

## Original Article

# Effects of lentiviral-mediated Wnt5a overexpression on biological functions of hepatoma cells and expression of Gli1 transcription factor

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**Abstract:** The aim of this study was to examine the effects of lentiviral-mediated Wnt5a overexpression on biological functions of the Huh7 human hepatoma cell line and on the expression of the glioma-associated oncogene homolog 1 (Gli1) transcription factor. We successfully constructed a lentiviral overexpression vector, pLVX-Wnt5a, containing the Wnt5a coding sequence. Huh7 cells stably transfected with the lentivirus harboring pLVX-Wnt5a were selected using puromycin. The Wnt5a protein level in pLVX-Wnt5a-harboring cells was found to be significantly higher than that in control cells transfected with the empty lentivirus vector. Wnt5a overexpression inhibited the cell cycle progression from the G1 to S phase, as well as the cell invasion and migration processes. In a nude mouse tumorigenesis experiment, the tumor formation rate and tumor sizes in the pLVX-Wnt5a group were significantly lower than those in the control group. The Gli1 mRNA and protein levels were significantly lower in control Huh7 cells than in pLVX-Wnt5a-harboring cells. Thus, the results demonstrated that the lentiviral-mediated Wnt5a overexpression inhibited the Huh7 cell cycle progression and tumorigenicity in nude mice. This suggests that Wnt5a plays a tumor suppressor-like role, which may be related to the decreased Gli1 expression. However, further studies are needed to elucidate the exact mechanism of tumor suppression by Wnt5a.

**Keywords:** Lentivirus, hepatoma cell, cell cycle, nude mice tumorigenesis, Wnt5a, Gli1

## Introduction

Hepatocellular carcinoma (HCC) is a common malignant tumor of the liver [1], and it is closely associated with hepatitis and cirrhosis. The pathogenesis of HCC is very complex, and various factors, such as oncogene expression, tumor suppressor gene inactivation, abnormal signaling pathways, and immunodeficiency, may cause HCC [2, 3]. Among these factors, abnormal signaling pathways play an important regulatory role in the development and progression of HCC and have clinical significance for HCC pathogenesis, prediction of the tumor biological behavior, and HCC treatment.

Wnt is a class of secreted glycoproteins, which regulate various *in vivo* biological functions, such as cell proliferation, differentiation, and migration, through transmembrane receptors and intracellular protein-mediated signal trans-

duction pathways [4]. To date, at least 19 Wnt family members have been identified, which can activate various downstream signaling pathways. There are two types of Wnt signaling pathways, canonical and non-canonical. The canonical Wnt pathway mainly involves  $\beta$ -catenin, which binds to transcription factors in the nucleus [3], thereby activating the target gene transcription. Non-canonical Wnt signaling pathways are represented by Wnt5a and Wnt11 and include the Wnt/Ca<sup>2+</sup> and Wnt/pla-nar cell polarity (PCP) pathways [5, 6]. The Wnt5a gene was first discovered by Gavin et al. in 1990 [7]. It is located in the chromosome region 3p14-p21 and encodes a cysteine-rich growth factor, which is involved in intra-and intercellular signal transduction [8], playing an important role in embryonic development and organ maturation. Its abnormal turnover is closely related to the development and progression of various diseases.

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The Wnt5a signaling pathway is closely associated with tumor development and progression; however, the mechanism is still under investigation. The main Wnt5a-involving signaling pathways discovered to date include (1) the inhibition or activation of the canonical Wnt/ $\beta$ -catenin signaling pathway; (2) the PCP signaling pathway; and (3) the Wnt/ $\text{Ca}^{2+}$  pathway. Although Wnt5a is a non-canonical Wnt protein, under some circumstances, Wnt5a can also exert its function through inhibition of the canonical Wnt signaling pathway by promoting  $\beta$ -catenin degradation or nuclear migration [9-11]. In the PCP signaling pathway, Wnt5a binds to the frizzled (Fzd) receptor and activates small G-proteins, forming a Dvl-Daam1-RhoA complex or Dvl-Rac complex, through which Wnt5 exerts its biological effects [12]. In the Wnt/ $\text{Ca}^{2+}$  pathway, Wnt5a plays different biological roles by influencing the intracellular  $\text{Ca}^{2+}$  concentrations. The Wnt5a binding to the Fzd receptor or to tyrosine kinase-like orphan receptor 2 (Ror2) activates a phosphodiesterase, increases the intracellular  $\text{Ca}^{2+}$  concentration, and promotes the cell adhesion [13]. Wnt5a plays different roles in different tumors, acting as an oncogenic protein or a tumor suppressor [14, 15].

