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Original Research

KLF5 is involved in regulation of IFITM1, 2, and 3 genes during H5N1 virus infection in A549 cells

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Abstract: The interferon-induced transmembrane (IFITM) proteins usually protect cells from many virus infections by viral entry and replication. Thus, the present study aimed to identification novel regulation factors of IFITM genes. Our data showed up-regulation of IFITM1, IFITM2, and IFITM3 genes in A549 by H5N1 virus infection. However, IFITM1 was not affected, IFITM2 and IFITM3 increases expression following different concentration H5N1 virus infection in HEK293T cells. This is the first time to find differences of IFITM1 transcription in different cells with the H5N1 virus infection. Moreover, experiments showed that H5N1 virus promotes expression of KLF4 and KLF5 in both A549 and HEK293T cells, but H5N1 virus NS1, M1, NP or PB2 alone has no obvious effect on transcription of KLF5. Overexpression of KLF5 increases transcription of IFITM1, IFITM2, and IFITM3 in A549 cells, however, it does not affect transcription of IFITM1, IFITM2, and IFITM3 in HEK293T cells. In addition, results for the first time displayed that KLF4 can up-regulate expression of IFITM5 in both A549 and HEK293T cells. In conclusion, we revealed KLF5 is involved in transcription of IFITM1, 2, and 3 thus making it a potential therapeutic activator in the future to treat viral infections through promoting the innate immune response.

Key words: KLF5, IFITM, transcription, H5N1, influenza virus.

Introduction

The IFITMs belong to a family of interferon-induced transmembrane proteins including five members of IFITM1, IFITM2, IFITM3, IFITM5, and IFITM10 in human. To date the expression and regulation of IFITM1, IFITM2, IFITM3 and IFITM5 is still far from clear. Actually only IFITM1, IFITM2, and IFITM3 were strongly up-regulated by stimulation on types I and II interferon, these proteins as innate antiviral cell-intrinsic restriction factors prevent viruses from traversing the lipid bilayer and accessing the cytoplasm, such as influenzaA virus (IAV), Japanese encephalitis virus (JEV), dengue virus (DENV), Ebola virus (EBOV), Rift Valley fever virus (RVFV), human immunodeficiency virus (HIV), et al (1-7). IFITM5 is a gene that encodes a membrane protein playing a key role in bone mineralization, IFITM5 mutations were associated with osteogenesis imperfect type 5 (8). Currently, the transcription patterns and function of IFITM10 have not been characterized. Krüppel-like factor 4 (KLF4) and Krüppel-like factor 5 (KLF5) are evolutionarily conserved zinc finger-containing transcription factors implicated in many biological processes in proliferation, differentiation, apoptosis, and development (9-11). However, up to date, no article has reported whether transcription factor KLF5 involves in regulation of IFITM1, IFITM2, and IFITM3 during H5N1 influenza virus infection. Thus, in the present study, we first found KLF5 can promotes transcription of IFITM1, IFITM2, and IFITM3 in A549 cells, but not in the HEK293T cells. Moveover, we further investigated the relationship between transcription of IFITM1, IFITM2, and IFITM3 and H5N1 influenza virus NS1, M1, NP, and PB2 protein expression in A549 and HEK293T cells.

Materials and Methods

Cells culture

A549 and HEK 293T cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM, Gibco) with 10% fetal bovine serum (FCS, Gibco), in a humidified 5% CO_2 and 95% air at 37°C.

Virus manipulation

Mouse adapted influenza virus A/environment/Qinghai/1/2008 (H5N1) was propagated in 10-day-old embryonated chicken eggs at 37 °C for 2 days. Allantoic fluid was purified by centrifugation and stored at −80 °C until use. Viral infection was performed when cells were 80% confluent. The serum-free DMEM medium containing allantoic fluid was added at 0.5, 1, 2 multiplicities of infection (MOI) for 1h. Then cells were washed thrice by PBS and cultured with DMEM containing 2% FBS. Virus titer was determined by plaque assay. All experiments with this infectious virus were carried out in a Biosafety Level 3 containment laboratory.

