Endogenous Retrovirus-Derived *lnc-ALVE1-AS1* Exerts Antiviral Defense Against ALV-J Infection in Chicken Macrophages

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Abstract

Endogenous retroviruses (ERVs) are remnants of ancient retroviral infections dating back many millions of years, and their derived transcripts with viral signatures are important sources of long noncoding RNAs (lncRNAs). We have previously shown that the chicken ERV-derived lncRNA *lnc-ALVE1-AS1* exerts antiviral innate immunity in chicken embryo fibroblasts. However, it is not clear whether this endogenous retroviral RNA has a similar function in immune cells. Here, we found that *lnc-ALVE1-AS1* was persistently inhibited in chicken macrophages after avian leukosis virus subgroup J (ALV-J) infection. Furthermore, overexpression of *lnc-ALVE1-AS1* significantly inhibited the proliferation of exogenous ALV-J, whereas knockdown of *lnc-ALVE1-AS1* promoted the proliferation of ALV-J in chicken macrophages. This phenomenon is attributed to the induction of antiviral innate immunity by *lnc-ALVE1-AS1* in macrophages, whereas knockdown of *lnc-ALVE1-AS1* had the opposite effect. Mechanistically, *lnc-ALVE1-AS1* can be sensed by the cytosolic pattern recognition receptor TLR3 and trigger the type I interferons response. The present study provides novel insights into the antiviral defense of ERV-derived lncRNAs in macrophages and offers new strategies for future antiviral solutions.

Introduction

Endogenous retroviruses (ERVs) are remnants of ancient retroviral infections dating back many millions of years, and they comprise nearly 8% of the human genome (Stoye 2012) and 3% of the chicken genome (Mason et al. 2016). Retroviral integration is an essential part of the endogenous retroviral lifestyle but is also a potential threat to the host. Recently, integrated chicken ERVs have been shown to have harmful impacts on poultry genetic traits and resistance (Mason et al. 2020). ERV activation has also been noted in various cancers, viral infections and autoimmunity, but whether ERVs contribute to these diseases is a highly controversial topic (Hayward and Katzourakis 2015). Because ERVs were once presumed to form much of the so-called redundant ‘junk’ DNA, without any function, or were recognized to have pathogenic potential and to be capable of causing harm to humans (Villarreal 2011), the biological effects of these endogenous retroviral elements beneficial to humans have largely been ignored and are currently not fully understood.

Recently, it has been shown that certain ERVs are activated in mammalian preimplantation embryos and embryonic stem cells (Liu et al. 2019; Xue et al. 2013; Yan et al. 2013). They can rewire the core regulatory network of human embryonic stem cells (Kunarso et al. 2010) and display essential roles in pluripotency (Fort et al. 2014; Macfarlan et al. 2012) and early mammalian embryonic development (Grow et al. 2015; Wang et al. 2014). Importantly, ERV activation can be sensed by innate immune receptors and trigger antiviral innate immunity (Grandi and Tramontano 2018; Hurst and Magiorkinis 2015). Surprisingly, ERVs not only promote T cell selection and improve the sensitivity with which T cells react to retroviral infection (Young et al. 2012) but also mobilize B cells to rapidly produce antibodies against pathogenic antigens (Grasset and Cerutti 2014; Zeng et al. 2014).

In general, ERVs have dual effects, harmful or beneficial, on host cell antiviral function. Only a few endogenous retroviral elements that have adapted to the evolution and plasticity of the host genome by positive selection have been adopted to regulate host gene expression and control cell function. These endogenous retroviral element-derived transcripts with viral signatures are important sources of noncoding RNAs (ncRNAs), which are sensed by innate immune receptors and trigger innate immunity. In mouse macrophages, a total of 1,278 full-length ERV-derived ncRNAs were identified, and among them, lnc-EPAV (ERV-derived lncRNA positively regulates antiviral responses) has been demonstrated to enhance host antiviral innate immunity (Zhou et al. 2019). The *lnc-ALVE1-AS1* is transcribed from chicken ERVs ALVE1 was also shown to activate antiviral innate immunity and inhibit the proliferation of exogenous retrovirus ALV-J replication in nonimmune cells (Chen et al. 2019). However, it is not clear whether this endogenous retroviral RNA has a similar function in immune cells. Therefore, individual ERV-derived lncRNAs with these immune functions need to be extensively investigated to reveal the impacts of these lncRNAs on antiviral innate immunity in host immune cells.

