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LABORATORY INVESTIGATION - HUMAN/ANIMAL TISSUE

Knockdown of cyclin D1 inhibits proliferation, induces apoptosis, and attenuates the invasive capacity of human glioblastoma cells

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Abstract Elevated cyclin D1 (CCND1) in human glioblastoma correlates with poor clinical prognosis. In this study, the human glioblastoma cell lines SHG-44 and U251 were stably transfected with short hairpin RNA (shRNA) targeting cyclin D1 or with ectogenic cyclin D1 by lentivirus-mediated transfection. Glioblastoma cells overexpressing or underexpressing cyclin D1 were then examined by in vitro growth assays, apoptosis assays, cell cycle analysis, and invasion assays. Cyclin D1 knockdown in SHG-44 cells inhibited cell proliferation, induced apoptosis, and attenuated migration across Matrigel, a model of invasive capacity. Western blot analysis and quantitative reverse-transcription polymerase chain reaction (RT-PCR) revealed that cells underexpressing CCND1 exhibited decreased multidrug resistance protein 1 (MDR1) and B-cell lymphoma-2 (Bcl-2) expression, but enhanced apoptosis effector caspase-3 expression. In contrast, cyclin D1 overexpression promoted cell proliferation, attenuated apoptosis, and enhanced invasive capacity. Furthermore, cyclin D1 overexpression was associated with increased expression of MDR1 and Bcl-2, and decreased caspase-3 expression. Results using the U251 cell line confirmed the effects of CCND1-targeted shRNA and lentivirus-mediated overexpression on proliferation and apoptosis of glioblastoma cells. Overexpression of cyclin D1 enhanced the proliferation and invasive potential of human glioblastoma cells, while reducing apoptosis. The ability to suppress the malignant phenotype by downregulating cyclin D1 expression may provide a new gene therapy approach for patients with malignant glioma.

Keywords Glioblastoma · Cyclin D1 · MDR1 · Bcl-2 · Caspase-3

Introduction

Cyclins are the regulatory subunits of a protein kinase family involved in cell cycle control and proliferation. Cyclins associate with catalytic cyclin-dependent kinase (CDK) subunits to regulate cell cycle progression [1, 2]. To date, 11 cyclins (A, B1–2, C, D1–3, E, F, G, and H) and seven CDKs (CDK1–7) have been identified [3]. The D-type cyclins (D1, D2, and D3) are responsible for integrating extracellular signals to regulate or suppress cell cycle progression [4]. Cyclin D1 is involved in regulation of the G_1 phase of the cell cycle [5, 6], an essential period in which cell differentiation is initiated and proliferation is suppressed [7]. The effects of cyclin D1 on cell cycle progression and gene expression patterns are key to the oncogenic function of cyclin D1 [8].

Abnormal overexpression of cyclin D1 caused by amplification of its genomic locus on chromosome 11q13 is involved in the pathogenesis of breast cancer and nasopharyngeal carcinoma [9, 10]. Rearrangement, amplification, or increased expression of the cyclin D1 gene and overexpression of its messenger RNA (mRNA) and proteins have been reported in several human cancers, including glioma, human parathyroid adenoma, breast, colon, and liver cancers, squamous carcinoma of the esophagus, and head and neck carcinoma [5, 11–14].

JunYu Wang and Qi Wang are equally contributed to this work.

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Furthermore, overexpression of cyclin D1 is associated with poor prognosis in laryngeal [15], esophageal [16], stomach [17], colorectal [18], and pancreatic cancer patients [19], as well as in patients with glioma [20]. Deregulated expression of cyclin D1 directly contributes to tumorigenesis in a number of animal models, further demonstrating a link between cyclin D1 and carcinogenesis and suggesting that inhibition of cyclin D1 may be a novel strategy for cancer prevention and therapy [21–23]. The strategy of antisense-mediated suppression of cyclin D1 has been assessed in human esophageal cancer cells, colon cancer cells, and squamous carcinoma [24–26]. These studies clearly demonstrated that inhibition of cyclin D1 lengthened the duration of the G_1 phase, inhibited the proliferation of tumor cells, and resulted in loss of tumorigenicity in nude mice [25, 26].

Glioblastoma is an aggressive and devastating disease with 5-year survival below 10% [27, 28]. Higher cyclin D1 expression was observed in grade IV astrocytomas than in grades II and III astrocytomas, and overexpression was correlated with shorter patient survival [12, 20, 28, 29]. However, the relationship between cyclin D1 overexpression and malignant behavior in glioblastoma cells has not yet been examined. We demonstrate that suppression of CCND1 reduces the proliferation and invasiveness of glioblastoma cells lines while promoting apoptosis, underscoring the importance of cyclin D1 in tumorigenesis and defining CCND1 as a promising molecular target for anticancer therapy.

Materials and methods

Cell culture

The human embryonic kidney cell line HEK293 and two human glioblastoma cell lines, SHG-44 and U251, were purchased from the Chinese Academy of Sciences (Shanghai). The SHG-44 and U251 cell lines were grown in a complete medium composed of Dulbecco's modified Eagle's medium (DMEM) supplemented with 8% fetal bovine serum (FBS), penicillin G (100 U/mL), and streptomycin (100 mg/ml), and maintained in monolayer culture at 37°C in humidified air with 5% CO_2. Viability of the cells was periodically monitored by trypan blue staining.

Constructs and transfection

Prior to lentivirus-mediated knockdown of cyclin D1, three different synthetic small interfering RNA (siRNA) sequences were tested for inhibitory activity by transient transfection into HEK293 cells, and the sequence of the most effective sequence was cloned into the pLVTHM vector [30]. Short hairpin sequences specific for CCND1 (5′-GATCCCC*GATCGTCGCCACCTGG ATG*ttcaagaga*CATCCAGG TGGCGACGATC*TTTTTGGAAA-3′) (Invitrogen) and, as a control, a shRNA directed against firefly luciferase (Luc, 5′-GATCCCC*CTTACGCTGAGTACTTCGA*ttcaagaga–*TC GAAGTACTCAGCGTAAG*TTTTTGGAAA-3′) were inserted into the *Mlu*I/*Cla*I restriction sites of pLVTHM. These complete vectors were named Lenti-shRNA and Lenti-GFP, respectively. A full-length human cyclin D1 complementary DNA (cDNA) (NM_001530) containing the entire coding sequence was subcloned into the BISVECTOR lentivirus vector (Invitrogen). The complete function vector was named Lenti-CCND1. Conditioned medium containing lentiviruses was harvested 48 h after transfection of HEK293 cells. This medium was filtered and used to infect recipient cells in the presence of 8 μg/ml polybrene. The SHG-44 cells were stably transfected with Lenti-shRNA, Lenti-CCND1, or lent-GFP according to the manufacturer's protocol (Invitrogen). After 48 h of incubation, transfected cells were selected in primary cell culture medium containing 200 μg/ml G418. After 2–3 weeks, single independent clones were randomly isolated and each individual clone was plated separately. After clonal expansion, cells from each independent clone were tested for cyclin D1 expression by immunoblotting. In this way, three SHG-44 lines were isolated either expressing green fluorescent protein (GFP), overexpressing CCND1, or expressing a CCND1-specific shRNA that led to stable CCND1 underexpression. Single independent clones of U251 transfected with Lenti-shRNA, Lenti-GFP, or Lenti-CCND1 were isolated as described above.

