Overexpression of microRNA gga-miR-1650 decreases the replication of avian leukosis virus subgroup J in infected cells

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INTRODUCTION

MicroRNAs (miRNAs) are a class of small regulatory non-coding RNAs that modulate gene expression at the post-transcriptional level, playing a crucial role in cell differentiation and development. Recently, some reports have demonstrated that a number of cellular miRNAs play a role during viral infection. In this study, a luciferase-reporter system carrying the 5' untranslated region (5' UTR) and 3' UTR of avian leukosis virus subgroup J (ALV-J) was used to determine whether cellular miRNAs are involved in ALV-J infection. The miRNA gga-miR-1650 was screened for its potential interaction with the 5' UTR of ALV-J and the ability to suppress luciferase-reporter activity. A mutational analysis of predicted gga-miR-1650-binding sites showed that the 5' and 3' ends of gga-miR-1650 contributed to the interaction between gga-miR-1650 and its target located at the 5' UTR. Overexpression of miRNA gga-miR-1650 was shown to downregulate the expression of the Gag protein and influence the replication of ALV-J through binding to the 5' UTR. Overall, this report provides the basis for the development of new strategies for anti-ALV-J intervention.

MicroRNAs (miRNAs) are small RNAs (~22 nt) that carry out critical functions for a wide variety of biological processes (Ambros, 2004; Carrington & Ambros, 2003; Sun et al., 2010). miRNAs have been detected in various animal species, plants and even some viruses (Glazov et al., 2008; Hussain et al., 2012; Kurihara et al., 2006). miRNAs perfectly, or imperfectly, bind to mRNAs, leading to mRNA degradation (Bartel, 2004). This is similar to the post-transcriptional repression activity of small interfering RNAs (Bhattacharyya et al., 2006; Ronemus et al., 2006; Yekta et al., 2004), or the decrease in translation in vertebrates. The decrease in translation is usually due to the ability of miRNAs to prevent their mRNA targets from serving as templates for protein synthesis (Eulalio et al., 2008; Wu et al., 2006). Generally, miRNAs target the 3' untranslated region (3' UTR) of mRNAs; however, the 5' UTR and mRNA ORFs have also been reported to serve as miRNA targets (Jopling et al., 2005; Roberts et al., 2011; Vella et al., 2004).

An increasing number of studies suggest that miRNAs are involved in host–virus interactions (Bakre et al., 2012; Poole et al., 2011; Scaria et al., 2006; Yao et al., 2009), mainly through two scenarios. In one scenario, virally encoded miRNAs mediate the interaction between the virus and host. For example, simian virus 40 encodes miRNAs that target the viral T-antigen, the main signal recognized by cytotoxic T-lymphocytes, resulting in a decrease in cytotoxic T-lymphocyte-mediated elimination of infected cells (Sullivan et al., 2005). In the second scenario, cellular miRNAs mediate the interaction between the virus and host. For example, the human cellular miRNA hsa-miR-29a interferes with viral Nef protein expression and human immunodeficiency virus type 1 (HIV-1) replication (Ahuwalia et al., 2008). In addition, miR-32 targets ORF2 of the primate foamy virus type 1 and decreases viral mRNA translation, restricting the accumulation of the retrovirus in cultured human cells (Lecellier et al., 2005). Several reports have shown that cellular miRNAs target viruses directly in infected cells, but most of these cases were in mammalian cells, and avian miRNAs that target viruses have not been explored extensively. To date, the number of avian mature miRNAs identified has reached 791 (http://www.mirbase.org/), which urgently prompts us to focus on the function of avian miRNAs.

