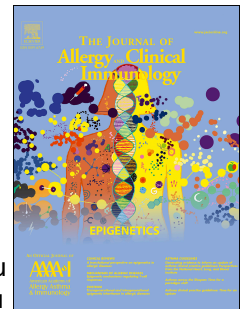


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Respiratory Syncytial Virus Nonstructural Protein 1

Downregulates Glucocorticoid Receptor Expression via miR-29a

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Key words: Respiratory syncytial virus nonstructural protein1; glucocorticoid receptor; microRNA 29a; glucocorticoid insensitivity

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Capsule Summary

Infants with bronchiolitis do not respond to steroids, and glucocorticoid receptor (GR) is downregulated in RSV infected airway epithelial cells (AECs). We show that RSVNS1 downregulates GR expression in amiR-29adependent manner.

To the Editor:

In contrast to asthma, the inflammatory response induced by human respiratory syncytial virus (RSV) infection is refractory to glucocorticoid (GC)treatment^{1, 2},for reasons that are not clear. Reduced corticosteroid responsiveness at a cellular and clinical level is associated with a reduced number of glucocorticoid receptor (GR)^{3, 4}. We previously showed that RSV infection downregulated GR mRNA and protein in RSV-infectedA549 cells and in BALB/c mice⁵. Other studies showed that downregulated GR level was associated with an increase in miRNAs miR-124-3p,miR-142-3p and miR-29a^{3, 6, 7}. Here, we investigated how RSV infection regulates GR expression, mediated by miRNAs. We hypothesized that miR-29a, regulated by RSV NS1 protein, is responsible for the downregulation of GR expression.

We first investigated hGR α and hGR β expression in nasopharyngeal aspirates (NPAs) from 26 infants with RSV infection and 24 healthy control infants. The data showed that both hGR α (Fig. 1A) and hGR β (Fig. 1B) mRNA expression and total GR protein levels were significantly decreased in the RSV group (Fig.1C). These results were confirmed by immunofluorescence (Fig.E1). We then quantified the expression of the three miRNAs, referred to above, in NPAs and found that only miR-29a levels were significantly increased in NPAs from RSV-infected infants (Fig. 1D). Relativity analysis of miR-29a and GR mRNA (Fig. E2A&B) and protein (Fig. E2C) in each RSV-infected infant showed significant correlations between miR-29a with GR α

($R^2=0.56$), GR β ($R^2=0.74$) and GR protein ($R^2=0.39$). The miR-29a also showed a significant correlation with NS1 gene expression in RSV-infected infants (Fig. E2D, $R^2=0.30$). There was also an association between NS1 expression and GR mRNA (Fig. E2E, $R^2=0.20$ & 0.19) and protein (Fig. E2F, $R^2=0.22$), which was significant at $P<0.05$ level. Together, the lower amounts of GR mRNA and protein in NPAs of RSV-infected infants were closely related to the enhancement of miR-29a, and both were in turn related to increased NS1 gene expression.

The majority of cells in NPAs were respiratory epithelial cells, which are the target cells of RSV infection. To detect the effect of RSV on GR and its transcription-regulated genes expression, A549 cells were infected with RSV (MOI=2) or UV-RSV for the indicated times. We found that RSV infection of A549 cells reduced their GR gene (Fig.1E) and protein (Fig.1F) expression and expression of its transcription-regulated genes, FKBP51 and GILZ (Fig.1K). RSV infection of A549 cells increased their relative miR-29a expression (Fig.1G). Infection of A549 cells with UV-irradiated RSV showed similar results to mock-infected cells (Fig. 1H-1K). We confirmed that these effects increased with time following RSV infection and that the increase in relative miR-29a expression in RSV-infected A549 cells was greater at higher MOI of infecting virus (Fig.E3). The data suggested that infection with live RSV upregulated miR-29a expression and downregulated GR mRNA and protein.

