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Original Article

Construction of human HepG-2 cells infected by lentivirus carrying green fluorescent protein gene

Wei Zang[1], Li-Hua Xie[2], Bao-Hua Zhu[1], Da-Wei Cui[1]

[1]Department of Hepatobiliary Surgery, Jilin Central Hospital, Jilin 132000, Jilin Province, China; [2]Department of Obstetrics and Gynecology, Jilin Second People's Hospital, Jilin 132000, Jilin Province, China

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Abstract: We constructed the human HepG2 cell line infected by lentivirus carrying green fluorescent protein (GFP) gene. After infection, the lentivirus basically had no impact on the growth, apoptosis and cell cycle of human HepG2 cell line. Using this cancer cell model, gene therapy of the human HepG2 cells is possible by integrating the shRNA targeting oncogene into human HeGp2 cells using lentivirus carrying GFP gene.

Keywords: Human HepG2 cell line, lentivirus carrying GFP gene, infection, model construction

Introduction

Exogenous genes or shRNA can be integrated into host chromosomes using lentiviral vector for effective expression of the relevant genes. The lentiviral vectors can infect a diversity of cells, including liver cells, myocardial cells, tumor cells, endothelial cells and stem cells. They can target specific genes in the cells and suppress their expression as a gene therapy [1, 2]. For primary cells and undifferentiated cells that are difficult to be infected with other vectors, lentiviruses can achieve more efficient delivery, integration and expression of target genes or shRNA [3]. Therefore, lentiviruses are among the most commonly used vectors in in vitro and in vivo experiments [4, 5]. Green fluorescent protein (GFP) is first isolated from a type of jellyfish inhabiting the cold waters of North Pacific Ocean. The bioluminescent protein aequorin found in the jellyfish emits blue light, which is turned green because of GFP. The fusion of GFP gene and the target protein gene can preserve both the activity of the target protein and the fluorescent ability of GFP. This luminescent label that can be observed under the microscope will indicate the position, movement, activity and interaction of the proteins [6]. GFP is now used for quantitative and dynamic study of expressions of target proteins in cells of eelworm, fruit flies and mammals.

Liver cancer is categorized as either primary or secondary liver cancer. Primary liver cancer usually originates from the epithelial or mesenchymal tissues of the liver and ranks as the most malignant tumors in China. Secondary liver cancer may evolve from cancers in other organs, such as stomach, bile duct, pancreas, colorectum, ovaries, uterus, lungs and breasts [7-9]. HepG2 is a perpetual cell line which was derived from the liver tissue of a 15-year-old Caucasian American male with a well-differentiated hepatocellular carcinoma liver cancer. HepG2 cells can secrete a variety of plasma proteins: albumin, α2-macroglobulin, profibrinolysin and transferrin [10-12]. In this study we constructed the human HepG2 cells infected by lenvirus carrying GFP gene and assessed the effect of infection on growth, apoptosis and cell cycle. The research paves the way for integrating shRNA targeting oncogenes into HepG2 cells using the lentivirus carrying GFP gene.

Materials and methods

Cells and lentivirus

Human HepG2 cell line (Cell Bank of Shanghai Institutes for Biological Sciences of Chinese Academy of Science), lentivirus carrying GFP label (Shanghai Innovation Biotechnology Co., Ltd.).

Reagents

50 mM PBS buffer, RPMI 1640 (GIBCO, USA), fetal bovine serum (TBD), double antibiotics (penicillin, streptomycin, Beyotime), trypsin (Beijing CellChip Biotechnology Co., Ltd., 0.25%, containing 0.02% EDTA. On a clean bench and using disposable filter, 250 mg trypsin and 20 mg EDTA were dissolved in 100 mL PBS buffer), cell cycle detection kit (Beyotime), and Annexin V-PE/7-AAD Apoptosis Detection Kit (Beyotime).

