Interaction between TGF-β1 and long non-coding RNA 4.9 in a model of latent infection with human cytomegalovirus

Xiaolian Yi
Children's Hospital affiliated to School of Medicine of Zhejiang University
Lifang Liu
Children's Hospital affiliated to School of Medicine of Zhejiang University
Ran Tao
Children's Hospital affiliated to School of Medicine of Zhejiang University
Wei Li
Children's Hospital affiliated to School of Medicine of Zhejiang University
Huamei Li
Children's Hospital affiliated to School of Medicine of Zhejiang University
lin He
Children's Hospital affiliated to School of Medicine of Zhejiang University
Yujie Liu
Children's Hospital affiliated to School of Medicine of Zhejiang University
Shiqiang Shang (shangsq0218@126.com)
Children's Hospital affiliated to School of Medicine of Zhejiang University

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Abstract

To investigate the interaction between long non-coding RNA4.9 (lncRNA4.9) and transforming growth factor-β1 (TGF-β1) in the model of HCMV DNA. The experiment was divided into 5 groups: NC group, PCDNA-lncRNA4.9 group, SH-lncRNA4.9 group, LV-TGF-β1) Group, and SH-lncRNA4.9 group. Compared with the NC group, overexpression of lncRNA4.9 could significantly increase (P <0.05) the expression of TGF-β1 and decrease with time. The relative expression of HCMV DNA was significantly decreased (P <0.05) with the overexpression of lncRNA4.9. Compared with the NC group, overexpression of TGF-β1 significantly increased the expression of lncRNA4.9 and decreased with time. The relative expression of HCMV DNA was significantly decreased (P <0.05) with the overexpression of TGF-β1. However, silencing TGF-β1 could not reduce the relative expression of lncRNA4.9, but increased the relative expression of lncRNA4.9 on day 5, and the difference was statistically significant (P <0.05). Silencing lncRNA4.9 could not reduce expression of TGF-β1, but increased expression of lncRNA4.9 on day 5 (P <0.05). Overexpression of TGF-β1 could increase the relative expression level of lncRNA4.9 and decrease with time, and reduce the relative expression level of HCMV DNA, thus promoting HCMV into the latent infection state.

1. Introduction

Human cytomegalovirus (HCMV) is a double-stranded DNA virus of the β subfamily of herpes virus, with an infection rate of over 90% in the human population (1). HCMV remains latent in most hosts after infection, allowing them to carry the virus for the rest of life. Long noncoding RNA (lncRNA) is a kind of non-coding RNA with a length > 200 nt and does not show any protein-coding potential (2). Although viruses are known for their compact genomes, with few regions that do not encode proteins, some highly expressed lncRNA has been identified in herpes viruses and confirmed to play key roles. These roles include regulation of chromatin structure (3), establishment, maintenance, and reactivation of latent infection periods(4), and recruitment of cellular transcription factors to viral DNA (5). HCMV mainly expresses four lncRNA during proliferative infection: lncRNA1.2, lncRNA2.7, lncRNA4.9, and lncRNA5.0 (6). The Immediate Early (IE) protein expressed by the *IE* gene is the most active cell regulatory protein after HCMV infects host cells, which can trans-activate a variety of cell genes and affect cell proliferation and apoptosis (7). IE1 (72kDa) and IE2 (86kDa) encoded by *UL123* and *UL122* are the most important IE proteins (8). *UL122* gene is one of the spliced genes after *IE* gene transcription, and its expression level directly affects the replication ability of the virus. In the process of initiating viral gene replication, IE2 protein can activate promoters of early and late HCMV genes alone or in collaboration with IE1, and IE2-deficient HCMV mutants cannot replicate and produce proliferative infections (7). Gatherer et al. found that the expression of lncRNA4.9 could not be detected until 72 hours after HCMV proliferative infection, and only a low expression level of lncRNA4.9 was maintained during HCMV proliferative infection (6). On the contrary, the study on the HCMV latent infection model found that high expression of lncRNA4.9 could be detected in infected cells, and the mRNA expression level of IE2 was up-regulated by nearly 150 times after inhibiting the expression of lncRNA4.9 by RNA interference (9). It indicates that latent

infection virus is activated (9). Previous studies suggested that immediate early response gene 1 (EGR1) is activated after binding to IE2, and the EGR1-IE2 complex specifically recognizes and binds CGCCCCGC sequence after entering the nucleus, thus promoting the expression of transforming growth factor-β (TGF-β) (10). Studies (11) have shown that in the HCMV latent infection model, lncRNA4.9 can directly reduce the expression level of IE1/IE2 to promote its entry into latent infection state, or indirectly inhibit the expression of nerve growth factor-induced gene A-binding protein 2 (NAB2) to promote the expression of EGR1 and its binding with IE2, and regulate the function of IE2 to initiate virus proliferation, thus promoting HCMV to enter latent infection.

The purpose of this study was to clarify the interaction between lncRNA4.9 encoded by HCMV and TGF-β1 in the HCMV latent infection model as well as the influence on the relative DNA expression of HCMV, to provide a reference for studying the pathogenesis and clinical treatment of HCMV latent infection.

2. Materials And Methods

2.1 Cell and virus culture

THP-1 cells (Cell Bank of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences) were cultured in RPMI-1640 (Hyclone) with 10% fetal bovine serum (FBS, Gibco, Gaithersburg, MD) and 0.05 mM β-mercaPtoethanol (Sigma, St. Louis, MO)., Logan, UT) medium at 37°C with 5% CO_2. HEF cells (Cell Bank of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences) were cultured in modified minimal essential medium (DMEM) containing 10% FBS at 37°C with 5% CO_2. When the HEF cells adhered to about 70%, the culture medium was discarded and washed once with PBS. Add an appropriate amount of serum-free DMEM medium and HCMV Towne virus to the culture flask at MOI = 2, and place it at 37°C in with 5% CO_2 incubator for cultivation. Shaking the culture flask lightly once every half hour. After 2 hours, the culture medium was discarded, washed twice with PBS, and an appropriate amount of DMEM medium containing 2% FBS was added to continue the culture. Add 1-2ml of culture medium to the flask the next day. When 90% of the HEF cells gradually swelled from spindle-shaped, thickened, rounded, and even fell off the wall of the culture flask, the virus was collected, and then freeze-thawed 3 times. Centrifuging at 12000rPm at 4°C for 15min, taking the supernatant, and storing it in a -80°C refrigerator for later use. The Towne virus was donated by Mr. Fan Jun from the First Affiliated Hospital of the School of Medicine of Zhejiang University.

