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Transcriptional and Post-Transcriptional Down-Regulation of Cyclin D1 Contributes to C6 Glioma Cell Differentiation Induced by Forskolin

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ABSTRACT

Malignant gliomas are the most common and lethal intracranial tumors, and differentiation therapy shows great potential to be a promising candidate for their treatment. Here, we have elaborated that a PKA activator, forskolin, represses cell growth via cell cycle arrest in the G0/G1 phase and induces cell differentiation characteristic with elongated processes and restoration of GFAP expression. In mechanisms, we verified that forskolin significantly diminishes the mRNA and protein level of a key cell cycle regulator cyclin D1, and maintenance of low cyclin D1 expression level was required for forskolin-induced proliferation inhibition and differentiation by gain and loss of function approaches. In addition, that forskolin down-regulated the cyclin D1 by proteolytic (post-transcriptional) mechanisms was dependent on GSK-3β activation at Ser9. The pro-differentiation activity of forskolin and related molecular mechanisms imply that forskolin can be developed into a candidate for the future in differentiation therapy of glioma, and cyclin D1 is a promising target for pro-differentiation strategy. J. Cell. Biochem. 112: 2241–2249, 2011. © 2011 Wiley-Liss, Inc.

KEY WORDS: CYCLIN D1; GSK-3β; GLIOMA; FORSKOLIN; DIFFERENTIATION; CELL CYCLE

Malignant gliomas are the most common primary tumors in the central nervous systems, and lead as the second most common cause of cancer-related deaths in children and young adults [Holland, 2000; Prados and Levin, 2000; Wechsler-Reya and Scott, 2001]. The infiltrative growth pattern of these tumors precludes curative neurosurgery, and tumor cells are usually resistant to irradiation, chemotherapy, or immunotherapy [Nicholas, 2007]. Additional considerations focus on developing novel modalities to increase anti-glioma effects and decrease side-effects.

Differentiation therapy changes the destructive behavior of cancer cells through induction of a proliferative capacity loss and terminal differentiation or apoptosis [Spira and Carducci, 2003]. This therapy has shown superiority to surgery and cytotoxic therapy to a certain extent and attracted a lot of interests. The most successful clinical application of differentiation therapy has been the introduction of all-trans-retinoic acid for the treatment of acute promyelocytic leukemia (APL) [Waxman, 2000]. Recently, induction of cellular differentiation has become promising therapeutic strategy against solid tumor including glioma [Cuevas et al., 2004; Kawamata et al., 2006].

Cyclin D1 is one of the positive cell cycle regulators necessary for the transition of cells from G1 to S phase and overexpression of cyclin D1 is one of the most commonly observed alterations in human cancer [Pines, 1999; Diehl, 2002]. It binds with cyclin-dependent kinases 4/6 (CDK 4/6) to form an activated complex that functions as a promoter of progression into S phase transition by phosphorylating retinoblastoma protein (Rb) and then releasing

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transcription factor E2F1 to initiate genes transcription required for S phase [Tashiro et al., 2007]. Recent studies have shown that D-type cyclins function not only as components of the cell-cycle core machinery but also function as co-factors for several transcription factors. In fact, it has been reported that cyclin D1 inhibits adipocyte differentiation by repressing the expression and transactivation of peroxisome proliferator-activated receptor-γ(PPAR-γ) [Wang et al., 2003]. In addition, the bioavailability of cyclin D1 is regulated at the genetic level by transcriptional modulation and also through proteolysis following phosphatidylinositol 3-kinase (PI3K)/Akt/glucose synthase kinase-3β (GSK-3β) pathway activation [Takahashi-Yanaga and Sasaguri, 2008]. Taken together, in the genesis and development of cancer, cyclin D1 is considered as a cellular proto-oncogene and may be developed as a candidate target to inhibit cell proliferation and induce cell differentiation in cancer therapy.

In our previous work, we discovered that cholera toxin, a cAMP/PKA pathway activator, induces C6 glioma cell differentiation [Li et al., 2007]. Herein, we demonstrate that an adenylate cyclase (AC) activator, forskolin, which also elevates the cAMP level and then initiates PKA pathway, caused cell growth inhibition and cell differentiation of C6 glioma cells via the dual regulation of cyclin D1 by genetic and proteolytic mechanisms.

MATERIALS AND METHODS

CELL CULTURE AND DRUG TREATMENT

C6 rat glioma cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in DMEM (Invitrogen, Grand Island, NY) supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37°C.

C6 cell differentiation was induced by the indicated concentration of forskolin (Sigma–Aldrich, St. Louis, MO) in DMEM containing 1% FBS for specific durations. Control was treated with an equivalent volume of DMEM containing 1% FBS.

MORPHOLOGICAL EVALUATION

The morphologiy of the cells was studied using an Olympus (Melville, NY) IX71 inverted microscope along with Olympus DP Controller software.

CELL VIABILITY ASSAY

The viability of cells was determined by the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich) assay. Briefly, cells were seeded in 96-well plates at 2,000 cells/well and incubated overnight. After different treatment for various times, 20 μl MTT solution (5 mg/ml in PBS) was added to each well to induce the production of formazan crystals. MTT solution was aspired off after 4 h and 100 μl DMSO was added to achieve solubilization of the formazan crystal. The optical density (OD) was determined at 570 nm using an EXL800 microimmunoanalyser (Bio-Tek Instruments, Burlington, VT). The cell viability rate = ODexperiment/ODcontrol × 100%.