Equipments

Incubator (Ai'purui), ultra-clean bench (Su-zhou Industrial Park Sanxing Purification Technology Co., Ltd.), biosafety cabinet (Suzhou Industrial Park Sanxing Purification Technology Co., Ltd.), inverted fluorescence microscope (Nikon), flow cytometer (FACSVantage SE, manufactured by Becton Dickinson, USA and provided by Chongqing Medical University), cell counting plate (Shanghai Medical Equipment Factory), fridge (4°C, -20°C, -80°C, Hair), inverted microscope (Olympus, Japan); culture flask, glass pipette, EP tube, pipette tip, 6-well plate, 12-well plate, 24-well plate, 96-well plate, 37°C water bath kettle, and centrifuge machine. All equipments were performed under sterilization.

Culture of human HepG-2 cells

The growth medium was prepared by adding RPMI 1640+10% fetal bovine serum into double-antibiotics stock solution (penicillin, streptomycin, final concentration 100 U/mL) at 1% volume fraction. The medium was subpackaged into 200 mL culture flasks. To quickly thaw the cells, the HepG2 cells were removed from liquid nitrogen and immediately immersed in 37°C water bath kettle with constant shaking. Inside the biosafety cabinet 8 mL of the culture medium was added into a 50 mL culture flask and the cells were transferred into the culture flask with sterile pipette. The inoculation density was about 10^4 cells/cm^2. The culture flask was then placed into a 5% CO_2 incubator at 37°C [13, 14] for about 24 h. Cell passage was performed when the cells grew to 90% confluence. The culture medium was removed, and the cells were washed with PBS three times. Into the culture flask approximately 1 ml of digestion solution containing 0.25% trypsin and 0.02% EDTA was added. The flask was gently shaken and placed into an incubator at 37°C for about 2 min. When the cells shrank and became round or shedded sporadically under the inverted microscope, the cells were completely detached from the bottom of the flask by gentle shaking. Next 3 mL of complete medium was added with gentle blowing, and the cells were collected into a 10 mL centrifuge tube. The cells were centrifuged at 1500 rpm for 4 min, with supernatant discarded. Culture medium was added, and the cells were dispersed by blowing to form a cell suspension. The cells were passaged by the ratio of 1:3 into the sterile culture flask and more culture medium was added. The HepG2 cells after centrifugation were cryopreserved and resuspended in culture medium containing 20% fetal bovine serum to reach the final concentration of about 10^6/ml. Then 10% DMSO was added, and the cells were subpackaged into a 2 mL cryogenic tube wrapped in heat insulating material. The tubes were first stored in a 4°C fridge and subsequently in a -20°C fridge and a -80°C fridge. The frozen cells were transferred to liquid nitrogen the next day [15].

Lentiviral packaging and titer determination

293T cells in log phase were digested and inoculated to a 10 cm culture dish at 2×10^6. The culture medium was replaced when the cells grew to 70% confluence. The mixture of lentiviral plasmid carrying GFP gene and calcium phosphate was added into the culture disk. Then polybrene was added (final concentration 5 μg/mL) with gentle mixing. And the culture medium was replaced after 6 h. The viral supernatant was collected after transfection for 48 h and centrifuged at 5000 rpm for 10 min at 4°C. The supernatant was collected, filtered and then centrifuged at 20000 rpm for 2 h at 4°C. Finally the precipitate was dissolved in serum-free medium, subpackaged and stored in a -80°C fridge [5].

Infection of human HepG-2 cells by lentivirus carrying GFP gene

The culture medium was removed, and the cells were washed with PBS three times. Into 50 ml culture flask, 1 mL of 0.25% trypsin digestion solution was added with gentle shaking and then placed into an incubator at 37°C for 2 min. The flask was gently shaken and placed into an incubator at 37°C for about 2 min. When the