2.2 Establishment of latent infection model and transfection of lentivirus

One day before transfection, THP-1 cells were planted at 2 × 105 cells/well into 2 very low-adherence 6-well plates in a volume of 1 ml culturing medium. On the day of transfection, count the cells again, 4 × 105 cells per well, adding serum-free 1640-diluted lentivirus (MOI = 30) according to the amount of discarded culture medium, and add HCMV Towne at MOI = 5 after 6 h of culture. After culturing for 2 hours, the cell suspension was collected, centrifuged at 1000 rpm for 5 minutes. The supernatant was

discarded, washed twice with PBS buffer, resuspended in 1640 medium containing 10% fetal bovine serum, and re-plated into each well for further culture. Transfection of lentiviral vectors using transfection solution HiTransGA A (GenePharma). The transfection solution used for the transfection of the plasmid overexpressing LncRNA4.9 was LiPofectamine 3000 (Invitrogen, LIFE, USA), which was used according to the instructions. All the aforementioned overexpression plasmids, lentiviral vectors, and empty viral vectors were purchased from GenePharma (Shanghai Genechem Co.ltd.). 3d and 5d after transfection of THP-1 cells, the cells were collected to extract protein samples and RNA samples to detect lncRNA4.9, TGF-β1 mRNA, and TGF-β1 protein expression levels, and changes in intracellular HCMV DNA copy number.

2.3 Experimental grouping

1) Plasmid group overexpressing lncRNA4.9 (PcDNA-lncRNA4.9 group): THP-1 cells + HCMV towne (MOI = 5) + plasmid overexpressing lncRNA4.9 (MOI = 30) (PcDNA-lncRNA4 .9); 2) Silencing lncRNA4.9 group (Sh-lncRNA4.9 group): THP-1 cells + HCMV Towne (MOI = 5) + silencing lncRNA4.9 lentiviral vector (MOI = 30) (Sh-lncRNA4.9); 3) NC group: THP-1 cells + empty lentiviral vector (MOI = 5) (MOI = 30) (NC); 4) Overexpression of TGF-β1 group (Lv-TGF-β1 group): THP-1 cellsl + HCMV Towne (MOI = 5) + TGF-β1 overexpression lentiviral vector (MOI = 30) (Lv-TGF-β1); 5) TGF-β1 silenced group (Sh-lncRNA4.9 group): THP-1 cells + HCMV Towne (MOI = 5) + TGF-β1 silenced group lentiviral vector (Sh-lncRNA4.9) (MOI = 30).

2.4. Transfection effect test

72h after transfection, the fluorescence expression was observed by an inverted fluorescence microscope to determine the transfection efficiency. Total RNA was extracted for real-time RT-PCR experiments to detect the mRNA expression and determine the transfection efficiency.

2.5 Detection of DNA copy number of HCMV in THP-1 cells by real-time PCR

The THP-1 cells in the culture plate were collected and centrifuged at 3000 rpm at room temperature to remove the supernatant. Washing three times with PBS and removing the supernatant. Total DNA was extracted using Diagnostic Kit for Quatification of Human cytomegalovirus DNA (PCR-Fluorescence, Da An Gene Co.,Ltd. Sun Yat-sen University). Add 50ul of DNA extraction solution to the pellet, shaking and mixing well, placing into100℃ metal bath for 10min, and centrifuging at 12000 rpm for 5min. Taking 36ul cytomegalovirus nucleic acid detection mixture and 0.4ul enzyme (Taq + UNG), shaking and mixing for several seconds, and centrifuging at 3000 rpm for several seconds. Add 4ul of the sample supernatant and standard to the PCR reaction tube, then add 36ul of the above mixture, and cover the thin-walled PCR reaction tube cap. Select FAM channel for PCR (ABI 7500 Real-Time PCR System, Thermo Fisher Scientific, Inc) amplification reaction: Stage 1: 37℃, 2min; 94℃, 2min; 1 cycle. Stage 2: 93°C, 15s; 60°C, 1min; 40 cycles. According to the fluorescence value of the standard substance and the DNA copy number, a standard curve was made, and the DNA copy number of the HCMV in the sample was calculated.

2.6. ELISA Essay

Cell culture supernatants were collected from the 5 experimental groups 2 and 4 days after infection to detect the protein expression of TGF-β1 by ELISA-Kit-for-Transforming-Growth- Factor-β1(Cloud-Clone corp.), and to observe the effect of lncRNA4.9 on TGF-β1 and the effect of TGF-β1 on lncRNA4.9. Centrifuge the cell culture supernatant at 1,000 × g for 20 minutes, and remove the supernatant for activation (refer to the instruction manual of ELISA-Kit-for-Transforming-Growth- Factor-β1). The activation steps are as follows: 1) Take 100 μL of cell culture supernatant, add 20 μL of 1M HCl, and mix well. 2) Age at room temperature for 10 minutes.3) Add 20μL of 1.2M NaOH and mix well. The detection steps were performed according to the ELISA instructions (cloud-clone corP.), and the samples were prepared for detection with a SynergyH1 multi-function microplate detector (BioTek).

2.7 RTqPCR

Cells were collected from the 5 experimental groups on days 3 and 5 after infection, and the mRNAs of lncRNA4.9 and TGF-β1 were detected by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted using UNIQ-10 column total RNA extraction kit (Shanghai Sangon Bioengineering Co., Ltd.) referred to the instruction manual. After extraction, the total RNA was measured with NanodroP2000 (UV-754 ultraviolet spectrophotometer, Shanghai Precision Instrument Co., Ltd.) to measure the RNA concentration and OD value. The RNA samples with A260/A280 ratio between 2.0 and 2.1 were used for follow-up experiments. The PrimeScriPt™ RT Master Mix Kit (Code NO. RR036A. TaKaRa BIO, Inc.) was used for reverse transcription of mRNA to cDNA according to the instruction manual.TB Green® Premix Ex Taq™ II kit (Code NO.RR820A, TaKaRa BIO, Inc) was utilized for RTqPCR via ABI 7500 Real-Time PCR System (Thermo Fisher Scientific, Inc.). The primers of lncRNA4.9, TGF-β1, and internal reference GAPDH were synthesized by Wuhan Qingke Biotech Co.Ltd. The relative expression levels of genes were analyzed by the $2^{-\triangle\triangle CT}$ method with GAPDH as the internal reference. The primer sequences are as follows (Table 1):

