

Glioma cell proliferation is inhibited by miR-342-3p, miR-377/E2F1 signaling pathway

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In recent years, microRNAs (miRNAs) have been reported to be critical regulators to influence tumor genesis or its further progression by directly targeting downstream tumor related genes in glioma. However, there are still many underlying mechanisms related to miRNAs signaling pathway that remain to be uncovered. In the present study, we found that miR-342-3p and miR-377 inhibited the glioma cell line proliferation and arrested the cell cycle at G1 phase. Inhibition of miR-342-3p and miR-377 function promoted the cell proliferation. miR-342-3p and miR-377 target the E2F1 3'UTR to repress its expression on both mRNA and protein level. Downregulation of E2F1 inhibited the cell proliferation and arrested the cell cycle. Overexpression of E2F1 blocked the proliferation repression caused by miR-342-3p or miR-377 in glioma cells. This study showed the function of miR-342-3p, miR-377/E2F1 axis in regulating glioma cells proliferation and provided the potential therapeutic target.

Key words: glioma cell, proliferation, cell cycle, miRNAs, E2F1

Malignant glioma is one of the most common center nervous system tumors worldwide. Glioma showed the rapid cell proliferation and lower apoptotic rate [1–3]. Although there is a remarkable development of clinical treatments, the glioma patients still show poor prognosis [4, 5]. Investigation of the molecular signaling that regulates the glioma cell proliferation will uncover the potential regulatory pathway and therapeutic targets in future.

MicroRNAs (miRNAs), endogenous short noncoding RNAs, have been reported to regulate many physiological processes including embryonic development, disease occurrence, ageing, and so on [6–10]. miRNAs also play important role in regulating tumor genesis and development. miR-132 suppresses the breast cancer proliferation by targeting Foxa1 [11]. miR-503 repressed cell proliferation and invasion in osteosarcoma [12]. In glioma cells, miRNA-485-5p repressed proliferation and invasion by targeting TPD52L2 [13]. Downregulation of miR-377 promotes proliferation and migration in oral squamous cells [14]. miR-377 also inhibits the cell proliferation in pancreatic cancer [15]. Additionally, miR-324 may be a diagnostic biomarker, treatment target and also relates to the survival rate in esophageal cancer [16]. However, more miRNAs regulating signaling pathway that can modulate the cell proliferation remain unclear in glioma.

E2F1, a member of the E2F family transcription factors, has been reported to play critical role in regulating cell cycle and apoptosis [17, 18]. In epithelial ovarian cancer, E2F1 regulates lncRNA RAD51 antisense RNA 1 to promote cell cycle progression and repress apoptosis. The level of E2F1 is closely related to the poor prognosis [19]. Transcription of E2F1 also can be upregulated by ANKRD22 to promote the cell proliferation [20]. Downregulation of E2F1 by miR-320 inhibits the glioma cell proliferation [21]. miR-98 targets E2F1 to increase chemosensitivity in leukemia cells [22]. It is of importance to investigate the upstream regulator of E2F1, especially miRNAs, to determine the critical miRNA-E2F1 pathway regulating glioma cell proliferation.

Here we found that both miR-342-3p and miR-377 significantly inhibited the cell proliferation of glioma cell lines U251 and U87. Inhibition of miR-342-3p or miR-377 promoted the proliferation. We also detected that G1 phase of the cell cycle was regulated by these miRNAs. We further found that both of miR-342-3p and miR-377 targeted the 3'UTR of E2F1 and downregulated the E2F1 expression. Downregulation of E2F1 in glioma cells inhibits the proliferation and arrests the cell cycle. Rescue experiments also confirmed that overexpression of E2F1 restored the proliferation repression caused by miR-342-3p or miR-377. This study indicated the

function of miR-342-3p/miR-377/E2F1 signaling pathway in regulating the cell cycle and proliferation in glioma cells, which also uncovered the potential function of this pathway for further clinical treatment.

