KCNE1L inhibits herpes simplex virus replication

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Abstract

Interferon(IFN) plays an essential role in the innate immune response by acting as an antiviral agent.Human KCNE gene family has five members,among them,KCNE4 and x chain KCNE5 (formerly known as KCNE1-like,KCNE1-L) are the least studied so far.The IFN regulatory factor IRF7 is a key transcription factor regulating the type I and III IFN genes.In this study,we aim to explore whether KCNE1L participates in the innate immune response;and which signaling pathway it may be involved in.We found that over-expression of KCNE1L significantly inhibited herpes simplex virus-1(HSV-1) replication and enhanced IL-29 production;while silencing KCNE1L led to enhanced HSV-1 replication and reduced IL-29 production.Furthermore, we found that KCNE1L interacted with IRF7,and enhanced IRF7-mediated signaling pathway.This study therefore identified that KCNE1L is a novel component of IRF7-mediated anti-viral immune pathway.

Keywords

Interferon(IFN);Herpes Simplex Virus 1(HSV-1);Innate immune.

1. Introduction

Natural ion channels are known to be formed by a large molecular complex formed by a number of alpha and beta subgroups, and a number of regulatory proteins are involved.[1]The current generated by these channel complexes is essential for almost all physiological processes of multicellular organisms. These include the high frequency action potential of the neurons, the homeostasis of the epithelial cells, and the contraction of the muscle, which stimulates the chemical ligand, membrane potential, membrane expansion and cell expansion/osmotic potential through the channel gate(open and closed).[2-5]KCNE protein is a single-layer transmembrane voltage gated potassium(Kv) channel auxiliary subunit with multiple physiological functions. Human KCNE gene mutation is related to various pathophysiological states, most notably arrhythmia. The KCNE subunit can be used to cross the plasma membrane once, and its main function is to serve as the subunit or the auxiliary subunit on the Kv channel.[6-8]KCNE protein can regulate all the basic properties of Kv channel, including the composition of alpha subunit, protein transport in the cell secretion pathway, and the recirculation and utilization of protein in anterograde transport. At the same time, the function properties of Kv channel can be adjusted, including ionic conductivity, ionic selectivity, gating kinetics, voltage dependence and the influence of some regulatory proteins on Kv channel.[9-11]

The signal transduction Pattern Recognition Receptors(PRRs) are used by the innate immune system to identify the Pathogen-Associated Molecular Patterns(PAMPs),and IRFs is widely concerned as an important regulatory factor to activate immune cells.[11]So far, a variety of PRRs have been identified: Toll-like receptors(TLRs),RIG-I-like receptors(RLRs),NOD-like receptors(NLRs),C-type lectin receptors(CLRs),and other nucleic acid sensing receptors.[12]

Most PRRs can cause type I interferon responses by activating IRFs, especially IRF3 and IRF7.Unlike IRF3, IRF7 is expressed in low levels in most cells. By combining with Interferon-Stimulated Gene Factor 3(ISGF3), signal transduction was performed on the promoter of the gene, which strongly induced I type Interferon.

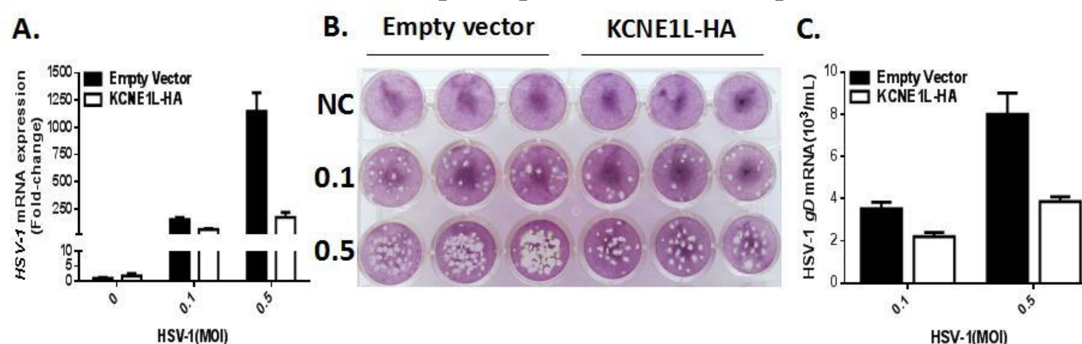
So far, at least 13 different TLRs (10 were found in humans, and 12 were found in mice) and a variety of PAMPs were found out, they come from bacteria, viruses, fungi and(or) protozoa, so as to trigger an immune response, including induction of type I interferon and proinflammatory cytokines.[17-18]All TLRs are membrane receptors, this type of receptors activate IRFs and other transcription factors by combining Myeloid Differentiation Factor88(MyD88) and(or) TIR-Domain-Containing Adaptor Inducing Interferon-β(TRIF).