Table 1 The primer

GAPDH	Forward:5'- GAAGGTGAAGGGTCGGAGTC-3'
	Reverse: 5'-GAAGATGGTGATGGGATTC-3'
lncRNA4.9	Forward: 5'-CCGCCATGACCACCAAAAAG-3'
	Reverse: 5'-GCCGCTCTCTTACGTATCCC-3''
UL122	Forward: 5'-CATACTGGGAATCGTGAAGG-3'
	Reverse: 5'-TTGGACAACGAGAAGGTGC-3'
UL138	Forward: 5'-TATCGTCTGTCCGACTCCCG-3'
	Reverse: 5'-TGGCACGACACCTTCAAACTGG-3'
TGF-β	Forward: 5'ACTGCGGATCTCTGTGTCATT
	Reverse: 5'GTGCCCAAGGTGCTCAATAA

2.8 Statistical methods

The software used for data analysis and graphing in this study was SPSS 25.0 and GraphPad Prism 9.0 (GraphPad Software, La Jolla California USA). T-test was used for comparison between two groups, and one-way analysis of variance (OneWay ANOVA) was used for pairwise comparison between different groups, and $P < 0.05$ indicated statistical difference.

3. Results

3.1 Evaluation of transfection efficiency of THP-1 cells

Constructing an experimental model for transfection of THP-1 cells with overexpressed lncRNA4.9 plasmid, and establishing an experimental model for lentivirus transfection into THP-1 cells. In the latent infection model of HCMV, the expression of TGF-β1 was positively correlated with the expression of lncRNA4.9. Overexpression of lncRNA4.9 and overexpression of TGF-β1 can reduce the relative expression of HCMV DNA, thereby promoting the latent infection state of HCMV. The visual field of THP-1 cells transfected with a lentiviral vector for 72 h was taken by an inverted fluorescence microscope, and the transfection efficiency of the lentiviral vector was observed (Fig. 1). The transfection efficiency of the lentiviral vector is as high as 60%. The most obvious transfection effect (MOI = 30) was selected for subsequent experiments.

3.2. Plasmid and lentiviral vector transfection efficiency were evaluated by RT-PCR

After 3 days of plasmid or lentivirus transfection in each group, RT-qPCR was used to detect the transfection effect, and the expressions of lncRNA4.9 and TGF-β1 were detected. Compared with the NC group, the pcDNA-lncRNA4.9 group could significantly increase the expression of lncRNA4.9, and the difference was statistically significant ($P < 0.01$) (Fig. 2A). Silencing lncRNA4.9 can significantly reduce the expression of lncRNA4.9, and the difference is statistically significant ($P < 0.01$) (Fig. 2B). Overexpression of TGF-β1 could significantly increase the expression of TGF-β1, and the difference was statistically significant ($P < 0.01$) (Fig. 2C). Silencing TGF-β1 could significantly reduce the expression of TGF-β1, and the difference was statistically significant ($P < 0.05$) (Fig. 2D).

3.3 The expression of TGF-β1 increased after overexpression of lncRNA4.9

After 3 days of plasmid and lentivirus transfection in each group, RT-qPCR was used to detect the expression of TGF-β1. Compared with the NC group, the pcDNA-lncRNA4.9 group could significantly increase the expression of TGF-β1, and the difference was statistically significant ($P < 0.01$) (Fig. 3A). Compared with the NC group, the expression of TGF-β1 in the Sh-lncRNA4.9 group was decreased, but the difference was not statistically significant ($P < 0.05$) (Fig. 3A).

Each group was transfected with plasmid and lentivirus for 5 days, and the relative expression of TGF-β1 was detected by RT-qPCR. Compared with the NC group, the relative expression of TGF-β1 in the pcDNA-lncRNA4.9 group, Sh-lncRNA4.9 group, Lv-TGF-β1 group, Sh-lncRNA4.9 group was significantly increased, and the difference was statistically significant ($P < 0.05$). (Fig. 3B).

The expression of TGF-β1 increased after overexpression of lncRNA4.9, but the expression of TGF-β1 decreased after transfected for 3 days and then increased after transfected for 5 days by silencing the lncRNA4.9 gene, which may be related to the decrease in the first three days and then increases of HCMV replication after silencing lncRNA4.9 gene. It may be related to the inability of the lentiviral silencing lncRNA4.9 gene vector to completely silence the lncRNA4.9 gene, and this result may be because the RNAi machinery is primarily located in the cytoplasm, with restricted access to nuclear-retaining lncRNAs [22].

3.4. Increased expression of lncRNA4.9 after overexpression of TGF-β1

After 3 days of lentivirus transfection in each group, RT-qPCR was used to detect the expression of lncRNA4.9. Compared with the NC group, the Lv-TGF-β1 group could significantly increase the expression of lncRNA4.9, and the difference was statistically significant ($P < 0.01$) (Fig. 4A). Compared with the NC group, the expression of lncRNA4.9 in the Sh-lncRNA4.9 group was significantly reduced, and the difference was statistically significant ($P < 0.05$) (Figure, 4A).

After each group was transfected with different lentiviruses and cultured for 5 days, the relative expression of lncRNA4.9 was detected by RT-qPCR. The relative expression of lncRNA4.9 in the Lv-TGF–

β1 group and Sh-lncRNA4.9 group increased, and the difference was statistically significant (P1 = 0.002, P2 = 0.003, P3 = 0.000, P4 = 0.003, all $P < 0.05$) (Fig. 4B).

(B) each group was transfected with different lentiviruses and cultured for 5 days, the relative expression of lncRNA4.9 was detected by RT-qPCR

3.5 The expression of lncRNA4.9 decreased with time after overexpression of TGF-β1

Each group was transfected with different plasmids and lentiviruses and cultured for 3 and 5 days. The cells were collected and the relative expression of lncRNA4.9 was detected by RT-qPCR. The relative expression of lncRNA4.9 in each group was significantly reduced at 5 days compared with 3 days, and the difference was statistically significant (P1 = 0.001, P2 = 0.001, P3 = 0.000, all $P < 0.05$) (Fig. 5A). However, in the Sh-lncRNA4.9 group, the relative expression of lncRNA4.9 did not change significantly at 5 days compared with 3 days, and the difference was not statistically significant (P = 0.694, $P > 0.05$) (Fig. 5A). On the contrary, the relative expression of lncRNA4.9 in the Sh-lncRNA4.9 group was significantly increased at 5 days compared with 3 days, and the difference was statistically significant (P = 0.003, $P < 0.05$) (Fig. 5A).