Gene cloning and plasmids

The open reading frame (ORF) of human KLF4, KLF5, SOX2 and MYC genes were generated by PCR and cloned into pEGFP-N2 vector for genes overex-

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pression experiments and fluorescence localization observed. The ORF sequences of the H5N1 influenza virus protein genes (NS1, M1, NP, and PB2) were generated by PCR and cloned into pcDNA3.1/myc-His(+)A vector for genes overexpression experiments. All constructs were verified by sequencing, and primers used in this study are listed in Table 1.

Transfection

Cells were seeded in 12-well plates overnight and transfected with 2 ug plasmids suspended in 3 ul Lipofectamine 3000 (Invitrogen) and 100 ul OPTI-MEM Reduced Serum Medium. After 6 h transfection, the cell medium was replaced by fresh DMEM medium with 10% FBS. A549 and HEK 293T cells were transfected for 36 h, then harvested and resolved in TRIzol Reagent for RNA extraction and reverse transcription.

RNA extraction and cDNA synthesis

Total RNA was extracted from A549 and HEK 293T cells using the TRIzol Reagent according to the supplier's specifications. Spectrophotometer (Thermo) was used to determined the concentration of RNA at 260/280 nm. cDNA was synthesized using 5ug of total RNA from A549 and HEK 293T cells, GoScript™ Reverse Transcription System (Promega) was used for first-stand synthesis following manufacturer's instructions and was stored at -80°C until used.

PCR and real-time PCR

Real-time PCR was performed in a ABI 7500 fast real-time system (Life Technology) with SYBR green real-time PCR master mix (CWBIO). Real-time PCR conditions were as follows: 95°C for 10 min, then 94°C for 30 s, 60°C for 1 min, 40 cycles, followed by 72°C for 7 min. Reactions were performed in a 20ul volume containing 10 ul SYBR green real-time PCR master mix, 8.5 ul double-distilled water, 1 ul forward and reverse primers, and 0.5 ul cDNA template. The primers are listed in Table 2 (12-15). β-actin as internal reference in order to normalize the mRNA expression levels. The generation of specific PCR products was confirmed by melting curve analysis. $2^{-\Delta\Delta CT}$ method was used to quantify the relative mRNA express levels. To analyze transcription level of IFITM5 the cDNA was amplified in a total volume of 20 ul with 35 cycles of PCR. All PCR products were confirmed on 1.5% agarose gels with ethidium bromide.

Statistical analysis

All data were expressed as the means ± S.E.M. (standard error of the mean). Student's t-test was used to

Table 1. Primer sequences of full-length gene annealing temperature used in vector constructs.

Gene Symbol	Sequence (5'-3')	Annealing (°C)	plamsid
KLF4	CCGGAATTCGCCACCATGAGGCAGCCACCTGG	61	N2
	CGCGGATCCTAAAATGCCTCTTCATGTGTAAGG		
KLF5	CCGGAATTCGCCACCATGGCTACAAGGGTGCTGAGCA	59	N2
	CGCGGATCCTGTTCTGGTGCCTCTTCATATGC		
SOX2	CCGGAATTCGCCACCATGTACAACATGATGGAGACGGAGC	63	N2
	CGCGGATCCTCATGTGTGAGAGGGGCAGTGTGC		
MYC	CCGGAATTCGCCACCATGCCCCTCAACGTTAGCTT	60	N2
	TGAGGATCCTCGCACAAGAGTTCCGTAGCTG		
M1	TACAAGCTTATGAGCCTTCTAACCGAGGTCGAA	58	3.1
	TACCTCGAGCTTGAATCGCTGCATTTGCACTC		
NS1	TACGAATTCATGGATTCCAACACTGTGTC	54	3.1
	TACCTCGAGCTTTGGAGAGAGTGGAGGTC		
NP	CCCGGATCCATGGCGTCTCAGGGCACCAAACGA	56	3.1
	CCCCTCGAGATTGTCATATTCCTCTGCATTGTC		
PB2	CCCAAGCTTATGGAGAGAATAAAAGAATTAAG	56	3.1
	CCCCTCGAGATAGATGGCCATCCGAATCCTTTTG		

Table 2. Primer sequences, amplicon size and annealing temperature used in Real-time PCR assays.