The avian leukosis virus subgroup J (ALV-J), which belongs to the genus Alpharetrovirus of the family Retroviridae, causes neoplastic diseases and immunosuppression...
in poultry worldwide, ALV-J has become a serious problem since it was first isolated in the UK in 1988 (Gao et al., 2010, 2012). ALV-J also has major public-health implications because it can be transmitted vertically from dam to progeny through an embryo; thus, it represents a potential threat for humans who receive vaccines that are produced in eggs or chicken embryonic fibroblasts (e.g. measles and mumps vaccine and yellow-fever vaccine) (Hussain et al., 2003). In this study, we investigated potential interactions between the 5′ UTR and 3′ UTR of ALV-J with avian miRNAs through reporter gene activity assays. We showed that avian miRNAs could suppress ALV-J replication by targeting the 5′ UTR. Our results indicate that the emerging hypothesis that cellular miRNAs are involved in viral infection, with positive or negative effects on virus replication, is relevant not only for mammalian cells (Zhang et al., 2010, 2011) but also for avian cells.

RESULTS

Luciferase-reporter activity of ALV-J 5′ UTR is decreased by cellular miRNAs

A luciferase-reporter assay was developed to determine whether cellular miRNAs could interact directly with the ALV-J genome. Two PCR fragments, containing the 5′ UTR or 3′ UTR of ALV-J, were cloned into luciferase-reporter plasmids between the Renilla luciferase ORF and the synthetic poly(A) sequence, respectively (Fig. S1, available in JGV Online). The recombinant plasmids were named psi-5′UTR and psi-3′UTR. The recombinant plasmids were then transfected into DF-1 cells, a continuous cell line of chicken embryo fibroblasts free of endogenous sequences related to ALV (Himly et al., 1998), and luciferase activity was measured 48 h after transfection.

As shown in Fig. 1, the relative luciferase activity (Renilla/firefly) in the DF-1 cells transfected with the psi-5′UTR plasmid was decreased relative to that of cells transfected with the empty psi-control vector (P=0.0028). The Renilla luciferase signal was normalized to the firefly luciferase signal. Decreased expression of the relative luciferase activity commonly reflects miRNA-mediated repression in luciferase-reporter assays (Song et al., 2010). These results suggested that the 5′ UTR of ALV-J harbours potential target sites to which cellular miRNA might directly bind.

Four potential miRNAs could target the 5′ UTR, but only gga-miR-1650 effectively decreases the luciferase-reporter activity of ALV-J 5′ UTR

To determine whether miRNAs were functionally decreasing the luciferase expression of psi-5′UTR, the 5′ UTR was selected using the RNA22 (http://cbsrv.watson.ibm.com/rna22.html) and miRanda (http://cbio.mskcc.org/) programs to search for putative miRNA-binding sites. The 5′ UTR harboured putative binding sites for miR-1456*, miR-1553, miR-1650, and miR-3537 (Fig. 2). To identify the cellular miRNAs responsible for the decrease in expression of the psi-5′UTR reporter gene, antisense miRNA inhibitors were used to block endogenous gga-miR-1456*, gga-miR-1553, gga-miR-1650 and gga-miR-3537. When the specific inhibitor of gga-miR-1650 was co-transfected with psi-5′UTR into DF-1 cells, the silencing effect of the psi-5′UTR was significantly reduced (P=0.0014) compared with that of psi-5′UTR co-transfected with the inhibitor control (Fig. 3a). To further confirm that the inhibitor was specific to gga-miR-1650, the antisense miRNA inhibitors were added to 293T cells (which do not express gga-miR-1650), and the results showed no difference between each group (Fig. S2). We also detected the effect of antisense sequences on the psi-control and there was no difference between each group (Fig. S3).