Each group was transfected with different plasmids and lentiviruses for 3 and 5 days, and the cells were collected to detect the relative expression of TGF-β1 by RT-qPCR. The relative expression of TGF-β1 at 5 days was significantly lower than at 3 days, and the difference was statistically significant (P1 = 0.035, P2 = 0.000, P3 = 0.001, $P < 0.05$) (Figure, 5B).In the Sh-lncRNA4.9 group, the relative expression of TGF-β1 did not change significantly between 5 days and 3 days, and the difference was not statistically significant (P = 0.208, $P > 0.05$) (Fig. 5B). The relative expression of Lv-TGF-β1 in the Sh-lncRNA4.9 groups was significantly increased at 5 days compared with 3 days, and the difference was statistically significant (P = 0.003, $P < 0.05$) (Figure, 5B). The relative expression of TGF-β1 in the Sh-lncRNA4.9 group was significantly increased at 5 days compared with 3 days, and the difference was statistically significant (P = 0.003, $P < 0.05$) (Figure, 5B).

3.6 Relative HCMV DNA expression

Each group was transfected with plasmid or lentivirus and co-cultured for 3 days. The cells were collected and the relative expression of HCMV DNA was detected by qPCR. Compared with the NC group, the difference was statistically significant ($P < 0.05$) (Fig. 6A). Compared with the NC group, the relative expression of HCMV DNA in the pcDNA-lncRNA4.9 group was significantly decreased, and the difference was statistically significant (P = 0.001, $P < 0.05$). Compared with the NC group, the relative HCMV DNA expression in the Lv-TGF-β1 group was significantly decreased, and the difference was statistically significant (P = 0.001, $P < 0.05$) (Fig. 6A). Compared with the NC group, the relative DNA expression of HCMV in the Sh-lncRNA4.9 group was decreased, and the difference was not statistically significant (P = 0.071, $P > 0.05$); compared with the NC group, the relative expression of HCMV DNA in the Sh-lncRNA4.9 group increased, and the difference was not statistically significant (P = 0.123, $P > 0.05$).

The HCMV DNA expression in each group was co-cultured with different plasmids and lentivirus for 5 days. Compared with the NC group, the relative DNA expression of HCMV in the pcDNA-lncRNA4.9 group was decreased, and the difference was statistically significant (P = 0.019, $P < 0.05$), and the relative DNA expression of HCMV in the Lv-TGF-β1 group decreased, and the difference was statistically significant (P = 0.028, P < 0.05) (Fig. 6B). Compared with the NC group, the relative DNA expression of HCMV in the Sh-lncRNA4.9 group was increased, and the difference was statistically significant (P = 0.020, P < 0.05); The relative expression of HCMV DNA in the Sh-lncRNA4.9 group was significantly increased when compared with the NC group and the difference was statistically significant ($P < 0.05$).

3.7 Detection of TGF-β1 protein levels by ELISSA assay

Each group was transfected with different plasmids and lentiviruses and co-cultured for 3 days. The supernatant of the cell culture was taken, and the protein expression of TGF-β1 was detected by the ELISA method. Compared with the NC group, the protein expression of TGF-β1 in the pcDNA-lncRNA4.9 group was increased, and the difference was statistically significant (P = 0.02, P < 0.05) (Fig. 7B). The protein expression of TGF-β1 in the Sh-lncRNA4.9 group decreased when compared with the NC group, but the difference was not statistically significant (P = 0.173, $P > 0.05$) (Figure, 7B).

Each group was transfected with different plasmids and lentiviruses and co-cultured for 5 days, the supernatant of cell culture was taken, and the protein expression of TGF-β1 was detected by the ELISSA method. Compared with the NC group, the protein expression of TGF-β1 in the pcDNA-lncRNA4.9 group, Lv-TGF-β1 group and, the Sh-lncRNA4.9 group was increased, and the difference was statistically significant (P1 = 0.002, P2 = 0.002, P3 = 0.001, $P < 0.05$) (Fig. 7B). Compared with the NC group, there was no significant change in the protein expression of TGF-β1 in the Sh-lncRNA4.9 group, and the difference was not statistically significant (P = 0.233, P < 0.05) (Figure, 7B).

Each group was co-cultured with different plasmids and lentivirus for 5 days, the supernatant of cell culture was taken, and the protein expression of was detected by the ELISA method. Compared with 3 days, the protein expression of TGF-β1 in the NC group, the pcDNA-lncRNA4.9 group, and the Lv-TGF-β1 group was decreased, and the difference was statistically significant ((P1 = 0.008, P2 = 0.007, P3 = 0.031, $P < 0.05$) (Fig. 7A). Compared with 3 days, the protein expression of TGF-β1 of the Sh-lncRNA4.9 group was increased, and the difference was statistically significant (P = 0.011, $P < 0.05$) (Fig. 7A). Compared with 3 days, the protein expression of TGF-β1 in the Sh-lncRNA4.9 group was increased, and the difference was statistically significant (P = 0.018, $P < 0.05$).

4. Discussion

Gatherer D et al. found that viral RNA products were mainly concentrated in lncRNA1.2, lncRNA2.7, lncRNA4.9, and lncRNA5.0 by sequencing depth of the transcriptome of HCMV-infected fibroblasts (6). These lncRNAs were expressed by low- and high-passage isolates during lytic and latent infection of HCMV (13–15). Studies have confirmed that in the CMV infection model, genes with low or moderate