CELL CYCLE ANALYSIS

A flow cytometry analysis of the DNA content of the cells was performed to assess the cell cycle phase distributions as described [Roz et al., 2002]. In brief, the cells were collected by trypsinization, washed in PBS, and fixed in 70% ethanol for 30 min at 4°C. After washing with PBS, cells were incubated with the DNA-binding dye propidium iodide (50 μg/ml) and RNase (1.0 mg/ml) for 30 min at 37°C in the dark. Finally, cells were washed and red fluorescence was analyzed by a FACSCalibur flow cytometer (Beckman Coulter Inc) using a peak fluorescence gate to discriminate aggregates.

WESTERN BLOT AND IMMUNOCOPRECIPITATION ANALYSIS

After lysis of cells and measurement of protein concentration, the cells were dissolved in SDS sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mM DTT and 0.1% bromophenol blue). Equal amount of proteins were analyzed by SDS–PAGE on 12% polyacrylamide gels. Proteins were electro-blotted onto a nitrocellulose membrane. The membranes were incubated in 5% nonfat dry milk in TBST (Tris-buffer saline, 0.05% Tween-20) and then overnight at 4°C with antibodies against GFAP, cyclin D1, p-GSK-3β, GSK-3β, CDK4, p27 (1:1,000; Cell Signaling Technology, Beverly, MA), p-Rb, ubiquitin, CDK6 (1:500; Santa Cruz, CA) and β-actin (1:1,000; Neomarker, CA).

The immunoprecipitation experiment was carried out as described [Othumpangat et al., 2005]. In brief, the cells were washed twice with ice-cold phosphate-buffered saline, and lysates were prepared. Immunoprecipitation was carried out for 1 h at 4°C using a polyclonal rabbit anti-mouse ubiquitin antibody (Santa Cruz) and protein A-Sepharose beads (Amersham Biosciences) following procedures as described elsewhere. The immunoprecipitated proteins conjugated to the protein A-Sepharose beads were washed twice with lysis buffer and then boiled with denaturing SDS–PAGE loading buffer. The supernatant was collected and used for Western blot analysis using rabbit cyclin D1 antibody.

RNA EXTRACTION AND REAL-TIME RT-PCR ANALYSIS

Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA) according to manufacturer's protocol. Purity and integrity of all isolated RNA samples were analyzed by using agarose gel electrophoresis. The first strand of the cDNA was synthesized using the Superscript™ II reverse transcriptase kit (Gibco-BRL, USA) with Oligo-dT as the primer following the manufacturer's instructions. Sequences of the PCR primers are as follows: Cyclin D1: 5′-GTG CAT GCC ACC ATG CT-3′ (sense primer), 5′-CTC AGC TAA GCT GGT GGT-3′ (antisense primer). GAPDH: 5′-TCA CCA TCT TCC AGG AGC GAG A-3′ (sense primer), 5′-ATG AGC CCT TCC ACG ATG C-3′ (antisense primer). The PCR amplification, detection of the amplified gene products, and their quantitation was performed with the SYBR green PCR kit (PE Applied Biosystems, Foster City, CA) and the ABI-PRISM 7300 sequence detection system following the instructions provided by the manufacturer. The thermal cycler conditions were as follows: 10 min at 95°C, followed by 40 cycles at 95°C for 15 s, 65°C for 30 s, 72°C for 15 s, followed by 60°C for 1 min. The mRNA expression levels of cyclin D1, calculated using the formula, 2-DDCT Method [Livak and Schmittgen, 2001], were normalized to the expression level of the housekeeping gene, GAPDH.

CONSTRUCTION OF TRANSGENIC CYCLIN D1-C6 CELL LINES

pcDNA 3.0 and pcDNA 3.0-cyclin D1 were purchased from Addgene company. Plasmid DNA prepared using the Qiagen maxi preparation kit (Qiagen) was used to transfect C6 cells utilizing Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's protocol. Stable transfectants were selected by culturing the transfected cells in medium containing G418 (Invitrogen) at a final concentration of 1 mg/ml, and cell lines were developed individually from surviving colonies. Overexpression of the cDNA-encoded protein was determined by Western blot analysis.

RNA SILENCING OF GSK-3β AND CYCLIN D1

The endogenous GSK-3β and cyclin D1 expression in C6 cells was silenced utilizing the small interfering RNA (siRNA). The target sequences of double-stranded nucleotides used for siRNA knock-down were GCA GCA AGG UAA CCA CAG U for GSK-3β (RIBOBIO, Guangzhou, Guangdong, China), CCA CAG ATG TGA AGT TCA T for cyclin D1 (RIBOBIO) and ATC CAA TGG CAC CGT CAA G for GAPDH (RIBOBIO). C6 cells with 30–50% confluence were transfected using 50 μM siRNA and Lipofectamine RNAi MAX reagent (Invitrogen) following the manufacturer's protocol. Thirty-six hours after the transfection, cells were treated with or without forskolin for another 48 h. The total protein extracts from the cells were used for Western blot analysis.

IMMUNOCYTOCHEMISTRY

Immunocytochemistry was performed as described [Zhang et al., 2008]. Briefly, cells were fixed with 4% paraformaldehyde, blocked with 5% BSA and incubated overnight with rabbit GFAP antibody (1:200; Sigma–Aldrich). A biotin-marked secondary antibody (Boster Biological Technology, Wuhan, China) was applied for 30 min, followed by peroxidase-marked streptavidin (Boster Biological Technology, Wuhan, China) for additional 10 min.

STATISTICAL ANALYSIS

Data are presented as mean ± standard deviation (SD) of three separate experiments. Statistical significance was determined by ANOVA analysis. A result with a P-value of <0.05 was considered statistically significant.

