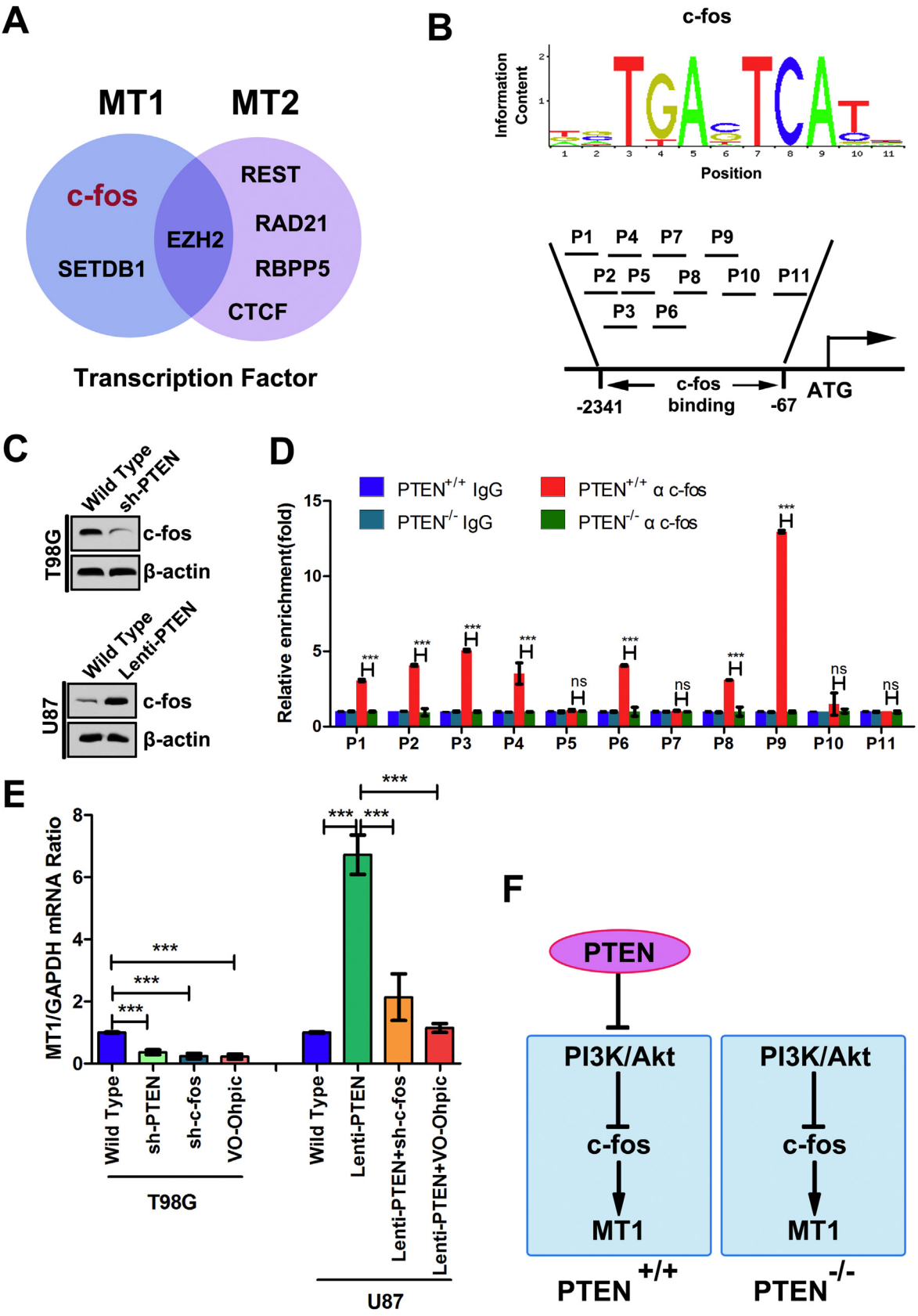

Fig. 4. PTEN upregulated MT1 transcription through inducing c-fos to binding to the MT1 promoter. (A) Bioinformatic analysis predicated that c-fos, SETDB1 and EZH2 are potential transcription factors for MT1 promoter, and REST, RAD21, RBPP5, CTCF and EZH2 are potential transcription factors for MT2 promoter. (B) Upper panel: Identification of CRE-containing genes in the human genome by the FIMO software tool. Lower panel: Bioinformatic analysis predicated that c-fos enrichment at the *MT1* promoter. P1–P11 indicate the different sub-regions. (C) Immunoblotting analysis of c-fos expression in T98G and U87 cells. β-Actin was used as loading control. (D) ChIP-qPCR analysis for c-fos enrichment at the MT1 promoter in T98G cells. P1–P11 indicates the different sub-regions. ***$P < 0.001$; ns, not significant. (E) The mRNA levels of MT1 in PTENWT, sh-PTEN, and sh-c-fos T98G

