



RESEARCH ARTICLE



Expression Profile and Function Analysis of Long Non-coding RNAs in the Infection of Coxsackievirus B3

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Abstract

The roles of lncRNAs in the infection of enteroviruses have been barely demonstrated. In this study, we used coxsackievirus B3 (CVB3), a typical enterovirus, as a model to investigate the expression profiles and functional roles of lncRNAs in enterovirus infection. We profiled lncRNAs and mRNA expression in CVB3-infected HeLa cells by lncRNA-mRNA integrated microarrays. As a result, 700 differentially expressed lncRNAs (431 up-regulated and 269 down-regulated) and 665 differentially expressed mRNAs (299 up-regulated and 366 down-regulated) were identified in CVB3 infection. Then we performed lncRNA-mRNA integrated pathway analysis to identify potential functional impacts of the differentially expressed mRNAs, in which lncRNA-mRNA correlation network was built. According to lncRNA-mRNA correlation, we found that XLOC-001188, an lncRNA down-regulated in CVB3 infection, was negatively correlated with NFAT5 mRNA, an anti-CVB3 gene reported previously. This interaction was supported by qPCR detection following siRNA-mediated knockdown of XLOC-001188, which showed an increase of NFAT5 mRNA and a reduction of CVB3 genomic RNA. In addition, we observed that four most significantly altered lncRNAs, SNHG11, RP11-145F16.2, RP11-1023L17.1 and RP11-1021N1.2 share several common correlated genes critical for CVB3 infection, such as BRE and IRF2BP1. In all, our studies reveal the alteration of lncRNA expression in CVB3 infection and its potential influence on CVB3 replication, providing useful information for future studies of enterovirus infection.

Keywords Coxsackievirus B3 (CVB3) · lncRNA-mRNA correlation network · Long non-coding RNA (lncRNA) · XLOC-001188 · NFAT5

Lei Tong and Ye Qiu these authors contributed equally to this work.

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Abbreviations

ARRDC3	Arrestin domain containing 3
BRE	Brain and reproductive organ-expressed
CCRN4L	Carbon catabolite repression 4-like
CDCA3	Cell division cycle associated 3
CVB3	Coxsackievirus B3
FIBP	FGF1 intracellular binding protein
HLA-DQA1	Major histocompatibility complex, class II, DQ alpha 1
hnRNPH3	Heterogeneous nuclear ribonucleoprotein H3
IRF2BP1	Insulin-like growth factor 2 mRNA-binding protein 1
KEGG	Kyoto Encyclopedia of Genes and Genomes
lncRNA	Long non-coding RNA
MDA5	Melanoma differentiation associated gene 5
MTX1	Metaxin 1

NEMF	Nuclear export mediator factor
NFAT5	Nuclear factor of activated T cells 5
SNHG11	Small nucleolar RNA host gene 11
TIMP1	TIMP metalloproteinase inhibitor 1
TMED9	Transmembrane p24 trafficking protein 9
ZNF295	Zinc finger protein 295

Introduction

Long noncoding RNAs (lncRNAs) are classically defined as RNA transcripts larger than 200 nucleotides without any protein-coding capacity (Cheetham *et al.* 2013; Ma *et al.* 2013), although recent publications have reported that several lncRNAs retain limited translational activity and code small peptides (Anderson *et al.* 2015; Matsumoto *et al.* 2017). In cells, lncRNAs play various regulatory roles via interacting with DNA, RNA or protein molecules, modulating gene expression and protein functions (Ponting *et al.* 2009; Benetatos *et al.* 2011; Fatica and Bozzoni 2014). Recently, accumulated studies demonstrate critical roles of host lncRNAs in viral infection (Liu and Ding 2017). For example, lnc-Lsm3b is up-regulated upon the infection of different viruses such as vesicular stomatitis virus, sendai virus and herpes simplex virus, inhibits the stimulation of IFN- α and - β , and thus blocks cellular antiviral response (Jiang *et al.* 2018). By contrast, lncIT-PRIP-1 induced by the infection of hepatitis C virus mediates MDA5-triggered production of IFNs and ISGs, leading to the suppression of HCV replication (Xie *et al.* 2018).

Coxsackievirus B3 (CVB3), a pathogenic enterovirus of *Picornaviridae* family, is a common causative agent of various inflammatory diseases including myocarditis (Zhang *et al.* 1997; Fairweather *et al.* 2012), meningitis (Wong *et al.* 2011), pancreatitis (Ursing 1973; Mena *et al.* 2000) and hepatitis (Liu *et al.* 2013). Especially, CVB3-induced myocarditis frequently progresses to dilated cardiomyopathy (DCM), which is a major cause of sudden unexpected death in patients less than 40 years of age (Kearney *et al.* 2001; Fung *et al.* 2016). Currently, there is no effective therapy available for acute CVB3 infection, and as for DCM, the only treatment is heart transplantation (Kearney *et al.* 2001). Therefore, it is critical to identify novel drug targets for therapy development of CVB3-related diseases.

During the life cycle of CVB3, the virus interacts with various cellular components of the host, which either benefits viral survival or is a defensive mechanism of host to counteract viral infection (Zhai *et al.* 2018). For instance, CVB3 promotes the expression of a molecular chaperone protein Hsp70 that stabilizes viral genomic

RNA and enhances viral translation (Qiu *et al.* 2016; Wang *et al.* 2017); CVB3 infection induces the up-regulation of a stress-responsive protein NFAT5 which inhibits CVB3 replication, but this antiviral protein is cleaved and inactivated by CVB3 proteases at later infectious stage (Qiu *et al.* 2017). In addition to host proteins, CVB3 also interplays with cellular non-coding RNAs, especially microRNAs. Our previous studies demonstrated that miR-342-5p suppresses CVB3 biogenesis via targeting the coding region of the viral protein 2C (Wang *et al.* 2012), while miR-10a* favors CVB3 biosynthesis via targeting the viral-3D-coding region (Tong *et al.* 2013). However, the interactions between CVB3 and lncRNAs have not been revealed to date, although lncRNAs are also closely related to several pathways critical for CVB3 replication.

To uncover the roles of lncRNA in CVB3 infection, we employed mRNA function analyses and the correlation network of lncRNA-mRNA expression. Firstly, we screened the expression profiles of lncRNAs and mRNAs in CVB3-infected and -uninfected HeLa cells by microarray analysis. Based on the data, we summarized the function of all differentially expressed mRNAs by Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses. Finally, we established the correlation network of differentially expressed lncRNAs and mRNAs, which facilitated the elucidation of the lncRNA-mRNA interactions and the functional roles of lncRNAs in CVB3 infection.