Wnt5a is moderately or highly expressed in hepatitis B, cirrhosis, liver cell dysplasia, and in HCC-adjacent tissue, whereas its expression is significantly downregulated or absent in HCC tissue. This suggests that Wnt5a may play a tumor suppressor-like role in HCC [16, 17]. This study used lentiviral-mediated Wnt5a overexpression in the human hepatoma cell line Huh7 to further elucidate the effect of Wnt5a on biological functions of liver cancer cells. In addition, we used microarray and western blot techniques and found that Wnt5a played a role in the expression of a key transcription factor, glioma-associated oncogene homolog 1 (Gli1), in the Hedgehog signaling pathway.

### Materials and methods

#### *Chemicals and reagents*

The human hepatoma cell line, Huh7, was obtained from the cell bank of the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. pLVX-EGFP-3FLAG-Puro was purchased from Shanghai SunBio, Inc., China. The Effectene transfection kit was purchased from Qiagen. Taq polymerase and dNTPs were

purchased from TaKaRa Bio. *EcoRI* and *BamHI* were purchased from New England Biolabs, and primers were synthesized by Shanghai Generay Biotech Co., Ltd. Rabbit polyclonal anti-Wnt5a and anti-Gli1 antibodies were purchased from Santa Cruz Biotechnology (USA). An HRP-conjugated goat anti-rabbit secondary antibody was purchased from Cell Signaling Technology, and Transwell 24-well chambers were purchased from Corning Costar.

#### *Construction of lentivirus vector for Wnt5a overexpression and selection of transfected cell lines*

The Wnt5a gene was amplified using polymerase chain reaction (PCR). The expression vector, pLVX-EGFP-3FLAG-Puro, was double digested with *EcoRI* and *BamHI* and gel-purified. The Wnt5a gene was inserted into the vector fragment via homologous recombination, followed by transformation of competent cells of *Escherichia coli*. Transformants were identified by colony PCR, and positive clones were subjected to DNA sequencing. Human embryonic kidney cells, 293T, were cultured until the logarithmic growth phase and transfected with the pLVX-Wnt5a plasmid. The supernatant was collected, and the ultracentrifugation sedimentation method was used to concentrate and purify the lentivirus. Quantitative PCR was used to determine the viral titer. When the confluence of Huh7 cells in the logarithmic growth phase reached approximately 50%, they were infected with the virus containing the pLVX-Wnt5a plasmid and with the empty virus control. Polybrene (8  $\mu\text{g}/\text{mL}$ ) was added simultaneously to increase the efficiency of transduction. Fluorescence was observed 48 h after the lentiviral infection. Puromycin at a final concentration of 8  $\mu\text{g}/\text{mL}$  was added to select stably transfected cells expressing Wnt5a and control cells transfected with the empty vector.

#### *Cell cycle analysis by flow cytometry*

Cells of each type were trypsinized, washed twice with phosphate-buffered saline (PBS), and centrifuged. Precooled 70% ethanol was added to the cells, and cell densities were adjusted by resuspending the cells in PBS. Propidium iodide and RNase A were added, and the samples were incubated in the dark for 30 min. Cell cycle progression was analyzed by flow cytometry using cell cycle analysis software. The experiment was performed in triplicate.

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### *Transwell cell invasion assay*

Matrigel was diluted with Dulbecco's modified Eagle's medium and added into Transwell (8- $\mu$ m pore size) polycarbonate membrane inserts. Cells of each type were trypsinized, diluted to a density of  $1 \times 10^5$  cells/mL, and added to the Transwell chambers, which were then immersed in the medium. After culturing in an incubator for 48 h, the cells were fixed with formaldehyde, stained with hematoxylin, and observed under a light microscope. Cells in the lower chamber were counted to represent the number of cells that penetrated through Matrigel. Each experiment was performed in triplicate.