Materials and methods

Cell culture. Human glioma cancer cell lines U251 and U87 cells were cultured in DMEM medium (Hyclone, Logan, UT) with 10% (v/v) fetal bovine serum (FBS) (Gibco, USA), at 37°C, 5% CO₂ atmosphere, supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin sulfate (Sigma, USA).

Transfection of miRNAs. We transiently transfected 40 pmol miRNA-342-5p, miR-377 miRNA mimics or miRNA inhibitors (Ribobio, China) by using Lipofectamine 2000 (Thermo, USA) into the glioma cancer cell line for miRNAs overexpression or functional inhibition respectively.

qRT-PCR. M-MLV Reverse Transcriptase kit (Takara, China) was used for producing cDNA from total RNA isolated by Rnaiso Plus (Takara). qRT-PCR included 40 cycles of amplification by SYBR Green qPCR Mix (Takara). Level of genes was normalized against GAPDH (2^{-ΔΔCt}). Primer sequences are as follows: GAPDH (forward: 5'-CTGGGCTACTGAG-CACC-3'; reverse: 5'-AAGTGGTCGTTGAGGGCAATG-3'), E2F1 (forward: 5'-CATCCCAGGAGGTCACCTTCTG-3'; reverse: 5'-GACAACAGCGGTTCTTGCTC-3'), Cyclin E (forward: 5'-CGCCTGCCGGGACTGGAG-3'; reverse: 5'-TCTTCTGGAGCGAGCCG-3'), Cyclin D (forward: 5'-GACCACCGAGGAGTTAATCG-3'; reverse: 5'-GGGTG-ATCCCCTGATCCTTTG-3'), p21 (forward: 5'-TGTCGGT-CAGAACCCATGC-3'; reverse: 5'-AAAGTCGAAGTTC-CATCGTC-3'), p27 (forward: 5'-TAATTGGGGCTCCGGC-TAACT-3'; reverse: 5'-TGCAGGTCGTTCTTATTCC-3').

Western blot. SDS buffer (Beyotime, China) was used to obtain cell proteins for further electrophoresis. Then the proteins were transferred onto PVDF membranes (Whatman, USA) and detected by antibodies listed as follows: E2F1 (ab14768, Abcam, USA), GAPDH (ab97626, Abcam). Levels of protein signals were detected by Chemistar ECL Western Blotting Substrate (Thermo).

Plasmid construction. E2F1 3'UTR was cloned from U251 cell DNA by PCR. The sequences of primers are as follows: PF: 5'-GGCGTCGACGCCAGAGAGCCACCGCCG-3' (SalI restriction enzyme); PR: 5'-GGCTCTAGAGCCCTGGCTCTGCCCTG-3' (XbaI restriction enzyme). The sequence was inserted into the pGL3cM vector (Promega).

Knockdown of E2F1. The sequences of siRNA of E2F1 are as follows: siRNA-1 of E2F1: 5'-AUGCUACGAAGG UCCUGACAGUCA-3'; siRNA-2 of E2F1: 5'-AAAGUU-CUCCGAAGAGUCCACGGCU-3'.

Overexpression of E2F1. The total RNA was isolated by using Rnaiso Plus (Takara, China) from the U251 cells and used for cDNA synthesizing using the Takara-PrimeScript RT reagent Kit (Takara). The fragment of CDS of E2F1 was amplified by following primers and inserted into the overex-

pression vector Fugw: PF: 5'-GGCGGATCCATGGCCTTG-GCCGGG-3' (BamHI site), PR: 5'-GGCGAATTCGAGT-GGGGGGACCTAAAGACT-3' (EcoRI site).

Cell proliferation analysis. U251 and U87 cells at the density of 1.2×10⁴ cells per well were seeded into 96-well plates. MTS proliferation assay was performed by Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega).

Cell counting. In order to detect the total cell number in different treatment groups, we cultured the cells in 6-well culture dishes. For cell counting, we prepared the cell suspensions digested by trypsin (Gibco, USA). Then number of cells per milliliter was detected by using Countstar cell count meter (Inno-Alliance Biotech, USA) to determine the total cell number in each group.