Consistent with ELISA result, qRT-PCR detection showed that with the overexpression of high concentration of KCNE1L,it can slow virus replication,293t cells were transfected with short hairpin RNA(shRNA) and then stimulated with high multiplicities of infection(MOI) conditions of Herpes Simplex Virus 1(HSV-1),the expression of KCNE1L in the negative control group was significantly higher than that in the silent group, while the expression of HSV-1 in the negative control group was significantly lower than that in the silent group.Together with our ELISA results, these data demonstrate that KCNE1L gene is involved in the antiviral process.The interaction of KCNE1L and IRF7 were shown in the results of immune co-precipitation.Importantly, qRT-PCR analysis revealed that the expression of IL-29 showed a trend under the stimulation of 1ug/ml poly(I:C).Collectively, our data demonstrate that KCNE1L plays an antiviral role in the immune system and may affect the expression of interferon by regulating IRF7.

2. Results

2.1 KCNE1L inhibits HSV-1 replication.

KCNE1L was overexpressed in 293t cells with different concentrations of HSV-1 stimulation after 24 hours, the experimental group(MOI 0.5 PFU/cell) showed a significant reduction in virus replication. The same is true of the viral plaque assay, see Fig1A-1C.so we speculate that KCNE1L may be involved in the antiviral process in natural immunity. Subsequently, we transfected 293t cells with KCNE1L shRNA, and then stimulated with different concentrations of HSV-1 in 24 hours, In the untreated group(MOI=0 PFU/cell),compared with the negative control group and the silent group, KCNE1L shRNA significantly reduced the expression level of KCNE1L,and the difference was statistically significant($P < 0.01$);With the increase of MOI, the expression of KCNE1L in the negative control group was significantly increased, and the expression of KCNE1L in the negative control group was significantly higher than that in the silent group(Fig1D),and the expression of hsv-1 in the negative control group was significantly lower than that in the silent group, and the difference was statistically significant($P < 0.01$)(Fig1E),which is consistent with the results of the viral plaque assay. These data show that both overexpression and knockdown experiments prove our previous conjecture that KCNE1L does somehow participate in the antiviral process in natural immunity.