cells shrank and became round or shedded sporadically under the inverted microscope, the cells were completely detached from the bottom of the flask by gentle shaking. Next 3 mL of complete medium was added with gentle blowing, and the cells were collected into a 10 mL centrifuge tube. The cells were centrifuged at 1500 rpm for 4 min, with supernatant discarded. Culture medium was added, and the cells were dispersed by blowing to form a cell suspension. The cell density was adjusted to about 10^5 cells/ml, and the cells were inoculated to a 12-well plate at 1 mL per well. The amount of viral solution (200 μL) was calculated according to multiplicity of infection (MOI) of 25 and the viral titer. Transfection was performed when the cells grew to 70% confluence. The culture medium was replaced and the viral solution was added with proper mixing. The cells were placed in an incubator [6] for 24 h and 48 h, the medium was replaced, and the cells were further cultured until 72 h. As a control, the normal HepG2 cells without infection were cultured.

Fixation of cells on coverslip before and after infection

The coverslip was sterilized, heated over the alcohol lamp using tweezers inside the biosafety cabinet and placed in the well of 6-well plate. The culture medium in the experimental group was removed, and the cells were washed with PBS three times. The cells were digested with 2 mL of 0.25% trypsin digestion solution and placed into an incubator at 37°C for about 2 min. When the cells shrank and became round or shedded sporadically under the inverted microscope, the cells were completely detached from the bottom of the flask by gentle shaking. After that 3 mL of complete medium was added with gentle blowing, and the cells were collected into a 10 mL centrifuge tube. The cells were centrifuged at 1500 rpm for 4 min, with supernatant discarded. Culture medium was added, and the cells were dispersed by blowing to form a cell suspension. The cell density was adjusted to 10^6 cells/ml, and the cells were inoculated to the coverslip of the 6-well plate at 0.5 mL per well. When the cells adhered to the wall after 12 h, the culture medium was removed and the cells were washed with PBS three times. Into each well 2 mL of culture medium was added. When the cells grew to 70% confluence, the cells on the coverslip were fixed. The culture medium was removed, and the cells were washed with PBS twice. After the cells were fixed in 1 mL 4% paraformaldehyde for over 30 min, the cells were dehydrated in a gradient series of alcohol (75%, 85%, 100%). After air dried, the cells were preserved in a -4°C fridge.

Detection of apoptosis and cell cycles by flow cytometry before and after infection

In normal cells, phosphotidylserine (PS) is localized to the inside surface of the cell membrane. But in early apoptotic cells, PS turns over from the inside to outside of plasma membrane and is exposed to the extracellular environment. Annexin-V is a 35-36 KD Ca^{2+}-dependent phospholipid binding protein. Displaying a high affinity with PS, Annexin-V can bind to early apoptotic cells. Annexin-V labeled by PE, red fluorescent dye (phycoerythrin), can be used as a probe to detect early apoptotic cells under the fluorescence microscope, flow cytometer or other fluorescence detectors. 7-AAD is a nucleic acid dye that has similar fluorescence features as PI. Since the emission spectrum of 7-AAD is narrower than that of propidium iodide (PI), it causes less interference to other channels. This feature makes 7-AAD an ideal alternative to PI in multicolor analysis, and it can be used in combination with Annexin V. However, 7-AAD cannot penetrate the membrane of normal cells or early apoptotic cells, but only the membrane of late apoptotic cells or necrotic cells, thereby binding to DNA within them. The problem with the combined use of AnnexinV-PE and 7-AAD is that 7-AAD remains outside the normal cells (AnnexinV-/7-AAD-) and early apoptotic cells (AnnexinV+/7-AAD-), whereas the late apoptotic cells or necrotic cells are simultaneously stained by AnnexinV-PE and 7-AAD (AnnexinV+/7-AAD+). Thus 7-AAD can be used to differentiate between early apoptotic cells and necrotic or late apoptotic cells [16, 17]. Fluorescent dye PI can be used to stain the DNA and applied to the detection of cell apoptosis. An analog of ethidium bromide, PI will emit red fluorescent after intercalating into double-stranded DNA. PI cannot penetrate the membrane of normal cells, but can stain the nuclei after penetrating the damaged cell membrane. Therefore PI staining is able to differentiate live and dead cells. The excitation and emission wavelength of PI-DNA complex is 535 nm and 615 nm, respectively.