To further investigate whether gga-miR-1650 downregulated the luciferase expression of psi-5′UTR, two additional experiments were carried out. The relative luciferase activity was downregulated when psi-5′UTR was co-transfected with a gga-miR-1650 expression vector (pcDNA-gga-miR-1650) into DF-1 cells (Fig. 3b). To confirm the gga-miR-1650 silencing activity on the psi-5′UTR, psi-5′UTR and pcDNA-gga-miR-1650 were co-transfected into 293T cells (mammalian cells without endogenous gga-miR-1650). The results showed a significant decrease in the luciferase activity of psi-5′UTR (Fig. 3c). These data clearly indicated that the silencing effect of psi-5′UTR was attributable to gga-miR-1650. Based on these results, gga-miR-1650 was selected as a candidate cellular miRNA involved in ALV-J and cellular miRNA
interaction. To further confirm that the mature strand of gga-miR-1650 is released, we carried out Northern blot analysis on gga-miR-1650 in DF-1 cells and 293T cells with transfection of pcDNA-gga-miR-1650 or a control (Fig. 3d), and the results showed that mature gga-miR-1650 existed in the DF-1 cells but not in 293T cells. The mature gga-miR-1650 was released after transfection of gga-miR-1650 expression vector in both DF-1 cells and 293T cells. We then focused on the target sequence of gga-miR-1650, 5’-AGGAGGGCAGAAGCUGAGUGGCGUCGGAGGGA-3’, at positions 448–479 of the SD1009 genome. The sequence was found to be highly conserved across a variety of ALV-J strains by alignment of strain genomes from the NCBI database (Table S1).

Identification of the interaction between gga-miR-1650 and the ALV-J 5’ UTR

To explore the interaction between gga-miR-1650 and the 5’ UTR of ALV-J, a series of mutations were introduced into the 5’ UTR. We defined the 5’ motif of the target sequence as 5U, the 3’ motif of the target sequence as 3U, the 5’ end of gga-miR-1650 as 5P and the 3’-end of gga-miR-1650 as 3P. As shown in Fig. 4(b, c), the mutations generated 3U5Pmu5 (mutation of five sites in the target sequence in the 3’ motif corresponding to the 5’ end of gga-miR-1650) and 5U3Pmu5 (mutation of five sites in the target sequence in the 5’-motif corresponding to the 3’-end of gga-miR-1650) in the 5’ UTR. psiCHECK-2 vectors containing the mutated 5’ UTR were named psi-5’UTR3U5Pmu5 and psi-5’UTR5U3Pmu5. The silencing activity of gga-miR-1650 against different mutated 5’ UTRs was evaluated after co-transfection with pcDNA-gga-miR-1650 and the reporter plasmid containing different mutated 5’ UTRs. The reporter activity of psi-5’UTR3U5Pmu5 and psi-5’UTR5U3Pmu5 was significantly higher than that obtained with the WT construct in both DF-1 cells and 293T cells (Fig. 4d, e), providing strong evidence for an interaction between gga-miR-1650 and its viral target. We also explored whether the reporter activity of psi-5’UTR5U3Pmu5 or psi-5’UTR5U3Pmu5 could be altered by endogenous levels of gga-miR-1650. The results showed that the endogenous gga-miR-1650 cannot decrease the reporter activity of psi-5’UTR5U3Pmu5 or psi-5’UTR5U3Pmu5 (Fig. S4). Mutations in the seed region usually influence binding (Potenza et al., 2011), as observed with the introduced mutations that generated 3U5Pmu5 in the 5’ UTR (Fig. 4b) in this study, which influenced the interaction between gga-miR-1650 and the ALV-J 5’ UTR. Interestingly, five mutations introduced into the 5U, corresponding to the 3’ end of gga-miR-1650, induced a decrease in reporter-silencing activity. These results revealed two important findings: an interaction between gga-miR-1650 and the ALV-J 5’ UTR, and that both the 3’-end and 5’-end of gga-miR-1650 influence the binding between gga-miR-1650 and the ALV-J 5’ UTR.