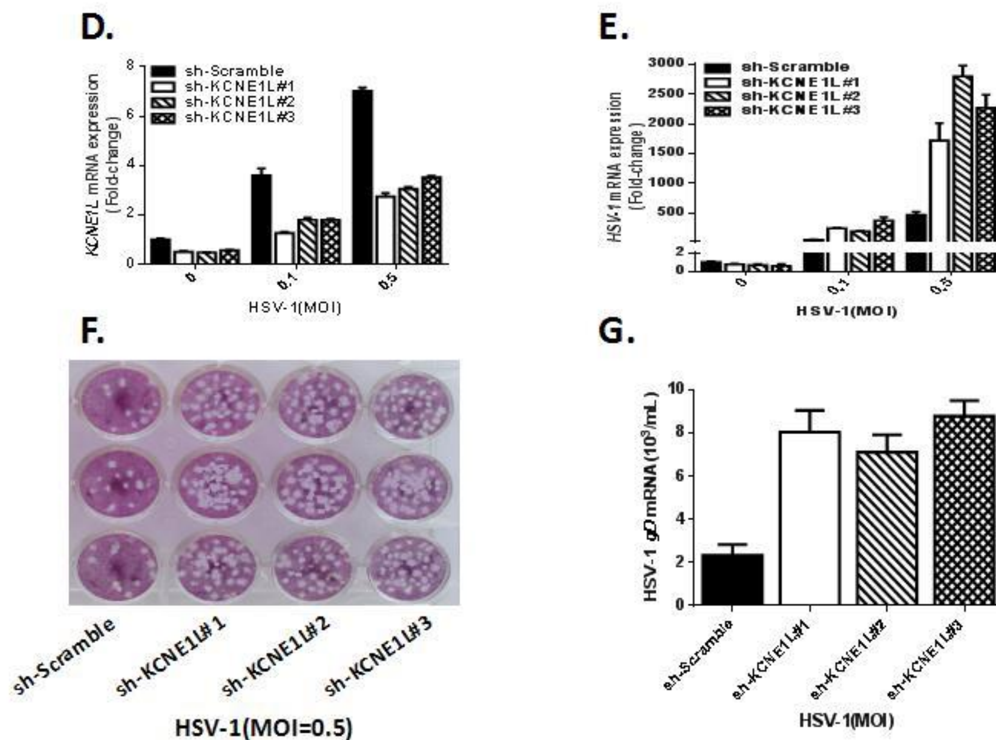


Fig.1 KCNE1L participates in the antiviral process.A: Overexpression of different concentrations of KCNE1L,HSV-1 infected at an MOI of 0.1 and 0.5 PFU/cel, respectively,then the mRNA expression level of HSV-1 was detected. B-C: We use vero cells to make the viral plaque assay, which can more intuitively detect the inhibition ability of KCNE1L to the virus.D-E:KCNE1L is proved to have antiviral function by knocking down KCNE1L.

2.2 KCNE1L affects the gene expression of IL-29

Interferon as the first important line of defense for antiviral infection, we have good reason to suspect that KCNE1L plays a role in the antiviral process by affecting the expression of interferon. We know the truth that IRF7 is an important transcription factor that regulates interferon. Therefore, we assume that KCNE1L interacts with IRF7 to influence the expression of IRF7, and then affecting the expression of downstream interferon. This is a way to play an important role in the antiviral process of natural immunity. Correspondingly, it is also very likely to participate in the IRF7-IFN signaling pathway. Therefore, it is very necessary for us to test whether the expression of KCNE1L to the downstream interferon has any effect, thus verifying our inference.KCNE1L was overexpressed in 293t cells, and different concentrations of HSV-1 were added to stimulate cells after 24h, then RNA was extracted, and mRNA expression of each cytokine was detected by qRT-PCR. Our data showed that the mRNA expression level of type III interferon IL-29 and TNF-α mRNA expression level were affected. The protein level of IL-29 was detected by ELISA, it is obvious that the change trend is basically the same as that of the mRNA level. Especially when under high MOI conditions (MOI 1.0 PFU/cel),the expression level of IL-29 was significantly higher in the experimental group transfection with KCNE1L than the empty vector group. Collectively, all the data are more validating our previous inference.

