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Original Article

Orphan nuclear receptor Nur77 is required for the differentiation of C6 glioma cells induced by cholera toxin

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Aim: To investigate a possible regulator gene involved in the cholera toxin-induced differentiation of rat C6 glioma cells.
Methods: The global changes in the mRNA expression pattern induced by cholera toxin were analyzed using gene chip microarray. The selected gene was then silenced by RNA interference or overexpressed with an ORF plasmid to determine its necessity in this process.
Results: Nur77, a member of the orphan nuclear receptor family (NR4A), was markedly up-regulated during the process of differentiation. Furthermore, RNAi of nur77 attenuated the induction effect of cholera toxin on C6 cells, whereas overexpression of nur77 led to similarly differentiated behavior, including morphologic and biomarker changes, as well as cell cycle arrest.
Conclusion: Nur77 participated actively and essentially as an important regulator in the cholera toxin-induced differentiation of C6 cells.

Keywords: cholera toxin; malignant glioma; differentiation; orphan nuclear receptor; nur77

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Introduction

Gliomas derived from astrocytomas are the most common primary brain tumor in humans and account for more than 60% of all primary brain tumors[1]. Treatments for most malignant glioblastoma multiforme (GBM, WHO grade IV) tumors, including chemotherapy, radiotherapy and surgery, are acknowledged to be palliative measures. The median survival time for GBM patients has shown little improvement over the past 25 years[2].

Differentiation therapy for primitive-status cancer is a concept that has only been recently proposed. Retinoic acid, the first identified differentiation agent, has a differentiation-inducing effect on acute promyelocytic leukemia (APL) and is used as a common differentiation therapy in APL[3]. Although new therapies that target certain molecules associated with other types malignant tumors have been successfully carried out[4], effective differentiation agents for some solid tumors such as GBM have not yet been found.

We previously demonstrated that cholera toxin, a traditional biotoxin, can induce cellular differentiation in both rat C6 cells and primary cultured human glioma cells[5]. Cholera toxin-induced differentiation was characterized by typical changes, *eg*, in morphology, bionomics and cell cycle. These findings indicated the potential of cholera toxin for the further development of glioma differentiation therapy. However, the molecular determinants of C6 cell differentiation remained unknown.

In the present work, we analyzed the global changes in mRNA expression patterns during the process of C6 differentiation induced by cholera toxin with microarray technology. Our results showed that the expression of more than 1000 genes (>3% of analyzed genes) was changed during differentiation and the expression of 29 genes was stable during the process. Among them, two members of the orphan nuclear receptor subfamily 4 group A (NR4A)—NR4A1 (also known as Nur77) and NR4A3 (Nor1)—were significantly up-regulated throughout the entire differentiation process. We found that down-regulation of nur77 expression could attenuate the induced differentiation effect, whereas overexpression of nur77 produced analogous change associated with differentiation. On the basis of these results, we propose that nur77 functions as a key regulator of the induced differentiation of C6 cells.

Materials and methods

Cell culture and treatment

Rat C6 glioma cells obtained from the American Type Culture

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Collection (Manassas, VA) were maintained in Dulbecco's modified Eagle's medium (DMEM, 4500 mg/L glucose; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, GIBCO, Carlsbad, CA, USA), penicillin (20 U/mL) and streptomycin (20 μg/mL), and were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. The culture medium was replaced with DMEM containing 1% fetal bovine serum before the experiment.

Cholera toxin (Sigma, St Louis, MO) was incubated with C6 cells at a final concentration of 10 ng/mL. Cells were collected before and 3, 6, 12, 24, and 48 h after cholera toxin was added.

RNA extraction and microarray analysis

Total RNA was extracted at the indicated times with TRIzol (Gibco/BRL, Life Technologies, Inc, Grand Island, NY) and then stored at -80 °C before use.

Hybridization assays and data collection were performed at Shanghai GeneTech Biotechnology Ltd, China. Briefly, 20 mg of total RNA from each sample was reverse transcribed using an oligo dT primer containing a T7 RNA polymerase binding site. *In vitro* transcription was performed on the cDNA, and the resulting cRNA was biotinylated through incorporation of biotinylated dUTP and dCTP. Samples were fragmented in 40 mmol/L Tris-acetate, 100 mmol/L potassium acetate, and 30 mmol/L $MgCl_2$ (pH 8.1) at 95 °C to reduce the secondary structure. A total of 15 mg cRNA was hybridized to an Affymetrix Rat Genome 230 2.0 GeneChip array (Santa Clara, CA) and then washed, stained, and scanned with a Hewlett Packard Gene Array scanner.

