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Conditional control of dendritic cell factor 1 expression by a tetracycline-inducible system

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Abstract

Dendritic cell factor 1 plays important roles in neural stem cells differentiation and in glioma cells proliferation, migration, and invasion. Here, we used a tetracycline-inducible system that regulates the expression of Dendritic cell factor 1 in glioma cells. We constructed two tet-inducible vectors, pTRE-EGFP-DCF1 and pTRE-LJM1-DCF1, by modifying the promoter P_{CMV}. In the absence of tetracycline or doxycycline, the expression of Dendritic cell factor 1 in cells co-transfected with pTRE-EGFP-DCF1 or pTRE-LJM1-EGFP-DCF1 and ptTS-Neo was suppressed through binding of the tetracyline-controlled transcriptional suppressor to tetracycline response element, and the suppression was released by the addition of doxycycline. Our work has laid foundations for potential clinical application of cancer therapy in realizing artificial regulation of gene.

Key words: Tetracycline, Inducible vector, Regulation, Gene expression, Glioma.

Introduction

Dendritic cell factor 1 (DCF1), also known as transmembrane protein 59 (TMEM59), is a membrane protein that modulates neural stem cells (NSCs) differentiation(1) and amyloid precursor protein (APP) glycosylation(2). Previously, our study has revealed that silencing DCF1 tends to differentiate NSCs into astrocytes (3, 4). Recently, we found that overexpression of DCF1 significantly inhibits cell proliferation, migration, and invasion and promotes apoptosis in the glioblastoma U251 cell line(5).

Gossen and Bujard firstly described the tetracycline (Tet) controlled gene expression system containing Tet-off or Tet-on in mammalian cells (6, 7), in which the gene expression is turned off by adding Tet or doxycycline (Dox), contrarily, the system is turned on after adding tetracycline. (8). For the tet-on system, it consists of two components: the tetracyline-controlled transcriptional suppressor (tTS) and the modified tet-responsive promoter ($P_{TREmod/cmv}$) derived from the P_{TREmod} and the cytomegalovirus (CMV). The tTS is a fusion of the Tet repressor protein (TetR) and the KRAB-AB silencing domain of the Kid-1protein (SDKid-1), a powerful transcriptional suppressor (9, 10). The P_{TREmod} contains a modified Tet response element (TRE_{mod}) which is composed of seven direct repeats of a 36 bp sequence including a 19 bp tet operator sequence (*tetO*). In the absence of Dox, tTS binds TREmod and suppresses transcription. The KRAB-AB domain then acts as a potent suppressor of transcription from any promoter downstream of the tetO sequences.

Here, we constructed a tetracycline-regulated system to modulate the expression of DCF1 in U251 cells. In the absence of doxycycline (Dox), a tetracycline derivative, down regulation is achieved through binding of the silencer tTS to *tet*O sequences. Addition of Dox relieves transcriptional suppression and activates pTRE-EGFP-DCF1.

Materials and methods

Materials

Plasmids pEGFP-N2, pSIREN-RetroQ-TetP, ptTS-Neo and pQC-tTS-IN were purchased from Clontech, and pLJM1-EGFP was purchased from Addgene. The HEK293T and U251 were purchased from the Cell Bank of Shanghai Institute of Biochemistry and Cell Biology (SIBS, CAS). Restriction enzymes *Ase*I, *Nde*I, *Age*GI, *Eco*RI, *Xba*I, *Bam*HI and *Sac*I were purchased from Fermentas. Doxycycline hyclate and propidiumiodide were purchased from Sigma. FBS and DMEM were purchased from Gibco. Lipofectamine™ 2000 Transfection Reagent was purchased from Invitrogen, 0.25% trypsin and BCA Protein Assay Kit were purchased from Beyotime. Taq polymerase was purchased from Tiangen. PrimeSTAR®HS DNA Polymerase and T4 DNA Ligase were purchased from Takara. IRDye®800CW infrared dyes were purchased from LI-COR.