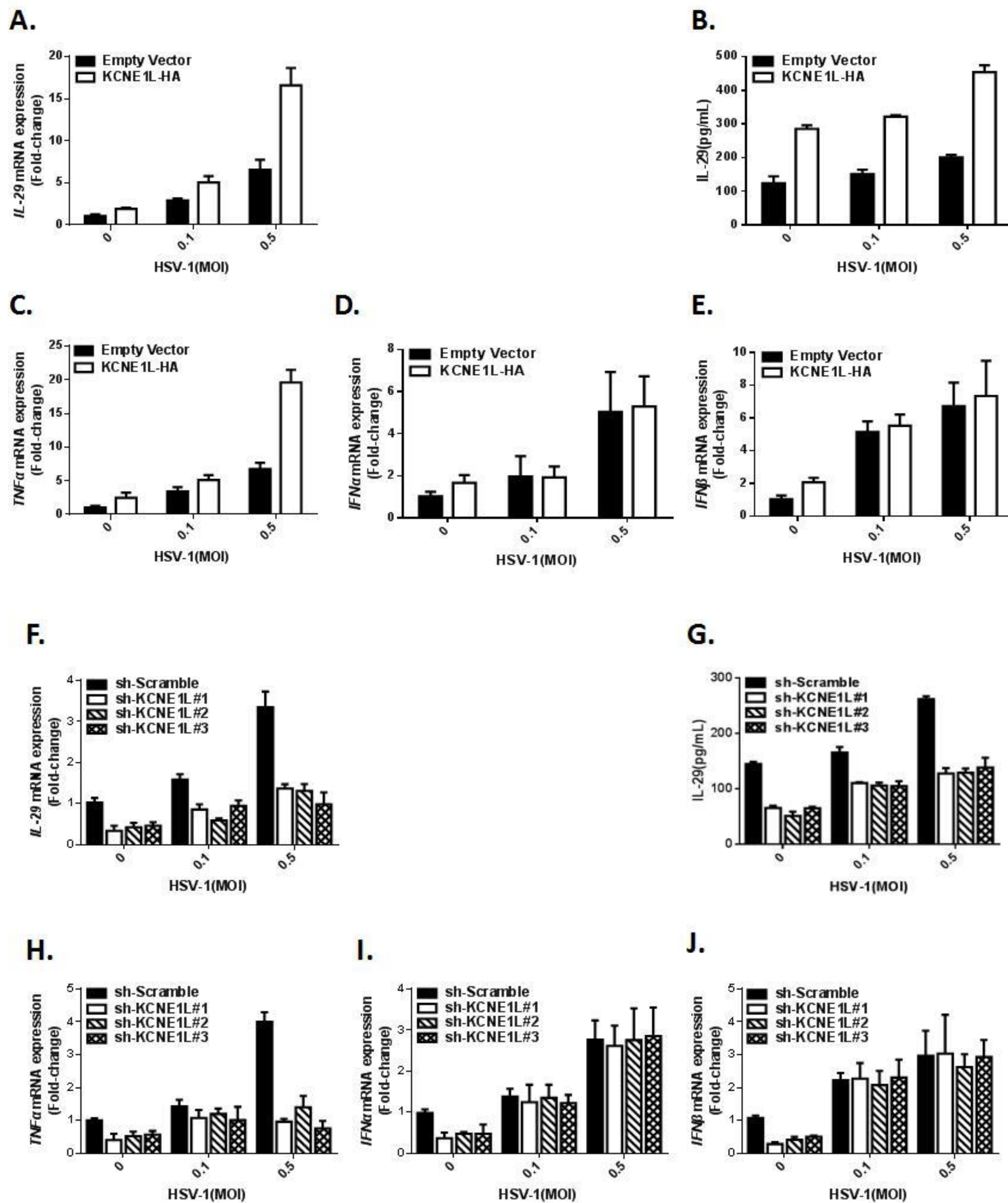


Fig.2 KCNE1L affects the expression of IL-29.Significant increases in IL-29 expression were observed following the addition of HSV-1(MOI 1.0 PFU/cel).

2.3 KCNE1L interacts with IRF7

Now that we know that KCNE1L is involved in the antiviral process, so we've picked a number of important virus-related molecules, here we effectively examine whether KCNE1L interacts with multiple important molecules associated with virus. The CO-IP method is used to detect whether KCNE1L interacts with these molecules. The results are very clear, and the data shows that KCNE1L interacts with IRF7.