Gene Symbol	Sequence (5'-3')	Amplicn (bp)	Annealing (°C)
β-actin	GCGGGAAATCGTGCGTGACATT	232	60
	GATGGAGTTGAAGGTAGTTTCGTG		
MYC	AAGTCCTGCGCCTCGCAA	249	60
	GCTGTGGCCTCCAGCAGA		
OCT4	GAGGAGTCCCAGGACATCAA	430	60
	ACACTCGGACCACATCCTTC		
SOX2	TGGACAGTTACGCGCACAT	215	60
	CGAGTAGGACATGCTGTAGGT		
KLF4	ACCTACACAAAGAGTTCCCATC	136	60
	TGTGTTTACGGTAGTGCCTG		
PTEN	CGAACTGGTGTAATGATATGT	330	60
	CATGAACTTGTCTTCCCGT		
KLF5	CTTCATCTTTCTGTCCCTAC	447	60
	GGGGTTACTCCTTCTATTGT		
IFITM1	ATCAACATCCACAGCGAGAC	253	60
	CAGAGCCGAATACCAGTAACAG		
IFITM2	TTCATAGCATTCGCGTACTCC	296	60
	GAATACAGGTCAAGGGCAGAG		
IFITM3	GAGAACCATCCCAGTAACCC	318	60
	CAACCATCTTCCTGTCCCTAG		
IFITM5	CCCTCTACCTGAATCTGTGTTG	268	60
	CAGTCATAGTCCGCGTCATC		

determine the significance of differences between two groups, while one-way analysis of variance (ANOVA) was used when there are more than two groups. P-value <0.05 was considered to be statistical significance.

Results

H5N1 virus promotes the transcription of IFITM1, 2, and 3 genes in A549 cells

In the present study, we detected the transcription of IFITM1, IFITM2, and IFITM3 genes in A549 and HEK293T cells. Our data showed that up-regulation of IFITM1, IFITM2, and IFITM3 genes in A549 cells during H5N1 virus infection, whereas IFITM1 did not change in HEK293T cells, but IFITM2 and IFITM3 increased transcription following different concentration H5N1 virus infection (Figure 1). This is the first time to find differences of IFITM1 transcription during the H5N1 virus infection. Furthermore, our survey displayed higher level expression of IFITM1 in the HEK293T cells than A549cells, but not for IFITM2 and IFITM3 (Figure 2). Above results showed that the background level of IFITM1 may cause distinct IFITM1 transcription sensitivity to H5N1 virus infection.

H5N1 virus Promotes the transcription of KLF4 and KLF5

Previous studies shown that interferon promotes IFITM1, IFITM2, and IFITM3 transcription, whereas very few reports illustrated other transcription factors involved in their regulation. Currently, our study showed that H5N1 avian influenza virus can up-regulate transcription factors KLF4 and KLF5 in HEK293T and A549 cells (Figure 3). In order to analyze the specificity of KF4 and KLF5, four plasmids were constructed to express NS1, M1, NP, and PB2 proteins. Overexpression of four H5N1 virus proteins only revealed that NP protein most significantly increased transcription of KLF4 in A549 and PB2 most significantly decreased transcription of KLF4 in HEK293T, no found other transcriptional regulation (Figure 4).

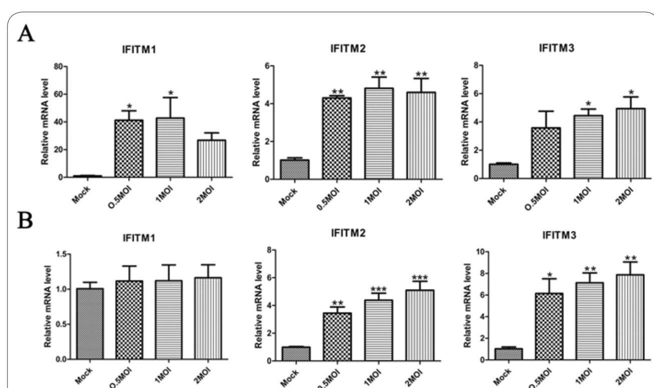

Figure 1. Transcription expression pattern of IFITM1, 2, and 3 in (A) A549 and (B) HEK293T cells by H5N1 influenza virus. Cells seeded on 12-well plates overnight were infected with 0.5 MOI, 1 MOI, and 2MOI H5N1 virus for 36 h. Then total RNAs were extracted to examine the mRNA levels of IFITM1, 2, and 3 by real-time PCR. Quantitative data (means±SEM) were normalized to β-actin, there data are based on at least three independent experiments. *p<0.05;**p<0.01;***p<0.001.

