# Construction of shRNA-PAX6 Lentiviral Vector and Its Effect on Proliferation of Glioma U251 Cells (Postprint)

**Authors:** Xiaohong Liao, Yin Weilan, Wang Fang, Wu Lixiang, Huang Baisheng

**Date:** 2018-06-15T00:00:00+00:00

## Abstract

Objective: To construct an shRNA-PAX6 lentiviral vector and observe its effect on the proliferation of glioma U251 cells. Methods Based on the reported PAX6 target sequences in literature, two siRNA target interference sequences for the PAX6 gene were designed. Primers were annealed to form double-stranded DNA with sticky ends, which were ligated with the linearized lentiviral vector after double digestion with BamH I and EcoR I, followed by transformation to construct the shRNA-PAX6 lentiviral recombinant vector. The recombinant vector was then identified by colony PCR and sequencing. The virus was packaged in 293T cells, and the packaged shRNA-PAX6 lentiviral recombinant vector was used to infect U251 cells. Real-time PCR was performed to detect the expression level of PAX6 mRNA, Western blot was used to detect PAX6 protein expression, and MTT assay was employed to assess U251 cell proliferation. Results After double digestion of the lentiviral vector pLKD-CMV-G&NR-U6-shRNA, a linearized large fragment of 8208 bp was observed. Colony PCR and sequencing identification confirmed the successful construction of the shRNA-PAX6 lentiviral recombinant vector. The constructed shRNA-PAX6 lentiviral vector was successfully packaged in 293T cells, and the lentiviral titer was measured to be 6.73×10^8 TU/mL. Real-time PCR results showed that silencing PAX6 expression led to significantly lower PAX6 mRNA expression levels in U251 cells compared with the normal control group and the empty lentiviral vector group ($P<0.05$). Western blot results demonstrated that PAX6 protein expression was also significantly lower than that in the normal control group and the empty lentiviral vector group ($P<0.05$). MTT results indicated that compared with cells in the normal control group and the empty lentiviral vector group, cells infected with the shRNA-PAX6 lentiviral recombinant vector showed significantly enhanced proliferative capacity ($P<0.05$). Conclusion The shRNA-PAX6 lentiviral recombinant vector targeting the human PAX6 gene

was successfully constructed, and silencing PAX6 gene expression enhanced the proliferative capacity of U251 cells.

## Full Text

## Preamble

LIAO Xiaohong, YIN Weilan, WANG Fang, WU Lixiang, HUANG Baisheng
Department of Physiology, Xiangya School of Medicine, Central South University, Changsha 410078, China

## Abstract

**Objective**: To construct a lentiviral vector for delivering short hairpin RNA (shRNA) targeting PAX6 and investigate its effect on the proliferation of glioma U251 cells in vitro. **Methods**: Two small interfering RNA sequences targeting PAX6 gene were designed based on the reported sequence of PAX6 and annealed to form a double-stranded chain, which was inserted into a lentiviral vector to construct the recombinant lentiviral vector shRNA-PAX6. The recombinant vector was infected into U251 cells, and the expression of PAX6 mRNA and protein in the cells was detected by real-time PCR and Western blotting, respectively. The changes in the proliferation of U251 cells after the infection was assessed using MTT assay. **Results**: Double enzyme digestion of the lentiviral vector pLKD-CMV-G&NR-U6-shRNA yielded an 8208-bp fragment, and colony PCR and sequencing analysis confirmed successful construction of the lentiviral vector shRNA-PAX6. Infection of the cells with shRNA-PAX6 caused a significant reduction of the expressions of PAX6 mRNA and protein ($P<0.05$) and resulted in obviously increased proliferation of U251 cells ($P<0.05$). **Conclusion**: We successfully constructed the recombinant vector shRNA-PAX6 for silencing PAX6 gene. PAX6 gene silencing results in increased proliferation of U251 cells in vitro.