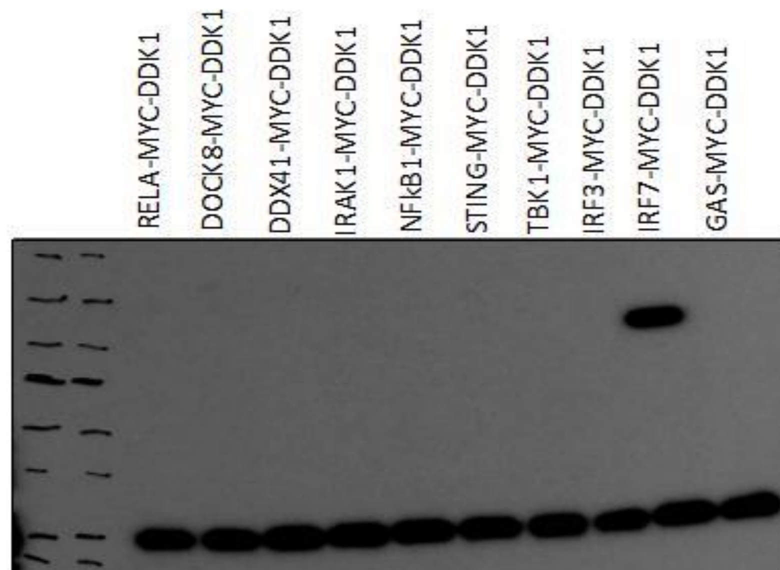


Fig.3 KCNE1L interacts with IRF7.293t(1x107) were transfected with empty or expression plasmids of KCNE1L,RELA,DOCK8,DDX41,IRAK1,NF-kB1,STING,TBK1,IRF3,IRF7,cGAS.Cells were lysed in protein lysis buffer(20mM Tris-HCl pH7.4,150 mM NaCl,1mM EDTA,1% Triton-X100) supplemented with protease inhibitors.

2.4 KCNE1L enhanced IRF7-mediated IL-29 gene expression

KCNE1L and IRF7 were transfected into 293t cells respectively.KCNE1L and IRF7 were co-transfected into 293t cells, After 24 hours, with different concentrations poly(I:C) (0.1μg/mL, 1μg/mL) stimulated cells, PRK was used as an empty vector control group, and then the mRNA expression level of IL-29,IFNα,IFN-β and TNF-α were detected. In the concentration of 1ug/mL poly(I:C),the expression of IL-29 in the co-transfection group was higher than that in the single transfection group, and the difference was statistically significant($P<0.05$).

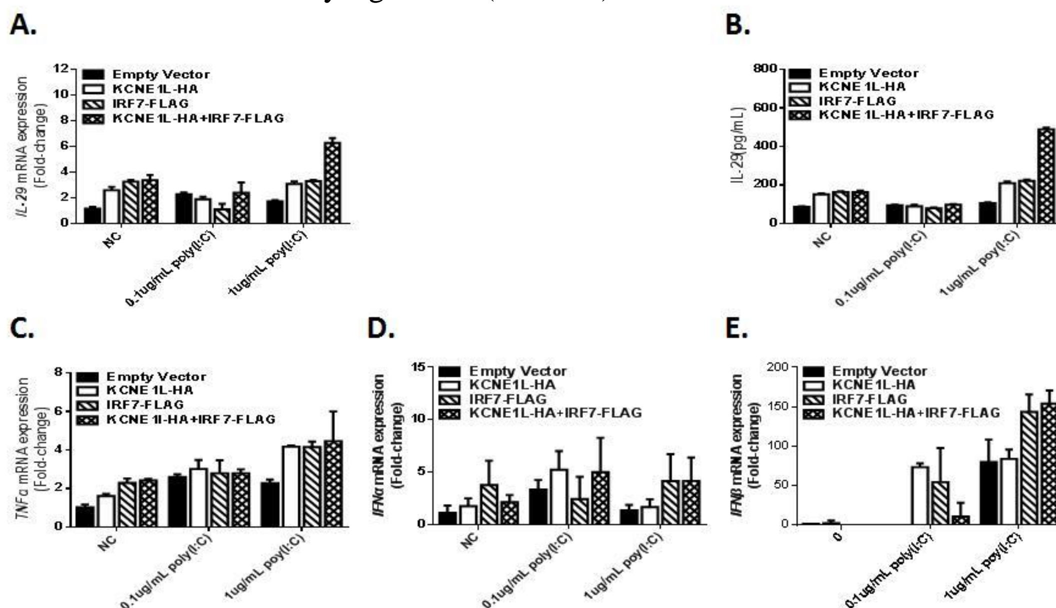


Fig.4 KCNE1L enhanced IRF7-mediated IL-29 gene expression.by way of contrast,IL-29 was induced by IRF7 which was effectively regulated by KCNE1L following 1ug/ml poly(I:C) stimulation.