Luciferase reporter assays. U251 cells at a density of 3×10⁴ cells per well were seeded into 48-well plates. Cells were co-transfected with 200 ng of the UTR luciferase reporter, 4 ng Renilla vector, and 25 pmol miRNA mimics (Ribobio) by Lipofectamine 2000 (Thermo, USA). Cells were harvested and lysed 48 h later for detecting the luciferase activity using Dual-Luciferase Assay system (Promega, USA).

Flow cytometry. Cells were harvested and fixed by cold 70% alcohol on ice for 2 h. Then the cells were washed with PBS and treated with RNase A (10 mg/ml). Propidium iodide (PI) staining solution (40 mg/ml) was added for 15 min incubation. Cell cycles were analyzed by Cytomics FC 500 instrument (Beckman Coulter).

Statistical analysis. Experiments were performed at least 3 times independently unless otherwise stated. Data statistical significance was determined between groups by Student's t-test or one-way Anova. Values were presented as the mean ± SD. *, **, *** represent p<0.05, p<0.01, p<0.001, respectively.

Results

miR-342-3p and miR-377 repressed the proliferation of glioma cells. We performed the MTS proliferation assay and found out that transfection with miR-342-3p and miR-377 mimics for overexpression significantly repressed the proliferation capability of U251 (Figure 1A) and U87 cells (Suppl. Figure S1A). The amounts of U251 cells transfected with miR-342-3p and miR-377 respectively were lower than control groups (Figure 1B). We further detected an increase of G1 phase cell proportion and decrease of cells in S, and G2/M phase proportion in U251 cells overexpressing miR-342-3p and miR-377 mimics, respectively (Figure 1C, Figure S1C). CCND1, CCNE1, cell cycle related genes, were downregulated. Cyclin-dependent kinase inhibitor 1A (p21) and cyclin-dependent kinase inhibitor 1B (p27) were upregulated in the U251 cells overexpressing miR-342-3p or miR-377 (Figure 1D). On the contrary, we found that transfection with the antisense of miR-342-3p or miR-377, miRNA inhibitor which combines with miRNAs to prevent miRNA-

