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Circadian gene Clock contributes to cell proliferation and migration of glioma and is directly regulated by tumor-suppressive miR-124

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ABSTRACT

Although the roles of circadian Clock genes and microRNAs in tumorigenesis have been profoundly studied, mechanisms of cross-talk between them in regulation of gliomagenesis are poorly understood. Here we show that the expression level of CLOCK is significantly increased in high-grade human glioma tissues and glioblastoma cell lines. In contrast miR-124 is attenuated in similar samples. Further studies show that Clock is a direct target of miR-124, and either restoration of miR-124 or silencing of CLOCK can reduce the activation of NF-κB. In conclusion, we suggest that as a target of glioma suppressor miR-124, CLOCK positively regulates glioma proliferation and migration by reinforcing NF-κB activity.

1. Introduction

In the living organisms, there exists circadian rhythm with a day/night alternating pattern. The circadian rhythm serves as an important regulatory system apart from the nervous, humoral and immune systems, and keeps the organisms in normal biological status. Disruption of the circadian rhythm may negatively affect cellular function, potentially leading to increased susceptibility to diseases, including cancers [1]. In normal circumstances, the core circadian genes, including Bmal1, Clock, Period (Per1, Per2, Per3), Cryptochrome (Cry1, Cry2), casein kinase Iε (CKIε), work in accurate feedback loops and keep the molecular clockworks in the hypothalamic suprachiasmatic nucleus (SCN) and controlling peripheral clocks [2,3]. The levels of mRNAs and proteins of circadian genes always oscillate throughout the 24 h period, with exceptions of Clock and CKIε [4]. Maywood et al. have also demonstrated the constitutive expression of mCLOCK as a nuclear antigen in the SCN [5]. It has been shown that up to 10% of all genes in the mammalian genome are under the regulation of circadian genes [6]. As the molecular clockworks regulate the gene expression related to cell cycle, apoptosis and other pathways in cells, aberration of circadian genes could conceivably result in deregulation of these processes and tumor development [7]. For example, the expression of all three Per genes is deregulated in breast cancer cells [8]. Targeted ablation of Per2 leads to the development of malignant lymphomas [9]. Breast tissue from healthy controls had significantly lower CLOCK gene expression than all breast tissue from patients with breast cancer, including adjacent normal tissue [1]. There are also evidences that circadian clock involves in gliomagenesis. The expression levels of Per1, Per2 and Cry1, Cry2 in glioma tissues were much lower than that in the adjacent normal tissues [10,11]. The expression of CLOCK in high-grade gliomas was found to be significantly higher than that of the low-grade gliomas and non-gliomas by RT-PCR and immunohistochemistry [12]. However, the roles of CLOCK in gliomagenesis remain totally unknown.

MiRNAs are small non-coding RNAs that play essential roles in post-transcriptional regulation of gene expression [13,14], and participate in many biological processes including cell proliferation, survival, and tumor angiogenesis, invasion, metastasis of cancers. They act either as tumor suppressor genes or oncogenes [15–17]. And alterations of miRNAs have also been reported in the initiation and progression of gliomas [18–22]. MiR-124 was frequently reported to be downregulated in glioma and play a glioma suppressive role by targeting specific genes such as Twist, Slug, Snai2, while miR-124 restoration inhibited glioblastoma cell proliferation and migration [23–26].

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In this study, we found that the core circadian clock gene, Clock, is up-regulated in glioma tissues and cell lines, consistent with an earlier study [12], and is related to the proliferation, survival, and migration of glioma cell. We also found that miR-124 can repress the expression of Clock by directly targeting its 3′ untranslated regions (3′UTR), and then we inferred that the attenuation of miR-124 may contribute to the high CLOCK protein level in high grade glioma tissue and cell lines. Furthermore, the clues indicated that restoration of miR-124 or silencing of CLOCK in glioma cell reduced the NF-κB activity, which implies a potential miR-124-CLOCK-NF-κB axial relationship in gliomagenesis.