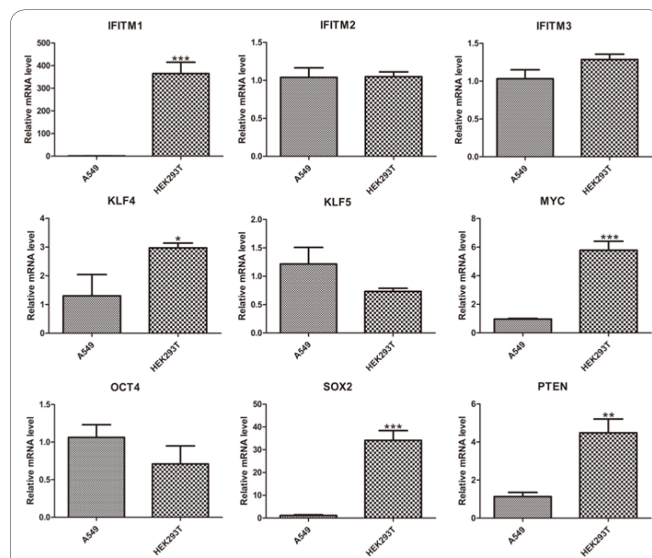

Figure 2. Comparison of antiviral factors and related transcription factors between A549 cells and HEK293T cells. A549 cells and HEK293T cells were infected with H5N1 influenza virus, RNA was extracted from A549 cells and HEK293T cells and subjected to real-time quantitative PCR analysis. Quantitative data (means±SEM) were normalized to β-actin, there data are based on at least three independent experiments.*p<0.05;**p<0.01;** *p<0.001.

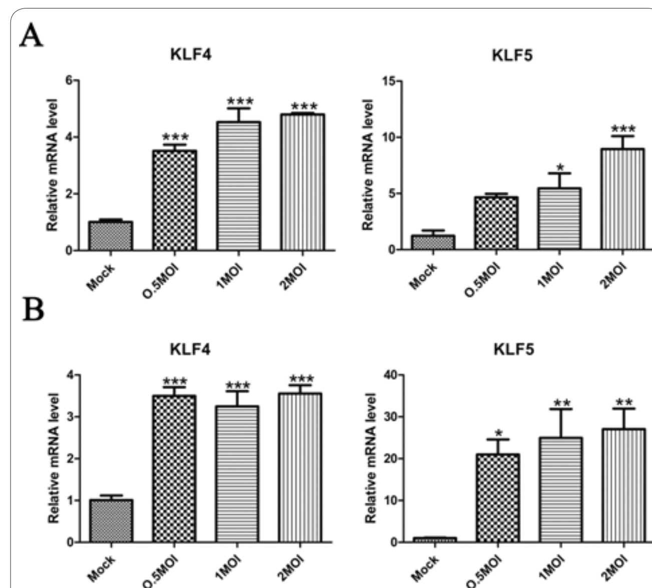

Figure 3. Gene expression of KL4 and KLF5 with H5N1 virus infection. (A) A549 cells and (B) HEK293T cells seeded on 12-well plates overnight were infected with 0.5 MOI, 1 MOI, and 2MOI H5N1 virus for 36 h. RNA was extracted from treated cells and subjected to real-time quantitative PCR analysis. Quantitative data (means±SEM) were normalized to β-actin, there data are based on at least three independent experiments. *p<0.05;**p<0.01;***p<0.001.