<table>
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<tr>
<th>Gallus gallus miRNA</th>
<th>ALV-J genomic target (nt)</th>
<th>Mfe (kcal mol(^{-1}))</th>
<th>miRanda score</th>
<th>miRNA–mRNA pairing</th>
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<tr>
<td>miR-1456*</td>
<td>404–428</td>
<td>-37.5</td>
<td>135</td>
<td>miRNA: 3’ CUCUCUCUCUCCUCCUGGGGGG5’ (Fig. 3d)</td>
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<td></td>
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<td>mRNA: 5’ UACUGGGACGGCGGAGAACCUG3’</td>
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<tr>
<td>miR-1553</td>
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<td>-29.4</td>
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<td>miRNA: 3’ UUGUGUGAGUGAGUCGUAUCG5’ (Fig. 3d)</td>
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<td>mRNA: 5’ UUGUGUGAGUGAGUCGUAUCG3’</td>
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<td>miR-1650</td>
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<td>mRNA: 5’ UUGUGUGAGUGAGUCGUAUCG3’</td>
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Fig. 2. Computational analysis of the 5’ UTR of the SD1009 genome by miRanda yielded four sites that are potential targets for Gallus gallus miRNAs. Mfe, minimal free energy.
Overexpression of miRNA gga-miR-1650 decreases ALV-J Gag protein expression and virus replication

The 5′ UTR of ALV-J regulates Gag protein expression, and expression of p27 from the Gag polyprotein could reflect Gag protein expression (Doria-Rose & Vogt, 1998). The gga-mir-1650 miRNA targets the 5′ UTR, and we detected the influence of gga-mir-1650 on p27 protein expression. Western blot analysis revealed that the p27 protein levels of WT rSD1009, in the cells transfected into pcDNA-gga-mir-1650, was lower than that of WT rSD1009 in the cells transfected into an miRNA control or with no treatment (Fig. 5a). This result indicated that gga-mir-1650 could decrease the expression of p27 by binding to the 5′ UTR of ALV-J.

To determine whether gga-mir-1650 influenced virus replication by interacting with the 5′ UTR, WT ALV-J, ALV-J containing the five mutation sites corresponding to the 3′-end of gga-mir-1650 (rSD10095U3Pmu5) and ALV-J containing the five mutation sites corresponding to the 5′-end of gga-mir-1650 (rSD1009U5P5mu5) were rescued. An immunofluorescence assay for the rescued viruses was carried out. DF-1 cells infected with the rescued viruses was stained using mAbs specific for the gp85 of ALV-J, JE9 (Qin et al., 2001) (data not shown). pcDNA-gga-mir-1650 or a control was transfected into the DF-1 cells. At 24 h post-transfection, the cells were infected with rSD1009, rSD10095U3Pmu5 or rSD1009U5P5mu5. Real-time PCR was used to detect the level of ALV-J replication at different time points. As shown in Fig. 5(b), gga-mir-1650 decreased WT rSD1009 replication, but rSD10095U3Pmu5 and rSD1009U5P5mu5 were unaffected by gga-mir-1650. The cell activity was monitored using an alamarBlue assay to ensure that the cell viability of each group was equal (Fig. 5c). We also detected the
gga-miR-1650 expression level after exogenous expression by transfection of pcDNA-gga-miR-1650, and the results showed that exogenous gga-miR-1650 expression was significantly upregulated compared with the endogenous gga-miR-1650 in DF-1 cells (Fig. S5).

Endogenous gga-miR-1650 changes during ALV-J infection

Because exogenous gga-miR-1650 decreased the replication of ALV-J in DF-1 cells, we investigated whether endogenous gga-miR-1650 was influential during the infection of ALV-J. To evaluate the relationship pattern of the expression of endogenous gga-miR-1650 after ALV-J infection, mature gga-miR-1650 was detected using real-time RT-PCR in DF-1 cells. We used two controls: DF-1 cells treated with Dulbecco’s modified Eagle’s medium (DMEM) and DF-1 cells treated with a reticuloendotheliosis virus (REV), named rHLJR0901 (described previously; Deng et al., 2012). As shown in Fig. 6, endogenous gga-miR-1650 was expressed in DF-1 cells and significantly upregulated following WT ALV-J and mutant ALV-J (rSD10095U3Pmu5 and rSD10093U5Pmu5) infection. The expression levels were normalized to the 5S small RNA of G. gallus. Also, we confirmed that the virus infected and replicated in the DF-1 cells in the experiments for quantification of mature gga-miR-1650 (data not shown).

