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Induction of the cellular microRNA-29c by influenza virus contributes to virus-mediated apoptosis through repression of antiapoptotic factors BCL2L2

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ABSTRACT

Influenza A virus is a cytolytic virus that induces apoptosis in numerous cell types, which contributes to cellular and organ dysfunction. MicroRNAs (miRNAs) represent a family of small noncoding RNAs controlling tanslation and transcription of many genes. Recent studies have revealed that miR-29c is involved in a variety of biological processes, including apoptosis. However, its role in influenza A virus infection is not well understood. Here, we report that miR-29c is involved in apoptosis induced by influenza A virus infection. We found that several apoptosis-associated miRNAs were stimulated in influenza A virus-infected A549 cells by miRNA array analysis. Within those, miR-29c was significantly up-regulated. In silico target prediction analysis revealed complementarity of miR-29c to the 3′-untranslated region (UTR) of BCL2L2 mRNA. Targeting of BCL2L2 3′ UTR by miR-29c was determined by luciferase assay. Functional overexpression of miR-29c with miR-29c precursor inhibited BCL2L2 protein expression. Transfection of miR-29c inhibitor abolished both suppression of BCL2L2 protein expression and A549 cells apoptosis induced by influenza A virus. Moreover, BCL2L2 overexpression rescued A549 cell death induced by influenza A virus infection. These findings indicate that miR-29c-mediated BCL2L2 suppression is involved in influenza virus-induced cell death in A549 cells.

1. Introduction

Influenza viruses are enveloped negative-stranded RNA viruses that cause approximately 500,000 deaths worldwide per year, and many animal species [1]. In recent history, highly pathogenic strains of influenza A virus with elevated case fatality rate have suddenly emerged [2]. Influenza A virus replicates throughout the respiratory tract, where the viral antigen is found predominantly in the epithelial cells. The clinical responses range from mild disease to fatal viral pneumonia.

One of the host cell responses to influenza infection involves the initiation of apoptosis. Influenza A virus is a cytolytic virus that induces apoptosis in numerous cell types [3]. The previous studies have shown that influenza virus induces apoptosis in bronchial and alveolar epidermal cells, the thymus, and spleen in a murine model [4]. Induction of apoptosis has been reported not only in adherent cells, but also in lymphocytes and monocytes in vivo [5]. The virus induces apoptosis in infected cells as part of the mechanisms contributing to cellular and organ dysfunction [6]. The reason why influenza virus induces apoptosis has been hotly debated [7]. It was thought previously that apoptosis is primarily a host defense mechanism limiting virus replication and that influenza virus overcomes this mechanism by rapid multiplication before apoptosis occurs [8]. However, there is now evidence to show that the induction of apoptosis is essential for influenza virus mRNA synthesis and virus propagation [9–11], as well as plays an important role in mediating inflammation and the subsequent tissue damage [6].

Apoptosis induction is multifactorial and highly regulated. MicroRNAs (miRNAs) as a new class of 18–23 nucleotide long non-coding RNA, play critical roles in a wide spectrum of biological processes. Several studies have indicated a regulatory role of miRNAs in apoptotic signaling by inducing increased mRNA degradation and translational inhibition of their cellular targets. For example, expression of miR-15a and miR-16-1 is inversely correlated to expression of anti-apoptotic factor BCL-2 [12]; The antiapoptotic effects of miR-155 could enhance macrophage resistance to apoptosis by binding to 3′ UTRs of Tspan14, Lpin1, and Pmaip1 [13]; miR-34c is involved in the regulation of germ cell apoptosis by targeting the ATF1 gene [14]; Several miR-29 targets are oncogenes or anti-apoptotic genes, including Tcl-1, p85- alpha, CDC42 and Mcl-1 [15–17].

Recent reports also throw light into the role of miRNAs as critical effectors in the intricate host-pathogen interaction networks.