Methods

Construction of the tetracycline-controlled expression vectors

The construction design of the tetracycline-controlled expression vectors, pTRE-EGFP-DCF1 and pTRE-LJM1-DCF1 was illustrated in Figure 1. The human *dfc*1 cDNA was amplified by PCR with *Pyrococcusfuriosus* DNA polymerase, and then subcloned into the *Eco*RI and *Bam*HI sites in the plasmid pEGFP-N2. The TRE, which derived from the P_{TREmod} in pSIREN-RetroQ-TetP, was inserted into the *Ase*I site at the 5' end of the promoter P_{CMV} of the plasmid pEGFP-DCF1 to give pTRE-EGFP-DCF1 (Fig.1A).The *egfp* between the *Eco*RI and *Age*GI sites was replaced with human *dfc*1 in the plasmid pLJM1-EGFP, and then the TRE was inserted into the *Nde*I site at the 5' end of the promoter P_{CMV} of the plasmid pLJM1-DCF1 to give pTRE-LJM1-DCF1 (Fig.1B).

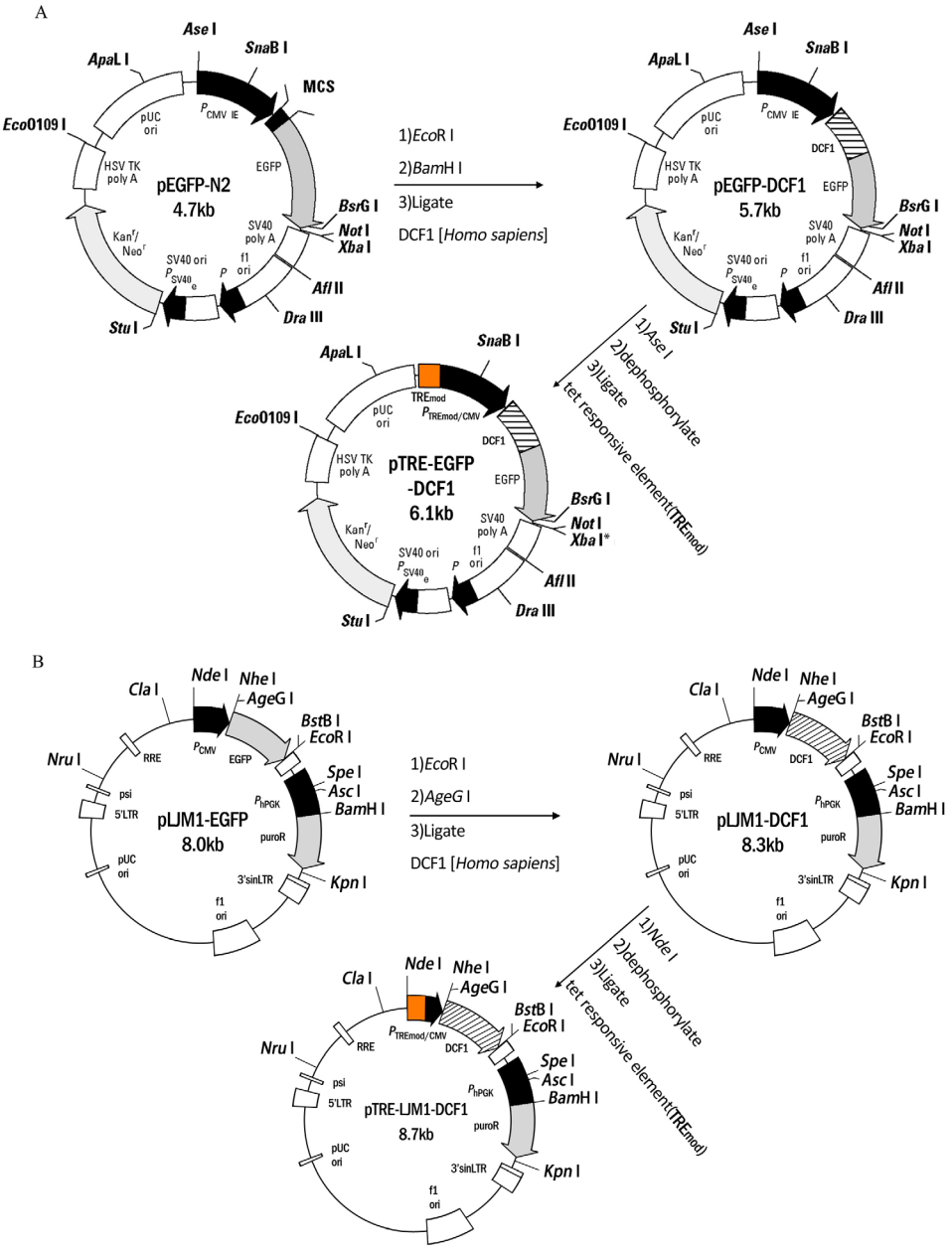

Figure 1. Construction of the vector pTRE-EGFP-DCF1 and pTRE-LJM1-DCF1 for tetracycline inducible DCF1 expression. The detailed procedure is in Materials and Methods. (A) The main steps in the construction of the vector pTRE-EGFP-DCF1. (B) The main steps in the construction of the viral vector pTRE-LJM1-DCF1.

Preparation of cells for cell cycle analysis using PI

After transfection for 48 hours, U251 cells were digested with trypsin (0.25% in D-Hanks solution without EDTA) and harvested in 10% FBS–DMEM. The cell suspension was centrifuged at 1000g for 5 minutes at room temperature (25°C). The supernatants were removed and cells were gently washed twice in ice-cold PBS. After removing the supernatants, 1ml of 70% cold ethanol was slowly added during vigorous mixing. Samples were stored at 4°C for 12 to 24 hours. When samples were to be analyzed, centrifuged the cell suspensions at 1000g for 5 minutes at room temperature and removed the supernatants. After washing the cells gently with ice cold PBS, 0.5ml stain solution (50μg/ml PI, 100μg/ml RNase A) was slowly added during vigorous mixing and the samples were incubated for 30 minutes at 4°C before flow cytometric analysis.