RESULTS

FORSKOLIN-INDUCED DIFFERENTIATION OF C6 GLIOMA CELLS

Differentiation of rat C6 glioma cells toward astrocyte type is characterized by the morphological transformation and expression of the differentiation marker. Microscopic observation of C6 glioma cells treated with 10 μM Forskolin for 48 h revealed alteration in morphology such as mature astrocytes with smaller round body and much longer, fine, tapering processes (Fig. 1A). We further examined the expressions of glial fibrillary acid protein (GFAP), a well-established marker of mature astrocytes. Immunocytochemistry with anti-GFAP antibody showed that few cells were stained positive in the C6 glioma cells, but numerous cells treated with forskolin were stained positive in the cytoplasm (Fig. 1B). Western blotting analysis confirmed that forskolin induced a significant up-regulation of GFAP protein expression in a concentration- (Fig. 1C) and time-dependent manner (Fig. 1D). Forskolin reached its maximal induction effect at 10 μM and kept GFAP at the maximal

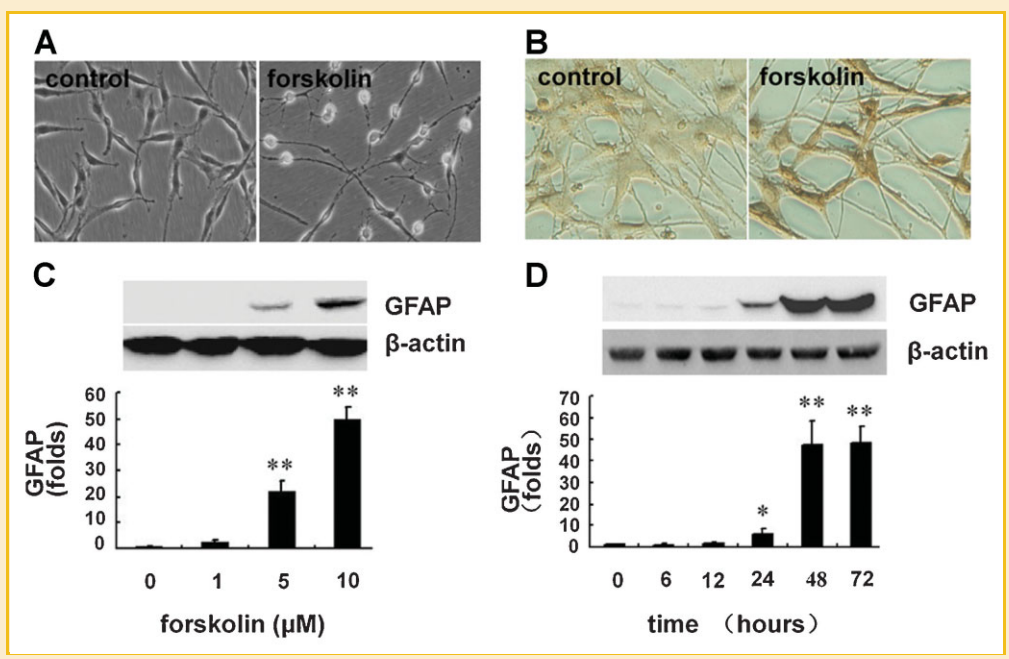

Fig. 1. Forskolin-induced differentiation of C6 glioma cells. A,B: C6 glioma cells were incubated with or without 10 μM forskolin for 48 h. A: Morphological transformation (original magnification: ×200). B: Immunocytochemistry of GFAP protein expression. C: Concentration-dependent effect of forskolin on GFAP protein by Western blot analysis. Cells were incubated with 0, 1, 5, and 10 μM forskolin for 48 h. D: Time-dependent effect of forskolin on GFAP protein. Cells were incubated with 10 μM forskolin for 6, 12, 24, 48, and 72 h. Results are means ± SD (n = 3). Statistical differences compared with the controls are given as $^{*}P<0.05$, $^{**}P<0.01$.

level after 48 h. According to the morphological alteration and the confirmation of differentiation marker, clearly, forskolin was capable of promoting C6 glioma cells differentiation into cells with astrocytic features.

FORSKOLIN INHIBITED PROLIFERATION OF C6 GLIOMA CELLS VIA INDUCING G0/G1-PHASE CELL CYCLE ARREST

Cell cycle progression and differentiation are opposing but tightly linked events. In particular, G1 exit is a critical stage of cell cycle where cells decide either to re-enter the next cell cycle or differentiate [Georgopoulou et al., 2006]. To test whether C6 cells slow down growth and accumulate in G0/G1 phase before differentiation, cell viability and cell cycle distribution was measured. As seen in Figure 2A, the C6 cells viability dropped to its minimum after 10 μM forskolin exposure for 48 h, and decreased within 72 h in a time-dependent manner. Then, the cell cycle analysis showed that the percentage of cells in the G1/G0 phase had significantly increased from 24 h, and kept the higher level after that time point. To state specifically, at 24, 48, and 72 h, the cell population in the G0/G1 phase accumulated to 86.7%, 84.1%, and 83.5% compared to the control 70.8%, while the proportion of cells in the S phase went down from 26.6% to 6.4%, 13.1%, and 14.5%, respectively (Fig. 2C). Obviously, forskolin inhibited cell growth of C6 cells and this inhibitory effect may be associated with induction of G0/G1 cell cycle exit.