2. Materials and methods

2.1. Samples and cell lines

All normal human brain tissue and glioma samples were obtained from the Department of Neurosurgery, Beijing Tiantan Hospital. All human materials were used in accordance with the policies of the institutional review board of Beijing Tiantan Hospital. Glioma cell lines U87MG, T98G, A172, U251 and normal astrocyte cell lines HASP, HEB were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan City, Utah).

2.2. Plasmids

The CLOCK-pcDNA3.1 plasmid is used in our lab earlier [24]. For the luciferase reporter assay, the 3′UTR of Clock (from nt 1 to nt 2019, with NotI/XhoI sites), and for the miR-124 target mutation experiment, the wild type of 3′UTR of Clock (from nt 930 to nt 1601, with XhoI/XbaI sites) were amplified from the cDNA of T98G cells. For mutagenesis, the miR-124 binding site was replaced with a random sequence by bridge PCR. Then the PCR products were cloned into the firefly luciferase reporter vector pcDNA3.1-Luc. The primers for plasmids construction are listed in Table S1. For NF-κB activity assay, sequences of four NF-κB target core site GGGAATTTCC (GGGRNNYYCC, R: purine, Y: pyrimidine, N: any nucleotide) in series were synthesized from Invitrogen (Beijing, China) and inserted into the BglII/HindIII sites of pGL3-basic plasmid.

2.3. siRNA and miRNA

The double-stranded siRNAs targeting human Clock and siRNA control, MiR-124 mimics and inhibitors, negative control or independent control mimics and inhibitors were commercially synthesized by GenePharma (Shanghai, China). The siRNAs and miRNA mimics/inhibiors are listed in Table S2. All the transfections were performed using the Lipofectamine 2000 reagent (Invitrogen, Shanghai, China) according to the manufacturer's instruction.

2.4. Growth, survival, colony formation

Cell growth was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) standard method. For survival assay, 24 h after transfection, the cells were maintained in serum-free medium, and the surviving cell number was determined using the MTT method. For colony formation assays, transfected cells were plated in 12-well plates at 200 cells per well. 10 days later, the cell colonies were stained and counted.

2.5. Transwell migration assays and wound healing migration

For transwell migration assays and wound-healing assay, T98G and U87MG cells were transfected with CLOCK siRNA or negative control (NC) and transwell migration assays were performed as described previously with small modification [24].

2.6. Luciferase assay

Dual-Luciferase reporter assays were performed according to the manufacturer's instructions (Promega, Madison, WI) as previously described [24].

2.7. RNA extraction and real-time quantitative PCR

Total RNA extraction, reverse transcription, and real-time PCR were performed as described previously [24]. The GAPDH mRNA was used as an internal control for normalization. Primers are listed in Supplementary Table S3.

2.8. Western blotting

Western blotting was performed as described previously [24]. The antibodies used include anti-CLOCK (Calbiochem, GER), anti-human β-actin antibodies (Sigma, USA), anti-CyclinD1 (Abcam, UK), anti-Vimentin, anti-E2F3a, anti-c-Myc (Santa Cruz Biotechnology, USA), anti-Bmi1, anti-p-p65 (Cell Signaling Technology, MA, USA).

2.9. Statistics

Data are expressed as mean ± S.D. Student's *t* test was used for analysis and *P* values <0.05 were considered significant.

2.10. Computational prediction

Two target prediction databases (TargetScan and PicTar) were used to predict the interaction between the miRNAs and the Clock 3′UTR.