expression may have the greatest impact on regulating the transition between viral latency and replication (16–17). Nerve growth factor-inducible gene A binding protein 2 (NAB2) is the target gene of lncRNA4.9, and NAB2 protein includes the N-terminal early growth response gene 1 (EGR1) binding region. NAB2 that conserved domain and C-terminal transcriptional repressor region is the downstream regulatory protein of transcription factor EGR1. The activation of EGR1 can up-regulate the expression level of NAB2 (18). At the same time, NAB2 can regulate the expression and activation of EGR1 via feedback, and the expression level of EGR1 is significantly increased after NAB2 is missing (18–19). Liu et al. confirmed that lncRNA4.9 can directly reduce the expression level of IE1/IE2 and promote its entry into a latent infection state, or indirectly by inhibiting the expression of NAB2, thereby promoting the expression of EGR1 and its combination with IE2, affecting the function of IE2 to initiate viral proliferation, thereby promoting HCMV to enter a state of latent infection(11).In this study, through overexpression and silencing of the lncRNA4.9 gene, it was found that the expression of TGF-β1 increased after overexpression of lncRNA4.9 and decreased with time, and the relative expression of HCMV DNA decreased, thereby promoting HCMV to enter a latent infection state. It is speculated that lncRNA4.9 may promote the expression of TGF-β1 by activating EGR1, thereby promoting HCMV to enter a latent infection state. TGF-β ligands bind with different affinities to the three TGF-β receptor subtypes (TGFBR).TGF-β1 exerts its biological activity by activating Smad-dependent and Smad-independent pathways (20).TGF-β1 binds to transforming growth factor-β type 2 receptor (Tβ-RII) on the cell membrane to activate and phosphorylate transforming growth factor-β type 1 receptor (Tβ RI), and TβRI phosphorylates Smad3 and Smad2 to transfer the signal from the cell membrane into the cell, and then Smad2, Smad3, and Smad4 combine to form a Smad complex, which promotes nuclear transcription and signal transmission (22). It has been reported that in addition to protein-coding genes, TGF-β1 also regulates the expression of non-coding RNAs, such as microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) (23). In this study, in the latent infection model of HCMV, the expression of TGF-β1 was positively correlated with the expression of lncRNA4.9. Overexpression of lncRNA4.9 can promote the relative expression of TGF-β1, and the expression of TGF-β1 decreased with time, which reduces the relative expression of HCMV DNA; Overexpression of TGF-β1 can promote the relative expression of lncRNA4.9, and the expression of lncRNA4.9 decreased with time, which reduces the relative expression of HCMV DNA, thereby promoting the latent infection state of HCMV. However, the experimental results also showed that silencing the lncRNA4.9 gene did not reduce the expression of TGF-β1, and silencing the TGF-β1 gene did not reduce the expression of lncRNA4.9. After silencing the lncRNA4.9 gene and TGF-β1 gene, the cytomegalovirus titer gradually increased, indicating that there is no negative feedback regulation between the lncRNA4.9 gene and TGF-β1 expression. This result may be due to the RNAi machinery being primarily located in the cytoplasm, with limited access to nuclear-retaining lncRNAs (12). Study showed that the lncRNA4.9 encoded by HCMV is located in the viral nuclear replication region, and its deletion restricts viral DNA replication and viral growth, while the expression of lncRNA4.9 is essential for viral DNA synthesis and growth (24). This study also demonstrates that the expression of lncRNA4.9 is critical for viral DNA synthesis and growth. The study found that long non-coding lncRNA4.9 is closely related to the viral origin of replication (oriLyt) and is transcribed at a higher level than any other

viral or host promoter (25). Therefore, it is speculated that it may be because TGF-β1 acts as a signal to promote the expression of the lncRNA4.9 gene and then affect the replication of HCMV.

In conclusion, TGF-β1 acts as a signaling pathway to promote the expression of the lncRNA4.9 gene, and it decreases with time. lncRNA4.9 can inhibit the replication of HCMV and reduce viral droplets, and ultimately promote the occurrence of HCMV latent infection. Similarly, overexpression of TGF-β1 can increase the relative expression of the lncRNA4.9 gene, which decreases with time, affects the replication of HCMV, reduces the virus droplets, and finally promotes the occurrence of latent HCMV infection.

Declarations

Acknowledgments

None.

Authors' contributions

X.Y. and S.S. made substantial contributions to the conception and design of the work; L.L., R.T., W.L., and H.L. substantial contributions to the acquisition and analysis; L.H. and Y.L. made substantial contributions to the interpretation of data; L.L. and Ran Tao made substantial contributions to the creation of new software used in the work; X.Y. perform the experiments and S.S. made substantial contributions to draft the work and substantively revise it.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

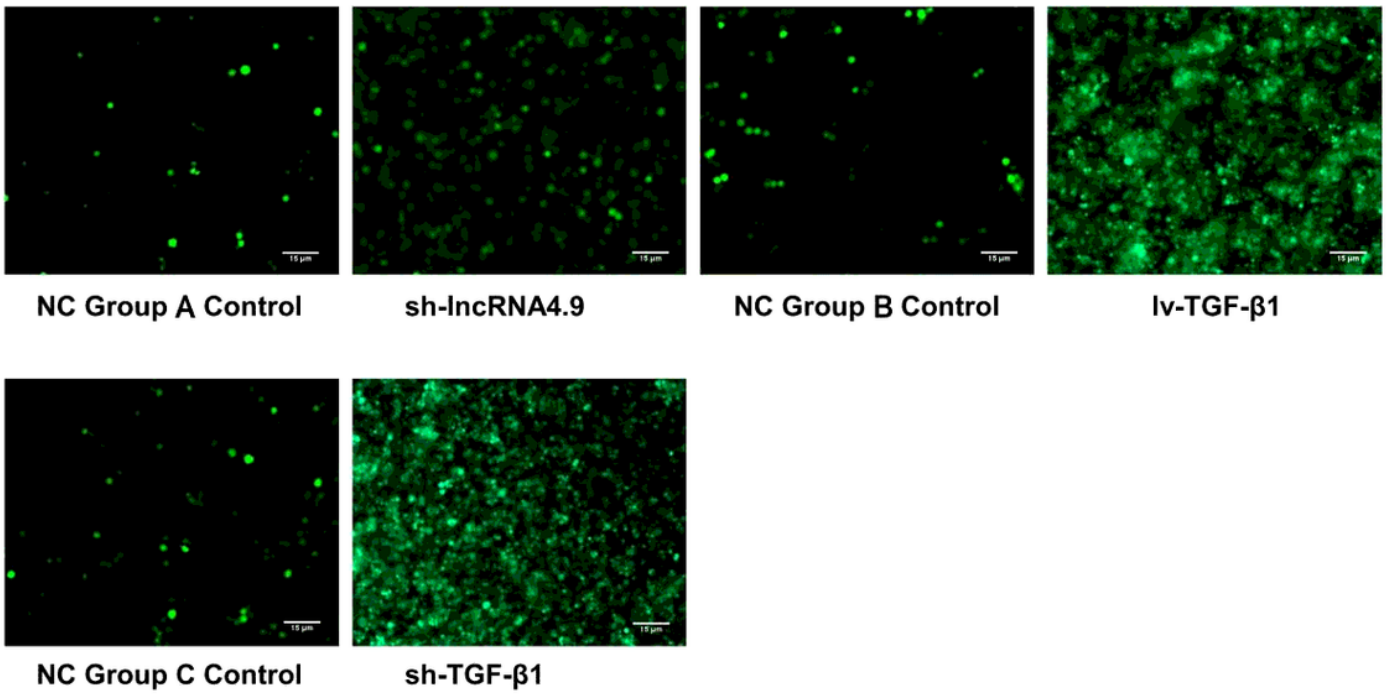

Figure 1

The visual field of THP-1 cells transfected with a lentiviral vector for 72 h was taken by an inverted fluorescence microscope.