Cell cycle analysis

Cell cycle was analyzed using flow cytometry. To acquire data on a FACScan or FACS Calibur, the cytometer was triggered on the PI signal. Primary gate was on forward scatter (FSC) against right angle light scatter (SSC). A secondary gate was placed around the single cell population on a pulse area versus pulse width dot plot.

Cell culture

The U251 and HEK293T cell lines were cultured in high-glucose DMEM with 10% fetal bovine serum (FBS), 1mM glutamine, 100U/ml penicillin and 100μg/ml streptomycin at 37°C and 5% CO_2.

Transient transfection and quantification of eGFP

Cell transfection using Lipofectamine®2000 or calcium phosphate precipitation was done according to the recommendations of the manufacturers. In general, 2-5μg plasmid was transfected into 10^5 cells in triplicate in 24-well culture plates and incubated for 1–2 days at 37°C and 5% CO_2. Transfection efficiency was analyzed by EGFP luminous intensity. The GFP expression was quantified by software Image-Pro Plus 6.0, the cells

expressing GFP were counted.

Western blotting

After transfection for 48 hours, the cells were washed twice with ice-cold PBS and protein was extracted according to the manufacturer's protocol (Beyotime). Protein concentrations were detected using the BCA Protein Assay Kit. The total protein (30μg) was separated by 12% SDS-PAGE and electroblotted onto nitrocellulose membranes, which were blocked with 5% BSA–PBS for 1 hour at room temperature, and then incubated with β-actin antibody (1:1000; Santa Cruz) for 2 hours, and then incubated with DCF1 (1:3000)antibody overnight at 4°C. Next, IRDye ® 800CW infrared dyesecondary antibodies were added and incubated for 1hour at room temperature. Immunoblotting bands were detected and quantified using the LI-COR Odyssey infrared imaging system (simultaneous two-color targeted analysis) and software (LI-COR).