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Cellular miRNA expression is profoundly influenced by viral infection, which can be attributed to both host antiviral defenses and viral factors altering the cellular environment, a likely common scenario for RNA viruses. For example, human T-cell lymphotropic virus type 1 (HTLV-1) modulates the expression of a number of cellular microRNAs in order to control T-cell differentiation [18]; human cytomegalovirus (HCMV) selectively manipulates the expression of miR-100 and miR-101 to facilitate its own replication [19]; EBV LMP1 induces miR-29b, which results in miR-29b mediated downregulation of T-cell leukemia gene 1 (TCL1), a protein with roles in cell survival and proliferation [20]; Influenza virus infection also induces expression signatures of cellular miRNAs and gene ontology analysis reveals that many of these target mRNAs play roles in immune response and cell death pathways, which suggests that virulence of influenza viruses may be mediated in part by cellular miRNA through dysregulation of genes [21].

Given the above mentioned information, this study was aimed to determine the as yet unraveled effect of miR-29c on influenza A virus-induced apoptosis in A549. We herein report that BCL2L2 expression is inversely correlated with miR-29c levels in influenza A virus-infected A549 cells. This repression of BCL2L2 at the protein level involves a release of miRNA-mediated translation repression by miR-29c. Furthermore, BCL2L2 expression repression confers apoptosis to A549 cells induced by influenza A virus. Thus, the miR-29c-mediated regulatory mechanism of BCL2L2 expression has been identified in influenza A virus-infected A549 cells, as part of the mechanisms contributing to cell death.

2. Materials and methods

2.1. Viruses and cell culture

Influenza viruses, A/Lufang/09/1993(H3N2) and A/Jingfang/01/1986(H1N1) were grown in 11-day-old embryonated eggs as previously described [22]. A549, a human pulmonary epithelial cell line (ATCC CCL-185) was cultured in RPMI 1640 (Invitrogen) medium with 2 mM glutamine, 10 U/ml penicillin, 10 μg/ml streptomycin, and 10% fetal bovine serum at 37 °C in a CO_2 incubator. Monolayers of A549 cells were inoculated at a 50% tissue culture infective dose ($TCID_{50}$) of 100/cell. After 1 h inoculation, cells were washed twice with phosphate buffer saline (PBS) and supplemented with maintenance media.

2.2. Microarray and data analysis

Total RNA was extracted from A549 cells infected with Influenza viruses, A/Jingfang/01/1986(H1N1) at 4 h and 24 h post-infection using TRIZOL reagent (Invitroge) and sent to LC Sciences for miRNA microarray analysis. Data were analyzed using ANOVA and *t* tests. Normalization of expression was performed using a cyclic LOWESS method [23]. Two databases, TargetScan (http://www.targetscan.org) and PicTar (http://pictar.mdc-berlin.de/), were used for miRNA target prediction.

2.3. Real-time PCR

For analysis of miR-29c, total RNA was isolated from cells with the mirVana miRNA Isolation kit (Ambion). Comparative real-time PCR was performed by using the TaqMan Universal PCR Master Mix (Applied Biosystems). Specific primers and probes for mature miR-29c and snRNA RNU6B were obtained from Ambion. All reactions were run in triplicate. The amount of miR-29c was obtained by normalizing to snRNA RNU6B and relative to control (untreated cell).

2.4. Anti-miR and precursor to miR-29c

To manipulate cellular function of miR-29c, we utilized an antisense approach to inhibit miR-29c function and transfection of cells with miR-29c precursor to increase miR-29c expression. For experiments, A549 cells were grown to 70% confluent and treated with anti-miR-29c or miR-29c precursor (Ambion) using the lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions.

2.5. Luciferase reporter assay

To create a luciferase reporter construct, 3′-untranslated region (UTR) of BCL2L2 that contained the putative binding site for miR-29c or mutant sequence was respectively cloned into pGL3-control (Promega). pRL-CMV plasmid (Promega) expressing Renilla luciferase was co-transfected to determine the transfection efficiency. A549 cells (5×10^3) were plated in a 96-well plate and then transfected with each reporter construct, as well as anti-miR-29c or miR-29c precursor, followed by assessment of luciferase activity 24 h after treatment. Luciferase activity was then measured using the Dual-Luciferase Reporter Assay (Promega). Each transfection was performed in triplicate.