3. Discussion

Innate immune defences are upregulated following the activation of PRR that detect PAMP unique to microbial pathogens, including foreign viral nucleic acids. PRR activation induces downstream signalling events that culminate in the expression of antiviral host genes, principally cytokines(including interferons) and interferon stimulated gene(ISG) products[20-24].This induced innate immune response confers a broadly refractory antiviral state that limits virus propagation and stimulates adaptive immune responses. Consequently,many viruses have evolved counter measures to antagonize intrinsic and innate immune defences to promote their efficient propagation and transmission to new hosts.[25-26]

In the immune system, macrophages and T lymphocytes and B lymphocytes also express KCNE, many KCNE protein expression is dependent on the cell cycle, and cells of different specific damage can also regulate KCNE expression. In this study, the replication of HSV-1 was determined by qRT-PCR detection of the overexpression/silence of KCNE1L.The results of detection of the relative plaque formation efficiency (PFE) also reached the conclusion.

Previous studies of mice and human trials have shown that type I interferon and type III interferon are the first line of defense against HSV infection. Mouse infection models showed that in the first few hours of infection, the production of type I interferon (IFN-α/β) was critical to the control of HSV-1 infection. Type I interferon can inhibit the early gene expression of HSV, limit the spread of the virus to the nervous system, and activate the natural killer cell defense system. The type III interferon IFN-L1(IL-29),IFN-L2(IL-28a),IFN-L3(IL-28b) has also been shown to protect human cells from HSV infection, which was discovered in 2003.[27]So we need to understand the connection between KCNE1L and interferon.

Consistent with knock down results,293t cells were transfected with empty vector or KCNE1L,we note that under high MOI conditions(MOI 1.0 PFU/cell),KCNE1L significantly affected the expression levels of IL-29 and TNF-α,and there was a positive correlation, as shown by qRT-PCR and ELISA.

Immunoprecipitation has identified the interaction between KCNE1L and IRF7,we show that IRF7,the important interferon regulatory factor of IRF7,plays functionally distinct role in the regulation of innate immune defence activated in response to HSV-1 infection through the induction of interferon expression. Activation of IRF7 is a prerequisite for its function as a transcription factor. Inactive IRF7 exists as a "latent" form in cytoplasm. The pathogenic infection triggers IRF7 phosphorylation and translocation into the nucleus, forming a transcriptional complex with other co-activators, binding to the promoter region of the target gene and activating transcription.

To verify whether KCNE1L interacts with IRF7 to affect the expression of interferon.KCNE1L,IRF7,and co-transfection of KCNE1L and IRF7 were transfected in 293t cells with the stimulation of poly(I:C), qRT-PCR detection of mRNA expression levels of IL-29,TNF-α,IFN-α, and IFN-β.Notably, qRT-PCR and ELISA analysis revealed that there was a difference in the expression of IL-29 in the single transfer group and the total transfer group at 1ug/mL poly(I:C) concentration. This suggests that KCNE1L may affect the expression of interferon by interacting with IRF7.by way of contrast,IL-29 was induced by IRF7 which was effectively regulated by KCNE1L following 1ug/ml poly(I:C) stimulation. The data demonstrate that KCNE1L may play a role in IRF7-IFN-mediated innate immunity signalling.

In addition, the studies on TLR- and the RIG-receptor signaling pathway indicate that TRIF-mediated TLR3 and TLR7/TLR8 signaling pathway through MyD88 also can activate the promoter of IFN-β and IL-29.[28]Further suggested that KCNE1L may participate in toll-like receptor signaling pathway and interact with transcription factor IRF7,thus affecting the expression of the downstream interferon. However, interferon does not directly kill or suppress the virus, but mainly through the cell surface receptors that cause the cells to produce antiviral proteins, thereby inhibiting the replication of the virus. It can also enhance the vitality of natural killer cells (NK cells), macrophages and T lymphocytes, thus play a role in immune regulation and enhance the antiviral ability.

With the deepening of the research, how to make KCNE1L intervene more thoroughly and more effectively in the development of HSV-1 will receive more and more attention. Importantly, it can also provide new perspectives and ideas for the treatment of herpes skin diseases caused by HSV-1.This experiment also laid a theoretical foundation for the further study of the specific molecular mechanism of KCNE1L for antiviral action, and will enrich people's understanding of the immune response, even can provide some reference with the research of anti-inflammatory drugs.