Reverse transcriptase PCR (RT-PCR)

For the detection of *nur77* and GAPDH RNA transcripts, one-step RT-PCR was performed. The following pairs of primers were used in the reaction:

nur77 forward: 5′-CCG AAC CGT GAC ACT TCC-3′, reverse: 5′-CTT GCA GCC CTC ACA GGT- 3′.

GAPDH forward: 5′-TCA CCA TCT TCC AGG AGC GAG-3′, reverse: 5′-ATG AGC CCT TCC ACG ATG-3′.

Expected amplification product sizes were as follows: 221 bp for *nur77* and 300 bp for GAPDH. The reverse transcription reactions included 27 cycles of PCR amplification (35 s at 94 °C, 35 s at 60 °C, and 35s at 74 °C), using a Superscript™ III First-strand Synthesis System kit (Invitrogen).

Western blotting

The expression of Nur77, glial fibrillary acidic protein (GFAP) and proliferating cell nuclear antigen (PCNA) in glioma cells was assessed by standard Western blot analysis. Affinity-purified anti-Nur77 antibodies (1:500), anti-GFAP antibodies (1:1000), anti-PCNA antibodies (1:20 000) and anti-GAPDH antibodies (1:2000; all antibodies were from Cell Signaling Technology, USA) were diluted in TBS-T supplemented with 0.5% nonfat dry milk and 0.1% Tween-20. Appropriate goat anti-rabbit or mouse IgG (Amersham Bioscience, USA), diluted 1:1000, was applied as the secondary antibody. Blots were visualized with enhanced chemiluminescence (ECL) and exposed on hypersensitive ECL film.

RNA interference (RNAi) of Nur77 on C6 cells

The *nur77* ShortCut siRNA Mix kit (New England Biolabs, Ipswich, MA, USA) was used to deplete *nur77*. Cells were transfected with si-*nur77* (20 nmol/L, 48 h) using Lipofectamine 2000 reagent (Invitrogen). Down-regulation of the total *nur77* was evaluated by RT-PCR, total cell lysates were collected, and the protein expression of Nur77, GFAP, and PCNA was analyzed by Western blotting.

Overexpression of Nur77 in C6 cells

An NR4A1 ORF cDNA clone of Mus musculus pCMV6 Entry Vector (OriGene Technologies Inc, Rockville, MD) was used. Vectors were transfected using Lipofectamine 2000 reagent following the recommended protocol. Six hours after the transfection, serum-free DMEM containing tetracycline was added. The medium was replaced with serum-containing DMEM without tetracycline 24 h after the transfection.

Cell cycle analysis

Cell cycle was assessed as described previously[5] with minor modifications. Briefly, cells (2×10^6–3×10^6) were harvested by trypsinization, washed twice with cool phosphate buffered saline (PBS) and fixed in 75% ethanol overnight at 4 °C. Cells were then incubated in a solution containing the DNA-binding dye propidium iodide (PI, 50 mg/mL), RNase (4 kU/mL), NaF (0.3 mg/mL) and sodium citrate (1 mg/mL) for 30 min at 37 °C in the dark. Finally, red fluorescence resulting from the laser-excited PI at 488 nm in each cell was analyzed by the EPICS ALTRA flow cytometer (Beckman Coulter, Fullerton, CA). A peak fluorescence gate was used to discriminate aggregates. The percentage of cells in G_0/G_1, S, and G_2/M were determined by DNA content histograms using Multicycle for Windows (Phoenix Flow Systems, San Diego, CA).

Statistical analysis

Data from three separate experiments were presented as mean±SD. Statistical significance was determined using Student's *t*-test. Results with a *P* value less than 0.05 were considered statistically significant.

Results

Cholera toxin-induced differentiation of C6 glioma cells

Differentiation of rat C6 glioma cells was first characterized by morphological transformation. Microscopic images of C6 glioma cells treated with 10 ng/mL cholera toxin for 0, 3, 6, 12, 24, and 48 h, respectively, are shown in Figure 1A. The morphology of the C6 cells gradually became similar to that of mature astrocytes during prolonged exposure to cholera toxin. Compared with the mainly polygonal morphology of the control cells, C6 cells treated with cholera toxin had smaller round nuclei and much longer, fine, tapering bodies. This result indicated that cholera toxin can induce glioma cell differentiation into more mature astrocytes.

