Induction of G0/G1 phase arrest and apoptosis by CRISPR/Cas9-mediated knockout of CDK2 in A375 melanocytes

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Abstract. Cutaneous melanoma is one of the most common malignant skin tumors, with a continuously increasing incidence. Cyclin-dependent kinase (CDK) 2 is a key regulator of G1-S transition and modulation of G2 progression; however, its role in cancer is a matter of debate. In the present study, a lentivirus expressing single-guide RNA (sgRNA) was constructed to knock out CDK2 using CRISP/Cas9 technology, in order to confirm the role of CDK2 in A375 human melanoma cells. The results demonstrated that CDK2 knockout induced G0/G1 phase arrest and early apoptosis by downregulating the expression of CDK4 and cyclin A2, and by upregulating the expression of cyclin D1. These results suggest that therapeutic strategies designed to target CDK2 using CRISP/Cas9 may improve the treatment outcome of cutaneous melanoma.

Introduction

Cutaneous melanoma is a common malignant skin tumor (1). The incidence of cutaneous melanoma is continuing to increase at an annual rate of 2.6% (2), and the mortality rates are projected to remain stable through 2019 (3). Currently, surgical or non-surgical treatment (chemotherapy, cryotherapy) are the gold standard or second-line options for the treatment of melanoma (4,5), but the 5-year survival rate, recurrence and side effects are major limitations. Furthermore, the incidence of melanoma and healthcare costs are expected to increase if suitable preventive measures are not undertaken.

The hallmark of malignant tumors is abnormal cell proliferation, and the regulation of the cell cycle is dependent upon the precise coordination of cyclins and cyclin-dependent kinases (CDKs) (6,7). CDKs (CDK2, 4, 6 and 1) are key regulatory enzymes of cell cycle phase transitions (8,9); CDK2, in particular, plays a crucial role in the regulation of G1-S transition and modulation of G2 progression (10,11). A CDK2 activity signature predicts outcome in CDK2-low cancers (12); hence, developing inhibitors targeting CDK2 may be a promising approach to cancer therapy (13). Other studies have indicated that the role of CDK2 in cancer is debatable, as CDK2 knockdown failed to inhibit the proliferation of colon cancer cells (14,15), and cell proliferation may occur in the absence of CDK2 (16,17). Whether CDK2 controls the cell cycle in cutaneous melanoma remains unknown.

CRISP/Cas9 technology is a novel tool for gene editing that has been used for identification of cancer genes (18,19). Our previous studies demonstrated that the proliferation of A375 cells was inhibited and apoptosis was promoted when CDK2 was downregulated by adenovirus-associated virus (AAV) or lentivirus-mediated shRNA (20,21). In an attempt to confirm the role of CDK2 in regulating the cell cycle, apoptosis, or as a therapeutic candidate in human melanoma, a CDK2-single-guide RNA (sgRNA)-expressing lentivirus was constructed in the present study, and the effects of CRISP/Cas9-mediated CDK2 knockout on A375 cell cycle progression and apoptosis were evaluated, aiming to provide novel insight into targeted therapy for cutaneous melanoma.

Materials and methods

Cell culture. A375 human melanocytes were obtained from the Cell Resource Center, Shanghai Institutes for Biological Science, Chinese Academy of Sciences. A375 cells were cultured in Dulbecco's modified Eagle's medium (HyClone; GE Healthcare) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), penicillin (100 U/ml) and streptomycin (100 U/ml), in a 5% CO_2 incubator at 37˚C.

Lentiviral CDK2-sgRNA vector construction and packaging. Two sgRNA target sequences [sgCDK2-108 (TGTTCG TACTTACACCCATG) and sgCDK2-110 (CAGAAACAA GTTGACGGGAG)] for the CDK2 gene (NM_001798) were designed, and a non-silencing sgRNA sequence (CGC TTCCGCGGCCCGTTCAA) was used as negative control (sgCDK2-NC). sgRNA constructs were synthesized and cloned into Lenti-CAS9-sgRNA-EGFP vector with *Bsm*BI sites (GeneChem). The CDK2-sgRNA plasmids were transfected into 293T cells using Lipofectamine 2000 (Invitrogen;

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Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The packaging lentivirus particles expressing CDK2-sgRNA were harvested after transfection for 72 h. Lentivirus was concentrated using the Centricon Plus-20 centrifugal ultrafiltration device (EMD Millipore), and stored at -80˚C for further use.