To detect the effect of miR-29a on GR and its transcription-regulated gene expression, A549 cells were transfected with plasmids expressing miRNA scramble control (miR-NC), miR-inhibitor, and miR-mimic for 16 h, infected with RSV (MOI=2) for another 24 h, and then GR mRNA, GR protein, FKBP51 and GILZ expression were measured. We first found that GR mRNA (Fig.2A) and protein (Fig.2B) expression were significantly increased in the miR-29a inhibitor group, compared to the control miRNA group. In line with those data, FKBP51 and GILZ levels increased in the miR-29a inhibitor group, compared with the miRNA control group (Fig. 2C). We then

106 explored whether GR is the target gene of miR-29a; to this end, A549 cells were
107 co-transfected with plasmids expressing the WT or mutant GR 3'UTR and miR-29a
108 mimics or miR-NC for 24 h, and cells were then harvested and the relative luciferase
109 activity was measured. The data documented a direct role of miR-29a in GR mRNA
110 downregulation (Fig. 2D). Together, these results demonstrated that miR-29a in fact
111 contributes to RSV-induced GR degradation.

112 Studies using quantitative liquid chromatography-tandem mass spectrometry
113 (LC-MS/MS) of A549 cells, infected with either HRSV subtype A or B isolates, have
114 found that NS1 is the most abundantly produced specific RSV protein^{8,9}. To detect
115 whether NS1 of RSV may affect GR expression, we first performed qRT-PCR to
116 determine which gene of RSV shows the greatest change, at early times (24 h)
117 post-infection (p.i.). Results (Fig. 2E) revealed that the NS1 mRNA, when normalized
118 to RSV N gene mRNA, is indeed the most abundantly increased, compared to the
119 other viral genes. To detect the effect of NS1 on GR, and miR-29a and its
120 transcription-regulated genes expression, A549 cells were infected with RSV (MOI of
121 2), then transfected with control vector (siCON) and siNS1 vector for another 24
122 h. The data showed that the amount of GR mRNA (Fig. 2F), GR protein (Fig. 2G),
123 GR-activated genes, FKBP51 and GILZ (Fig. 2I), were all upregulated in the siNS1
124 group. In parallel, miR-29a expression was downregulated in siNS1 group (Fig.
2H). These data suggested that RSV NS1 was the main viral protein that
downregulates GR mRNA and protein expression and upregulates miR-29a
expression. Finally, to inquire whether GR downregulation by NS1 is mediated by
miR-29a, A549 cells were co-transfected with empty vector or plasmids expressing
NS1-knockdown siRNA and miR-29a mimics or miR-NC, then infected with RSV.
We found that increased miR-29a could partially rescue the enhancement of GR
expression level by NS1 knockdown (Fig. 2J-2M). FKBP51 and GILZ mRNA
expression also decreased in the miR-mimic transfected group (Fig. 2N&2O).
Collectively, these data suggest that the downregulation of GR by NS1 is mediated via
miR-29a.

In summary, RSV infection decreases the amount of GR in AECs of RSV-infected infants. The biological effect of this decreased GR may account for GC-insensitivity in RSV infection. This study is the first to show that NS1 protein of RSV, through induction of miR-29a, mediates GR reduction in AECs. Our observations suggest that the GR, the RSV NS1 protein and miR-29a may be important targets for therapy in RSV infection. Further studies should be performed to clarify the specific mechanism whereby NS1 induces miR-29a and the role of NS1-induced miR-29a during RSV infection.

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Figure legends

Fig. 1. GR was reduced while miR-29a enhanced in nasopharyngeal aspirates (NPAs) of infants with RSV infection as well as in RSV-infected A549 cells.