suspension in high-glucose PBS was injected into the hemi-striatum of immunocompromised NOD/SCI mice vie stereotactic injection. The following coordinate parameters were used: antero-posterio r = 0; medio-lateral = +2.5 mm; dorso-ventral = −3.5 mm. The mice were then divided into two groups (n = 10 per group). After 3 days, one group was treated with melatonin (20 mg/kg daily) in the late afternoon, whereas the other was treated with a vehicle (PBS). Approximately 6 weeks post-injection, luciferin was injected into the peritoneal cavity to trace tumor cells *in vivo*. Animals were anesthetized and bioluminescence imaging was performed using the IVIS Lumina system (Perkin-Elmer). Animal experiments were performed according to the guidelines of the Animal Use and Care Committees at Hefei Institutes of Physical Science, CAS.

2.5. Statistical analysis

Data (mean ± SD, 2–3 experiments) were analyzed for statistical significance by performing unpaired, two-tailed Student's t tests; $p < 0.05$ was considered to indicate statistical significance. We performed statistical analyses using GraphPad Prism software (San Diego, CA, USA). All cell-line experiments were repeated a minimum of three times, unless stated otherwise. Pearson's Chi square tests and Fisher's exact tests were performed to compare frequencies between groups. The Kaplan–Meier method was applied for survival analysis. Probability values were obtained from two-sided tests, with a statistical significance of $p < 0.05$.

3. Results

3.1. Melatonin inhibits viability of PTEN-expressing glioma cells

To study the role of melatonin in glioma cell biology, we derived primary glioma cell cultures from 50 unrelated human samples. Subsequently, primary cells were subjected to *in vitro* treatment with 1 mM melatonin, which was previously shown to be sufficient for disrupting the malignant progression of glioma cells [16]. After 72 h, viability of seven (3.5%) cultures was inhibited by 75%, 21 cultures (42%) by 50%, and 40 cultures (80%) by 25% (Suppl. Fig. 1A). We then compared radio- or chemo-therapy-related genes status and the inhibitory effect of melatonin in 50 primary glioma cell cultures. Inhibition was negatively correlated with *PTEN* gene mutations (deletion of PTEN protein; $\chi^2 = 20.06$, $p < 0.01$), but was not correlated with the status of other genes such as *p53*, *Rb*, and *IDH1* (Suppl. Tab. 1).

To further investigate the relationship between *PTEN* status and the inhibitory effect of melatonin, glioma cell lines T98G ($PTEN^{WT}$) and U87 ($PTEN^{-/-}$) were used. The results showed that melatonin significantly inhibited the viability of T98G cells, and this inhibitory effect was suppressed after *PTEN* knockdown (Suppl. Fig. 1B and C). Consistent with these results, melatonin had no obvious effect on $PTEN^{-/-}$ U87 cells, and introduction of PTEN into U87 partially improved the effect of melatonin (Suppl. Fig. 1B and C). Taken together, these *in vitro* experiments demonstrated that *PTEN* status influences the inhibitory effects of melatonin on glioma cell viability.

3.2. Melatonin Represses Tumor Growth in PTEN-expressing glioma cells

Next, we measured cell proliferation by a colony formation assay, and found that melatonin could significantly reduce colony growth; this was dependent on the presence of PTEN (Fig. 1A and B). To explore the anti-cancer mechanism of melatonin, we analyzed the cell cycle by flow cytometry. Our results revealed that melatonin induced G2/M phase arrest in *PTEN*-expressing glioma cells, and *PTEN*-deficient or -knockdown glioma cells showed no obvious effect in G2/M cell cycle distribution (Fig. 1C and D).