### *Scratch assay for cell motility analysis*

Cells were trypsinized, diluted to the desired density, and seeded in a 6-well plate. After 24 h, the cell layer was scratched using a 20- $\mu$ L tip. The detached cells were washed off with PBS. After culturing the plate for an additional 12 h, cell growth was observed and photographed.

### *Flat-plate clone formation assay*

Huh7 cells in the logarithmic growth phase were trypsinized, seeded into the wells ( $10^3$  cells/well) of a 6-well plate, and cultured for 10 days. Then, the cells were fixed with formaldehyde and stained with Giemsa stain to determine the number of clones in each group.

### *Nude mouse tumorigenesis experiment*

BALB/C-nu/nu (nude) mice were randomly divided into two groups, pLVX-Wnt5a and control. Cells transfected with the blank lentivirus and lentivirus harboring the pLVX-Wnt5a plasmid were collected, and  $5 \times 10^6$  cells were injected subcutaneously into the right side of the back of the BALB/C-nu/nu nude mice from the respective group. After 15 days, the mice were sacrificed by cervical dislocation and photographed. The tumor size and weight were measured.

### *Agilent whole genome oligo microarrays*

Total RNA from each sample was quantified using the NanoDrop ND-1000, and the RNA integrity was assessed using standard denaturing agarose gel electrophoresis. For microarray

analysis, the Agilent array platform was employed. Sample preparation and microarray hybridization were performed based on the manufacturer's standard protocols. Briefly, total RNA from each sample was amplified and transcribed into fluorescent cRNA using the manufacturer's Quick Amp labeling protocol (version 5.7, Agilent Technologies). The labeled cRNAs were hybridized onto the whole human genome oligo microarray ( $4 \times 44$  K, Agilent Technologies). After washing the slides, the arrays were scanned by the Agilent scanner G2505C.

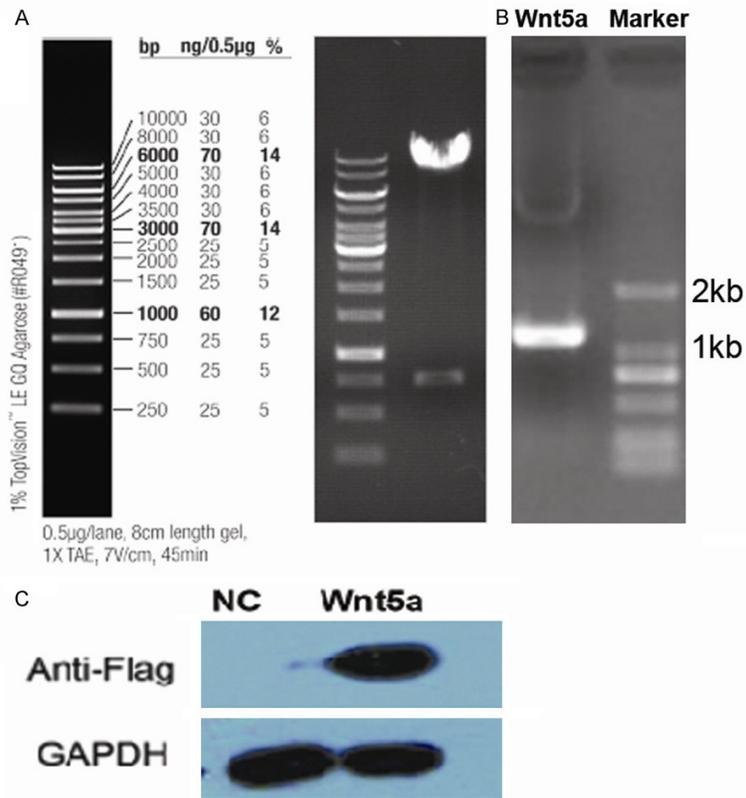
### *RNA extraction and real-time reverse transcription-polymerase chain reaction*