NPA from 26 infants with RSV infection and 24 uninfected controls were used and processed as described in Materials and Methods. (A, B) qRT-PCR was performed to detect GR α and GR β mRNA expression in NPAs. (C) Immunoblotting analysis of GR protein expression in NPAs. (D) qRT-PCR was performed to detect miR-29a, miR124a-3p and miR142a-3p expression in NPAs. Values of each experiment are presented as the mean $\pm$ SEM, ** P <0.01, *** P <0.001, comparisons of the RSV groups to the control group by the unpaired Student's t test. 'ns' represents 'not statistically significant'. A549 cells were infected with RSV (MOI=2) for the indicated times. (E) GR α and GR β mRNA expressions were quantified by qRT-PCR. (F) Immunoblotting analysis of GR protein expression. (G) miR-29a expression was quantified by qRT-PCR. Effect of UV-RSV on GR mRNA (H), GR protein (I), miR-29a (J), FKBP51 and GILZ mRNA (K) expression in RSV- or UV-RSV-infected A549 cells at 24 h p.i.. Values of each experiment are presented as the mean $\pm$ SD of tests performed in triplicate. Experiments were repeated at least three times. *** P <0.0001; ** P <0.001; * P <0.05. P values were obtained by one-way ANOVA with Bonferroni test.

Fig. 2. GR level was downregulated by RSV NS1 protein via mediating miR-29a.

A549 cells were transfected with plasmid containing miR-NC, miR-inhibitor, and miR-mimic for 16 h, then infected with RSV (MOI=2) for another 24 h. (A) qRT-PCR was performed to quantify GR α and GR β mRNA expression. (B) Immunoblotting analysis of GR protein expression. (C) FKBP51 and GILZ mRNA expression determined by qRT-PCR. Values of each experiment are presented as the mean $\pm$ SD of tests performed in triplicate. ** P <0.001, *** P <0.001 for comparisons of the RSV infection groups to the mock group by the unpaired Student's t test. $^{\wedge}P$ <0.05, $^{\wedge\wedge}P$ <0.01, $^{\wedge\wedge\wedge}P$ <0.001 for comparison of the miR-inhibitor and miR-mimic groups to

the controls, using one-way ANOVA with Bonferroni test. Experiments were repeated at least 3 times. (D) A549 cells were co-transfected with plasmids containing the WT or mutant GR 3'UTR and miR-29a mimics or miRNA scramble control (miR-NC) for 24 h, and cells were then harvested and the relative luciferase activity measured in the lysate. Data are expressed as the mean $\pm$ SD of tests performed in triplicate. Experiments were replicated three times. $**P<0.001$ was obtained by one-way ANOVA with Bonferroni test. (E) A549 cells were infected with RSV (MOI of 2) for the indicated times, and the cells and supernatants were collected to quantify the relative amounts of specific viral gene mRNA using qRT-PCR with customized ORF-specific TaqMan assay and the RSV N gene as reference. Data are shown as mean $\pm$ SD of duplicates, experiments being repeated at least three times. $***P<0.001$ for comparisons of the NS1 gene to the other 8 genes by one-way ANOVA with Bonferroni test. A549 cells were infected with RSV (MOI of 2), then transfected with control vector and siNS1 vector. (F) qRT-PCR analysis for GR α , GR β mRNA expression. (G) Immunoblotting analysis for N, NS1 and GR expression. qRT-PCR analysis for miR-29a (H) and FKBP51, GILZ (I) mRNA expression. $**P<0.01$, $***P<0.001$ for comparison siNS1 to siCON. Values of each experiment are presented as the mean $\pm$ SDs of tests performed in triplicate. Experiments were replicated three times. A549 cells were co-transfected with empty vector or plasmids encoding NS1-knockdown siRNA and miR-29a mimics or miR-NC, followed by infection with RSV at a MOI of 2 for 24 h. (J, K) GR α and GR β mRNAs were quantified with qRT-PCR. (L, M) Immunoblotting analysis for GR protein level. qRT-PCR was performed to detect GILZ (N) and FKBP51 (O) mRNA expression levels. Values of each experiment are presented as the mean $\pm$ SD of tests performed in triplicate. $*P<0.05$, $**P<0.01$, $***P<0.001$ for comparisons of the siNS1 groups to the siCON group by the unpaired Student's t test. $^{\wedge}P<0.05$, $^{\wedge\wedge}P<0.01$, $^{\wedge\wedge\wedge}P<0.001$ for comparison of the miR-mimic groups to miR-NC group by the unpaired Student's t test. Experiments were repeated at least three times.