We then examined the expression of GFAP, a well-estab-

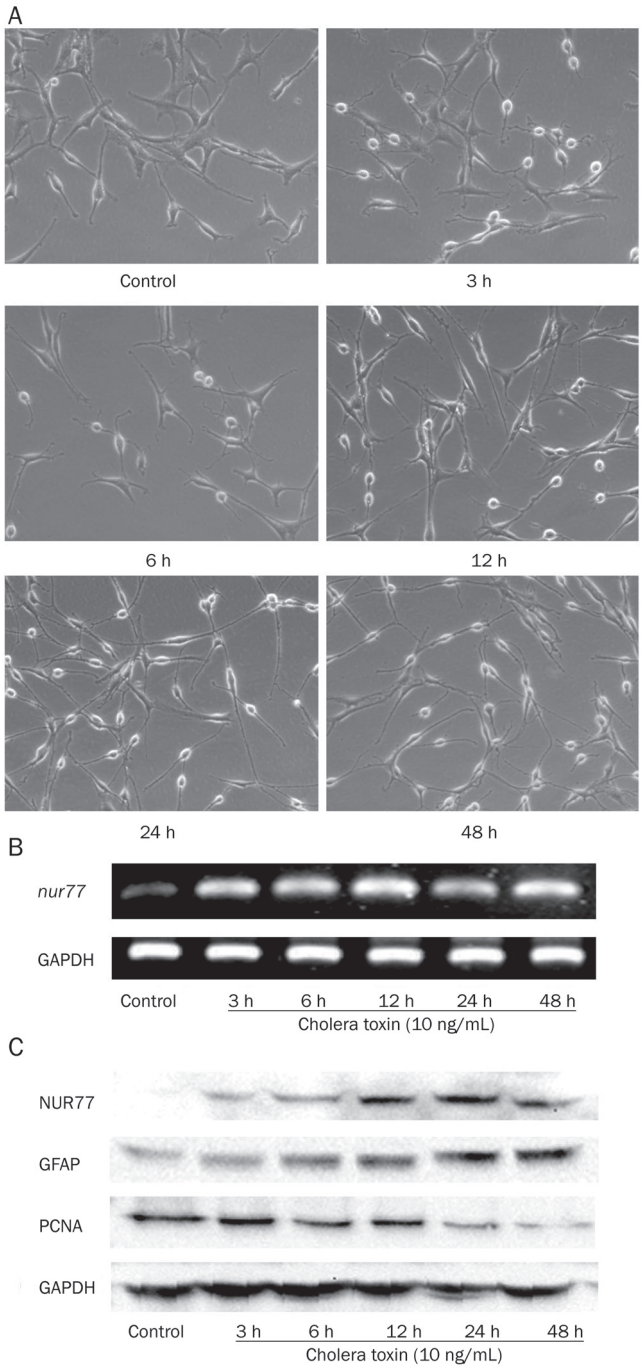

Figure 1. Induction of *nur77* expression during C6 glioma cells differentiation. A) C6 glioma cell differentiation was induced by cholera toxin in a time-dependent manner. Time represents the number of hours the cells were incubated with cholera toxin (CT, 10 ng/mL). B) Nur77 was up-regulated during the induced differentiation of C6 cells. C) Nur77 and GFAP were up-regulated and PCNA was down-regulated during the induced differentiation of C6 cells.

lished marker of mature astrocytes, and PCNA, a typical proliferation-associated protein in malignant proliferating cells. Compared with the control, Western blot analysis confirmed a significant up-regulation of GFAP expression in cholera toxin-treated cells in a positive time-dependent manner (Figure 1C), whereas the expression of PCNA was notably restrained in a negative time-dependent manner. The results indicated that the malignant proliferation of C6 cells slowed down and the characteristics of normal astrocyte cells emerged.

Gene expression changed rapidly and robustly in cholera toxin-induced differentiation

C6 cells displayed a robust transcription response, and the expression of many genes was changed. At the 3, 6, 12, 24, and 48 h time-points, we found 61, 149, 266, 682, and 506 up-regulated genes and 123, 192, 247, 491, and 291 down-regulated genes, respectively. In response to cholera toxin, the cascade of expression changes gradually became obvious. In the meantime, the number of involved pathways and Gene Ontology database count was also increased (Figure 2).