Materials and Methods

Cell Culture and CVB3 Infection

HeLa cells (ATCC) were cultured using Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 100 μ g/mL penicillin-streptomycin and 10% fetal bovine serum (FBS, Sigma) in 37 °C supplied with 5% of CO₂.

CVB3 (CG) strain was a kind gift from Dr. Charles Gauntt (University of Texas Health Science Center) and propagated in HeLa cells. Virus stock was prepared from the cells by three freeze-thaw cycles followed by centrifugation to remove cell debris and stored at -80 °C. The titer of virus stock was determined by plaque assay as described below. Cell cultures were infected with CVB3 at 0.5 MOI for 1 h in serum-free medium, washed with phosphate-buffered saline (PBS, Thermo Fisher), and then replenished with fresh medium containing fetal bovine serum (FBS) followed by 23 h culture. Cells treated with the same amount of PBS in the same process served as uninfected control.

RNA Preparation

Total RNA was extracted and purified using TRIzol™ Plus RNA Purification Kit (Invitrogen). Total RNA then was reverse-transcribed to cDNA using SuperScript™ IV First-Strand Synthesis System (Invitrogen) according to the manufacturer's instructions.

Microarray Processing and Analysis

Total RNA from each sample was quantified by the NanoDrop ND-1000 and RNA integrity was assessed by standard denaturing agarose gel electrophoresis. For microarray analysis, Agilent Array platform was employed. The sample preparation and microarray hybridization were performed based on the manufacturer's standard protocols with minor modifications. Briefly, mRNA was purified from total RNA after removal of rRNA (mRNA-ONLY™ Eukaryotic mRNA Isolation Kit, Epicentre). Then, each sample was amplified and transcribed into fluorescent cRNA along the entire length of the transcripts without 3' bias utilizing a random priming method. The labeled cRNAs were hybridized onto the Human lncRNA Array v3.0 (8 × 60K, Arraystar). After having washed the slides, the arrays were scanned by using the Agilent Scanner G2505C.

Agilent Feature Extraction software (version 11.0.1.1) was used to analyze acquired array images. Raw signal intensities were normalized in quantile method and subsequent data processing were performed using the GeneSpring GX v11.5.1 software package (Agilent Technologies). Expression of GAPDH (glyceraldehyde phosphate dehydrogenase) was used as an internal control. lncRNAs or mRNAs with at least two-fold changes in the expression levels and FDR (false discovery rate) < 0.05 were deemed to be differentially expressed. The differentially expressed lncRNAs and mRNAs with statistical significance between two groups were identified through Volcano Plot filtering. Pathway analysis was applied to determine the roles of these differentially expressed mRNAs played in these biological pathways. Finally, Hierarchical clustering was performed to show the distinguishable lncRNAs and mRNAs expression pattern among samples.

siRNA Transfection

Three siRNAs targeting XLOC_001188 were designed by the supplier (Suzhou GenePharma), among which only one effectively knocked down XLOC_001188 and was chosen to conduct the following experiments (Sense: 5'-GGU-GAGUCUGGAAAGCCUUTT-3'; Antisense: 5'-

AAGCCUUUCCAGACUCACCTT-3'). The siRNA was transfected to HeLa cells using Lipofectamine 2000 per the manufacturer protocol. At 48 h post transfection, the cells were subjected to CVB3 infection.

Quantitative Real-Time PCR (qPCR)

qPCR was performed in triplicate to validate the expression of significantly altered lncRNAs and mRNAs in CVB3-infected HeLa cells compared to uninfected control cells. Quantitative PCR analysis and data collection were performed on the ViiA™ 7 Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) using the primer pairs listed (Supplementary Table S1). GAPDH served as an endogenous control for normalization. For relative quantification, $2^{-\Delta\Delta C_t}$ was calculated and used as an indication of gene relative expression.

Pathway Analysis

Pathway analysis was conducted according to Kyoto Encyclopedia of Genes and Genomes (KEGG: <http://www.genome.ad.jp/kegg/>), two-sided Fisher's exact test and χ^2 test were used to select significant pathways, and the False Discovery Rate (FDR) was calculated to correct the *P* value (Draghici *et al.* 2007).

lncRNA-mRNA Correlation Network

The lncRNA-mRNA correlation network was constructed by correlation calculation based on the values of the normalized signal intensity of specific expression in the differentially expressed genes. For each pair of mRNA-lncRNA, Pearson correlation was calculated to determine the significance of correlation and the correlation value cutoff was 0.92. Correlation degrees of lncRNAs and mRNAs were calculated by counting their correlated counterparts. The *P*-value denoted the significant level of gene co-expression and the threshold of significance was *P*-value < 0.05.

Statistical Analysis

SPSS 20.0 software (SPSS Inc., Chicago, IL, USA) was used to do statistical analysis. All data were shown as mean ± SD of three independent experiments with each experiment in triplicate. The student *t* test was used to evaluate the expression differences of lncRNAs and mRNAs between CVB3-infected cells and uninfected control cells. *P* < 0.05 was considered as statistically significant.

Results

lncRNA and mRNA Expression Profile in CVB3 Infection

By microarray quantifying host lncRNAs and mRNAs in CVB3-infected HeLa cells and uninfected controls, we found 700 differentially expressed lncRNAs (Fig. 1A, Supplementary Table S2) and 665 differentially expressed mRNAs (Fig. 1B, Supplementary Table S3). Among the differentially expressed lncRNAs, 431 were up-regulated and 269 were down-regulated upon CVB3 infection; as for mRNAs, 299 were up-regulated and 366 were down-regulated (fold change > 2.0, $P < 0.05$). To evaluate the

reliability of microarray results, we randomly selected five up-regulated and three down-regulated lncRNAs, and verified their expression by qPCR. The qPCR results consisted with the microarray results, indicating a high confidence level of our data (Fig. 1C). To further reveal the DE profile of lncRNAs and mRNAs upon CVB3 infection, we employed hierarchical clustering analysis. The results showed that the differentially expressed lncRNAs and mRNAs perfectly distinguished CVB3-infected cells from uninfected control cells (Fig. 1D, 1E).