Moreover, we explored whether PTEN deficiency is linked to the effect of melatonin on glioma tumorigenesis. For this, $PTEN^{WT}$ and $PTEN^{-/-}$ T98G cells expressing luciferase were implanted into the brains of immunocompromised NOD/SCID mice. Mice were injected with 20 mg/kg daily in the late afternoon via the tail vein, whereas the control group was treated with a vehicle (PBS). *PTEN*-deficient cells were able to efficiently grow and form intracranial tumors *in vivo*, as evidenced by positive luminescence signals (Fig. 2A and B). Melatonin obviously suppressed tumor growth with $PTEN^{WT}$ cells (Fig. 2A and B). Consistent with these results, injection of $PTEN^{-/-}$ cells resulted in animal death within 45–55 days, with no benefit from melatonin treatment (Fig. 2C). In contrast, melatonin injection significantly extended the survival rate of all animals implanted with $PTEN^{WT}$ cells. These *in vivo* results suggested the direct involvement of melatonin in suppressing tumor growth of gliomas harboring wild type *PTEN*.

3.3. Melatonin-mediated inhibition of PTEN-expressing glioma growth requires the MT1/2 receptor

Melatonin is a ligand of several G protein-coupled receptors, and there are two mammalian isoforms of the melatonin receptor, MT1 and MT2, in brain and peripheral tissues [31,32]. $PTEN^{WT}$ glioma growth inhibition with melatonin could be mediated by a traditional ligand-receptor interaction, or by a combination of the two. To directly evaluate whether the effects of melatonin require receptor binding, we determined whether the receptor antagonist luzindole could counter melatonin-mediated inhibition of *PTEN*-expressing glioma growth. In the *in vitro* system, luzindole (a melatonin-receptor antagonist) almost completely mitigated the inhibitory effect of melatonin on $PTEN^{WT}$ glioma cell growth, indicative of a receptor-mediated effect (Suppl. Fig. 2A). Notably, 2-iodomelatonin (a melatonin-receptor agonist) partially improved the inhibitory effect of melatonin on $PTEN^{-/-}$ glioma cells (Suppl. Fig. 2B). In glioma cell lines, the results also confirmed that melatonin-mediated T98G and U87 cell growth inhibition requires the MT1/2 receptor (Suppl. Fig. 2C and D). These results suggested that differences in the effect of melatonin on $PTEN^{WT}$ and $PTEN^{-/-}$ glioma cell growth might be attributed to the different levels of the melatonin receptors MT1/2.

3.4. PTEN-deficient Glioma Cells Show Selectively Downregulation of the MT1 receptor

We next investigated whether PTEN levels correlate with changes in the expression of melatonin receptors (MT1, MT2, or both). We found that levels of MT1 mRNA and protein were significantly lower in $PTEN^{-/-}$ glioma cells, compared to that in $PTEN^{WT}$ gliomas or normal brain tissues (Fig. 3A). However, in the same tissues, the level of MT2 mRNA or protein was not altered (Fig. 3B). In $PTEN^{-/-}$ glioma cells, re-introduction of MT1 or PTEN improved the inhibitory effect of melatonin on cellular viability (Fig. 3C), similar to that observed with 2-iodomelatonin. In contrast, transfection with MT2 had no obvious effect on

cells with or without PTEN inhibitor VO-Ohpic trihydrate (VO-Ohpic), and in PTEN-deficient and Lenti-PTEN U87 cells with or without transfected with sh-c-fos or VO-Ohpic treatment were analyzed by RT-PCR. Data for each system are from at least three independent experiments. Data are shown as mean ± s.e.m. ***$P < 0.001$. (F) In $PTEN^{WT}$ cells, PTEN upregulated MT1 transcription through antagonizing PI3K/Akt to induce c-fos expression. In PTEN-deficient cells, c-fos and MT1 expression would be downregulated.

melatonin-mediated inhibition of *PTEN*$^{-/-}$ glioma cells.

In glioma cell lines T98G and U87, the MT1 receptor was less abundant with lower expression of PTEN (Suppl. Fig. 3A and B). In contrast, levels of the MT2 receptor were unchanged. As observed at the protein level, *MT1* mRNA was downregulated in *PTEN*-knockdown T98G or *PTEN*-deficient U87 cells, compared to levels in *PTEN*^{WT} T98G or *PTEN*-expressing U87 cells (Suppl. Fig. 3C and D). Remarkably, treating *PTEN*-knockdown T98G or *PTEN*-deficient U87 cells with melatonin rescued the loss of MT1 receptor mRNA (Suppl. Fig. 3A and B), although MT1 mRNA was also upregulated in *PTEN*^{WT} T98G or *PTEN*-expressing U87 cells. This increase in MT1 mRNA level was also reflected by a significant increase in MT1 protein (Suppl. Fig. 3C and D).