Lentivirus infection. A375 cells were seeded in 6-well plates and infected with sgCDK2 lentivirus or sgCDK2-NC lentivirus at multiplicity of infection (MOI) of 10, supplemented with polybrene (Santa Cruz Biotechnology, Inc.) when the confluency of A375 cells reached 30%. The culture medium was refreshed after 12 h. The lentiviral infection efficiency was determined through observing GFP fluorescence expression under a fluorescence microscope (Olympus Corporation) at 72 h post-infection.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was extracted using TRIzol reagent (15596026; Thermo Fisher Scientific, Inc.) from A375 cells following infection by lentivirus for 72 h. cDNA was synthesized by a high-capacity cDNA reverse transcription kit (4368814; Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. The primers used for qPCR were as follows: CDK2, forward 5'-TTCTATGCCTGATTACAAGCC-3' and reverse 5'-CTGGCTTGGTCACATCCT-3'; CDK4, forward 5'-CTGGTGACAAGTGGTGGAACAGTC-3' and reverse 5'-GGTGTAAGTGCCATCTGGTAGCTG-3'; cyclin D1, forward 5'-TACCGCCTCACACGCTTCCTC-3' and reverse 5'-ACCTCCTCCTCCTCCTCTTCCTC-3'; cyclin A2, forward 5'-AGAAACAGCCAGACATCACTAA-3' and reverse 5'-TTCAAACTTTGAGGCTAACAGC-3'; and GAPDH, forward 5'-AAGAAGGTGGTGAAGCAGGC-3' and reverse 5'-TCCACCACCCAGTTGCTGTA-3'. qPCR was performed using the Quantstudio3 platform (Applied Biosystems; Thermo Fisher Scientific, Inc.) with FS Universal SYBR Green Master (4913914001; Roche Diagnostics). The relative expression of the targets was determined by the $2^{-\Delta\Delta Cq}$ method in triplicate samples (22).

Western blot analysis. Total proteins were extracted using RIPA lysis buffer (Beyotime Institute of Biotechnology) supplemented with protease inhibitors (Roche Diagnostics). Protein concentrations were determined by a BCA protein assay kit (P0010; Beyotime Institute of Biotechnology). Proteins (40 μg per lane) were separated by SDS-PAGE and then transferred onto a polyvinylidene fluoride membrane. The membrane was blocked in TBST buffer containing 5% BSA for 2 h at room temperature, and then incubated with primary antibodies at 4˚C overnight. The primary antibodies used in the study were as follows: CDK2 (1:1,000; ab32147; Abcam), CDK4 (1:2,000; ab199728; Abcam), cyclin A2 (1:2,000; ab181591; Abcam), cyclin D1 (1:2,000; ab134175; Abcam), GAPDH (1:1,000, TA-08; ZsBio). The membrane was then incubated with horseradish peroxidase (HRP)-goat anti-rabbit IgG (1:5,000; ZB-2301; ZsBio) or HRP-goat anti-mouse IgG (1:5,000; ZB-2305; ZsBio) after washing three times with TBST buffer. The blots were developed using the BeyoECL Plus kit (P0018S; Beyotime Institute of Biotechnology). The bands of target protein were subjected to densitometric analysis using Image-Pro Plus 6.0 software (Media Cybernetics).