**Keywords**: PAX6 gene; glioma cells; proliferation; RNA interference; lentivirus vector

## Introduction

Gliomas are the most common primary brain tumors in clinical practice. These tumors lack clear boundaries with adjacent normal brain tissue, exhibit highly aggressive behavior, and are prone to recurrence, resulting in high mortality rates. Even with combined treatment modalities including surgery, chemotherapy, and radiotherapy, the median survival period is only slightly over one year [1-2], and the five-year survival rate is below 3% [3-4]. Therefore, identifying novel therapeutic targets for gliomas is of great significance for prolonging patient survival. The transcription factor PAX6 is a member of the evolutionarily conserved PAX gene family that plays crucial roles in the development and differentiation of various tissues and organs, including the eye, pancreas, central

nervous system, and endocrine system [5]. For instance, PAX6 participates in mouse retinal development [6], differentiation of corneal epithelial cells [7], and directional differentiation of limbal stem cells [8]. This gene is also extensively involved in various biological processes such as cell proliferation, migration, adhesion, embryonic development, and tumorigenesis [9]. In Parkinson' s disease, PAX6 exerts protective effects on dopaminergic neurons [10]. In tumor growth, invasion, and angiogenesis, PAX6 may function as either an oncogene or tumor suppressor depending on the tissue type [11]. Studies have shown that PAX6 expression levels vary across different tumor tissues, with expression in glioma tissues being significantly lower than in adjacent tissues [12]; however, the specific role of PAX6 in glioma tissues remains unclear. This study aims to construct a lentiviral recombinant vector expressing short hairpin RNA (shRNA) targeting PAX6, transfect it into glioma U251 cells, observe the effects of PAX6 on U251 cell proliferation, explore the role of PAX6 in gliomas, and provide insights for identifying novel therapeutic targets for glioma treatment.

## Materials and Methods

### 1.1 Materials

The materials used in this study included DMEM medium (Hyclone), fetal bovine serum (Invitrogen), Trizol (Invitrogen), PCR TaqMix (Guangzhou Dongsheng Biotech), agarose (Spanish), gel extraction kit (OMEGA), pLKD-CMV-G&PR-U6-shRNA lentiviral vector (Neuron Shanghai Biotech), DNA molecular weight markers (Fermentans), AgeI (NEB), EcoRI (Fermentans), BamHI, EcoR I, T4 ligase, and reverse transcription kit (Fermentas), ViraPower Lentiviral Packaging Mix (Invitrogen), PAX6 primary and secondary antibodies from Santa Cruz, 293T and U251 cells (Shanghai Cell Bank), and *E. coli* DH5 strain preserved in our laboratory.

### 1.2 Methods

**1.2.1 Construction of shRNA-PAX6 Vector** Based on the human PAX6 gene sequence (NM_000280) and reference [13], two siRNA target sequences for PAX6 were designed using the reported target sequence ATGGGCGGAGTTATGATACCTAC. The sequences were as follows: PAX6-shRNA-1: CCGGATGGGCGGAGTTATGATACCTACCTCGAGAGGTATCATAACTCCGCCCATTTTTTG, and PAX6-shRNA-2: AATTCAAAAAATGGGCGGAGTTATGATACCTACCTCGAGTAGGTATCATAACTCCGCCCA. The primers were annealed to form double-stranded fragments with sticky ends and ligated into the BamHI and EcoR I double-digested linearized lentiviral vector. A universal negative control sequence (AATTCTCCGAACGTGTCACGT) was also ligated into the lentiviral vector as a negative control (NC).

**1.2.2 Colony PCR and Sequencing Identification of Positive Clones** Single colonies from the transformation plates were picked and

resuspended in 10 µL LB liquid medium, and 6 µL of this suspension was used as template for PCR amplification. The primers used were: forward 5' -CCTATTTCCCATGATTCCTTCATA-3' and reverse 5' -GTAATACGGTTATCCACGCG-3' , with expected product sizes of 294 bp for the empty vector and 316 bp for positive clones. PCR products were analyzed by 1% agarose gel electrophoresis and photographed using a gel imaging system. Additionally, 200 µL of bacterial culture from positive clones was sent to BGI for sequencing, and the results were analyzed using CLUSTAL X 1.8 software.