Total RNA was digested with DNase I (Invitrogen) and used for the first-strand cDNA reaction. Real-time reverse transcription-PCR (RT-PCR) was carried out using SYBR Green dye in a Rotor Gene 3000 detection system (Corbett Research, Sydney, Australia). Each SYBR Green reaction (25  $\mu$ L) contained 1  $\mu$ L of diluted cDNA, 10.5  $\mu$ L of SYBR Green PCR master mix, and 5 pmol each forward and reverse primer [Gli1: forward 5'CCTTCCTACCAGAGTCCAAAGT3', reverse 5'GCCCTATGTGAAGCCCTATT3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH): forward 5'GAAGGTGAAGGTCGAGATC3', reverse 5'GAAGATGGTGATGGGATTT-C3']. The samples were activated by incubation at 95°C for 5 min and denatured at 95°C for 20 s, followed by 40 cycles of annealing at 60°C for 20 s and extension at 72°C for 20 s. GAPDH was used as an endogenous control, and relative levels of Gli1 expression were normalized to that of the GAPDH housekeeping gene.

### *Detection of Wnt5a and Gli1 expression by Western blot analysis*

Huh7 cells transfected with the blank lentivirus and lentivirus harboring the pLVX-Wnt5a overexpression vector were collected and lysed with RIPA buffer. Protein concentrations were determined in the cell lysates, and the proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, followed by the transfer to a polyvinylidene difluoride membrane. The membranes were blocked with 5% skim milk. Primary antibodies were added and incubated with the membranes overnight at 4°C. Secondary antibodies were added on

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**Figure 1.** Construction and confirmation of the pLVX-Wnt5a lentiviral expression vector. A. Restriction enzyme digestion for lentiviral vector confirmation. B. Electrophoresis of the PCR-amplified Wnt5a gene product. C. Western blot detection of viral-mediated FLAG expression.

inserted into the expression vector via homologous recombination. Positive clones were selected and subjected to DNA sequencing. The results showed that the cloned sequence was correct. The pLVX-Wnt5a vector was packaged using 293T cells, and the viral titer was determined to be  $1.44 \times 10^8$  transducing units per milliliter. 293T cells were transfected with the lentivirus harboring pLVX-Wnt5a. The Wnt5a gene was fused with a FLAG tag, and the expression was determined using an anti-FLAG antibody. The western blot result showed that FLAG was positively expressed in the pLVX-Wnt5a-harboring cells (**Figure 1C**), indicating that Wnt5a was successfully overexpressed.

*Construction of Huh7 cell line overexpressing Wnt5a by lentiviral transfection and determination of its biological behavior*

the next day, followed by visualization of color development.

### Statistical analysis

Data were analyzed using the SPSS 22.0 software. Numerical data are presented as the mean  $\pm$  standard deviation. A Student's *t*-test was performed for comparison between groups. *P*-values of  $< 0.05$  were considered statistically significant.