3. Results

3.1. CLOCK is up-regulated in glioma tissues and cell lines

We first measured the protein level of CLOCK in 16 glioma tissues and two normal brain tissues and found that CLOCK expressed in glioma tissues of grade III and grade IV is higher comparing with normal brain tissues and grade II glioma tissues (Fig. 1A). An increase of CLOCK protein was also found in glioma cell lines U87MG, T98G, A172, and U251 compared to the human normal glial cell lines, HASP and HEB (Fig. 1B). The up-regulation of CLOCK

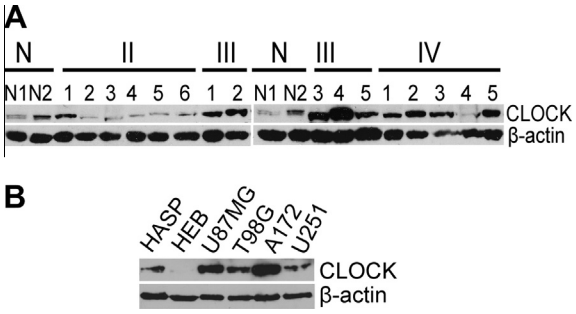

Fig. 1. CLOCK is up-regulated in both tissues and cell lines of glioma. (A) Western blotting analysis was used to detect CLOCK in normal brain tissues and glioma tissues. N: normal brain tissue; II–IV: grade II/III/IV glioma tissues. (B) Western blotting analysis was used to detect CLOCK in human normal glial cell lines (HASP, HEB) as well as in glioma cell lines (U87MG, T98G, A172 and U251).

in glioma suggests that CLOCK may have an important role in gliomagenesis.

3.2. Knocking-down CLOCK suppresses glioma cell growth, survival, and migration

It has been reported that the circadian clock can regulate the cell cycle, DNA damage responses, ageing and metabolism and alterations in clock function could lead to aberrant cellular proliferation [27]. To study the function of CLOCK in glioma cell growth and survival, we performed a loss-of-function analysis using CLOCK-targeting siRNA oligos (siCLOCK) in U87MG and T98G cells. CLOCK knockdown was first verified by Western blotting. Since siCLOCK2 is more efficient than siCLOCK1 (Fig. S1), siCLOCK2 was used for functional assays in Fig. 2. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT) assays showed that the reduction of CLOCK slowed the growth of glioma cells significantly and made them more sensitive to serum starvation than controls. The data showed that the growth of U87MG and T98G with 10% FBS medium were down-regulated by about 17% and 9.4%, respectively (siCLOCK vs. siNC), while with 0% FBS medium were down-regulated by about 29% and 30% respectively (siCLOCK vs. siNC) (Fig. 2A and B). In addition, the reduction of CLOCK decreased the colony formation ability of T98G cells (Fig. 2C). Thus, up-regulated CLOCK may have important roles in facilitating glioma cell growth, survival and colony formation. To determine the effect of CLOCK on glioma cell migration, we conducted Scratch Wound Healing Migration and Transwell Migration assays. The results indicated that depletion of CLOCK suppressed the migration of T98G and U87MG cells, in other words, CLOCK promoted migration of glioma cells. (Figs. 2D and S2).

3.3. Attenuation of miR-124 might contribute to high CLOCK expression in glioma cells

Using two algorithmic methods (TargetScan and PicTar) in common use, miR-124 and miR-181b were predicted to putatively bind to the 3′UTR of Clock (Fig. 3A and B). Previous study in our lab indicated that miR-124 is significantly lower in glioma cells (U87MG, T98G, A172, U251) than normal astrocyte HEB [28]. To validate the potential interaction between Clock 3′UTR and miR-124 or miR-181b, we adopted luciferase reporter assays and found that miR-124 but not miR-181b or miR-128 could significantly repress the luciferase activity of Clock 3′UTR in glioma cell T98G (Fig. 3C), and this effect were absent in normal glial cell HEB (Fig. 3D). We also confirmed the CLOCK protein repression by miR-124 in two glioma cell lines (U87MG and T98G). Moreover, the inhibition of miR-124 increased the CLOCK expression (Fig. 3E). The above results suggested that CLOCK was a downstream target of miR-124.