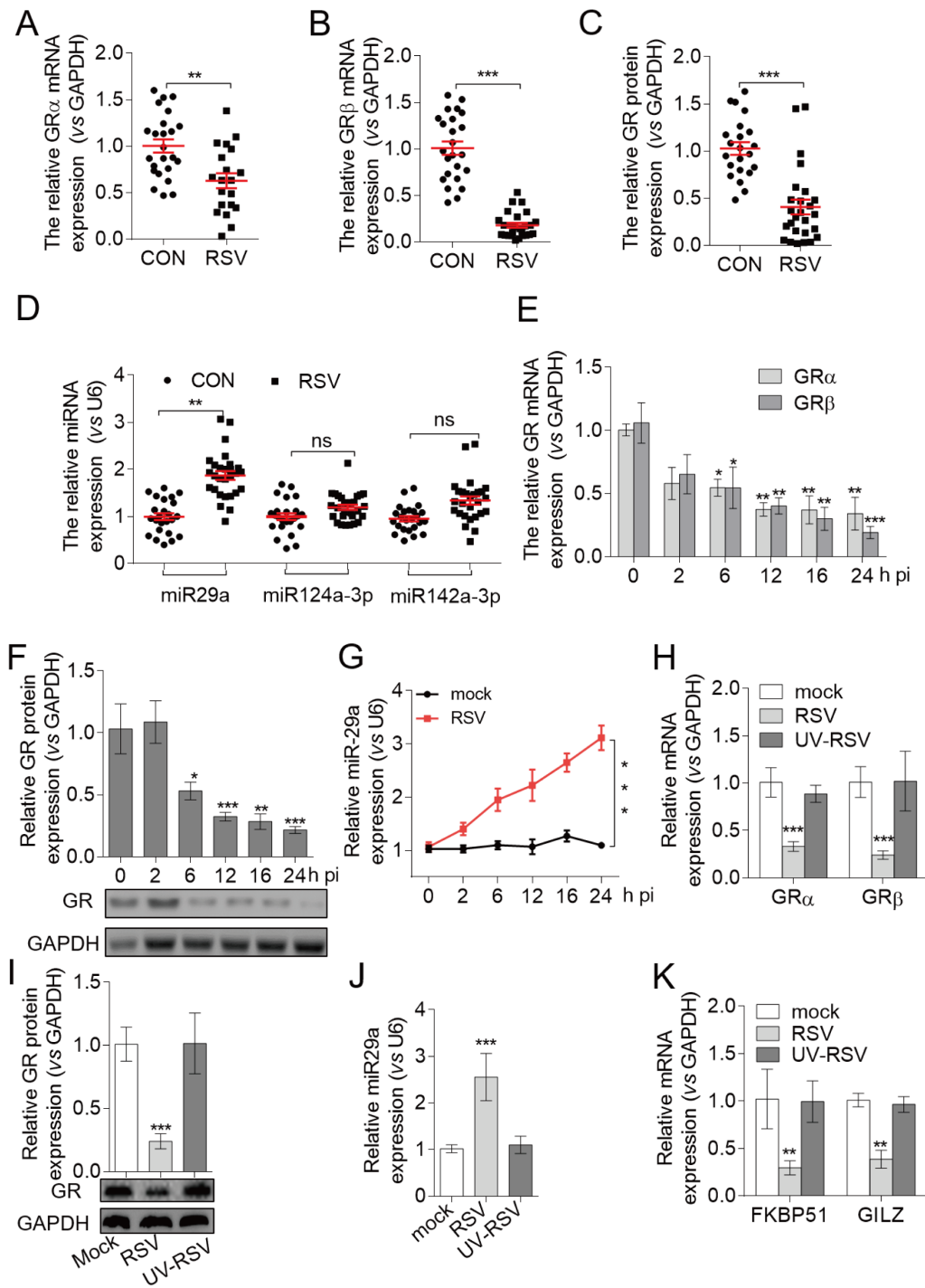
Reference

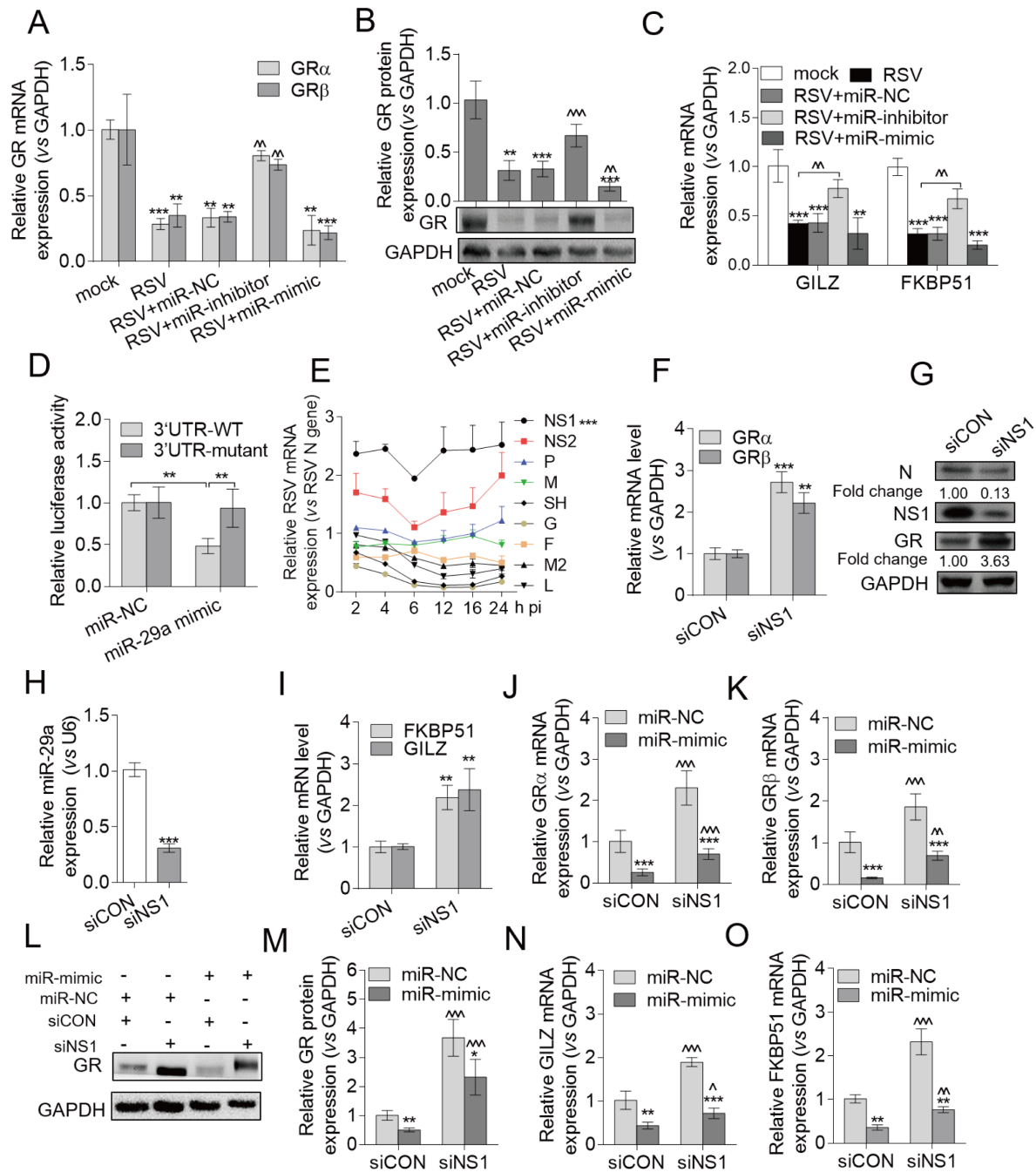
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1 **Supplementary**