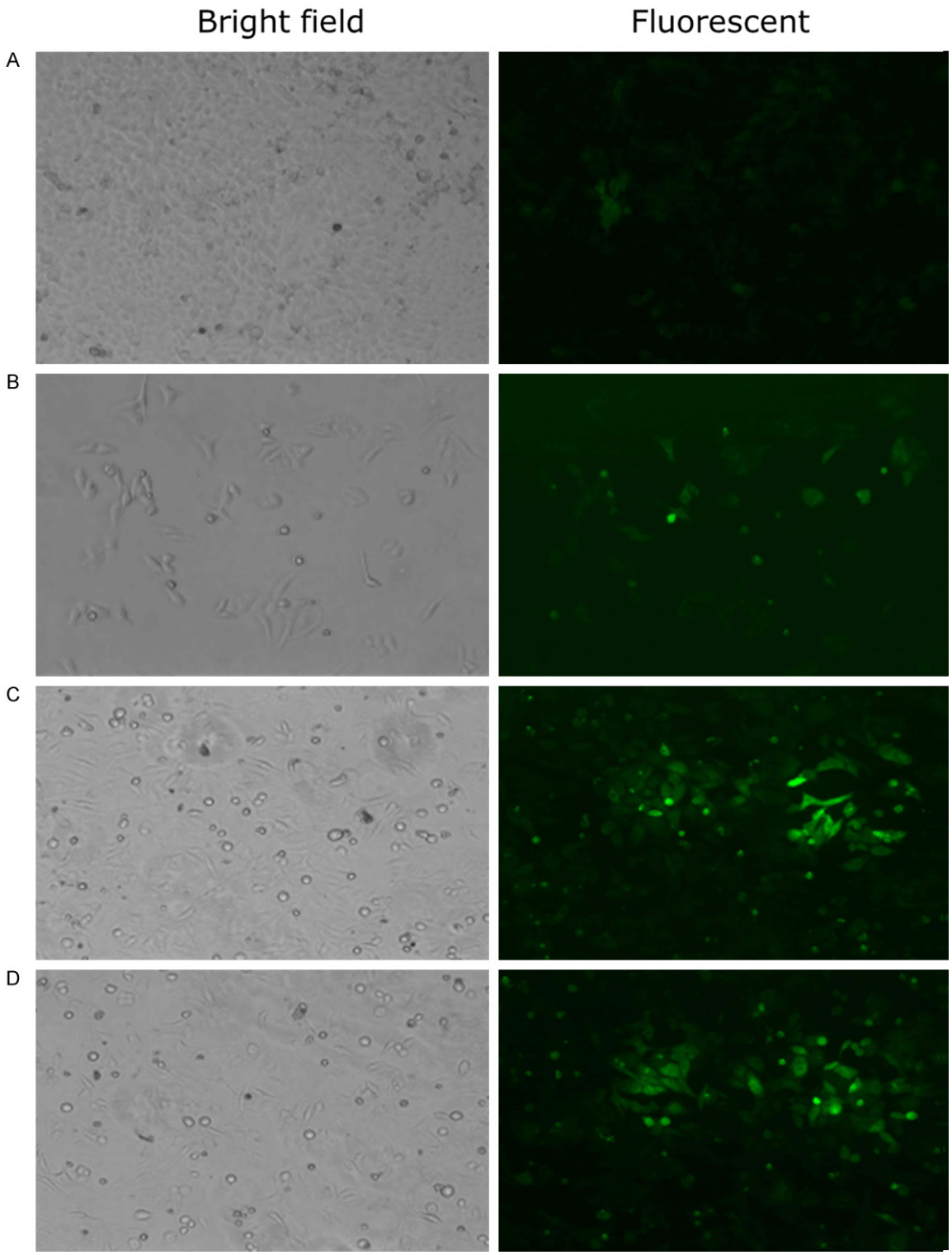


Figure 1. HepG2 cells before and after infection. A: Coverslip before infection (×10); B: Coverslip 24 h after infection (×10); C: Coverslip 48 h after infection (×10); D: Coverslip 72 h after infection (×10).