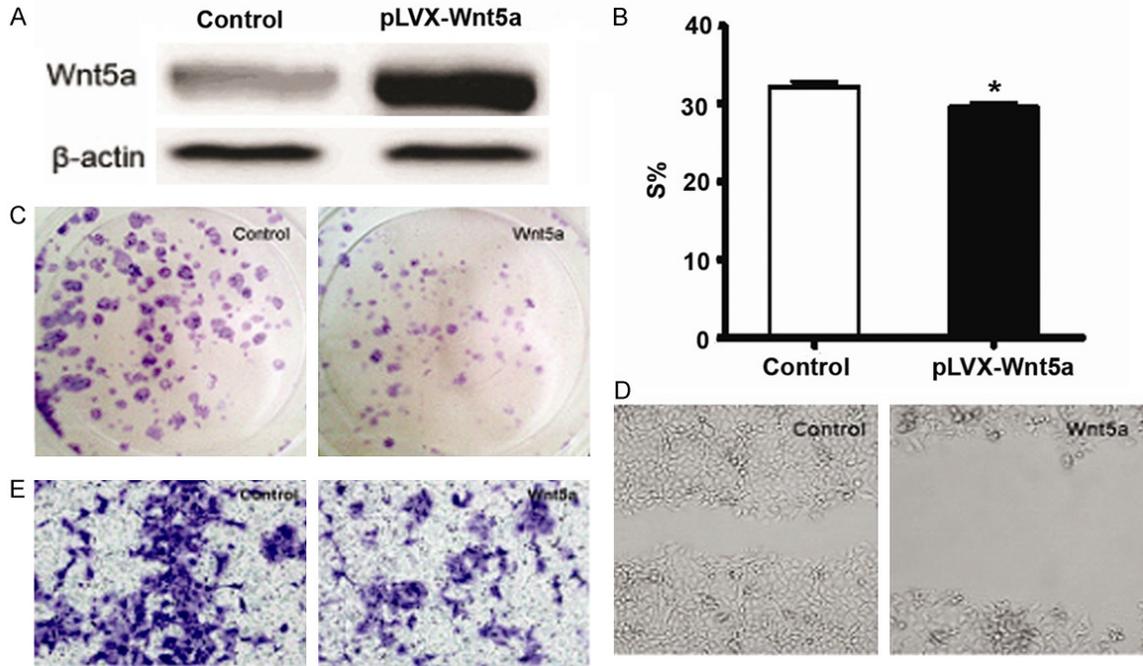
### Results

#### *Construction, packaging, and identification of the lentiviral Wnt5a expression vector*

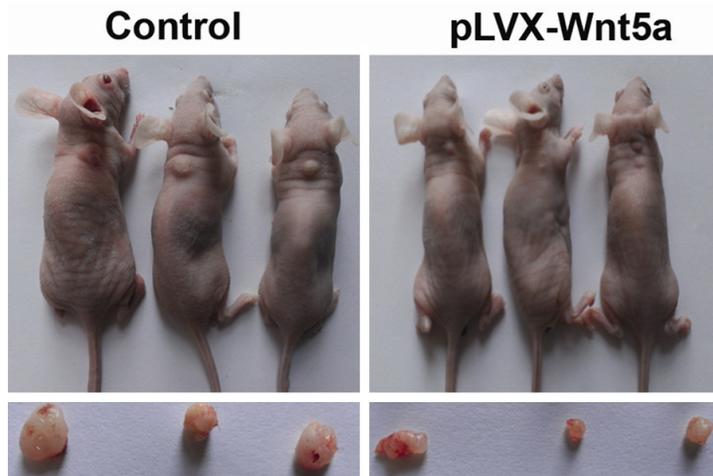
The pLVX-EGFP-3FLAG-Puro vector was double digested with *EcoRI* and *BamHI*. The 729-bp enhanced green fluorescent protein (EGFP) fragment was removed, and the 8,140-bp fragment was gel-purified (**Figure 1A**). The target gene fragment was amplified by PCR with the primers designed for Wnt5a (**Figure 1B**) and

The packaged lentivirus for Wnt5a overexpression and the blank lentivirus were used to infect Huh7 cells. Puromycin was used for selection of stably transfected cell lines. The results of western blot analysis showed that the Wnt5a protein expression in Huh7 cells stably transfected with the pLVX-Wnt5a lentivirus was significantly higher than that in control cells (**Figure 2A**). Flow cytometric analysis performed to determine the cell cycle progression revealed a higher percentage of cells in the G1 phase and a lower percentage of cells in the S phase in the cell population of pLVX-Wnt5a-harboring cells compared to that of control cells. The differences were statistically significant, suggesting that Wnt5a inhibited the cell cycle transition from the G1 to S phase (**Figure 2B**). In the flat-plate clone formation assay, pLVX-Wnt5a-harboring cells showed a significantly lower ability to form colonies than control cells (**Figure 2C**). The scratch assay revealed that pLVX-Wnt5a cells had significantly lower cell motility than control cells (**Figure 2D**). The Transwell assay

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**Figure 2.** Establishment of stably transfected Huh7 cells overexpressing Wnt5a and examination of their biological behavior. A. The Wnt5a protein is highly expressed in cells stably transfected with the recombinant lentivirus. B. Histograms of flow cytometry cell-cycle analysis. The number of cells in the S phase in the Wnt5a-overexpressing population was significantly lower than that in the control. C. Colonies on flat plates. The number of clones and colony diameters in the Wnt5a-overexpressing culture were smaller than those in the control culture. D. Photographs of the scratch experiment. The motility of Wnt5a-overexpressing cells was lower than that of control cells. E. Photographs of the Transwell experiment. The cell migration rate of Wnt5a-overexpressing cells was lower than that of control cells.