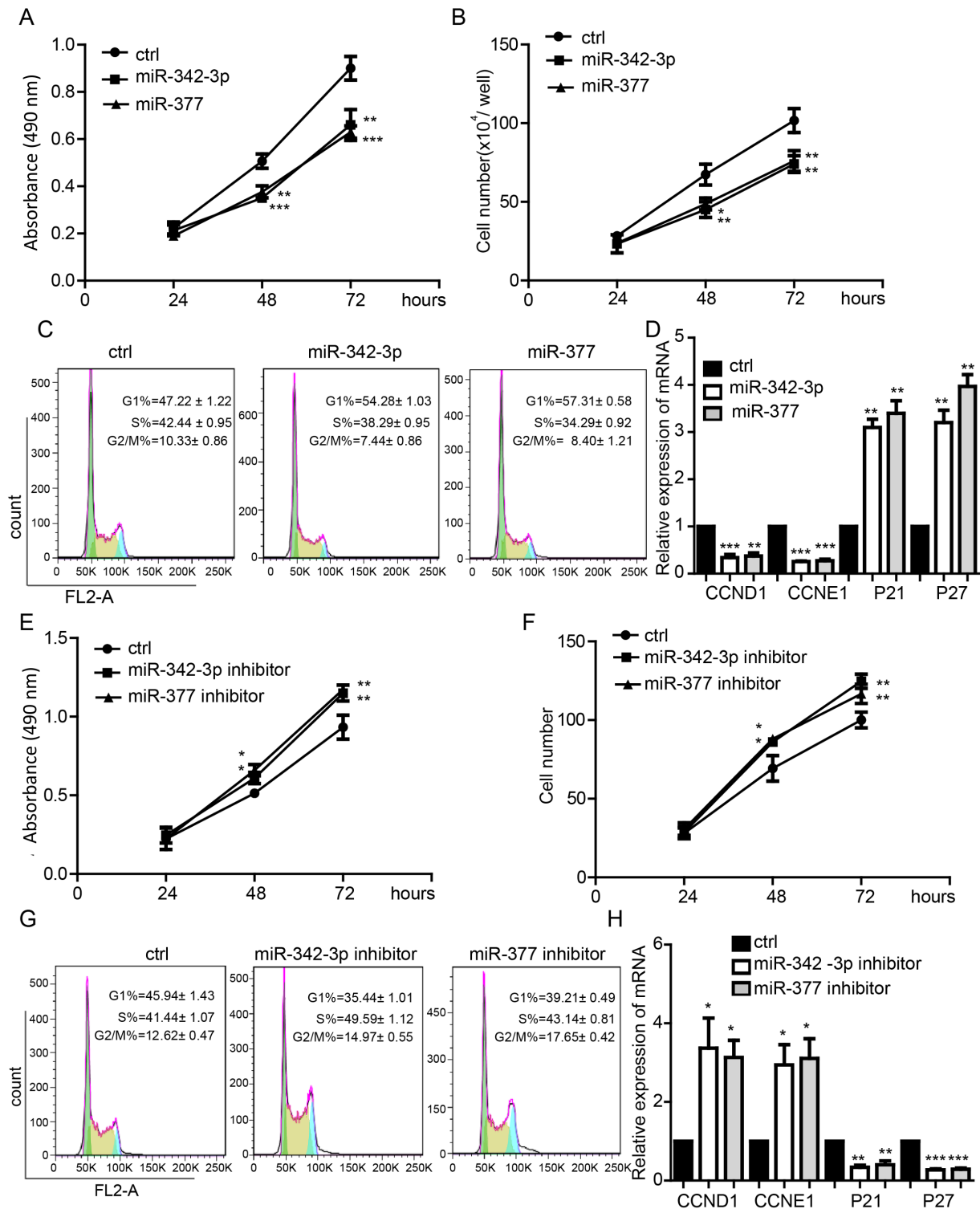


Figure 1 miR-342-3p and miR-377 repressed the cell proliferation of glioma cell line U251. **A)** miR-342-3p and miR-377 repressed the cell proliferation detected by MTS assay during 24, 48, 72 h in U251 cells. Data (n=5) shown are means ± SD. **B)** Counting of the U251 cell numbers transfected with miR-342-3p or miR-377 during 24, 48, 72 h. **C)** Flow cytometry assay detection indicated the G1 phase arrest via overexpressing miR-342-3p or miR-377. **D)** miR-342-3p and miR-377 regulated the cell cycle related genes expression. Data (n=5) shown are means ± SD. **E)** Transfection of miR-342-3p or miR-377 inhibitor promoted the cell proliferation detected by MTS assay. **F)** Total cell counts were increased by transfecting miRNA inhibitor in U251 cells. **G)** Flow cytometry assay detection of cells transfected with miR-342-3p or miR-377 inhibitor. **H)** miR-342-3p or miR-377 inhibitor increased the expression of CCND1 and CCNE1 and decreased the p21 and p27. Data shown are means ± SD (n=5).