2 **Materials and Methods**

3 **Research subjects**

4 Twenty-six children with RSV infection, hospitalized from November 2016 to
5 January 2017 in the Department of Respiratory Medicine, Children's Hospital,
6 Chongqing Medical University, were enrolled in this study. The nasopharyngeal
7 aspirates (NPAs) were prospectively collected from all subjects within the first days
8 after hospital admission. Immunofluorescence assays were performed to detect the
9 presence of RSV, adenovirus, influenza A and B virus, and parainfluenza virus 1, 2,
10 and 3 in the NPAs. Infants that carried viruses other than RSV were not included in
11 the study. Those considered positive for bacterial infection on the basis of published
12 criteria (Yu Deng, Enmei Liu et al, 2010/PMID: 20041894) were excluded as well.
13 The control group was selected from infants with no evidence of infection and
14 underwent surgical therapy only to clear secretions from the airway.

15 **NPAs analysis**

16 NPAs were collected and analyzed as previously described (Yu Deng, Enmei Liu et al,
17 2010/PMID: 20041894). In brief, the NPAs were collected gently and mixed
18 uniformly. To 0.5 mL of the aspirate, transferred to a new tube, 2 mL of 0.1%
19 dithiothreitol was added. The mixture was vortexed three times, 15 sec each, and
20 rocked on a bench rocker for 15 min. The suspension was collected and subsequently
21 centrifuged at 306 g for 10 min. Cell-free supernatants were collected and aliquots
22 stored at -80°C . A portion of the cell pellet was used to prepare slides for GR
23 immunofluorescence analysis, and the rest was used for total RNA, protein and
24 miRNA extraction.

25 **Reagents and antibodies**

26 The primary antibodies used in this study included a goat polyclonal antibody against
27 RSV (Millipore) and rabbit GR and mouse monoclonal GAPDH antibody (CST).
28 DAPI was purchased from Sigma-Aldrich. The secondary antibodies used were Alexa
29 Fluor 568/488-conjugated duck anti-goat IgG and anti-rabbit IgG from Biyuntian, and

horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG or anti-mouse IgG from CST.

Cell culture, virus, and RSV infection

Human lung carcinoma epithelial cell line A549 (ATCC CCL-185) and human epithelial type 2 cell line HEp-2 (ATCC CCL-23) were cultured in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum. RSV-A2 strain was obtained from ATCC. For all experiments, RSV was grown in HEp-2 cells with 5% FBS and purified by density gradient as previously described (Toms GL et al, 2008/PMID: 18029032).

Virus titration

To quantify cell-associated and released virus, the supernatants were collected at indicated time. The infected cells were scraped into the cell culture medium, vortexed three times with glass beads, and centrifuged at 1000 rpm for 5 min. Infectious RSV was titrated by plaque assay on HEp-2 cells.

Plasmids construct assay

The pCMV-MIR plasmid was purchased from OriGene (USA), Pre-miR-29a was amplified from natural genome sequences with using PCR. The insertion sequence included the stem ring structures of pre-miR-29a and contain the flanking sequences. Then inserted into the *BamH I/Not I* sites. miR-29a knockdown plasmid: pEZX plasmid was purchased from GeneCopoeia (USA), shRNA specific for miR-29a chemosynthesis according to miR-29a sequence, and inset into the pEZX plasmid in *BamH I/EcoR I* sites; miR-29a scramble control: a negative control insert was obtained by scrambling the sequences of miR-29a and inserted into the *BamH I/Not I* sites.