**Figure 3.** Data from the nude mouse tumorigenesis experiment. The tumor sizes in the mice from the pLVX-Wnt5a group were significantly smaller than those in the mice from the control group.

revealed that Wnt5a-overexpressing cells had a lower migration rate than control cells (**Figure 2E**). The nude mouse tumorigenesis experiment was conducted to determine the effect of

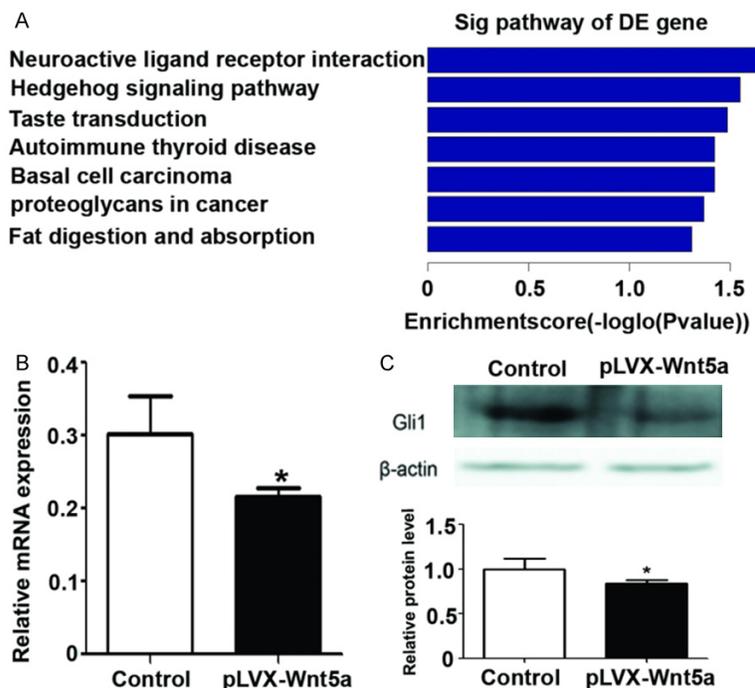
Wnt5a overexpression on the tumorigenicity of liver cancer cells, and the results showed that the tumor formation rate and tumor sizes in the pLVX-Wnt5a group were significantly lower than those in the control group (**Figure 3**).

*The inhibitory effect of Wnt5a in hepatoma cells is related to Gli1 expression*

Microarray differential expression analysis was conducted on both control cells and pLVX-Wnt5a lentiviral-transfected cells, and the results showed that various signaling pathways were affected (**Figure 4A**). Among these pathways, the Hedgehog signaling pathway showed altered expression

of many genes. Therefore, RT-PCR and western blot analysis were performed to determine the expression level of Gli1, a key transcription factor of the Hedgehog signaling pathway. The Gli1

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**Figure 4.** Microarray gene expression analysis of cells transfected with the pLVX-Wnt5a-harboring lentivirus and control cells and analysis of Gli1 expression in both types of cells. **A.** Pathway analysis of downregulated and differentially expressed genes. **B.** RT-PCR analysis of Gli1 mRNA expression in hepatoma cells transfected with the pLVX-Wnt5a lentivirus and control lentivirus. **C.** Western blot detection of Gli1 protein expression in hepatoma cells transfected with the pLVX-Wnt5a lentivirus and control lentivirus.

expression in the pLVX-Wnt5a-harboring cells was found to be significantly lower than that in the control cells (**Figure 4B** and **4C**), suggesting that the tumor inhibitory function of Wnt5a in HCC is related to altered Gli1 expression.