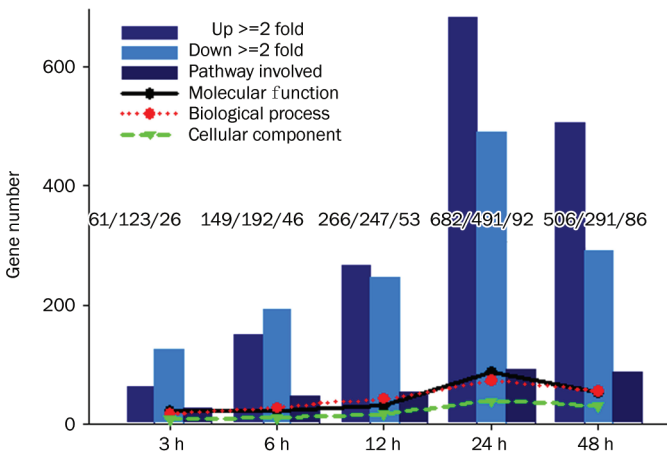

Figure 2. Gene expression changed rapidly and robustly during cholera toxin-induced differentiation. At the 3, 6, 12, 24, and 48 h time-points, there were 61, 149, 266, 682, and 506 up-regulated genes and 123, 192, 247, 491, and 291 down-regulated genes, respectively. Related pathways were increased from 26, 46, 53, 92, to 86 (shown with column). When examined in a gene ontology (GO) database, the genes with altered expression could be classified into three main functional groups: molecular function, biological process and cellular component. The number of gene in each group (shown with a line, detail not shown) was correspondingly changed during the induced differentiation process.

We then observed the gene with stably expression profiles (in a different time course) during the differentiation of C6 cells. The results revealed that 22 genes were consistently up-regulated and 7 genes were down-regulated during the entire differentiation process (Table 1). Most of the genes were transcribed loci and early response genes. Of these, two members of the nuclear receptor subfamily 4 group A, NR4A1 (Nur77) ,and NR4A3 (Nor1), were dramatically up-regulated from the beginning and persisted throughout the differentiation process. Due to its quick response and important function, NR4A1 (Nur77) was chosen for further analysis.

Table 1. Genes with constant expression change during the whole course.

Probe Set ID	Gene Symbol	Gene Title	3 h	6 h	12 h	24 h	48 h
1386935_at	Nr4a1	Nuclear receptor subfamily 4, group A, member 1	1.6	1.4	1.2	1.7	1.5
1390969_at	---	Transcribed locus	1.0	1.2	1.4	1.5	1.5
1375043_at	Fos	FBJ murine osteosarcoma viral oncogene homolog	1.3	1.2	1.4	2.8	2.5
1371091_at	Irs2	Insulin receptor substrate 2	1.3	1.5	1.5	2.1	1.5
1393478_at	---	Transcribed locus	1.5	2.4	1.5	1.6	1.0
1383954_at	---	Transcribed locus	1.1	1.8	1.6	1.9	1.9
1395023_at	---	Transcribed locus	1.9	2.6	1.7	1.6	1.4
1377508_at	Igsf11	Immunoglobulin superfamily, member 11	1.5	2.0	1.8	1.9	2.6
1383137_at	Sox4_p	SRY-box containing gene 4 (predicted)	1.3	1.8	1.9	1.8	1.2
1384260_at	---	---	1.3	1.9	1.9	2.2	2.1
1369067_at	Nr4a3	Nuclear receptor subfamily 4, group A, member 3	3.4	2.9	1.8	1.7	1.0
1369737_at	Crem	cAMP responsive element modulator	1.5	2.1	1.9	1.1	1.1
1373860_at	Sox4_p	SRY-box containing gene 4 (predicted)	1.0	1.8	2.0	1.7	1.0
1393571_at	---	Transcribed locus	1.7	2.6	2.0	2.2	1.4
1392791_at	Egr3	Early growth response 3	1.5	1.9	2.1	3.7	3.2
1374816_at	LOC363091	Similar to hypothetical protein FLJ30973	1.1	1.9	2.2	2.4	1.6
1384000_at	Sox4_p	SRY-box containing gene 4 (predicted)	1.5	2.2	2.2	2.0	1.3
1374157_at	Rgs8	Regulator of G-protein signaling 8	2.2	2.3	2.2	2.5	1.8
1374176_at	RGD1308059	Similar to DNA segment, Chr 4, Brigham & Womens Genetics 0951 expressed	1.7	2.7	2.3	2.1	1.5
1369044_a_at	Pde4b	Phosphodiesterase 4B, cAMP specific	2.0	2.4	2.4	2.5	1.9
1382072_at	LOC690485	Similar to cis-Golgi matrix protein GM130	2.3	3.7	4.2	3.7	3.4
1382819_at	Tyrp1	Tyrosinase-related protein 1(mapped)	2.4	4.5	6.3	8.0	7.0
1373777_at	Rgs16	Regulator of G-protein signaling 16	-2.9	-2.9	-2.9	-2.9	-3.3
1372213_at	LOC500300	Similar to hypothetical protein MGC6835	-1.8	-3.4	-2.4	-2.4	-2.2
1370834_at	Hs3st1	Heparan sulfate (glucosamine) 3-O-sulfotransferase 1	-1.4	-1.7	-2.1	-1.9	-1.5
1379470_at	---	Transcribed locus	-1.2	-1.1	-1.9	-1.1	-1.0
1378001_at	---	Transcribed locus	-1.1	-1.5	-2.0	-2.2	-3.2
1378867_at	RGD1307366	Similar to CG32425-PA (predicted)	-1.1	-1.9	-1.6	-1.4	-1.1
1387260_at	Klf4	Kruppel-like factor 4 (gut)	-1.0	-1.6	-1.9	-1.2	-1.1
1391948_at	Bcl11b_p	B-cell leukemia/lymphoma 11B (predicted)	-1.0	-1.2	-1.6	-2.1	-1.8
1395775_at	---	Transcribed locus	-1.0	-2.0	-1.6	-1.8	-1.7