Cell cycle analysis. A375 cells were seeded in 6-well plates and infected with sgCDK2-108 or sgCDK2-NC lentivirus. The cells were harvested by digesting with 0.25% trypsin and centrifuged at 1,000 x g at 4˚C for 5 min, washed twice with PBS and centrifuged at 1,000 x g at 4˚C for 5 min to remove the supernatant, then fixed with 70% ethanol at 4˚C overnight. The cells were resuspended in PBS containing RNase A (100 μg/ml) at 37˚C for 30 min, then stained with propidium iodide (WLA010a; Wanleibio) at 4˚C for 30 min. The stained cells were analyzed using the BD Accuri C6 flow cytometer (BD Biosciences).

Detection of apoptosis. The A375 cells were handled as described above. The cells were resuspended in 500 μl binding buffer; subsequently, 5 μl Annexin V-Light 650 and 10 μl propidium iodide (WLA002c; Wanleibio) were added and gently mixed. The cells were stained at 37˚C for 15 min and the apoptosis was determined using the BD Accuri C6 flow cytometer (BD Biosciences).

Statistical analysis. All data are expressed as the mean ± standard deviation of three independent experiments. The data were analyzed with Student's t-test between two groups, whereas multiple groups were analyzed with Student Newman Keuls (S-N-K) and one-way ANOVA using SPSS software, version 13.0 (SPSS, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

CDK2 knockout in A375 cells. Fluorescence microscopy was used to investigate the infection efficiency of recombinant lentivirus in A375 cells. The results demonstrated that >80% of A375 cells expressed GFP among the sgCDK2-110, sgCDK2-108 and sgCDK2-NC groups (Fig. 1A). The qPCR results revealed that the mRNA levels of CDK2 were significantly downregulated in A375 cells infected by lentiviruses sgCDK2-110 and sgCDK2-108 compared with sgCDK2-NC ($P<0.05$; Fig. 1B). The results were further confirmed by western blotting, which demonstrated that the CDK2 was knocked out in A375 cells infected by lentiviruses sgCDK2-110 and sgCDK2-108, particularly sgCDK2-108 ($P<0.05$; Fig. 1C and 1D). These results indicated that CDK2 is depleted in A375 cells.

CDK2 knockout induces G0/G1 phase arrest in A375 cells. The effect of CDK2 knockout on the cell cycle in A375 cells was examined by flow cytometric analysis. The lentivirus sgCDK2-108 was selected for the following experiments. The results revealed an increased percentage of cells in the G0/G1 phase among A375 cells infected by lentivirus sgCDK2-108 (Fig. 2A). The percentage of cells in the G0/G1 phase among lentivirus sgCDK2-108-infected A375 cells was significantly higher (81.78%) compared with lentivirus sgCDK2-NC (69.06%); however, the percentage of S phase cells in the sgCDK2-108 group was significantly reduced (7.85%) compared with the sgCDK2-NC group