To identify whether the suppressive effect of miR-124 on Clock expression is direct, we fused wild type (WT) and mutated (MT) 3′UTR of miR-124 target site of Clock to luciferase reporter vector pcDNA3.1-LUC. The luciferase reporter assays revealed that the luciferase activities were significantly reduced at the presence of

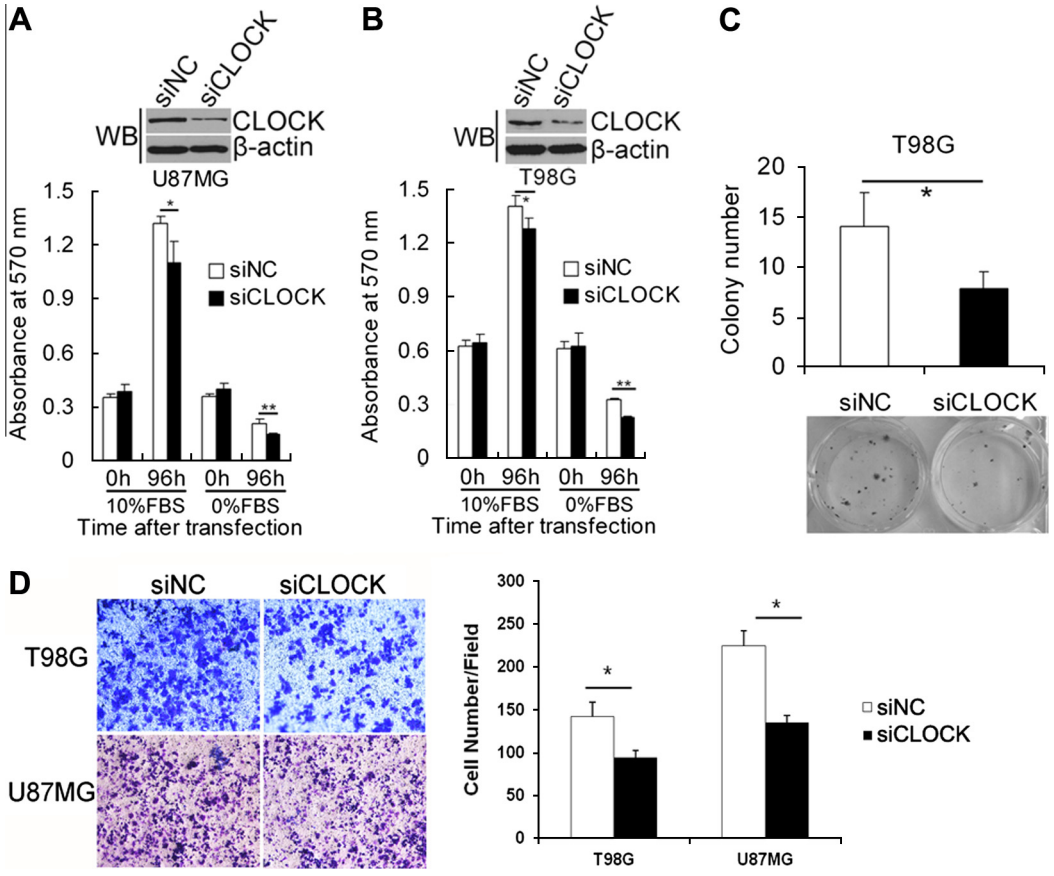

Fig. 2. Knocking down CLOCK suppresses glioma cell growth and migration. (A and B) MTT assays were performed to investigate the effect of CLOCK knockdown on the growth and survival of glioma cells U87MG (A) and T98G (B) cells. Data are represented as mean ± S.D., $n = 4$. On the top are the Western blotting results showing the CLOCK protein levels of the U87MG and T98G cells transfected with siNC or siCLOCK (siCLOCK2). (C) Colony formation assays were utilized to test the effect of knocking-down CLOCK on the colony formation ability of T98G. Data are represented as mean ± S.D., $n = 4$. (D) Cell migration was determined after transfection with siNC or siCLOCK (siCLOCK2) in T98G and U87MG cells. Photos were taken under inverse microscope (Nikon, Eclipse, TE2000-U), 10×. Data are represented as mean ± S.D., $n = 3$. $^{*}P < 0.05$; $^{**}P < 0.01$. SiCLOCK2 was used here for knockdown; NC: negative control.