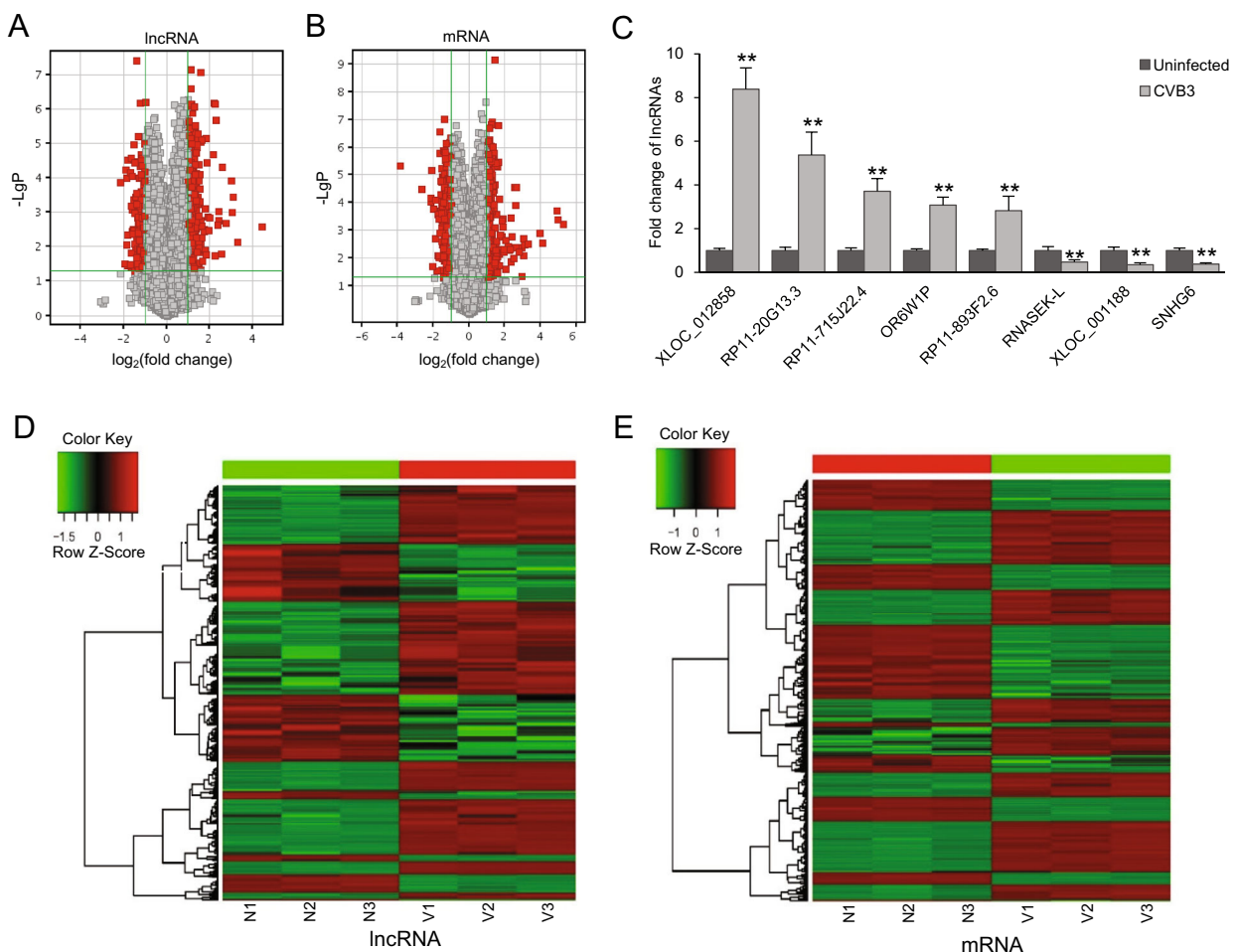


Fig. 1 Differentially expressed lncRNAs and mRNAs shown in microarray and qPCR verification. Volcano plots are used to present the changes of lncRNAs (A) and mRNAs (B) in CVB3-infected cells. The vertical axis corresponds to the negative logarithm of p value with base 10 ($-\text{LgP}$) and the horizontal axis represents the logarithm of fold change with base 2 ($\log_2(\text{fold change})$). The red points represent the differentially expressed lncRNAs or mRNAs with statistical significance ($P < 0.05$). C qPCR verification for the expression of five up-regulated and three down-regulated lncRNAs

in uninfected and CVB3-infected cells. GAPDH served as an endogenous control and the results were presented as the fold change of the corresponding uninfected control (** $P < 0.01$). D lncRNA hierarchical clustering and E mRNA hierarchical clustering. Red color indicates up-regulation and green color indicates down-regulation. Every column represents a tissue sample and every row represents an lncRNA/mRNA probe. V represents CVB3-infected cells and N represents uninfected control cells.

Pathway Analysis for Differentially Expressed mRNAs

In order to demonstrate the altered signaling pathways involving the differentially expressed mRNAs, we performed KEGG pathway analyses. The significance of a pathway was evaluated by an index of enrichment (IE) equal to “ $-\log_{10}(P\text{-value})$ ”. Therefore, the larger IE and the smaller P -value indicated the higher significant level of the pathway alteration. We counted the number of differentially expressed mRNAs in each pathway groups. According to the results, the most up-regulated pathways included asthma, rheumatoid arthritis, antigen processing and presentation, allograft rejection, and graft-versus-host disease (Fig. 2A). The down-regulated pathways included glutathione metabolism, arginine and proline metabolism, platinum drug resistance, non-alcoholic fatty liver disease, and drug metabolism–cytochrome P450 (Fig. 2B).

Fig. 2 Pathway analysis of differentially expressed mRNAs in CVB3 infected cells. A total of 665 differentially expressed mRNAs were chosen in pathway analysis. The dot plots present the enrichment of these mRNAs in every pathway. The color of each dot corresponds to the P -value which indicates the significant level of change of each pathway. The size of each dot show the number of mRNAs involved in the corresponding pathway. The horizontal axis represents the enrichment level of the pathways. A higher enrichment level means larger change in CVB3 infection when the P -values are the same.
A Pathway enrichment for up-regulated (UR) mRNAs.
B Pathway enrichment for down-regulated (DR) mRNAs.