Annexin V-PE/7-AAD apoptosis detection kit was used to detect the apoptosis of HepG2 cells before and after lentiviral infection. The culture medium was removed, and the cells

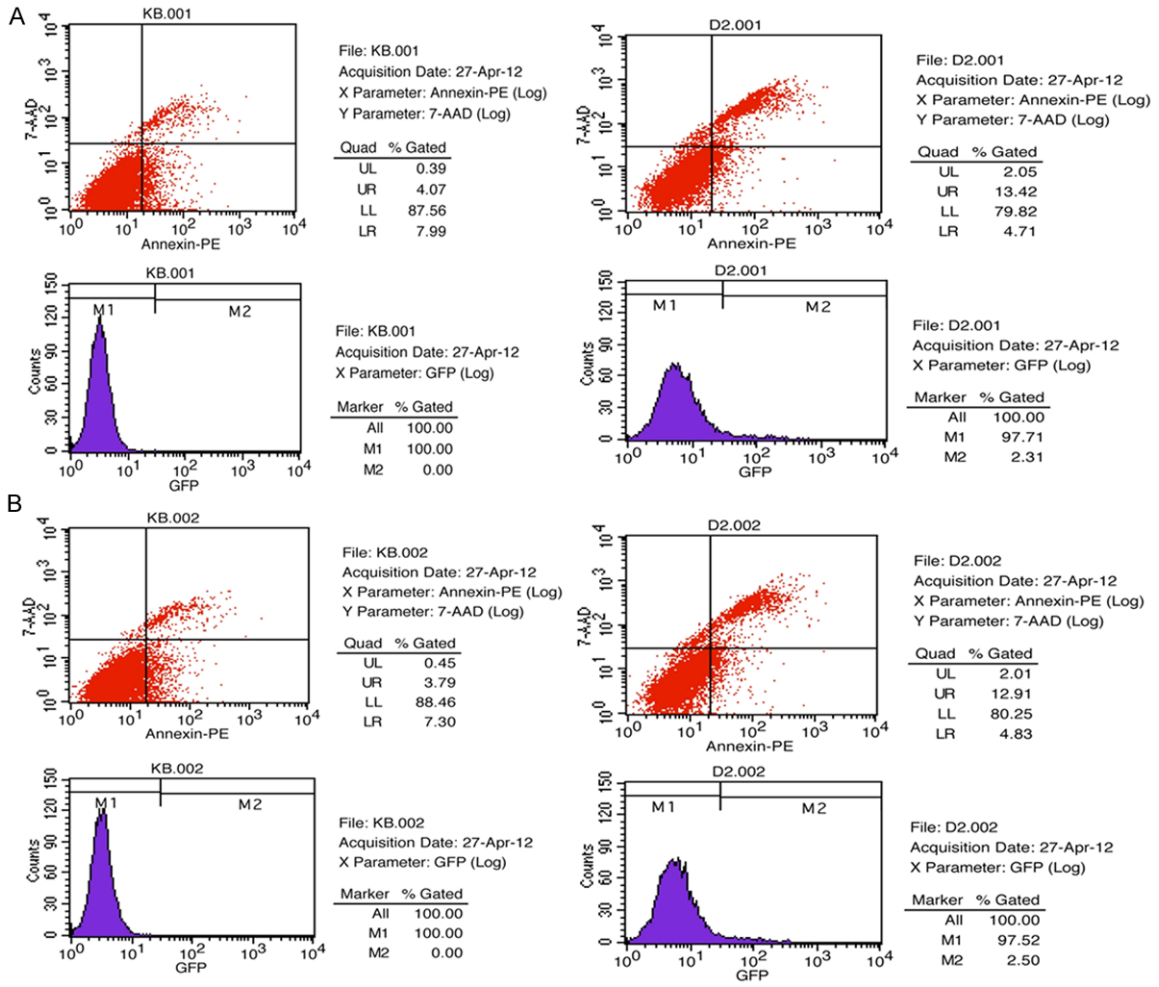
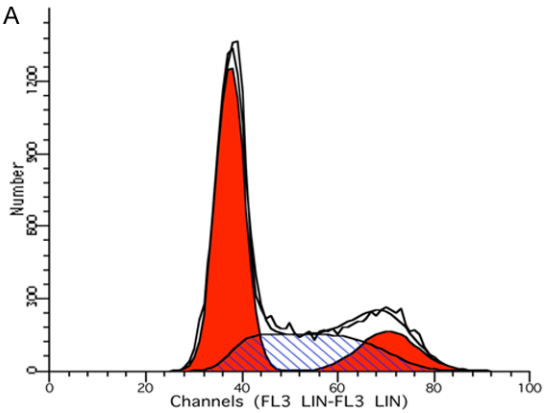