Protein extraction and Western blotting

Proteins were extracted from subconfluent cultures and subjected to Western blot analysis. After blocking with 5% nonfat milk in phosphate-buffered saline with Tween (PBS-T) for 1 h at room temperature, the membranes (Protran; Schleicher & Schuell, Dassel, Germany) were blotted with primary antibody, followed by incubation with a peroxidase-conjugated secondary antibody as described previously [30]. Bound antibodies were visualized using enhanced chemiluminescence (Bio-Rad). The primary antibodies used were rabbit polyclonal antibody to cyclin D1 (Santa Cruz, sc-717, 1:1,000 dilution), mouse monoclonal antibody to MDR1 (Santa Cruz, sc-59593, 1:1,000), goat polyclonal antibody to multidrug resistance-associated protein 1 (MRP1) (Santa Cruz, sc-104398, 1:1,000), goat polyclonal antibody to caspase-3 (Santa Cruz, sc-1225, 1:1,000), mouse monoclonal antibody to Bcl-2 (Santa Cruz, sc-130307, 1:1,000), goat polyclonal antibody to matrix metalloproteinase (MMP)-2 (Santa Cruz, sc-34014, 1:1,000), mouse monoclonal antibody to

MMP-9 (Santa Cruz, sc-12759, 1:1,000), and a rabbit polyclonal antibody to β-actin used as a gel loading control (Santa Cruz, sc-130656, 1:1,000).

Quantitative RT-PCR analysis

Total RNA was extracted from untreated SHG-44 cells, and from SHG-44-derived cell lines (Lenti-CCND1, Lenti-shRNA, and Lenti-GFP) using the RNeasy mini kit (Qiagen). First-strand cDNA was reverse transcribed from 1 μg total RNA using the SuperScript First-Strand cDNA system (Invitrogen) and amplified by Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). For each PCR reaction, a master mix was prepared that included Platinum SYBR Green qPCR SuperMix-UDG, forward primer, reverse primer, and 10 ng template cDNA (Table 1). The PCR conditions were 5 min at 95°C followed by 40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s.

Cell proliferation assay

Cell growth was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. Untransfected SHG-44 and U251 cell lines and the stably transfected lines Lenti-CCND1, Lenti-shRNA, and Lenti-GFP were replated onto 96-well plates at 4×10^3 cells/well and cultured overnight to allow for cell attachment. At daily intervals (24, 48, 72, 96, and 120 h), the number of viable cells was determined by MTT assay. Briefly, cells were incubated with 0.2 μg/ml MTT for 4 h in the dark at 37°C. After removal of the medium, the formazan crystals produced from MTT by live cells were dissolved in 150 μl dimethyl sulfoxide (DMSO), and the

Table 1 List of gene-specific primers, expected product sizes, and reaction conditions for qRT-PCR analysis

Gene name	Primer sequences (5′→3′)	Product size (bp)	PCR cycle parameters
CCND1	F: 5′ AGAAGCTGTGCATCTACACCGACA 3′	168	94 (5 min); 55°C (30 s); 72°C (30 s) 72°C (10 min) 72°C
CCND1	R: 5′ TGATCTGTTTGTTCTCCTCCGCCT 3′		
MDR1	F: 5′ AACAACGCATTGCCATAGCTCGTG 3′	183	94°C (5 min); 56°C (30 s); 72°C (30 s) 72°C (10 min) 72°C
MDR1	R: 5′ AGTCTGCATTCTGGATGGTGGACA 3′		
MRP1	F: 5′ CATCGTTCTGTTTGCTGCCCTGTT 3′	104	94°C (5 min); 56°C (30 s); 72°C (30 s) 72°C (10 min) 72°C
MRP1	R: 5′ AGTACGTGGTGACCTGCAATGAGT 3′		
Bcl-2	F: 5′ TTGTGGCCTTCTTTGAGTTCGGTG 3′	167	94°C (5 min); 55°C (30 s); 72°C (30 s) 72°C (10 min) 72°C
Bcl-2	R: 5′ GTACAGTTCCACAAAGGCATCCCA 3′		
Caspase 3	F: 5′ TCATTATTCAGGCCTGCCGTGGTA 3′	178	94°C (5 min); 56.5°C (30 s); 72°C (30 s) 72°C (10 min) 72°C
Caspase 3	R: 5′ TGGATGAACCAGGAGCCATCCTTT 3		
MMP-2	F: 5′ AGAAGGATGGCAAGTACGGCTTCT 3′	125	94°C (5 min); 56°C (30 s); 72°C (30 s) 72°C (10 min) 72°C
MMP-2	R: 5′ AGTGGTGCAGCTGTCATAGGATGT 3′		
MMP-9	F: 5′ TACCACCTCGAACTTTGACAGCGA 3′	193	94°C (5 min); 55.5°C (30 s); 72°C (30 s) 72°C (10 min) 72°C
MMP-9	R: 5′ GCCATTCACGTCGTCCTTATGCAA 3′		
β-Actin	F: 5′ ACCAACTGGGACGACATGGAGAAA 3′	192	94°C (5 min); 56°C (30 s); 72°C (30 s) 72°C (10 min) 72°C
β-Actin	R: 5′ TAGCACAGCCTGGATAGCAACGTA 3′		