In this study, we explored the antiviral function of lncRNA *lnc-ALVE1-AS1* derived from ALVE1 in macrophages. We found that *lnc-ALVE1-AS1* was persistently inhibited in chicken macrophages after ALV-J infection. Overexpression of *lnc-ALVE1-AS1* significantly inhibited the proliferation of ALV-J through the induction of antiviral innate immunity. Mechanistically, *lnc-ALVE1-AS1* can be sensed by the cytosolic pattern recognition receptor TLR3 and trigger the type I interferons response. The present study provides novel insight into the antiviral defense of ERV-derived lncRNAs in macrophages. These results have important implications for the study of the antiviral function of ERVs and the development of new antiviral vaccines.

Materials And Methods

Cells, virus, and plasmids

The chicken macrophage-like line HD11 were obtained from the Laboratory of Avian Preventive Medicine, Yangzhou University, China. HD11 cells are derived from chicken bone marrow and transformed with the avian myelocytomatosis virus MC29 (Beug et al. 1979). HD11 cells was cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) with 5% fetal bovine serum (FBS) at 41°C in 5% CO_2 and 95% humidity. Primary chicken embryo fibroblasts (CEFs) were prepared from 10-day-old specific pathogen-free (SPF) embryos of ALV-resistant G1 or ALV-susceptible G3 chickens obtained from Harbin Veterinary Research Institute (HVRI), the Chinese Academy of Agricultural Sciences. CEFs were cultured in DMEM with 5% FBS at 37°C in 5% CO_2 and 95% humidity. The JS09GY3 strain of ALV-J was obtained from the Laboratory of Avian Preventive Medicine, Yangzhou University, China. The plasmids pcDNA3.1-*lnc-ALVE1-AS1* and pcDNA3.1-EGFP came from the plasmid bank in our laboratory.

Viral infection

HD11 cells were seeded into six-well plates and infected with the JS09GY3 strain of ALV-J at a multiplicity of infection (MOI) of 5. At 6, 12 and 24 h post infection (hpi), cells were collected for ALV-J proliferation analysis. CEFs were infected with the JS09GY3 strain of ALV-J at an MOI of 5 and then collected at 24 and 96 hpi for ALV-J proliferation analysis.

Plasmid transfection

HD11 cells were transfected with the control or *lnc-ALVE1-AS1* plasmid using Lipofectamine™ 3000 Transfection Reagent (Thermo Fisher Scientific, USA) for 36 h, and then total RNA and protein were collected for gene expression analysis. For viral infection experiments, HD11 cells were first infected with the ALV-J virus at a multiplicity of infection (MOI) of 5 for 12 h. Cells were then transfected with *lnc-ALVE1-AS1* or the control plasmid using Lipofectamine™ 3000 Transfection Reagent (Thermo Fisher Scientific, USA) for another 36 h and then collected for ALV-J proliferation analysis.

RNA interference assays

For knockdown of *lnc-ALVE1-AS1*, HD11 cells were transfected with *lnc-ALVE1-AS1* or control siRNA using Lipofectamine™ RNAiMAX Transfection Reagent (Thermo Fisher Scientific, USA) for 36 h, and then total RNA and protein were collected for gene expression analysis. For knockdown of TLR3, HD11 cells were first transfected with the control or TLR3 siRNA using Lipofectamine™ RNAiMAX Transfection Reagent for 12 h. Cells were then transfected with *lnc-ALVE1-AS1* or control plasmid using Lipofectamine™ 3000 Transfection Reagent (Thermo Fisher Scientific, USA) for another 36 h, and total RNA and cell supernatant were collected for gene expression analysis. The Stealth RNAi™ siRNAs specific for *lnc-ALVE1-AS1* and chicken TLR3 siRNAs from previous studies (Chen et al. 2019; Hu et al. 2015; Hu et al. 2016a) and the sequences of the Stealth siRNAs are described in Table 1.