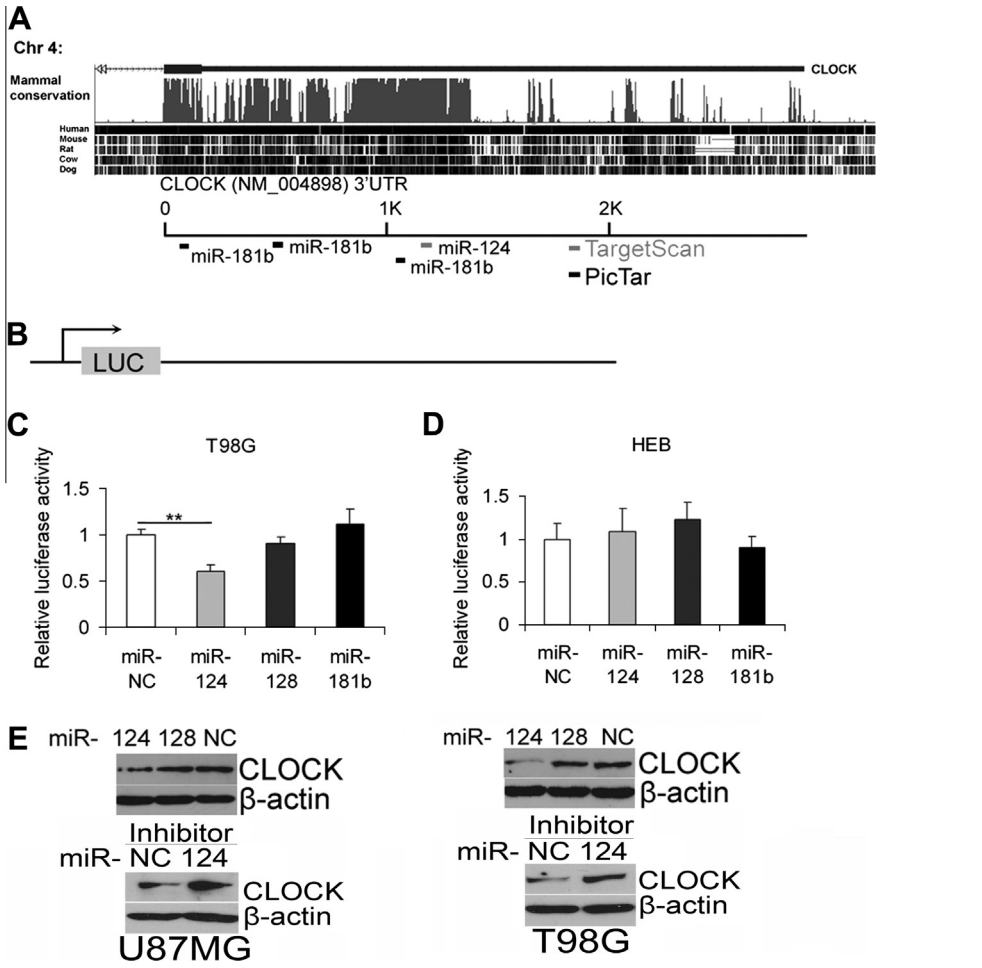

Fig. 3. MiR-124 inhibits the expression of CLOCK. (A) The 3′UTR of Clock gene is conserved in multiple species and harbors predicted miR-124 and miR-181b binding within the first 2 kb region. (B) The first 2010 bp of Clock 3′UTR containing miR-124 and miR-181b binding sites were cloned downstream of luciferase reporter gene cassette (gray box). (C) Luciferase reporters were cotransfected with synthetic miRNA mimics (miR-124, miR-128 and miR-181b or control miRNA) into T98G cells. Forty-eight hours later, the normalized luciferase activity was determined (mean ± S.D., $n = 4$). $^{**}P < 0.01$. (D) Luciferase reporters were cotransfected with synthetic miRNA mimics (miR-124, miR-128 and miR-181b or control miRNA) into HEB cells. Forty-eight hours later, the normalized luciferase activity was determined (mean ± S.D., $n = 4$). (E) U87MG or T98G cells were transfected with synthetic mimics of control, miR-124, miR-128 or inhibitors of control, miR-124. Forty-eight hours later the whole cellular proteins were extracted and the protein level of CLOCK was detected by Western blotting.

the wild type 3′UTRs and the repression effect could be abolished by mutations in seed complementary site. Based on the mutation experiments, we came to the conclusion that miR-124 could directly target Clock 3′UTR. Taken together, our results indicated that down-regulation of miR-124 contributed to high CLOCK expression in glioma cells (Fig. 4).