**1.2.3 Lentiviral Packaging** Positive clones identified by colony PCR were amplified, and plasmids were extracted and co-transfected with the ViraPower Lentiviral Packaging Mix into 293T cells. Culture supernatants were collected at 24 h and 48 h after transfection and medium change. The viral stocks collected at both time points were mixed and centrifuged at 3000 r/min for 30 min at 4°C to remove cells and debris, and the supernatant was filtered through a 0.45 µm filter. Finally, the lentiviral stock was concentrated by ultracentrifugation at 12,000 g for 20 min at 4°C, the supernatant was discarded, and the pellet was resuspended in DMEM containing 2% FBS.

**1.2.4 Lentiviral Titer Determination** The viral stock was serially diluted (10, 1, and 0.1 µL) and used to infect logarithmically growing 293T cells. After 72 h, the number of fluorescent cells in each well was observed under a fluorescence microscope. The viral titer was calculated as the average number of fluorescent cells per well divided by the volume of lentiviral fluid in each well.

**1.2.5 Detection of PAX6 Expression 1.2.5.1 Real-time PCR Detection of PAX6 mRNA Expression**: The packaged PAX6 lentiviral vector (shRNA-PAX6) and empty lentiviral vector (shRNA-NC) were used to infect cultured glioma U251 cells, and cells were collected after 72 h. Real-time PCR primers for PAX6 were: forward AGACACAGCCCTCACAAAC and reverse ATCATAACTCCGCCCATTC (product size 157 bp); for -actin: forward AGGGGCCGGACTCGTCATACT and reverse GGCGGCACCACCATGTACCCT (product size 202 bp). The reaction system was 20 µL, with cycling conditions of 94°C for 3 min, followed by 40 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s, with final extension at 72°C for 10 min.

**1.2.5.2 Western Blot Detection of PAX6 Protein Expression**: At 72 h after viral infection, cells were digested with 0.25% trypsin and collected by centrifugation at 1500 r/min. After washing with PBS, cell pellets were used for protein extraction. Total protein samples were subjected to SDS-PAGE, transferred to PVDF membranes, blocked with TBST at room temperature for 1 h, and incubated overnight with diluted primary antibodies against PAX6 or -actin. After washing three times with TBST, horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody was added and incubated at room

temperature for 1 h. Following three TBST washes, protein bands were detected using an ECL Western blot kit.

**1.2.6 MTT Assay for Cell Viability** At 0, 24, 48, and 72 h after lentiviral infection, 20 µL of 5 mg/mL MTT solution was added to each well of a 96-well plate (final concentration 0.5 mg/mL) and incubated for 4 h. After removing the supernatant, 200 µL DMSO was added to each well, shaken for 20 min, and absorbance (A) was measured at 570 nm using a microplate reader. Cell viability in the normal group was set as 100%, and cell viability in experimental groups was calculated using the formula: Cell viability (%) = (absorbance of experimental group / absorbance of control group) × 100%.

**1.2.7 Statistical Analysis** Experimental data were analyzed using SPSS 16.0 software, and results are expressed as mean ± standard deviation. Two-way ANOVA was used for comparison between groups. Real-time PCR data were analyzed using the 2^-ΔΔCt method to calculate relative gene expression ratios. $P<0.05$ was considered statistically significant.

## Results

### 2.1 Agarose Gel Electrophoresis of Double-Digested pLKD-CMV-G&NR-U6-shRNA Lentiviral Vector

The pLKD-CMV-G&NR-U6-shRNA lentiviral vector was digested with AgeI and EcoRI, and agarose gel electrophoresis revealed an 8208 bp fragment. After gel purification, the linearized pLKD-CMV-G&NR-U6-shRNA vector fragment was obtained [Figure 1: see original paper].

### 2.2 Colony PCR Identification

After transformation of competent *E. coli*, scattered single colonies were observed on the plates. Single colonies were picked as template DNA for colony PCR, and PCR products were analyzed by 1% agarose gel electrophoresis. The results showed that among transformants 1-7, colonies 2-7 were positive clones with amplification bands consistent with the expected product size of 316 bp [Figure 2: see original paper].