Table 1
Primers used in this study

Primer name	Nucleotide sequence 5'-3'
lnc-ALVE1-AS1 fwd	GACTACTGCCATAACTAAGG
lnc-ALVE1-AS1 rev	CAGAAGTCACAGCCAGAT
lnc-ALVE1-AS1 RT1	GATGGACAGACCGTTGAG
lnc-ALVE1-AS1 RT2	CCTCATCCGTCTCGCTTA
lnc-ALVE1-AS1 siRNA AS	GACUCUGGAGCGGACAUCACUAUUA
lnc-ALVE1-AS1 siRNA S	UAAUAGUGAUGUCCGCUCCAGAGUC
TLR3 Stealth_1 siRNA AS	AAGUAAAUCAGAUUGCUGUACUCGG
TLR3 Stealth_1 siRNA S	CCGAGUACAGCAAUCUGAUUUACUU
TLR3 Stealth_2 siRNA AS	UGUUGCUGCAAUCCUAAAUUUGCUG
TLR3 Stealth_2 siRNA S	CAGCAAAUUUAGGAUUGCAGCAACA
IFIH1 fwd	GATTACCAGATGGAAGTTGC
IFIH1 rev	GGTAATGTAAACAGCCACTC
TLR7 fwd	CACACATTCAACTGGGGCAA
TLR7 rev	GGGAACGGTAGTCAGAAGGT
MB21D1 fwd	CGACACTGGAGCCTATTATT
MB21D1 rev	TTCCACGCTTTTTCCTTTTC
IRF7 fwd	GAGCCTCCTCCCTCAACAGT
IRF7 rev	AGGGACACAGGAAGGGAGTG
IFN-α fwd	TACGGCATCCTGCTGCTCAC
IFN-α rev	AGAGAAGGTGGCATCCTGGG
IFN-β fwd	GCCCACACACTCCAAAACACTG
IFN-β rev	TTGATGCTGAGGTGAGCGTTG
MX1 fwd	CCGCAACACAGAAATACAG
MX1 rev	TTATCTTGTGGCTGGTTCC
OASL fwd	CTGAAGGGGGAGATAGAGAA
OASL rev	TCTTGTTACTGTCCTTCACC
IFITM3 fwd	ATCGCCAAGGACTTCGTA

Primer name	Nucleotide sequence 5'-3'
IFITM3 rev	ATGGAGAGGATGGTCACAA
ALV-J *env* fwd	TGCGTGCGTGGTATTATTTC
ALV-J *env* rev	AATGGTGAGGTCGCTGACTGT
GAPDH fwd	GAGAAACCAGCCAAGTATGA
GAPDH rev	CTGGTCCTCTGTGTATCCTA

For viral infection experiments, HD11 cells were first transfected with the control or *lnc-ALVE1-AS1* siRNA using Lipofectamine™ RNAiMAX Transfection Reagent (Thermo Fisher Scientific, USA) for 12 h. Cells were then infected with the ALV-J virus at a multiplicity of infection (MOI) of 5 for another 48 h and then collected for ALV-J proliferation analysis.

Cell treatment

After treatment with 100 μM Amlexanox (InvivoGen, California, USA) for 2 h, HD11 cells were then transfected with control or *lnc-ALVE1-AS1* plasmid using Lipofectamine™ 3000 Transfection Reagent for another 36 h. Cell supernatant was then collected to detect the expression of the IFN-β gene, and total RNA was extracted to measure the expression of antiviral innate immunity-related genes. For TLR3 stimulation, HD11 cells were incubated for 24 h in medium containing TLR3 ligand (InvivoGen, California, USA), a synthetic analog of dsRNA poly (I:C) with a high molecular weight, and then collected for gene expression analysis.

Reverse transcription-quantitative PCR (RT–qPCR)

RT–qPCR assays were performed according to previous studies (Chen et al. 2019). Briefly, total RNA was extracted from chicken cells or tissues using TRIzol™ reagent (Thermo Fisher Scientific, USA) according to the manufacturer's recommendations. The gDNA Eraser-treated RNA samples were reverse-transcribed with RT primers at 37°C for 15 minutes or strand-specific RT primers at 42°C for 15 minutes with PrimeScript® Reverse Transcriptase (TaKaRa, Japan). Quantitative PCR was then performed with gene-specific primers and SYBR Green Master Mix (TaKaRa, Japan) on the CFX Connect™ Real-Time PCR Detection System (Bio–Rad, California, USA). GAPDH RNA levels were used as internal controls to normalize gene expression. The strand-specific RT primers and gene-specific primers are listed in Table 1.

Protein extraction and immunoblotting

Whole-cell lysates were prepared with Cell Lysis Buffer (Cell Signaling Technologies, USA), separated by 12% SDS–PAGE at 120 V for 90 min and transferred to polyvinylidene difluoride membranes at 50 V for 150 min. Membranes were blocked in TBST containing 5% nonfat dry milk (Bio–Rad, California, USA). Primary antibodies were incubated overnight at 4°C with agitation. The following antibodies were used to determine protein expression: rabbit anti-TLR3 (Novus Biologicals, USA), anti-GAPDH (Abcam, United Kingdom) and mouse monoclonal antibody JE9, which is specific to the envelope protein of ALV-J. After

washing extensively with TBST, secondary antibodies (anti-rabbit or anti-mouse horseradish peroxidase conjugate) were incubated for 1 h at room temperature. After washing extensively with TBST, blots were developed using enhanced chemiluminescent detection reagents on the FluorChem Q imaging system (Protein Simple, USA).