3.4. MiR-124 modulates NF-κB activity by regulating CLOCK

We have shown that CLOCK promotes the cell growth, survival and migration of glioma cells. Spengler et al. found that CLOCK can bind to p65 as a complex for up-regulating NF-κB-mediated transcription in the absence of BMAL1, and indicated that its modulation of NF-κB activity may occur through increasing specific phosphorylated and acetylated active forms of p65, but not its role of traditional circadian control [29]. And it has been shown that NF-κB pathway plays a role in cellular transformation and tumorigenesis by up-regulating the expression of some antiapoptosis genes [30–32]. However, little is known regarding its role in the pathophysiology of glial cells. In order to explore whether miR-124 cooperating with CLOCK affects NF-κB activity in glioma, we inserted NF-κB target core site sequences into luciferase reporter vector pGL3-basic. In the luciferase reporter assays, we found that

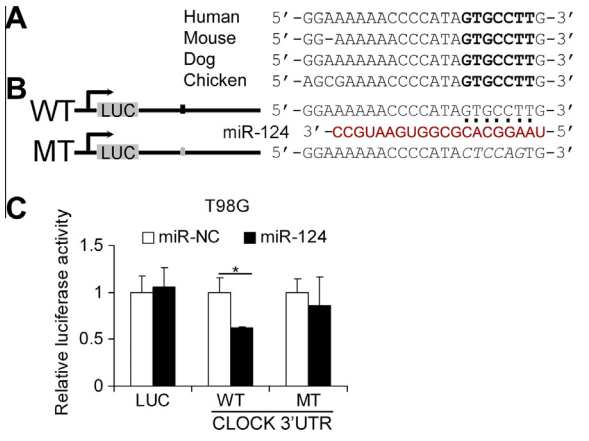

Fig. 4. MiR-124 directly targets Clock 3′UTR. (A) The miR-124 targeting sequence in 3′UTR of Clock is conserved. (B) Either a wild-type (WT) or a mutated (MT) Clock 3′UTR was cloned downstream of the luciferase reporter gene, the mutated nucleotides were shown in italic. (C) The interactions between miR-124 and the 3′UTRs of Clock were tested by luciferase reporter assays. The normalized luciferase activity is expressed as the mean ± S.D., $n = 4$. $^{*}P < 0.05$.