Results

Low dcf1 expression in co-transfected HEK293TN cells in the absence of dox

To study the suppression efficiency of gene expression, HEK293TN cells were transiently co-transfected with pTRE-EGFP-DCF1 and ptTS-Neo. As control, pEGFP-DCF1 without the TRE_{mod} was co-transfected with ptTS-Neo, and pEGFP-DCF1 or pTRE-EGFP-DCF1 was also transfected alone. The expression of fusion protein eGFP-DCF1 was observed through a fluorescence microscope. As shown in Figure 2A, the background of expression was significant lower in cells co-transfected with pTRE-EGFP-DCF1 and ptTS-Neo than others in the absence of Dox. The population of fluoresce cells were counted and statistically analyzed. There was a significant reduction in cell number between the experimental group and other groups (Fig. 2B). It indicated that the repression of the fusion protein eGFP-DCF1 expression in the off-state was sufficiently efficient. However, the inserted TRE_{mod} might have a negative effect on promoter $P_{CMV\ IE}$.

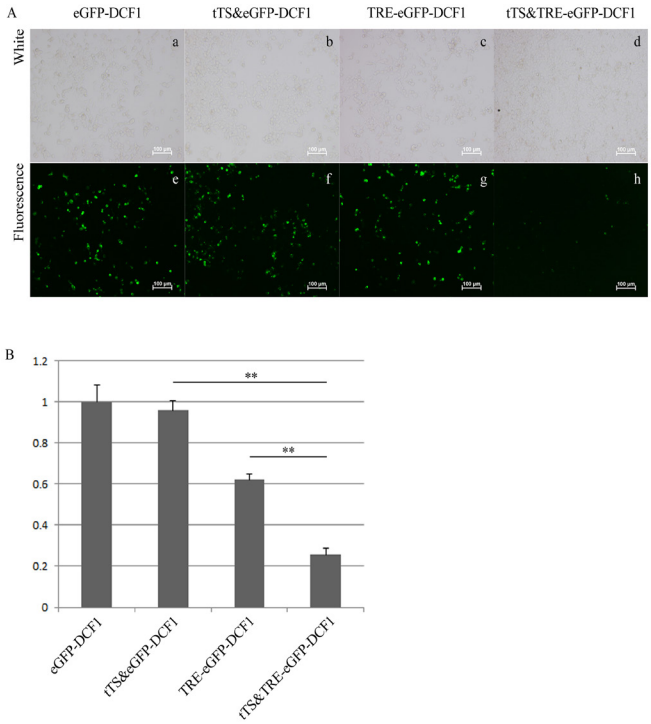

Figure 2. Efficient suppression of gene expression by tTS in HEK293T cells. (A) The eGFP-DCF1 was observed with fluorescence microscope. The expression in the co-transfected cells was significant lower than other control groups. (B) Statistical analysis of the fluorescence cell population by software Image-Pro Plus. It indicated that the suppression is significant, and there was a negative effect in modifying P_{CMV} (n=3, *p<0.05, **p<0.01).

Inducible expression of DCF1 in co-transfected HEK293T cells in the presence of Dox

To demonstrate that the gene expression was controlled by Dox, the HEK293T cells were treated with different concentration of Dox. After co-transfected with pTRE-EGFP-DCF1 and ptTS-Neo for 24 hours, the expression of eGFP-DCF1 increased with the concentration of Dox and the maximal expression efficiency was achieved in presence of 1μg/ml Do x (Fig. 3A).

DCF1 expression level was tracked with Western blot analysis (Fig.3B). In the absence of Dox, DCF1 expression level reduced in the cells co-transfected with pTRE-LJM1-DCF1 and ptTS-Neo compared to cells transfecting with pTRE-LJM1-DCF1 alone (Fig.3B lane 4 and lane 5). Although the background of DCF1 in HEK293T cells was tracked, it also indicated that the suppression was released and DCF1 expression was induced by the gradual addition of Dox (Fig.3B lane 1 to lane 3).