2.6. Western blot

Whole cell lysates were obtained with RIPA buffer (150 mM NaCl, 10 mM Tris, pH 7.2, 0.1% SDS, 1.0% Triton X-100, 5 mM EDTA, pH 8.0) containing 1 × protease inhibitor cocktail (Roche). Cell lysates were then loaded at each line (a total of 40 μg lysate proteins) in 10–12% SDS–PAGE gel to separate proteins and transferred to polyvinylidene fluoride membranes (Millipore). Antibodies to BCL2L2 (Cell Signaling) and actin (Sigma–Aldrich) were used. Densitometric levels of ICAM-1 signals were quantified and expressed as their ratio to actin.

2.7. Apoptosis assay

After treatment, cells were resuspended in binding buffer containing annexin V-FITC and propidium iodide (PI) according to manufacturer's instructions (KeyGEN) and assessed by flow cytometry. Results were expressed as percentage of the vehicle control levels set at 100%. Each sample was run in triplicate.

2.8. Cell viability assay

Cell viability was analyzed by Cell Counting Kit-8 (Dojindo). Briefly, the A549 cells were seeded into the 96-well plate. After treatment, 10 μl of Cell Counting Kit-8 solution was added into each well. The OD450 values were measured with the Spectra Microplate Reader (BIOTEC). Results were expressed as percentage of the vehicle control levels set at 100%. Each treament was performed in triplicate.

2.9. Statistical analysis

Data were expressed as mean ± standard deviation (SD) from at least three separate experiments. Significance of differences between groups was determined by Student's *t* test. Values of $P < 0.05$ were taken as statistically significant.

3. Results

3.1. miR-29c is significantly up-regulated in influenza virus-infected A549 cells

In a systematic approach to identify miRNAs involved in the pathogenesis of influenza viruses infection, we detected host miRNAs expression profiling of influenza virus Infection by using A549 cell line as host. We compared miRNA expression profiles in A549 infected with influenza A virus (H1N1) for 4 and 24 h to control by performing microarray analysis. miRNAs were considered as differentially expressed when differences in expression levels were significant both in Student's *t* test ($P < 0.01$) and analysis of microarray test (*q* value < 5%). Among the individual miRNAs represented on the microarray, 9 apoptosis-associated miRNAs reported from previous studies were significantly overexpressed on induction of influenza virus infection. miR-29 family members showed a striking relationship to influenza virus infection, including miR-29b and miR-29c.(Fig. 1A and B). The most up-regulated miRNA was miR-29c. Then the up-regulation of miR-29c was confirmed in A549 cell infected with H1N1 and H3N2 influenza A virus by qPCR (Fig. 1C). Collectively, these data demonstrate that miR-29 family members are up-regulated during influenza virus infection, especially miR-29c.

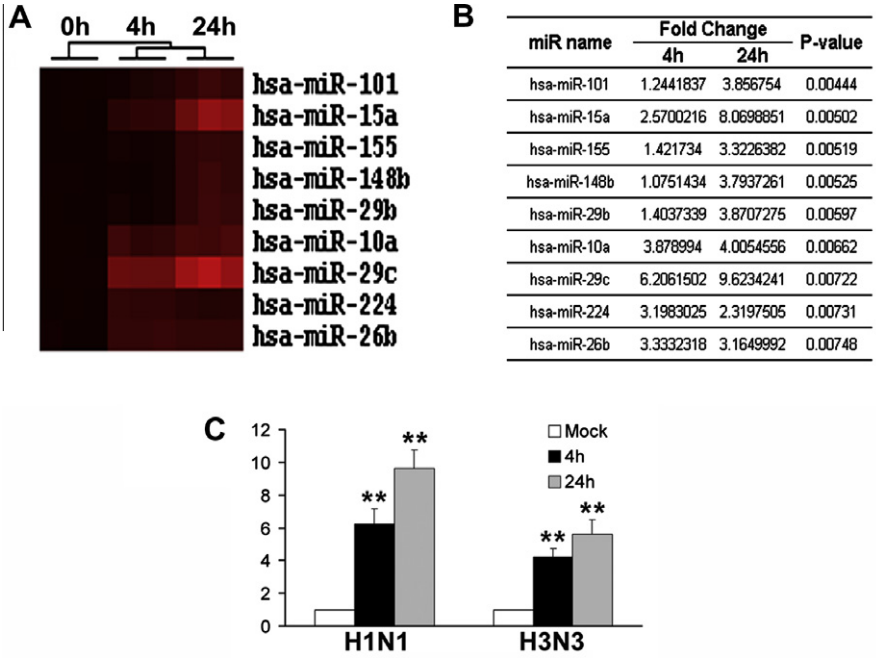
3.2. miR-29c targets and inhibits BCL2L2 protein expression