Dual-luciferase reporter assay

Luciferase reporter plasmids pmirGLO was purchased from Promega (USA). To construct wild-type (WT) or mutant pmirGLO-GR 3'UTR, GR 3'UTR (WT) fragment or GR 3'UTR mutant fragment (A) was first synthesis as template to obtain the PCR fragment with primers (sense, 5'-

59 GTTTAAACACTATGATACATCCAGATGATGGAATACTATTGAG-3', anti-sense,
 60 5'-GAGCTCAATGGCCTAAGGTTTATAATAACCAACTAGTTCA -3', the underline
 61 is *PmeI* and *XhoI* cutting site). The *PmeI* and *XhoI*-cut PCR fragment was then inserted
 62 into the *PmeI/XhoI* sites. A549 cells were grown in 6-well flasks for 12 h before
 63 transfection. The next day, cells were co-transfected with WT or mutant GR 3'UTR
 64 luciferase reporter plasmid and miRNA scramble control (miR-NC), miR-29a mimic
 65 using Lipofectamine 2000 (Invitrogen). Cells were lysed with Passive Lysis Buffer
 66 (Promega) at 24 h post-transfection time. Programed dual-luciferase reporter system
 67 (Promega) was used to measure the luciferase activity according to the manufacturer's
 68 instructions.

69 **siRNA transfection**

70 NS1 siRNA (siNS1) of RSV and negative control siRNA, tagged internally with
 71 mCherry, were purchased from Genechem (Shanghai, China). The siRNA sequences
 72 were as follows (only the sense strand is shown): siNS1, 5'-
 73 GGCAGCAAUUCAUUGAGUAUG-3'; siCON (negative control), 5'-
 74 UUCUCCGAACGUGUCACGU-3'. The oligonucleotide sequences were inserted
 75 into the GV298 plasmid at appropriate sites. RSV-infected cells were transfected with
 76 siNS1 or siCON with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA)
 77 according to manufacturer's instructions. A549 cells in 6-well plates were transfected
 78 with siRNA or siCON using Lipofectamine 2000 for 24 hours.

79 **Western blot (immunoblot) analysis**

80 Fifty micrograms of total protein were separated on 5-15% Bis-tris SDS
 81 polyacrylamide gels, and transferred onto PVDF membranes. The membranes were
 82 incubated with primary antibodies against GR and GAPDH, followed by incubation
 83 with appropriate secondary antibodies. The protein bands were visualized with using a
 84 chemiluminescence kit (Bio-Rad).

85 **RNA isolation and quantitative (q) RT-PCR**

86 A549 cells, transfected with plasmids or infected by RSV, were harvested and
 87 intracellular RNA were purified at indicated times post-transfection (BioTake). The

miRNA was isolated using a miRNA isolation Kit (BioFlux, China, Cat. No BSC64S1) in accordance with the manufacturer's instructions. The viral RNA was isolated from the mixture of cells and supernatants or NPAs. RNA (1 µg) was used for first-strand cDNA synthesis (Takara) and the products were amplified using the VeriQuest Fast SYBR Green qPCR (Invitrogen) with appropriate primers (Table E1). The PCR cycle conditions were as follows: 50°C for 2 min, 95°C for 10 min, 40 cycle at 95°C for 15 s, and 60°C for 30 s. The fold change was obtained by the 2- $\Delta\Delta C_t$ method using GAPDH as a calibrator. RNA (1 µg) of miRNA was used for first-strand cDNA synthesis (GeneCopoeia, Cat.No:QP013) and the products were amplified using the VeriQuest Fast SYBR Green qPCR (Invitrogen) with appropriate primers (miR-29a Cat#HmiRQP0371, miR-124a-3p Cat#HmiRQP0074, miR-142a-3p Cat#HmiRQP0186). The fold change was obtained by the 2- $\Delta\Delta C_t$ method using U6 as a calibrator. The ten RSV ORFs was quantified by qRT-PCR using customized ORF-specific TaqMan assays.

Indirect immunofluorescence assays

The cell pellets from NPAs were fixed with 4% paraformaldehyde for 30 min at room temperature and permeabilized with 0.2% Triton X-100 (sigma) for an additional 20 min. After washing and blocking with 2% BSA for 1 h, the cells were incubated with GR antibody overnight at 4°C. The cells were then washed three times followed by incubation with Alexa Fluor 488-conjugated secondary antibodies (Molecular Probes) for 1 h at room temperature and then with DAPI (sigma) for another 15 min. The stained cells were visualized and photographed in a Nikon laser microscope. All images were analyzed with NIS-Elements BR software, version 4.11.