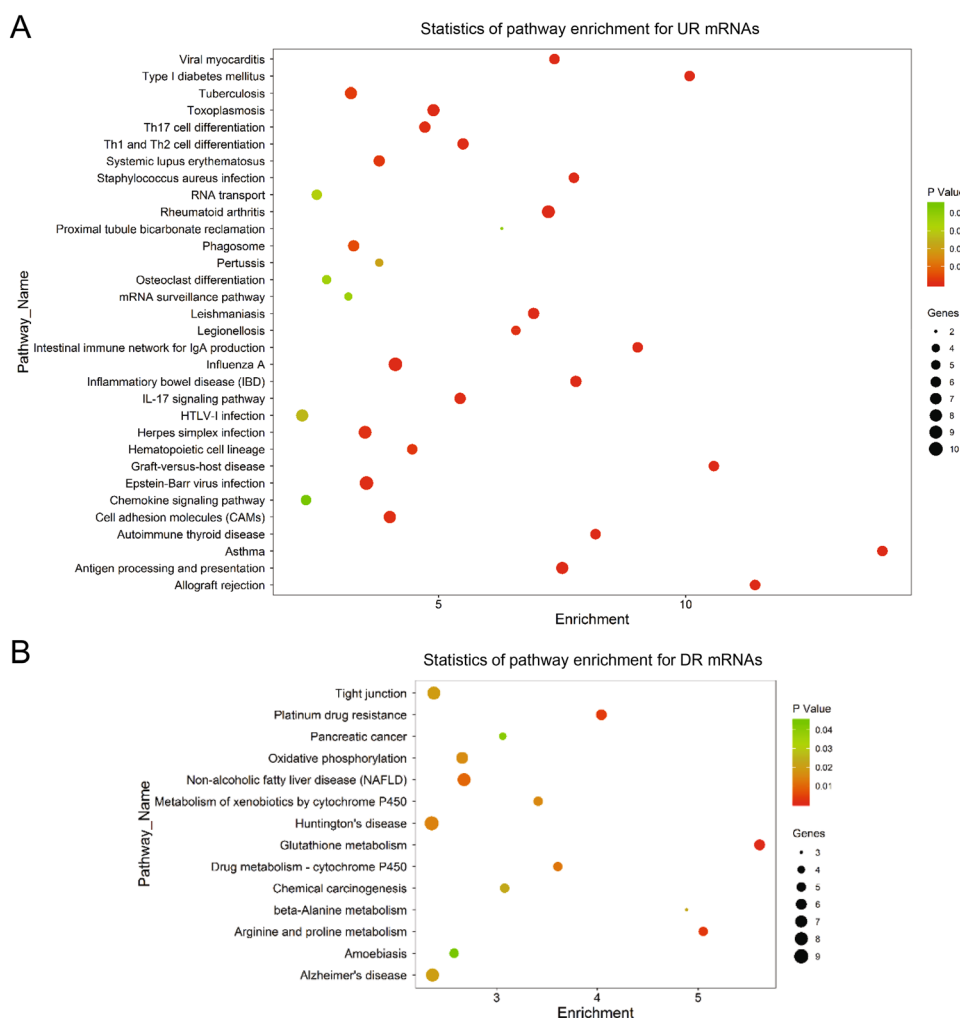
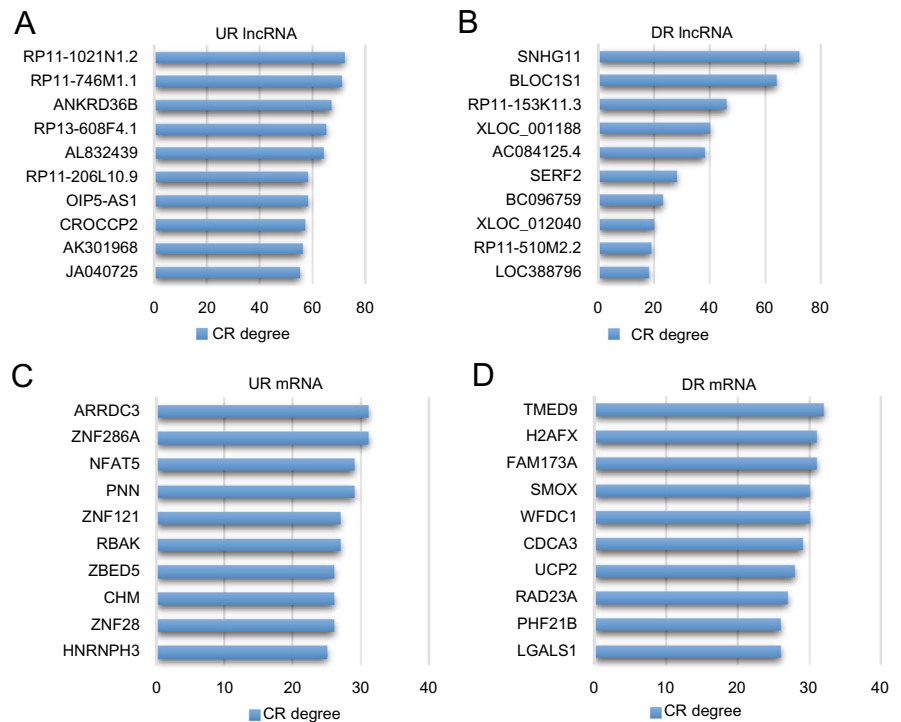


Fig. 3 The top correlated lncRNA-mRNA pairs. Correlation levels of 203 lncRNAs and 401 mRNAs were evaluated by correlation degrees (CR degrees) which is equal to counts of significantly correlated lncRNA-mRNA pairs ($P < 6 \times 10^{-6}$) involving the corresponding lncRNA or mRNA. **A** Up-regulated (UR) and **B** down-regulated (DR) lncRNAs with top 10 CR degrees. **C** UR and **D** DR mRNAs with top 10 CR degrees.



mRNA was correlated with only 7.89 lncRNAs. Among the up-regulated lncRNAs, RP11-1021N1.2 was the one with the highest mRNA correlation degree, and for the down-regulated lncRNAs, SNHG11 was the top one regarding the mRNA Correlation degree. As for the mRNA, TMED9 was correlated with the most lncRNAs. Based on the correlation profile, we built a correlation network of lncRNA-mRNA expression (Supplementary Figure S1).

Verification of the Correlation between lncRNAs and NFAT5 mRNA

To verify the reliability of the correlation network, we selected the mRNA of NFAT5, a protein significantly inhibiting CVB3 infection (Qiu *et al.* 2016), as the central mRNA to build a sub-network of lncRNA-mRNA correlation. In this sub-network, 27 lncRNAs positively correlated to NFAT5 expression were up-regulated in CVB3 infected cells while 3 lncRNAs negatively correlated to NFAT5 expression were down-regulated (Fig. 4A), according with the up-regulation of NFAT5 mRNA shown in our mRNA expression profile.