Validation of the Affymetrix expression data by RT-PCR and Western blot analysis

To validate the data obtained from the Affymetrix gene expression array, both mRNA expression and protein levels of *nur77* in C6 cells were examined. The results showed that *nur77* was rapidly and stably induced in response to cholera toxin both in RT-PCR and Western blot (Figure 1B, 1C). As we anticipated, the mRNA and the protein levels of *nur77* were concordant with the mRNA microarray results.

Silencing *nur77* attenuated the differentiation of C6 glioma cells induced by cholera toxin

In order to address the role of *nur77* in cholera toxin-induced differentiation, the expression of *nur77* was inhibited by RNA interference. Small synthetic interfering RNA targeting rat *nur77* and non-sense siRNA were transfected into C6 cells 24 h before the cholera toxin was added. Transfection efficiency, as monitored by transfection of FAM-labeled siRNA in a separate well, was approximately 90%.

After incubation with cholera toxin for 24 h, morphological changes induced by cholera toxin were attenuated due to the silencing of *nur77*. Down-regulating *nur77* before cholera toxin was added reversed the development of a maturate morphology (Figure 3A), compared to the induced C6 cells, which were similar to the mature astrocytes. The flat polygonal appearance of si-*nur77* cells was more similar to the shape of normal C6 cells than that of astrocytes. Meanwhile, the up-regulation of GFAP and down-regulation of PCNA protein induced by cholera toxin were markedly reduced by si-*nur77* (Figure 3C).

Cholera toxin-induced differentiation resulted in G_1/S arrest with an increase from 66.9% to 90.4% in the proportion of cells in G_1 phase and a decrease from 28% to 4.3% in the S phase, (Figure 3D, 3E). After we down-regulated *nur77*, the cell cycle arrest was abolished and the percentage of S phase cells returned to 22.7% (P<0.01 *vs* cholera toxin group), indicating that a high cell proliferation rate was recovered. In all groups, no obvious sub-G_1 peak (apoptotic peak) was shown by flow cytometry.