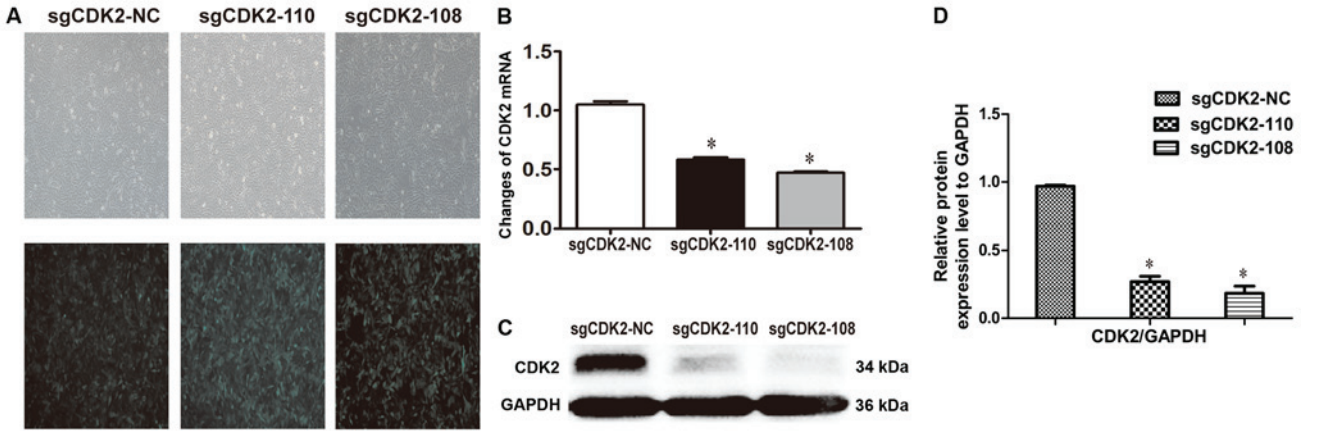

Figure 1. CDK2 expression was knocked out by lentivirus in A375 cells. A375 cells were infected with sgCDK2-110, sgCDK2-108 and sgCDK2-NC lentiviruses. (A) Infection efficiency of A375 cells was determined by fluorescence microscopy; original magnification, x100. (B) The levels of CDK2 mRNA were assessed by reverse transcription-quantitative polymerase chain reaction analysis. (C) Protein levels of CDK2 in A375 cells infected with lentivirus. (D) Densitometric analysis of CDK2 protein identified by western blotting. *P<0.05 vs. sgCDK2-NC control. CDK, cyclin-dependent kinase; sg, single-guide.

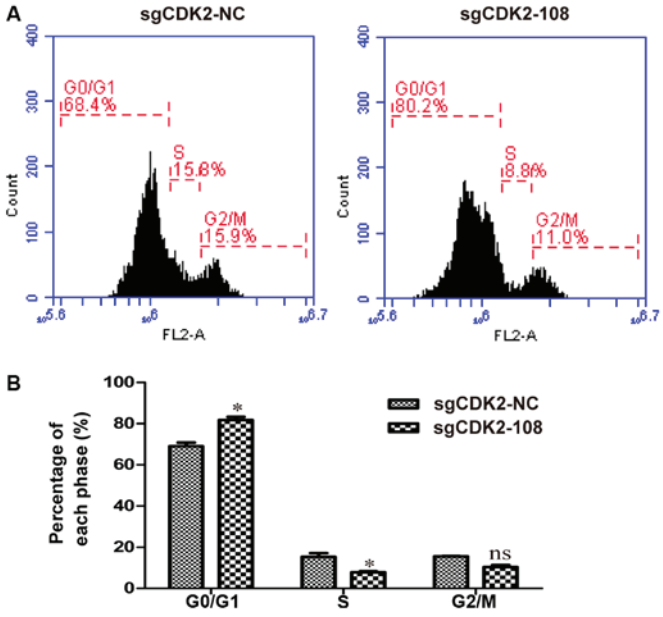

Figure 2. Knockout of CDK2 induced G0/G1 phase arrest in A375 cells. (A) The cell cycle distribution of A375 cells infected with sgCDK2-108 or sgCDK2-NC lentivirus was determined by staining with propidium iodide through flow cytometry at 72 h. (B) The percentages of G0/G1, S and G2/M phase cells are displayed as histograms. The data are expressed the mean ± standard deviation of three independent experiments. *P<0.05 vs. sgCDK2-NC control. CDK, cyclin-dependent kinase; sg, single-guide; ns, not significant.

(15.38%) (P<0.05; Fig. 2B). These results indicated that CDK2 knockout induces G0/G1 phase arrest in A375 cells.

CDK2 knockout induces early apoptosis in A375 cells. Apoptosis was then analyzed in A375 cells infected with lentiviruses sgCDK2-108 or sgCDK2-NC. Compared with the sgCDK2-NC group, early apoptosis of A375 cells infected with lentivirus sgCDK2-108 was observed (Fig. 3A). The rate of early apoptosis in A375 cells infected with lentivirus sgCDK2-108 was 11.76%, and the rate of total apoptosis reached 12.47% (P<0.05; Fig. 3B), suggesting that knockout of CDK2 induces early apoptosis in A375 cells.