Tet-inducible system for DCF1 expression in U251 glioma cells

To verify the performance of Tet-inducible system in glioma cells, U251 cells were transiently transfected or co-transfected with pEGFP-N2, pEGFP-DCF1,

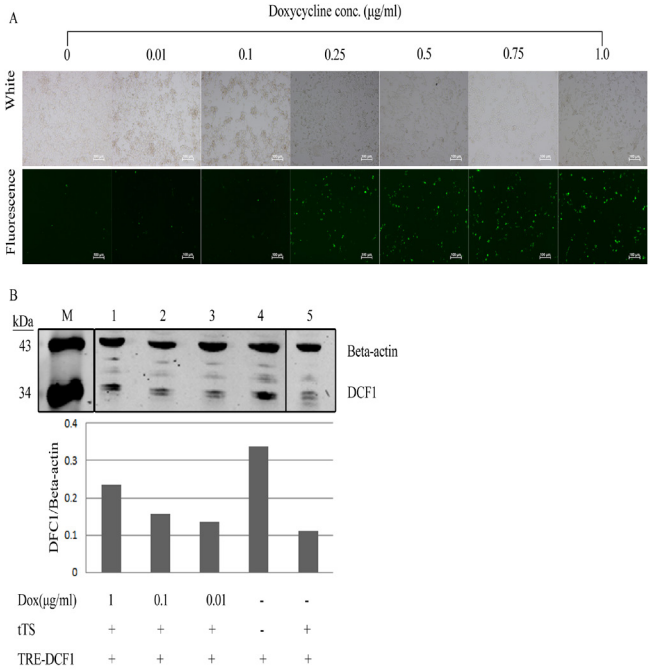

Figure 3. Gradual induction of gene expression by Dox in HEK293T cells. (A) The eGFP-DCF1 was observed with fluorescence microscope. Addition of increasing concentration of Dox had revealed a dose-dependent increase in the number of fluorescence cells. (B) Western blotting showed that the suppression was released by the gradual addition of Dox.