KLF5 increase transcription of IFITM1, 2, and 3 in A549 cells

To investigate whether KLF4 and KLF5 were associated with transcription regulation of IFITM genes, KLF4 and KLF5 plasmids were constructed in this study, with SOX2 and MYC plasmids also constructed as controls. Fluorescence microscope indicated that KLF4, KLF5, SOX2, and MYC localize to nuclear region and display diverse localization shapes (Figure 5 and 6). Overexpression of KLF5 up-regulated transcription of

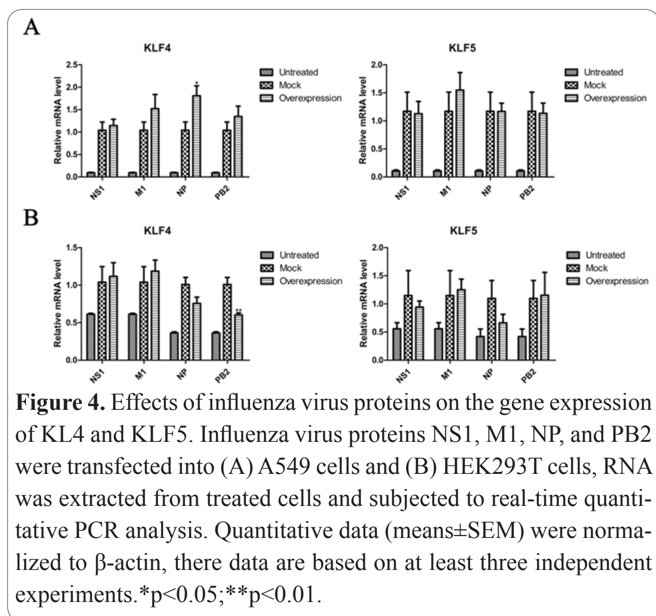

Figure 4. Effects of influenza virus proteins on the gene expression of KL4 and KLF5. Influenza virus proteins NS1, M1, NP, and PB2 were transfected into (A) A549 cells and (B) HEK293T cells, RNA was extracted from treated cells and subjected to real-time quantitative PCR analysis. Quantitative data (means±SEM) were normalized to β-actin, there data are based on at least three independent experiments.*p<0.05;**p<0.01.

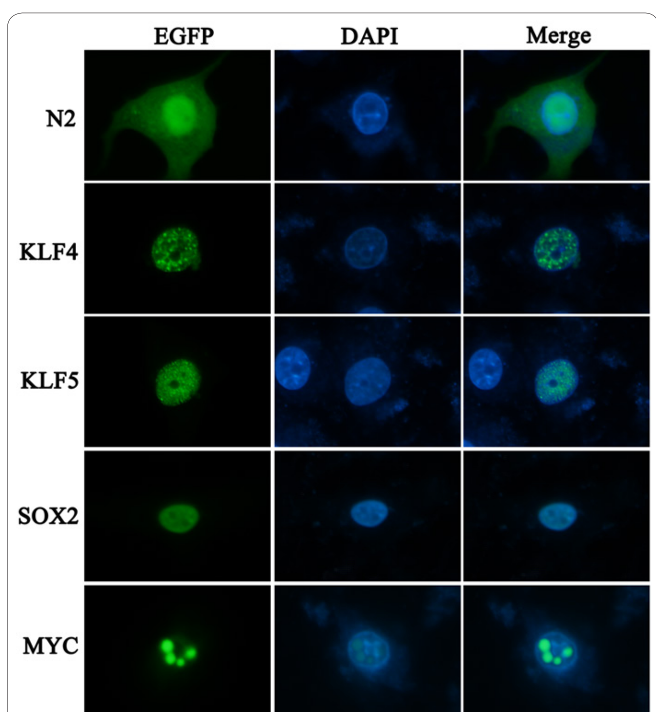

Figure 5. Intracellular localization of the KLF4, KLF5, SOX2, and MYC in A549 cells. A549 cells were transiently tranfected with pEGFP-N2-KLF4, pEGFP-N2-KLF5, pEGFP-N2-SOX2, and pEGFP-N2-MYC plasmids, 36h later, A549 cells were observed under the fluoreacence microscope, photograph indicated these transcription factors localize to nuclear region.