Base-pairing between 3′ UTR of mRNA and the "seed sequence" located in the 5′ end of miRNA are essential to determining whether targeting miRNA results in degradation of mRNA or inhibition of translation [24]. To identify the target genes of miR-29c in regulating cell death, we searched for candidate genes using Targetscan 5.1 (http://www.targetscan.org) and PicTar (http://pictar.mdc-berlin.de) microRNA database. BCL2L2 is an important antiapoptotic gene and, as a target gene of miR-29c, can be predicted (Fig. 2A). Then, we constructed a firefly luciferase reporter containing the potential binding site for miR-29c in BCL2L2 3′ UTR or the mutant (Mut) sequence (GGTGCT to CCACGA) (Fig. 2A). We co-transfected A549 cells with the 3′-UTR construct and miR-29c precursor. The reporter assay showed that miR-29c was able to significantly suppress luciferase expression, and mutation in the miR-29c target sequence led to abrogation of the suppressive effect (Fig. 2B). Moreover, we observed that influenza virus infection decreased BCL2L2 protein levels in A549 cells (Fig. 2C). Meanwhile, we also found miR-29c overexpression suppressed BCL2L2 protein expression (Fig. 2D). These observations suggest that miR-29c targets BCL2L2 3′ UTR in A549 cells.

3.3. Influenza virus infection decreases BCL2L2 expression through upregulation of miR-29c

Because miR-29c targets BCL2L2 3′ UTR and induces translation suppression, we expect that influenza virus infection can induce a inhibiton of BCL2L2 translation repression through upregulation of miR-29c. To test this possibility, we transfected A549 cells with the pGL3-control luciferase construct containing the BCL2L2 3′ UTR with the binding sites for miR-29c. Sequential exposure of transfected cells to influenza virus for 24 h and the viral infection decreased BCL2L2 3′ UTR-associated luciferase activity compared with the mock infection. No significant change of luciferase activity was found in influenza virus-infected cells transfected with mutant and empty vector control (Fig. 3A).

To test whether the miR-29c-mediated BCL2L2 translation repression is involved in influenza virus-induced the suppression of BCL2L2 protein expression in A549 cells, we transfected A549 cells with various doses of miR-29c inhibitor for 24 h and then infected those cells with influenza virus for 24 h followed by western blot for BCL2L2 protein. As shown in Fig. 3B, miR-29c inhibitor significantly reversed influenza virus-induced the suppression of BCL2L2 protein expression in A549 cells. A control RNA sequence did not show any effects. Coupled with upregulation of celluar miR-29c in response to influenza virus infection, the above data



miR name	Fold Change 4h	Fold Change 24h	P-value
hsa-miR-101	1.2441837	3.856754	0.00444
hsa-miR-15a	2.5700216	8.0698851	0.00502
hsa-miR-155	1.421734	3.3226382	0.00519
hsa-miR-148b	1.0751434	3.7937261	0.00525
hsa-miR-29b	1.4037339	3.8707275	0.00597
hsa-miR-10a	3.878994	4.0054556	0.00662
hsa-miR-29c	6.2061502	9.6234241	0.00722
hsa-miR-224	3.1983025	2.3197505	0.00731
hsa-miR-26b	3.3332318	3.1649992	0.00748