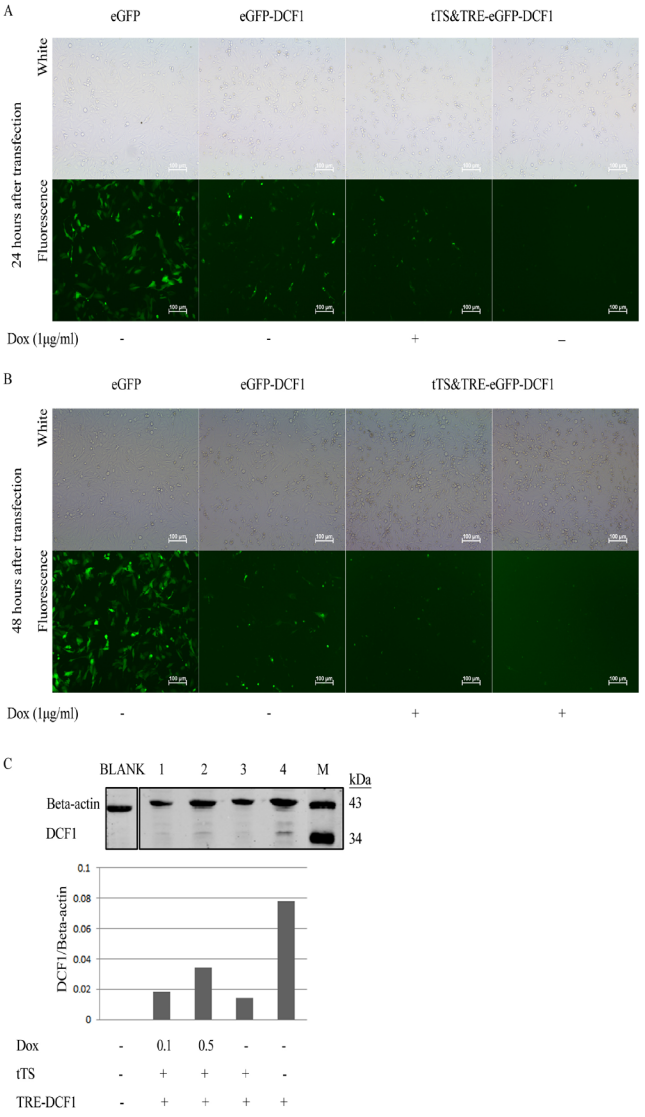

Figure 4. Verification of Tet-inducible system in U251 glioma cells. (A) The eGFP and eGFP-DCF1 was observed with fluorescence microscope after transfection 24 hours. The suppression was obviously effective and the gene expression could be induced by the addition of Dox (1μg/ml). (B) The eGFP was observed with fluorescence microscope after transfection for 48 hours. The eGFP-DCF1expression didn't increased as same as eGFP. (C) Western blotting showed that the suppression was efficient and could be released by the addition of Dox.

pTRE-EGFP-DCF1 and ptTS-Neo respectively and were treated with Dox (1μg/ml) for 48 hours. The cells were observed every 24 hours in a total of twice through a fluorescence microscope (Fig.4A). As shown, the suppression was obviously effective and eGFP-DCF1 expression could be induced by the addition of Dox (1μg/ml).We also observed that eGFP distributed in the whole cell and the eGFP expression increased with time, whereas the fusion protein eGFP-DCF1distributed like a spot and the eGFP-DCF1expression maintained almost the same level (Fig. 4B).

U251 cells were also transiently transfected with pTRE-LJM1-DCF1 and ptTS-Neo, and DCF1 expression was detected with Western blot analysis (Fig.4C), indicating a significant suppression. However, the induction of expression by Dox in U251 cells was not as efficient as it was in HEK293T cells.

The cell cycle was analyzed by flow cytometry (Fig. 5). Compared with controls (Fig. 5A and Fig. 5B), the frequency of G1 phase increased significantly in the cell overexpressing DCF1 (Fig. 5C and Fig. 5D). As DCF1 was induced by adding Dox to overexpression in the cells co-transfected with pTRE-EGFP-DCF1 and ptTS-Neo, the frequency of G1 phase increased significantly comparing to the co-transfected cells in the absence of Dox (Fig.5E and Fig.5F).

Discussion

Thanks of DCF1 plays very important roles in regulating the differentiation of neural stem cells and promoting the apoptosis of glioma cells (5), establishment of an induced DCF1 expression system *in vitro* has potential clinical application value to realize artificial regulation of gene. In this study, we constructed a tetracycline-inducible system and confirmed its efficiency in HEK293T cells and U251 glioma cells. And we also analyzed the cell cycle change under dcf1 gene drive.

Our results show that the modified promoter $P_{TREmod/CMV}$ is suppressed by tTS and the suppression is relieved by the addition of Dox. As expected, the background is low, and the inducibility is high in the presence of Dox, indicating a dose dependent relationship between the DCF1 expression and Dox concentration. However, the performance of the Tet-inducible system in U251 glioma cells is not perfect as expected, and the induction rate is lower in U251 glioma cells than in HEK293T cells. It may be the cell properties difference for the Tet-inducible system.

The pTRE-LJM1-DCF1 is an inducible retroviral DCF1 expression vector, and pQC-tTS-IN is a retroviral vector that can be used to generate stable tTS-expressing cell lines by retroviral infection(11). They can be used to generate virus by transfection into a suitable packaging line. The virus generated can be used to infect U251 cells and other cells of interest. Additionally, the inducible virus provides the better security and spatiotemporal specificity than normal virus.