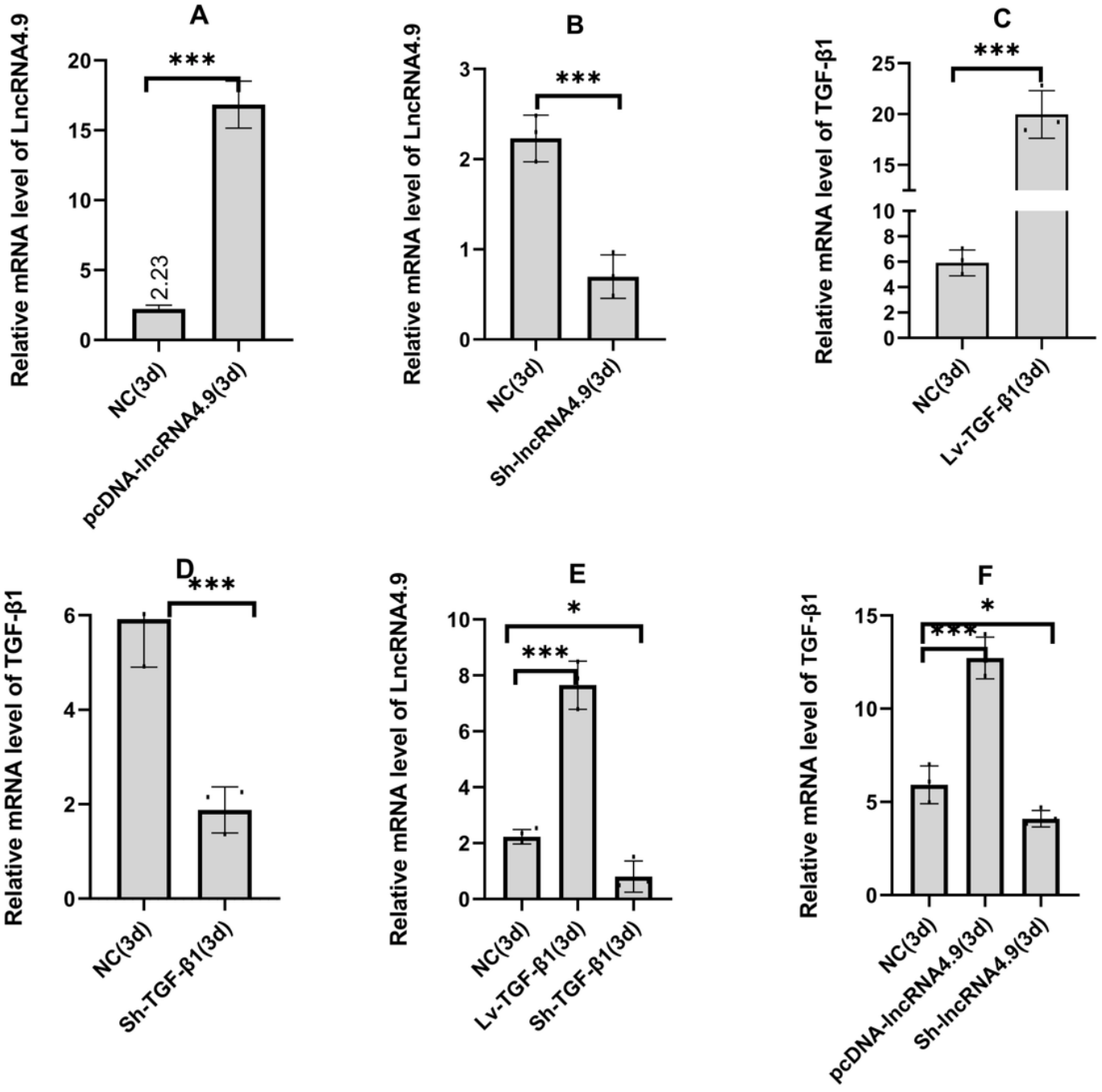

Figure 2

RT-qPCR at 3 days after treatment. Expression of lncRNA4.9 and TGF-β1 was detected by RT-qPCR. Compared with the NC group, the lncRNA4.9 Expression levels of HCMV (Fig. 2A) and the TGF-β1 Expression levels of HCMV (Fig. 2C) were significantly higher in HCMV (both P<0.05). Compared with NC group, the lncRNA4.9 Expression levels (Fig. 2B) and TGF-β1 (Fig. 2D) were significantly decreased in HCMV (both P<0.05).***P<0.001; **P<0.01,*P<0.05