Fig. 1. Apoptosis-associated miRNA expression in influenza virus-infected A549 cells. (A) Microarray analysis for miRNA was performed with RNA extracts from influenza virus-infected A549 cells. Heat map represented the significantly upregulated apoptosis-associated miRNAs. (B) Listing of miRNAs in heat map with fold change and p values. Of note, miR-29c was the highest expressed miRNA in influenza virus-infected A549 cells compared to mock-infected cells. (C) miR-29c expression was validated by qPCR in A549 cells infected with H1N1 and H3N2 influenza A viruses (100TCID50). Bars are represented from three independent experiments. $^{**}P < 0.01$ vs. mock infection.

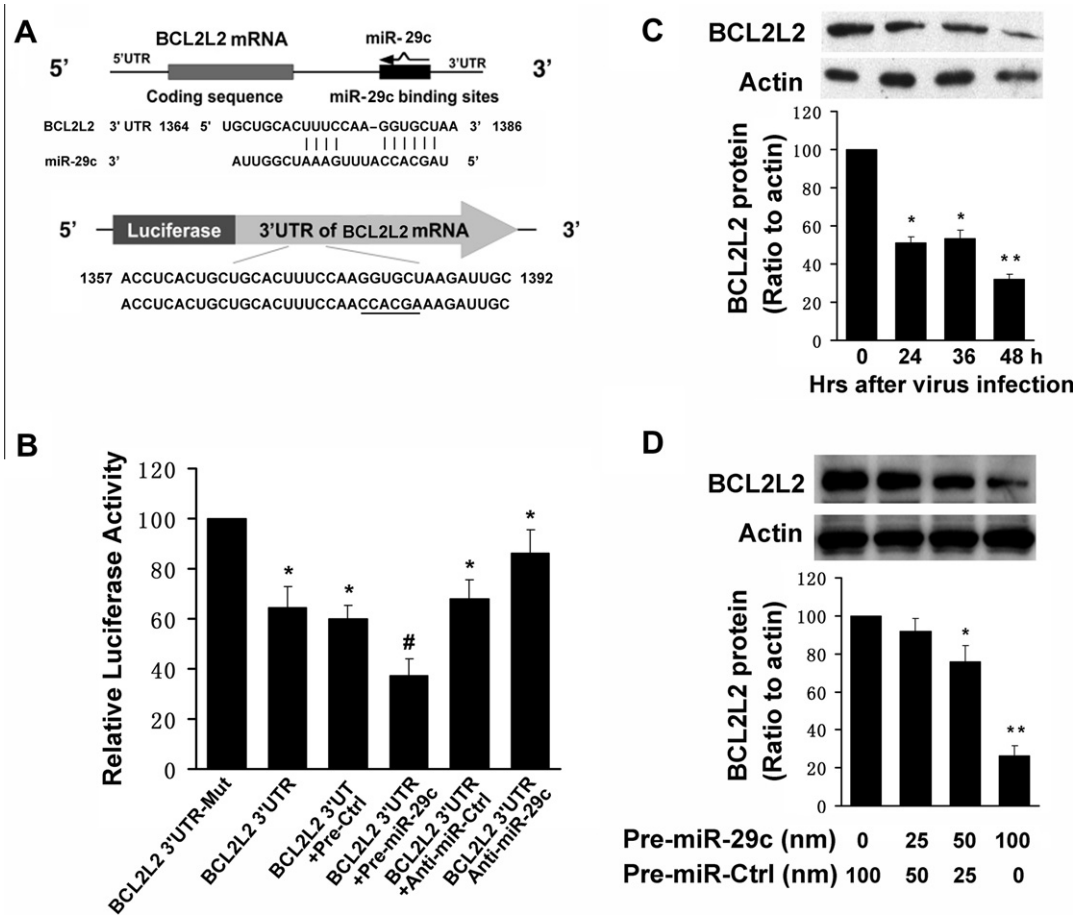


Fig. 2. miR-29c targets BCL2L2 3′ UTR and causes protein expression suppression. (A) human BCL2L2 mRNA shows a potential binding site in the 3′ UTR for miR-29c. (B) targeting of BCL2L2 3′ UTR by miR-29c resulted in transcriptional suppression. A549 cells were transiently cotransfected with the reporter construct, pRL-CMV Vector and the miR-29c precursor or anti-miR-221 for 24 h. Luciferase activities were measured and normalized to pRL-CMV (Ctrl) *Renilla* luciferases level. A nonspecific precursor (precursor-Ctrl) and anti-miR (anti-miR-Ctrl) were used as the controls. Bars represent the means ± SD from 3 independent experiments. $^{*}P < 0.05$ vs. 3′ UTR mutant; $^{\#}P < 0.05$ vs. BCL2L2 3′ UTR reporter construct. (C) Western blot analysis for endogenous BCL2L2 protein level after 100TCID50 influenza virus infection at different times