Fig. 4. Analysis of the interaction between mature gga-miR-1650 and its target 5’ UTR. (a) Predicted interactions between gga-miR-1650 and WT 5’ UTR sequences. (b) Five mutations introduced into the 3’ motif of the target (3U), corresponding to the 5’ end of gga-miR-1650 (5P). (c) Five mutations introduced in 5U, corresponding to 3P. The mutations are underlined. (d) The silencing effect of psi-5’UTR in DF-1 cells was decreased by gga-miR-1650 when 3U5Pmu5 and 5U3Pmu5 mutations were introduced into psi-5’UTR. (e) The silencing effect of psi-5’UTR in 293T cells was decreased by gga-miR-1650 when 3U5Pmu5 and 5U3Pmu5 mutations were introduced into psi-5’UTR. The mutated vectors were named psi-5’UTR3U5Pmu5 and psi-5’UTR5U3Pmu5. The results are given as means ± SEM of three independent experiments.
Endogenous gga-miR-1650 could be altered by the WT and mutant ALV-J, but virus replication was similar between the WT ALV-J and mutant ALV-J in DF-1 cells without exogenous gga-miR-1650 (Fig. 5b). To clarify the phenomenon, we further transfected decreasing amounts of pcDNA-gga-miR-1650 and found that the decrease in virus replication was significantly reduced (Fig. S6).

**DISCUSSION**

Recent reports have shown that cellular miRNAs play critical roles in host–virus interactions (Berkhout & Jeang, 2007; Sun et al., 2012). Studies exploring the interaction between ALV-J and cellular miRNAs are important for providing insight into cellular defences against ALV-J. In this study, we screened the cellular miRNA gga-miR-1650 and found that it decreased the replication of ALV-J by binding to the 5’ UTR of ALV-J. This study demonstrated that the host-encoded gga-miR-1650 might serve as an antiviral tool to regulate ALV-J replication.

The discovery that host gga-miR-1650 has targets in the genome of ALV-J indicates that gga-miR-1650 is part of the host’s antiviral defences. However, given that viruses evolve much faster than host miRNAs, it is surprising that viral variants lacking these ‘antiviral’ miRNA target sequences have not become established (Table S1). Furthermore, as shown in Fig. 5(b), WT ALV-J replicated equally as well as mutant ALV-J in DF-1 cells, despite increased cellular endogenous gga-miR-1650 expression by virus infection (Fig. 6). Altogether, these findings may support an
alternative view that viruses co-opt the host miRNAs to suppress virus replication but also to aid in evading immunity-based elimination and in establishing a persistent infection (Mahajan et al., 2009). For example, HIV is suppressed by cellular miRNAs such as miR-28, miR-125b, miR-223 and miR-382 in resting CD4⁺ T-cells, and it has been speculated that these miRNAs help in maintaining viral latency by shutting off virus replication, allowing the viruses to escape the immune system (Finzi et al., 1997; Huang et al., 2007). Also, it seems that the virus should have the potential to escape selective pressure, but viral escape seems to be less of an issue than we would have predicted. For example, in a study that exploited the ubiquitous miRNA let-7a to target poliovirus, the selective pressure was so great that no viral escape mutants appears (Barnes et al., 2008).