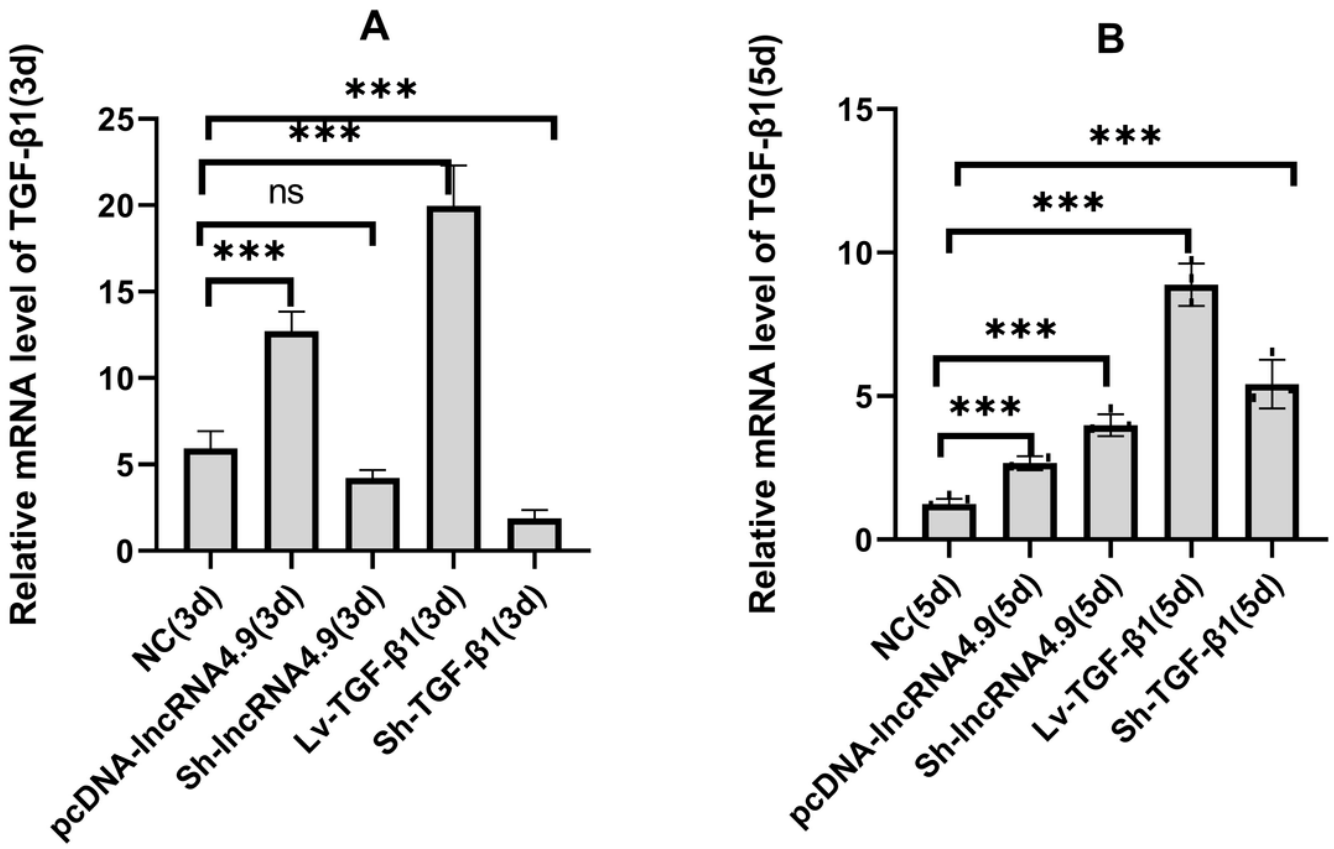

Figure 3

Expression of TGF-β1 was detected by RT-qPCR.

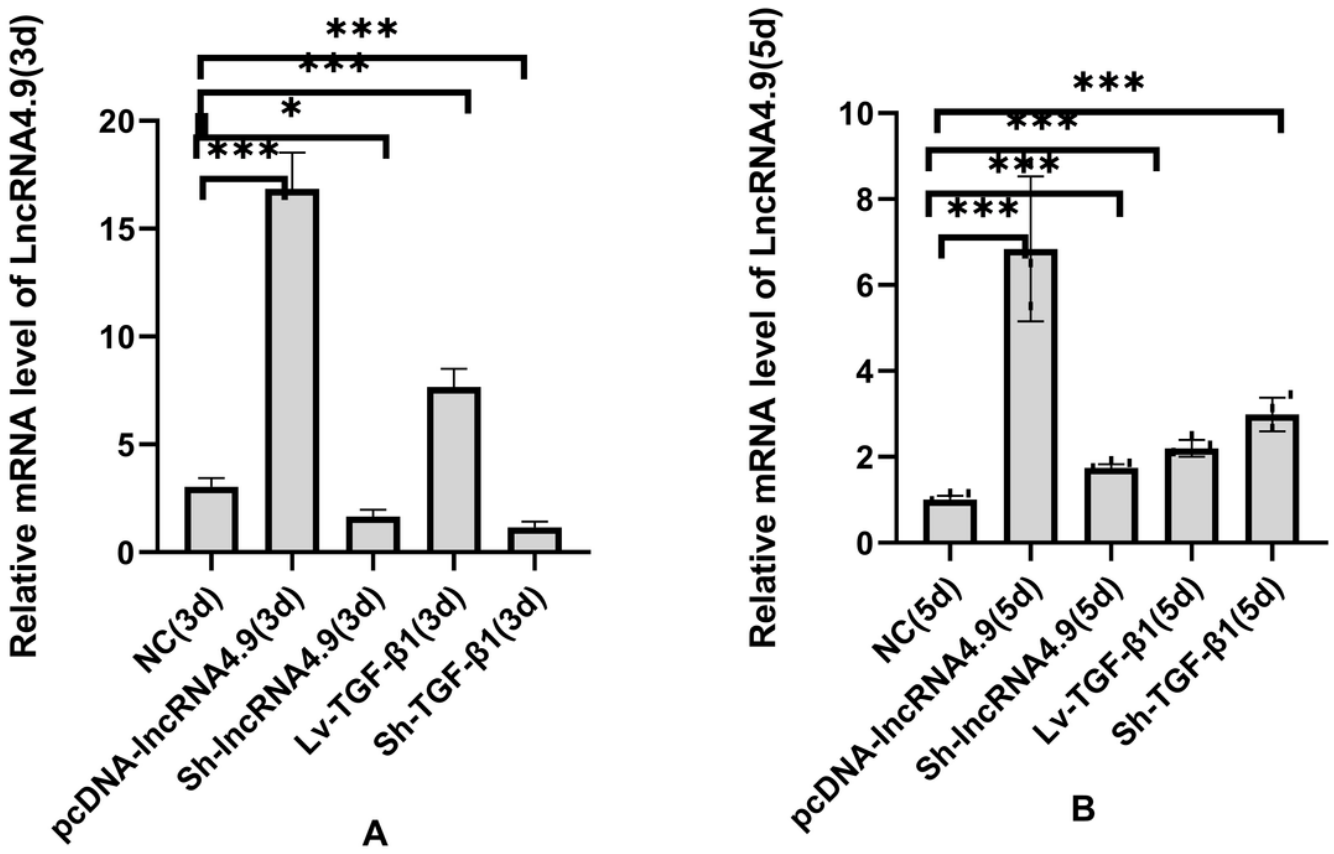

Figure 4

The expression of lncRNA4.9. (A) each group was transfected with different lentiviruses and cultured for 3 days, the relative expression of lncRNA4.9 was detected by RT-qPCR.

Figure 5

The expression of lncRNA4.9 and TGF-β1 decreased with time. (A) the relative lncRNA4.9 mRNA expression. (B) the relative TGF-β1 mRNA expression.