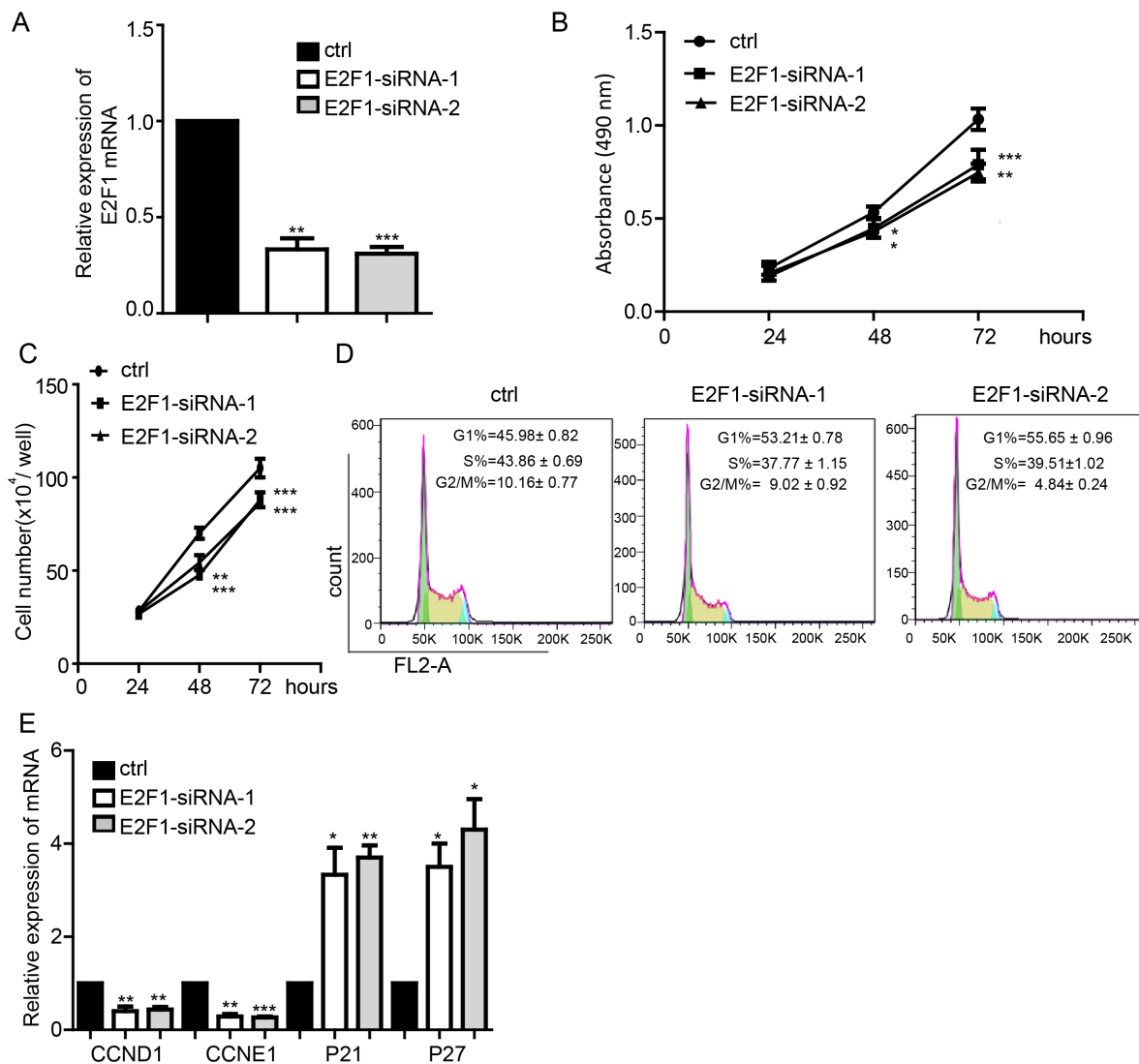


Figure 3 Downregulation of E2F1 inhibited the glioma cell proliferation. A) Downregulation of expression of E2F1 by siRNA. B) Knockdown of E2F1 repressed the cell proliferation. C) Total cell counts were decreased by downregulating of E2F1 expression in U251 cells. D) Flow cytometry analysis indicated that downregulation of E2F1 induced the G1 phase arresting. E) Downregulation of E2F1 influenced the cell cycle related genes expression.

Discussion

Rapid proliferation of glioma cells, which ensures the subsequent invasion and migration, has become one of the great challenges of clinical treatment [23]. Investigation of glioma proliferation molecular signaling pathway is of great importance in order to develop new diagnostic markers and therapeutic targets [24, 25].