### Discussion

Recombinant lentiviral vectors are a very effective tool for transducing genetic material into the cell genome. Lentivirus can affect the G0/G1 phase of the cell cycle and can be used in primary cell cultures. Genes of interest carried by a lentiviral vector can be integrated into the genome of the target cell and stably expressed in the host cell for a long time. The virus is safe and induces weak immune responses [18, 19]. In this study, we successfully constructed a lentivirus vector for overexpression of Wnt5a and obtained a stably transfected Huh7 cell line. Wnt5a protein overexpression was confirmed using western blot analysis. This was considered a basis for further studies aimed to determine the biological function and mecha-

nism of Wnt5a in HCC, and the results were reliable and well reproducible.

Wnt5a has been suggested to contribute to tumor formation and development through various functions. Wnt5a can affect the cell migration, invasion, and angiogenesis. It is upregulated in cancers of the lung, breast, stomach, and pancreas, and this upregulation is closely related to tumor invasiveness, disease progression, and prognosis [14, 20]. It has been suggested that Wnt5a expression results in increased invasiveness of non-small cell lung cancer [21]. Kurayoshi et al. have found that Wnt5a also plays an important role in gastric cancer cell adhesion, invasion, and metastasis [22]. The results of this study provided further information on the biological function of Wnt5a in liver cancer cells. It was found that Wnt5a significantly

inhibited the liver cancer cell proliferation, invasion, and migration. This study employed flow cytometry to determine the effect of Wnt5a overexpression on the progression of cell cycle in liver cancer cells. The results indicated that Wnt5a overexpression significantly inhibited the cell cycle progression from the G1 to S phase. The study adopted a flat-plate clone formation assay to assess the cell migration ability. The results showed that the cell migration ability of Wnt5a-overexpressing cells was significantly lower than that of control cells, suggesting that Wnt5a is closely related to the liver cancer migration ability. The nude mouse tumorigenesis experiment indirectly reflected the effect of Wnt5a on HCC. We found that the tumorigenicity in the nude mice infected with Wnt5a-overexpressing cells was significantly lower than that in the control group, further suggesting that Wnt5a is closely related to HCC development and can inhibit the development and progression of HCC, thus playing a tumor suppressor-like role.

To further elucidate the inhibitory mechanism of Wnt5a in HCC, we performed microarray gene expression analysis on lentiviral-mediated, Wnt5a-overexpressing and control cells. The results indicated that Wnt5a was associated with several signaling pathways in liver cancer cells. In particular, we found that Wnt5a overexpression resulted in expression changes of many key factors in the Hedgehog signaling pathway. The role of the Hedgehog signaling pathway in HCC is especially notable. Several groups have reported that the Hedgehog signaling pathway was abnormally activated in HCC [23-25]. However, the mechanism of its activation in HCC is unclear. Lu et al. [25] have found that the treatment of liver cancer cells with 0.5 µg/mL of the sonic hedgehog protein triggered the expression of the Gli1 transcription factor and thereby promoted the liver cancer cell invasion and metastasis. This study employed a western blot method to further examine the expression level of the Gli1 protein. The results indicated that compared to the control cells, the lentiviral-mediated Wnt5a-overexpressing cells showed a significantly decreased level of the Gli1 protein, suggesting that the effect of Wnt5a on the biological function of liver cancer cells is related to the altered Gli1 expression.

Gli1 is a zinc finger transcription factor located downstream of the Hedgehog signaling pathway [26]. Its abnormal activation can cause liver cancer and is related to the liver cancer prognosis [27]. Studies have suggested that the Gli1 expression in liver cancer tissues was significantly higher than that in the adjacent tissue, indicating that Gli1 was positively associated with liver cancer development. At the cellular level, Gli1 expression was significantly higher in several hepatoma cell lines than in normal liver cells, suggesting its important role in liver cancer development [23, 28]. In this study, we found that Wnt5a overexpression was negatively associated with Gli1 expression, and therefore, we speculated that Wnt5a might play a tumor suppressor-like role via inhibiting the expression of the Gli1 transcription factor. Both Wnt5a and Gli1 participate in HCC development and progression. However, Wnt5a is a secreted protein, and it remains to be determined whether the effect of Wnt5a on Gli1 expression is direct or indirect.

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### Disclosure of conflict of interest

None.

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