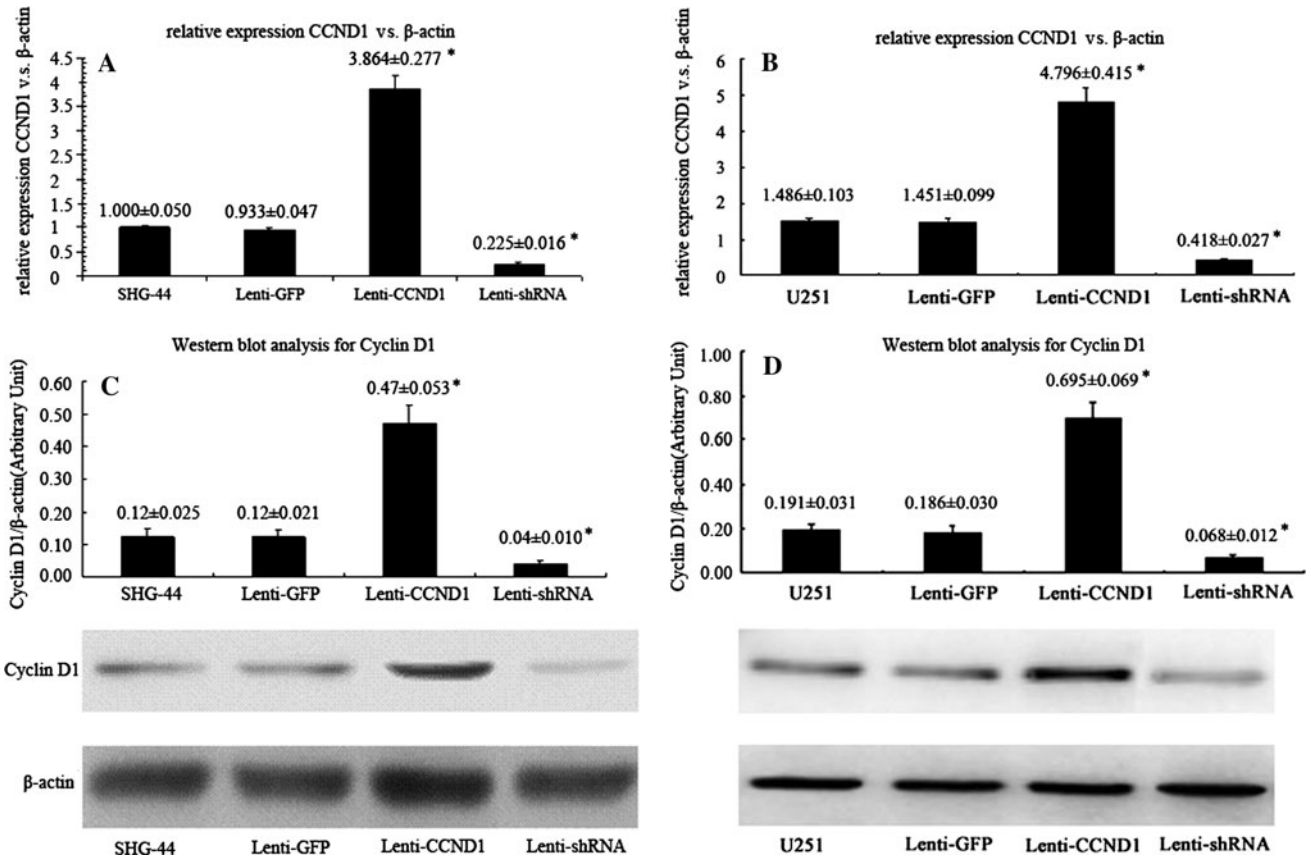

Fig. 1 Effects of Lenti-CCND1 and Lenti-shRNA transfection on cyclin D1 expression (**a**, **b**). Quantitative RT-PCR analysis demonstrates that CCND1 mRNA was significantly overexpressed in glioblastoma cells stably transfected with Lenti-CCND1 and suppressed in glioblastoma cells stably transfected with Lenti-shRNA compared with untransfected glioblastoma cells and cells stably transfected with Lenti-GFP. Expression of cyclin D1 protein in SHG-44 (**c**) and U251 cells (**d**) was examined by Western blotting. β-Actin served as a loading control. Results shown are representative of three independent experiments. $^{*}P < 0.01$ as compared with control groups

absorbance was measured at 570 nm with an Ultra multifunctional microplate reader (Tecan, Durham, NC). Three independent experiments were performed in quadruplicate wells.

Cell cycle assay

Subconfluent cultures of the three stably transfected SHG-44 cell lines and untransfected control cells were trypsinized, collected, washed, and suspended in cold PBS. On the day of analysis, cells were centrifuged at 1,000 rpm for 5 min, resuspended in 0.5 ml PBS, fixed in 4.5 ml 70% ice-cold ethanol, and stored at 4°C. The cell suspension was then incubated in 0.2 mg/ml propidium iodide containing 0.1% Triton X-100 and RNase A (1 mg/ml, both from Sigma) in the dark for 30 min at room temperature. Cell cycle distribution was determined using fluorescence-activated cell sorting (FACS) analysis (FACSCalibur, BD Biosciences).

Apoptosis assay

Apoptosis was measured using an Annexin V/fluorescein isothiocyanate (FITC) apoptosis detection kit (Bender Med System, CA). Briefly, cells cultured in 6-cm dishes were trypsinized, washed, stained with FITC-conjugated anti-Annexin V antibody under darkness for 15 min at room temperature, and then analyzed by flow cytometry (FACSCalibur; Becton–Dickinson).

Invasion assay

Equal numbers (1×10^5) of untransfected cells as well as cells stably transfected with Lenti-shRNA, Lenti-CCND1, or Lenti-GFP were plated onto separate 24-well cell culture inserts coated with Matrigel with 8-μm pores. Minimum essential medium (MEM) with 10% FBS was added to the lower chamber as chemoattractant. After 24 h incubation at 37°C under a 5% CO_2 atmosphere, cells still