FORSKOLIN DOWN-REGULATED THE CYCLIN D1 PROTEIN LEVEL BY TRANSCRIPTIONAL AND PROTEOLYTIC MECHANISMS

Cyclin D1 is believed to be one of the cell cycle checkpoints from G0/G1 phase to S phase, so we measured the protein expression of cyclin D1 first. In response to forskolin, the cyclin D1 protein decreased with dose (1–10 μM) and time (6–48 h, Fig. 3A,B). In the paralleled experiment, we observed that the phosphorylation of downstream protein Rb conspicuously decreased to a nearly undetected level, concurrent with the unaltered CDK4/6, indicating that the reduced cyclin D1 protein may be the main cause to repress the cyclin D1-CDK4/6-Rb pathway. Furthermore, the inhibitory protein to cyclin D1-CDK4/6 complex, p27 levels strikingly went up, which would further potentiate the suppression of the cyclin D1-CDK4/6-Rb pathway (Fig. 3C).

The down-regulation of cyclin D1 expression can occur by various mechanisms [Agami and Bernards, 2000]. To better delineate the mechanism of forskolin-mediated cyclin D1 down-regulation, amounts of cyclin D1 mRNA were analyzed by quantitative PCR. As seen in Figure 3D, forskolin-reduced cyclin D1 mRNA in a time-dependent manner from 3 to 12 h. Cyclin D1 mRNA dropped to 19.6% of control at 12 h after exposure to 10 μM forskolin, and then increased gradually. However, as compared with Figure 3B, the trend of cyclin D1 mRNA level with time was not completely consistent with that of protein level. Even when mRNA level began to climb up, protein level still kept going down, suggesting that forskolin may also regulate proteolysis of cyclin D1 besides inhibiting the transcriptional initiation.

Considering that proteasome-mediated proteolysis is an important post-translational regulation to cyclin D1 stabilization, we wished to determine the involvement of proteasome function in the degradation of cyclin D1. As shown in Figure 4A, a proteasome inhibitor, MG-132, led to a dramatic increase of cyclin D1 in cells untreated with forskolin, and resulted in abrogation of forskolin-mediated cyclin D1 inhibition that implied the involvement of proteasome activity. As proteins that are selected for proteasomal degradation are commonly marked by polyubiquitination [Hershko and Ciechanover, 1998; Pickart, 2001], we examined whether forskolin induced the ubiquitination of cyclin D1. As expected, we

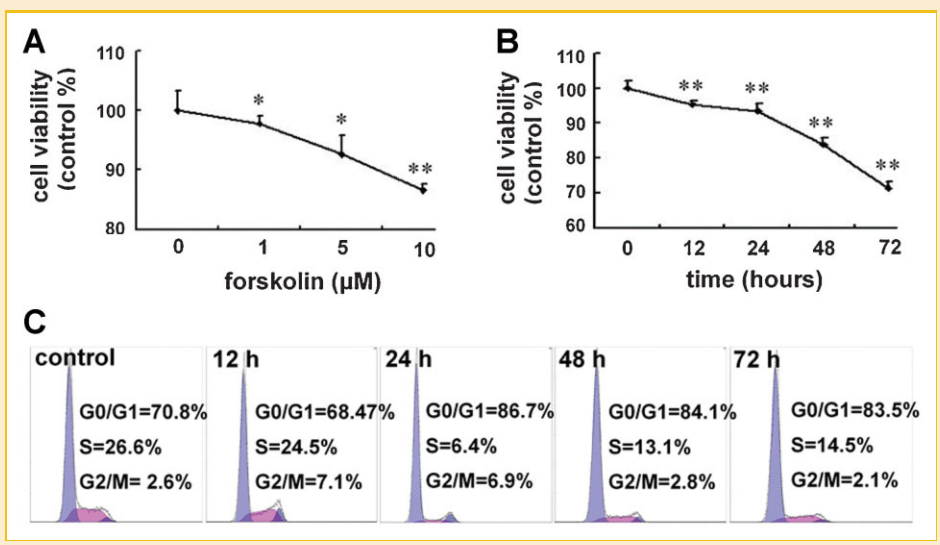

Fig. 2. Forskolin inhibited proliferation of C6 cells. A: Concentration-dependent effect of forskolin on the cell viability. C6 cells were treated with 10 μM forskolin for 0, 12, 24, 48, and 72 h. B: Time-dependent effect of forskolin on the cell viability. C6 cells were treated with 0, 1, 5, and 10 μM forskolin for 48 h. Results are means ± SD (n = 3). Statistical differences compared with the controls are given as $^{*}P < 0.05$, $^{**}P < 0.01$. C: Forskolin caused G0/G1 phase cell cycle arrest in C6 cells. The experiments were repeated three times, and the result of a representative experiment was presented.