### 2.3 Determination of shRNA-PAX6 Lentiviral Recombinant Vector Titer

The lentivirus exhibited infectivity toward 293T cells in a dilution-dependent manner, with infectivity directly reflected by the number of green fluorescent cells. Under fluorescence microscopy, higher dilutions of the lentiviral stock resulted in fewer infected cells [Figure 3: see original paper]. After counting GFP-positive cells, the average viral titer obtained from this production system was determined to be 6.73 × 10^8 TU/mL.

### 2.4 PAX6 mRNA Expression

Real-time PCR results showed that 48 h after infection with the shRNA-PAX6 lentiviral recombinant vector, PAX6 expression levels in glioma U251 cells were significantly lower than in the uninfected group and the shRNA-NC group ($P<0.05$) [Figure 4: see original paper].

### 2.5 PAX6 Protein Expression

After infection with the shRNA-PAX6 lentiviral recombinant vector, Western blot analysis revealed that PAX6 protein expression in U251 cells was significantly lower than in the uninfected group and the shRNA-NC group ($P<0.05$) [Figure 5: see original paper].

### 2.6 Effect of PAX6 on U251 Cell Proliferation

U251 cells were infected with the shRNA-PAX6 lentiviral recombinant vector, and cell proliferation was assessed at different time points. MTT results showed that compared with uninfected cells and shRNA-NC cells, cell proliferation was significantly enhanced at 72 h ($P<0.05$) [Figure 6: see original paper].

## Discussion

The transcription factor PAX6 is an important member of the PAX gene family. This gene regulates the transcription of downstream target genes by activating or inhibiting the activity of promoters and enhancers [14], and may function as either an oncogene or tumor suppressor in the growth, invasion, and angiogenesis of different cancer types. Research has reported [15] that PAX6 is highly expressed in pancreatic cancer tissues, with expression levels positively correlated with tumor malignancy, and that inhibiting PAX6 expression may promote apoptosis and reduce growth capacity in pancreatic cancer cells through activation of tyrosine kinase receptors. In breast cancer tissues, PAX6 expression levels are associated with prognosis [16]. Suppression of PAX6 expression can promote proliferation of human retinoblastoma cells by increasing Bcl-2, CDK1, and PCNA expression while decreasing BAX and p21 expression, thereby reducing apoptosis [17]. However, other studies have reported that targeted silencing of PAX6 may inhibit proliferation and invasion of retinoblastoma cells by regulating the PI3K/AKT signaling pathway [18]. PAX6 is highly expressed in non-small cell lung cancer A549 and H1299 cells, and its downregulation inhibits cancer cell proliferation and cell cycle progression by suppressing the MAPK signaling pathway [19], while PAX6 overexpression promotes proliferation and invasion of A549 cells through ERK and MAPK signaling pathways [20]. In prostate cancer tissues and cells, PAX6 expression is lower than in normal epithelial cells, and PAX6 overexpression inhibits prostate cancer cell proliferation and colony formation [21]. PAX6 is also associated with bladder cancer and may function as a tumor suppressor and molecular marker in cancer development. PAX6 expression is significantly lower in glioma tissues than in

adjacent normal brain tissues [11], and PAX6 overexpression can inhibit glioma cell growth and invasion, possibly by suppressing the G1-to-S phase transition or by inhibiting VEGFA expression to suppress tumor growth, angiogenesis, and metastasis [22]. PAX6 overexpression may also regulate glioma stem cell growth and invasion through the PI3K/Akt signaling pathway [23], suggesting that low PAX6 expression in glioma tissues may contribute to abnormal proliferation and growth.