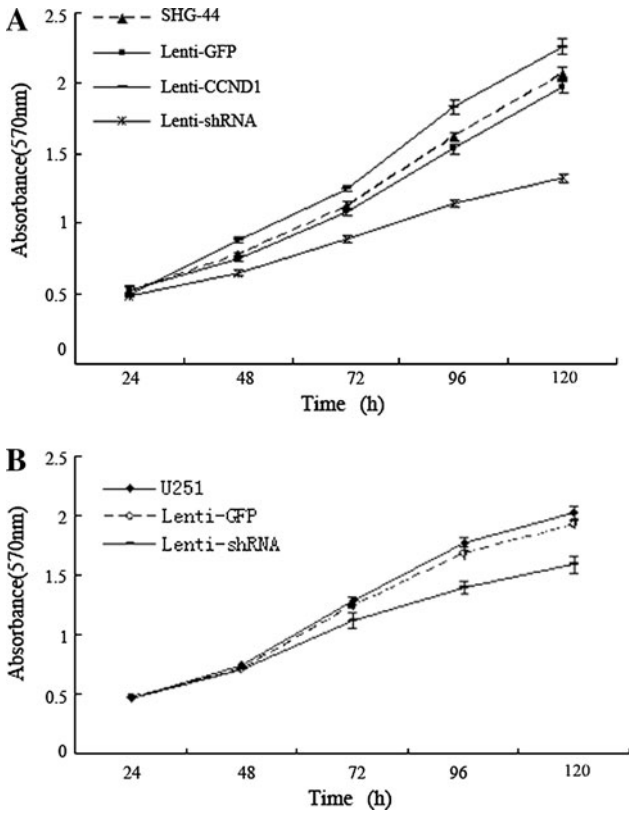

Fig. 2 Effects of cyclin D1 expression on glioblastoma cell proliferation. **a** Effect of cyclin D1 overexpression and underexpression on SHG-44 cell proliferation as measured by MTT assay. Absorbance was read at 570 nm with averages from triplicate wells. ($P < 0.05$ at 48, 72, 96 and 120 h for Lenti-CCND1 versus Lenti-GFP or untransfected SHG-44 cells; $P < 0.05$ at 48 h, and $P < 0.01$ at 96 and 120 h for Lenti-shRNA versus Lenti-GFP or untransfected parental SHG-44 cells). **b** Effect of cyclin D1 inhibition on U251 cell proliferation ($P < 0.01$ at 72, 96, and 120 h for Lenti-shRNA versus Lenti-GFP or parental U251 cells)

adhered to the upper surface of the filter were removed using a cotton applicator. The cells on the lower surface of the membrane (the migrated cells) were fixed with 3.7% formaldehyde, stained with hematoxylin, and counted. The invasion rate was determined from three independent experiments.

Statistics

All data are expressed as mean ± standard deviation (SD) of three independent experiments. Statistical analysis between two groups was performed using Student's *t* test, and the comparison between three or more groups was performed using analysis of variance (ANOVA) analysis followed by Dunnett's *t* test. Immunoblot bands were visualized and quantified by ImageJ software (NIH, USA). *P* value <0.05 was considered statistically significant.

Results

Effects of Lenti-shRNA or Lenti-CCND1 on cyclin D1 expression

After transfection, qRT-PCR analysis for CCND1 expression demonstrated a 3.8-fold increase in CCND1 transcript in SHG-44 cells stably transfected with Lenti-CCND1 and a 3.3-fold increase in stably transfected U251 cells compared with untransfected parental controls (Fig. 1a, b). Western blot analysis confirmed the increase in cyclin D1 protein expression in both SHG-44 Lenti-CCND1 transfectants (Fig. 1c) and U251 Lenti-CCND1 transfectants (Fig. 1d). In contrast, CCND1 protein was reduced by 66.7% in SHG-44 Lenti-shRNA transfectants and by 63% in U251 Lenti-shRNA transfectants compared with CCND1 expression in the control lines (Fig. 1c, d).

Cell proliferation assay

As shown in Fig. 2a, Lenti-shRNA that targeted CCND1 significantly inhibited tumor cell proliferation in stably transfected SHG-44 cells compared with negative controls transfected with Lenti-GFP or left untreated ($P < 0.05$ at 72 and 96 h; $P < 0.01$ at 120 h compared with untransfected and Lenti-GFP SHG-44 cells). In contrast, Lenti-CCND1 transfectants exhibited a markedly higher proliferation rate compared with negative control cells ($P < 0.05$ at 48, 72, 96, and 120 h), whereas a significant difference was not observed between untreated (parental) and Lenti-GFP SHG-44 cells ($P > 0.05$). Similarly, Lenti-shRNA transfection inhibited growth of U251 cells compared with both untransfected U251 cells and U2512 cells stably transfected with Lenti-GFP (Fig. 2b, $P < 0.01$ at 72, 96, and 120 h).

Cell apoptosis assay

The rate of cellular apoptosis in these six different stably expressing lines was examined using flow cytometric analysis of Annexin V staining. The cellular apoptosis rate of SHG-44 cells was 16.4 ± 2.45% in Lenti-shRNA transfectants, 7.5 ± 1.63% in Lenti-GFP transfectants, and 5.9 ± 0.94% in Lenti-CCND1 transfectants at 72 h post transfection. In U251 cells, apoptotic rates were 1.87 ± 0.15% in untransfected controls, 2.23 ± 0.49% in Lenti-GFP transfectants, and 10.2 ± 1.20% Lenti-shRNA transfectants at 96 h post transfection (Fig. 3b, 1.87 ± 0.15 versus 2.23 ± 0.49%, $P = 0.286$; 2.23 ± 0.49% versus 10.2 ± 1.20%, $P < 0.01$). Thus, underexpression of CCND1 significantly reduced apoptotic cell death in both the SHG-44 and U251 glioblastoma cell lines.