In summary, the above results indicated that the induced

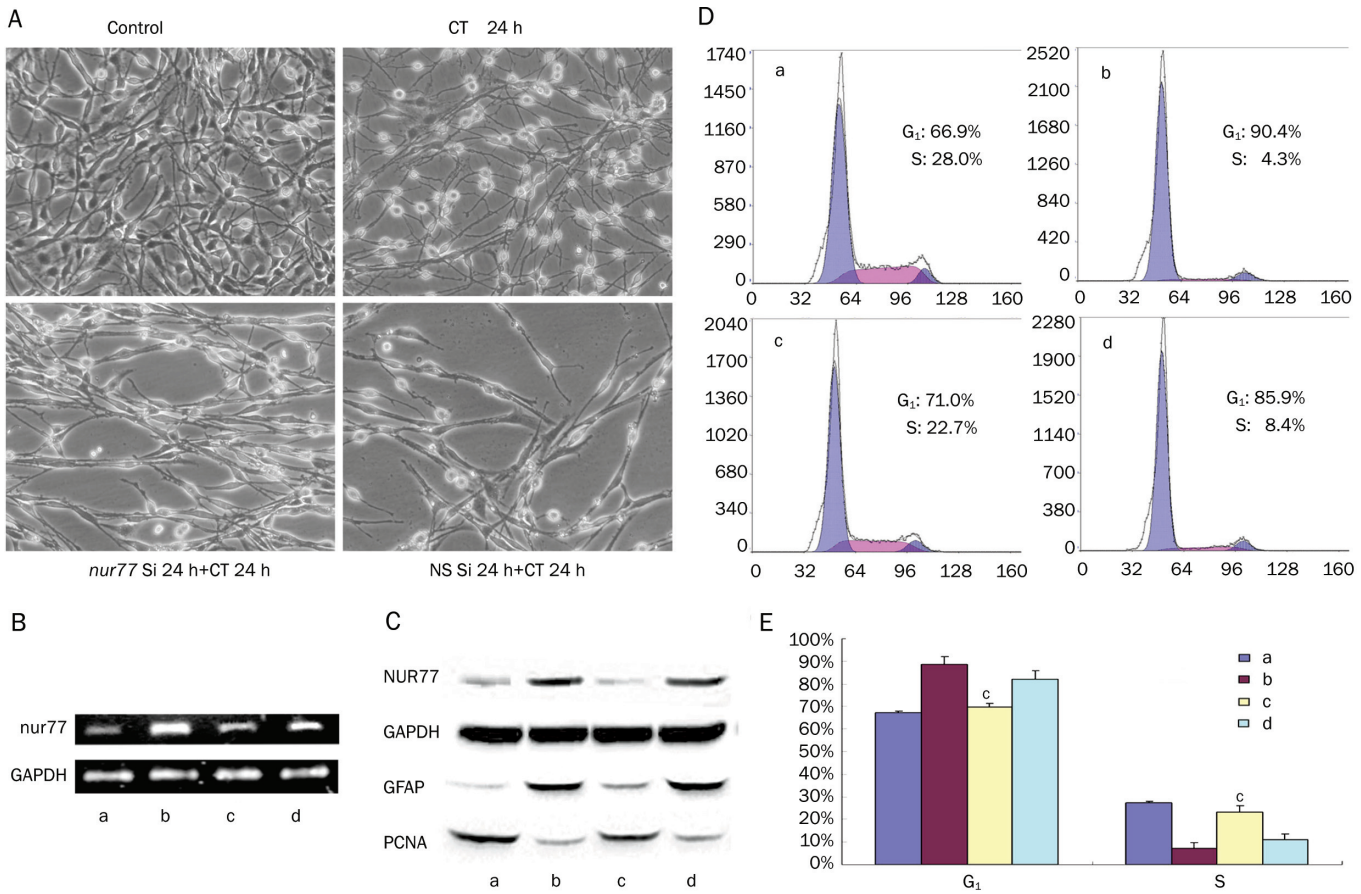

Figure 3. Nur77 was required for cholera toxin-induced differentiation of C6 glioma cells. C6 cells were grown for 24 h without (group a) or with cholera toxin (10 ng/mL, group b). Cells were transfected with *nur77* siRNA (group c) or non-sense siRNA (group d) for 24 h and then incubated with cholera toxin (10 ng/mL) for another 24 h. (A) Morphological changes induced by cholera toxin were attenuated by down-regulation of *nur77*. (B) Nur77 expression was inhibited by RNA interference. (C) Down-regulating Nur77 inhibited cholera toxin-induced GFAP expression, and cells recovered the high level of PCNA. (D, E) Down-regulating Nur77 abolished cholera toxin-induced cell cycle arrest. Results are expressed as means±SD (n=3) for control. ^cP<0.01 *vs* cholera toxin group.

differentiation effect of cholera toxin was attenuated by down-regulating *nur77*.

Overexpression of Nur77 induced differentiation behavior in C6 cells

To determine whether *nur77* leads to differentiation, we introduced a *nur77* ORF plasmid into C6 cells to achieve a robust expression of *nur77*. Overexpression of *nur77* was able to mimic the cholera toxin-induced differentiation phenotype (Figure 4). In the *nur77* overexpression group, most C6 cells were morphologically similar to the cholera toxin-treated group, showing smaller, round nuclei and fine stretched arms (Figure 4A). The increase of GFAP and the decrease of PCNA protein (Figure 4C) were coincident with that change. The S phase fraction decreased from the normal 21.8% to 9.0% (P< 0.01 *vs* control Figure 4D, 4E), and the fraction of G_1 phase cells rose from 69.0% to 87.5% (P<0.01 *vs* control). These changes indicated that most cells had exited the active proliferating phase and the cell cycle was arrested.

The results showed that the overexpression of nur77 could induce the differentiation of C6 glioma cells, which was analogous to the effects of cholera toxin.