CLOCK knockdown and miR-124 over-expression in T98G could down-regulate the NF-κB activity. Additionally, ectopically expressing CLOCK could reverse the repression effect of miR-124 on NF-kB activity (Fig. 5A). Furthermore, transfecting miR-124 mimics or CLOCK siRNA into T98G both could lead to down-regulated mRNA expression of NF-κB target genes (Fig. 5B). And the protein level of five NF-κB target genes: Bmi1, C-Myc, Vimentin, CyclinD1, and E2f3a, which play key roles in cell cycle, apoptosis, cellular transformation, were also verified at protein level after knocking down CLOCK in T98G, the results indicated that the five genes were down-regulated while CLOCK and activated p65 (p-p65) were decreased (Fig. 5C). All these data suggested that the aberrant expression of CLOCK may be associated with NF-κB pathway in glioma.

4. Discussion

Gliomas are the most common and lethal malignant tumors in the central nervous system [33]. Despite intensive therapeutic strategies on glioma [2,3], the prognosis remains extremely poor [33–35]. Further studies on the molecular mechanism for gliomagenesis and glioma progression are crucial for developing specific treatment strategies.

In our present study, we demonstrate that CLOCK is increased in grade III and IV glioma tissues and glioma cell lines by Western blotting, reinforced the earlier study detected the expression of Clock though RT-PCR and immunohistochemistry techniques by Chen et al. [12], suggesting that disturbed CLOCK expression may be associated with glioma progression. However, the roles of CLOCK in glioma generation and progression are poorly understood.

It has been shown that Per2 function as tumor suppressor by regulating DNA damage-responsive pathways [9]. But how CLOCK influence the tumor progression, especially glioma here, is unclear. Aiming to investigate this question, in this study we first examined the impact of CLOCK in proliferation though MTT assay and colony formation, and its role in glioma cell migration by transwell assay. The results revealed that repressed CLOCK by siRNA led to the retardation of cell growth and migration of glioma cells, thus we infer CLOCK may acts as a tumor enhancer in gliomas. All the functional experiments were performed in cell lines, so using nude mouse transplantation tumor experiment in vivo will fine down the growth-promoting function of CLOCK in the future.

MiRNAs can usually regulate their target genes at post-transcriptional level. To find out the mechanism of CLOCK up-regulation in gliomas, we hypothesized that attenuation of miRNAs in glioma may lead to aberrant expression of CLOCK. After predicting the miRNAs target to Clock with softwares, we found two potential miRNAs (miR-124 and miR181b), then we identified that miR-124 can directly target to Clock 3′UTR. MiR-124 is one of the most widely studied tumor suppressive miRNAs in glioma and has been shown to target several important transcription factors such as SNAI2 [23]. MiR-124 is frequently silenced in glioma [23], possiblely due to the hypermethylation of the miR-124-1 promoter [36]. We deduced that depressed miR-124 may be responsible for high expression of CLOCK in glioma cells. Thus, miR-124-1 promoter hypermethylation might be one of the factors determining the expression level of miR-124 and its target CLOCK in glioma.

To understand the mechanism of the roles of CLOCK in glioma proliferation and migration, we analyzed the NF-κB activity and alteration of NF-κB target genes after knocking-down CLOCK. Our results indicated that NF-κB activity was reduced and NF-κB target