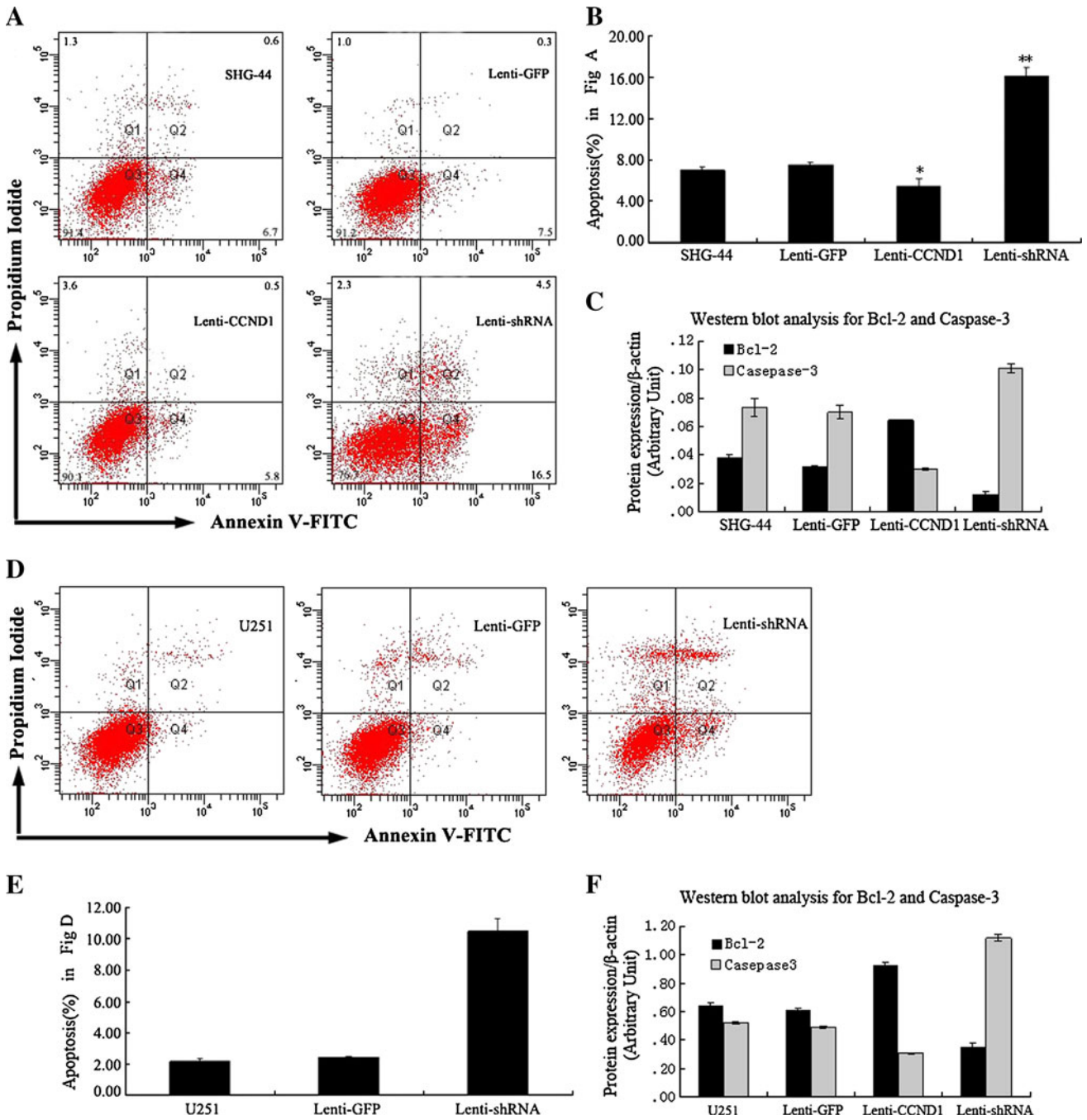

Fig. 3 Effect of cyclin D1 expression on apoptosis in glioma cell lines. **a** Apoptosis in SHG-44 cells was measured by Annexin V/propidium iodide (PI) staining following Lenti-shRNA or Lenti-CCND1 transfection. **b** Early apoptotic cell populations were significantly increased ($P < 0.01$) after Lenti-shRNA transfection and decreased ($P < 0.05$) after Lenti-CCND1 transfection. **c** Western blotting analysis for antiapoptotic Bcl-2 and effector caspase-3. Increased Bcl-2 ($P < 0.01$) and decreased caspase-3 ($P < 0.01$) expression were observed in SHG-44 cells stably overexpressing CCND1, whereas decreased Bcl-2 ($P < 0.03$) and increased caspase-3 ($P < 0.05$) expression were observed in SHG-44 cells 72 h after transfection as compared with the control groups. **d** The effect of CCND1-targeted shRNA on U251 cell apoptosis was similar to that in SHG-44 cells. **e** Early apoptotic cell numbers were significantly increased after Lenti-shRNA transfection ($P < 0.01$) in U251 cells. **f** Sample Western blot of Bcl-2 and caspase-3 expression (densitometric analysis in Fig. 7) demonstrated that decreased Bcl-2 ($P < 0.01$) and increased caspase-3 ($P < 0.001$) were observed in Lenti-shRNA transfected U251 cells 72 h after transfection compared with control cultures

We then examined the expression of the antiapoptotic protein Bcl-2 and the apoptosis effector caspase-3 in these different glioblastoma transfectants by Western blotting. As shown in Figs. 3c, 6, and 7, mRNA and protein levels of Bcl-2 were significantly lower in Lenti-shRNA transfectants compared with control cells, whereas caspase-3

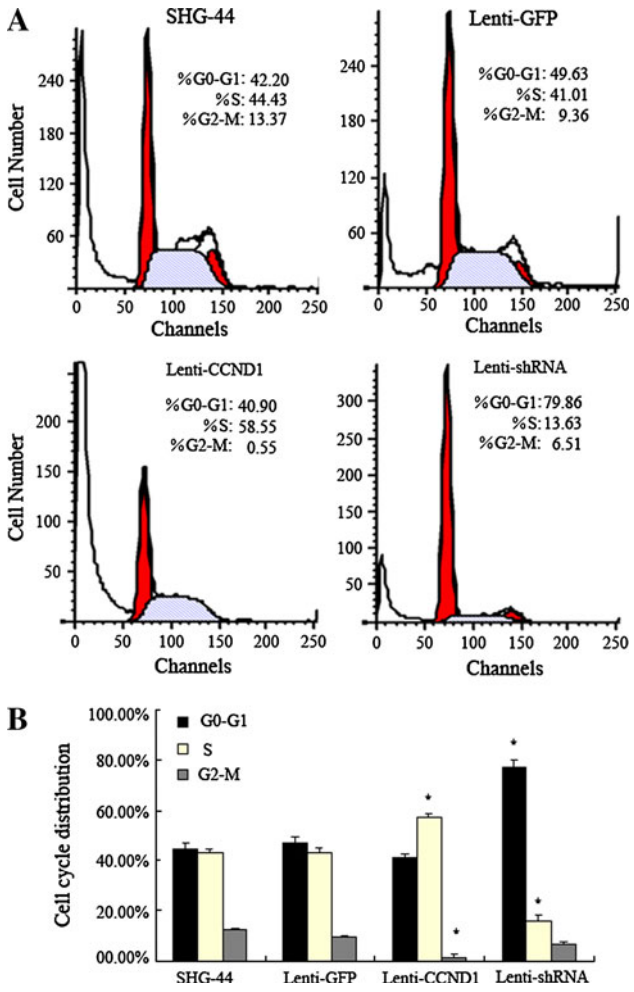

Fig. 4 Effect of CCND1 expression on the cell cycle distribution. **a** FACS of the different exponentially growing cyclin D1 transfectants. **b** *Histograms* showing G_0/G_1 phase arrest and a decline in S phase in SHG-44 cells underexpressing CCND1 through targeted shRNA expression. In contrast, cells overexpressing CCND1 showed an increase in cell fraction S phase and a decline in G_2–M phase cell numbers compared with control groups ($^*P < 0.05$)

expression was significantly higher than in control groups. Conversely, mRNA and protein levels of Bcl-2 were significantly higher and caspase-3 expression lower in Lenti-CCND1 transfectants of both lines. Furthermore, Western blot analysis (Figs. 3f, 7b) of U251 cells was consistent with results from SHG-44 cells. Thus, underexpression of CCND1 led to significantly enhanced cell apoptosis concomitant with enhanced effector caspase-3 expression and reduced expression of the seminal antiapoptotic protein Bcl-2.