Among the lncRNAs correlated with NFAT5, XLOC_001188 was a most down-regulated one and was preliminarily verified by qPCR (Fig. 1C). Therefore, we selected XLOC_001188 and NFAT5 as a representative lncRNA-mRNA pair to do further verification. HeLa cells were infected with CVB3 of 0.5 MOI. At 6, 8, 10, 12 and 24 h post infection (hpi), the cells, together with the

uninfected control, were subjected to RNA extraction and qPCR quantification for the levels of XLOC_001188, NFAT5 mRNA and CVB3 genomic RNA. As shown in Fig. 4B, the genomic RNA of CVB3 was observed at 8 hpi and increased at the later time points, while the decrease of XLOC_001188 started at the same time point (Fig. 4C), coinciding with the increase of NFAT5 mRNA (Fig. 4D). Nevertheless, the NFAT5 mRNA was decreased at 24 hpi, probably due to the severe cell death at the late point of viral infection. These results indicate a negative correlation between XLOC_001188 and NFAT5 mRNA in a time-dependent pattern during CVB3 infection, which is consistent with the prediction. Similar results were also observed on NALCN-AS1 and NALCN mRNA (data not shown), together supporting the reliability of the lncRNA-mRNA correlation network.

To further elucidate the relationship between XLOC_001188 and NFAT5 and its role in CVB3 infection, we extracted the sequence of XLOC-001188 (Fig. 5A) from a database of human large intergenic noncoding RNAs (Cabili *et al.* 2011). Based on the sequence, a specific siRNA was designed to knock down XLOC-001188 expression in CVB3-infected HeLa cells. Then, we detected the levels of NFAT5 mRNA and CVB3 genomic RNA. Upon siRNA challenge, we observed a dramatic decrease of XLOC-001188 (Fig. 5B), sustaining the efficiency of the siRNA we used. When XLOC-001188 was knocked down, the level of NFAT5 mRNA (Fig. 5C) was significantly increased and CVB3 genomic RNA was

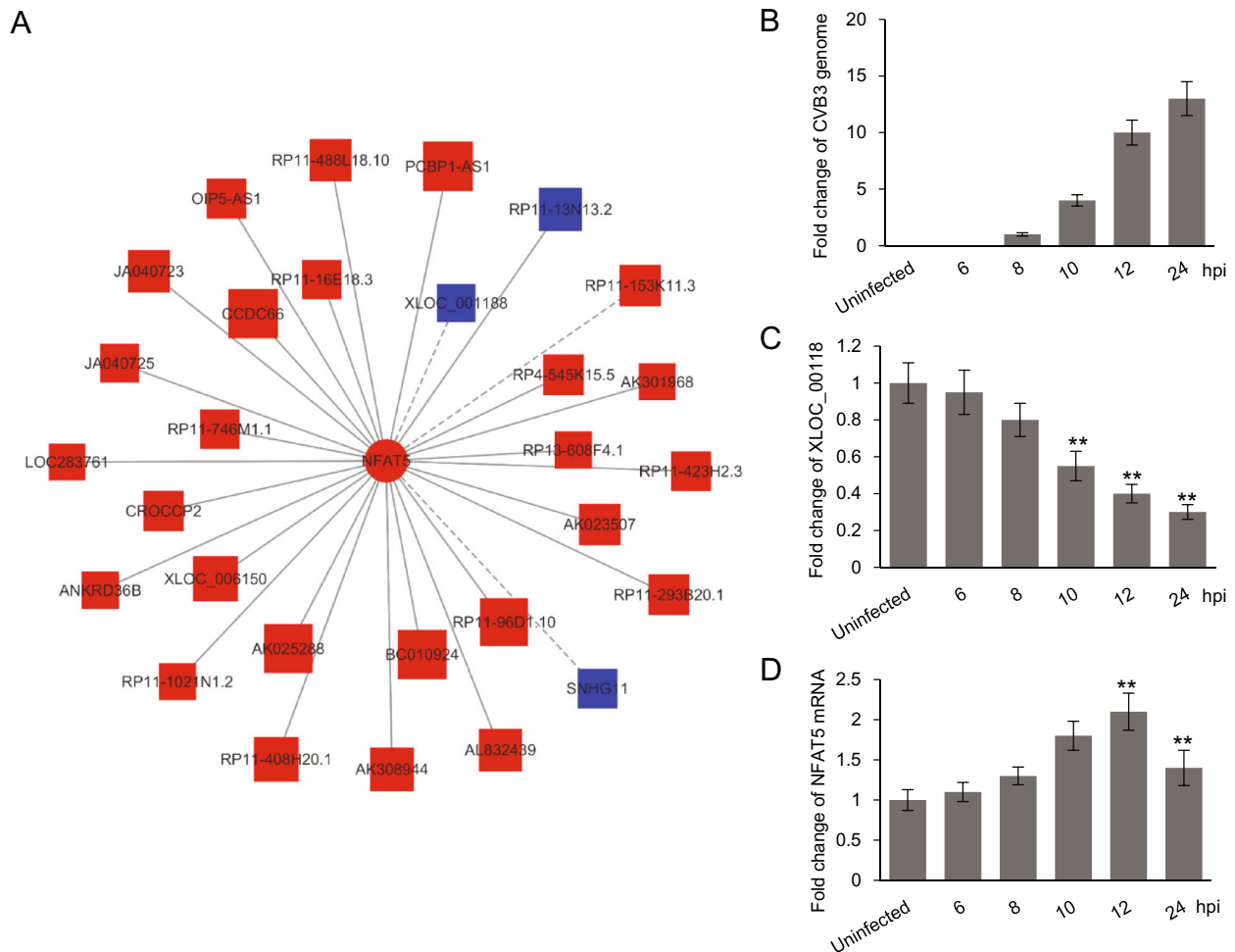


Fig. 4 The correlation between lncRNA XLOC_001188 and NFAT5 mRNA. **A** The lncRNAs correlated to NFAT5 mRNA. The red color indicates up-regulation while the blue color indicates down-regulation. The solid lines mean positive correlation and the dashed line mean negative correlation. **B–D** HeLa cells were infected with CVB3 at 0.5 MOI. At indicated time points, the RNA was extracted from the cells and subjected to qPCR analysis for the levels of CVB3 genomic

RNA (**B**), XLOC_001188 (**C**) and NFAT5 mRNA (**D**). Every time point was repeated for three times. The results were normalized to the corresponding mRNA levels of GAPDH, presented as the fold change to the 8 hpi sample (CVB3 genome) or uninfected group (XLOC_001188 and NFAT5 mRNA), and then subjected to student *t* test (***P* < 0.01).

reduced (Fig. 5D), supporting our hypothesis that XLOC_001188 is negatively correlated with NFAT5 and NFAT5-mediated antiviral property.