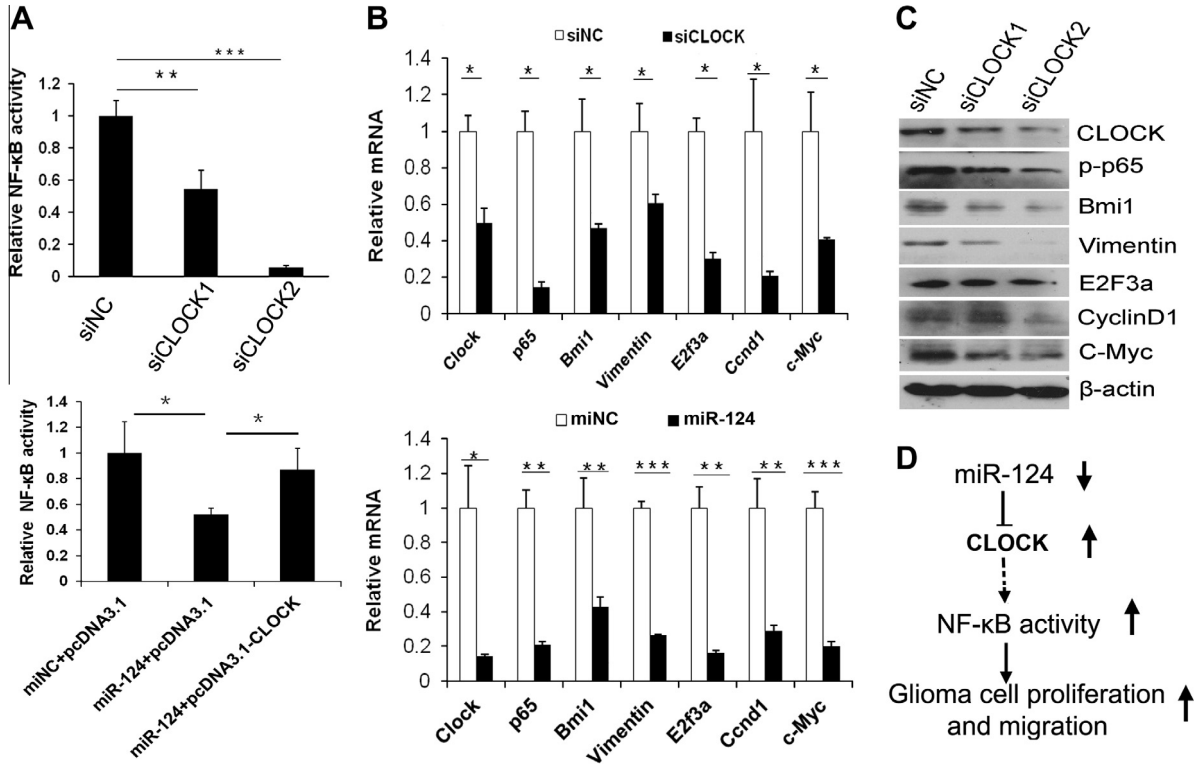

Fig. 5. MiR-124 modulates NF-κB activity by regulating CLOCK. (A) NF-κB activity was detected by luciferase reporter assays. The upper data shows relative NF-κB activity after knocking down CLOCK. The bottom data shows the relative NF-κB activity after transfecting the T98G cells with NF-κB reporters together with synthetic miR-124 (or miR-NC) and pcDNA3.1-CLOCK (or pcDNA3.1). (B) The relative mRNA level of NF-κB target genes were quantified by realtime-PCR after knocking-down CLOCK with siCLOCK (siCLOCK2) or overexpressing miR-124 in T98G. Statistical data are represented as mean ± S.D., $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (C) The CLOCK, p-p65 and proteins of five NF-κB target genes were detected in CLOCK suppressed T98G or control cells by Western blotting. (D) A potential miR-124-CLOCK-NF-κB axis in glioma. NC: negative control.

genes were repressed after CLOCK knockdown. For abundant evidences demonstrate that constitutive activation of NF-κB in glioma plays an important role in the regulation of genes involved in cellular adhesion, migration, and invasion [3737], we hypothesized that aberrant expression of CLOCK may disrupt the NF-κB pathway in glioma. Consistently, we found miR-124 restoration also significantly reduced the NF-κB activity and the expression of the bona fide NF-κB target genes.

Taken together, we presented evidences for a glioma enhancive role of CLOCK in glioma cells for the first time and showed that the attenuation of glioma suppressor miR-124 contributes to the high CLOCK expression. Moreover, we confirmed that miR-124 and CLOCK modulate the NF-κB activity in glioma cells, suggesting a potential miR-124-CLOCK-NF-κB relationship (Fig. 5D). These findings further our understanding on the mechanism of gliomagenesis and may provide a new perspective for the clinical therapy.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.06.018.

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