Cell cycle analysis

Transfectants of the glioblastoma cell line SHG-44 cells were analyzed by flow cytometry at 48 h after transfection to assess the cell cycle distribution. As shown in Fig. 4, Lenti-shRNA cells exhibited a significant increase in the cell fraction in G_1 and a corresponding reduction in the fraction of cells in S phase compared with the control groups, while the percentage of cells in G_2–M phase was unaffected by cyclin D1 inhibition. The SHG-44 cells overexpressing cyclin D1 (Lenti-CCND1 transfectants) exhibited a significant increase in the percentage of cells in the S phase compared with the control groups, indicating that cyclin D1 does indeed influence the cell cycle distribution of glioblastoma cells and shifts cells to the proliferative S phase.

Effects of Lenti-CCND1 or Lenti-shRNA on invasion in vitro in glioma cells

To examine the effect of cyclin D1 inhibition or overexpression on glioma cell invasion, Transwell migration was performed. Reducing CCND1 expression by Lenti-shRNA transfection reduced Transwell migration compared with the other transfectant groups (Fig. 5). The processes of cell migration and infiltration that characterize metastasis require several extracellular matrix metalloproteinases (MMPs), so we examined the expression of MMP-2 and MMP-9. As shown in Figs. 5b, 6, and 7, glioblastoma cells transfected with Lenti-CCND1 demonstrated significantly elevated MMP-2 and MMP-9 mRNA and protein expression, while Lenti-shRNA transfectants exhibited decreased expression. Thus, underexpression of CCND1 reduced MMPs expression, consistent with the reduced invasive capacity in the Matrigel invasion model (Fig. 5).

Discussion

Elevated expression of cyclin D1 is associated with degree of malignancy, rapid cell proliferation, and poor patient survival in malignant astrocytoma [20, 29, 31], suggesting that cyclin D1 expression may have significant value as a prognostic indicator in glioma patients. Indeed, we provide compelling evidence that cyclin D1 expression levels regulate cell proliferation, apoptosis, and migration, the principle hallmarks of malignancy. Underexpression of cyclin D1 in a human glioblastoma SHG-44 cell line by shRNA transfection significantly inhibited cell proliferation, suppressed invasive capacity, and promoted apoptosis, possibly by inhibiting expression of antiapoptotic Bcl-2 and enhancing expression of apoptotic effector caspase-3. Furthermore, shRNA-mediated knockdown of cyclin D1 also downregulated the mRNA and protein levels of multidrug resistance protein 1 (MDR1). Conversely, overexpression of cyclin D1 attenuated apoptosis, promoted cell proliferation, and enhanced invasiveness across Matrigel scaffold membranes. Further study using the U251 glioblastoma cell