post-infection. (D) miR-29c precursor inhibited BCL2L2 protein expression in A549 cells. Cells were transfected with precursor with various doses of miR-29c precursor for 24 h followed by Western blot for BCL2L2. Bars represent the means ± SD from 3 independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. mock-infected control or nonspecific precursor control-treated cells.

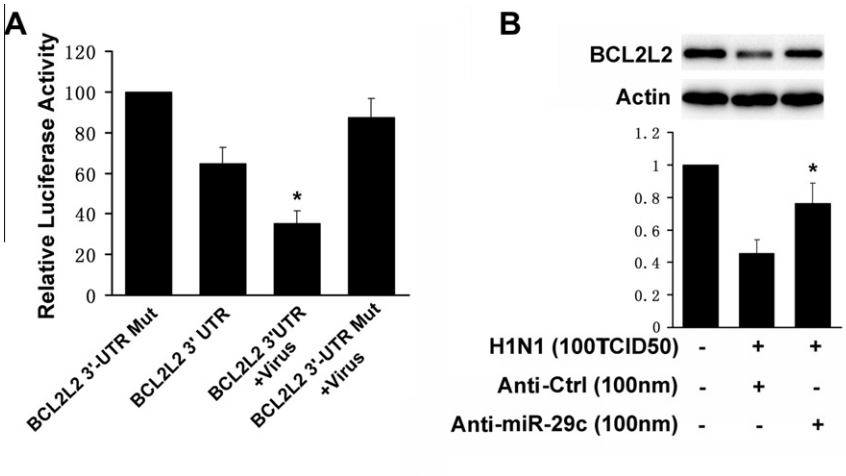


Fig. 3. Influenza virus infection induces BCL2L2 suppression and apoptosis through upregulation of miR-29c. (A) Influenza virus infection inhibited the luciferase reporter translational activity in A549 cells transfected with the construct with BCL2L2 3′ UTR encoding miR-29c binding site. Cells were transfected with the pGL3 luciferase construct containing the BCL2L2 3′ UTR with the putative miR-29c binding site and then infected with influenza virus for 24 h. Luciferase activity in cells after influenza virus infection was then measured and normalized to pRL-CMV (Ctrl) *Renilla* luciferases level. (B) miR-29c inhibitor blocked influenza virus-induced BCL2L2 protein suuppression. A549 cells were transfected with the miR-29c inhibitor or a control nonspecific inhibitor for 24 h and then infected with 100TCID50 influenza virus for 24 h followed by Western blot for BCL2L2. Bars represent the means ± SD from 3 independent experiments. $^{*}P < 0.05$ vs. BCL2L2 3′ UTR (in A) or Anti-Ctrl (in B).

suggest that miR-29c-mediated translational repression of BCL2L2 is involved in influenza virus–induced BCL2L2 expression repression in A549 cells.