4. Materials and methods

4.1 Materials

The following reagents were used: Dulbecco's Modified Eagle Medium (DMEM, Gibco, C11995500BT), fetal bovine serum (FBS, Gibco, 10270-106), penicillin/ streptomycin (P/S, 10,00μg/ml,Sigma,P0781),phosphate buffered saline(PBS,Hyclone,SH30256.02),Easypure RNA Kit(TRANSGEN Biotech, Beijing),PrimeScriptTM RT Reagent Kit(TaKaRa Bio, Japan, Shiga),SYBR Green qPCR master Mix(Bimake,B21202), StarPure Endo-free Plasmid Miniprep Kit(GenStar,D207-01),Endofree Maxi Plasmid Kit(TIANGEN,DP117),QlAquick Gel Extraction Kit(QIAGEN,28704), lipofectamine 2000 (Invitrogen,Carlsbad,CA),Human IL-29(IFN lambda 1)ELISA Ready-SET-Go!(eBioscience,887296).

4.2 Methods

4.2.1 Cells culture

293t and Vero cells were obtained from American Type Culture Collection(ATCC),and both cell lines were grown in DMEM medium supplemented with 10% FBS and 1%P/S at 37℃ with 5% CO_2.In addition, Cells grow up to 70% to propagation,and cell digestion using Trypsin/EDTA Solution for Primary Cells and Trypsin Neutralizing Solution.

4.2.2 Virus propagation and infection

HSV-1 wildtype was originally obtained by ATCC.The virus was propagated on Vero cell in complete DMEM without P/S. Infected Vero culture was harvested at 80%-100% cytopathic effect and subjected to three freeze-thaw cycles. Cellular debris was removed by low-speed centrifugation(1000×g) at 4℃.The supernatant was removed and centrifuged at 23000×g for 2h at 4℃ in 35% sucrose cushion buffer(in Tris-buffered saline;50 mM Tris-HCl and 150 mM NaCl; pH7.4).The virus pellet was resuspended in DMEM and aliquots were stored at −80℃.Virus titers were determined on Vero cells.

Infection studies were performed with purified preparations of HSV-1 wildtype which used at a multiplicity of infection (MOI=1.5,3,6).After adding the virus, culture plates were incubated at 37℃ for 2h followed by washing with plain medium without FBS.Then, the culture was maintained in fresh complete medium for up to 24h.

4.2.3 Viral infection in cell

293t cells were seeded in 48 well dishes at $5x10^4$/well that transfected with an KCNE1L and control vector (PRK) or shRNA knockdown KCNE1L gene expression for 24 h,then HSV-1 infection (MOI=0.1,0.5) for 2 h.The cells were then washed with plain medium without FBS and maintained in fresh complete medium for up to 24 h.After the cell treatment,supernatant collection for ELISA and the total cell media were extracted RNA.IFN-α,IFn-β,IL-29,TNF-α and HSV-1 replication was analyzed by the qRT-PCR.

4.2.4 shRNA knockdown gene expression

KCNE1L shRNA Lentiviral vector plko.1 puro were purchased from (Transheep Bio).The sequence for KCNE1L shRNA are: KCNE1L-1: CCGGCGCACATCCAACTGCACTAAACTCGAGTTTAG TGCAGTTGGATGTGCGTTTTTG(TRCN0000188497);KCNE1L-2:CCGGGACCAGTCTGTGGGATAGAATCTCGAGATTCTATCCCACAGACTGGTCTTTTTT G(TRCN0000163580);KCNE1L-

3:CCGGGATTACCATTACTGGGTGTATCTCGAGATACACCCAGTAATGGTAATCTTTTTG(TRCN0000158768);KCNE1L-4:CCGGCTATCTCTACATCCTGCTCATCTCGAGATGAGCAGGATGTAGAGATAGTTTTTG(TRCN0000203770).293T were plated in 48 well plates at $5x10^4$ per well the day before transfection. Cells were transfected with shRNA duplexes at final concentration of 10 nM using lipofectamine 2000 according to the manufacturers' instructions (Invitrogen, Carlsbad,CA).After 6 hours incubation, the cells were replaced with DMEM containing 10% FBS without P/S for up to 24 hours. Then the cells were harvested for total RNA extraction and supernatant collection for ELISA.