Figure 2. Cell apoptosis before and after infection. A: Before infection; B: 72 h after infection.

were washed with PBS three times. Into the culture flask approximately 1 mL of digestion solution containing 0.25% trypsin and 0.02% EDTA was added. The flask was gently shaken and placed into an incubator at 37°C for about 2 min. When the cells shrank and became round or shedded sporadically under the inverted microscope, the cells were completely detached from the bottom of the flask by gentle shaking. Later 3 mL of complete medium was added with gentle blowing, and the cells were collected into a 10 mL centrifuge tube. The cells were centrifuged at 1500 rpm for 4 min, with supernatant discarded. Culture medium was added, and the cells were dispersed by blowing to form a cell suspension. The cell density was adjusted to 10^5 cells/ml, and the cells were inoculated to a 6-well plate at 2 mL per well. The culture medium was removed after the cells grew to 70% confluence, and the culture was continued until 72 h.

The culture medium was sucked out into a sterile centrifuge tube. The HepG2 cells were washed with PBS twice and digested with 1 mL of trypsin digestion solution. The cells were inoculated at room temperature. The adherent cells were blown off the wall, and the digestion solution was sucked out. The initially collected cell culture was added, mixed well and transferred into the centrifuge tube. After centrifugation at 1500 rpm for 5 min, the supernatant was discarded and the cells were collected. The cells were resuspended in PBS and counted. Then 10^4 cells were taken and added with 5 μL Annexin V-PE with proper mixing. After incubation at room temperature (about 25°C) in the dark for 10 min, the cells were centrifuged at 1500 rpm for 2 min, with supernatant discarded. The cells were resuspended in 190 μL Annexin V-PE binding buffer and added with 10 μL 7-AAD stock solution. After treatment in ice bath in the dark, the cells were immediately detected by flow cytometry.

A



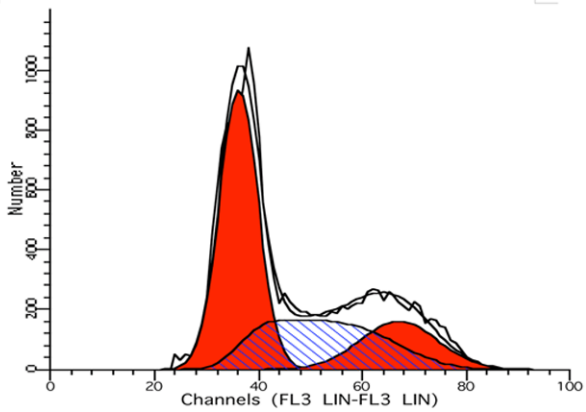
File analyzed: Cycle-1.LMD
Date analyzed: 2-May-2012
Model: 1nn0A_DSF
Analysis type: Manual analysis

Diploid: 100.00 %
Dip G1: 56.19 % at 37.54
Dip G2: 14.53 % at 70.41
Dip S: 29.27 % G2/G1: 1.88
%CV: 8.02