Immunofluorescence confocal microscopy

Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min at room temperature, permeabilized with 0.25% Triton X-100 for 5 min, and blocked with 2% BSA for 30 min. Cells were then incubated with the mouse anti-ALV-J envelope protein (JE9 antibody) at room temperature for 1 h, followed by incubation with goat anti-mouse IgG conjugated with Alexa Fluor 488 dye (ab150081 Abcam, 1:200) at room temperature for 45 min. After five washes in PBST (PBS with 0.05% Tween), cells were stained with DAPI dye (Sigma, Shanghai, China) at room temperature for 10 min. Images were acquired using a Leica TCS SP8 confocal microscope, and data analysis was carried out with Leica LAS AF Lite (Leica Microsystems).

RNA fluorescence in situ hybridization (FISH)

RNA FISH was performed according to the manufacturer's recommendations (RIBOBIO, Guang Zhou, China) and the previous study (Chen et al. 2019). Briefly, HD11 Cells were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature, permeabilized with 0.25% Triton X-100 for 5 min, and blocked with 2% BSA for 30 min. Cells were then incubated overnight with the *lnc-ALVE1-AS1* probe mix at 37°C. For colocalization studies, after RNA FISH, cells were subjected to immunofluorescence for TLR3 protein. The pictures were captured and merged with a Leica SP8 confocal microscope.

Enzyme-linked immunosorbent assay (ELISA)

The cytokine concentrations in the cell supernatants were measured with ELISA kits for chicken type I interferons IFN-α and IFN-β according to the manufacturer's instructions (Shanghai Hengyuan Bioscience & Technology Company, China). The p27 antigen of ALV-J in the cell supernatants was measured by ELISA (IDEXX, Beijing, China) following the manufacturer's instructions.

Statistical analyses

The statistical analysis was performed with the Statistical Package for the Social Sciences (version 16.0) software. Statistical significance was assessed using a two-tailed unpaired Student's *t*-test with a *P* value threshold of < 0.05.

Results

ALV-J inhibits the expression of lnc-ALVE1-AS1 in macrophages

The influence of exogenous virus ALV-J infection on the expression of *lnc-ALVE1-AS1* in macrophages was measured by RT–qPCR. As shown in Fig. 1, the expression of *lnc-ALVE1-AS1* in the chicken

macrophage cell line HD11 was persistently downregulated from 6 to 48 hpi and significantly downregulated at 24 h and 48 h after ALV-J infection. These results suggest that *lnc-ALVE1-AS1* might be involved in the antiviral response to ALV-J infection in chicken macrophages.

Inhibition of ALV-J proliferation by lnc-ALVE1-AS1 in macrophages

To confirm the role of *lnc-ALVE1-AS1* in antiviral defense, macrophages were transfected with *lnc-ALVE1-AS1* after ALV-J infection, and then the influence of *lnc-ALVE1-AS1* on ALV-J proliferation was assessed. Compared with the control group, transfection of *lnc-ALVE-AS1* significantly inhibited the expression of the ALV-J *env* gene at both the mRNA and protein levels in HD11 cells (Fig. 2a and b). $TCID_{50}$ and ELISA analysis results also showed that overexpression of *lnc-ALVE-AS1* led to significant reductions in viral titers and viral protein in the culture medium of HD11 cells infected with ALV-J (Fig. 2c and d). The confocal immunofluorescence microscopy analysis results shown in Fig. 2e further confirmed the inhibition of *lnc-ALVE-AS1* on ALV-J proliferation in chicken macrophages.

Knockdown of lnc-ALVE1-AS1 can promote the replication of ALV-J in macrophages

Knockdown of *lnc-ALVE-AS1* by RNAi in macrophages was performed to further confirm the role of *lnc-ALVE1-AS1* in antiviral defense. Conversely, knockdown of *lnc-ALVE-AS1* significantly increased the expression of the ALV-J *env* gene at both the mRNA and protein levels in HD11 cells (Fig. 3a and b). $TCID_{50}$ and ELISA analysis results also showed that knockdown of *lnc-ALVE-AS1* led to significant increases in viral titers and viral protein levels in the culture medium of HD11 cells infected with ALV-J (Fig. 3c and d). Our data collectively indicate that *lnc-ALVE1-AS1* may possess a function in antiviral defense in chicken macrophages.