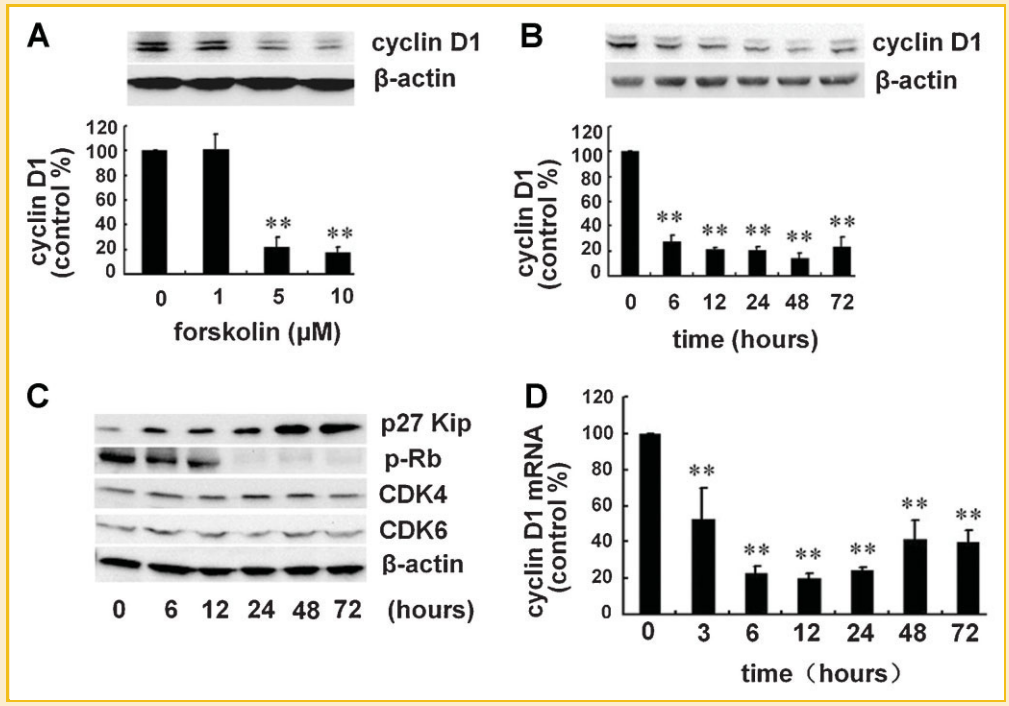

Fig. 3. Forskolin decreased the protein and mRNA levels of cyclin D1 in C6 cells. A,B: Concentration- and time-dependent effect of forskolin on cyclin D1 protein by Western blot analysis. Cells were treated as seen in Figure 1C,D. Results are means ± SD (n = 3). Statistical differences compared with the controls are given as $^{**}P < 0.01$. C: Effect of forskolin on $p27^{kip}$, p-Rb, CDK4, and CDK6 protein levels. C6 cells were incubated with 10 μM forskolin for indicated duration. D: Time-dependent effect of forskolin on cyclin D1 mRNA by RT-PCR. Results are means ± SD (n = 3). Statistical differences compared with the control are given as $^{**}P < 0.01$.

observed an increased accumulation of high molecular weight ubiquitinated proteins after forskolin treatment (Fig. 4B). Subsequently, using the immunoprecipitation assay, the conspicuously increased ubiquitination of cyclin D1 in forskolin-treated cells provided further evidences for the notion that forskolin down-regulated cyclin D1 protein via ubiquitination-dependent proteolysis (Fig. 4C). Collectively, these results suggest that forskolin accelerates cyclin D1 turnover at the protein level by inducing

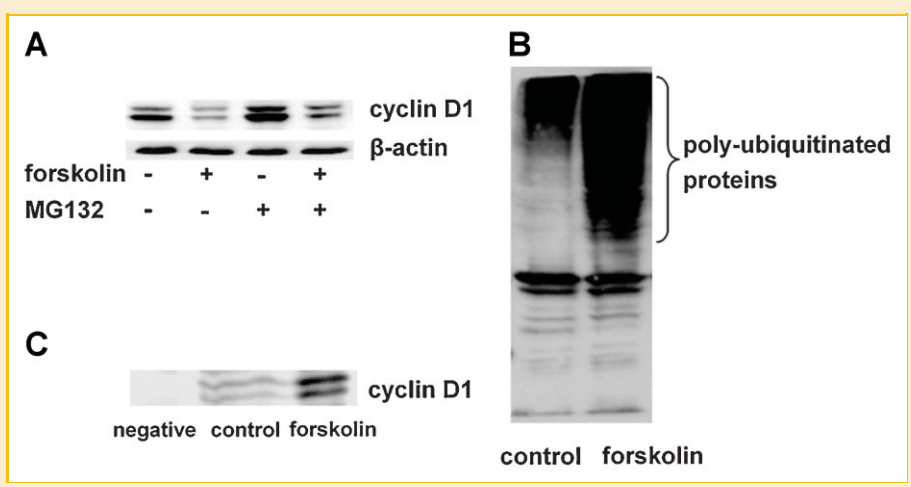

Fig. 4. The forskolin-induced degradation of cyclin D1 protein was due to protosome-mediated proteolysis. A: The decrease of cyclin D1 protein was partially reversed by proteasome inhibitor, MG132. C6 cells were pretreated with 10 μM MG132 for 1 h and treated with 10 μM forskolin for another 12 h. B,C: Exposure of C6 cells to 10 μM forskolin for 24 h increased the ubiquitination of cyclin D1. B: The level of ubiquitin in forskolin treated and untreated cells by Western blot analysis. C: The level of ubiquitinated cyclin D1 in forskolin treated and untreated cells by immunoprecipitation. Cell lysates were subjected to immunoprecipitation using an antibody for ubiquitin, and the presence of cyclin D1 protein in the immunoprecipitate was determined by Western blot analysis using a cyclin D1 antibody.

ubiquitination- and proteasome-dependent degradation of cyclin D1.

Taken together, all these observations indicated that the forskolin-induced reduction of cyclin D1 levels in C6 cells was mediated via transcriptional and proteolytic mechanisms.