Pathway Analysis of the Differentially Expressed lncRNAs

Based on the profiles of lncRNA-mRNA correlation and KEGG pathways of the differentially expressed mRNAs, we predicted the potential pathways related to the differentially expressed lncRNAs (Supplementary Table S5). Similar to the correlation network above, the correlation degrees of lncRNAs and pathways were calculated by the number of correlated counterparts linked (Supplementary Table S6). According to the correlation degree, the most significantly up-regulated pathways included those related

to mRNA processing, viral infection and immune response (Fig. 6A), while the top-ranked down-regulated pathways included those related to protein turnover and pro-survival signals (Fig. 6B). RP11-1023L17.1 and RP11-145F16.2 were the up-regulated lncRNAs with the highest pathway correlation degree (Fig. 7A). Interestingly, SNHG11, the lncRNA with the top mRNA correlation degree, also had the highest pathway correlation degree among all down-regulated lncRNAs, indicating a central role in the regulatory network (Fig. 7B). To investigate the molecular correlations within the pathways in detail, we selected RP11-145F16.2, RP11-1023L17.1 and SNHG11, as well as RP11-1021N1.2, the lncRNA with the top mRNA degree, as the core lncRNAs to establish a sub-network of correlation (Fig. 7C). According to the sub-network, we found that SNHG11 and RP11-1021N1.2 showed a perfect

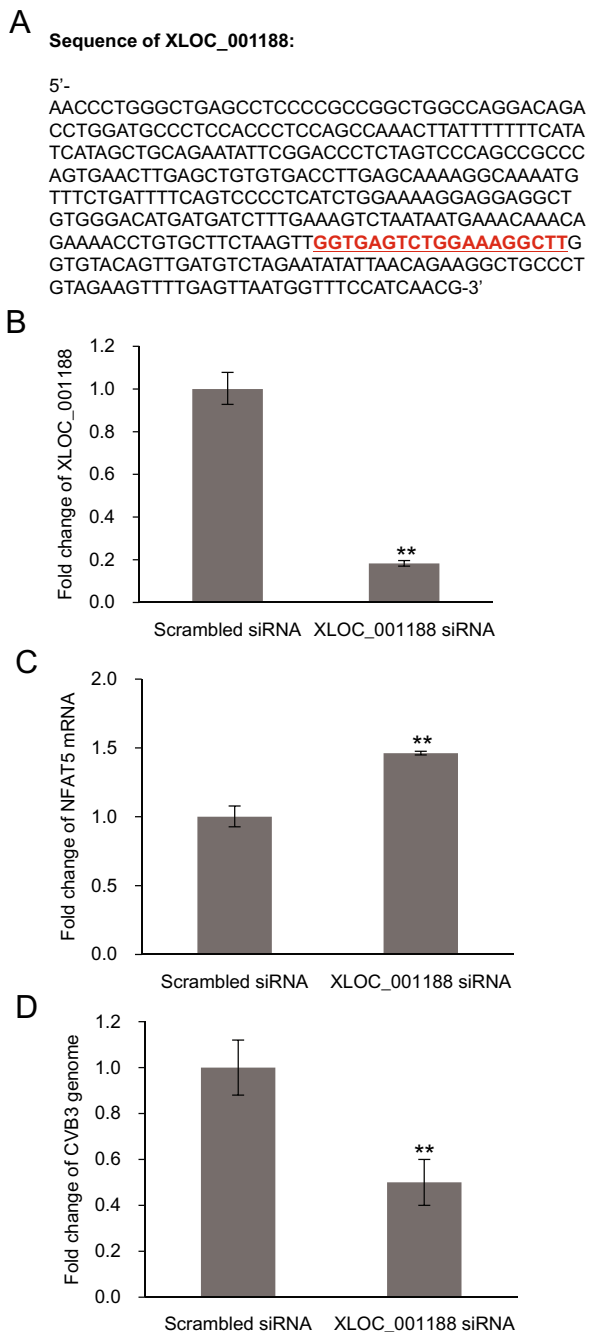


Fig. 5 The negative correlation between lncRNA XLOC_001188 and NFAT5 mRNA and its influence on CVB3 replication. **A** Partial sequence of lncRNA XLOC_001188. The red underlined sequence is the target sequence of the XLOC_001188 siRNA. HeLa cells infected by CVB3 at 0.5 MOI for 10 h. HeLa cells were transfected with XLOC_001188 siRNA or scrambled control siRNAs, and then were subjected to CVB3 infection at 0.5 MOI for 10 h. The RNA levels of XLOC_001188 (**B**), NFAT5 mRNA (**C**), and CVB3 genome (**D**) were detected by qPCR. All RNA levels were normalized to the level of GAPDH mRNA. Every time point had three duplicates and the results are displayed as mean \pm SD (** $P < 0.01$).

overlap on their correlated mRNAs that these two top-degree lncRNAs are correlated with the same mRNAs but in opposite directions, indicating a strong correlation between these two lncRNAs in the regulatory network. Also, RP11-1023L17.1 was partially embedded in the SNHG11-RP11-1021N1.2 network by sharing several common correlated mRNAs such as ARRDC3, BRE, CCRN4L, CDCA3, hnRNPH3 and ZNF295. RP11-145F16.2 was comparatively isolated but was still linked to the above network by sharing correlated mRNAs with RP11-1023L17.1, including HLA-DQA1, NEMF, IRF2BP1, FIBP, TIMP1 and MTX1. These results indicated potential synergistic regulation or mutual regulation of these lncRNAs on gene expression.

Discussion

lncRNAs are an important subgroup of non-coding RNAs and actively transcribed in large numbers in human genome (Carninci *et al.* 2005; Birney *et al.* 2007). Attributing to the rapid development of RNA-seq in the past decade, more than 27,000 lncRNAs have been identified in human and at least 27% of transcription across the human genome is believed to produce lncRNAs (Kapranov *et al.* 2007; Hon *et al.* 2017). lncRNAs are widely involved in various physiological and pathological processes, including viral infection. The sophisticated interplay between lncRNAs and viruses is likely to provide novel biomarkers or drug targets for diagnosis or treatment of viral infectious diseases. However, the role of lncRNAs in picornavirus infection has barely been reported. Therefore, in this study, we used CVB3, a typical picornavirus, as a model to provide a first glance of lncRNA involvement in picornavirus infection.

lncRNA-mRNA integrated microarrays screened 700 differentially expressed lncRNAs in CVB3-infected human cells compared to uninfected controls, most of which have not been described in current lncRNA database (Amaral *et al.* 2011; Quek *et al.* 2015). Unlike microRNAs, the mode of action of lncRNA has not been systematically demonstrated. Previous reports indicated that lncRNAs function through various mechanisms such as chromatin remodeling (Li *et al.* 2013; Orom and Shiekhattar 2013; Lam *et al.* 2014; Ounzain and Pedrazzini 2015), microRNA sponges (Ebert *et al.* 2007), ceRNAs (Karreth *et al.* 2011; Tay *et al.* 2011; Kallen *et al.* 2013) and antisense transcripts (Katayama *et al.* 2005). In fact, usually the functional mechanism of lncRNAs in a certain scenario could be quite different from others. Thus, it is hard to establish a bioinformatic program, which is widely used in microRNA study to predict the targets and function of lncRNAs. To circumvent this dilemma, we established a