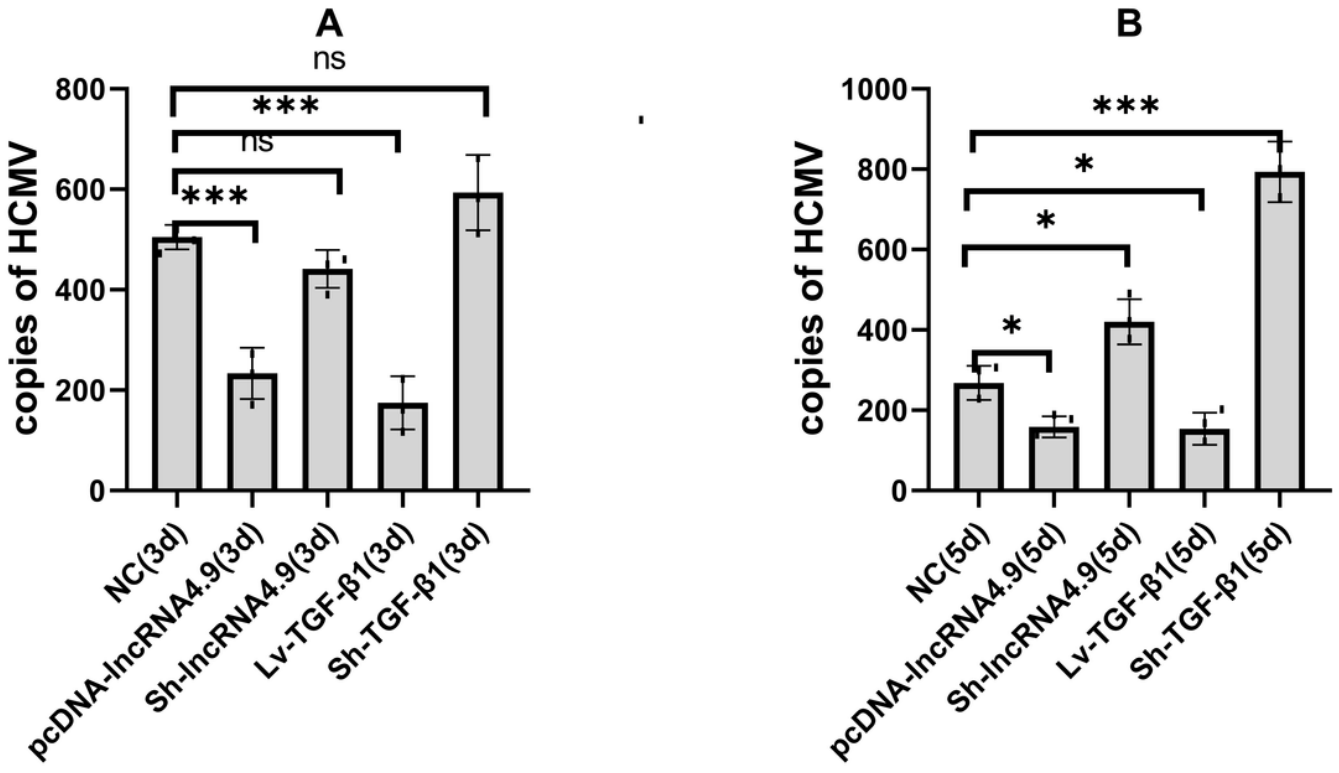

Figure 6

the HCMV DNA Expression. (A) The HCMV Expression levels by qPCR at 3 days after treatment. (B) The HCMV Expression levels by qPCR at 5 days after treatment.

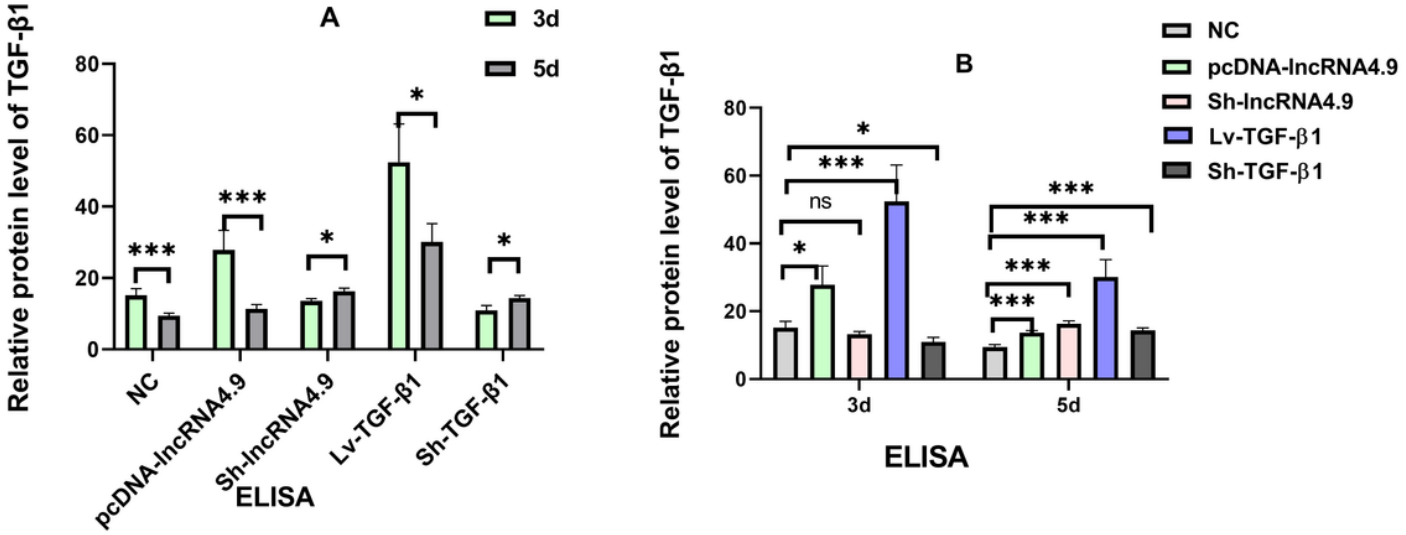

Figure 7

the protein expression levels of TGF-β1 by ELISA at 3 and 5 days after treatment. (A) The comparison of TGF-β1 expression levels between 3 days and 5 days. (B) the TGF-β1 Expression levels by ELISA at 5 days after treatment.