MicroRNAs have been reported to be the critical regulators in cancer cells proliferation. miR-429 suppresses the glioblastoma multiforme cell proliferation by directly targeting SOX2 [26]. Repression of miR-155 in glioma cells inhibited the proliferation [27]. miR-592 targeted the IGF2BP2 to be a tumor suppressor in glioma [28]. miR-202 inhibits

cell proliferation by targeting metadherin [29]. These studies showed that miRNAs play important roles as the key switch to regulate glioma proliferation by targeting downstream cancer related regulators. In this study, we also detected that both miR-342-3p and miR-377 could inhibit glioma cells proliferation. These two miRNAs induced the cell cycle arrest at G1 phase, inhibition of the function of miR-342-3p and miR-377 promoted cell proliferation and decreased the proportion of cells in G1 phase, inducing more cells into the cell division cycle of G2/M phase. Our study indicated the function of miR-342-3p and miR-377 in the induction of cell cycle arrest thus inhibiting the cell proliferation, which suggested that the two miRNAs might be the potential clinical treatment targets.

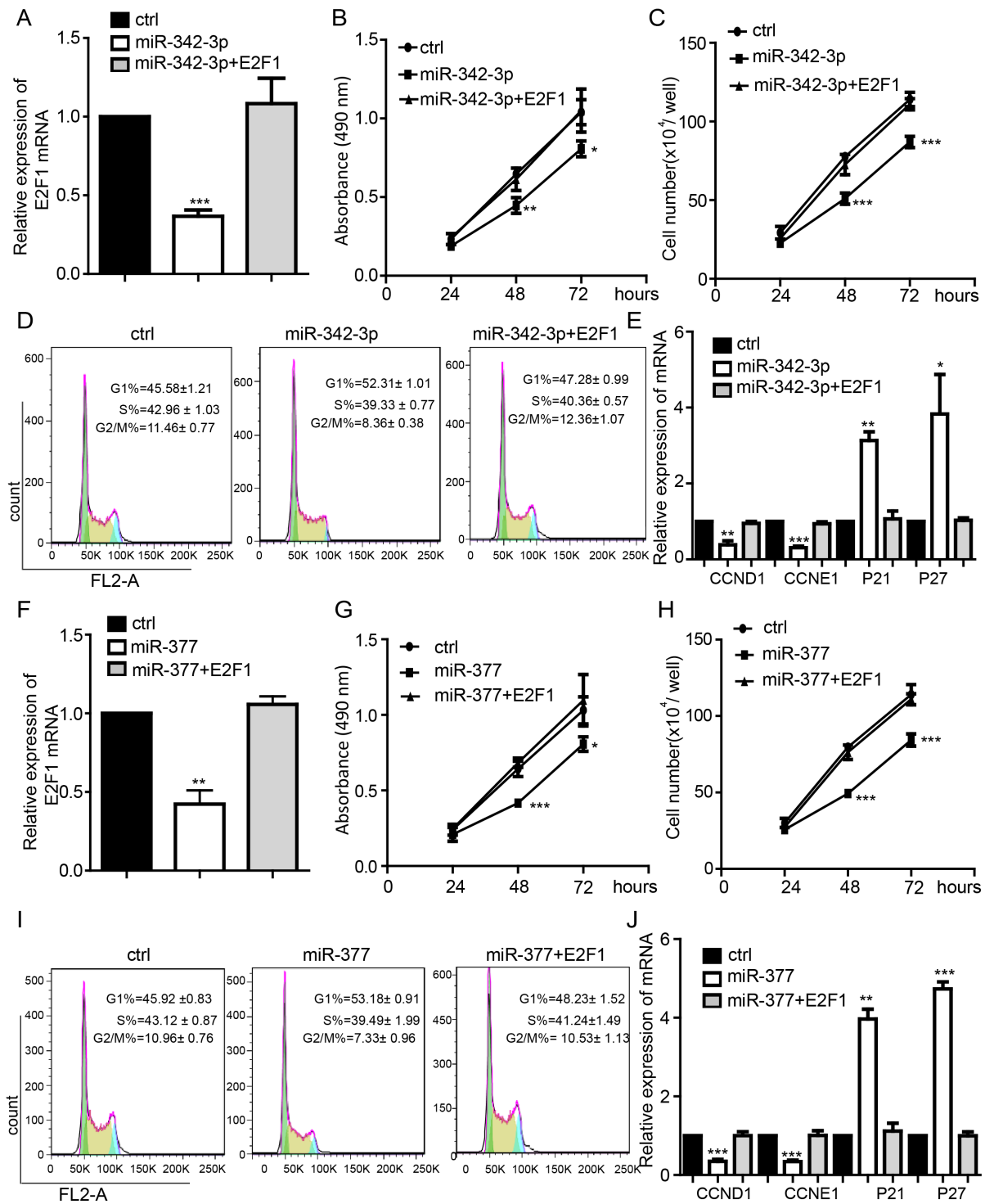


Figure 4. E2F1 mediated the function of miR-342-3p and miR-377 on regulating the glioma cell proliferation. A) Detection of the expression of E2F1 in the U251 cells co-overexpressed with E2F1 and miR-342-3p compared with control group. Data shown are means \pm SD (n=5). B) Overexpression of E2F1 restored the proliferation repressed by miR-342-3p. Data shown are means \pm SD (n=4). C) Total cell counting showed the overexpression of E2F1 rescue the inhibition of miR-342-3p. D) Overexpression of E2F1 rescued the G1 phase arresting caused by miR-342-3p. Data shown are means \pm SD (n=5). E) E2F1 restored the regulation of cell cycle related genes expression. F) Overexpression of E2F1 in the U251 cells transfected with miR-377. Data shown are means \pm SD (n=5). G) E2F1 restored the proliferation repressed caused by miR-377. Data shown are means \pm SD (n=4). H) Overexpression of E2F1 restored the inhibition of miR-377. I) E2F1 restored the cell cycle regulation influenced by miR-377. J) E2F1 rescued the cell cycle related genes expression regulated by miR-377.