In this study, we employed RNA interference technology and selected two interference target sites as PAX6 siRNA sequences based on reference [13]. Using the pLKD-CMV-G&NR-U6-shRNA lentiviral vector as a gene delivery tool, we constructed the shRNA-PAX6 lentiviral recombinant vector. Since establishing a packaging cell line is a critical step for obtaining intact viral particles to infect target cells, we packaged the lentivirus in 293T cells using the recombinant vector confirmed by colony PCR and sequencing. Observation of GFP expression under fluorescence microscopy and measurement of an average viral titer of 6.73 $\times$ 10^8 TU/mL demonstrated successful transfection of 293T cells, indicating that the shRNA-PAX6 lentiviral vector could be used to infect target cells. Infection of glioma U251 cells with the shRNA-PAX6 lentiviral vector resulted in significantly reduced PAX6 mRNA and protein expression compared to uninfected cells and shRNA-NC infected cells, demonstrating effective silencing of the target gene at the molecular level. MTT assays showed that shRNA-PAX6 infected cells exhibited enhanced proliferation capacity compared to uninfected and shRNA-NC infected cells, indicating that silencing PAX6 expression promotes proliferation of glioma U251 cells.

Recent studies have shown that PAX6, as a target gene of microRNA-335, inhibits proliferation of glioma U251 and U87 cells [24] as well as breast cancer MCF-7 cells [25]. However, as a target of microRNA-7, PAX6 promotes proliferation of colorectal cancer Caco-2 and SW480 cells [26], and as a target of microRNA-433, it promotes proliferation and metastasis of retinoblastoma cells [27]. PAX6 also serves as a target of microRNA-375 to enhance the viability of breast cancer MCF-7 cells [28]. Since PAX6 regulates numerous genes and is itself a target of multiple microRNAs, its regulatory mechanisms remain incompletely understood. How PAX6 influences glioma cell proliferation and growth requires further investigation. The shRNA-PAX6 lentiviral recombinant vector constructed in this study can effectively silence the target gene at the molecular level, providing an experimental foundation for further functional studies of PAX6. PAX6 may represent a potential therapeutic target for clinical treatment of glioma patients.

## References

[1] Badhiwala JH, Nassiri F, Almenawer SA. GBM surgery in the elderly-time to be more aggressive [J]? Clin Neurol Neurosurg, 2016, 141(2): 131-2.
[2] Riedmann EM. Agenus cancer vaccine extends survival of GBM patients[J]. Hum Vaccin Immunother, 2014, 10(2): 252-3.

[3] Qu ST, Yao YL, Shang C, et al. MicroRNA-330 is an oncogenic factor in glioblastoma cells by regulating SH3GL2 gene[J]. PLoS One, 2012, 7(9): e46010.
[4] Dolecek TA, Propp JM, Stroup NE, et al. CBTRUS statistical report: primary brain and central nervous system tumors diagnosed in the United States in 2005-2009[J]. Neuro Oncol, 2012, 14(Suppl 5): v1-49.
[5] Paixão-Côrtes VR, Salzano FM, Bortolini MC. Origins and evolvability of the PAX family[J]. Semin Cell Dev Biol, 2015, 44(8): 64-74.
[6] Kim CH, An MJ, Kim DH, et al. Histone deacetylase 1 (HDAC1) regulates retinal development through a PAX6-dependent pathway[J]. Biochem Biophys Res Commun, 2017, 482(4): 735-41.
[7] Kitazawa K, Hikichi T, Nakamura TA, et al. PAX6 regulates human corneal epithelium cell identity[J]. Exp Eye Res, 2017, 154(1): 30-38.
[8] Ouyang H, Xue YC, Lin Y, et al. WNT7A and PAX6 define corneal epithelium homeostasis and pathogenesis[J]. Nature, 2014, 511(7509): 358-61.
[9] Blake JA, Ziman MR. Pax genes: regulators of lineage specification and progenitor cell maintenance[J]. Development, 2014, 141(4): 737-51.
[10] Thomas MG, Welch C, Stone L, et al. PAX6 expression may be protective against dopaminergic cell loss in Parkinson' s disease[J]. CNS Neurol Disord Drug Targets, 2016, 15(1): 73-9.
[11] Zhou YH, Tan F, Hess KR, et al. The expression of PAX6, PTEN, vascular endothelial growth factor, and epidermal growth factor receptor in gliomas: relationship to tumor grade and survival[J]. Clin Cancer Res, 2003, 9(9): 3369-75.
[12] Lin J, Teo S, Lam DH, et al. MicroRNA-10b pleiotropically regulates invasion, angiogenicity and apoptosis of tumor cells resembling mesenchymal subtype of glioblastoma multiforme[J]. Cell Death Dis, 2012, 3(10): e398.
[13] Zong XY, Yang HJ, Yu Y, et al. Possible role of Pax-6 in promoting breast cancer cell proliferation and tumorigenesis[J]. BMB Rep, 2011, 44(9): 595-600.
[14] Gregory-Evans CY, Wang X, Wasan KM, et al. Postnatal manipulation dosage reverses congenital tissue malformation defects[J]. J Clin Invest, 2014, 124(1): 111-6.
[15] Lai JP, Mertens RB, Mirocha J, et al. Comparison of PAX6 and immunohistochemical markers pancreatic neuroendocrine tumors[J]. Endocr Pathol, 2015, 26(1): 54-62.
[16] Xia XH, Yin WJ, Zhang XP, et al. PAX6 overexpression is associated with the poor prognosis of invasive ductal breast cancer[J]. Oncol Lett, 2015, 10(3): 1501-6.
[17] Meng B, Wang YS, Li B. Suppression of PAX6 promotes cell proliferation and inhibits apoptosis in human retinoblastoma cells[J]. Int J Mol Med, 2014, 34(2): 399-408.
[18] 祁海燕, 冷非, 苏学刚. 靶向沉默 Pax6 基因表达抑制视网膜母细胞瘤增殖侵袭的机制研究[J]. 临床和实验医学杂志, 2017, 16(16): 1604-1608.
[19] Zhao XT, Yue WT, Zhang LN, et al. Downregulation of PAX6 by shRNA inhibits proliferation and cell cycle progression of human non-small cell lung cancer cell lines[J]. PLoS One, 2014, 9(1): e85738.
[20] Luo JS, Li H, Zhang CF. MicroRNA-7 inhibits the malignant phenotypes of non-small cell lung cancer in vitro by targeting Pax6[J]. Mol Med Rep, 2015,