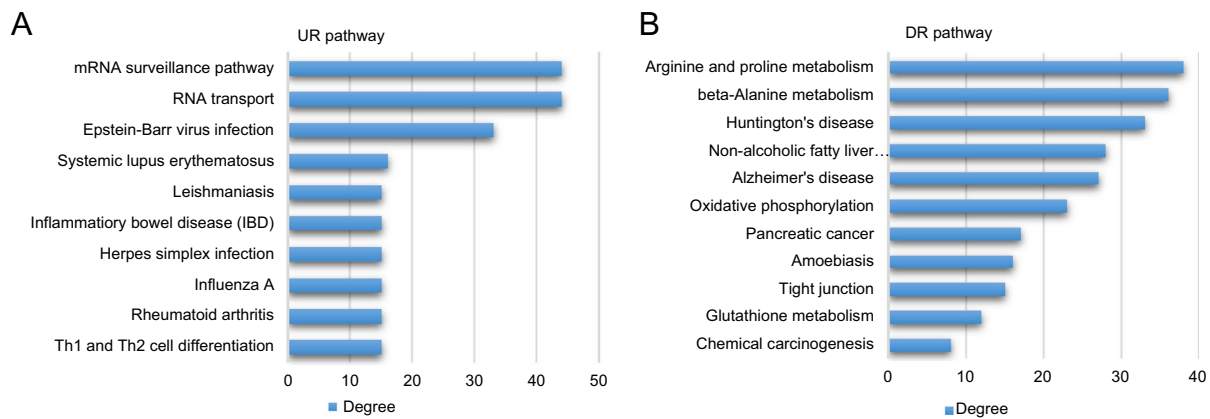


Fig. 6 The pathways correlated to differentially expressed lncRNAs. The correlation between a pathway and a lncRNA was established based on their common mRNAs. The correlation levels of pathways to lncRNAs were evaluated by correlation degrees (CR degrees)

correlation network to pair differentially expressed lncRNAs and differentially expressed mRNAs in our study based on their expression profiles. In this way, we could indirectly predict the function of lncRNAs according to the mRNA-function analysis.

Our functional study revealed a high consistency of pathways involving differentially expressed lncRNAs and differentially expressed mRNAs. Both up-regulated lncRNAs and mRNAs are mainly involved in cellular pathways related to nucleic acid processing, immune response and inflammation, while down-regulated lncRNAs and mRNAs are mainly involved in pathways related to protein degradation and pro-survival signals. This consistency implies potential synergetic action of lncRNA and mRNA, which may be direct interaction, mutual regulation or signaling transduction. The overlap of overall functional profile well supports our strategy which predicts lncRNA function based on the lncRNA-mRNA correlation. In addition, the pathway alterations revealed by differentially expressed lncRNAs and mRNAs also match our current knowledge about CVB3 infection. For instance, CVB3 infection up-regulates RNA protective proteins to stabilize its own genomic RNA (Qiu *et al.* 2016). CVB3-induced up-regulation of pro-inflammatory factors is one of the major causes of inflammatory diseases (Fung *et al.* 2016). Also, the protein degradation via autophagy is significantly inhibited during CVB3 infection (Wong *et al.* 2008), which leads to protein overload and activates unfolded protein response (Zhang *et al.* 2010). As a cytolytic virus, it is widely accepted that CVB3 induces apoptosis to favor its viral progeny release (Yuan *et al.* 2003; Zhang *et al.* 2010). These facts sustain the reliability of our functional analysis.

According to the correlation network and pathway analysis of lncRNAs and mRNAs, lncRNAs generally have

higher correlation degrees than mRNAs, indicating that lncRNA may have larger interacting range and play more regulatory roles. Considering the much lower abundance of lncRNA than that of mRNAs in cells (Ravasi *et al.* 2006; Cabili *et al.* 2011) and the high specificity of lncRNAs in different tissues (Cabili *et al.* 2011) or developmental stages (Yan *et al.* 2013), we reasonably speculate that lncRNAs play regulatory roles on multiple signaling pathways rather than execute housekeeping functions like most proteins coded by mRNAs. Therefore, lncRNAs are probably biomarkers or drug targets with more specificity and effectivity than proteins.

One lncRNA-mRNA correlation verified in our study is the pair of XLOC_001188 and NFAT5 mRNA. We have showed in a previous study that NFAT5 plays critical roles in inhibiting CVB3 replication (Qiu *et al.* 2017). In that study, we observed the increase of NFAT5 mRNA during CVB3 infection but could hardly explain the phenomenon. Here, according to the lncRNA-mRNA correlation network, we have found and preliminarily verified that XLOC_001188, a lncRNA down-regulated in CVB3 infection, is negatively correlated with NFAT5 mRNA, which could potentially explain the mechanism underlying the increase of NFAT5 mRNA in CVB3 infection. Considering the anti-viral activity of NFAT5, the XLOC_001188-NFAT5 co-regulation might be a host cell defense against CVB3 infection, which is an interesting direction for the future study of lncRNAs' roles in CVB3 infection.

In the correlation and pathway analysis of lncRNAs, we found four lncRNAs with the highest Correlation degrees for mRNAs or pathways, including SNHG11, RP11-1023L17.1, RP11-145F16.2 and RP11-1021N1.2, none of which were previously reported in viral infection. SNHG11 holds the highest level for both correlation degrees for