CDK2 knockout alters the expression of CDK4 and cyclin A2. In order to investigate whether the cell cycle-related proteins were differentially regulated, the relative changes in CDK4, cyclin A2 and cyclin D1 expression were evaluated with RT-qPCR and western blot analysis. The results revealed that the transcriptional level of CDK4 was slightly downregulated (P>0.05), the level of cyclin A2 was significantly downregulated (P<0.05), whereas the level of cyclin D1 was significantly upregulated (P<0.05) (Fig. 4A). The protein levels of CDK4 and cyclin A2 were downregulated; in particular, cyclin A2 was significantly decreased. However, the protein expression of cyclin D1 was significantly upregulated (P<0.05) (Fig. 4B and 4C).

Discussion

Previous studies demonstrated that the colony-forming ability and cell viability were markedly inhibited by knockdown of myosin VI using lentivirus-mediated shRNA in A375 melanocytes (23). Knockdown of cyclin-dependent kinase-like 1 (CDKL1) by CDKL1-siRNA-expressing lentivirus in A375 and MV3 cells also significantly inhibited cell growth and colony-forming ability (24). Therefore, molecular-targeted therapy using lentivirus-mediated shRNA has become a focus in anti-melanoma research, as it is not associated with genotoxicity compared with conventional chemotherapeutic agents. However, the outcome of lentivirus-mediated gene knockdown may be affected by the off-target effects using a single shRNA. CRISP/Cas9 technology is a reliable method for gene editing, and CRISP/Cas9-mediated knockout of the PDEF gene significantly inhibited the migration and invasion of AGS human gastric cancer cells by transfection with pX459-PDEF-sgRNA plasmids (25). Therefore, CRISP/Cas9-based genome editing may provide novel insight into cancer therapy (26-28).

CDK2 is a key regulator of the G1/S and S/G2 cell cycle transitions; however, genetic deletion of CDK2 in p27 (Kip1)-null mice failed to suppress the development of pituitary tumors (29). CDK2 was also found to be a key mediator of epidermal growth factor-induced cell transformation through directly phosphorylating ELK4 and regulating *c-fos*