Total S-Phase: 29.27 %
Total B.A.D.: 0.00 % no debris no aggs

Apoptosis: % Mean:

Debris: %
Aggregates: 0.00 %
Modeled events: 17081
All cycle events: 17081
Cycle events per channel: 504
RCS: 3.348



File analyzed: Cycle-5.LMD
Date analyzed: 2-May-2012
Model: 1nn0A_DSF
Analysis type: Manual analysis

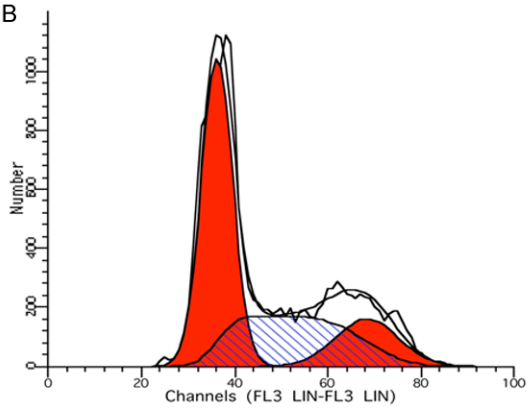
Diploid: 100.00 %
Dip G1: 51.80 % at 36.20
Dip G2: 17.75 % at 66.98
Dip S: 30.46 % G2/G1: 1.85
%CV: 10.29

Total S-Phase: 30.46 %
Total B.A.D.: 0.00 % no debris no aggs

Apoptosis: % Mean:

Debris: %
Aggregates: 0.00 %
Modeled events: 16874
All cycle events: 16874
Cycle events per channel: 531
RCS: 4.357

B



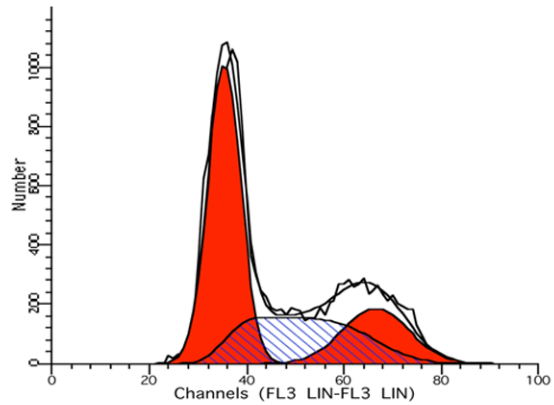
File analyzed: Cycle-3.LMD
Date analyzed: 2-May-2012
Model: 1nn0A_DSF
Analysis type: Manual analysis

Diploid: 100.00 %
Dip G1: 51.82 % at 36.20
Dip G2: 15.96 % at 68.32
Dip S: 32.22 % G2/G1: 1.89
%CV: 9.32

Total S-Phase: 32.22 %
Total B.A.D.: 0.00 % no debris no aggs

Apoptosis: % Mean:

Debris: %
Aggregates: 0.00 %
Modeled events: 17047
All cycle events: 17047
Cycle events per channel: 515
RCS: 5.457



File analyzed: Cycle-4.LMD
Date analyzed: 2-May-2012
Model: 1nn0A_DSF
Analysis type: Manual analysis

Diploid: 100.00 %
Dip G1: 51.69 % at 35.38
Dip G2: 19.02 % at 66.49
Dip S: 29.29 % G2/G1: 1.88
%CV: 9.60

Total S-Phase: 29.29 %
Total B.A.D.: 0.00 % no debris no aggs

Apoptosis: % Mean:

Debris: %
Aggregates: 0.00 %
Modeled events: 16657
All cycle events: 16657
Cycle events per channel: 519
RCS: 3.845

Figure 3. Cell cycles before and after infection. A: Before infection; B: 72 h after infection.