12(4): 5443-8.
[21] Shyr CR, Tsai MY, Yeh S, et al. Tumor suppressor PAX6 functions as androgen receptor co-repressor to inhibit prostate cancer growth[J]. Prostate, 2010, 70(2): 190-9.
[22] Qiu JF, Zhang ZQ, Wang Y, et al. Lentivirus-mediated RNAi knockdown of VEGFA in RKO colorectal cancer cells decreases tumor formation and growth in vitro and in vivo[J]. Int J Clin Exp Pathol, 2012, 5(4): 290-8.
[23] Huang BS, Luo QZ, Han Y, et al. MiR-223/PAX6 axis regulates glioblastoma stem cell proliferation and the chemo resistance to TMZ via regulating PI3K/Akt pathway[J]. J Cell Biochem, 2017, 118(10): 3452-61.
[24] Cheng Q, Cao H, Chen ZG, et al. PAX6, a novel target of miR-335, inhibits cell proliferation and invasion in glioma cells[J]. Mol Med Rep, 2014, 10(1): 399-404.
[25] Meng YB, Zou QQ, Liu TQ, et al. microRNA-335 inhibits proliferation, cell-cycle progression, colony formation, and invasion via targeting PAX6 in breast cancer cells[J]. Mol Med Rep, 2015, 11(1): 379-85.
[26] Li YW, Li YH, Liu YH, et al. PAX6, a novel target of microRNA-7, promotes cellular proliferation and invasion in human colorectal cancer cells[J]. Dig Dis Sci, 2014, 59(3): 598-606.
[27] Li XH, Yang L, Shuai TH, et al. MiR-433 inhibits retinoblastoma malignancy by suppressing Notch1 and PAX6 expression[J]. Biomed Pharmacother, 2016, 82(8): 247-55.
[28] Zou QY, Yi WJ, Huang JH, et al. MicroRNA-375 targets PAX6 and inhibits the viability, migration and invasion of human breast cancer MCF-7 cells[J]. Exp Ther Med, 2017, 14(2): 1198-204.

*Note: Figure translations are in progress. See original paper for figures.*