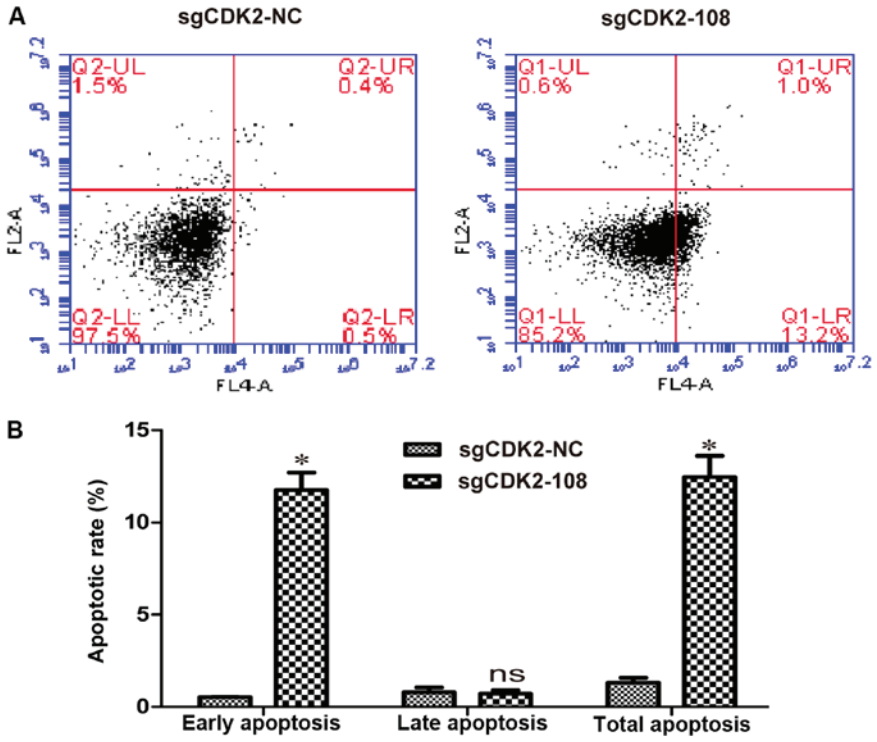

Figure 3. Knockout of CDK2 induced early apoptosis in A375 cells. (A) Apoptosis of A375 cells infected with lentiviruses sgCDK2-108 or sgCDK2-NC was determined by staining with Annexin V-Light 650 and propidium iodide through flow cytometry at 72 h. (B) The percentages of early, late and total apoptotic cells are displayed as histograms. The data are expressed the mean ± standard deviation of three independent experiments. *P<0.05 vs. sgCDK2-NC control. CDK, cyclin-dependent kinase; sg, single-guide; ns, not significant.

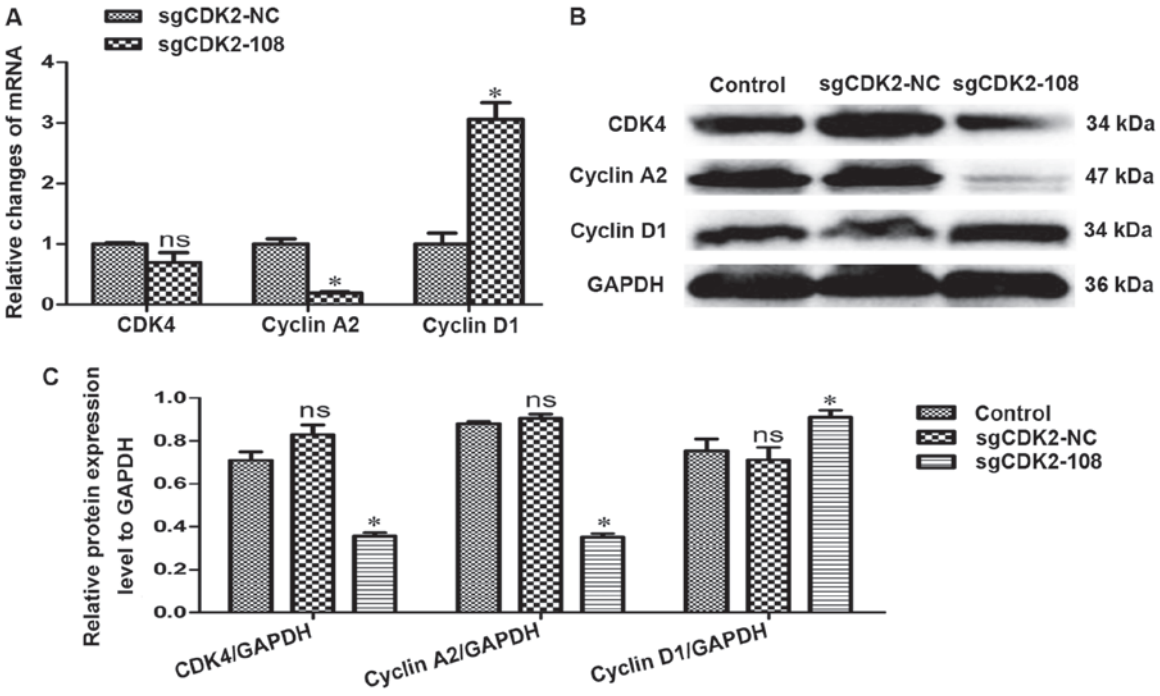

Figure 4. CDK2 knockout altered the expression of cell cycle-related proteins. (A) Relative changes in CDK4, cyclin A2 and cyclin D1 levels were determined by reverse transcription-quantitative polymerase chain reaction analysis. (B) Expression of CDK4, cyclin A2 and cyclin D1 in A375 cells infected with sgCDK2-108 or sgCDK2-NC lentiviruses. (C) Densitometric analysis of CDK4, cyclin A2 and cyclin D1 identified by western blotting. The data are expressed as the mean ± standard deviation of three independent experiments. *P<0.05 vs. sgCDK2-NC control. CDK, cyclin-dependent kinase; sg, single-guide; ns, not significant.

expression (30). Additionally, ablation of CDK2 significantly delayed S-M progression and downregulated the expression of CDK6 (31). Therefore, the suitability of CDK2 as a therapeutic target remains controversial.