Bioinformatic methods were used to predict miRNA pairing with targets in this study, identifying four miRNAs predicted to target the 5’ UTR of ALV-J; however, only gga-miR-1650 was found to have an effect on the luciferase-reporter activity of the ALV-J 5’ UTR. To provide further evidence of the effects of gga-miR-1650 on its viral targets, mutations were introduced into 5’ UTR target sites. The results provided strong evidence for an interaction between gga-miR-1650 and the 5’ UTR of ALV-J. Notably, gga-miR-1650 was not the best-scoring miRNA, as predicted by miRanda, to bind to the 5’ UTR (Fig. 2). The reason that the other miRNAs did not affect the luciferase-reporter activity is unknown. One possible reason is that the RNA secondary structures of the other miRNA targets might prevent miRNA binding.

In this study, p27 protein expression and virus replication after overexpression of gga-miR-1650 were investigated, and the results showed that gga-miR-1650 could decrease p27 protein expression and virus replication by binding to the 5’ UTR of ALV-J (Fig. 5). Although the 3’ UTR is the target of miRNAs in most cases, the 5’ UTR can also be a target for miRNAs. For example, microRNA-10a binds the 5’ UTR of ribosomal protein mRNAs (Orom et al., 2008), and miR-122 activates the translation of hepatitis C virus through 5’ UTR binding (Jopling et al., 2005). In addition, the expression levels of gga-miR-1650 could be altered during ALV-J infection of DF-1 cells (Fig. 6). However, the endogenous level of gga-miR-1650 does not have a significant effect on the replication of WT virus in infected DF-1 cells, as we observed that WT and mutant viruses had a similar replication activity without exogenous gga-miR-1650 (Fig. 5b). This is potentially because the level of gga-miR-1650 was not high enough, even after viral infection, to exert a significant effect. As shown in Fig. S5, the decrease in virus replication was significantly reduced by transfection with decreasing amounts of DNA vector expressing gga-miR-1650. These results indicated that a certain amount of gga-miR-1650 is needed to decrease the replication of WT ALV-J.

The introduction of mismatches into the seed region of a presumptive miRNA–mRNA duplex can be used to validate the predicted target sites. The seed principle is that the contiguous 2–7 nt at the 5’ end of the miRNA pair with their target by Watson–Crick base paring. Initially, this idea was based on evolutionary evidence (Lewis et al., 2003). The numerous seed-based target predictions that have been validated in some studies, showing that miRNA–mRNA interactions obey the seed rule, are helpful for characterizing the biological functions of miRNA (Hussain et al., 2012; Potenza et al., 2011). However, some reports have shown that seed pairing is not the only mechanism for target recognition (Grimson et al., 2007). In this study, mismatches were introduced not only into the seed region but also into the 5’ motif of the target, corresponding to the 3’ end of the non-seed region. We defined the 5’ motif of the mRNA as 5U, the 3’ motif of the mRNA as 3U, the 5’ end of gga-miR-1650 as 5P and the 3’-end of gga-miR-1650 as 3P. Reporter activity was significantly influenced by five mutations in 3U, corresponding to the 5P seed region. In addition, five mutation sites in 5U, corresponding to 3P, demonstrated that the 3’ end of gga-miR-1650 could play a role in the interaction between gga-miR-1650 and the target. This finding illustrated that the ‘non-seed’ target sites were involved in the binding activity of gga-miR-1650. Non-seed target sites have been described previously. For example, cytomegalovirus encodes a miRNA that uses a non-seed target site to repress an MHC-related gene,
avoiding the destruction of infected cells (Stern-Ginossar et al., 2007). miR-10a targets ribosomal protein transcripts via non-seed sites in the 5′ UTR (Orom et al., 2008) and Caenorhabditis elegans lin-4 and let-7 use many functional non-seed sites in their lin-41 and lin-14 target miRNAs (Ha et al., 1996). The 3′ end of gga-miR-1650 influenced binding activity in this study, which supports the hypothesis that 3′-end pairing could contribute to target recognition when targets are located in the 5′ UTR of genes (Lee et al., 2009). This result indicates that seed pairing is very important but not the only way for miRNAs to recognize their targets. Non-seed pairing, such as through the 3′ end of miRNAs, is also very important for guiding miRNAs to their targets (Lee et al., 2009).