FORSKOLIN DEGRADED CYCLIN D1 PROTEIN VIA INHIBITING GSK-3β PHOSPHORYLATION AT SER 9

Cyclin D1 gene expression can be regulated by Wnt/β-catenin/GSK-3β signaling, and proteasome-dependent proteolysis of cyclin D1 is well-known in a PI3K/Akt/GSK-3β-dependent mode [Diehl et al., 1998], suggesting that GSK-3βplays a critical role in sustaining cyclin D1 stability. The phosphorylated GSK-3β at Ser 9 is inactive to degrade cyclin D1 protein [Cohen and Frame, 2001], which means that the reduction of GSK-3β phosphorylation would restore its capability to degrade protein. Hence, we determined the amount of phosphorylated GSK-3β at Ser 9 and found that forskolin treatment decreased this level by 75.9% and 89.1% at 12 and 24 h, respectively (Fig. 5A), indicating that GSK-3β was activated by forskolin.

To probe the role of the GSK-3β pathway in cyclin D1 down-regulation mediated by forskolin, we first introduced a PI3K inhibitor LY294002, which can repress the phosphorylation of GSK-3β (Fig. 5B), to mimic the inhibitory effect of forskolin on pGSK-3β at Ser9, and the result is as we had expected. We further introduced LiCl, a known inhibitor of GSK-3β activation [Klein and Melton, 1996; Coghlan et al., 2000]. As shown in Figure 5C, LiCl almost completely inhibited the decline of cyclin D1 induced by forskolin via promoting GSK-3β phosphorylation. Moreover, GSK-3β siRNA inhibited the ability of GSK-3β to degrade cyclin D1 protein and thus prevented forskolin mediated decreases in cyclin D1 protein (Fig. 5D).

These findings provided molecular evidence indicating that GSK-3β was necessary for forskolin-induced down-regulation of cyclin D1.

CYCLIN D1 DOWN-REGULATION IS A REQUISITE FOR THE DIFFERENTIATION AND PROLIFERATION LOSS IN C6 GLIOMA CELLS

To verify whether cyclin D1 down-regulation was a determinant factor in C6 glioma cells differentiation, gain-and-lose function analysis was used. We found that the forced over-expression of cyclin D1 was able to absrogate the GFAP up-regulation by forskolin (Fig. 6A), indicating that cyclin D1 attenuated foskolin-induced differentiation. Meanwhile, cyclin D1 was knocked down with synthetic siRNA with siRNA for GAPDH used as a control. Data in Figure 6B demonstrates that the knockdown of cyclin D1 resulted in the same effects as forskolin including GFAP expression elevation, morphological transformation to an astrocyte-like shape (Fig. 6C) and G0/G1 phase accumulation from 72% to 83.5% with a reduction of cells in the S phase from 19.1% to 7.2% (Fig. 6D). These data provide direct evidence indicating that cyclin D1 is a crucial target

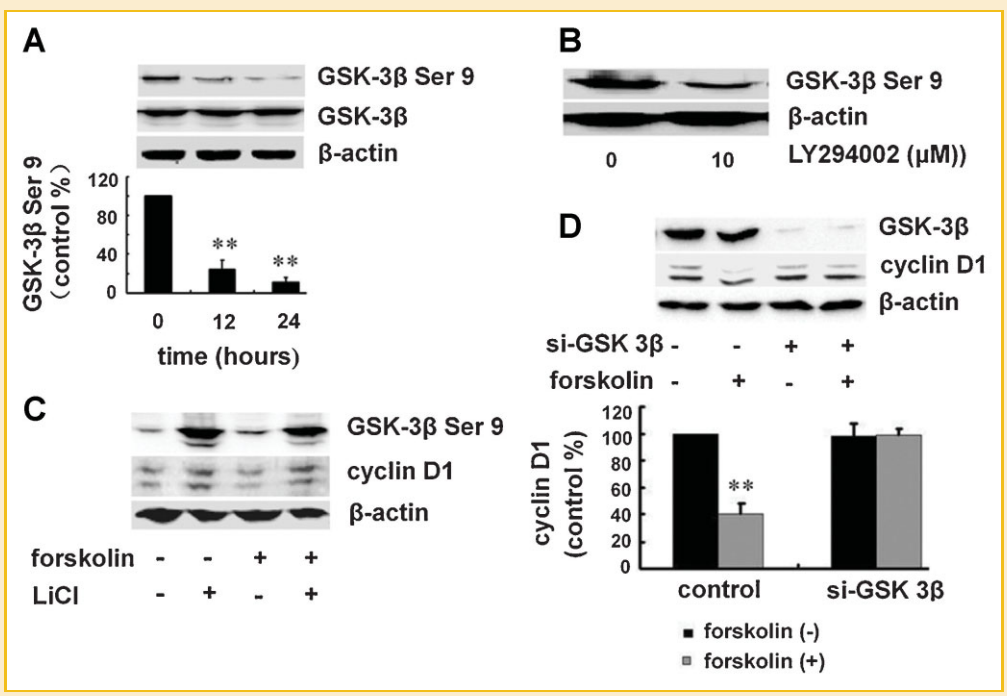

Fig. 5. Forskolin-induced cyclin D1 protein degradation via inhibiting GSK-3β phosphorylation at Ser 9. A: Forskolin decreased the level of phosphorylated GSK-3β at Ser 9. C6 cells were treated with 10 μM forskolin for 12 and 24 h. B: PI3K activator, LY294002, elevated the level of phosphorylated GSK-3β at Ser 9. C: GSK-3β inhibitor, LiCl, abrogated the forskolin induced decrease of cyclin D1 via promotion of GSK-3β phosphorylation. C6 cells were pretreated with 10 mM LiCl for 1 h, and incubated in the absence or presence of 10 μM forskolin for another 24 h. D: GSK-3β siRNA prevented the forskolin mediated decrease in cyclin D1 protein. C6 glioma cells were transfected with 50 μM GSK-3β siRNA for 24 h and treated with 10 μM forskolin for another 48 h. Results are means ± SD (n = 3). Statistical differences compared with the controls are given as $^{**}P < 0.01$. Cyclin D1 levels were presented as a relative value of control.