Cell cycle was detected using cell cycle detection kit before and after lentiviral infection. The precipitate collected after centrifugation at 1500 rpm for 5 min was resuspended in 1 mL of PBS precooled in ice bath. Then the cells were transferred to a 1.5 mL centrifuge tube and centrifuged at 1500 rpm for 4 min. The supernatant was removed (leaving about 50 μL PBS to prevent the cells from being sucked out), and the bottom of the centrifuge tube was gently knocked to disperse the cells. Then 1 mL of 70% ethanol precooled in ice bath was added with proper mixing, and the cells were fixed at 4°C for 3 h. The cells were precipitated after centrifugation at 500 rpm for 4 min, and the supernatant was removed (leaving about 50 μL 70% ethanol to prevent the cells from being sucked out). After that 1 mL of 70% ethanol precooled in ice bath was added again and centrifugation was performed once more. The cells were collected and the supernatant was removed (leaving about 50 μL PBS). A proper amount of PI staining solution was prepared according to the manufacture's instruction and the amount of sample to be detected. Into each tube 0.5 mL of PI staining solution was added. The cell precipitate was slowly but completely resuspended and treated in warm bath at 37°C for 30 min in the dark (later stored at 4°C or in ice bath in the dark) [18]. Flow cytometry was performed within 24 h after staining.

Results

Infection of human HepG-2 cells by lentivirus carrying GFP gene

When the cells grew to 70% confluence, lentiviral infection of the cells was performed at MOI of 25 with 200 μL viral solution. The coverslips were prepared and observed under the inverted fluorescence microscope before infection, 24 h, 48 h and 72 h after infection, respectively, to observe the infection efficiency and growth status of cells. The results are shown in **Figure 1**.

Fluorescence was observed 24 h after infection. The growth status of the cells infected for 24 h, 48 h and 72 h was not significantly different from that before infection. The fluorescence emitted by GFP became stable after 72 h. Thus the lentiviruses successfully infected HepG2 cells.

Cell apoptosis after infection by lentivirus carrying GFP gene

Cell apoptosis was detected using Annexin V-PE/7-AAD apoptosis detection kit using the flow cytometer before and after infection, with results shown in **Figure 2**. The red fluorescence was attributed to Annexin V-PE, and the blue fluorescence to GFP.

As indicated by flow cytometry, the lentiviruses carrying GFP gene successfully entered the cells and had no adverse impact on the cells 72 h after infection. The cell apoptosis was not significantly different before and after infection (**Figure 2**).

Cell cycles after infection by lentivirus carrying GFP gene

The cell cycles were detected using the cell cycle detection kit by flow cytometry, and the results are shown in **Figure 3**. Red fluorescence was emitted by PI after intercalating into double-stranded DNA.

According to cell cycle detection, lentiviruses carrying GFP gene had no obvious impact on the cell cycle of HepG2 cells 72 h after infection as compared with that before infection.

Discussion

Biotherapy for cancer treatment mainly works by mobilizing the natural anti-cancer capacity of the human body. The cancer cells can be killed and inhibited by human immune response activated by the biotherapy, thereby restoring the balance of human body's inner environment [19]. Tumors will produce stimulating factors that facilitate the formation of new blood vessels to provide nutrients for tumor growth. Gene targeting therapy is intended to block the effect of the stimulating factors and thus inhibit the tumors [20, 21]. Gene therapy is the process of introducing genetic materials into an organism's genome. It targets the root cause of diseases, which is genetic disorder. Gene targeting therapy brings new hope for cancer treatment. In this study, we successfully constructed the HepG2 cells infected by lentiviruses carrying GFP gene. The growth status, apoptosis and cell cycle were basically unaffected 72 h after infection. In the future, shRNA targeting the oncogenes can be integrated into the

lentiviral vector carrying GFP gene and then delivered into the HepG2 as a gene therapy. This research provides the cell model and theoretical basis for gene targeting therapy for liver cancer.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Da-Wei Cui, Department of Hepatobiliary Surgery, Jilin Central Hospital, No. 4 Nanjing Street, Chuanying District, Jilin 132000, Jilin Province, China. Tel: +86 0432-62165655; Fax: +86 0432-62165655; E-mail: jldrzang@163.com

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