In summary, we showed that the cellular miRNA gga-miR-1650 decreased the efficient replication of ALV-J by binding to the 5′ UTR. This finding suggested that cellular miRNAs are vital factors in host resistance to viral infection. Our results further enrich our understanding of the mechanism of host cell defence systems. This study demonstrated that the viral genome is a target of cellular miRNAs, and that this process is an important component of virus–host interactions.

**METHODS**

**Cell culture.** DF-1 cells and 293T cells were cultured in DMEM (Invitrogen) supplemented with 10% FBS plus penicillin and streptomycin (100 μg ml⁻¹). The cultures were incubated at 37 °C in a humidified 5% CO₂ incubator.

**Luciferase-reporter plasmid construction.** The luciferase-expression vector psiCHECK-2 (Promega) was used as the parent vector for 5′ UTR and 3′ UTR reporter-analysis experiments. The 5′ UTR (397 nt) and 3′ UTR (643 nt) of ALV-J were amplified using PCR of the ALV-J isolate SD1009, which was isolated from a layer chicken in the Shandong province in China (Wang et al., 2011). To facilitate cloning, NotI and XhoI restriction sites were added to the 5′ and 3′ primers. The primers used to amplify the 5′ UTR were: forward: 5′-CCGCCTGAGCCCATTTTACCTCCCACCACAT-3′, and reverse: 5′-AAGGGCTAGGCCTACAGAGGA-3′. The PCR products were ligated to the psiCHECK-2 vector directly through gel retrieval (BioFlux), and these constructs were named psi-5′ UTR and psi-3′ UTR and confirmed by sequencing.

**Expression vector and miRNA inhibitors.** The pcDNA6.2-GW/EmGFp-miR vector (Invitrogen) was used to express gga-miR-1650 (Kim et al., 2009). The pre-miRNA sequence of gga-miR-1650 was obtained from mirBase (http://www.mirbase.org/), synthesized by Invitrogen, inserted into the pcDNA6.2-GW/EmGFp-miR vector and named pcDNA-gga-miR-1650. In addition, we used a non-G. gallus miRNA (C. elegans mir-239b) as a control and named it Ctrl-miRNA. miRNA inhibitors from GenePharma were used for the sequence-specific inhibition of mature miRNA using 2′-O-methyl-modified antisense oligonucleotides to bind to mature miRNAs (Bagga et al., 2005; Davis et al., 2006) and the gga-miR-1650 inhibitor sequence was 5′-AGAGGCAGAGCTGAGTCAGAGGA-3′. The inhibition control was the antisense of C. elegans mir-239b and was named Ctrl-anti-miRNA.

**Transfection and luciferase assays and data analysis.** The plasmid was transfected into cells using Lipofectamine 2000 (Invitrogen). DF-1 cells and 293T cells were plated in six-well plates. At 80% confluency, the cells were transfected with reporter plasmid alone or co-transfected with 2 μg pcDNA-gga-miR-1650 and inhibitors. After 48 h, the cells were harvested for a relative luciferase-activity assay. Luciferase assays were performed using a Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s protocol. In brief, the harvested cells were washed with PBS. Passive lysis buffer (100 μl) was added, and after 10 min the supernatant was collected by centrifugation at 12 000 g for 5 min. The relative luciferase-expression values were analysed using EnVision (PerkinElmer).

**Northern blotting of miRNAs.** RNA samples (10 μg each) was analysed using a MiRNA Northern Blot assay kit (Signosis) following the manufacturer’s instructions. Northern blots were hybridized with biotin-labelled gga-miR-1650 and mir-221 probe. The sequence of miR-221 is conserved between G. gallus and Homo sapiens, and thus miR-221 was used as a loading control (Burnside et al., 2008). The probe sequences were as follows: gga-miR-1650, 5′-AGAGGC- GAAGCTGAGTCAGAGGA-3′, and mir-221, 5′-GAACCCCA-GCACGACATTGAGCT-3′.