3.4. Upregulated miR-29c promotes cell death induced by influenza virus infection

To study the physiological role of miR-29c in cell death of A549 cells induced by influenza virus, the apoptosis and cell viability of A549 cells infected with influenza virus were analyzed after transfection with miR-29c inhibitor or precursor. The results showed that miR-29c, downregulated by its inhibitor, inhibited cell death induced by influenza virus (Fig. 4A and C). Furthermore, transfection with miR-29c mimics was significantly increased miR-29c levels in A549 cells (data not shown), and promoted influenza virus-induced cell death (Fig. 4B and D). These results suggest that upregulated miR-29c negatively regulates cell death after influenza virus infection.

3.5. BCL2L2 overexpression rescues A549 cells death induced by influenza virus infection

BCL2L2 is an important determinant of cell apoptosis, and downregulated expression of BCL2L2 results in abnormal cell death. To further determine the role of miR-29c in influenza virus-induced cell death by regulating BCL2L2, we overexpressed BCL2L2 in influenza virus-infected A549 cells and assayed cell death. As shown in Fig. 4E, A549 cell death was prevented. The result confirm that miR-29c promote A549 cell death induced by influenza virus, at least in part, by repressing BCL2L2.

4. Discussion

The complex and intricate cellular apoptosis pathway activated by influenza virus infection are not completely understood, but are known to include activation of pro-death and inhibition of anti-death signaling cascades which converge on the mitochondria. As