In order to evaluate the role of CDK2 in regulating cell cycle and mediating apoptosis of cutaneous melanoma cells, we selected a single lentiviral vector to deliver nuclease Cas9, a sgRNA, and a puromycin selection and enhanced green fluorescent protein (EGFP) markers into target cells. A previous study using a single lentiviral vector (lentiCRISPR) to deliver Cas9 and sgRNA into target cells demonstrated that lentiCRISPs could abolish EGFP fluorescence in 93±8% of infected cells at a low MOI of 0.3 for 11 days; however, lentiviral vectors expressing EGFP-targeting shRNA were unable to completely knock down EGFP (32). Further study also demonstrated a significant reduction in the diversity of sgRNAs in surviving human melanoma A375 cells and human HUES62 stem cells transduced with the GeCKO library at an MOI of 0.3 (32). This lentiviral SCRISP/Cas9 genome editing system have been using in human cells (33). Two lentiviruses were constructed to knock out CDK2 using CRISP/Cas9 technology. The results revealed a successful lentivirus-mediated knockout of CDK2 using CRISP/Cas9 technology; the expression of CDK2 was also completely knocked out in A375 cells. Although the nuclease Cas9 and sgRNA (sgCDK2-108) were delivered into A375 cells by sgCDK2 lentivirus and CDK2 expression was abolished at the mRNA and protein levels, single colonies and PCR identification at the DNA level were not conducted. The homozygosity of the cells was not known, and the lack of a precise genetic investigation is a limitation of the study.

Further study demonstrated that the loss of CDK2 function significantly increased the percentage of cells in the G0/G1 phase and induced G0/G1 phase arrest. The percentage of early apoptotic A375 cells was also increased. These results indicated that CDK2 plays a pivotal role in the regulation of cell cycle transition, and may be associated with the progression of cutaneous melanoma. Our study also demonstrated that the expression of CDK4 and cyclin A2 was downregulated, whereas the expression of cyclin D1 was upregulated at the transcriptional and translational levels. This result indicates that G0/G1 phase arrest is induced by downregulated expression of CDK4 and cyclin A2, and upregulated expression of cyclin D1. Subsequently, apoptosis occurs as a result of G0/G1 phase arrest. Apoptosis as a protective mechanism ensures homeostasis of host cells through cell shrinkage, fragmentation of cellular DNA and formation of 'apoptotic bodies' leading to cell death. Two pathways, namely 'extrinsic' and 'intrinsic' pathways, activate caspases to cleave vital cellular proteins, and BCL-2 protein, as the first inhibitor of apoptosis, controls cell death first though directly regulating the integrity of the outer mitochondrial membrane (34,35). In the present study, apoptosis of A375 cells occurred following knockout of CDK2 by flow cytometry, but the changes of apoptotic-related proteins, such PARP, caspase-3 and BCL-2, were not evaluated by western blotting, which is another limitation of this study. Further research will focus on the mechanism of apoptosis of A375 cells following CDK2 knockout by a lentiviral CRISP/Cas9 system. Elucidating the changes in whole cellular proteins by proteomic analysis and investigating the role of caspases or BCL-2 may provide more evidence regarding the role(s) of CDK2 in human melanoma.

In conclusion, the results of the present study demonstrated that CDK2 is crucial for cell cycle regulation through controlling the G1/S transition in A375 human melanoma cells. Therefore, knockout of CDK2 by CRISPR/Cas9 technology may provide a novel therapeutic approach to cutaneous melanoma.

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Availability of data and materials

All the datasets generated and analyzed in the present study are available from the corresponding author on reasonable request.

Authors' contributions

HL conceived, designed, supervised the study and wrote the manuscript. ZL and SH performed the experiments, QW and LG analyzed the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors have no commercial or other associations that may pose a conflict of interest.

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