Many critical cancer-related genes were regulated by miRNAs. miR-374c-5p targets Foxc1/Snail pathway to regulate the invasion of cervical cancer cell [30]. miR-503-3p induces apoptosis by targeting p21 and CDK4 of lung cancer cells [31]. miR-155 directly targets PDCD4 to inhibit proliferation of lung cancer [32]. Activation of E2F1 by DEPDC1 promotes cell proliferation in prostate cancer cells [33]. E2F1 also mediated the function of Klf4 on promoting melanoma cell growth [34]. In our study we detected that E2F1 expression was repressed by miR-342-3p and miR-377. Knockdown of E2F1 inhibited the proliferation of glioma cells and arrested the cell cycle at G1 phase, which was similar with the function of miR-342-3p or miR-377 overexpression. Both miR-342-3p and miR-377 directly targeted the E2F1. In general, miRNA can repress multiple target genes and one gene can be repressed by multiple miRNAs to form the complex regulatory networks [35]. Previous study showed that miR-342-3p and miR-205-5p work together to repress E2F1 to be the anticancer regulators in human non-small cell lung carcinoma cell line [36]. This study suggested the complex regulation of E2F1 expression by miRNAs and this regulatory pathway maybe participates also in the glioma cell proliferation, which remains for further investigation. In our study, overexpression of E2F1 restored the cell proliferation and cell cycle regulation that was inhibited by overexpression of miR-342-3p or miR-377. These results indicated that E2F1 is the critical modulator mediated by the miR-342-3p and miR-377 function. The glioma cell proliferation can be regulated by miR-342-3p and miR-377/ E2F1 signaling axis. Our work suggested that miR-342-3p and miR-377/ E2F1 could also be therapeutic target or diagnostic marker for future clinical treatment.

Supplementary information is available in the online version of the paper.

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