lnc-ALVE1-AS1 triggers antiviral innate immunity in macrophages

Since *lnc-ALVE1-AS1* can block the proliferation of ALV-J in macrophages, we next investigated the influence of *lnc-ALVE1-AS1* on the expression of host genes involved in antiviral innate immunity. In chicken macrophages, *lnc-ALVE1-AS1* triggered an interferon response, which included type I interferons (IFN-α and IFN-β) and a panel of interferon-stimulated genes (ISGs; MX1, OASL and IFITM3) (Fig. 4a and b). Each ISG functions predominantly in antiviral innate immunity. Generally, the key upstream gene IRF7 in the type I interferon pathway was also upregulated by *lnc-ALVE1-AS1* overexpression in macrophages. ELISA analysis further confirmed that the expression of IFN-α and IFN-β was induced by overexpression of *lnc-ALVE1-AS1* (Fig. 4c). However, knockdown of *lnc-ALVE1-AS1* by RNAi led to significant decreases in the expression levels of IFN-α, IFN-β, IRF7, MX1, OASL and IFITM3 (Fig. 4d and e). Knockdown of *lnc-ALVE1-AS1* also decreased the concentrations of IFN-α and IFN-β in macrophages (Fig. 4f). These results suggested that *lnc-ALVE1-AS1* is involved in antiviral innate immunity, especially the antiviral interferon response.

lnc-ALVE1-AS1 is involved in antiviral innate immunity by inducing TLR3 signaling

We then sought to determine the molecular mechanism by which lnc-ALVE1-AS1 induced an antiviral interferon response. Several studies in mammals have reported that endogenous retroviral RNAs can trigger signaling by the cytosolic pattern recognition receptors IFIH1 and TLR3 (Chiappinelli et al. 2015; Licht 2015; Yu et al. 2012), which recognize double-stranded RNA (dsRNA) associated with virus infection. In HD11 cells, we found that *lnc-ALVE1-AS1* only increased the transcript levels of cytosolic sensors for dsRNA (TLR3) but not IFIH1, MB21D1 or TLR7 (Fig. 5a and b). Consistently, knockdown of *lnc-ALVE1-AS1* caused a significant decrease in the expression levels of the TLR3 gene (Fig. 5c). Western blot results further confirmed that overexpression of *lnc-ALVE1-AS1* induced the expression of TLR3 protein in HD11 cells (Fig. 5d), whereas knockdown of *lnc-ALVE1-AS1* had the opposite effect (Fig. 5e). RNA FISH combined with confocal immunofluorescence further confirmed the colocalization of *lnc-ALVE1-AS1* and TLR3 in HD11 cells (Fig. 5f). Thus, TLR3 could be an important dsRNA recognition receptor for *lnc-ALVE1-AS1* to induce an interferon response.

We next investigated whether *lnc-ALVE1-AS1* affects the expression of antiviral innate immunity genes through TLR3 signaling. We observed that knockdown of TLR3 by RNAi significantly blunted the *lnc-ALVE1-AS1*-induced type I interferon (IFN-α and IFN-β) response in HD11 cells (Fig. 5g). Furthermore, inhibition of the TLR3-type I interferon signaling pathway with a TBK1/IKKε inhibitor (Amlexanox) also significantly blunted the expression of IFN-α and IFN-β induced by *lnc-ALVE1-AS1* (Fig. 5h). In addition, activation of TLR3 by the TLR3 ligand induced *lnc-ALVE1-AS1* expression in HD11 cells (Fig. 5i). These results indicate that *lnc-ALVE1-AS1* can be sensed by the innate immune receptor TLR3 and trigger the TLR3-induced interferon response.

lnc-ALVE1-AS1 exerts innate immune resistance to ALV-J in chickens

Next, we investigated the antiviral innate immune function of *lnc-ALVE1-AS1* in chickens. First, we found that the expression level of *lnc-ALVE-AS1* in ALV-resistant (G1) chicken immune organs (thymus, spleen, and bursa of Fabricius) was significantly higher than that in ALV-susceptible (G3) chickens (Fig. 6a). Viral infection experiments showed that the ALV-J replication level in CEFs of ALV-resistant G1 chickens at 24 and 96 hpi was significantly lower than that in CEFs of ALV-susceptible G3 chickens, whereas the *lnc-ALVE1-AS1* expression level showed the opposite results (Fig. 6b and c). Furthermore, the expression of *lnc-ALVE1-AS1* was significantly downregulated in ALV-resistant G1 and ALV-susceptible G3 chicken CEFs infected with ALV-J at 24 and 96 hpi (Fig. 6d and e). However, the decrease in *lnc-ALVE1-AS1* expression was more obvious in ALV-susceptible G3 chicken CEFs. It was further confirmed that overexpression of *lnc-ALVE1-AS1* could inhibit ALV-J replication in ALV-susceptible G3 chicken CEFs (Fig. 6f). Overexpression of *lnc-ALVE1-AS1* also increased the expression levels of the dsRNA recognition receptor TLR3 and antiviral innate immune genes, including IFN-α, IFN-β and IFITM3 (Fig. 6g-i). These results suggested that high-level expression of *lnc-ALVE1-AS1* in ALV-resistant chickens is associated with host viral resistance.