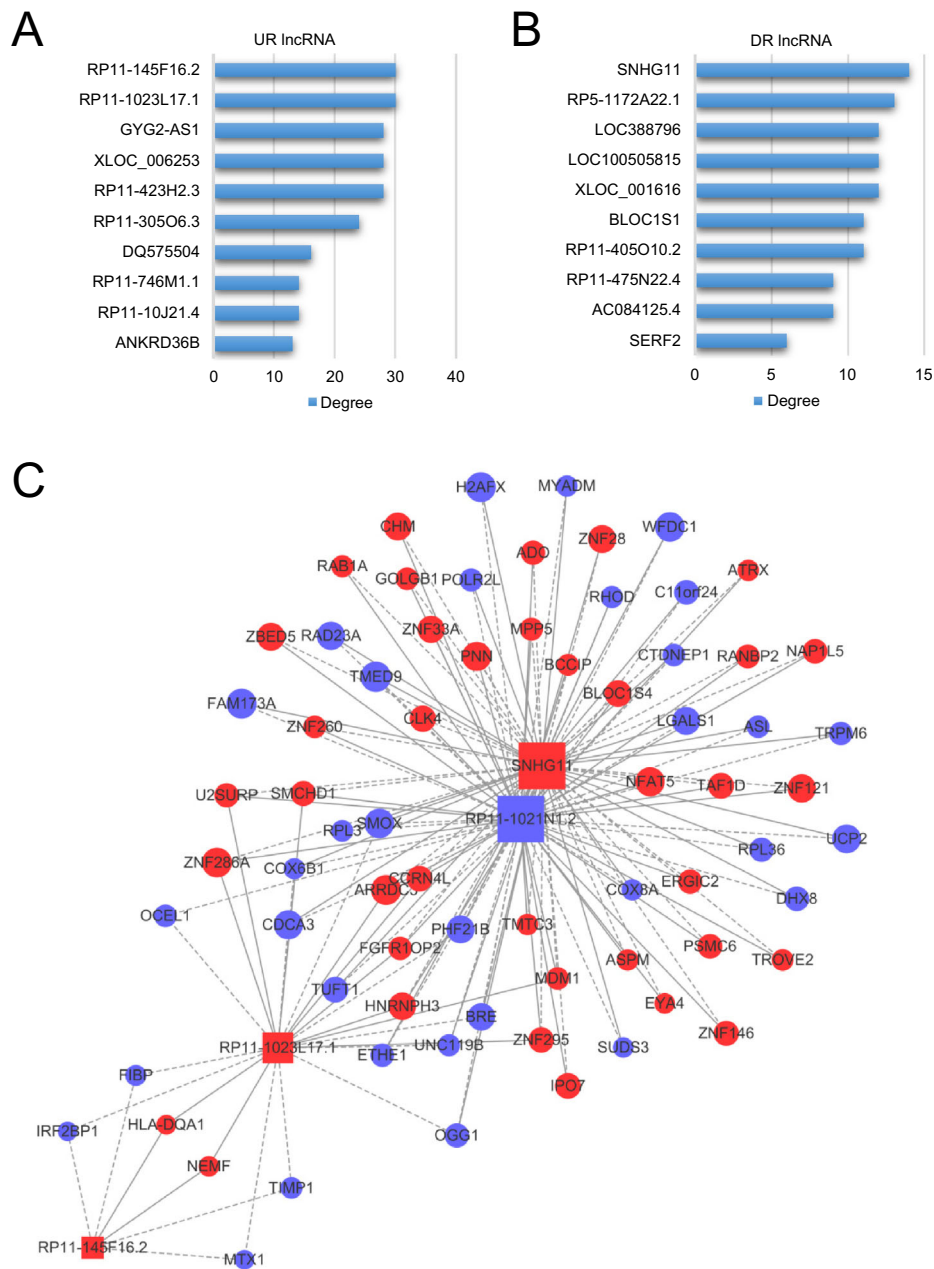


Fig. 7 Pathway analysis of differentially expressed lncRNAs in CVB3 infection. The significance of lncRNAs regarding pathways were evaluated with correlation degrees (CR degrees) which is equal to counts of pathways correlated. **A** Up-regulated (UR) lncRNAs with

top 10 CR degrees. **B** Down-regulated (DR) lncRNAs with top 10 CR degrees. **C** The lncRNA-mRNA correlation network derived from four core lncRNAs, RP11-145F16.2, RP11-1023L17.1, SNHG11 and RP11-1021N1.2.

mRNAs and pathways among all down-regulated lncRNAs, implying critical position in the regulatory network. SNHG11 is a small nucleolar RNA (snoRNA) positively regulating cell proliferation. It is true that cell proliferation is slowed down during CVB3 infection (Luo *et al.* 2003), but the direct interaction between CVB3 and SNHG11 is quite unlikely, since the intracellular life cycle of CVB3 is exclusively located in the cytoplasm and viral components can barely enter the nucleus (Garmaroudi *et al.*

2015). Thus, CVB3 probably interplays with SNHG11 via an intermediate protein.

Interestingly, SNHG11 and RP11-1021N1.2 were correlated to the same group of mRNAs exactly, but each mRNA was oppositely correlated to the two lncRNAs. Since SNHG11 was down-regulated in CVB3 infection while RP11-1021N1.2 was up-regulated, thus the alterations of these two lncRNAs caused the same change of mRNAs during CVB3 infection. Considering the

extraordinary coincidence of SNHG11 and RP11-1021N1.2, it is reasonable to speculate that one lncRNA was probably upstream to the other one in a regulatory pathway. This result indicates a potential mutual regulation between the two lncRNAs which was barely reported in previous studies, though more evidence is needed to support this speculation.

RP11-145F16.2, RP11-1023L17.1 and RP11-1021N1.2 were significantly up-regulated during CVB3 infection, but their functions remain obscure to date. Our correlation network has revealed several interesting genes correlated to these lncRNAs. For instance, BRE mRNA was negatively correlated to RP11-1023L17.1 and RP11-1021N1.2, indicating an up-regulation of BRE in CVB3 infection. BRE protein was reported to promote the degradation of p53, a classical tumor-suppressor protein (Jin *et al.* 2017). Interestingly, CVB3 infection indeed induces degradation of p53 which benefits CVB3 replication (Gao *et al.* 2010). These findings imply that RP11-1023L17.1 and RP11-1021N1.2 may be involved in the CVB3-p53 mutual regulation. In addition, IRF2BP1 was negatively correlated to RP11-145F16.2 and RP11-1023L17.1, indicating a down-regulation of IRF2BP1 in CVB3 infection. IRF2BP1 protein is a transcriptional corepressor binding to the C-terminal repression domain of IRF2, a negative regulator of many interferon (IFN)-responsive genes (Childs and Goodbourn 2003). IFN response is a critical antiviral pathway. Thus, down-regulation of IRF2BP1 may be a cellular protective response against CVB3 infection in which RP11-1023L17.1 and RP11-1021N1.2 are involved.

In conclusion, the strategy utilizing lncRNA-mRNA correlation is reliable to predict lncRNA function in CVB3 infection, providing a useful tool for the lncRNA studies. By this approach, we have revealed a negative correlation between the lncRNA XLOC_001188 and the antiviral gene NFAT5. In addition, we have also identified four critical lncRNAs, SNHG11, RP11-145F16.2, RP11-1023L17.1 and RP11-1021N1.2, which potentially affect CVB3 replication. Although the results are quite preliminary and more verification is required, this is the first study indicating the involvement of lncRNAs in CVB3 infection. These findings bring a new perspective for enterovirus studies, which may provide potential drug targets for therapy development against CVB3 infection.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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