To control DCF1 expression spatially and temporally in genetically modified mice, several techniques should be used, like the tetracycline-inducible system and the Cre/loxP recombination system (12, 13). Generating a transgenic mouse line can provide a tool, which can be used for inducible overexpression of DCF1 efficiently, stably and safely (14), to study the potential functions of DCF1*in vivo.*

The proteins involved in the control of the cell cycle, which is driven by cyclins and cyclin-dependent kinases (CDks), regulate cell proliferation (15). Tumor cell proliferation is often associated with genetic or epigenetic alterations in key cell cycle molecules that regulate the activity of CDKs(16). The mitotic CDKs inactivation prevents progression throughout the mitotic cell cycle efficiently (17). Our prior research has made clear that overexpression of DCF1 inhibits cell proliferation significantly. And now we find that overexpression of DCF1can inhibit the G1/S transition in U251 glioma cells. There may be a potential relationship between DCF1 overexpression and CDks inactivation in U251 cells. We need a better understanding of the functions of DCF1in cell cycle regulation to design efficient cancer therapy.

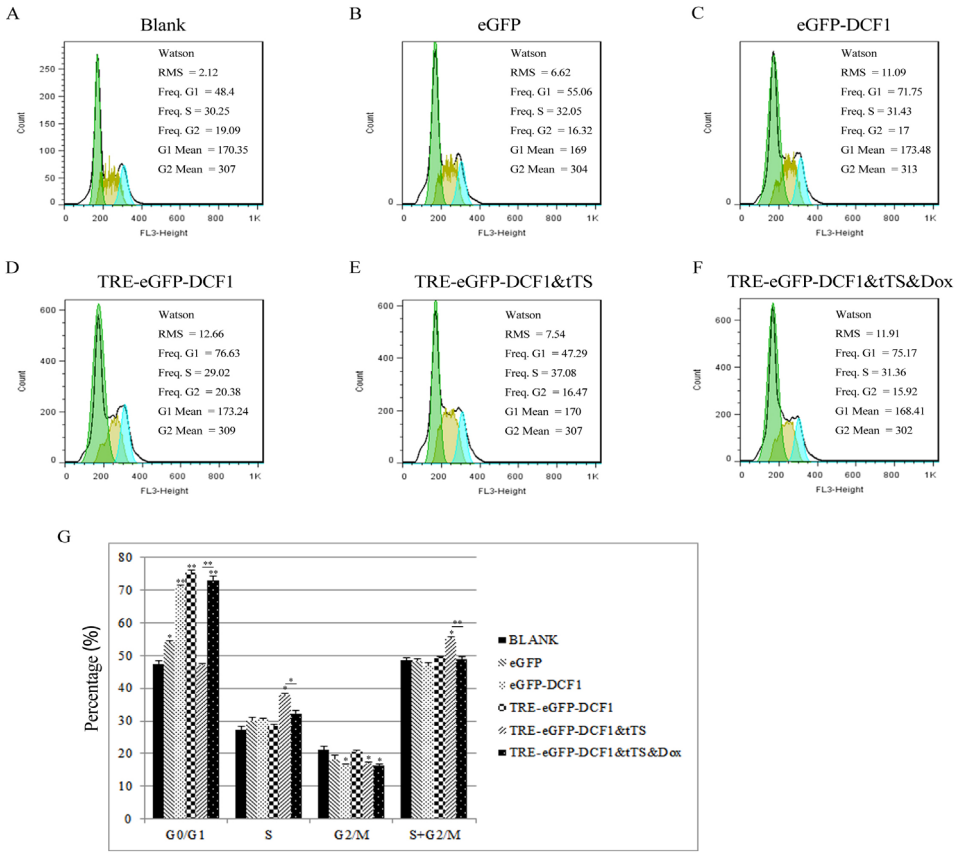

Figure 5. Cell cycle analysis of U251 cells. (A, B and E) In the absence of DCF1, the frequency of G1 phasewas low. (C, D and F) In the presence of DCF1, the frequency of G1 phase increased by average 20 percent. It indicated that DCF1 inhibited the proliferation of U251 cells. (E, F) Adding Dox or not, the contrast of the frequency of G1 phase was obvious. (G) Statistical analysis of cell cycle. It showed the significant differences between blank and treatments (n=3, *P<0.05, **P<0.01).

Acknowledgements

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