Discussion

ERVs have adapted long-term evolutionary selection by the host and are important sources and evolutionary origins of various regulatory non-coding RNAs, including lncRNAs, microRNAs, and piRNAs. When these ERV-derived ncRNAs are activated by exogenous infections or other stimuli, they may activate the functions of immune cells, including B cells, T cells and macrophages. The long noncoding RNA *lnc-ALVE1-AS1* is derived from chicken ERV ALVE1 (Chen et al. 2019), which contains LTR, gag, pol and env regions and is 7.5 kb in length. It is located on chromosome 1 (Tereba et al. 1979) and is regulated by DNA methylation (Groudine et al. 1981). Chicken ERV ALVE1 is highly homologous to exogenous retrovirus ALVs and thus provides a unique model for investigating the interaction between endogenous and exogenous retroviruses as well as the symbiotic relationship and interplay between ERVs and innate immunity (Chen et al. 2019; Hu et al. 2016b; Hu et al. 2017).

In this study, we found that *lnc-ALVE1-AS1* inhibited the proliferation of ALV-J by activating antiviral immunity in chicken macrophages. Inhibition of virus proliferation by lncRNAs derived from ERVs was also observed in mouse macrophages (Zhou et al. 2019). The piRNA derived from the chicken endogenous retrovirus ALVE may be involved in resistance to ALV (Sun et al. 2017). Therefore, noncoding RNAs such as lncRNAs and piRNAs derived from the chicken endogenous retrovirus ALVE may be an important part of the cellular immune response to enhance the host's resistance to foreign viral infections.

Activation of the type I interferon response mediated by TLR3 signaling may be an important mechanism by which *lnc-ALVE1-AS1* inhibits the proliferation of ALV-J in chicken macrophages (a working model is shown in Fig. 7). It has been shown that *lnc-ALVE1-AS1* activates TLR3 signaling in chicken CEFs (Chen et al. 2019). In this study, we further found that *lnc-ALVE1-AS1* significantly activates the expression of antiviral innate immune genes such as TLR3 and type I interferons (IFN-α and IFN-β) in chicken macrophages. However, the effect of *lnc-ALVE1-AS1* is significantly reduced after interfering with TLR3 or suppressing TLR3 signaling. Confocal localization analysis showed that lnc-ALVE1-AS1 can directly bind to TLR3 protein in macrophages. In addition, studies have also found that TLR3 ligand stimulation can induce abnormal expression of a large number of lncRNAs (Wang et al. 2016), suggesting that lncRNAs could be an important signal for TLR3 recognition and a regulator of innate immunity (Murphy and Medvedev 2016). These results indicate that the induction of TLR3 signaling is an important mechanism by which *lnc-ALVE1-AS1* activates antiviral innate immunity.

TLR3 is an important double-stranded RNA (dsRNA) recognition receptor and participates in the antiviral immune response (Beutler 2004). Studies have shown that RNAs derived from endogenous retroviruses can be recognized by TLR3 protein and activate antiviral innate immunity. Several studies in mammals have reported that endogenous retroviral RNAs can trigger interferon signaling by the cytosolic pattern recognition receptors MDA5 and TLR3 (Chiappinelli et al. 2015; Licht 2015; Yu et al. 2012). The past study has been shown that *lnc-ALVE1-AS1* can be sensed by TLR3 in chicken CEFs (Chen et al. 2019). We also found that the key dsRNA recognition receptor TLR3 was significantly upregulated in macrophages transfected with *lnc-ALVE1-AS1*, indicating that *lnc-ALVE1-AS1* may form a dsRNA structure that can bind to TLR3. The long noncoding RNA *lnc-ALVE1-AS1* is an antisense lncRNA that may form dsRNA with the

sense RNA complementary to its sequences. In addition, some short dsRNA fragments may be formed in the secondary structure of *lnc-ALVE1-AS1*. These dsRNAs may be recognized by TLR3 and activate antiviral innate immunity. This phenomenon is very similar to the observation that the DNA methylation inhibitor 5-Aza-dC induces an interferon response by activating endogenous retroviral dsRNA (Chiappinelli et al. 2015; Roulois et al. 2015). In addition, TLR3 can also recognize the virus-derived single-stranded RNA segments harboring stem structures with bulge/internal loops (Tatematsu et al. 2014; Tatematsu et al. 2013).