4.2.5 Total RNA extraction and quantitative RT-PCR

Total RNA was extracted from cell cultured in vitro using Easypure RNA Kit. High-fidelity cDNA was generated from each RNA sample using PrimeScriptTM RT Reagent Kit with the Bio-Rad S1000 Thermal Cycler system. Quantitative RT-PCR reaction samples were prepared as a mixture with Quantitect SYBR Green PCR kit following the manufacturers' instructions. Reactions were performed using Bio-Rad CFX Connect Real-Time System. The PCR conditions were as follows:95℃ for 7min followed by 39 cycles of 95℃ for 15s and 60℃ for 30s,and then 95℃ for 20s.After the expansion, the dissolution curve was analyzed, and the dissolution curve showed a single peak in the product Tm, indicating that the amplification specificity was good. Because the target gene is consistent with the amplification efficiency of the internal reference gene, its relative quantitative value is calculated using the comparative Ct method, the calculation formula is $2^{-\Delta\Delta Ct}$.

The primer sequences used are as shown follows:Human KCNE1L (Forward: GGCTGCGAACCCTTCTGAG;Reverse:GCCAAGCAGGCGTAGAAGA);HSV-1(Forward: CGGCCG TGTGACA CTATCG; Reverse: CTCGTAAAATGGCCCCTCC); Human IFNα1(Forward: GCCTCGCC CTTTGCTTTACT;Reverse:CTGTGGGTCTCAGGGAGATCA); Human IFNβ (Forward: GCTTGGA TTCCTA CAAAGAA GCA;Reverse: ATAGA TGGTCA ATGCGGCGTC);Human IL-29 (Forward: TCCTAGACCA GCCCCTTCA;Reverse: GTGG GC TG AGGCTGGATA);Human TNFα (Forward: C CTCTC TCTAAT CAGCCCTCTG;R everse: GAGGAC CTGGGA GTAGA TGAG);Human HPRT (Forwa rd:CCTG GCGTCG TGAT TAGT GAT; Reverse: AGA CGTTCAGTCCTGTCCATAA).

4.2.6 ELISA

293t transfected with KCNE1L or knockdown KCNE1L gene expression with shRNA,then were stimulated with viruses for the indicated times.The culture media were collected for measurement of IL-29 by ELISA kit following the manufacturers' instructions.

4.2.7 CO-IP

293t($1x10^6$) were transfected with empty or expression plasmids of KCNE1L,RELA, DOCK8, DDX41,IRAK1,NF-kB1,STING,TBK1,IRF3,IRF7,GAS.Cells were lysed in protein lysis buffer(20mM Tris-HCl pH7.4,150 mM NaCl,1mM EDTA,1% Triton-X100) supplemented with protease inhibitors.

4.2.8 Viral plaque assay

Subconfluent 293t monolayers were cultivated in 24 well plates up to 70-80% that transfected with an KCNE1L and control vector (PRK) or shRNA knockdown KCNE1L gene expression for 24 h.Then infected with HSV-1(MOI=0.1,0.5) and incubated for 2h at 37 ℃ to allow virus adsorption.The culture medium was then replaced with newly prepared medium.After 24 h,HSV-1 collection were previously described.To prepaved into a single layer of background vero cells in 24 well then adding diluted 100 times HSV-1 50ul/well of over-expression treatment but for knockdown experiment that were added diluted 100 times HSV-1 with 25ul/well.Cells were incubated for 2h at 37℃ to allow virus adsorption with DMEM containing 2% FBS without P/S.Next,to replaced with newly prepared medium with 2% FBS and 0.2% agar.Sixty-eight hours later,the viral plaque formation was visualized by 5% crystal violet staining.

4.2.9 PRRs agonist stimulate Cells

293t cells were transfected with KCNE1L,IRF7,co-transfected with KCNE1L and IRF7 in 24 well dishes at 1x10^5/well,stimulated by poly(I:C)(0.1μg/mL,1μg/mL) after 24h.and then,RNA was extracted after 6 hours.The mRNA expression of IFN-α,IFN-β,IL-29,and TNF-α were detected by qRT-PCR.

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