**Prediction of miRNA-binding sites.** miRNA-binding sites were predicted according to the rules for binding miRNA targets (Rajewsky, 2006; Yoon & De Micheli, 2006). In brief, the 5′ UTR of ALV-J was paired with G. gallus miRNAs using miRanda (http://cbio.mskcc.org/). The results were restricted to targets conserved in ALV-J and exhibited a minimal free energy of binding less than −29 kcal mol⁻¹ and a score >130. The resulting miRNA–target pairs were confirmed using the RNA22 miRNA target predictor (http://cbsrv.watson.ibm.com/ma22.html).

**Mutations in miRNA-binding sites.** Mutations were generated directly on the psi-5′ UTR plasmid. The mutant plasmid was generated by PCR using 20 ng template and primers containing the mutation sites under the following conditions: 95 °C for 5 min, followed by 16 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 7 min. The mixture was digested with 1 μl DpnI (NEB) for 30 min at 37 °C to remove the parental DNA. The remaining DNA was used to transform Escherichia coli DH5α (TaKaRa). The resulting colonies were sequenced to identify those containing the desired mutation.

**Rescue of WT and mutant ALV-J and analysis of ALV-J p27 protein expression.** The strategy for constructing an infectious clone has been described previously by our laboratory (Wang et al., 2012). The WT-rescued ALV-J was named rSD1009, and the other rescued viruses that contained mutant sites were named according to the mutation sites. Western blot analysis was performed using a mouse anti-p27 antibody (Yun et al., 2013) on cell extracts obtained using Protein Extraction Reagent (Thermo Fisher Scientific).

**Real-time RT-PCR analysis of ALV-J and gga-miR-1650.** To assess the influence of gga-miR-1650 on the replication of ALV-J, a real-time RT-PCR assay was used to detect ALV-J. The primers and method were designed and performed as described previously by our laboratory (Qin et al., 2013). Because the quantification of ALV-J is an absolute measurement, an alamarBlue assay (Invitrogen) was performed to confirm that each experimental sample contained an equal number of viable cells.

To explore the influence of ALV-J on gga-miR-1650, real-time PCR measurements of gga-miR-1650 were performed using a Hairpin-it miRNAs qPCR Quantification kit (GenePharma) according to the manufacturer’s instructions. The cycling conditions for the real-time PCR were as follows: 94 °C for 3 min, followed by 40 cycles of 95 °C for 15 s and 62 °C for 40 s. Expression of 5S RNA was used as a control with the primers provided in the kit. The reactions were
performed in a Roche 480 PCR machine (Roche Applied Science). The expression level of gga-miR-1650 in DF-1 cells treated with REV (named rHLJR0901) was used as one control when exploring the influence of ALV-J on gga-miR-1650. rHLJR0901 was rescued by our group (Deng et al., 2012) and we used 0.1 ml rHLJR0901 (10^3 TCID$_{50}$ ml$^{-1}$) to infect DF-1 cells to explore gga-miR-1650 expression levels.

**Confirmation of replication of ALV-J and REV in infected DF-1 cells.** To confirm that the virus infected and replicated in the DF-1 cells in the experiments for quantification of mature gga-miR-1650, virus-infected cell cultures were harvested at 24, 48, 72 and 96 h after infection and a real-time RT-PCR assay was used to detect ALV-J and REV, as described previously (Li et al., 2012; Qin et al., 2013).

**Statistical analysis.** The significance of the variability among the trials was analysed using GraphPad Prism (version 5.0) software. Western blot bands were quantified with Labworks 4.0 software.

**ACKNOWLEDGEMENTS**

This study was funded by the National Natural Science Foundation of China (31072146 and 31201923) and the earmarked fund for the Modern Agro-industry Technology Research System (no. nycytx-42-G3-01).

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