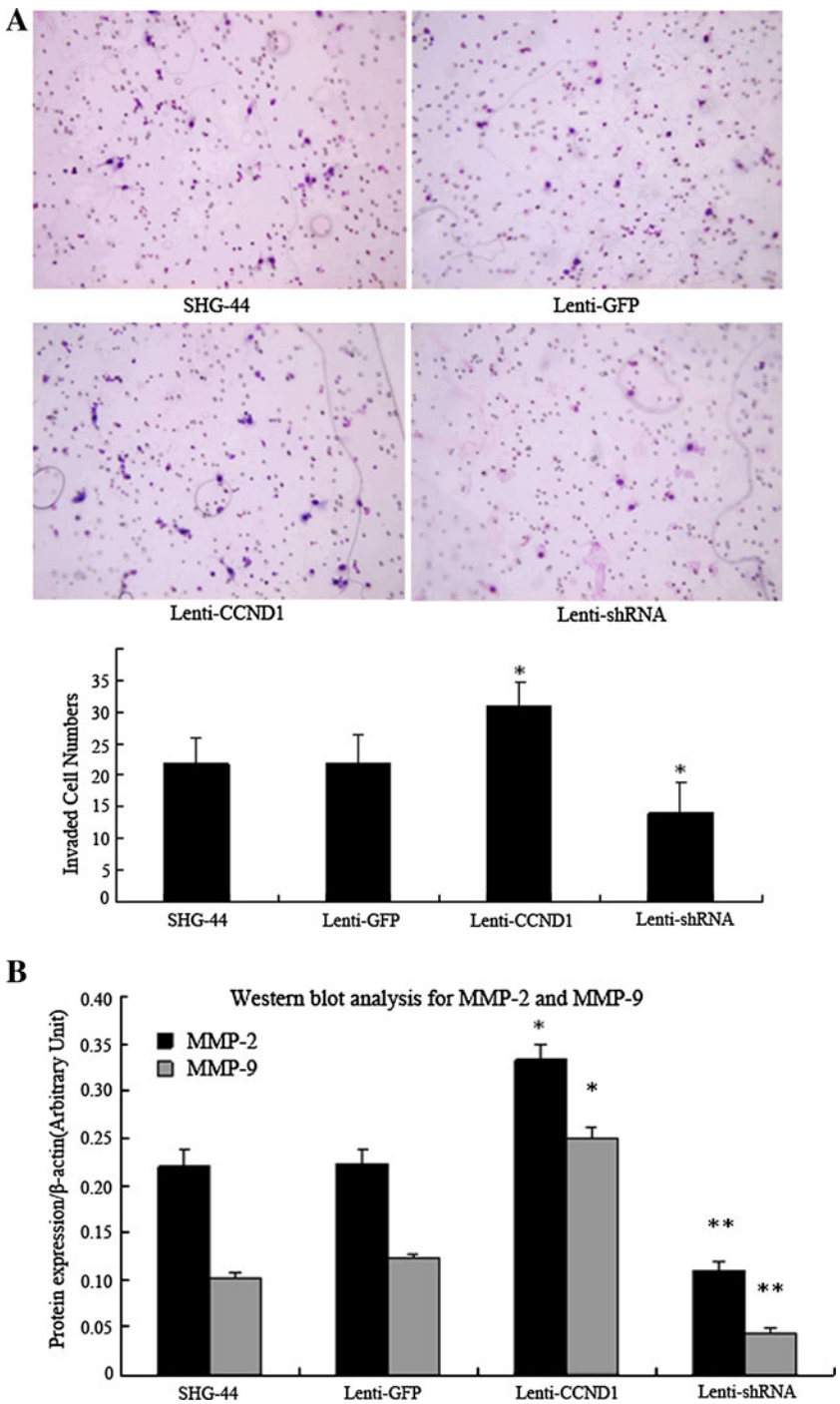

Fig. 5 Cyclin D1 expression regulates cell invasive capacity in the Matrigel model. **a** Matrigel assays showing the effect of Lenti-shRNA and Lenti-CCND1 on the invasive potential of SHG-44 cells. The *bar graph* indicates the mean number of invaded cells at 24 h after top-side cell seeding. Results demonstrated that Lenti-shRNA suppressed invasion of SHG-44 cells, while Lenti-CCND1 potentiated invasion of SHG-44 cells compared with control groups ($*P < 0.05$). **b** Western blot of MMP-2 and MMP-9 expression (densitometric analysis in Fig. 7). Increased MMP-2 and MMP-9 expression was observed in Lenti-CCND1-transfected SHG-44 cells, whereas Lenti-shRNA-transfected cells exhibited decreased MMP-2 and MMP-9 protein expression 48 h after transfection. ($*P < 0.05$ and $**P < 0.02$)

line confirmed the effects of cyclin D1 suppression on cell proliferation and apoptosis. Collectively, these findings demonstrate that cyclin D1 performs multiple functions as an oncogene through enhancement of proliferation and resistance to apoptosis, and may also contribute to chemoresistance in glioma.

In agreement with previous studies on a wide variety of tumor cells, including esophageal cancer cells [24], colon cancer cells [25], pancreatic cancer cells [32], and squamous carcinoma [26], inhibition of cyclin D1 by shRNA attenuated cell proliferation of glioma cells in vitro. Thus, SHG-44 cells stably transfected with CCND1-targeted shRNA showed significantly lower cell numbers at 48, 72, 96, and 120 h compared with control transfectants and untransfected cells seeded at the same initial cell density. In contrast, SHG-44 cells overexpressing CCND1 exhibited increased cell proliferation compared with untransfected SHG-44 cells. Similarly, U251 cells stably

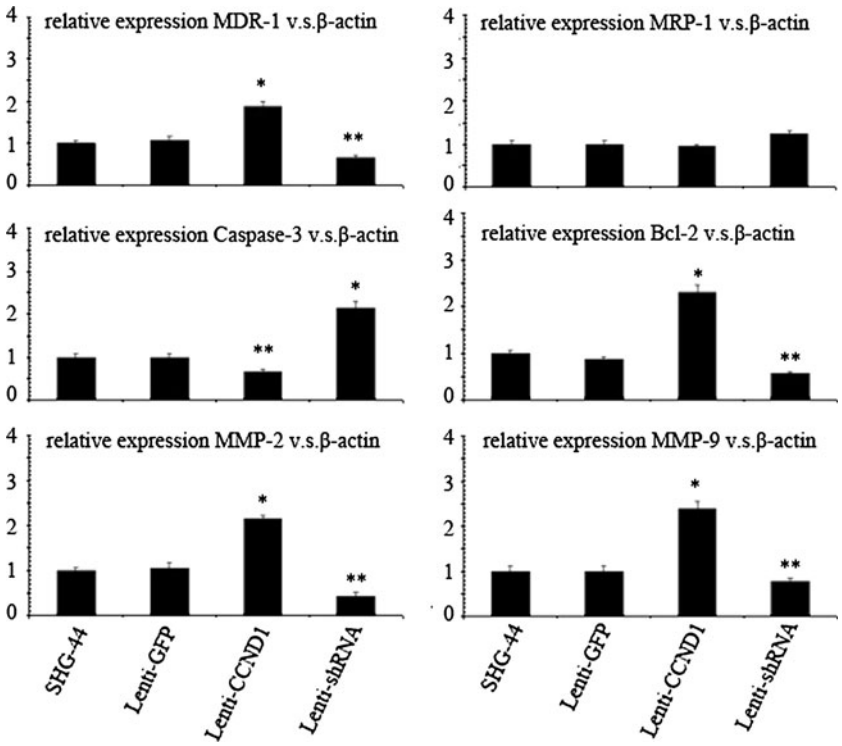

Fig. 6 Effect of Lenti-CCND1 and Lenti-shRNA on mRNA expression of MDR1, MRP1, Bcl-2, caspase-3, MMP-2, and MMP-9 in SHG-44 cells. qRT-PCR showing the effect of cyclin D1 inhibition or overexpression on mRNA levels of genes related to chemoresistance (MDR1 and MRP1), apoptosis (Bcl-2 and caspase-3), and cell invasion (MMP-2 and MMP-9) (*$P < 0.01$, **$P < 0.05$, ***$P > 0.05$)