Discussion

The rat C6 cell line is one of the most well-established glioma cell lines and has an undifferentiated phenotype and oligodendrocytic, astrocytic, and neuronal properties. Therefore, C6 cells are a useful model for studies of glial cell differentiation[6]. Cholera toxin triggers C6 cells to transform into a more mature astrocytic state, and this transformation can be measured by the biomarkers GFAP and PCNA.

We first analyzed the gene expression profiles of C6 glioma cells during cholera toxin-induced differentiation. The results provided us with an overall gene expression database for further studies on C6 glioma differentiation.

The gene expression profile revealed that the expression levels of more than 1000 genes (>3% of analyzed genes) changed during the C6 differentiation process. Massive numbers of

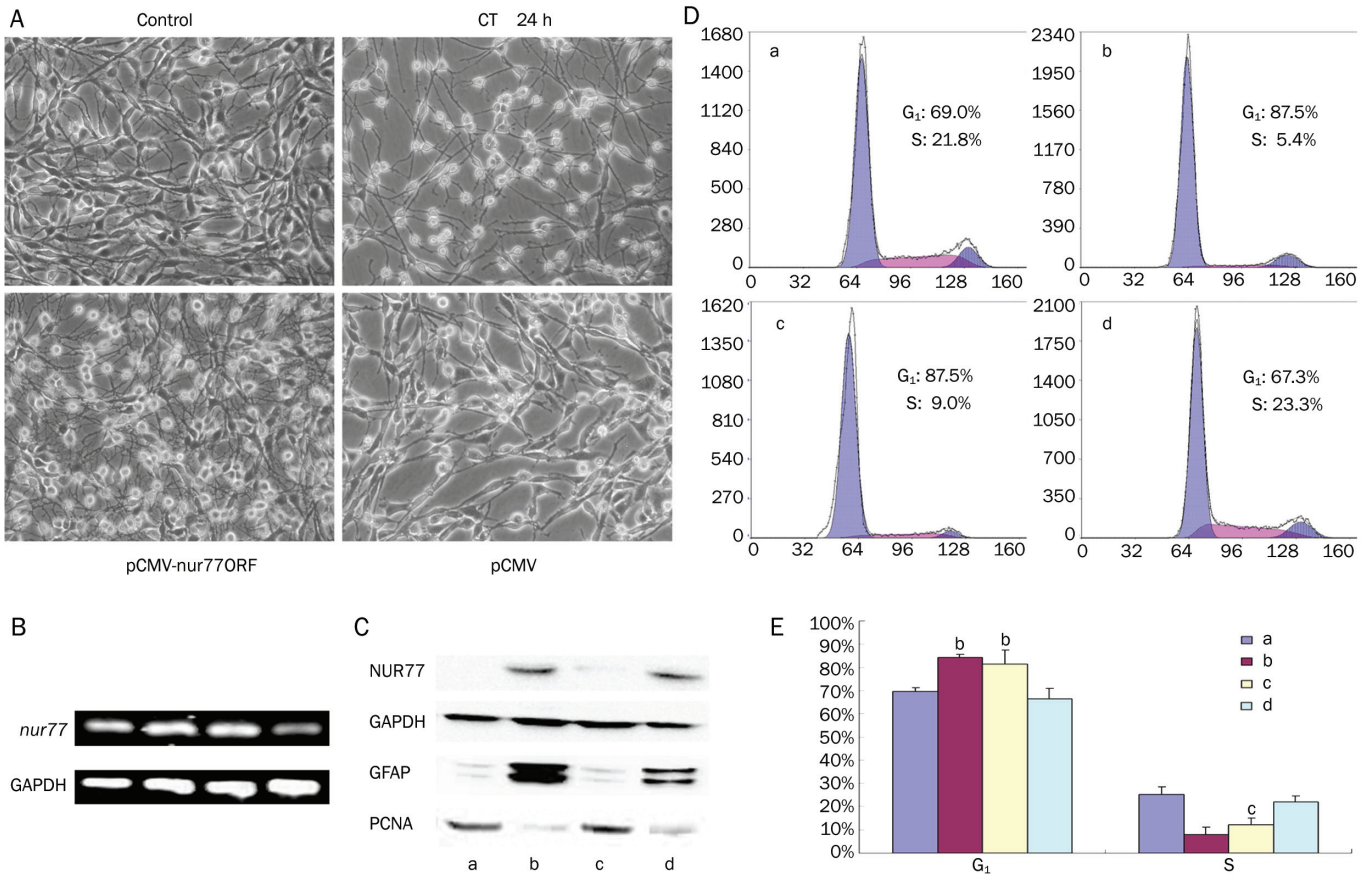

Figure 4. Overexpression of *nur77* promoted differentiation of C6 glioma cells. C6 cells were grown for 24 h without (group a) or with cholera toxin (10 ng/mL, group b). Cells were transfected with *nur77* ORF pCMV6 vector (group c) or empty pCMV6 vector (group d) for 6 h, and then changed to normal DMEM (+1% FBS) and analyzed 24 h later. (A) When *nur77* was overexpressed, the C6 glioma cells in group C adopted a differentiated morphology that was distinguishable from normal C6 cells. (B) Nur77 was overexpressed using the NR4A1 ORF cDNA clone of the pCMV6 entry vector. (C) Overexpression of *nur77* enhanced GFAP expression and down-regulated PCNA expression. (D, E) Overexpression of *nur77* resulted in cell cycle arrest similar to the cholera toxin effect. Results are expressed as means±SD (n=3) for control. $^{b}P<0.05$, $^{c}P<0.01$ *vs* control.

consecutive downstream genes and pathways were involved in the induced differentiation. At this point, further studies were required to demonstrate how these genes were involved in the differentiation. Among these differentially expressed genes, stable changes were less common. Only 22 genes were stably up-regulated and 7 genes were stably down-regulated. Most of these genes were involved in transcription or were regulator genes. Based on their original functions and the stable high expression, it is reasonable to assume that they bear more responsibility for C6 cell differentiation.

Among the genes involved in transcription regulation, nuclear receptors were particularly important in coordinating the changes in the environmental milieu. The ability to sense and rapidly respond to changes in the cellular environment appears to be a hallmark of this subfamily. The up-regulated expression of *nur77* and *nor1* was 1.6- and 3.4-fold that of the control at the third hour, respectively. Our RT-PCR results coincided with the microarray data. The change was significant throughout the differentiation process, indicating that they were actively involved in the induced differentiation of C6 cells.