We also analyzed the relationship between PTEN and MT1 or MT2 expression in 50 glioma samples. Our analysis showed that MT1 levels significantly correlated with the expression of PTEN in patients (Cor = 0.4508, $P = 0.001$; Suppl. Fig.3E). In contrast, no significant association between MT2 and PTEN was observed (Cor = 0.1508, $P = 0.2957$; Suppl. Fig.3F). These data strongly suggest that MT1 is a significant and independent mediator of glioma growth in PTEN^{WT} cells, and that MT1 is a key modulator of the effect of melatonin on PTEN^{WT} gliomas.

3.5. PTEN upregulated MT1 transcription through inducing c-fos to binding to the MT1 promoter

PTEN associates with chromatin DNA and its signaling is involved gene transcription regulation [33,34], we thus reason that PTEN may induce MT1 transcription through regulating its transcription factors for MT1 promoter. Bioinformatic analysis predicated that c-fos, SETDB1 and EZH2 are potential transcription factors for MT1 promoter, and REST, RAD21, RBPP5, CTCF and EZH2 are potential transcription factors for MT2 promoter (Fig. 4A). Of note, c-fos is a MT1-own factor, and PTEN could regulate c-fos expression in human glioma cells via PI3-kinase/Akt pathway [35]. Bioinformatics analysis predicted several binding sites at MT1 promoter for transcription factor c-fos (Fig. 4B). Thus, we hypothesized that interplay between PTEN and c-fos account for the PTEN-mediated transcriptional repression of MT1. Supporting this hypothesis, *PTEN*-deficient gliomas demonstrated downregulated levels of c-fos (Fig. 4C). As for *MT1* gene, ChIP-quantitative-PCR showed that c-fos occupied within the 2244-bp DNA region at the *MT1* promoter in PTEN^{WT} glioma cells (Fig. 4D). Moreover, depletion of *PTEN* in glioma cells resulted in a downregulated occupation of c-fos at the *PAX7* promoter (Fig. 4D). Consistent with these results, knockdown of PTEN or c-fos, or treatment of PTEN inhibitor, VO-Ohpic would suppress the MT1 transcription in T98G cells (Fig. 4E). In contrast, induction of PTEN would increase the MT1 expression, and re-repression of c-fos or treatment with VO-Ohpic would inhibit the MT1 expression again (Fig. 4E). These results support a model that PTEN led to induction of c-fos machinery at the *MT1* promoter, resulting in activation of *MT1* transcription (Fig. 4F).

We also analyzed the role c-fos-mediated MT1 in glioma tumor growth (Suppl. Fig. 4). The results showed that melatonin significantly inhibited the viability of T98G cells, and this inhibitory effect was suppressed after *PTEN*, *c-fos* or *MT1* knockdown. Moreover, re-introduction of c-fos or MT1 partially improved the effect of melatonin, but MT2 had not this effect. Consistent with these results, melatonin had no obvious effect on *PTEN*$^{-/-}$ U87 cells, and introduction of *PTEN* into U87 partially improved the effect of melatonin. But, re-repression of c-fos or MT1 would reduce the effect of melatonin. These data strongly suggest that c-fos mediated MT1 was a significant and independent factor for melatonin suppressing PTEN^{WT} glioma tumor growth.

4. Discussion

In the present study, we showed that the antioxidant indolamine melatonin can inhibit glioma cell growth in both cell culture and *in vivo*. This inhibition was associated with the level of PTEN, which significantly correlated with the expression of MT1 in patients. c-fos mediated MT1 was shown to be a key modulator of the effect of melatonin on PTEN-mediated glioma. Taken together, these data suggested that melatonin-MT1 receptor complexes could represent a potential target for the treatment of glioma.