IFITM1, IFITM2, and IFITM3 in A549 cells, but not in HEK293T cells (Figure 7). In addition, our experimental displayed that KLF4 can also promote transcription of IFITM1 in A549 cells, interestingly, transcription of IFITM2 and IFITM3 were not increased by KLF4 in A549 cells (Figure 7).

IFITM5 was upregulated by KLF 4 in both A549 and HEK293T cells

The exact transcription mechanism of IFITM5 by which it contributes to bone mineralization and osteogenesis imperfect type 5 are still unknown. Our results first display KLF4 can up-regulate expression of IFITM5 in A549 and HEK293T cells (Figure 8). This result will increase the knowledge about the regulation of IFITM5 during bones formation and related disease development.

Discussion

The important function of IFITM protein family is to inhibit the invasion of many pathogenic viruses, such as HIV, IAV, and RVFV, et al (4). Previous studies have shown that IFITM 1, 2, and 3 can be induced by interferon, while viral infection usually promotes production of interferon as an innate immune response. Further research revealed that IFITM 1, 2, and 3 are important innate immune proteins to mainly resist invasion of virus (1, 6, 7). However, there is limited information about the transcription regulation of IFITM family, IFITM3 was expressed at higher levels in colon tumors than normal colon tissue, and the loss of KLF4 expression up-regulates IFITM3 in human colon tumors, indi-

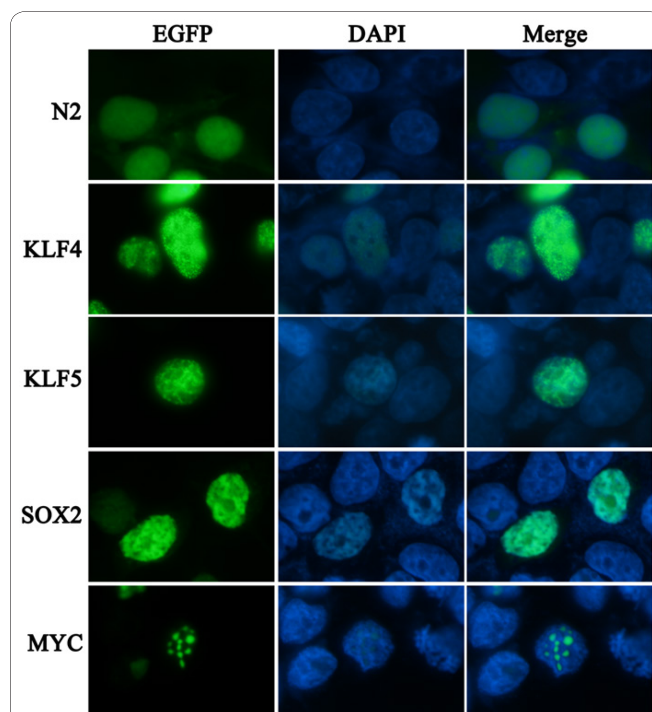

Figure 6. Intracellular localization of the KLF4, KLF5, SOX2, and MYC in HEK293T cells. HEK293T cells were transiently tranfected with pEGFP-N2-KLF4, pEGFP-N2-KLF5, pEGFP-N2-SOX2, and pEGFP-N2-MYC plasmids, 36h later, HEK293T cells were observed under the fluoreacence microscope, photograph indicated these transcription factors localize to nuclear region.

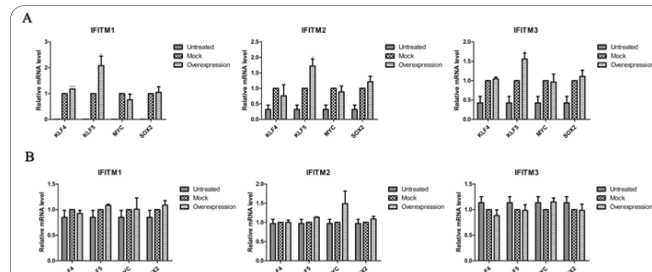

Figure 7. Effects of transcription factors on the gene expression of IFITM1, 2, and 3 in A549 and HEK293T cells. (A) A549 cells and (B) HEK293T cells were transfected with four transcription factors KLF4, KLF5, SOX2 and MYC, RNA was extracted from treated cells and subjected to real-time quantitative PCR analysis. Quantitative data (means±SEM) were normalized to β-actin, there data are based on at least three independent experiments.*p<0.05;**p<0.01.