Statistical analysis

All statistical tests were performed using the SPSS 19.0 (IBM, US), and the results are expressed as mean values $\pm$ standard or mean values $\pm$ standard errors of the mean. Unpaired Student's *t* test or ANOVA were used to determine differences between all groups. Spearman's tests were used to detect differences in correlation analysis. Differences were considered to be significant when *P* values were <0.05.

Ethics Statement

Use of NPA samples of infants were approved by the Ethics Committee of the Children's Hospital, Chongqing Medical University (permit number 2015–77), and the parents or legal guardians offered written informed consent to participate in the study before the infants were enrolled. All procedures were performed in accordance with the approved guidelines and obey to the principles of the Declaration of Helsinki.

Figure legends

Fig.E1. GR was reduced in nasopharyngeal aspirates (NPAs) of RSV-Infected infants. NPA from 26 RSV-infected infants and 24 uninfected controls were used and processed as described in Materials and Methods. A portion of the cell pellet was used to prepare slides for immunofluorescence analysis (IFA), and the rest was used for total protein extraction. (A) GR protein expression in NPAs. (B) IFA of GRs in NPA cells, randomly selected from 5 of 26 RSV-infected or 24 control infants, and quantitation of endogenous GR MFI in NPA cells (C). Values are means $\pm$ standard errors of the mean. $**P < 0.01$, comparisons of the RSV groups to the control group by the unpaired Student's test.

Fig. E2. The miR-29a, GR mRNA and protein, and NS1 gene mRNA correlation analysis.

NPAs from the E1 sample collected from RSV-positive cases were correlated for: 1) each sample's miR-29a with GR α , GR β or GR protein; 2) each sample's miR-29a with NS1 gene expression; 3) each sample's NS1 gene with GR α , GR β or GR protein (A) Scatter clustered correlation between miR-29a and GR α mRNA levels; $R^2 = 0.56$. (B) Scatter clustered correlation between miR-29a and GR β mRNA levels; $R^2 = 0.74$. (C) Scatter clustered correlation between miR-29a and GR protein levels; $R^2 = 0.39$. (D) Scatter clustered correlation between miR-29a and NS1 mRNA level; $R^2 = 0.30$. (E) Scatter clustered correlation between NS1 mRNA and GR α /GR β mRNA levels, with R^2 values of 0.20 and 0.19, respectively. (F) Scatter clustered correlation between NS1 mRNA and GR protein level; $R^2 = 0.22$. Correlation was assessed by Spearman's tests.

Fig. E3. RSV infection upregulated miR-29a expression in A549 cells. A549 cells were infected with RSV at an MOI of 0.8, 2 or 4. qRT-PCR was performed to determine the expression levels of miR-29a at indicated infection time. Data are shown as mean $\pm$ SD of triplicates from at least three independent experiments.

*** $P < 0.001$ for comparisons of the RSV infection groups to the mock group by one-way ANOVA with Bonferroni test. $^{\wedge\wedge}P < 0.01$, $^{\wedge\wedge\wedge}P < 0.001$ was obtained by one-way ANOVA with Bonferroni test.

Table E1 Primers used in qRT-PCR

Gene Name	Accession Number	Direction	Primer sequence (5'-3')
NR3C1 α (GR α)	NM_001204258	F	ACCTGCTGGGTAAACTGTGG
		R	AGACAGGGCCTCTTGGTAGT
NR3C1 β (GR β)	NM_001204258	F	GCATGCCATGAAGGAAAGCC
		R	GGAGTGACCAGCCAAGATGG
GILZ	NM_004089	F	GCACAATTTCTCCATCTCCTTCTT
		R	TCAGCATGATTCTTCACCAGATCCA
FKBP5	NM_004117	F	TCCCTCGAATGCAACTCTCT
		R	AAACATCCTTCCACCACAGC
GADPH	NM_002046.3	R	ATCAAGAAGGTGGTGAAGCAGGC
		F	TCAAAGGTGGAGGAGTGGGTGTC
		R	