However, it must be emphasized that the present study is not a simple repetition of past study although the results of *lnc-ALVE1-AS1* inhibits ALV-J proliferation and activates TLR3 signaling in chicken macrophages are consistent with those in chicken CEFs (a non-immune cell). First, it is not clear whether this endogenous retroviral RNA has a similar function in immune cells before this study. Secondly, this study more comprehensively evaluated the resistance of *lnc-ALVE1-AS1* to ALV-J in macrophages when compared with the single result of ALV-J inhibition by *lnc-ALVE1-AS1* overexpression in chicken CEFs. Finally, the role of *lnc-ALVE1-AS1* in the antiviral defense against ALV-J infection *in vivo* through ALV-resistant/susceptible chickens was evaluated only in this study.

In conclusion, the present findings collectively show that *lnc-ALVE1-AS1* exerts antiviral protective roles by triggering TLR3-induced antiviral innate immunity in macrophages. Individual lncRNAs with these immune functions remain to be extensively revealed in future research. Future advances in our understanding of ERV-derived lncRNA functions in immune cells are bound to generate new insights into the roles of these peaceful genome inhabitants in infection, inflammatory disease, autoimmunity, and cancer.

Authors' Contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Huan Luo, Xuming Hu, Huixian Wu, Gul Zaib, Wenxian Chai and Hengmi Cui. The first draft of the manuscript was written by Huan Luo and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Declarations

Authors' Contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Huan Luo, Xuming Hu, Huixian Wu, Gul Zaib, Wenxian Chai and Hengmi Cui. The first draft of the manuscript was written by Huan Luo and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Code or data availability All data and materials are available for publication.

Ethics approval Animal experiment was performed in strict accordance with the recommendations provided in the Guide for the Care and Use of Laboratory Animals of Yangzhou University. The protocol was approved by the Committee on the Ethics of Animal Experiments of Yangzhou University (licence number: 06R015).

Consent to participate Not applicable.

Consent for publication Not Applicable.

Conflicts of interest The authors declare that they have no competing interests.

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Figures

Figure 1

ALV-J inhibited the expression of *lnc-ALVE1-AS1* in chicken macrophage cell line HD11. RT-qPCR **(a)** and Western blotting (**b**) analysis of ALV-J *env* gene expression in HD11 cells at 6, 12, 24 and 48 hpi. **(c)** RT-qPCR analysis of *lnc-ALVE1-AS1 expression* in HD11 cells at 6, 12, 24 and 48 hpi. Error bars represent the s.d., n=3. *P < 0.05 and **P < 0.01 (two-tailed Student's t-test).

Figure 2

Overexpression of *lnc-ALVE1-AS1* inhibited the proliferation of ALV-J in HD11 cells. HD11 cells were firstly infected with the ALV-J virus at MOI of 5 for 12 h and then transfected with *lnc-ALVE1-AS1* or the control for another 36 h. RT-qPCR **(a)**, Western blotting (**b**) and $TCID_{50}$ **(c)** analysis of ALV-J *env* gene expression in HD11 cells. **(d)** ELISA analysis of ALV-J p27 protein expression in the supernatant of HD11 cells. **(e)** Confocal immunofluorescence microscopy analysis of ALV-J *env* gene expression in HD11 cells. HD11 cells were incubated with the anti-ALV-J envelope protein (JE9 antibody) and then stained with goat anti-mouse IgG conjugated with the Alexa Fluor 488 dye (Sigma-Aldrich). The nuclei were stained with DAPI dye (Sigma-Aldrich). The pictures were captured and merged with a Leica SP8 confocal microscope (20×). Error bars represent the s.d., n=3. *P < 0.05 and **P < 0.01 (two-tailed Student's t-test).

Figure 3

Knockdown of *lnc-ALVE1-AS1* promoted the proliferation of ALV-J in HD11 cells. HD11 cells were firstly transfected with the control or lnc-ALVE1-AS1 siRNA for 12 h and then infected with the ALV-J virus at a MOI of 5 for another 48 h. RT-qPCR **(a)**, Western blotting **(b)** and $TCID_{50}$ **(c)** analysis of ALV-J *env* gene expression in HD11 cells. **(d)** ELISA analysis of ALV-J p27 protein expression in the supernatant of HD11 cells. Error bars represent the s.d., n=3. *P < 0.05 and **P < 0.01 (two-tailed Student's t-test).

Figure 4

lnc-ALVE1-AS1 triggered antiviral innate immunity in macrophage. Relative expression analysis of *lnc-ALVE1-AS1* **(a)** and innate immunity genes **(b)** in HD11 cells transfected with *lnc-ALVE1-AS1* for 36 h. **(c)**

ELISA analysis of IFN-α and IFN-β expression in HD11 cells transfected with *lnc-ALVE1-AS1* for 36 h. Relative expression analysis of *lnc-ALVE1-AS1* **(d)** and innate immunity genes **(e)** in HD11 cells transfected with the *lnc-ALVE1-AS1* siRNA or control siRNA for 48 h. **(f)** ELISA analysis of IFN-α and IFN-β expression in HD11 cells transfected with the *lnc-ALVE1-AS1* siRNA or control siRNA for 48 h. Error bars represent the s.d., n=3. *P < 0.05 and **P < 0.01 (two-tailed Student's t-test).