Nur77 (also referred to as NR4A1, NGFI-B, or TR3) belongs to the orphan nuclear receptor subfamily 4, group A (NR4A). The subfamily consists of two other isotypes, commonly known as Nurr1 (NR4A2) and Nor1 (NR4A3). Functions of NR4As include neuroendocrine regulation, neural differentiation, liver regeneration, apoptosis, and mitogenic and inflammatory stimulation[7–10]. Nur77, Nurr1, and Nor1 are typical immediate-early response genes, which can be quickly induced by a variety of extracellular stimuli[11, 12]. Although Nur77 has often been reported as an important apoptosis-promoting factor in the negative selection of T cells[12, 13] or tumor cell lines such as melanoma cells[14] and ovarian cancer cells[15], evidence for its role in cell apoptosis was not found in our induced differentiation model, indicating that the expression of nur77 did not cause apoptosis here.

Nur77 is expressed mainly in the adult mammalian brain[16] and can be induced by many growth factors in different cell types. The different expression patterns of Nur77 during brain development and tissue distribution indicate that it

might reflect a different transcriptional role in the brain[16, 17]. For example, as a transcription factor, Nur77 plays an important role in nerve growth factor(NGF)-induced PC12 cell differentiation[18]. We demonstrated that *nur77* was rapidly and constantly up-regulated during the differentiation of C6 glioma cells induced by cholera toxin. These findings indicated that *nur77* exerted its considerable differentiation-promoting effect not only in normal brain cells, but also in malignant tumor cells, *eg*, in C6 glioma cells.

When we silenced *nur77* by RNAi, the induced differentiation was weakened significantly. Overexpression of *nur77* reliably promoted differentiation in C6 cells, resulting in the display of the appropriate cellular phenotype. These bidirectional experimental results proved that *nur77* was necessary and indispensable for the induced differentiation of C6 cells.

Recently, a natural compound, *n*-butylidenephthalide, isolated from the chloroform extract of *Angelica sinensis*, has been investigated for its antitumor effects on GBM both *in vitro* and *in vivo*. Nur77 was found to be a possible target gene of this effect[19], identifying *nur77* as a potential gene target in differentiation research and therapy.

In conclusion, we found by using microarray analyses that the nuclear receptor gene *nur77* was highly induced during the differentiation of C6 cells. We demonstrated the importance of *nur77* by down- and up-regulation experiments, leading us to speculate that it may play an important role in the induced differentiation of glioma cells. Further exploration into the regulated cascade may help to reveal the actual role of *nur77* in the differentiation of C6 glioma cells.

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Author contribution

Guang-mei YAN designed research; Dong XU and Yi-jun HUANG performed research; Yan LI and Wei YIN contributed new analytical tools and reagents. Dong XU analyzed data and wrote the paper.

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