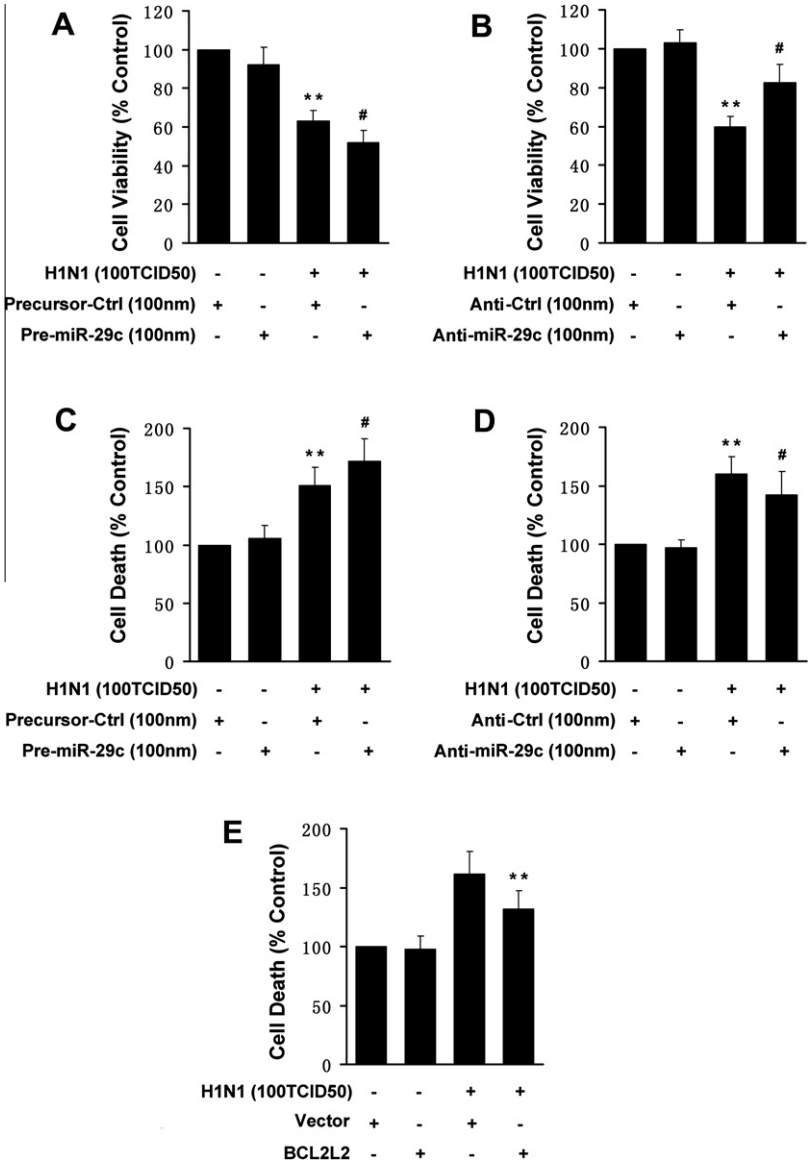


Fig. 4. Upregulated miR-29c promotes apoptosis induced by influenza A virus infection by regulating BCL2L2 expression. Functional manipulation of miR-29c influenced cell viability and apoptosis of A549 cells infected with influenza virus. A549 cells were first treated with miR-29c precursor (A, C), anti-miR-29c (B, D) or BCL2L2 construct (E) for 24 h. After being washed, cells were challenged with influenza virus for 48 h. Cell viability was detected by CCK-8 assay. Cell apoptosis was measured by annexin V–FITC/PI double staining with the use of flow cytometry analysis. Bars represent the means ± SD from 3 independent experiments. $^{**}P < 0.01$ vs. mock group (in A, B, C, D) or empty vector (in E); $^{\#}P < 0.05$ vs virus-pre-Ctrl or virus-anti-Ctrl.

the BCL-2 family members reside upstream of irreversible cellular damage and focus much of their efforts at the level of mitochondria, they play a pivotal role in deciding whether a cell will live or die [25]. Previous studies have shown that influenza virus induce PK-15 and HeLa cells death by downregulation of anti-apoptotic Bcl-2 and upregulation of pro-apoptotic Bax [26] and influenza virus-induced apoptosis is inhibited by expression of Bcl-2 [27]. Thus, Bcl-2 family may be important modulators of cell death in influenza virus infection.

There is increasing evidence that post-transcriptional regulation of gene expression, mediated by miRNAs, plays an important role in the control of cell apoptosis [28]. The human miR-29 family of miRNAs has three mature members, miR-29a, miR-29b, and miR-29c, which share a common seed region sequence and are predicted to target largely overlapping sets of genes. The miR-29 family have also been shown to be proapoptotic and involved in the regulation of cell differentiation via inhibiting expression of target genes, such as Mcl-1, a member of the ntiapoptotic Bcl-2 family [17], p85a (the regulatory subunit of PI3 K) [16], cell division cycle 42 (CDC42) [16] and tumor necrosis factor alpha-induced protein 3 (TNFAIP3) [29]. However, the miR-29 family members exhibit differential regulation in several cases and different subcellular distribution [30]. It remains to be explored how various cellular effects of miR-29s determine functional relevance of miR-29s to specific diseases.

Here, we provided evidence that miRNA-mediated posttranscriptional mechanism is involved in influenza virus-mediated BCL2L2 expression repression in A549 cell line. Firstly, we detected a significant increase of miR-29c expression in A549 cells after influenza virus infection by miRNA array and real-time PCR. Secondly, we identified that miR-29c targets 3′ UTR of BCL2L2, resulting in translational suppression in A549 cells. A significant decrease of the luciferase reporter translation was detected in the miR-29c precursor-treated cells using a luciferase reporter vector containing BCL2L2 3′ UTR with the putative miR-29c binding site. Moreover, treatment of cells with miR-29c inhibitor abolished influenza virus-induced the decrease of BCL2L2 expression. Because a control precursor showed no effect, we speculate that inhibition of BCL2L2 protein expression by miR-29c is attributable to suppress translational expression induced by influenza virus infection. BCL2L2 is an anti-apoptotic member of the Bcl-2 potein family. The mechanism by which BCL2L2 suppresses apoptosis is that BCL2L2 interacts directly with pro-apoptotic members to block their apoptotic activities [31,32]. Finally, we confirmed that BCL2L2 overexpression abolished A549 cell death induced by influenza virus infection.

In conclusion, our data indicate that influenza virus infection suppresses BCL2L2 expression in A549 cells via inducing miR-29c. Moreover, influenza virus promotes A549 cell death, at least in part, by repressing BCL2L2.

Acknowledgments

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