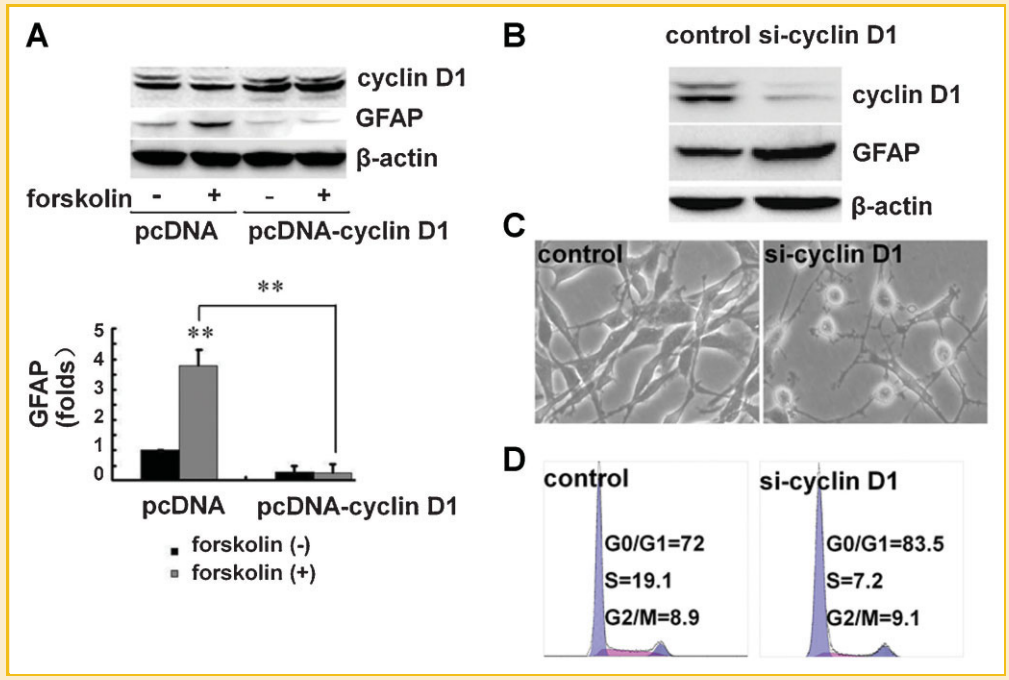

Fig. 6. Cyclin D1 down-regulation is requisite for proliferation loss and differentiation in C6 glioma cells. A: Overexpression of cyclin D1 abolished the forskolin-induced C6 glioma cells differentiation. C6 cells were transfected stably with a pcDNA 3.0 vector control or pcDNA 3.0 cyclin D1, and incubated in the absence or presence of 10 μM forskolin for 48 h. B–D: Knockdown of cyclin D1 induced C6 glioma cells differentiation and G0/G1 phase cell cycle arrest. Cells were transfected transiently with 50 μM cyclin D1 siRNA or control siRNA for 48 h. B: Cellular levels of cyclin D1 and GFAP. C: Morphological transformation (original magnification: ×200). D: Cell cycle distributions.

in the forskolin-induced differentiation and proliferation inhibition in C6 glioma cells.

DISSCUSSION

Differentiation-inducing therapy is the most successful therapy for the treatment of acute myelocytic leukemia and is a promising therapy in treating certain osteosarcoma. Recently, this therapy has been proposed to be a novel potential approach to treat malignant gliomas [Dai and Holland, 2003]. In this study, we demonstrated that forskolin, a small molecule (MW 410.5), induced cell growth inhibition and differentiation in C6 glioma cells.

Forskolin, a diterpene extracted from the roots of the plant *Coleus forskohlii*, directly activates AC and catalyzes synthesis of cAMP from ATP to activate protein kinase A (PKA). cAMP/PKA pathway regulates a wide range of processes, including cell proliferation, differentiation, microtubules dynamics, chromatin condensation and decondensation, nuclear envelop disassembly and reassembly, and exocytosis [Insel and Ostrom, 2003; Tasken and Aandahl, 2004]. Accumulating evidences have reported that forskolin induces the differentiation of human monoblast U937 cells, neurocytoma cells and neuroblastoma × rat glioma cell line NG108-15 cells via elevating cAMP [Ammer and Schulz, 1997; Brodsky et al., 1998; Kim et al., 2004], suggesting forskolin to be a potential candidate of differentiation inducing agents for glioma. In our work, we found that forskolin-promoted C6 cell differentiation via regulating the transcription and proteolysis of cyclin D1, and the inhibitor of PKA abolished this effect (data not shown). In addition, our work confirmed that cholera toxin-induced C6 cell differentiation was mediated via PKA/CREB pathway [Li et al., 2007]. All this information supports the notion that forskolin-induced differentiation is greatly associated with cAMP elevation and the subsequent PKA activation.