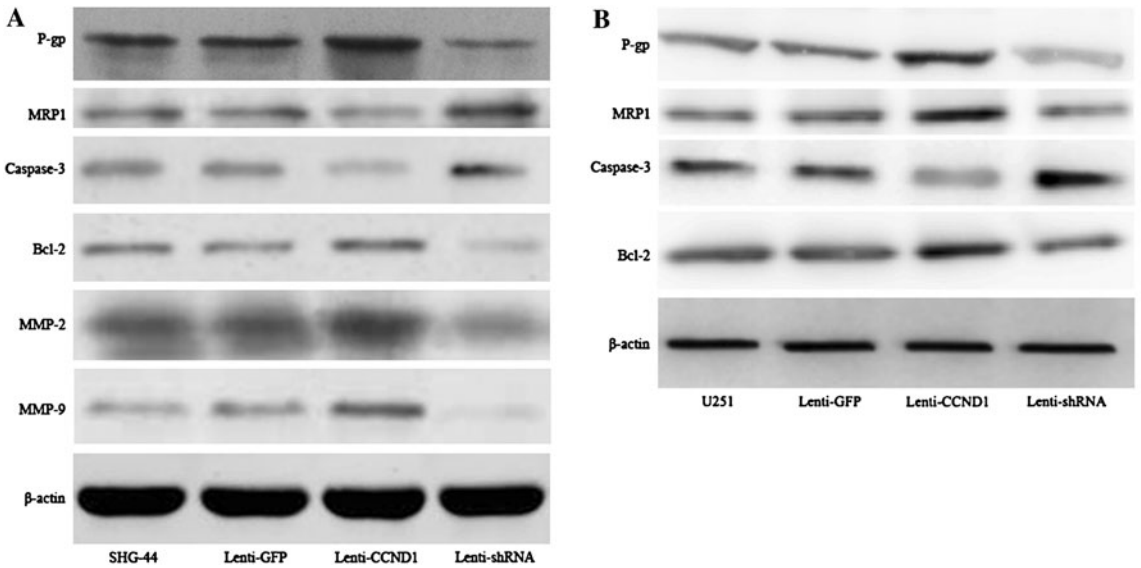

Fig. 7 **a** Effect of Lenti-CCND1 and Lenti-shRNA on protein expression of MDR1, MRP1, Bcl-2, caspase-3, MMP-2, and MMP-9 in SHG-44 cells. Densitometric analysis of Western blots revealed that cyclin D1 inhibition decreased MDR1/P-gp ($P < 0.01$), Bcl-2 ($P < 0.03$), MMP-2 ($P < 0.02$), and MMP-9 ($P < 0.02$) expression, while increasing caspase-3 expression ($P < 0.05$). Cyclin D1 overexpression increased MDR1/P-gp ($P < 0.05$), Bcl-2 ($P < 0.01$), MMP-2 ($P < 0.05$), and MMP-9 ($P < 0.05$) expression, and decreased caspase-3 ($P < 0.01$) expression. **b** Effect of altered cyclin D1 expression on protein expression of MDR1, MRP1, Bcl-2, and caspase-3 in U251 cells. Cyclin D1 inhibition decreased P-gp ($P < 0.01$), MRP1 ($P < 0.001$), and Bcl-2 ($P < 0.03$) expression, while increasing caspase-3 expression ($P = 0.01$). Cyclin D1 overexpression increased MDR1/P-gp ($P < 0.012$), MRP1 ($P = 0.006$), and Bcl-2 ($P < 0.01$) expression and decreased caspase-3 expression ($P < 0.001$)

transfected with CCND1-targeted shRNA showed significantly lower cell numbers at 72, 96, and 120 h compared with controls, demonstrating that CCND1 underexpression inhibited cell proliferation in human glioblastoma cell lines. Inhibition of cyclin D1 arrested SHG-44 cells in the G_0/G_1 phase of the cell cycle, while overexpression of cyclin D1 increased the percentage of cells in S phase and reduced the cell fraction in the G_0/G_1 phase. This result is

consistent with previous studies [3] showing that cyclin D1 inhibition prevented normal cells from entering the S phase while overexpression of cyclin D1 increased the percentage of cells in the S phase without altering the length of the cell cycle.

Furthermore, shRNA-mediated knockdown of cyclin D1 promoted apoptosis in both SHG-44 cells and U251 cells as indicated by the increase in the number of Annexin V/PI-positive cells measured by flow cytometry. To address the molecular mechanisms of shRNA-mediated apoptosis in SHG-44 and U251 cells, we investigated Bcl-2 and caspase-3 expression and demonstrated that reduced cyclin D1 expression was associated with high effector caspase-3 expression and lower antiapoptotic Bcl-2 expression. Conversely, overexpression of cyclin D1 increased Bcl-2 expression and decreased caspase-3 expression.

High invasive capacity is one of the hallmarks of malignant glioma cells [33]. Degradation of the stromal extracellular matrix (ECM) is the first step required for cancer cell invasiveness. Matrix metalloproteinases are required for degradation of the physical barrier of the ECM; in particular, MMP-2 and MMP-9 play a critical role in the degradation of both denatured collagen and type IV collagen [34–36]. In the present study, we demonstrated that overexpression of cyclin D1 led to enhanced invasive capacity in SHG-44 cells, possibly through increased expression of MMP-2 and MMP-9. Attenuation of the invasive capacity in Lenti-shRNA cells may be correlated with the observed decrease in MMP-2 and MMP-9 expression. The molecular mechanisms regulating cell migration through the artificial Matrigel scaffold have not been determined, however, and clearly warrant further study.

A second hallmark of cancer cells, including glioma, is multidrug resistance mediated by expression of the MDR/transporter associated with antigen processing (TAP) superfamily of membrane transporters. Development of multidrug resistance can severely reduce the efficacy of chemotherapies, but a possible relationship between cyclin D1 and MDR protein expression has not been established in glioma cells. In testicular germ cell tumors, ovarian tumors, and prostate tumors, cyclin D1 was overexpressed in drug-resistant cells compared with drug-sensitive samples [37]. Moreover, combined CCND1 knockdown with chemotherapy inhibited cell growth in vitro more effectively than either treatment alone [37–39]. Our data suggest that cyclin D1 knockdown induces apoptosis in glioblastoma cell lines through upregulation of caspase-3. It has been well documented that downregulation of Bcl-2 and caspase-3 activation may mediate apoptosis during temozolomide therapy [40, 41]. Downregulation of MDR1 concomitant with cyclin D1 knockdown suggests that cyclin D1 inhibition could promote temozolomide-induced apoptosis and thus enhance the clinical efficacy of chemotherapy, but this intriguing possibility remains to be examined.

One mechanism involved in chemoresistance is the expression of chemoresistance-associated proteins, such as MDR1/P-glycoprotein [42, 43] and MRP1 [44]. In this study, analysis of MDR1 and MRP1 expression revealed that cyclin D1 suppression decreased MDR1 expression at both mRNA and protein levels. In contrast, increased MDR1 mRNA and proteins were found in SHG-44 cells overexpressing cyclin D1. The mRNA and protein levels of MRP1 were unrelated to cyclin D1 expression, however, in contrast to results of Kornmann et al. [39] showing that downregulation of cyclin D1 by RNA interference (RNAi) decreased both MDR1 and MRP1 expression in human pancreatic cells. In U251 glioblastoma cell line, however, downregulation of cyclin D1 reduced expression of both MDR1 and MRP1, while overexpression of cyclin D1 increased both MDR1 and MRP1 expression. Schaich et al. [45] revealed that temozolomide is a target for the MDR1 efflux pump and that MDR1 participates in the resistance of tumor cells to temozolomide, again suggesting that the increased MDR1 observed in cyclin D1-overexpressing SHG-44 and U251 cells may confer greater resistance to temozolomide.

In summary, our data indicate that cyclin D1 knockdown in SHG-44 cells inhibits proliferation by arresting cell cycle progression, induces apoptosis by increased caspase-3 expression, and attenuates invasiveness, possibly by reducing the expression of MMP-2 and MMP-9. Conversely, cyclin D1 overexpression promotes cell proliferation, potentiates cell invasiveness, and inhibits apoptosis. Further study in another glioblastoma cell line U251 confirmed the effects of CCND1-targeted shRNA on cell proliferation and apoptosis. Collectively, these data indicate that cyclin D1 knockdown may provide a potential gene therapy approach for patients with malignant glioma.

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