Figure 5

lnc-ALVE1-AS1 involved in antiviral innate immunity induced by TLR3 signalling. **(a)** Relative expression analysis of IFIH1, MB21D1 and TLR7 genes in HD11 cells transfected with *lnc-ALVE1-AS1* for 36 h. **(b)** Relative expression analysis of TLR3 gene in HD11 cells transfected with *lnc-ALVE1-AS1* for 36 h. **(c)** Relative expression analysis of TLR3 gene in HD11 cells transfected with the *lnc-ALVE1-AS1* siRNA or control siRNA for 48 h. Western blotting analysis of TLR3 protein expression in HD11 cells transfected with *lnc-ALVE1-AS1* for 36 h **(d)** or with the *lnc-ALVE1-AS1* siRNA or control siRNA for 48 h **(e)**. **(f)** Co-localization of the *lnc-ALVE1-AS1* RNA with TLR3 protein in HD11 cells detected by the RNA FISH and immunofluorescence confocal microscopy assay. The pictures were captured and merged with a Leica SP8 confocal microscope (100×). **(g)** Gene expression analysis of IFN-α and IFN-β genes in HD11 cells, which were firstly transfected with the control or TLR3 siRNA for 12 h and then transfected with *lnc-ALVE1-AS1* or the control for another 36 h. **(h)** Gene expression analysis of IFN-α and IFN-β genes in the Amlexanox-mediated TLR3 pathway inhibition of HD11 cells, which firstly treated with 100 μM Amlexanox (TBK1/IKKε inhibitor) for 2 h and then transfected with the control or *lnc-ALVE1-AS1* for another 36 h. **(i)** Relative expression analysis of *lnc-ALVE1-AS1* in HD11 cells incubated with TLR3 ligand (a synthetic analogue of dsRNA poly (I:C) with a high molecular weight) for 24 h. Error bars represent the s.d., n=3. *P < 0.05 and **P < 0.01 (two-tailed Student's t-test).

Figure 6

lnc-ALVE1-AS1 exerts innate immune resistance to ALV-J in chickens. **(a)** RT-qPCR analysis of *lnc-ALVE1-AS1 expression* in the immune organs (thymus, spleen, and bursa of fabricius) of ALV resistant (G1) and susceptible (G3) chickens. **(b)** RT-qPCR analysis of ALV-J *env* gene expression in CEF cells from ALV resistant (G1) and susceptible (G3) chickens. **(c)** RT-qPCR analysis of *lnc-ALVE1-AS1 expression* in CEF cells from ALV resistant (G1) and susceptible (G3) chickens. **(d)** RT-qPCR analysis of *lnc-ALVE1-AS1 expression* in the ALV resistant (G1) chicken CEF cells infected with ALV-J at 24 and 96 hpi. **(e)** RT-qPCR

analysis of *lnc-ALVE1-AS1 expression* in the susceptible (G3) chicken CEF cells infected with ALV-J at 24 and 96 hpi. **(f)** RT-qPCR analysis of ALV-J *env* gene expression in the ALV susceptible (G3) chicken CEF cells, which firstly infected with the ALV-J virus at MOI of 5 for 12 h and then transfected with *lnc-ALVE1-AS1* or the control for another 36 h. RT-qPCR analysis of *lnc-ALVE1-AS1* **(g)** and innate immunity genes **(h)** expression in the ALV susceptible (G3) chicken CEF cells transfected with *lnc-ALVE1-AS1* or the control for 36 h. **(i)** ELISA analysis of IFN-α and IFN-β expression in the ALV susceptible (G3) chicken CEF cells transfected with *lnc-ALVE1-AS1* for 36 h. Error bars represent the s.d., n=3. *P < 0.05 and **P < 0.01 (two-tailed Student's t-test).

Figure 7

A working model of TLR3-mediated antiviral interferons response triggered by *lnc-ALVE1-AS1* in macrophages. Chicken ERVs derived lncRNA *lnc-ALVE1-AS1* are sensed by TLR3, which recruit TRIF (Toll-IL1 receptor domain-containing adaptor inducing IFN-β) protein to the TIR domain of the receptor. This is followed by activation of IRF7 and led to production of interferons, which further induces the expression of interferon stimulated genes (ISGs). Ultimately, signals from TLR3 sensor promote antiviral innate immunity and inhibit the proliferation of ALV-J in chicken macrophages.