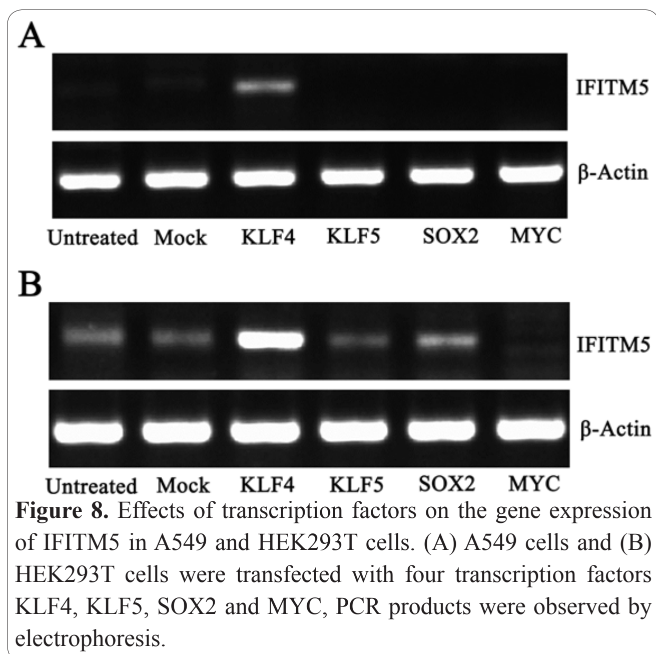

Figure 8. Effects of transcription factors on the gene expression of IFITM5 in A549 and HEK293T cells. (A) A549 cells and (B) HEK293T cells were transfected with four transcription factors KLF4, KLF5, SOX2 and MYC, PCR products were observed by electrophoresis.

cating that IFITM3 is a direct transcriptional target of KLF4 (16). Previous study has shown KLF4 as a critical tumor repressor associated with the initiation and progression of gastrointestinal cancers through interaction with Wnt/β-catenin signaling pathway (17). In addition, KLF4 also play an important role in the establishment or maintenance of pluripotency (18). KLF4 knockdown promotes cell migration and invasion through suppressing TIMP-1 and TIMP-2 expression in hepatocellular carcinoma (HCC) (19).

KLF4 and KLF5 belong to the same family of transcription factors involved in the regulation of many cellular biochemical processes. KLF4 and KLF5 were associated with regulation of anti-apoptotic signaling which mediates lapatinib resistance in breast cancer (20). Moreover, KLF5 inhibits angiogenesis through attenuating AKT activation and subsequent HIF1α accumulation in PTEN-deficient prostate cancer, that result demonstrating that PTEN can make up for role of KLF5 (21). KLF5 promotes gastric cancer development by engaging in mutual crosstalk with both GATA4 and GATA6 (22). Single nucleotide polymorphisms (SNPs) of KLF5 can alter the response to angiotensin II playing an important role in the pathogenesis of hypertension (23). Furthermore, both KLF5 and the p300 acetylase can interact via TGF-β, and acetylation of KLF5 was prerequisite for Smad4 to associate with p300 (23). KLF5 plays important roles in development, stemness and tumorigenesis, however no information is available on the detailed function of KLF5 in transcription regulation of IFITM family genes. Interestingly, our study did not find KLF4 to be significantly associated with transcription of IFITM3 in A549 and HEK293T cells, but IFITM1, IFITM2, and IFITM3 were found to be upregulated by KLF5 in A549 cells.

Above results illustrated that KLF5 regulates can involve in expression of IFITM family genes, but different types of cell can induce specificity regulated. In this study, we first found KLF5 regulate transcription of IFITM1, IFITM2, and IFITM3 in A549 cells. Moreover, up-regulation of IFITM5 was also detected by overexpression of KLF4 either in A549 cells or HEK293T cells. These results not only expanded the knowledge of transcriptional regulation of IFITM family genes but also provided the basis for revealing the mechanism of viral diseases development.

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