Cyclin D1, one of the checkpoints in the G1 to S phase transition, was down-regulated by forskolin in C6 glioma cells that proved to be responsible for proliferation loss and differentiation. The function of cyclin D1 in growth inhibition is very clear: reduced cyclin D1 first binds to altered CDK4/CDK6, and then inhibits the phosphorylation of Rb, which affected free E2F1 release and eventually hampered the entry of cells into S phase. In the mechanism of forskolin to repress proliferation, the inhibitory activity of p27 on cyclin D1-CDK4/6 complexes cannot be neglected. In fact, not only p27 but also p21 can inactivate these complexes, but the p21 expression was not altered in forskolin treatment (data not shown). Compared with the definite role of cyclin D1 in cell cycle regulation, the action of cyclin D1 in cell differentiation is not so clear. However, increasing number of reports have demonstrated that inhibition of cyclin D1 was able to induce differentiation of tumor cells such as melanoma cells and neuroblastoma cells as well as normal cells like keratinocyte and skeleral muscle cells [Strasberg Rieber and Rieber, 1995; Guttridge et al., 1999; Georgopoulou et al., 2006; Nishi et al., 2009]. With regard to the mechanism of how cyclin D1 regulates cell differentiation, some researches considered that it is greatly possible because of the negative regulation of cyclin D1 to transcription factors, such as mucin MUC1 and B-myb, required for transcription of differentiation genes [Cesi et al., 2002; Mayo and Mayol, 2009]. In forskolin-induced cell cycle arrest and differentiation, the essential

role of cyclin D1 as a dual modulator was determined via the gain and loss function analysis, in consistency with previous reports [Takahashi-Yanaga et al., 2006; Zhu et al., 2006; Nishi et al., 2009; Ma et al., 2011], which unified with the notion that cells have a chance to differentiate before entering into G0/G1 phase. As for determining the mechanism of how cyclin D1 promotes differentiation, we need to perform further experiments.

Accumulated evidences demonstrate that cyclin D1 level is tightly regulated through multiple mechanisms, including promoter activation, mRNA stability, initiation of translation, and protein stability [Kroger et al., 2007]. Regulation at the mRNA level can occur through destabilizing elements in its 3′-untranslated region. AU-rich elements on the distal end of the cyclin D1 mRNA are positively regulated by prostaglandin A2 and negatively regulated by PI3K [Dufourny et al., 2000; Lin et al., 2000]. Post-translational control of cyclin D1 levels is mediated by phosphorylation-dependent polyubiquitination and degradation by the 26S proteasome [Diehl et al., 1998]. Here, we showed that forskolin decreased cyclin D1 mRNA by using real-time RT-PCR analysis, indicating that forskolin-regulated cyclin D1 transcription. Furthermore, the regulatory mode of forskolin at the proteolytic level was evidenced by increased levels of ubiquitinated cyclin D1 protein, the increased binding of ubiquitin with cyclin D1, and the reversal of cyclin D1 inhibition by proteasome inhibitor. The dual regulation of forskolin in transcription and proteolysis contributed to the noticeable reduction of cyclin D1 and subsequent cell cycle exit and differentiation.

In the ubiquitin-dependent proteolysis of cyclin D1, the activation of PI3K/Akt/GSK-3β pathway is believed to be the main regulatory mechanism. A series of researches verified that inhibition of PI3K/Akt/GSK-3β pathway, especially the GSK-3β activity, enhanced cyclin D1 protein stability and caused cyclin D1 accumulation [Chen et al., 2005; Dal Col and Dolcetti, 2008; Ong et al., 2011]. GSK-3β, a serine/threonine protein kinase, has been shown to negatively influence cyclin D1 expression by regulating mRNA transcription and protein degradation [Takahashi-Yanaga and Sasaguri, 2008]. In the regulation of gene expression, GSK-3β is transformed to its inactive form by phosphorylating specific amino terminal residues such as Ser 33, Ser 37, and Thr 41, which first causes the attenuated degradation of cytoplasmic β-catenin, then activates Wnt/β-catenin signaling and finally enhances the cyclin D1 promoter activity [Takahashi-Yanaga and Sasaguri, 2008]. In proteasome-mediated cyclin D1 degradation, GSK-3β phosphorylation at Ser 9 facilitates the sustainment of cyclin D1 stability, which means that dephosphorylation would promote cyclin D1 degradation [Cohen and Frame, 2001]. In our study, we tested the effect of forskolin on GSK-3β phosphorylation at Ser 9 but not at other sites and found that the inactive form of GSK-3β diminished. Interestingly, inhibition of GSK-3β activity by LiCl and deletion of endogenous GSK-3β by siRNA attenuated the forskolin-induced cyclin D1 degradation. These data sufficiently reveal that forskolin disrupted the stability of cyclin D1 by activating GSK-3β. In our previous work [Li et al., 2010], we demonstrated that another cAMP-elevating stimuli cholera toxin also activated GSK-3β at Ser 9 to cause the remarkable reduction of the cellular cyclin D1 protein levels but not mRNA and thus triggered C6 glioma cell differentiation. Obviously, all these information support the notion that both forskolin- and cholera toxin-induced differentiation are greatly associated with cAMP/PKA signaling activation and the subsequent regulation of GSK-3β in cyclin D1 proteolysis.

In conclusion, a PKA activator forskolin induces cell proliferation inhibition and differentiation of C6 glioma cells in a time- and dose-dependent manner. We found that cyclin D1 is down-regulated at mRNA and protein level, and the latter is mediated by GSK-3β activation. Furthermore, overexpression of cyclin D1 in C6 cells attenuated the differentiation induced by forskolin; however, knockdown of cyclin D1 by interfering RNA imitated forskolin to trigger C6 differentiation and cell cycle arrest. These findings suggest that cyclin D1 is a dual-function molecule to regulate cell cycle exit and differentiation, and shed new light on the target therapy target of malignant gliomas.

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