Melatonin, an indolamine produced and secreted predominantly by the pineal gland, mediates a variety of physiological functions and possesses antioxidant and antitumor properties [11,13,15]. Regarding its anticancer properties, melatonin was shown to inhibit the development and/or growth of various experimental animal glioma tumors and some human glioma cell lines *in vitro* [16–18]. These results also suggested that the mechanism through which melatonin regulates cell growth is affected by cell type, as well as the duration and the concentration of melatonin. In our study, we found that differences in the effect of melatonin on glioma cells might be attributed to the different levels of PTEN. *PTEN*-expressing glioma cells are more sensitive than *PTEN*-deficient cells. In fact, melatonin was shown to inhibit viability and tumor growth and induce G2/M phase arrest in *PTEN*-expressing glioma cells; however, *PTEN*-deficiency or knockdown resulted in no obvious effect. Primary glioma frequently harbors loss-of-function mutations in the *PTEN* gene, and more than 40% of glioma patients have tumors with mutant *PTEN*. Loss-of-function mutations in *PTEN* result in the aberrant function of the phosphatidylinositol-3′-kinase (PI3K)/Akt signaling pathway, loss of cell-cycle control and uncontrolled cell proliferation, escape from apoptosis, brain invasion, and aberrant neoangiogenesis [36–39]. These processes might be the reason that *PTEN*-deficient gliomas are less responsive to melatonin.

Physiologically, melatonin is secreted at low (nanomolar) concentrations, and has been shown to be an effective antioxidant, free radical scavenger, and regulator of antioxidant genes [40–42]. In addition to being a broad-spectrum antioxidant, melatonin is a ligand of several G protein-coupled receptors [43,44]. There are two mammalian isoforms of the melatonin receptor, MT1 and MT2. MT1 and MT2 can be found in brain and peripheral tissues [32,45]. In this study, we found that differences in the effect of melatonin on *PTEN*-expressing and *PTEN*-deficient glioma cells might be attributed to different levels MT1. This receptor was shown to be less abundant in *PTEN*-deficient glioma cells than in *PTEN*-expressing cells. In contrast, levels of MT2 receptors were unchanged. Analysis of a glioma database showed that MT1 levels significantly correlate with PTEN expression in patients. Notably, treating *PTEN*-knockdown or -deficient glioma cells with melatonin countered the loss of MT1 receptor mRNA. This increase in MT1 mRNA level was also reflected by the fact that the regulation of cell growth by melatonin is affected by the concentration and duration of treatment.

The PTEN tumor suppressor gene modulates MT1 expression and cell survival through mechanisms that are incompletely understood. In this study, we investigated the possibility that PTEN mediates MT1 transcription through modulation of transcription factor c-fos, which was dependent on PI3kinase activity. Consistent with a induction in the MT1 levels, an c-fos dependent reporter gene was positively induced in the PTEN expressing cell lines. The aberrant expression of PTEN contributes to the activation of the PI3kinase/Akt pathway and its transcription factor mediators in glioma. In PTEN^{WT} cells, PTEN upregulated MT1 transcription through antagonizing PI3K/Akt to induce c-fos expression. In *PTEN*-deficient cells, c-fos and MT1 expression would be downregulated.

In conclusion, we have demonstrated that therapies that target

melatonin-MT1 receptor complexes could provide a potential avenue for suppressing glioma tumor growth. MT1 is a significant and independent factor of PTENWT glioma growth, and MT1 is a key modulator of this effect. The low cytotoxicity of melatonin and its ability to cross the blood-brain barrier suggest that it is a potentially useful compound for the treatment of PTEN-expressing glioma. In addition, 2-iodomelatonin, an agonist of MT1 and MT2, could improve the inhibitory effect of melatonin on PTEN-deficient glioma cells. Thus, the combination of melatonin and 2-iodomelatonin could provide a new therapeutic strategy to treat gliomas.

Conflicts of interest

The authors of this paper declare that they have no conflicts of interest related to this work.

Acknowledgments

The authors thank the Center of Medical Physics and Technology, Hefei Institutes of Physical Science, Chinese Academy of Sciences for technical assistance and Dr. Aijun Hao at Shandong University School of Medicine for providing glioma cell lines. This work was supported by grants from the National Natural Science Foundation of China (nos. 31501171, 31571433, and 81773131), Anhui Provincial Natural Science Foundation (nos. 1608085MH180 and 1508085SMC214) and Youth Innovation Promotion Association of Chinese Academy of Sciences.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.bbrc.2018.02.010.

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